PREDICTING THE OUTCOME OF ORAL POTENTIALLY MALIGNANT DISORDERS USING A COMPOSITE CLINICAL, HISTOPATHOLOGICAL AND MOLECULAR CLASSIFIER

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Abstract

Oral squamous cell carcinoma (OSCC) is associated with a high degree of morbidity and mortality. OSCCs are often preceded by oral potentially malignant disorders (OPMD) which have a higher propensity to undergo malignant transformation compared to clinically normal oral mucosa. However, traditional methods such as clinical and histopathological assessment of OPMD are unable to accurately predict clinical outcome. The search to identify the perfect biomarker for prognosticating malignant transformation in OPMD is still ongoing. This study was undertaken to construct a prognostic classifier for patients with OPMD by integrating clinical, histopathological and molecular factors as well as to discover a gene expression signature that characterises OPMD with a high risk of undergoing malignant transformation. The demographic and clinical features of a cohort of OPMD patients were studied in detail. Assessment and analysis of clinico-demographic features, histopathological features, differential gene expression, loss of heterozygosity (chromosomal regions 3p14.2, 9p21 and 17p13) and DNA ploidy status were performed on archived formalin-fixed paraffin-embedded tissue material from this cohort of patients. Gene expression studies revealed several genes that had statistically significant differential expression between cases that underwent malignant transformation and those that did not. A novel gene-signature for cases that had a propensity for undergoing malignant transformation was developed. Statistical model building was performed to construct a prognostic classifier for OPMD patients. A prognostic model composed of age at diagnosis, site of index OPMD, binary oral epithelial dysplasia grading and the novel gene-signature was found to be highly prognostic of clinical outcome for OPMD patients (concordance index: 0.85). Our findings show that a molecular biomarker driven prognostic classifier outperformed conventional methods for predicting clinical outcome in patients with OPMDs. The findings from this study have also reinforced that formalin-fixed paraffin-embedded tissue can be used to generate a molecular classifier with clinical utility.

Dedication

This thesis is dedicated to,

My wife, Prethiba Yugaraj

The love of my life. Her unwavering love and support are the reasons why I have been able to complete the PhD whilst maintaining my sanity.

My boys, Mathavan and Mahendran

For adding colour and spice to my life.

My father, Sathasivam NSS Maniam

For always reminding me of what is important in life.

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Declaration

I declare that this thesis is my own work and that I have acknowledged the contribution and work of others appropriately. This work has not been submitted for any degree or examination elsewhere.

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Abbreviations

A	:	Adenine
ACTB	:	Actin beta
ALDH	:	Aldehyde dehydrogenase
AUC	:	Area under the curve
BH	:	Benjamini-Hochberg
bp	:	Base pair
C	:	Cytosine
CaCl2	:	Calcium chloride
CCND1	:	Cyclin D1
CCNE1	:	Cyclin E1
CDK	:	Cyclin-dependent kinase
CDKN2A	:	Cyclin dependent kinase inhibitor 2A
cDNA	:	Complementary deoxyribonucleic acid
CHC	:	Chronic hyperplastic candidiasis
CO2	:	Carbon dioxide
CRA	:	Cox proportional-hazards regression analysis
CSV	:	Comma-separated value
CV	:	Coefficient of variation
df	:	Degrees of freedom
DGE	:	Differential gene expression

DMSO	:	Dimethyl sulfoxide
DNA	:	Deoxyribonucleic acid
DNase	:	Deoxyribonuclease
EDTA	:	Ethylenediaminetetraacetic acid
EGF	:	Epidermal growth factor
EGFR	:	Epidermal growth factor receptor
ERCC	:	External RNA Controls Consortium
FAM	:	Fluorescein
FBS	:	Foetal bovine serum
FDR	:	False discovery rate
FFPE	:	Formalin-fixed paraffin-embedded
FHIT	:	Fragile histidine triad
FISH	:	Fluorescence *in situ* hybridisation
fM	:	Femtomolar
G	:	Guanine
GAPDH	:	Glyceraldehyde 3-phosphate dehydrogenase
H&E	:	Haematoxylin and eosin
HCl	:	Hydrochloric acid
HEX	:	Hexachlorofluorescein
HGNC	:	Human Genome Organisation Gene Nomenclature Committee
HNSCC	:	Head and neck squamous cell carcinoma

HPV	:	Human papillomavirus
HR-HPV	:	High-risk human papillomavirus
HR-HPV ISH	:	High-risk human papillomavirus DNA *in situ* hybridisation
IARC	:	International Agency for Research on Cancer
IBC	:	Image-based cytometry
ICD	:	International Classification of Diseases
IHC	:	Immunohistochemistry
IOD	:	Integrated optical density
IQR	:	Interquartile range
ISH	:	*In situ* hybridisation
ITGB8	:	Integrin subunit beta 8
JAK	:	Janus kinase
KEGG	:	Kyoto Encyclopaedia of Genes and Genomes
KSFM	:	Keratinocyte serum-free medium/media
LASER	:	Light amplification by stimulated emission of radiation
LCM	:	LASER capture microdissection
lncRNA	:	Long non-coding ribonucleic acid
LOH	:	Loss of heterozygosity
MAPK	:	Mitogen activated protein kinase
MCM2	:	Minichromosome maintenance protein 2
mins	:	Minutes
miRNA	:	Micro RNA

ml	:	Millilitres
mM	:	Millimolar
MMD	:	Manual microdissection
MMP	:	Matrix metalloproteinase
mRNA	:	Messenger ribonucleic acid
MT	:	Malignant transformation
N	:	Nodal category
NA	:	Not available/Not applicable
ncRNA	:	Non-coding ribonucleic acid
ng	:	Nanograms
NGS	:	Next generation sequencing
NI	:	Non-informative
NT	:	Non-transforming/No malignant transformation
OCRG	:	Oral Cancer Research Group
OED	:	Oral epithelial dysplasia
OPAL	:	OPMD associated leukocytes
OPALS	:	OPMD associated leukocytes score
OPMD	:	Oral potentially malignant disorder
OPSCC	:	Oropharyngeal squamous cell carcinoma
OSCC	:	Oral squamous cell carcinoma
PBS	:	Phosphate buffered saline
PCA	:	Principal component analysis

PCNA	:	Proliferating cell nuclear antigen
PCR	:	Polymerase chain reaction
PEN	:	Polyethylene naphthalate
PI3K	:	Phosphatidylinositol-4,5-bisphosphate 3-kinase
PVL	:	Proliferative verrucous leukoplakia
PWS	:	Ploidy Work Station
QC	:	Quality control
qPCR	:	Quantitative real-time polymerase chain reaction
RB	:	Retinoblastoma
RIN	:	Ribonucleic acid integrity number
RNA	:	Ribonucleic acid
RNase	:	Ribonuclease
RNASeq	:	Ribonucleic acid sequencing
ROC	:	Receiver operating characteristic
rpm	:	Revolutions per minute
RT-PCR	:	Reverse-transcription polymerase chain reaction
SCC	:	Squamous cell carcinoma
SD	:	Standard deviation
secs	:	Seconds
SNOMED CT	:	Systematised Nomenclature of Medicine-Clinical Terms
SPT	:	Second primary tumour
STAT	:	Signal transducer and activation of transcription protein

T	:	Thymine
TBE	:	Tris-borate-EDTA
TBS	:	Tris-buffer saline
TGF	:	Transforming growth factor
TLX1	:	T-cell leukaemia homeobox 1
TNM	:	Tumour, Node, Metastasis
TP53	:	Tumour protein P53
TP63	:	Tumour protein P63
TRIPOD	:	Transparent reporting of a multivariable prediction model for Individual Prognosis or Diagnosis
TSG	:	Tumour suppressor gene
UK	:	United Kingdom
vs	:	Versus
WHO	:	World Health Organisation
µg	:	Micrograms
µl	:	Microlitres
µm	:	Micrometre
µM	:	Micromolar

Chapter 1. Introduction

1.1 Cancer

A recently completed epidemiological project to assess the global incidence and prevalence of major types of cancers, GLOBOCAN 2012, estimated that globally around 14 million new cancers occurred in 2012 resulting in eight million cancer-related deaths (Ferlay *et al.*, 2015; Torre *et al.*, 2015). Worldwide, lung cancer was the most common malignancy (excluding non-melanoma skin cancers) with regard to incidence and caused the majority of cancer-related deaths in 2012. Breast cancer was the most frequently diagnosed malignancy in women and second most common malignancy (excluding non-melanoma skin cancers) overall (Ferlay *et al.*, 2015; Torre *et al.*, 2015). In 2016, one in four deaths in the United Kingdom (UK) were cancer-related and a recent study estimated that around 50% of individuals born since 1960 in the UK will develop some form of cancer during their lifetime (Ahmad *et al.*, 2015; *Cancer Research UK*, 2018).

Cancers are the end result of a multistep process of accumulated genetic alterations (Balmain *et al.*, 1993). Fearon and Vogelstein (1990) through their study of colorectal cancers highlighted a molecular genetic model of carcinogenesis that is based on a multi-step progression from adenoma to carcinoma (Fearon and Vogelstein, 1990). This concept for carcinogenesis has also been adapted for other anatomical sites including the oral cavity (Califano *et al.*, 1996; Kim and Califano, 2004). Despite the heterogeneity of cancers, there are key molecular genetic events in carcinogenesis defined as the “Hallmarks of Cancer” (Figure 1.1) (Hanahan and Weinberg, 2000; Hanahan and Weinberg, 2011).

1.2 Oral Cancer

1.2.1 Introduction

Tumours arising in the upper aerodigestive tract can be broadly classified by site into tumours of the oral cavity, pharynx (nasopharynx, oropharynx, laryngopharynx), sinonasal complex, salivary glands, oesophagus and larynx. Currently, there is no standardised definition for oral cancer and researchers report epidemiological data using differing combinations of International Classification of Diseases (ICD) codes

(ICD 10 C00-C06, C09-C10 and C14) (World Health Organisation, 2016). In previous years, due to the close proximity of the oral cavity and oropharynx, tumours involving these two sites were often discussed together (Barnes *et al.*, 2005). This may cause some inconsistency in clinical coding and care is required when comparing different epidemiological studies. The recently published 4th edition of the World Health Organization (WHO) Classification of Head and Neck Tumours however has classified oropharyngeal squamous cell carcinoma (OPSCC) as a distinctly separate disease to conventional oral cavity squamous cell carcinoma (OSCC) (El-Naggar *et al.*, 2017). The oral cavity extends from the palatoglossal folds to the lips. Oral cavity cancers are usually further subdivided into two anatomical sub-sites: lip cancers and intra-oral cancers. This study will focus on intra-oral cancers as most lip cancers have a different aetiology (solar radiation) and better prognosis compared to oral cavity cancers (Moore *et al.*, 1999; Barnes *et al.*, 2005; Warnakulasuriya, 2009; El-Naggar *et al.*, 2017).

Epidemiological studies estimate that more than 300,000 new cases and 145,400 deaths from oral cancers (inclusive of lip cancers) occur (Torre *et al.*, 2015; Bray *et al.*, 2018). Approximately two-thirds of the new oral cancer cases occurred in men and around 77% of oral cancer (inclusive of lip cancers) deaths were in less-developed nations (Ferlay *et al.*, 2015). In 2012, it was the 15th most common cancer globally and was the most prevalent cancer in Indian men (Bray *et al.*, 2013; Ferlay *et al.*, 2015; Torre *et al.*, 2015).

In the United Kingdom (UK), in 2013, there were 7,591 new cases of oral and oropharyngeal cancers reported (around 21 people per day per annum) accounting for 2% of all new cancer cases (*Cancer Research UK*, 2015; *Welsh Cancer Intelligence and Surveillance Unit*, 2018; *ISD Scotland*, 2018; *Northern Ireland Cancer Registry*, 2018; *Office for National Statistics*, 2018). Furthermore, oral and oropharyngeal cancers have increased by more than 92% since the late 1970s (*Cancer Research UK*, 2015; *ISD Scotland*, 2018; *Northern Ireland Cancer Registry*, 2018; *Office for National Statistics*, 2018; *Welsh Cancer Intelligence and Surveillance Unit*, 2018). The north of England has higher levels of oral and oropharyngeal cancer than the south, possibly due to the complex relationship between lifestyle factors and socio-economic determinants (Johnson and Warnakulasuriya, 1993; Edwards and Jones, 1999; Quinn *et al.*, 2001; Conway *et al.*, 2006). Over the past few decades, an

increasing incidence of oral and oropharyngeal cancers has also been recorded in younger, middle aged adults (Macfarlane *et al.*, 1996; Mork, 1998; Koch *et al.*, 1999; Barnes *et al.*, 2005; Parkin *et al.*, 2005).

Most oral cancers are oral squamous cell carcinomas (OSCCs) (Barnes *et al.*, 2005). OSCC is a malignant epithelial neoplasm exhibiting varying degrees of squamous differentiation and has a propensity for regional lymph node metastasis (Barnes *et al.*, 2005; El-Naggar *et al.*, 2017). Most OSCC arise in the floor of the mouth, ventro-lateral surface of the tongue, sublingual sulcus and retromolar region (Johnson and Warnakulasuriya, 1993). The five-year survival rate for lip cancers is in the order of 80 - 95%, which is much better than the 30 - 50% five-year survival rate for intra-oral cancers (Johnson and Warnakulasuriya, 1993; Moore *et al.*, 1999; Moore *et al.*, 2000; Barnes *et al.*, 2005; Warnakulasuriya, 2009). This is possibly because SCCs of the lip are usually detected early due to their location as well as due to their differing aetiology.

It has been established that OSCC has a high rate of mortality (Moore *et al.*, 1999; Moore *et al.*, 2000; Barnes *et al.*, 2005; Warnakulasuriya, 2009; El-Naggar *et al.*, 2017). Survivors have high morbidity and risk of developing further upper aerodigestive tract cancers (Barnes *et al.*, 2005; El-Naggar *et al.*, 2017). Around 30-50% of patients with OSCC die from the disease within five years and survival rates have not improved over the last 30 years (Barnes et al., 2005; Warnakulasuriya, 2009). These trends have mostly been attributed to delayed detection of the disease, because improved outcomes are achievable if the disease is treated in its earlier stages (Barnes *et al.*, 2005; Goodson and Thomson, 2011; El-Naggar *et al.*, 2017). Delayed detection is often attributed to the fact that most OSCC are painless during the early stages and tumours may have reached a considerable size before the patient notices them or seeks medical attention (Barnes *et al.*, 2005). Early detection is feasible because many OSCC are preceded by clinically identifiable lesions termed ‘oral potentially malignant disorders’ (OPMDs) (Barnes *et al.*, 2005; Warnakulasuriya *et al.*, 2007; van der Waal, 2009; El-Naggar *et al.*, 2017). A good opportunity for early detection of OSCC exists as the mouth is easily accessible for regular routine screening by healthcare professionals (Sankaranarayanan *et al.*, 2013).

However, more frequently these tumours would have already metastasized to the regional lymph nodes by the time the patient presents at a clinic due to delayed

detection (Barnes *et al.*, 2005; El-Naggar *et al.*, 2017). Patients with advanced or late stage lesions are challenging to manage as complete surgical clearance is often not feasible due to the complex anatomy of the head and neck region. Inadequate clearance increases the risk of recurrent loco-regional disease (Woolgar *et al.*, 1999; Slootweg *et al.*, 2002).

Furthermore, the phenomenon of “field cancerisation” first described by Slaughter more than 60 years ago, is thought to predispose these patients to development of second primary tumours (SPTs) (Slaughter *et al.*, 1953; Day and Blot, 1992; Braakhuis *et al.*, 2003; Barnes *et al.*, 2005; El-Naggar *et al.*, 2017). Patients treated successfully for early primary OSCC may eventually succumb to SPTs. The concept of field change suggests that although the oral mucosa may appear normal clinically (and even histologically normal) in these patients, the mucosa has undergone genetic and molecular change that increases its propensity for malignant transformation. This concept is supported by accumulating evidence on the molecular genetic features of oral carcinogenesis (Bedi *et al.*, 1996; Scholes *et al.*, 1998; Braakhuis *et al.*, 2003; Wood *et al.*, 2015; Wood *et al.*, 2017).

Despite advances in management strategies, treatment outcomes are still rather poor and neither surgery, radiotherapy nor chemotherapy separately or in combination can effect a cure from OSCC (Johnson and Warnakulasuriya, 1993; Moore *et al.*, 1999; Moore *et al.*, 2000; Barnes *et al.*, 2005; Warnakulasuriya, 2009; El-Naggar *et al.*, 2017). This fact combined with the increased morbidity associated with the treatment for OSCC highlight the importance of early detection and more effective management of OSCC (Barnes *et al.*, 2005; Goodson and Thomson, 2011; El-Naggar *et al.*, 2017).

1.2.2 Aetiology

The aetiopathogenesis of OSCC is complex and multifactorial in nature, characterised by clinical and pathologic heterogeneity. As such, a multitude of environmental factors are thought to be involved in triggering the genetic aberrations and modifications involved in the initiation, progression and development of OSCC (Califano *et al.*, 1996; Forastiere *et al.*, 2001; Barnes *et al.*, 2005; Leemans *et al.*, 2011).

Historically, tobacco, alcohol and betel-quid use have been considered as the most contributory risk factors in the development of OSCC (Blot *et al.*, 1988; Johnson and Warnakulasuriya, 1993; Gupta *et al.*, 1996; Moore *et al.*, 1999; Balaram *et al.*, 2002). All forms of tobacco use have been strongly implicated in the aetiology of OSCC with cigar and pipe smoking being linked to a greater risk than cigarette smoking (Smith, 1973; Maier *et al.*, 1992; Johnson and Warnakulasuriya, 1993; Gupta *et al.*, 1996; McDowell, 2006). Tobacco use can be divided into either being smokeless (snuff, chewing) or smoke-producing (cigar, cigarette, pipe). Smoke-producing usage creates a higher number and level of carcinogens than smokeless tobacco as the majority of carcinogens are produced during combustion (Hecht, 2003). Tobacco products contain numerous carcinogenic elements that can act as either initiators or promoters of carcinogenesis (Maier *et al.*, 1992; Gupta *et al.*, 1996; Hecht, 2003; McDowell, 2006). Alterations at guanine residues of DNA induced by carcinogenic derivatives of tobacco products can cause nucleotide modifications in key genes involved in carcinogenesis such as the tumour-suppressor gene *TP53* (Brennan *et al.*, 1995; Gupta *et al.*, 1996; Hecht, 2003).

Increased intake of alcohol is a well-known risk factor for the development of OSCC (Smith, 1973; Johnson and Warnakulasuriya, 1993; Rodriguez *et al.*, 2004; Hashibe *et al.*, 2009). Although not fully understood yet, alcohol is believed to exert its deleterious effects through several different ways (Boffetta and Hashibe, 2006; Seitz and Stickel, 2007). The primary metabolite of ethanol, acetaldehyde, is thought to be the primary alcohol derived carcinogen involved with development of OSCC. Acetaldehyde is believed to interfere with DNA synthesis as well as forming DNA adducts. Acetaldehyde also binds to various proteins such as enzymes and glutathione, resulting in altered structure and function (Boffetta and Hashibe, 2006; Seitz and Stickel, 2007; Hashibe *et al.*, 2009).

Alcohol is also believed to facilitate absorption of other carcinogens such as nitrosonornicotine (a tobacco-specific nitrosamine) by increasing the permeability of the oral mucosa (Squier *et al.*, 1986). Alcohol also induces the cytochrome p450 enzyme CYP2E1 dependent microsomal biotransformation system in the liver and mucosa that is believed to be associated with the activation of several pro-carcinogens found in tobacco and other substances (Boffetta and Hashibe, 2006; Seitz and Becker, 2007; Hashibe *et al.*, 2009). Combined use of both alcohol and

tobacco increases the risk of OSCC by a factor of 15 to 20, compared with individuals who use neither; but the effects of tobacco and alcohol are time and dose dependent (Smith, 1973; Maier *et al.*, 1992; Johnson and Warnakulasuriya, 1993; Rodriguez *et al.*, 2004; McDowell, 2006).

Heavy alcohol intake alone was shown by some studies to increase the risk of developing OSCC by about three- to five-fold (Rodriguez *et al.*, 2004; Hashibe *et al.*, 2009). This dose dependent influence is possibly due to several reasons; one being the increased amount of acetaldehyde found in the saliva, derived primarily from alcohol metabolism and the other being nutritional deficits due to reduced intake of micronutrients, impaired intestinal absorption and changes in the metabolic pathways (Maier *et al.*, 1992; Boffetta and Hashibe, 2006; Seitz and Becker, 2007; Hashibe *et al.*, 2009). The recent implication that alcohol containing mouthwashes may play some role in oral carcinogenesis is controversial with no conclusive evidence (McCullough and Farah, 2008; La Vecchia, 2009).

The relationship between areca/betel nut and OSCC has long been established (Muir and Kirk, 1960; Hoffmann et al., 1994; Zain et al., 1999; Jeng et al., 2001). Areca nut used in combination with a number of different substances such as tobacco, slaked lime and betel leaf is prevalent in the Indian sub-continent as well as parts of Eastern Asia (Zain et al., 1999). The main alkaloid in areca nut, arecoline, is believed to alter gene expression, repress DNA repair and inhibit p53 in human epithelial cells (Sundqvist et al., 1989; Tsai et al., 2008). Slaked lime when used as a component of the betel-quid may act as a tissue-eroding agent due to its alkalinity (Zain et al., 1999).

High-risk human papillomavirus (HR-HPV) infection, specifically HPV-16, has been classified by the International Agency for Research on Cancer (IARC) as a causative agent for a subset of OSCC and OPSCC (IARC, 2007). Several studies have demonstrated the presence of HR-HPV in a subset of OSCC and OPMD, however its role in OPMD is still inconclusive especially since clinically “normal” mucosa has also been shown to be positive for HR-HPV (Miller and White, 1996; Elamin *et al.*, 1998; McKaig *et al.*, 1998; Smith *et al.*, 1998; McCord *et al.*, 2013; Woo *et al.*, 2013; El-Naggar *et al.*, 2017; Lerman *et al.*, 2017). Although HR-HPV associated oral epithelial dysplasia (OED) has been recognised as a distinct entity characterised by unique histopathological features as well as the presence of transcriptionally active

HR-HPV, the natural history of such lesions is as yet unclear (McCord *et al.*, 2013; Woo *et al.*, 2013; El-Naggar *et al.*, 2017).

Candida albicans has also been postulated to be a possible causative agent due to its ability to produce N-nitrobenzylmethylamine, a carcinogen. Chronic hyperplastic candidiasis (CHC) is a well-recognized oral lesion that has been considered as an OPMD (Speight and Morgan, 1993). Another infective agent thought to be associated with OSCC is *Treponema pallidum* which causes syphilis. Tertiary syphilis is known to cause changes to the oral epithelium making it more atrophic and susceptible to the effects of carcinogens. Sir Jonathan Hutchinson suggested the possible association between syphilis and tongue cancer (Hutchinson, 1887). A possible reason for the increased rate of tongue cancer in these patients may be due to the fact that the chemical agents used to “treat” tertiary syphilis decades ago have now been recognised as being carcinogenic (Michalek *et al.*, 1994). As such, it is quite difficult to conclude that syphilis is a possible causative agent for OSCC.

Nutritional deficiencies have also been linked to the development of OSCC. The most well-known nutritional factor associated with SCC of the upper aerodigestive tract is iron deficiency, seen in sideropenic dysphagia (Patterson – Kelly/Plummer-Vinson syndrome) (Watts, 1961). This syndrome typically affects middle-aged women and the presenting signs and symptoms are usually: a painful red tongue, mucosal atrophy and dysphagia caused by oesophageal webs (Watts, 1961; McDowell, 2006). The atrophic changes seen in the oral mucosa of sideropenic patients may adversely affect the epithelium making it more susceptible to carcinogens. It has been shown that diets rich in fresh fruit and vegetables confer a protective effect against OSCC due to the effects of vitamins A, C and E (Tavani *et al.*, 2001; Marchioni *et al.*, 2002).

Inheritable conditions such as ataxia telangiectasia, xeroderma pigmentosum, Fanconi anaemia, dyskeratosis congenita, systemic sclerosis, Li-Fraumeni syndrome and Bloom syndrome (to name a few) are known to predispose individuals to an increased risk of developing head and neck SCC (HNSCC) (Jacobsen *et al.*, 1998; Prime *et al.*, 2001; Kutler *et al.*, 2003; Kuo *et al.*, 2012; Kutler *et al.*, 2016; Martínez and Blasco, 2017; Furquim *et al.*, 2018). Genetic predisposition is also considered to be an important risk factor as first-degree relatives of HNSCC patients appear to

have a higher chance of developing HNSCC compared to the general population (Copper *et al.*, 1995; Foulkes *et al.*, 1996; Jefferies *et al.*, 1999).

Other factors historically considered to be significant in the aetiology of OSCC such as chronic trauma/irritation and poor oral hygiene are thought to be “modifiers or promoters” rather than initiators of OSCC, meaning that if a cancer is initiated from another reason, these promoters could hasten the process (Brandner *et al.*, 1986; Perez *et al.*, 2005).

1.2.3 Molecular pathogenesis

Califano *et. al.* (1996) in a landmark paper proposed a model for head and neck squamous cell carcinoma (HNSCC) based on the premises outlined by Fearon and Vogelstein (1990) for the development of colorectal carcinomas (Figure 1.2) suggesting a progressive change from hyperplasia through dysplasia to squamous cell carcinoma implicating the involvement of several cancer-related genes (Fearon and Vogelstein, 1990; Califano *et al.*, 1996). The two most widely studied groups of cancer related genes are oncogenes and tumour suppressor genes (Field, 1992; Scully, 1993; Ha *et al.*, 2009; Leemans *et al.*, 2011).

Oncogenes are genes that promote growth, survival and proliferation of cells as well as allowing cells to escape normal growth control mechanisms (Field, 1992; Scully, 1993; Scully *et al.*, 2000b; Ha *et al.*, 2009; Leemans *et al.*, 2011). Over-activity or over-expression of these genes can lead to the development of a tumour. Several oncogenes have been established as being commonly associated with OSCC; epidermal growth factor receptor (*EGFR*), receptor tyrosine kinase *MET* and Cyclin D1 (*CCND1*), with *EGFR* being the most widely studied (Ozanne *et al.*, 1986; Inaba *et al.*, 1992; Opitz *et al.*, 2001; Hama *et al.*, 2009; Seiwert *et al.*, 2009; Sheu *et al.*, 2009; Leemans *et al.*, 2011; Bates *et al.*, 2016).

The *EGFR* gene is located on chromosome 7p11 and it encodes a cell surface tyrosine kinase receptor expressed in squamous epithelia. Activation of *EGFR* can initiate numerous signalling pathways that contribute to cell proliferation and angiogenesis (Herbst, 2004; Normanno *et al.*, 2006; Sheu *et al.*, 2009). Several studies have shown that *EGFR* is over-expressed in many HNSCC suggesting an

important role of *EGFR* in carcinogenesis of HNSCC (Ozanne *et al.*, 1986; Grandis and Tweardy, 1993; Bonner *et al.*, 2006; Hama *et al.*, 2009).

Tumour suppressor genes are normally involved with the control of growth through cell cycle regulation, apoptosis, cell adhesion and DNA repair (Field, 1992; Sugerman *et al.*, 1995; Leemans *et al.*, 2011). A defect or inhibition of these genes may result in the proliferation of tumour cells. At present, the two most widely studied tumour suppressor genes in OSCC are *TP53* and *CDKN2A* (Field, 1992; Burns *et al.*, 1993; Brennan *et al.*, 1995; Sugerman *et al.*, 1995; Reed *et al.*, 1996; Rheinwald *et al.*, 2002; Gasco and Crook, 2003; Angiero *et al.*, 2008; Ha *et al.*, 2009; Agrawal *et al.*, 2011; Stransky *et al.*, 2011; Cancer Genome Atlas, 2015). *TP53* is located at chromosome 17p13 and genetic aberrations have been identified in the majority of OSCC (Somers *et al.*, 1992; Scully *et al.*, 2000b; Gasco and Crook, 2003; Leemans *et al.*, 2011). *TP53* is involved in cell cycle regulation mainly in the G1 phase of the cell cycle detecting DNA damage and initiating apoptosis if the damage is irreparable (Scully *et al.*, 2000b; Gasco and Crook, 2003; Leemans *et al.*, 2011). Changes in the *TP53* gene usually represent an early stage in carcinogenesis especially in tobacco related OSCC (Brennan *et al.*, 1995; Leemans *et al.*, 2011).

CDKN2A is located on chromosome 9p21 and has been found to be inactivated in many head and neck squamous cell carcinomas (HNSCCs) (Reed *et al.*, 1996; Agrawal *et al.*, 2011; Stransky *et al.*, 2011; Cancer Genome Atlas, 2015). The inactivation usually occurs through a combination of promoter methylation defects, gene mutations, chromosomal loss or homozygous deletion (Reed *et al.*, 1996; Forastiere *et al.*, 2001). *CDKN2A* encodes $p16^{INK4a}$, an inhibitor of cyclin-dependant kinases involved in cell cycle regulation and acts as a checkpoint in cellular growth control (Reed *et al.*, 1996; Forastiere *et al.*, 2001; Leemans *et al.*, 2011).

Aside from the above-mentioned genes, numerous candidate genes are being studied to develop a better understanding of the molecular events in oral carcinogenesis (Leemans *et al.*, 2011; Cancer Genome Atlas, 2015; Leemans *et al.*, 2018). By using gene expression profiling, Chung *et al* (2004) found that HNSCC could be categorised into four subgroups with differing molecular profiles and prognoses (Chung *et al.*, 2004). Rapid advances in the field of next-generation sequencing and proteomics has led to improved understanding of the diverse pathways involved in the various sub-types of HNSCC (Chung *et al.*, 2004; Molinolo

et al., 2009; De Cecco *et al.*, 2015; Keck *et al.*, 2015). The recent genomic characterization of HNSCC has also reinforced the heterogeneous nature of HNSCC (Cancer Genome Atlas, 2015).

The original genetic progression model proposed by Califano *et al* in 1996 (Figure 1.2) though remarkable in its own right, is now considered to be rather simplistic. The availability of next-generation sequencing and proteomics have made it possible to further expand this original model by elucidating the various pathways involved in the aetiopathogenesis of OSCC.

1.2.4 Clinical and histopathological features

OSCC has a heterogeneous clinical presentation ranging from ulcers to white plaque-like lesions (Barnes *et al.*, 2005). The primary tumour site is typically dependent on the aetiological agent involved, with the floor of mouth, retromolar area and latero-ventral surface of tongue being more commonly involved in tobacco-associated OSCC whilst the buccal mucosa and gingiva are more frequently affected in betel-quid chewers (Gupta *et al.*, 1980; Moore *et al.*, 2000; Barnes *et al.*, 2005; Warnakulasuriya and Ariyawardana, 2016; Speight *et al.*, 2018).

As OSCC is a malignant tumour of the mucosal epithelium, the key histological feature is invasion of underlying connective tissue by malignant epithelial cells with squamous differentiation. Grading of OSCC is performed according to the degree of similarity of the tumour cells to normal squamous epithelium (histological differentiation; Broders classification), however it is a poor prognostic predictor of clinical outcome (Broders, 1920; Bryne *et al.*, 1989; Roland *et al.*, 1992; Barnes *et al.*, 2005). Several other histological parameters such as nuclear pleomorphism, mitotic activity, pattern of invasive front, positive surgical resection margins and perineural or vascular invasion, provide a more accurate prognostic indicator when used in combination as a multi-factorial grading system (Bryne *et al.*, 1989; Close *et al.*, 1989; Odell *et al.*, 1994; Slootweg *et al.*, 2002; Rahima *et al.*, 2004; Barnes *et al.*, 2005).

1.2.5 Staging of oral squamous cell carcinoma

Oral squamous cell carcinoma (OSCC) is usually staged using the “Tumour, Node & Metastasis” (TNM) system developed by the International Union Against Cancer

(Barnes *et al.*, 2005; Sobin *et al.*, 2009; Brierley *et al.*, 2017). By combining the clinical and radiological findings for each of the TNM component, an overall clinical stage for the specific patient is obtained. The clinical stage is then used to formulate a treatment plan. The clinical stage may eventually be modified following the findings from the histopathological assessment of resection specimens; pathologic staging (Sobin *et al.*, 2009; Brierley *et al.*, 2017; El-Naggar *et al.*, 2017).

1.2.6 Treatment and outcome

Surgery with or without radiotherapy is still the favoured treatment modality for most OSCC (Woolgar *et al.*, 1999; Barnes *et al.*, 2005; Scully and Bagan, 2009; Shah and Gil, 2009; El-Naggar *et al.*, 2017). However, treatment planning is highly dependent on the clinical staging, patient factors as well as anatomical location of the tumour with management being directed towards the elimination of the lesion while attempting to preserve function and quality of life.

Patients with low stage tumours (Stage I & II) are usually treated with surgery alone while patients with higher clinical staging (Stage III & IV) usually require adjuvant radiotherapy with or without chemotherapy (Mazeron *et al.*, 2009; Scully and Bagan, 2009; Shah and Gil, 2009; Specenier and Vermorken, 2009). Treatment of OSCC is debilitating due to the complex anatomy of the craniofacial region. Although advances in treatment techniques with the addition of organ-preservation protocols have improved post-treatment quality of life, there are still some unavoidable side effects resulting from surgery and/or radiotherapy (Vissink *et al.*, 2003; Vergeer *et al.*, 2009; Tolentino Ede *et al.*, 2011).

As TNM staging of OSCC is related to prognosis (Sciubba, 2001; Barnes *et al.*, 2005; Sobin *et al.*, 2009; Brierley *et al.*, 2017; El-Naggar *et al.*, 2017; Tirelli *et al.*, 2018), an effective method to improve patient outcome would be detection of OSCC whilst still in Stage I or when the lesions are at a “pre-invasive” stage (Sciubba, 2001; Goodson and Thomson, 2011). A proportion of OSCC are thought to be preceded by these “pre-invasive” lesions that were previously called “precancerous lesions”. As not all of these types of lesions progress or transform to OSCC, the terminology of “oral potentially malignant disorder” (OPMD) was suggested (Warnakulasuriya *et al.*, 2007; van der Waal, 2009).

1.3 Oral Potentially Malignant Disorders (OPMD)

1.3.1 Introduction

A number of OSCC are preceded by clinical entities termed 'oral potentially malignant disorders' (OPMDs) (Warnakulasuriya *et al.*, 2007; van der Waal, 2009; El-Naggar *et al.*, 2017; Speight *et al.*, 2018). OPMDs are defined as clinical disorders having an increased risk of developing OSCC in oral mucosa; either in recognisable lesions or clinically "normal" oral mucosa (van der Waal, 2009; El-Naggar *et al.*, 2017). The term OPMD was adopted following a workshop coordinated by the World Health Organisation (WHO) Collaborating Centre for Oral Cancer and Precancer held in London in 2005 to replace previously used terminology such as 'oral precancer', 'precursor lesions' or 'premalignant lesions' (Warnakulasuriya *et al.*, 2007).

OPMD is thought to be a more accurate terminology to include all lesions and conditions/diseases that have a risk of malignant transformation to OSCC as it also indicates that not all patients with such lesions/conditions will undergo malignant transformation (Warnakulasuriya *et al.*, 2007; Napier and Speight, 2008; van der Waal, 2009; El-Naggar *et al.*, 2017; Speight *et al.*, 2018). Even in cases where there is an obvious identifiable lesion, OSCC may arise at a different site in the oral cavity due to the "field-change" phenomenon. It also must be emphasised that the majority (around 90%) of these lesions/conditions either remain unchanged or actually undergo regressive changes (Mehanna *et al.*, 2009; Shariff and Zavras, 2015; Warnakulasuriya and Ariyawardana, 2016; Thomson *et al.*, 2017b). The precise number of OSCC arising from OPMD however is still unclear. A new terminology, "potentially premalignant oral epithelial lesion" (PPOEL), was introduced by Nikitakis et al (2018) as an alternative to OPMD, once again showing that terminology of such lesions still continues to be a contentious area (Nikitakis, 2018; Speight *et al.*, 2018). For this study, the term OPMD will be used to represent these precursor lesions as implemented in the recently published 4th edition of the WHO Classification of Head and Neck Tumours (2017) (El-Naggar *et al.*, 2017).

OPMD encompasses numerous entities that are clinically and histologically diverse (Table 1.1) (El-Naggar *et al.*, 2017). Most OPMDs present as distinct oral mucosal lesions such as leukoplakia, erythroplakia, chronic hyperplastic candidosis and erythroleukoplakia, but more generalised disorders including oral submucous fibrosis

and proliferative verrucous leukoplakia also come under this umbrella term of OPMD (Barnes *et al.*, 2005; Warnakulasuriya *et al.*, 2007; van der Waal, 2009; El-Naggar *et al.*, 2017). The distinction between oral potentially malignant conditions and oral potentially malignant lesions is thought to be redundant as both are associated with field change (Warnakulasuriya *et al.*, 2007; van der Waal, 2009).

1.3.2 Epidemiology

Due to the diversity of OPMDs, truly representative epidemiological data on OPMDs is lacking. The majority of studies and reports concerning OPMDs have been about oral leukoplakia, oral submucous fibrosis (OSMF) and oral lichen planus. Oral leukoplakia and OSMF are the most frequently encountered OPMDs, whilst erythroplakia is thought to have the highest risk for malignant transformation (Petti, 2003; Reichart and Philipsen, 2005; Warnakulasuriya *et al.*, 2007; van der Waal, 2009; Warnakulasuriya *et al.*, 2011; El-Naggar *et al.*, 2017; Mello *et al.*, 2018). According to a recent systematic review and meta-analysis, OPMDs have an estimated global prevalence of 4.47% (CI: 2.43, 7.48) (Mello *et al.*, 2018).

Although clinicians are able to detect OPMD and obtain histopathological information, accurately predicting the clinical behaviour of these lesions is still difficult (Napier and Speight, 2008). Several articles have highlighted that the natural history of OPMD is poorly understood (Gupta *et al.*, 1980; Speight, 2007; Napier and Speight, 2008; Speight *et al.*, 2018), nevertheless, a recent systematic review looking at malignant transformation (MT) in oral leukoplakia focusing on observational studies estimated the overall MT rate to be around 3.5% (ranging from 0.13 – 34%) (Warnakulasuriya and Ariyawardana, 2016). This figure is similar to the MT rate of 4.8% in a hospital-based cohort of OPMD patients from the north-east of England (Thomson *et al.*, 2017a; Thomson *et al.*, 2017b). Additionally, a range of between 10 – 12 % rate of malignant transformation in lesions with oral epithelial dysplasia (OED) was calculated from two recent meta-analysis (Mehanna *et al.*, 2009; Shariff and Zavras, 2015).

However, the findings from these systematic reviews and meta-analyses must be read with caution as the studies included were not randomised controlled trials and were mostly hospital-based studies (Mehanna *et al.*, 2009; Shariff and Zavras, 2015). Hospital based studies also have an inherent referral bias of high-risk OPMD patients

and thus may not reflect the true incidence of malignant transformation in dysplastic OPMDs which is likely to be much lower.

1.3.3 Clinical and histopathological features

Clinically, OPMDs can appear as either white, red or mixed red and white lesions. They can also be homogenous or non-homogenous. It must be remembered that terminology such as leukoplakia or erythroplakia are clinical and a biopsy is required to ascertain the presence of epithelial dysplasia or malignant change (Barnes *et al.*, 2005; El-Naggar *et al.*, 2017; Speight *et al.*, 2018). Other OPMDs that are commonly encountered are oral lichen planus/lichenoid reaction (a chronic inflammatory muco-cutaneous disorder demonstrating an immune-mediated pathogenesis) and oral submucous fibrosis (a chronic disorder usually seen in betel-quid chewers) (Barnes *et al.*, 2005; Warnakulasuriya *et al.*, 2007; van der Waal, 2009; Speight *et al.*, 2018).

Several studies have reported that non-homogenous leukoplakia (speckled, nodular, verruciform *etc.*) undergo malignant transformation more frequently than homogenous lesions (Pindborg *et al.*, 1963; Silverman *et al.*, 1976; Gupta *et al.*, 1980; Holmstrup *et al.*, 2006). Even though the clinical features of OPMD may give some indication as to the risk of malignant transformation, they are non-specific and overlap with other disease processes. As such, clinico-pathologic correlation is always necessary (Barnes *et al.*, 2005; van der Waal, 2009; Warnakulasuriya *et al.*, 2011; El-Naggar *et al.*, 2017; Speight *et al.*, 2018).

Histopathological examination of OPMD can show a diverse spectrum of findings ranging from hyperkeratosis to severe oral epithelial dysplasia. Due to the difficulty and relatively subjective nature of interpreting histological features in OPMDs, assigning a precise diagnosis is known to be liable to intra- and inter-observer inconsistencies (Barnes *et al.*, 2005; Kujan *et al.*, 2007; El-Naggar *et al.*, 2017). The presence of oral epithelial dysplasia (OED) has been widely considered as an indicator of the risk of malignant transformation in OPMD (Reibel, 2003; van der Waal, 2009; Liu *et al.*, 2011; Warnakulasuriya *et al.*, 2011; Sperandio *et al.*, 2013). OED is characterised by cytological and architectural changes. The diagnosis and grading of oral epithelial dysplasia (OED) as such is based upon the presence and degree of individual criteria within the cytological and architectural categories (Table 1.2) (Barnes *et al.*, 2005; El-Naggar *et al.*, 2017). However, it must be remembered

that such changes can also be seen in reactive and reparative epithelium, once again emphasizing the need for clinico-pathological correlation (Macdonald and Rennie, 1975; Barnes *et al.*, 2005; El-Naggar *et al.*, 2017; Speight *et al.*, 2018).

Several grading systems have been proposed for grading OED with the most widely used grading system being the World Health Organization (WHO) classification system that originally sub-divided OED into hyperplasia, mild dysplasia, moderate dysplasia, severe dysplasia and carcinoma *in-situ* (Barnes *et al.*, 2005). However, the most recent version of the WHO grading system has merged the severe dysplasia and carcinoma *in-situ* categories into a single category of severe dysplasia and dropped the hyperplasia category thus making it a three-tiered grading system (El-Naggar *et al.*, 2017).

In recent years, there has been a suggestion to replace this classification scheme with a binary system consisting of “low-grade” and “high-grade” lesions (Kujan *et al.*, 2006; Warnakulasuriya *et al.*, 2008; Nankivell *et al.*, 2013; Gale *et al.*, 2014). The cut-off point between low-grade and high-grade OED as originally suggested by Kujan et al (2006) was OED having ≥ 4 architectural and ≥ 5 cytological changes (Table 1.2), however Nankivell et al (2013) suggested that a cut-off point of ≥ 4 architectural and ≥ 4 cytological changes may improve the predictive strength of the binary grading system (Kujan *et al.*, 2006; Nankivell *et al.*, 2013). However, as this is a relatively new grading system for OED, it requires further validation before routine clinical application.

The binary grading system is believed to have better intra- and inter-observer variation as there are fewer categories/levels (Kujan *et al.*, 2006; Kujan *et al.*, 2007). Regardless of the classification system used, there is always the issue of inter-observer variability (Kujan *et al.*, 2006; Kujan *et al.*, 2007). Diagnostic reliability however, may be improved via a consensus grading after review by more than one pathologist and a well-outlined set of criteria (Fleskens *et al.*, 2011; Speight *et al.*, 2015). Even though the presence/severity of OED is thought to be indicative of a higher risk of malignant transformation, non-dysplastic OPMD such as oral lichen planus have been reported to undergo malignant change as well (Mattsson *et al.*, 2002; Hsue *et al.*, 2007; van der Meij *et al.*, 2007; Bagan *et al.*, 2011). As such, there are no specific histopathological features that accurately predict the progression of OPMD to OSCC (van der Waal, 2009; van der Waal, 2014).

Although the role of HR-HPV in oral carcinogenesis is unclear, several research groups have described a histopathologically distinct subset of OED harbouring transcriptionally active HR-HPV (McCord *et al.*, 2013; Woo *et al.*, 2013; El-Naggar *et al.*, 2017). Diagnosis of HPV associated OED is through a defined set of histopathological features in conjunction with demonstration of HR-HPV through p16 immunohistochemistry and molecular techniques (McCord *et al.*, 2013; Woo *et al.*, 2013; El-Naggar *et al.*, 2017). Due to the relative rarity of HPV associated OED, the natural history and prognosis of this entity is still unclear.

1.3.4 Prognosis and outcome

There are several possible outcomes for OPMD, the lesion remains unchanged, it increases in size, it regresses in size, it disappears completely or undergoes malignant transformation (Banoczy and Sugar, 1972; Gupta *et al.*, 1980; Schepman *et al.*, 1998; Napier and Speight, 2008). Several epidemiological studies conducted in different areas of the world have shown that most OPMD do not undergo malignant transformation although they may persist (Banoczy and Sugar, 1972; Mehta *et al.*, 1972; Silverman *et al.*, 1976; Gupta *et al.*, 1980; Liu *et al.*, 2011).

Many of the studies on malignant change in OPMD are focused on leukoplakia and OED. The reported rates of malignant transformation from these studies have a wide variation ranging from 0.13% to 26.8% (Banoczy and Sugar, 1972; Mehta *et al.*, 1972; Silverman *et al.*, 1976; Silverman *et al.*, 1984; Lumerman *et al.*, 1995; Liu *et al.*, 2011). The presence of oral epithelial dysplasia in OPMDs is indicative of an increased risk for malignant transformation, with one meta-analysis reporting a mean overall malignant transformation rate of 12.1% in OED from a combined cohort of 992 patients from 14 studies (Mehanna *et al.*, 2009). A recent systematic review and meta-analysis however concluded that the pooled MT rate of OED was 10.5% in a combined cohort of 3708 patients from 16 studies (Shariff and Zavras, 2015). The difference in MT rate between the two meta-analysis is possibly due to the increased number of studies included in the meta-analysis by Shariff & Zavras (2015). However, the studies included in both meta-analyses were considerably heterogeneous with regard to study design making it difficult to draw definitive conclusions (Mehanna *et al.*, 2009; Shariff and Zavras, 2015).

Although malignant transformation may not be the most frequently encountered outcome, the fact that there is no precise way to predict which OPMD will undergo malignant transformation underlies the principle that all patients with OPMD should be on long-term follow up regardless of the type of OPMD as well as the treatment received (Barnes *et al.*, 2005; Napier and Speight, 2008; van der Waal, 2014; Warnakulasuriya and Ariyawardana, 2016; El-Naggar *et al.*, 2017).

1.3.5 Management

Currently, there is no consensus for the management of OPMD with treatment approaches differing between centres and even different specialists in a single centre (Brennan *et al.*, 2007; Mehanna *et al.*, 2009; van der Waal, 2009; Nankivell and Mehanna, 2011; Arnaoutakis *et al.*, 2013; Balasundaram *et al.*, 2014; Thomson *et al.*, 2017a). Treatment can be divided into either surgical or non-surgical. For surgical treatment, excision is most often based on the severity of dysplasia (if present) despite not having much clinical evidence. Some surgeons prefer using carbon dioxide (CO_2) LASER to excise and/or ablate OPMD (Thomson and Wylie, 2002). The evidence for surgical intervention in preventing malignant transformation is rather weak with most studies being retrospective or observational in nature and some studies reporting no significant advantage of surgical intervention over non-surgical management (Einhorn and Wersall, 1967; Vedtofte *et al.*, 1987; Chiesa *et al.*, 1993; Schepman *et al.*, 1998; Thomson and Wylie, 2002; Holmstrup *et al.*, 2006; van der Waal, 2009; Nankivell and Mehanna, 2011; Diajil *et al.*, 2013; Balasundaram *et al.*, 2014; Lodi *et al.*, 2016; Thomson *et al.*, 2017a).

A recent meta-analysis on OED composed mostly of level II to III evidence revealed that a lower number of malignant transformations were seen in cases treated with surgical excision (5.4%) compared to cases not treated surgically (14.6%) (Mehanna *et al.*, 2009). However, surgical treatment may not always be feasible due to the anatomical location and extent of the lesion in the oral cavity.

Non-surgical modalities include modification of risk factors such as tobacco cessation, elimination of other possible causes, topical/systemic retinoids and photodynamic therapy (van der Waal, 2009; Ribeiro *et al.*, 2010; van der Waal, 2010; Kumar *et al.*, 2013; Villa and Woo, 2017). Many clinicians also tend to follow up these patients on a regular basis (at least 6 monthly) for the rest of their lives. Several

research groups have also undertaken clinical trials using anti-inflammatory agents and molecular targeted therapy as chemo-preventive measures for the management of OPMD, however none have proven to be very successful (Mulshine *et al.*, 2004; Papadimitrakopoulou *et al.*, 2008; William *et al.*, 2016). Regardless of the treatment modality employed, continued follow-up and active surveillance is advisable due to the phenomenon of "field change" and because no single treatment is completely effective in eliminating the chance of malignant transformation in these patients (Holmstrup *et al.*, 2006; Brennan *et al.*, 2007; Napier and Speight, 2008; Mehanna *et al.*, 2009; van der Waal, 2009; van der Waal, 2010).

1.3.6 Prognostic factors for clinical outcome

Several demographic and clinical factors (Table 1.3) have been associated with a higher risk of malignant transformation in OPMDs (van der Waal, 2009; Warnakulasuriya *et al.*, 2011; Speight *et al.*, 2018). Studies have shown that there is an increased risk of malignant transformation in older patients which may be due to the longer period of exposure to risk factors and increased opportunity for cumulative genetic/molecular damage predisposing to carcinogenesis (Banoczy and Sugar, 1972; Mehta *et al.*, 1972; Schepman *et al.*, 1998; Napier and Speight, 2008; Warnakulasuriya *et al.*, 2011). The literature appears to be divided when it comes to gender differences as some studies have shown that malignant transformation occurs more frequently in women (Silverman *et al.*, 1984; Schepman *et al.*, 1998; Cowan *et al.*, 2001; Amagasa *et al.*, 2006), others show a male preponderance (Gupta *et al.*, 1980; Hsue *et al.*, 2007; Warnakulasuriya *et al.*, 2011; Goodson *et al.*, 2015) and a number of studies showed no obvious gender difference when it comes to clinical outcome (Einhorn and Wersall, 1967; Arduino *et al.*, 2009; Ho *et al.*, 2012; Bates *et al.*, 2016).

Certain anatomical sub-sites are thought to have a higher risk of malignant transformation compared to other oral sub-sites with two recent systematic reviews highlighting the fact that tongue lesions have a higher risk than other oral sub-sites (Banoczy, 1977; Silverman *et al.*, 1984; Mehanna *et al.*, 2009; Warnakulasuriya and Ariyawardana, 2016). However, several studies have shown that oral sub-sites are not statistically significantly associated with malignant transformation (Schepman *et al.*, 1998; Holmstrup *et al.*, 2006; Liu *et al.*, 2011; Brouns *et al.*, 2014). The size of OPMD lesions is also believed to have some degree of relationship to malignant

transformation with larger lesions having a higher risk than smaller lesions (Napier *et al.*, 2003; Ho *et al.*, 2012; Brouns *et al.*, 2014).

One of the most commonly studied prognostic features of malignant transformation is the presence and degree of OED. Some studies have suggested that dysplastic lesions have a higher transformation rate when compared to non-dysplastic lesions and several studies have also evaluated the risk of malignant transformation with the degree of OED suggesting that the grade of dysplasia is somewhat prognostic of malignant change (Mincer *et al.*, 1972; Banoczy and Csiba, 1976; Pindborg *et al.*, 1977; Silverman *et al.*, 1984; Lumerman *et al.*, 1995; Lee *et al.*, 2000; Cowan *et al.*, 2001; Mehanna *et al.*, 2009; Warnakulasuriya *et al.*, 2011; Sperandio *et al.*, 2013). However, studies by Dost *et al* (2014) and Holmstrup *et al* (2006) found no statistically significant relationship between degree of OED and malignant change indicating that the current grading system for OED lacks clinical utility as a predictor of malignant change (Holmstrup *et al.*, 2006; Dost *et al.*, 2014).

Consequently, to compensate for the limitations of histopathological features in predicting malignant change, biomarkers have been sought based on an improved understanding of the underlying molecular pathogenesis of OSCC. Biomarkers are molecular, biochemical or genetic features that can be assessed to identify presence or progress of pathological processes. Numerous studies have assessed the prognostic ability of various biomarkers in OPMD and OED, however none have proved to be particularly useful in clinical practice (Reibel, 2003; Pitiyage *et al.*, 2009; Smith *et al.*, 2009; Lingen *et al.*, 2011; Nankivell and Mehanna, 2011; Nikitakis *et al.*, 2018; Speight *et al.*, 2018). Discovering a biomarker that is altered in OPMD and indicative of the early stages of oral carcinogenesis as well as being quantifiable in small incisional biopsies is highly desirable to affect appropriate early intervention. This may pave the way for more personalized management protocols for individual patients with OPMD underpinning improved clinical outcomes. A list of promising prognostic biomarkers is shown in Table 1.4.

Two of the more promising biomarkers for OPMDs are loss of heterozygosity (LOH) and DNA ploidy analysis. Loss of heterozygosity (LOH) refers to allelic loss from one chromosomal locus in a chromosomal pair and can be identified by assessing DNA polymorphism patterns between normal and abnormal tissue (Scully *et al.*, 2000a; Beder *et al.*, 2003; Kasamatsu *et al.*, 2011). LOH at chromosomal regions 3p14,

9p21 and 17p13 have been shown by some studies to be a useful adjunct to predict malignant transformation of OPMD (Califano *et al.*, 1996; Mao *et al.*, 1996; Rosin *et al.*, 2000; Zhang and Rosin, 2001; Zhang *et al.*, 2012). A systematic review however highlighted the fact that many of these studies have a small sample size, were conducted in single centres and most are retrospective in nature (Smith *et al.*, 2009). Interestingly, a recently concluded randomised clinical trial showed that LOH profiling was useful as a prognostic marker of oral cancer risk in patients with OPMDs (William *et al.*, 2016) . The increasing body of evidence supporting the use of LOH has prompted the WHO to recommend LOH as an adjunctive/complimentary biomarker to be used in conjunction with traditional methods for assessment of OPMDs, however there is currently insufficient evidence to recommend LOH as a sole prognostic biomarker for OPMDs (El-Naggar *et al.*, 2017).

Irregular nuclear DNA content is a feature of malignant cells and their precursors (Sen, 2000; Danielsen *et al.*, 2016). Aneuploidy can be detected in tumours at a very early stage and is considered to be a marker of genomic instability, an enabling characteristic for tumour development and progression (Pihan and Doxsey, 1999; Duesberg *et al.*, 2000; Leemans *et al.*, 2011; Danielsen *et al.*, 2016). Several studies have shown that DNA ploidy can be a useful tool in identifying malignant change in OPMD (Klanrit *et al.*, 2007; Torres-Rendon *et al.*, 2009b; Bradley *et al.*, 2010; Bremmer *et al.*, 2011; Sperandio *et al.*, 2013). The study by Sperandio *et al* (2013) demonstrated that image-based DNA ploidy analysis worked as well as conventional dysplasia grading in predicting clinical outcome and combining both OED grading and DNA ploidy status gave a slightly better prognostic value (Sperandio *et al.*, 2013). An even more recent study showed that DNA ploidy analysis has some value in prognosticating clinical outcome in oral lichen planus, a non-dysplastic OPMD (Sperandio *et al.*, 2016). However, gross alterations of DNA content are not demonstrable in all cases of malignant change and this subset of patients may have a different oncogenic pathway or profile (Bradley *et al.*, 2010; Bremmer *et al.*, 2011; Sperandio *et al.*, 2013; Danielsen *et al.*, 2016).

Tumour suppressor genes have also been studied as potential biomarkers with many studies focusing on *TP53* that encodes for the transcription factor p53 (Opitz *et al.*, 2001; Gasco and Crook, 2003; Angiero *et al.*, 2008; Pitiyage *et al.*, 2009; Smith *et al.*, 2009; Denaro *et al.*, 2011; Lingen *et al.*, 2011; Nasser *et al.*, 2011; de Oliveira *et al.*,

2012). Although extensively studied, the prognostic utility of p53 protein expression in malignant transformation of OPMD is poor as shown in a recent systematic review on biomarkers in OED (Smith *et al.*, 2009). In the review, the pooled relative risk for malignant transformation amongst OED in p53 positive cases was calculated to be 0.96 (CI 0.65, 1.42) (Smith *et al.*, 2009). *MDM2* a gene that has a regulatory role with regard to *TP53* has also been studied in relation to head and neck cancers with overexpression of *MDM2* protein being seen in some OPMDs suggesting a role for *MDM2* in oral carcinogenesis (Agarwal *et al.*, 1999; Lothaire *et al.*, 2006; Perez-Ordonez *et al.*, 2006; Denaro *et al.*, 2011).

The roles of two other members of the p53 family of transcription factors, p63 and p73, in oral carcinogenesis and OPMD have also been investigated though not as extensively as p53. ΔNp63 has been reported to be overexpressed in OPMDs that transformed to OSCC by some studies (Chen *et al.*, 2005; Saintigny *et al.*, 2009; Matsubara *et al.*, 2011; Varun *et al.*, 2014). However, the study by Bortoluzzi et al (2004) could not demonstrate any obvious relationship between OED grading and p63 staining patterns (Bortoluzzi *et al.*, 2004). Chen et al (2004) attempted to assess the relationship between p73 expression in OED of the buccal mucosa and OSCC and found that there was increased suprabasal expression in dysplastic epithelium compared to normal (Chen *et al.*, 2004). However, the association with malignant transformation was inconclusive (Chen *et al.*, 2004). The incremental expression pattern of p63 and p73 described by Choi et al (2002) in head & neck squamous carcinoma led the authors to suggest that p63 and p73 may play a role in carcinogenesis, however this has yet to be convincingly replicated in studies on OSCC/OPMD (Choi *et al.*, 2002). The relative paucity of studies regarding the usefulness of ΔNp63 and p73 as potential biomarkers for malignant change in OPMD necessitate the need for more research to determine their utility as prognostic markers of MT (Smith *et al.*, 2009; Lingen *et al.*, 2011; Nankivell and Mehanna, 2011).

The *CDKN2A* gene which encodes the tumour suppressor protein p16 has also been studied in OSCC and OPMD. p16 is involved in cell cycle regulation through inhibition of the formation of the cyclin D and CDK4/6 complex which phosphorylates the RB protein. Though studied extensively, the prognostic value of *CDKN2A* and p16 in OPMD for clinical outcome is inconclusive (Reed *et al.*, 1996; Kresty *et al.*,

2002; Angiero *et al.*, 2008; Lingen *et al.*, 2011; Nasser *et al.*, 2011; Nankivell *et al.*, 2014). The recognition of HPV-associated OED further complicates assessment of p16 as a prognostic biomarker for clinical outcome in OPMD as the natural history of HPV-associated OED is as yet unclear (McCord *et al.*, 2013; Woo *et al.*, 2013; El-Naggar *et al.*, 2017; Lerman *et al.*, 2017).

Biomarkers associated with cellular proliferation and cycle such as proliferating cell nuclear antigen (PCNA), Ki-67, minichromosome maintenance protein 2 (MCM2), and cyclin D1 (CCND1) have also been studied in relation to their role in oral carcinogenesis (Kodani *et al.*, 2001; Rousseau *et al.*, 2001; Shintani *et al.*, 2002; Turatti *et al.*, 2005; Kovesi and Szende, 2006; Pitiyage *et al.*, 2009; Smith *et al.*, 2009; Torres-Rendon *et al.*, 2009a; Lingen *et al.*, 2011; Poh *et al.*, 2012). Although most studies agree that there is a correlation between dysplasia grade and Ki-67 expression, the association between its expression and malignant transformation is not very clear (Kodani *et al.*, 2001; Bortoluzzi *et al.*, 2004; Takeda *et al.*, 2006; Pitiyage *et al.*, 2009; Torres-Rendon *et al.*, 2009a; Vered *et al.*, 2009; Lingen *et al.*, 2011). As expected of a proliferation marker, reactive lesions showed an almost similar proliferation index to dysplastic tissue (Takeda *et al.*, 2006; Pitiyage *et al.*, 2009; Vered *et al.*, 2009; Lingen *et al.*, 2011). Expression of CCND1 has been shown to be increased in both dysplasia and OSCC, however, similar to Ki-67, its utility as a prognostic marker of MT is not very clear (Rousseau *et al.*, 2001; Turatti *et al.*, 2005; Kovesi and Szende, 2006). Both PCNA and MCM2 have been shown to have some association with progressive changes in epithelial tissue from normal to dysplasia to malignancy (Kodani *et al.*, 2001; Shintani *et al.*, 2002; Torres-Rendon *et al.*, 2009a; Lingen *et al.*, 2011), however due to the limited evidence and obvious overlap with proliferative/reactive lesions, the role of proliferation markers would need further assessment before refuting or supporting their utility as prognostic biomarkers.

Another widely studied biomarker is the epidermal growth factor receptor (*EGFR*). *EGFR* has roles in cellular proliferation, apoptosis, angiogenesis, invasion and metastasis. *EGFR* is known to have many downstream carcinogenesis associated signalling targets and increased *EGFR* expression is seen in many OSCC with its expression correlating to poorer outcome (Nicholson *et al.*, 2001; Ciardiello and Tortora, 2003; Kalyankrishna and Grandis, 2006; Normanno *et al.*, 2006; Taoudi Benchekroun *et al.*, 2010). *EGFR* may also play a role in OPMDs that undergo

malignant change (Nagatsuka *et al.*, 2001; Taoudi Benchekroun *et al.*, 2010; Bates *et al.*, 2016). Nagatsuka *et al* (2001) reported the amplification of *EGFR* in OED that appeared to increase with the grade of dysplasia and in a more recent study, OPMD with irregular *EGFR* gene copy number had a higher tendency to undergo malignant transformation (Nagatsuka *et al.*, 2001; Bates *et al.*, 2016). Although these results suggest that *EGFR* gene copy number gain and amplification may be associated with malignant transformation in OPMD, large prospective studies are required to verify these findings (Grandis and Tweardy, 1993; Pitiyage *et al.*, 2009; Smith *et al.*, 2009; Taoudi Benchekroun *et al.*, 2010; Lingen *et al.*, 2011; Nankivell and Mehanna, 2011; Poh *et al.*, 2012; Bates *et al.*, 2016).

The association between OPMDs and matrix metalloproteinases (MMPs) specifically MMP1, MMP2 and MMP9 have been investigated by several groups (Sutinen *et al.*, 1998; Jordan *et al.*, 2004; Chen *et al.*, 2008b; de Carvalho Fraga *et al.*, 2014; Chandolia *et al.*, 2016). MMP9 was found to be the more promising biomarker amongst the MMPs and has been shown to have altered expression between normal tissue and OPMDs as well as some association with OPMDs that undergo MT (Jordan *et al.*, 2004; Chen *et al.*, 2008b; Chandolia *et al.*, 2016; Venugopal and Uma Maheswari, 2016). The role of MMPs in MT of OPMD is however still not confirmed due to the relative scarcity of studies examining this association.

Podoplanin, a transmembrane glycoprotein that is a stem cell marker, has been studied as a potential prognostic biomarker in OED. Podoplanin overexpression has been shown to be associated with lymphatic metastasis and poorer outcome in HNSCC (Yuan *et al.*, 2006). It has also been shown that podoplanin in expressed in OED and may be of value as a prognostic indicator of malignant change in OPMD (Kawaguchi *et al.*, 2008; Inoue *et al.*, 2012; Kreppel *et al.*, 2012; Swain *et al.*, 2014). Kawaguchi *et al* (2008) demonstrated that podoplanin expression was a statistically independent factor in predicting malignant change in OPMD (Kawaguchi *et al.*, 2008). But yet again, these findings have yet to be replicated in larger cohorts.

An inhibitor of apoptosis, survivin, has recently emerged as a protein of interest in OSCC with a recent meta-analysis concluding that expression of survivin has prognostic value in OSCC (Xie *et al.*, 2015). Although there is limited data on its role in MT of OPMD, several studies have indicated that an overexpression of survivin is associated with MT in OPMD and OED (Lo Muzio *et al.*, 2003; Tanaka *et al.*, 2003;

Zhou *et al.*, 2010). A protease, cathepsin L, was shown to be overexpressed in oral carcinomas using gene expression profiling by Alevizos et al (2001) (Alevizos *et al.*, 2001). Assessment of both mRNA and protein levels of cathepsin L by Macabeo-Ong et al (2003) however showed no statistically significant difference between OED that underwent MT and those that did not (Macabeo-Ong *et al.*, 2003). There is very scarce evidence in the literature to refute or support the prognostic utility of cathepsin L in OPMDs.

The relatively recent discovery of non-coding RNAs (ncRNA) have added another paradigm to the altered gene regulation process in carcinogenesis. There is some evidence that non-coding RNA are associated with development and progression of malignant neoplasms (Guttman *et al.*, 2009; Li *et al.*, 2013; Lv *et al.*, 2013; Wu *et al.*, 2014; Conway *et al.*, 2015). The two members of the ncRNA family that have been studied most extensively with regard to carcinogenesis are microRNA (miRNA) and long non-coding RNA (lncRNA).

MicroRNA are small (18-22 nucleotide in length) non-coding molecules and to date, more than one thousand miRNA have been identified (Ambros, 2004; Griffiths-Jones, 2004). In carcinogenesis, studies have shown that miRNAs can behave as oncogenes or tumour suppressor genes and exert their effects by affecting the translation of the target mRNA (Calin and Croce, 2006; Esquela-Kerscher and Slack, 2006; Osada and Takahashi, 2007). In a recent review on epigenetics in oral cancer, D'Souza and Saranath (2015) discussed the deregulated expression of miRNA in oral cancers and highlighted several miRNA that may be regulators of invasion and metastasis in OSCC (D'Souza and Saranath, 2015). A study by Cervigne et al (2009) showed that the overall miRNA expression profiles could distinguish progressing and non-progressing oral leukoplakia (Cervigne *et al.*, 2009). MiR-31 has been recently shown to be upregulated in OPMDs compared to normal oral mucosa and OPMDs that underwent malignant transformation had a further increased expression of miR-31 (Hung *et al.*, 2014; Hung *et al.*, 2016). Research investigating the possibility of using miRNA to predict malignant transformation in OPMD has led to several groups creating panels using miRNA expression to improve prediction of malignant transformation in OPMD (Philipone *et al.*, 2016; Chang *et al.*, 2018). However, a recent systematic review concluded that there is currently insufficient evidence to

validate the utility of miRNA expression profile as a prognostic marker of malignant transformation in OPMDs (El-Sakka *et al.*, 2018).

Long non-coding RNAs (lncRNA) have recently emerged as possible biomarkers in carcinogenesis. LncRNA have been shown to be dysregulated during carcinogenesis and metastasis (Lv *et al.*, 2013; Wu *et al.*, 2014; Zhang *et al.*, 2014; Conway *et al.*, 2015). Conway *et al* (2015) highlighted several anti-sense HOX transcripts as being differentially expressed between “normal” and tumour samples as well as between “normal” and dysplastic samples (Conway *et al.*, 2015). However, at this point in time there is very limited data available concerning its role in the development and malignant transformation of OPMD.

Numerous other biomarkers have been studied with little progress being made. Though promising initially, most biomarkers have yet to be validated as prognostic indicators of malignant change in OPMD with LOH and DNA ploidy analysis being the two most promising biomarkers thus far (Pitiyage *et al.*, 2009; Smith *et al.*, 2009; Lingen *et al.*, 2011; Zhang *et al.*, 2012; Sperandio *et al.*, 2013; Nikitakis *et al.*, 2018; Speight *et al.*, 2018). With the advent of next generation sequencing techniques, newer biomarkers are being discovered and studied.

Despite the extensive research that has gone into the discovery and validation of prognostic biomarkers, the standard and quality of reporting of such studies have been found to be lacking in detail (McShane *et al.*, 2005; Sauerbrei *et al.*, 2018). To address this issue, a set of recommendations for reporting such findings was developed; the “Reporting Recommendations for Tumour Marker Prognostic Studies (REMARK)” (McShane *et al.*, 2005). This set of recommendations is composed of 20 suggestions that would improve the standard of reporting biomarker-related studies (Appendix A) (McShane *et al.*, 2005). These recommendations however, are not only suitable for tumour marker studies but can be extended to any biomarker study in the biomedical field (McShane *et al.*, 2005; Sauerbrei *et al.*, 2018). Although the recommendations made are for reporting study findings, a more robust study can be designed by taking into consideration these recommendations during the early stages of a research.

1.4 Differential Gene Expression Profiling

From a developmental point of view, cells are thought to differentiate through differential gene expression (DGE). As such, by studying DGE between normal and abnormal tissue, in-depth understanding of the genetic pathways involved in carcinogenesis can be elucidated. Studies based on DGE have allowed researchers to dissect and examine the cancer transcriptome in a way that was not possible using conventional molecular biological methods (Perou *et al.*, 2000; Sorlie *et al.*, 2001; Sorlie *et al.*, 2003; Molinolo *et al.*, 2009; Conway *et al.*, 2015; De Cecco *et al.*, 2015; Wood *et al.*, 2015; Zhu *et al.*, 2015; Makarev *et al.*, 2017; Wood *et al.*, 2017).

Rapid advances in genomic laboratory techniques and bioinformatic analysis have enabled the analysis of thousands of expressed genes simultaneously in various tissues providing a unique way to identify molecules with varying expression between normal, dysplastic and malignant tissue. Such studies may also help improve our understanding of the complex nature of malignancies (Perou *et al.*, 2000; Sorlie *et al.*, 2001; Sorlie *et al.*, 2003; Carey *et al.*, 2015; De Cecco *et al.*, 2015). DGE has also contributed to the paradigm shift away from single biomarkers for diagnosis or prognosis (Perou *et al.*, 2000; Sorlie *et al.*, 2001; Sorlie *et al.*, 2003). Rather than depending on a single prognostic variable, combining several variables to construct a prognostic model seems more robust (Harrell *et al.*, 1996; Royston *et al.*, 2009; Steyerberg *et al.*, 2013; Moons *et al.*, 2015). One way to add strength to conventional risk-stratification methods is by integrating or combining gene-signatures with clinico-pathologic parameters to add a further dimension in enhancing individualised patient care (Saintigny *et al.*, 2011; Sperandio *et al.*, 2013).

The ability to identify patient clusters with similar molecular patterns in different tumour types have enabled researchers to define new molecular cancer sub-types enhancing better targeted therapy and patient care. The paradigm is breast cancer where at least five molecular sub-types with prognostic correlation were discovered (Perou *et al.*, 2000; Sorlie *et al.*, 2001; Sorlie *et al.*, 2003; Hu *et al.*, 2006). The findings were then further refined and validated resulting in the predictive PAM50 gene signature (Hu *et al.*, 2006; Parker *et al.*, 2009).

The lack of prognostic biomarkers in OPMD is a cogent reason to perform DGE based studies to identify gene-signatures for early diagnosis, therapy or prognosis in

OPMD to inform targeted therapy (Chung *et al.*, 2004; Chen *et al.*, 2008a; Saintigny *et al.*, 2011; van Hooff *et al.*, 2012; Abdulmajeed and Farah, 2013; Sumino *et al.*, 2013). A recent meta-analysis performed by De Cecco et al. (2015) demonstrated the usefulness of DGE studies in stratifying HNSCC into six sub-types characterized by their respective clinico-pathological features and dysregulation of relevant signalling pathways (De Cecco *et al.*, 2015).

There are only a few DGE studies on OPMD/OED (Chen *et al.*, 2008a; Saintigny *et al.*, 2011; Conway *et al.*, 2015; Zhu *et al.*, 2015). As yet, only one truly investigated DGE between OPMD that transformed to OSCC and those that did not (Saintigny *et al.*, 2011). Saintigny et al (2011) proposed gene expression-based prediction models that showed superior prognostic accuracy when compared to models using clinico-pathologic risk factors (Saintigny *et al.*, 2011). Though highly interesting, a major limitation of this study that may have influenced the prognostic gene-signature was that all the patients were enrolled in a clinical chemo-prevention trial, which may have influenced the outcome of the OPMD as well as the gene expression. As such, further studies in DGE between OPMD that undergo malignant transformation versus those that do not would provide much needed insight into the molecular mechanisms that translate into malignant transformation in OPMDs.

Whole transcriptome sequencing methods such as total RNA sequencing (RNASeq) provide a method to detect both coding and non-coding RNA. This enables investigators to measure transcript abundance and identify both known and novel genes that are differentially expressed between malignant transforming and non-transforming OPMDs.

Formalin-fixed paraffin-embedded (FFPE) tissue are an invaluable resource for molecular biology research as they are linked to various patient and disease related information. Though FFPE may contain ample genetic material, formalin fixation is known to adversely affect the quality of nucleic acids hampering downstream applications such as gene expression microarrays (von Ahlfen *et al.*, 2007). A relatively new gene expression profiling system that relies on direct measurement of transcripts using colour coded probe-based technology, the NanoString nCounter platform (NanoString Technologies, Seattle, USA), has been able to provide accurate gene expression data using RNA obtained from FFPE material (Geiss *et al.*, 2008; Reis *et al.*, 2011).

Recent studies have shown that mRNA expression analysis using the NanoString platform were equivalent to that achieved through quantitative real-time polymerase chain reaction (qPCR) and possibly superior to microarrays (Geiss *et al.*, 2008; Reis *et al.*, 2011; Balko *et al.*, 2012; Veldman-Jones *et al.*, 2015a; Veldman-Jones *et al.*, 2015b). There are several advantages of NanoString compared to PCR-based techniques: NanoString is hybridisation-based which means that there is no reverse transcription, no amplification and no enzymes involved. This reduces handling of the sample, minimizing errors and amplification bias. An additional advantage of this platform is that the mRNA level is determined for every individual sample and normalised against "housekeeping/internal reference" genes within the same sample (Geiss *et al.*, 2008; Reis *et al.*, 2011; Veldman-Jones *et al.*, 2015a). NanoString allows detection of up to 800 targets (genes or regions) in a single reaction. Recently a genomic assay based on the PAM50 gene-signature for assessing long-term risk of breast cancer recurrence using NanoString technology, Prosigna (NanoString Technologies, Seattle, USA), has been approved for use in the UK by the National Institute for Health and Care Excellence (NICE) (National Institute for Health and Care Excellence, 2015b). NanoString technology has also been used successfully by a group studying molecular classification of B-Cell lymphomas (Carey *et al.*, 2015).

1.5 Multivariate Prognostic Models

Prognostic models are composed of two or more variables (or determinants) which convert observed values into prognostic scores by assigning relative weightage to each factor (Moons *et al.*, 2009; Steyerberg, 2009). In a healthcare setting, such models are constructed to aid in diagnosing disease or to predict whether a specific event will occur in the future. From a prognostic aspect, such models are useful when planning interventions or treatments based on a specific risk of developing a particular condition (Moons *et al.*, 2009; Steyerberg, 2009). Risk estimates for treatment planning are almost never based on a single predictor as clinicians will invariably integrate patient parameters and other predictive/prognostic markers (Steyerberg *et al.*, 2013). Some well-known predictive and prognostic models include the Nottingham Prognostic Index for breast cancers (Haybittle *et al.*, 1982), Ottawa Ankle Rules for ankle/foot fractures (Stiell *et al.*, 1992), Framingham Risk Score for cardiovascular disease risk (Anderson *et al.*, 1991), PREDICT for prognosis following surgery for breast cancer (Wishart *et al.*, 2010) and EuroSCORE for risk of mortality

following cardiac surgery (Nashef *et al.*, 1999). To be of clinical use as a prognostic tool in personalised medicine, prognostic models need to be converted into a statistically relevant numerical value.

Although such prognostic models are invaluable, developing, validating and reporting multivariate prognostic models can be challenging. The quality of reports on prediction models have been critically reviewed and evaluated several times with most reviews concluding that the quality of reporting is poor with inadequate detail being provided regarding all areas of model development and validation (Mallett *et al.*, 2010; Collins *et al.*, 2011; Bouwmeester *et al.*, 2012; Collins *et al.*, 2013; Collins *et al.*, 2014; Moons *et al.*, 2014). Appropriate assessment of prediction models can only be performed if reports provide clear and sufficient information on all aspects of the model construction and validation.

To overcome this problem, a guideline for reporting studies developing and/or validating multivariate prediction/prognostic models, the "Transparent Reporting of a multivariable prediction model for Individual Prognosis or Diagnosis (TRIPOD) Statement" was developed recently and published in several medical journals simultaneously (Collins *et al.*, 2015; Moons *et al.*, 2015). The TRIPOD statement is composed of a 22-item checklist (Appendix B) that covers a minimum set of recommendations focusing on how the study was designed, performed, analysed and interpreted (Collins *et al.*, 2015; Moons *et al.*, 2015).

Despite the global health burden and relatively poor prognosis associated with OSCC, a robust prognostic biomarker or prognostic model for predicting malignant transformation in OPMD has yet to be developed and validated. The clinical, histopathological and genetic heterogeneity of OPMD and OSCC are major contributing factors to this problem. DGE provides a suitable method to explore and discover novel biomarkers as well as genetic signatures that may be useful not only as prognostic indicators of disease outcome but also in furthering our understanding of the pathogenesis of OPMD and OSCC. Instead of focusing on single prognostic biomarkers, models made up of several factors may be more suitable in providing personalised assessment/prognostication for patients with OPMDs (Moons *et al.*, 2009; Royston *et al.*, 2009; Collins *et al.*, 2011; Steyerberg *et al.*, 2013; Moons *et al.*, 2015).

1.6 Aims

This study will test the hypothesis that a multi-parameter biomarker driven prognostic classifier for OPMD is better than conventional prognostic methods that are based only on clinical and histopathological findings. The aims of this study are:

1. To identify two separate cohorts (training and validation cohorts) of OPMD patients with known clinical outcomes and adequate archived FFPE tissue for relevant tests.
2. To compare efficacy of two different OED grading systems as prognostic indicators for clinical outcome in OPMDs.
3. To assess the utility of two molecular techniques (DNA ploidy analysis and LOH analysis) as prognostic indicators for clinical outcome in OPMD using archived FFPE tissue.
4. To identify differentially expressed genes between OPMD that undergo malignant transformation and OPMD that do not undergo malignant transformation using archived FFPE tissue.
5. To discover a gene expression signature that characterises OPMDs with a high risk of undergoing malignant transformation.
6. To develop a composite clinical, histopathological and molecular prognostic classifier for patients with OPMD.
7. To validate the developed classifier on an external cohort of OPMD patients with known clinical outcomes (validation cohort).

Table 1.1 List of Oral Potentially Malignant Disorders (OPMD).

OPMD
Leukoplakia
Erythroplakia
Erythroleukoplakia
Proliferative verrucous leukoplakia
Oral submucous fibrosis
Oral lichen planus
Palatal lesions in reverse smoking
Actinic cheilitis
Dyskeratosis congenita
Fanconi anaemia
Sideropenic dysphagia
Discoid lupus erythematosus
Cheilitis glandularis
Syphilis
Chronic hyperplastic candidosis

*Adapted from Barnes et al (2005) and El-Naggar et al (2017).

Table 1.2 Histological features of Oral Epithelial Dysplasia.

Cytological changes	Architectural changes
Abnormal variation in nuclear size	Irregular epithelial stratification
Abnormal variation in nuclear shape	Loss of polarity of basal cells
Abnormal variation in cell size	Drop shaped rete ridges
Abnormal variation in cell shape	Increased number of mitotic figures
Increased nuclear to cytoplasmic ratio	Abnormally superficial mitotic figures
Atypical mitotic figures	Premature keratinization of single cells
Increased number and size of nucleoli	Loss of epithelial cell cohesion
Hyperchromasia	Keratin pearls in rete processes

*Adapted from Barnes et al (2005) and El-Naggar et al (2017).

Table 1.3 Demographic and clinical risk factors associated with increased risk of malignant transformation in OPMD.

Demographic risk factor	Clinical risk factor
Age (> 50 years)	Clinical appearance (homogenous vs non-homogenous, leukoplakia vs erythroplakia, etc)
Sex (female)	Anatomical sub-site (tongue and floor of mouth)
Risk habits (tobacco usage, betel quid usage, alcohol consumption, etc)	Size of lesion (> 200mm^2)

*Adapted from Speight et al (2018).

Table 1.4 Promising prognostic biomarkers for malignant transformation in OPMD.

Marker	RR	HR	OR	95% CI	*P* value	Relevant studies
LOH 3p +/- 9p	3.92	NA	NA	1.50 - 10.25	0.006	(Rosin *et al.*, 2000; Zhou *et al.*, 2005)
DNA ploidy	3.90	NA	NA	1.30 - 11.62	0.01	(Torres-Rendon et al., 2009)
Survivin	30.00	NA	NA	4.25 - 197.73	< 0.001	(Lo Muzio et al., 2003)
MMP9 mRNA	19.00	NA	NA	1.56 – 209.38	0.02	(Jordan, 2004)
Podoplanin	NA	3.09	NA	1.53 - 6.23	0.02	(Kawaguchi et al., 2008)
ΔNp63	NA	3.31	NA	1.66 - 6.58	0.0007	(Saintigny et al., 2009)
EGFR copy number	NA	3.62	NA	1.44 - 9.10	0.006	(Taoudi Benchekroun et al., 2010)

*Adapted from Smith et al (2009) and Nankivell and Mehanna (2011).

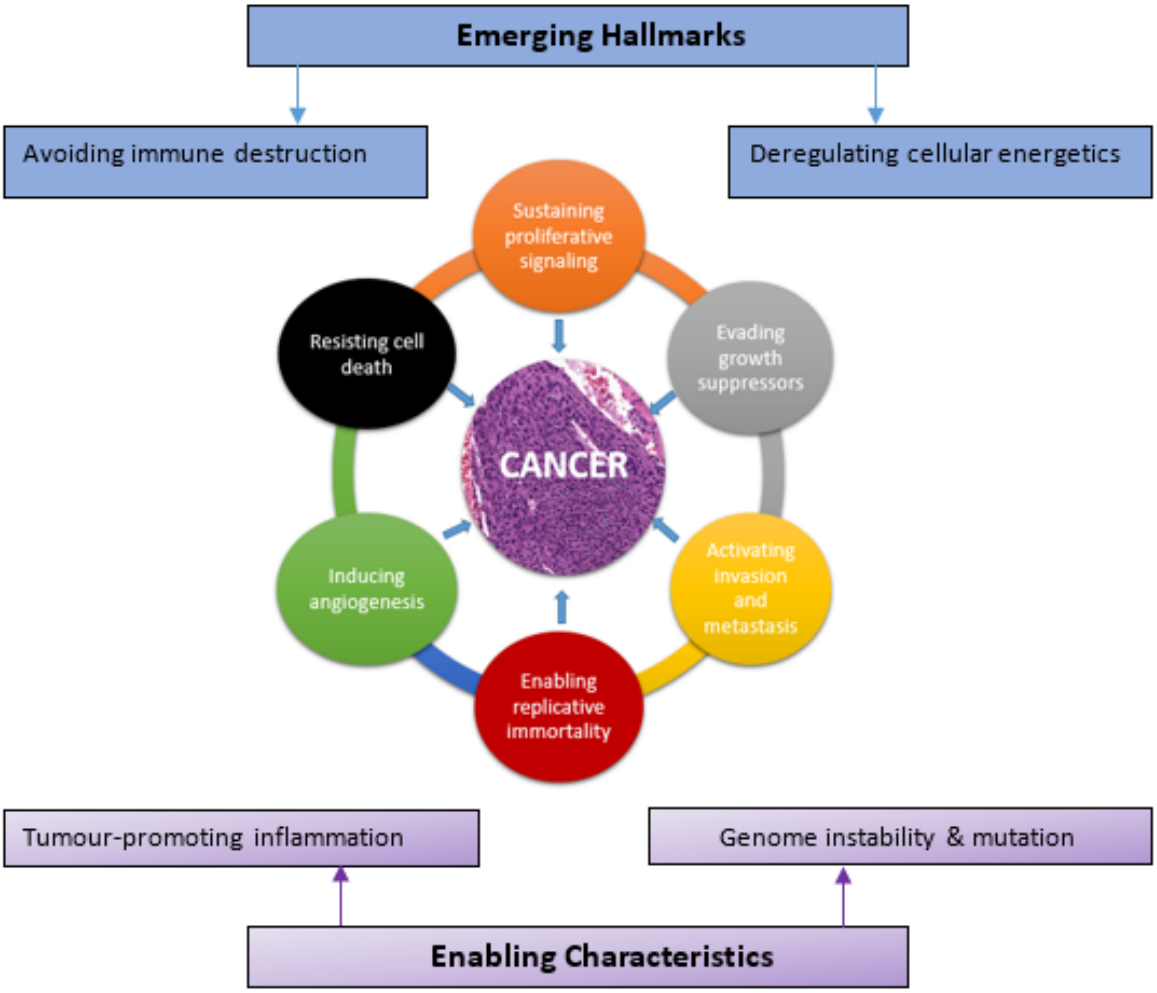


Figure 1.1 The hallmarks of cancer comprising six capabilities acquired by neoplastic cells during carcinogenesis.

Two new emerging hallmarks; deregulating cellular energetics and avoiding immune destruction are also included. Genomic instability & mutation and tumour-promoting inflammation are two factors which have been considered as enabling characteristics for cancer development. Adapted from Hanahan & Weinberg (2011) (Hanahan and Weinberg, 2000; Hanahan and Weinberg, 2011).

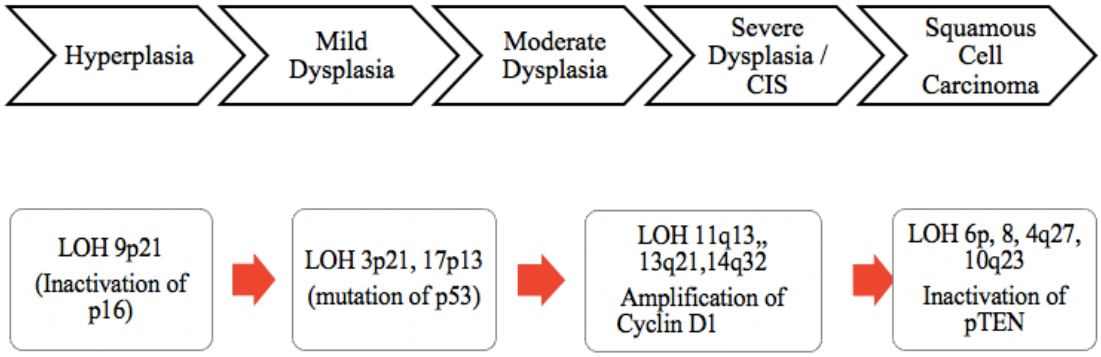


Figure 1.2 Multistep genetic progression model for oral carcinogenesis.

LOH: Loss of heterozygosity. Adapted from Califano et al (1996) and Forastiere et al (2001).

Chapter 2. Materials and Methods

2.1 Case Selection

Suitable patients for the "training cohort" were identified from a pre-existing oral potentially malignant disorder (OPMD) database. All the patients were diagnosed with OPMD between 1st August 1996 and 31st December 2015, had a diagnostic biopsy, were seen at a specialist clinic and have been followed-up for at least 12 months. Demographic and clinico-pathologic features as well as outcome data were recorded.

Patients for the "validation cohort" were identified through a systematic search of the Royal Victoria Infirmary Cellular Pathology Database for OPMD cases originating from Sunderland Royal Hospital diagnosed between 1st January 2001 and 31st December 2015. The search was performed using the Systematised Nomenclature of Medicine-Clinical Terms (SNOMED CT) codes. Demographic and clinico-pathologic features as well as outcome data were obtained from the Oral and Maxillofacial Surgery Department at Sunderland Royal Hospital.

2.1.1 Inclusion and exclusion criteria

The following inclusion and exclusion criteria were applied to the cases found from the initial search:

- Inclusion criteria:
    - Clinical diagnosis of OPMD
    - Clinical follow-up for at least 12 months after initial diagnosis of OPMD
    - FFPE tissue available for analysis
- Exclusion criteria:
    - Previous history of head and neck cancer
    - Less than six months between index biopsy for OPMD and diagnosis of OSCC (the initial biopsy may be a false negative)

- Patients with hereditary/acquired conditions that are linked to an increased risk of head and neck SCC (such as ataxia telangiectasia, xeroderma pigmentosum, Fanconi anaemia etc)
- Patients that were diagnosed as having chronic hyperplastic candidosis/chronic candidosis. Such cases were excluded as the literature is divided as to whether these lesions are truly OPMDs (El-Naggar *et al.*, 2017; Speight *et al.*, 2018)
- Patients with clinical follow up of less than 12 months
- Cases with incomplete/inconsistent records
- Cases with inadequate/damaged/unavailable FFPE tissue for analysis

2.1.2 Clinical outcomes

The cases were categorized into one of two clinical outcomes:

- Malignant transformation (MT) or
- No malignant transformation (NT)

A case was classified as having undergone MT when there was progression from an OPMD to oral squamous cell carcinoma (OSCC) after a period of six months or more from the time of the initial diagnosis of OPMD. Those patients with OPMD who were recorded as not having developed OSCC at their last known follow-up appointment were classified as NT cases with the caveat that the patients were followed up for at least 12 months.

2.1.3 Data management

For each selected case, the following data points were collected and entered into a Microsoft Excel spreadsheet:

- age at first diagnosis of OPMD
  - continuous and
  - dichotomised into $\leq$ 50 or > 50 years of age (Einhorn and Wersall, 1967; Speight *et al.*, 2018)
- sex
- clinical diagnosis of lesion

- site of index OPMD
- clinical management of OPMD
- clinical outcome of OPMD
- date of MT (diagnostic biopsy with OSCC) or last clinical follow-up.

All data were coded, link anonymised and stored in password protected computer files.

2.2 Ethical Approval

This project has favourable ethical opinion ("Evaluation of the prognostic potential and functional significance of biomarkers in oral cancer"; NRES Committee Northeast – Sunderland 11/NE/0118).

2.3 Histopathological Specimens

Archived haematoxylin and eosin (H&E) stained sections of the cases identified from the OPMD database were retrieved and assessed to choose suitable FFPE blocks from each case. Subsequently, selected FFPE blocks were retrieved from the Royal Victoria Infirmary Department of Cellular Pathology archives. Blocks with adequate material for the planned experiments were then selected. In case a FFPE block for a specific case was unavailable or had inadequate tissue, an alternative FFPE block from the case was chosen if available. Serial 4µm sections were taken from the selected FFPE blocks using a Leica RM 2135 microtome (Leica Microsystems, Germany) and individual sections were mounted onto microscope slides (Superfrost Plus, Thermo Fisher Scientific, UK).

All histopathological assessments (oral epithelial dysplasia grading; OPMD associated leukocytic infiltrate scoring; high-risk HPV assessment) were performed using a 3-tiered assessment protocol involving three oral and maxillofacial pathologists; Dr Max Robinson (MR), Hans Sathasivam (HS) and Professor Philip Sloan (PS). The pathologists were blinded to clinical outcome of OPMD patients during the assessment and grading exercise.

2.3.1 Oral epithelial dysplasia (OED) assessment and grading

Following FFPE block selection, 4µm sections were taken from each block and individual sections were mounted onto microscope slides. Haematoxylin & eosin (H&E) staining was performed on the DAKO CoverStainer (Agilent Technologies,

USA). The slides for each of the cases were reviewed, assessed and graded following a modified three-tier system adapted from the work published by Speight *et. al.* (2015).

Briefly, the slides were first graded independently by two oral and maxillofacial pathologists (MR & HS). Following which, if there was discordance in the grade between the two pathologists, the cases were sent to a third pathologist (PS), who assumed the role of an adjudicator and independently graded the case. The majority diagnosis (agreement by two out of three pathologists) was accepted as the final grading. For cases where the grading of the adjudicator was different from both pathologists, a consensus meeting was convened to derive a final grade. The workflow for this process is outlined in Figure 2.1.

The cases were graded using two different grading systems:

I. Three-tiered (mild, moderate or severe) World Health Organization (WHO) 2017 classification (El-Naggar *et al.*, 2017)
II. Binary (low-grade or high-grade) classification (Kujan *et al.*, 2006; El-Naggar *et al.*, 2017)

2.3.2 Assessment of oral potentially malignant disorders (OPMD) associated leukocytes (OPAL)

Subepithelial leukocytic inflammatory infiltrate was assessed and scored for each case in a binary manner depending on whether there was a high or low level of leukocytic infiltrate. An OPMD associated leukocytes (OPAL) score (or OPALS) was given for each case. When there was presence of a well-defined leukocytic infiltrate in the lamina propria, the case was scored as being OPALS positive and if there was either no/very minimal leukocytic infiltrate in the lamina propria, it was scored as OPALS negative. All cases were assessed in a manner similar to the earlier mentioned 3-tiered OED grading method (Figure 2.1).

2.3.3 Assessment for high-risk human papillomavirus (HR-HPV)

Cases chosen for molecular and gene expression experiments were all assessed for the presence of HR-HPV due to the fact that HR-HPV positive cases may have a different aetiopathogenesis and gene expression profile compared to negative cases. A two-tiered testing method was employed:

- Initial screening with p16 staining via immunohistochemistry (IHC)
- High-risk human papillomavirus DNA *in situ* hybridisation (HR-HPV ISH) for cases that were considered to be positive for p16 staining.

Immunohistochemistry for p16 was performed on 3µm thick FFPE sections using a proprietary kit (CINtec Histology, Roche mtm laboratories AG, Germany) on a Ventana Benchmark Autostainer (Ventana Medical Systems Inc, USA). Oropharyngeal squamous cell carcinoma (OPSCC) tissue with high p16 expression was used as a positive control whilst histologically normal tonsillar tissue was used as a negative control. A case was considered as being p16 positive when there was strong and diffuse nuclear and cytoplasmic staining of OED involving more than 1/3 of the thickness of the involved epithelium, excluding keratin (Singhi and Westra, 2010; McCord *et al.*, 2013; Woo *et al.*, 2013).

HR-HPV ISH was performed on 3µm thick FFPE sections using proprietary reagents (Inform HPV III Family 16 Probe (B), Ventana Medical Systems Inc, USA) on a Benchmark Autostainer (Ventana Medical Systems Inc, USA). The following control samples were used: FFPE CaSki cells (HPV-16 positive), HeLa cells (HPV-18 positive) and C-33A (HPV negative; Ventana Medical Systems Inc, USA). The test was recorded as being positive if any blue reaction products were observed within the nucleus of epithelial cells (either punctate or homogenous pattern) (Thavaraj *et al.*, 2011; McCord *et al.*, 2013; Woo *et al.*, 2013). All cases were assessed in a manner similar to the earlier mentioned 3-tiered OED grading method (Figure 2.1). Cases were only considered HR-HPV positive if there was positivity for both p16 IHC and HR-HPV ISH.

2.4 Image-based Cytometry (IBC) DNA Ploidy Analysis

All image-based cytometry (IBC) DNA ploidy analysis procedures were performed at Guy's Hospital Head & Neck Pathology Laboratory London, in collaboration with Professor Edward Odell (EO). Cases with adequate tissue for analysis were selected from the OPMD cohort. Sample preparation was performed by HS with the kind assistance of Mrs. Deepa Nayar, Senior Biomedical Scientist at the Guy's Hospital Head & Neck Pathology Laboratory.

2.4.1 Sample preparation

The relevant H&E stained slides were reviewed to delineate suitable areas for DNA ploidy analysis (areas with worst OED). Whole sections were taken for small tissue samples whilst larger samples were macrodissected to obtain the areas of interest. The relevant areas for macrodissection were marked on the H&E slides. The preliminary decision as to whether ploidy analysis could be performed for each specimen was dependent largely on visual inspection of the amount of tissue in each FFPE block. Samples were prepared and analysed according to the steps outlined by Diwakar et al (2005).

Macrodissection was performed on large samples by outlining/scoring the corresponding areas (previously marked on the H&E slides) using a scalpel blade. The blocks were then cut into multiple 50μm sections (approximately 4 – 7 sections depending on the length of area of interest; ideally 8mm epithelial length x 6 sections) using a microtome (Jung RM2055, Leica) (Table 2.1). The sections were then placed into a 15ml labelled polypropylene centrifuge tube.

For deparaffinisation, tissues were incubated twice for 30 minutes in 4ml of xylene. Following this, rehydration in a series of aqueous ethanol solutions of decreasing concentrations was performed: two washes of 5 minutes in 4ml absolute ethanol and sequential immersion in 96%, 85%, 74% and 50% ethanol for 10 minutes each. The supernatant was then removed and replaced with 4ml of ice-cold PBS and left standing for 5 minutes. The supernatant was subsequently removed, and enzymatic digestion was performed by incubating samples with 2ml 0.05% protease (Sigma Protease type XXIV [Proteinase]) in a shaking (250 rpm) water bath (37°C) for 90 minutes. After which, the tubes were placed on ice and 2ml of ice-cold PBS was added and tissue pellets were re-suspended using a 1ml pastette.

The suspension was then filtered through a 60μm Nylon mesh filter into labelled 5ml Falcon tubes. The tubes were then centrifuged at 3300 rpm (Immufuge II, Dade Behring, USA) for 10 minutes, following which the supernatant was removed using a 1ml Gilson pipette and 0.5 – 2 ml (depending on size of pellet) of PBS was added to re-suspend the pellet. For dispersion of a monolayer of nuclei onto a glass microscope slide, 200μl of the cell suspension was spun for 5 minutes at 600 rpm

using a cytospin cytocentrifuge (Thermo Shandon Cytospin 4, Thermo Fisher Scientific, UK).

A light microscope was used to determine the nuclear concentration. A monolayer containing between 10 and 20 nuclei per x40 magnification field was considered to be suitable for analysis. If the monolayer was deemed to be inadequate for analysis, a new monolayer was made from the remaining suspension. The monolayer was then fixed in 4% buffered formalin overnight in a fume cupboard.

The next day, the slides with the fixed monolayers were washed in distilled water for 2 minutes before being placed in 5N HCl for one hour at room temperature to remove the purine bases of DNA. They were then washed in distilled water for two minutes and stained in Schiff's reagent (in the dark) for two hours. This was then followed by placing the slides in three changes of sodium bisulphite for 30 minutes (10 minute per change). The slides were then gently rinsed three times in tap water and three times in distilled water, following which the slides were dehydrated through a series of graded ethanols (96%, 85%, 74% and 50% ethanol) and transferred to xylene (5 minutes each immersion). The slides were then mounted with DPX mounting medium and coverslips were placed. The slides were then dried flat in an oven at 60°C for 10 minutes.

2.4.2 Measurement and analysis of DNA content

The Ploidy Work Station (PWS) Grabber system (Room4, Sussex UK) was used for scanning and capturing the images of the monolayer slides. The system was composed of an automated scanning microscope with a 546nm green barrier filter (Zeiss Axioplan II, Zeiss, Oberkochen, Germany), a black and white digital camera that provided a resolution of 162nm per pixel using a 40x lens (AxioCam MRm, Zeiss, Oberkochen, Germany) and a computer work-station.

Nuclear area and optical density of each nucleus was measured and corrected to the background optical density. By integrating the measured optical density of each pixel across the area of each nucleus, the software is able to provide the integrated optical density (IOD) value. The software automatically sorts the nuclei into separate galleries (epithelial nuclei, small lymphocyte nuclei, large lymphocyte nuclei,

fibroblast nuclei and unclassified objects) for analysis using the PWS Classifier software (Room4, Sussex, UK).

Using the PWS Classifier, the contents of each gallery were manually edited and refined to ensure the contents were accurate. For each case, at least 300 epithelial nuclei were assessed to arrive at ploidy status. Samples with < 300 nuclei of interest were repeated if there was adequate remaining material or excluded from further analysis. The software was then used to create DNA ploidy histograms from the integrated optical density (IOD) of the nuclei; the lymphocyte and fibroblast nuclei were used as the internal control/reference nuclei (diploid nuclei) by the software.

Classification of the histograms were performed according to a published set of criteria (Haroske *et al.*, 2001; Sperandio *et al.*, 2013):

- A sample was classified as being diploid if;
    - the epithelial nuclei formed only one 2c peak (G_0/G_1),
    - the 4c peak (G_2) has ≤ 10% of the total number of epithelial nuclei
    - the percentage of epithelial nuclei with DNA content of more than 5c was ≤ 1%.
- A sample was considered as tetraploid if;
    - A 4c peak with > 10% of the total number of epithelial nuclei was present with no other abnormal values
- A sample was classified as being aneuploid if;
    - there were one or more peak/s containing > 10% of the total epithelial nuclei outside the range of the diploid or tetraploid peaks or
    - number of epithelial nuclei with a DNA content of 5c and above was > 1% of the total number of epithelial nuclei

The ratio of standard deviation to mean DNA content for nuclei in the diploid peak (in percentage) was obtained through the software. This ratio, known as the coefficient of variation (CV), provides a measure of the detection limit or resolution for the analysis; peaks that are narrower (low CV with a low degree of scatter) are easier to assess and discriminate compared to peaks that are broad (high CV with a high degree of scatter) (Haroske *et al.*, 2001; Danielsen *et al.*, 2016). In best case scenarios a 1% change (corresponding to a CV of 1%) of DNA content is detectable

(Haroske *et al.*, 2001; Danielsen *et al.*, 2016). For the current study, cases with a CV that was > 5% for the diploid peak were excluded. The cut-off of 5% was chosen based on previously published studies and guidelines on DNA ploidy analysis (Haroske *et al.*, 2001; Sperandio *et al.*, 2013; Sperandio *et al.*, 2016). All image galleries were edited, and histograms were diagnosed by HS and Professor Edward Odell (EO).

2.5 Loss of Heterozygosity (LOH) Analysis

Sample preparation from FFPE tissue for LOH analysis was performed at the Institute for Genetic Medicine, Newcastle University, whilst the molecular analysis portion for LOH analysis was performed by medical scientists at an ISO 15189:2012 accredited molecular diagnostics laboratory, NewGene Limited (Centre for Life, Newcastle upon Tyne). Patient samples in the form of FFPE material were selected from the OPMD cohort identified earlier. The entire oral epithelium was selected as the "test" sample whilst the underlying connective tissue component was used as a comparator ("control") to judge loss of heterozygosity in the overlying oral epithelium. Two different methods were assessed to determine the most suitable method for sample preparation:

- LASER capture microdissection (LCM) and
- manual microdissection

The two methods were assessed using six cases. The more pragmatic method, manual microdissection, was then used for the remaining samples.

The number of 4μm thick sections per sample was dependent on the size of the tissue; 12 - 14 sections for small sized samples, 8 – 12 sections for medium sized samples, 6 – 8 sections for large sized samples and 3 – 6 sections for very large sized samples (Table 2.1). To facilitate microdissection, the sections were stained with haematoxylin and eosin (H&E) on a DAKO CoverStainer (Agilent Technologies, USA) without coverslips. All sample collection tubes were labelled with unique patient identifiers before the start of the procedure. The matched "test" and control tubes had the same identifier numbers with the exception of the addition of "C" as the last letter for tubes used for controls. The oral epithelium was collected into the "test" sample

tubes whilst the matching underlying connective tissue for each case was collected into the control sample tubes.

2.5.1 Sample preparation using LASER capture microdissection (LCM)

FFPE blocks were cut to produce 4μm thick sections using a Leica RM2135 microtome (Leica Microsystems, Germany) and mounted onto special Polyethylene Naphthalate (PEN) membrane slides (MembraneSlide) (Carl Zeiss, Munich, Germany). LASER capture microdissection (LCM) was performed using the PALM MicroBeam LASER capture microdissection system (Zeiss, Germany). As the LCM procedure is a non-contact procedure, a specialised collection device with an adhesive material filled microcentrifuge tube cover known as AdhesiveCap (Zeiss, Munich, Germany) was used as the collection device for the procedure.

2.5.2 Sample preparation by manual microdissection

The manual microdissection method was adapted from the methodology described by Mao et al (1996). FFPE blocks were cut to produce 4μm thick sections using a Leica RM2135 microtome (Leica Microsystems, Germany) and mounted onto glass microscope slides. The relevant areas for microdissection were annotated and subsequently microdissected manually using sterile 25-gauge needles and sterile scalpel blades. The laboratory bench and microscope were wiped clean with 70% ethanol before starting microdissection for each new case and new disposable gloves, needles and scalpel blades were used for each case. Dissected samples were placed in labelled sterile collection tubes.

2.5.3 Microsatellite markers

A panel of microsatellite markers located on chromosomes 3p, 9p, and 17p were chosen based on relevant studies which have shown that LOH involving these regions were useful as early predictors of malignant change in OPMDs (Partridge *et al.*, 1998; Zhang and Rosin, 2001; Bremmer *et al.*, 2008; Bremmer *et al.*, 2009). LOH at 3p14 (*FHIT*), 9p21 (p16/*CDKN2A*) and 17p13 (*TP53*) has been shown by some studies to be a useful adjunct to predict malignant transformation of OPMD (Califano *et al.*, 1996; Mao *et al.*, 1996; Partridge *et al.*, 1998; Partridge *et al.*, 2000; Rosin *et al.*, 2000; Zhang and Rosin, 2001; Bremmer *et al.*, 2008; Zhang *et al.*, 2012). The

chosen markers also have been shown to have a high percentage of LOH in head & neck SCC (Lippman and Hong, 2001; Tabor *et al.*, 2001; Braakhuis *et al.*, 2003).

The nine microsatellite markers are listed in Table 2.2. The microsatellite markers were initially assessed in 24 paired samples (test & control) to evaluate the performance of each of the microsatellite. Based on the results from the 24 paired samples, the best performing microsatellite marker for each chromosomal region was chosen for the remaining samples.

2.5.4 DNA extraction

DNA extraction was performed at an ISO 15189:2012 accredited molecular diagnostics laboratory (NewGene Limited, Centre for Life, Newcastle upon Tyne). Automated DNA extraction was performed using the Promega Maxwell MDx (Promega Corporation, Wisconsin, USA) extraction robot and the Promega FFPE DNA extraction kit AS1135 (Promega Corporation, Wisconsin, USA).

Briefly, following an overnight digestion in incubation buffer with proteinase K at 70°C (provided in the kit), lysis buffer was added to the sample and it was then loaded onto the DNA extraction robot. For samples obtained via LCM, prior to addition of proteinase K, 15μl of lysis buffer was added to the microdissected sample in the AdhesiveCap and the tubes were centrifuged at 10000xg for 5 minutes. DNA was extracted using magnetic beads. The quantity and quality of the DNA in each sample was determined using a NanoDrop Spectrophotometer (ThermoFisher Scientific, United Kingdom).

2.5.5 Polymerase chain reaction (PCR)

Polymerase chain reaction (PCR) was performed using the HotStarTaq DNA polymerase Master Mix Kit (QIAGEN, Germany). Sample DNA was diluted to 10ng/μl to dilute any contaminants from the FFPE tissue that may inhibit PCR. PCR was performed using specific primers labelled with either fluorescein (FAM) or hexachlorofluorescein (HEX) depending on the specific microsatellite marker. The PCR cycling protocol is shown in Table 2.3.

2.5.6 Loss of heterozygosity (LOH) analysis

PCR products from the samples were subjected to capillary electrophoresis using the ABI 3130*xl* genetic analyser (Applied Biosystems, Thermo Fisher Scientific, UK). Analysis of the outputs were performed using the GeneMarker software (SoftGenetics, LLC, State College, USA). Electropherograms produced by the GeneMarker software were assessed for LOH in the test sample using the control sample as the comparator and final results were verified by an appropriately qualified staff member of NewGene Limited, Centre for Life, Newcastle upon Tyne. LOH was defined as a 50% decrease in the peak height of the test sample compared to the peak height of the relevant control sample.

2.6 Cell Culture Techniques

All the cell culture work for the project was performed at the Craniofacial & Molecular Biology Research Laboratory at the Faculty of Dentistry, University of Malaya, Malaysia. The OED cell lines were cultured in keratinocyte serum-free medium (KSFM) supplemented with 25ug/ml bovine pituitary extract, 0.2ng/ml EGF and 0.3mM calcium chloride ($CaCl_2$). Cells were cultured in a CO_2 incubator (5%; Binder Inc, NY, USA) at 37°C. All the cell culture media and reagents were obtained from Gibco (Thermo Fisher Scientific, UK).

2.6.1 Characteristics of oral epithelial dysplasia (OED) cell lines

Four OED cell lines (D4, D19, D20 and D35) were used in this study. The characteristics of cell lines grown are shown in Table 2.4. The characterisation and maintenance of these cell lines have been previously described (McGregor *et al.*, 1997; McGregor *et al.*, 2002). Cryopreserved cells were revived by quickly thawing them at 37°C. The thawed cells were then re-suspended in 5ml of culture medium and centrifuged using Rotofix 32A (Hettich Instruments, Tuttlingen, Germany) at 13,000xg for 10 minutes before growing them in 25 cm^3 flasks containing 5ml of medium.

2.6.2 Subculture and cell number determination

The cells were subcultured at approximately 60 - 70% confluence. Cells were washed with phosphate buffered saline (PBS) followed by incubation with 4ml

trypsin/EDTA (Thermo Fisher Scientific, USA) for 20 minutes at 37°C. Trypsinised cells were then resuspended in 4ml of medium in a universal tube and centrifuged at 13,000xg using Rotofix 32A (Hettich Instruments, Tuttlingen, Germany) for 10 minutes. Cell pellets were re-suspended in fresh medium and the number of cells was determined. To assess the number of dead cells, cell suspensions were mixed 1:1 with Trypan Blue (Sigma-Aldrich, UK) and 10µl of the mixture was pipetted onto the Luna cell counting slide (Logos Biosystem Inc., South Korea). The Luna Automated Cell Counter (Logos Biosystem, South Korea) was then used to determine the cell concentration from the glass slide. Cells were then seeded out at the required density. The necessary volume of cell suspension was added to the culture flask containing pre-warmed medium and the flask was then placed in an incubator at 37°C with 5% CO_2. Cell lines were subcultured at least once before RNA extraction.

2.6.3 Storage of cells

Approximately $1x10^6$ cells were resuspended in foetal bovine serum (FBS) with 10% dimethyl sulfoxide (DMSO) (Sigma-Aldrich, Gillingham, UK). Cells were then transferred to cryopreservation tubes (Nunc® Cryo Tubes®; Sigma-Aldrich, UK) and cooled overnight to -80°C in a cryo-container (Nalgene® Mr. Frosty; Sigma-Aldrich, Gillingham, UK). Following which, the cells were stored in liquid nitrogen.

2.6.4 Total RNA extraction from cell lines

RNA extraction was performed using the QIAGEN RNeasy® Mini kit following the manufacturer's protocol (QIAGEN, Manchester, UK). Adherent cells were collected by trypsinisation (as described in Section 2.6.2), washed once with ice-cold PBS, transferred to a polypropylene centrifuge tube and centrifuged at 300xg for 5 minutes. The recommended volume of RLT buffer supplemented with β-mercaptoethanol was added prior to vortexing for one minute to homogenise samples. Alcohol (70%) was added to the cell lysates and then transferred to the RNeasy Mini spin column for centrifugation. Subsequently, on-column deoxyribonuclease (DNase) digestion was performed as per the manufacturer's instructions using the ribonuclease (RNase) Free DNase Set (QIAGEN, Manchester, UK). Following multiple washes with the supplied buffers, RNA was eluted from the columns using 40µl of RNase-free water pipetted directly onto the membrane. The

concentration and the quality of the isolated RNA were measured using a NanoDrop 2000 Spectrophotometer (Thermo Fisher Scientific, UK). RNA with a 260/280 ratio of 1.7 - 2.3 as well as a 260/230 ratio in the range of 1.8 -2.3 were considered to be of acceptable quality for downstream assays (NanoString, 2016).

2.7 Differential Gene Expression Experiments

Differential gene expression (DGE) experiments were performed using RNA extracted from FFPE and cell line material. Two different gene expression profiling platforms were used to determine differential gene expression profiles between OPMD that underwent malignant transformation (MT) and those that had not undergone malignant transformation (NT):

- whole transcriptome sequencing using Illumina's Next Generation Sequencing RNASeq platform (Illumina, USA)
- targeted transcriptome profiling using the NanoString nCounter platform (NanoString Technologies, Seattle, USA)

RNA extraction and purification from FFPE tissue for differential gene expression analysis was performed at the Institute for Genetic Medicine, Newcastle University. The RNA sequencing was performed by biomedical scientists at the Genome Centre, Queen Mary University of London. NanoString sample processing was performed with the kind assistance of Ms. Anastasia Resteu from the Human Dendritic Cell Laboratory, Institute of Cellular Medicine, Newcastle University.

2.7.1 Total RNA extraction from formalin-fixed paraffin-embedded (FFPE) tissue

After trimming excess paraffin off the sample block, 10µm sections were cut from the FFPE blocks and placed in 2ml microcentrifuge tubes after discarding the first two sections. Whole sections that included both epithelium and underlying connective tissue were used. The number of sections per sample was dependent on the size of the tissue; 4 sections for small sized samples, 3 - 4 sections for medium sized samples, 2 - 3 sections for large sized samples and 1 – 2 sections for very large sized samples (Table 2.1). RNA extraction and purification were performed using the QIAGEN RNeasy FFPE kit following the manufacturer's protocol (QIAGEN, Manchester, UK). A brief outline of the protocol is listed in Appendix C. Following RNA extraction, the concentration and the quality of the isolated RNA were

measured using a NanoDrop 2000 Spectrophotometer (Thermo Fisher Scientific, UK). The samples were then stored in a -80°C freezer prior to utilisation in downstream experiments.

2.7.2 Whole transcriptome sequencing - RNA sequencing (RNASeq)

Total RNA sequencing (RNASeq) was performed using RNA extracted from 20 FFPE samples (10 MT vs 10 NT). RNA samples were assessed for quantity and integrity using the NanoDrop 8000 spectrophotometer V2.0 (Thermo Fisher Scientific, USA) and Agilent 2100 Bioanalyser (Agilent Technologies, Waldbronn, Germany). From each sample, 100ng of total RNA was used to prepare RNA libraries using the KAPA Stranded RNASeq Kit with RiboErase (KAPA Biosystems, Massachusetts, USA). Prior to first strand cDNA synthesis, fragmentation was carried out using incubation conditions recommended by the manufacturer for degraded samples (65°C for 1 minute), and 14 cycles of PCR were performed for final library amplification. The libraries produced were quantified using the Qubit 2.0 spectrophotometer (Life Technologies, California, USA) and assessment of the average fragment size was performed using the Agilent 2200 Tapestation (Agilent Technologies, Waldbronn, Germany). The Illumina NextSeq®500 (Illumina Inc., Cambridge, UK) was used to generate 75bp paired-end reads for each library.

2.7.3 Bioinformatic analysis of RNASeq data

Bioinformatic analysis was performed by Mr. John Casement from the Bioinformatics Support Unit of Newcastle University. FastQ files generated from the sequencing runs were downloaded from the Illumina server using BaseMount, the command line interface for Illumina BaseSpace. Read quality of the FastQ files generated from the sequencing run were assessed using FastQC (http://www.bioinformatics.babraham.ac.uk/projects/fastqc) and MultiQC (http://multiqc.info) was used to obtain summary statistics for quality control tests on the read quality. Reads were quantified against transcripts using “Kallisto” (Bray *et al.*, 2016). Kallisto is a program for quantifying abundances of transcripts from RNASeq data, which determines the compatibility of reads with targets without the need for alignment. The summarised Kallisto workflow is as follows:

- (1) Build index file using the “kallisto index” command. The index was built from

Gencode transcript FASTA files (Gencode version 24: http://www.gencodegenes.org/releases/24.html)

- (2) Run the quantification algorithm “kallisto quant” for each pair of forward (R1) and reverse (R2) FastQ files against the index.

Kallisto was used to quantify reads against transcripts. To obtain gene-level counts, a package from the R statistical programming language (R Foundation for Statistical Computing, Vienna, Austria), “tximport” was used. Gene annotation was obtained from Ensembl transcript IDs using the R package “biomaRt” (Durinck *et al.*, 2005).

The R package DESeq2 was used for normalisation and testing for differential gene expression by use of negative binomial generalised linear models (Love *et al.*, 2014). Genes were considered to be significantly differentially expressed when the False Discovery Rate (FDR) using the Benjamini-Hochberg method corrected p-value was less than 0.05.

2.7.4 NanoString experiments

The NanoString nCounter system (NanoString Technologies, Seattle, USA) uses hybridisation of short length probes (35- to 50- base sequence) that are subsequently fixed to a biotin-coated cartridge which is then digitally imaged and counted to quantify mRNA expression. In-depth details regarding NanoString technology can be obtained from Geiss et. al. (2008). NanoString sample processing was carried out at the Human Dendritic Cell Laboratory, Institute of Cellular Medicine, Newcastle University using the nCounter MAX/FLEX system (NanoString Technologies, Seattle, USA) with the kind assistance of Ms. Anastasia Resteu. The experiment involved two stages:

- Stage 1: Differential gene expression experiment using the PanCancer Pathways Panel Plus of target genes
- Stage 2: Differential gene expression experiment using a customised list of target genes

Experiments were performed using previously extracted RNA from selected FFPE blocks as described in section 2.7.1. Each assay comes with engineered External

RNA Controls Consortium (ERCC) synthetic internal negative and positive control probes.

For the PanCancer Pathways Panel Plus, an additional ten probes targeting specific mRNA (Appendix D) were added to the pre-existing 770 gene list available in the PanCancer Pathways Panel. The additional targets were chosen from statistically significant differentially expressed genes from the earlier RNASeq experiment (FDR < 0.05 and fold change greater than 2). The selection of these additional candidate genes was based upon biological relevance and review of the relevant literature. This was discussed with and finalised through consensus by members of the Newcastle University Oral Cancer Research Group (OCRG). This experiment was performed using RNA extracted from 48 FFPE samples (25 NT and 23 MT cases). RNA samples were assessed for quantity and quality using the NanoDrop 2000 Spectrophotometer (Thermo Fisher Scientific, UK). Samples were considered suitable for the NanoString experiment if the A260/A280 ratio was between 1.7 – 2.3 and the A260/A230 ratio was between 1.8 – 2.3 (NanoString, 2016). RNA content for all samples was normalised to 30ng/μl and 5 μl (150ng of total RNA) per sample was used for the experiment [Dr Jim White, Senior Field Application Specialist, NanoString Technologies; personal communication]. The summarised laboratory workflow for the NanoString nCounter assay using the PanCancer Pathways Panel Plus according to the manufacturer's protocol is listed in Appendix E (NanoString, 2016).

For the Customised CodeSet Panel experiment, a list of target genes was compiled based on the results from the RNASeq experiment, NanoString PanCancer Pathways Panel Plus experiment and review of relevant scientific literature. The selection of candidate genes for this customised panel was discussed and finalised through consensus by members of the Newcastle University Oral Cancer Research Group (OCRG) and the gene list is shown in Appendix F. This experiment was performed using RNA extracted from 44 FFPE samples (24 NT and 20 MT) and four OED cell lines. RNA samples were assessed for quantity and quality using the NanoDrop 2000 Spectrophotometer (Thermo Fisher Scientific, UK). Samples were considered suitable for the NanoString experiment if the A260/A280 ratio was between 1.7 – 2.3 and the A260/A230 ratio was between 1.8 – 2.3 (NanoString, 2016). RNA content for all samples was normalised to 30ng/μl and 5 μl (150ng of

total RNA) per sample was used for the experiment. The summarised laboratory workflow for the Customised CodeSet Panel gene expression assay according to the manufacturer's protocol is listed in Appendix G (NanoString, 2016).

Output from the nCounter Platform was quality assured using the quality control (QC) function in the nSolver analysis software 3.0 (NanoString Technologies, Seattle, USA). The following parameters were assessed during the QC function for each sample:

- Imaging QC: measure of the percentage of requested fields of view successfully scanned in each cartridge lane (75% cut-off)
- Binding Density QC: measure of reporter probe density on the cartridge surface within each sample lane (range between 0.05 – 2.25)
- Positive Control Linearity QC: measure of correlation between the counts observed for the ERCC synthetic positive control probes and the concentrations of the spike-in synthetic target nucleic acids (0.95 cut-off)
- Positive Control Limit of Detection QC: measures the limit of detection by comparing results from positive control probes and negative control probes (0.5fM positive control probe should produce raw counts of > 2 standard deviations higher than the mean of the negative control probes)

2.7.5 Differential gene expression analysis of NanoString data

Differential gene expression data analysis between MT and NT groups was performed using the nSolver Analysis Software 3.0 (NanoString Technologies, Seattle, USA). Prior to the DGE analysis, the raw data were normalised in a two-step manner. Firstly, the raw counts were background subtracted using the geometric mean of the internal negative controls followed by technical normalisation using the geometric mean of the internal positive controls. Subsequently, the data were then normalised using the geNorm algorithm that chooses only the most stable housekeeping genes in the analysed dataset (Vandesompele *et al.*, 2002).

For the PanCancer Pathways Panel Plus experiment, genes were considered to be significantly differentially expressed when the False Discovery Rate (FDR) using the Benjamini-Hochberg method corrected p-value was < 0.1. A False Discovery Rate (FDR) of < 0.1 was chosen to be significant for this experiment as this was an

exploratory experiment to find genes with altered expression between OPMD that undergo MT and those that do not. Setting the FDR rate too stringently could exclude key genes that may have been statistically significant if the cohort was larger. A hypergeometric test was carried out to identify Kyoto Encyclopaedia of Genes and Genomes (KEGG) pathways in which differentially expressed genes were over-represented. KEGG pathways were rendered using Pathview (Luo and Brouwer, 2013).

For the Customised CodeSet Panel experiment, genes were considered to be significantly differentially expressed when the False Discovery Rate (FDR) using the Benjamini-Hochberg method corrected p-value was < 0.05. Raw and log2 normalised counts of significant differentially expressed genes were then exported in a CSV file to be used for statistical analysis and model building. Log2 normalised counts of the significant differentially expressed genes between MT and NT cases were then dichotomised using the respective medians into low-expression and high-expression sub-groups for further analysis [Dr Kim Pearce and Dr Syed Haider; personal communication].

2.7.6 Development of gene-signature for clinical outcome in OPMD

Development of a gene-signature was performed by fitting the dichotomised (low-expression; high-expression) log2 normalised gene expression of significant differentially expressed genes between MT and NT cases from the Customised CodeSet experiment into a Cox regression (proportional hazards regression) model using a stepwise method. The β-coefficients from the gene-signature model were used to obtain risk scores and the resultant risk scores were then dichotomised into risk groups (low-risk and high-risk) using the median of the risk scores [Dr Kim Pearce and Dr Syed Haider; personal communication].

2.8 Statistical Analysis and Prognostic Model Building

Statistical analysis and prognostic model building were performed using IBM SPSS for Windows (version 24, IBM-SPSS Inc., Chicago, Illinois, USA) and the R Environment for Statistical Computing version 3.2 (R Foundation for Statistical Computing, Vienna, Austria). The selection and interpretation of specific tests was

informed by discussions with Dr Kim Pearce from Newcastle University and Dr Syed Haider from the Institute of Cancer Research, London.

A variety of methods were used to assess and analyse the data. Continuous data was always assessed for normality of distribution prior to choosing appropriate statistical tests. Parametric and non-parametric tests were used for initial analysis of demographic, clinical, pathological and molecular variables. For continuous data, descriptive results were appropriately expressed as either median with interquartile range (IQR) or mean with standard deviation (SD). For crosstabulations and chi-squared tests, exact p-values were calculated where possible. Cohen's kappa coefficient statistics was used to measure the level of agreement for OED grading between Pathologist A and Pathologist B (Cohen, 1960; Cohen, 1968). The kappa scores were interpreted based on the study by Landis and Koch (1977) (Landis and Koch, 1977).

Differential gene expression data analyses between MT and NT groups were carried out and genes were considered to be significantly differentially expressed where the False Discovery Rate (FDR) using the Benjamini-Hochberg method corrected p-value was < 0.05 except for the PanCancer Pathways Panel Plus experiment, where the FDR value was set at the 10% level (< 0.10). Principal component analysis (PCA), an unsupervised method to summarise complex data that has high dimensionality into fewer dimensions whilst attempting to find patterns without knowing the clinical outcome of the samples was also performed.

Univariate analysis and multivariate analysis were performed using Cox regression (proportional hazards regression) analysis. The proportional hazard assumption for each variable was also assessed prior to Cox regression analysis. Multivariate analysis was performed using variables that were considered to be clinically relevant and/or statistically significant from preliminary statistical analyses. Multivariate analysis was performed to determine the variables that independently contributed to clinical outcome as well as to formulate a suitable prognostic model composed of different variables (clinical, pathological or molecular variables). During multivariate regression analysis, covariates were selected using a stepwise procedure. Statistical significance was defined at the 5% level. The concordance index (Harrel's c-index) for each model was also calculated (Harrell *et al.*, 1996). Confidence intervals (CI) at the 95% confidence level were reported where relevant. Statistically significant

variables from the regression analysis were then used to build a prognostic model for patients with OPMD. Assessment of biomarkers was performed guided by the 20-point recommendations made in the “Reporting Recommendations for Tumour Marker Prognostic Studies (REMARK)” checklist (Appendix A) (McShane *et al.*, 2005).

Only complete-case analysis was performed for multivariate analysis; cases with missing data-points were excluded from statistical model building. Multivariate prognostic statistical model construction and validation were performed guided by the recommendations in the “Transparent Reporting of a multivariable prediction model for Individual Prognosis or Diagnosis (TRIPOD) Statement” checklist (Collins *et al.*, 2015; Moons *et al.*, 2015). The TRIPOD statement is composed of a 22-item checklist (Appendix B) that covers a minimum set of recommendations focusing on how the study was designed, performed, analysed and interpreted (Collins *et al.*, 2015; Moons *et al.*, 2015).

Receiver operating characteristic (ROC) curves were generated by plotting the cumulative frequency distribution of a variable/model score for predicting MT. ROC curves plot sensitivity (true positive rate) against 1 – specificity (false positive rate) and provide a graphical representation of the performance of a prognostic variable/model in a binary outcome situation.

Area under the curve (AUC) from the ROC curves generated from different independent variables and statistical models were used to assess the performance/efficacy of the variables or models. Time to event analysis was also performed and Kaplan-Meier curves were generated to assess the influence of independent variables as well as prognostic models on time to event (MT) using the Log Rank test. All statistical tests were two-sided, and results were considered statistically significant at $p < 0.05$ value unless stated otherwise; the Exact method was used for calculating p-values where relevant.

Table 2.1 Amount of formalin-fixed paraffin-embedded tissue used for molecular experiments according to sample size

Sample size	DNA ploidy analysis	Loss of heterozygosity (LOH) analysis	Differential gene expression (DGE) experiments
Small sized samples	50μm x (4 to 7) sections*	4μm x (12 to 14) sections	10μm x 4 sections
Medium sized samples		4μm x (8 to12) sections	10μm x (3 to 4) sections
Large sized samples		4μm x (6 to 8) sections	10μm x (2 to 3) sections
Very large sized samples		4μm x (3 to 6) sections	10μm x (1 to 2) sections

* Ideally 8mm epithelial length x 6 sections

Table 2.2 Microsatellite markers for LOH analysis

Marker	Loci	Position on chromosome
D3S1766	3p14.2	58956715-58956927
D3S1029	3p21.33	44110861-44111030
D3S1293	3p24.3	21902207-21902338
D9S171	9p21	24524210-24524384
D9S162	9p22.1	19669807-19669992
D9S157	9p22.2	17618382-17618526
CHRNB1	17p13.1	7290301-7290466
TP53	17p13.1	7558143-7558252
D17S1866	17p13.3	82571-82745

Table 2.3 Optimised PCR cycling protocol

Step	Temperature	Time	Cycle(s)
Initial activation	95°C	15 minutes	1
Denaturation	95°C	1 minute	35
Annealing	60°C	1 minute	
Extension	72°C	1 minute	
Final extension	72°C	10 minutes	1

Table 2.4 Characteristics of OED cell lines cultured.

Cell line	Age (years)	Sex	Smoker	Site	Lesion	OED grade
D4	51	M	Yes	FOM/VT	Leukoplakia	Severe
D19	53	M	Yes	LT	Erythroleukoplakia	Severe
D20	50	M	No	LT	Leukoplakia	Moderate
D35	68	M	Yes	FOM/VT	Erythroleukoplakia	Severe

* M = Male; FOM = floor of mouth; VT = ventral tongue; LT = lateral tongue; OED = oral epithelial dysplasia;

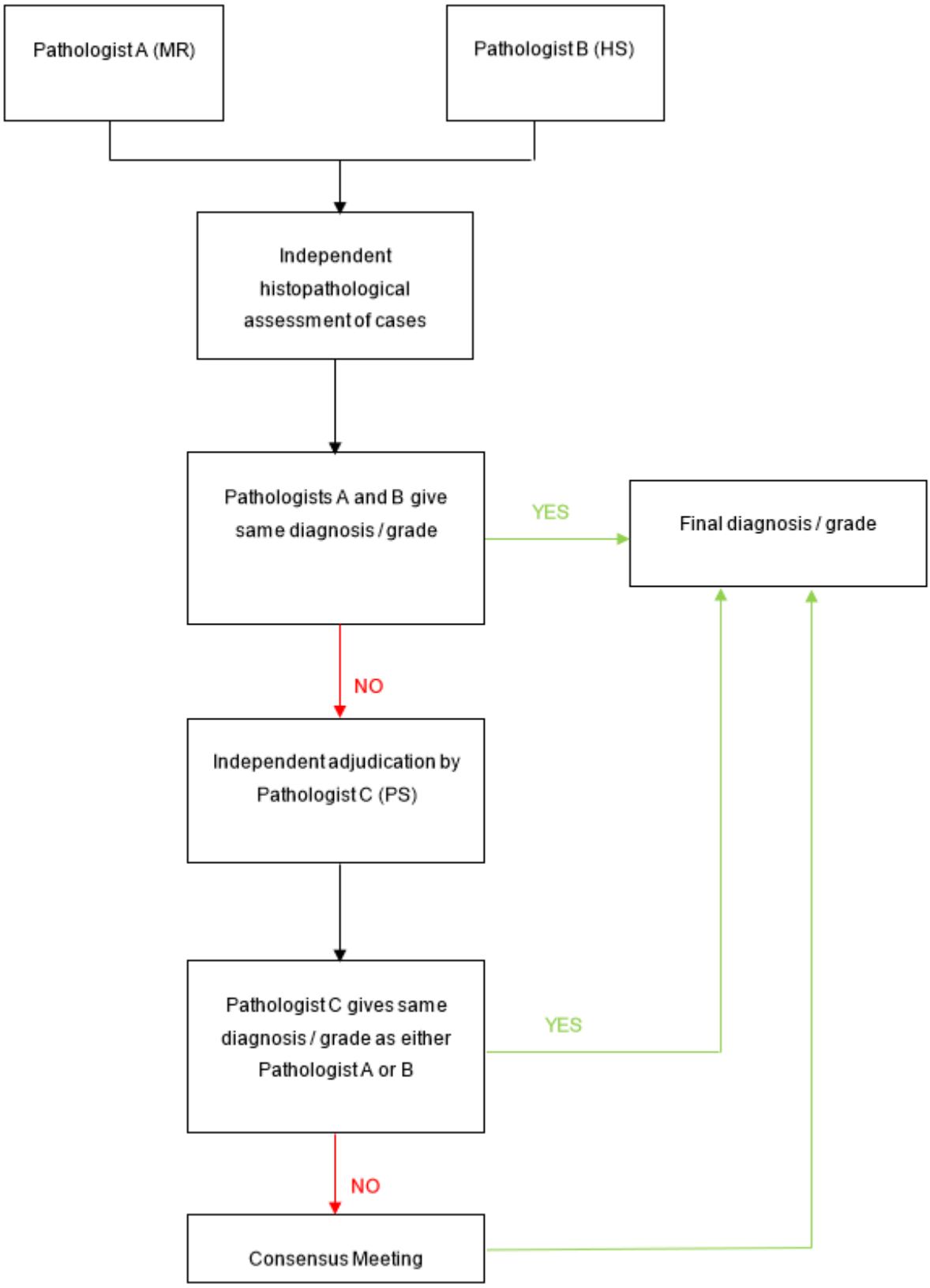


Figure 2.1 Workflow of the histopathological assessment process.

MR: Dr Max Robinson; HS: Hans Sathasivam; PS: Professor Phil Sloan. Adapted from Speight et al (2015).

Chapter 3. Clinical and Histopathological Parameters

3.1 Introduction

Risk stratification and management strategies for patients with OPMD at most centres are based on the demographic and clinico-pathological features of the patient such as age, sex, smoking history, type of OPMD and OED grading (van der Waal, 2009; Awadallah *et al.*, 2018; Speight *et al.*, 2018). However, such methods are not very accurate as some patients stratified as being low-risk using such parameters still experience malignant transformation (Holmstrup *et al.*, 2006; van der Waal, 2009; Warnakulasuriya *et al.*, 2011; Sperandio *et al.*, 2013; Dost *et al.*, 2014; van der Waal, 2014). This chapter examines the relationship between demographic as well clinico-pathological parameters of the study cohort and clinical outcome.

3.1.1 Demographic and clinical parameters

A few demographic and clinical factors have been found to be correlated to increased risk of malignant transformation (MT) in OPMDs (Table 1.4) (Speight, 2007; van der Waal, 2009; Warnakulasuriya *et al.*, 2011; El-Naggar *et al.*, 2017; Speight *et al.*, 2018). However, many of these factors are not particularly good as prognostic indicators especially when used individually. Combining the demographic and clinical parameters for risk stratification of OPMD patients though imperfect is commonplace. Clinical parameters are usually augmented by histopathological findings from incisional biopsies to guide patient management (Epstein *et al.*, 2012; Forman *et al.*, 2015; National Institute for Health and Care Excellence, 2015a). Data of several demographic and clinical parameters were obtained and analysed to ascertain correlation with clinical outcome. Unfortunately, it was not possible to obtain complete information regarding risk habits (tobacco usage, alcohol consumption etc) for all patients and as such these parameters were excluded from further analysis.

3.1.2 Oral epithelial dysplasia (OED)

There have been several classification systems that have been proposed over the years for oral epithelial dysplasia (OED) grading (Warnakulasuriya *et al.*, 2008) with the recently published WHO reference text on head and neck tumours elaborating on

two systems; the three-tiered WHO grading system and the binary grading system (El-Naggar *et al.*, 2017). Grading systems developed thus far attempt to mirror the perceived spectrum of histologic changes in oral epithelium undergoing oral carcinogenesis from normal to severe OED to cancer. However, oral carcinogenesis is not likely to be a simple stepwise linear progression model. There is no established evidence of it following a simple progressive model of changes from normal epithelium to OED followed by oral squamous cell carcinoma (OSCC). This is proven by studies that show that OSCC can arise in oral mucosa that is histologically non-dysplastic/mildly-dysplastic and conversely, severely dysplastic oral epithelium may regress or remain unchanged (Gupta *et al.*, 1980; Silverman *et al.*, 1984; Holmstrup *et al.*, 2006; Arduino *et al.*, 2009; Dost *et al.*, 2014; Goodson *et al.*, 2015; Kuribayashi *et al.*, 2015). For this study, both the WHO 2017 and binary OED grading systems were used to grade OED. Data were then analysed to ascertain the relationship between OED grading and clinical outcome.

3.1.3 Oral potentially malignant disorder (OPMD) associated leukocytes score (OPALS)

A prominent sub-epithelial leukocytic infiltrate is not an infrequent finding in conjunction with OPMD or oral epithelial dysplasia (OED) and its presence can at times mimic inflammatory conditions affecting the oral cavity such as oral lichen planus histologically (Eisenberg and Krutchkoff, 1992; Eisen, 2002; Muller, 2011). Previously the term “lichenoid dysplasia” was introduced by Krutchkoff and Eisenberg (1985) to describe dysplastic oral lesions with lichenoid features (Krutchkoff and Eisenberg, 1985). This term however is quite misleading and such lesions are now more commonly described as either oral epithelial dysplasia with lichenoid inflammation/lichenoid features/lichenoid mucositis/interface mucositis. Fitzpatrick et al (2014) in a recent study showed that up to 39% of mild to moderate dysplasia and 16% of severe dysplasia specimens in their study had a positive overall score for focal lichenoid features ($\geq$ 3 lichenoid features) (Fitzpatrick *et al.*, 2014b). The most frequently encountered lichenoid feature in their study was the presence of a band-like inflammatory infiltrate (Fitzpatrick *et al.*, 2014b). This is suggestive that a prominent sub-epithelial inflammatory infiltrate is not specific for oral lichen planus and may arise in conjunction with OED. It is hoped that through the assessment of

OPMD associated leukocytes (OPAL) in this cohort of patients, a clearer picture regarding the prognostic significance of OPAL in OPMD patients can be obtained.

3.1.4 High-risk human papillomavirus (HR-HPV) infection

The association between high-risk human papillomavirus (HR-HPV) infection and oropharyngeal squamous cell carcinoma (OPSCC) has been well established and in recent years several groups have managed to identify and characterise HPV-associated oral epithelial dysplasia (OED) (McCord *et al.*, 2013; Woo *et al.*, 2013). HPV-associated OED is defined by specific histopathological features together with the demonstration of HR-HPV by molecular methods such as DNA/RNA in situ hybridisation (ISH) (McCord *et al.*, 2013; El-Naggar *et al.*, 2017). Strong and diffuse nuclear and cytoplasmic expression of p16 by immunohistochemistry is consistently observed in HPV-related cervical intraepithelial neoplasia and OPSCC, as such, it is a useful surrogate marker for HR-HPV in HPV-associated OED but secondary testing for HR-HPV using molecular methods is currently recommended to diagnose HPV-associated OED (McCord *et al.*, 2013; El-Naggar *et al.*, 2017). The influence and role of HR-HPV infection on development and clinical outcome of OPMD is still unclear as there are no studies with long-term follow up data (Angiero *et al.*, 2010; Lopes *et al.*, 2011; Lingen *et al.*, 2013; McCord *et al.*, 2014; Nankivell *et al.*, 2014; El-Naggar *et al.*, 2017; Lerman *et al.*, 2017; Muller, 2018; Speight *et al.*, 2018). HPV-related oral squamous cell carcinoma may actually be a completely different disease from traditional non-HPV related OSCC. As such, identifying HPV-associated OED and performing sub-group analysis for clinical studies on OPMD/OED is desirable. All the cases chosen for the molecular arm of this study were assessed for HR-HPV infection using both p16 immunohistochemistry (IHC) and HR-HPV DNA *in-situ* hybridisation (DNA ISH).

3.2 Aims

The aims of this chapter are:

1) To summarise the case selection process of OPMD patients that were suitable for this study as well as selection of the cohort to be assessed using molecular methods (training cohort) described in Chapters 4, 5 and 6.

2) To describe the demographic and clinical features of the overall OPMD cohort as well as the sub-groups.

3) To assess and correlate demographic and clinical features as prognostic parameters for OPMD patients.

4) To assess and correlate oral epithelial dysplasia (OED) grading as a prognostic instrument for OPMD as well as to compare the efficacy of two different OED grading systems in prognosticating clinical outcome in patients with OPMDs.

5) To describe and assess the relationship between OPAL and clinical outcome in OPMDs.

6) To describe and assess the prevalence and association between high-risk human papillomavirus (HR-HPV) infection and clinical outcome in OPMDs.

3.3 Results

3.3.1 Case selection

OPMD cases for the training cohort were obtained from a clinical database. Briefly, 301 cases were assessed for eligibility and 135 cases were excluded. Ninety-eight cases were excluded as they did not meet the inclusion criteria and a further 37 cases were excluded as the FFPE tissue blocks could not be obtained from the archives. There were 166 cases that were suitable for this study. Sixty-five cases were selected for downstream molecular experiments based on the amount of FFPE tissue available. A balanced number of malignant transforming and non-transforming cases were selected for the molecular experiments; 30 malignant transforming (MT) vs 35 non-transforming (NT). The flow chart displayed in Figure 3.1 summarises the case selection process for this study.

3.3.2 Demographic and clinical parameters

The demographic and clinical features of the 166 cases that were found suitable for this study are shown in Table 3.1. Mean patient age at diagnosis of the index lesion was 58.79 (SD ±12.81) years of age. There was a male predominance in this cohort (62.7%). Leukoplakia was the most frequently encountered OPMD (85.5%) and the

tongue was the most frequent site for the index lesions (34.3%). Only time to malignant transformation/last-follow-up was significantly different between the MT and NT cases (p = 0.015; Mann-Whitney U test), with the median time to MT being 41 months (range: 7 – 128 months) and for NT cases the median follow-up time was 61 months (range: 12 – 215 months). No other clinical variable was significantly associated with clinical outcome when analysis was performed using the 166-patient cohort (p > 0.05).

Table 3.2 shows the demographic and clinical characteristics of the 65 cases chosen for the molecular experiments in this study. There were 30 MT cases in the selected cohort versus 35 NT cases. The mean age at diagnosis of OPMD was 60.00 years (SD = 13.29) and the majority were males (n = 44; 67.7%). The cohort of patients with OPMD that underwent MT was older with a male preponderance although these findings were not statistically significant (Table 3.2 and Figure 3.2). When cases were divided according to those aged $\leq$ 50 and > 50, there was a statistically significant relationship between age at diagnosis and clinical outcome (p = 0.046; χ^2= 4.74; df = 1). The non-transforming (NT) cases were followed-up for a median of more than 10 years whilst the median of MT cases occurred within 3 years of diagnosis.

There was a statistically significant relationship between site of index OPMD and clinical outcome (p = 0.028; χ^2= 10.18; df = 4). The tongue was the most commonly involved site that underwent MT, however, when analysis was performed after re-categorising site into tongue versus at all other sites, site of index OPMD was not statistically significant (p = 0.065). Most of the cases that underwent MT were clinically diagnosed as leukoplakia, but this was not statistically significant (p = 0.650).

3.3.3 Oral epithelial dysplasia (OED)

Results from the oral epithelial dysplasia (OED) grading assessment are shown in Tables 3.3 to 3.5. OED for each case was graded according to the worst area in the specimen.

There were five cases that had no obvious OED in the overall cohort, one of which underwent MT. The one non-dysplastic case that underwent MT only transformed to cancer after more than 10 years of being diagnosed with an OPMD. When analysis

was performed on all 166 cases (Table 3.3; including non-dysplastic cases), only the binary grading system was significantly correlated with clinical outcome (p = 0.016; χ^2= 8.41; df = 2). Inter-rater agreement between the two pathologists (MR and HS) using weighted kappa scoring was 0.822 (*p* < 0.001; Cohen's κ) for the WHO 2017 OED grading system and 0.833 (*p*-value of < 0.001; Cohen's κ) for the binary OED grading system. The binary OED grading was found to outperform the WHO 2017 grading system when it comes to prognosticating clinical outcome for OPMD and this finding was statistically significant (Figure 3.3). Time to event analysis on the cohort of patients with OED (n = 161) showed that binary grading system was statistically significant (Figure 3.4; Log rank test; p = 0.003; χ^2= 9.05; df = 1).

Analysis of the grading systems showed that there were statistically significant associations between OED grading (binary and WHO 2017 systems) and site of index OPMD as well as type of OPMD (Tables 3.4 and 3.5). The association between "tongue" as the site of index OPMD and OED grading systems was statistically significant after sub-analysis was performed by combining the other oral sub-sites (Tables 3.4 and 3.5).

Results of the OED grading exercise on the 65 cases selected for downstream assays are shown in Table 3.6. Inter-rater agreement between the two pathologists (MR and HS) for the WHO 2017 OED grading system was 0.709 (*p* < 0.001; weighted Cohen's κ) and 0.814 (*p*-value of < 0.001; weighted Cohen's κ) for the binary OED grading system. There was statistically significant correlation between the binary OED grading system and clinical outcome (p = 0.026; χ^2= 5.464; df = 1). The WHO 2017 grading system however was not statistically significant (p > 0.05). Time to event analysis on this cohort of patients (Figure 3.5) again showed that the binary grading system was statistically significant (Log rank test; p = 0.012; χ^2= 6.34; df = 1). The binary grading system was shown to have good negative predictive values for clinical outcome (Table 3.7).

3.3.4 Oral potentially malignant disorder (OPMD) associated leukocyte score (OPALS)

The summarised results of OPALS from the 166 and 65 patient cohorts is shown in Table 3.8 and Table 3.9 respectively. There was a statistically significant relationship between OPALS and both the WHO 2017 grading system (p = 0.001; χ^2= 15.841; df

= 3) and binary grading system (p = 0.004; χ^2= 10.384; df = 2) in the 166-patient cohort. Higher grades of OED were found to be more frequently OPALS positive (Table 3.8). Clinical features were not statistically significantly associated with OPALS in the 166-patient cohort (p > 0.05). Time to event analysis showed that there was no statistically significant relationship between OPALS and clinical outcome in the 166-patient cohort (Figure 3.6).

However, when analysis was performed using the 65-patient cohort, only the association between clinical outcome and OPALS was statistically significant (p = 0.026; χ^2= 5.251; df = 1) and other clinico-pathological features such as type of OPMD, site of index OPMD and OED grading were found to have no statistically significant association with OPALS (p > 0.05).

3.3.5 High-risk human papillomavirus (HR-HPV) infection

Assessment for high-risk human papillomavirus (HR-HPV) infection was performed in a two-tiered manner as described in Section 2.3.3. All 65 cases selected for the molecular arm of the study were tested for p16 using IHC. Only seven cases showed p16 staining. These cases were then tested for HR-HPV using DNA ISH and only three cases (4.6 %) were positive, two from the NT and one from the MT sub-groups. HR-HPV infection was not correlated to clinical outcome in this cohort of 65 patients (p = 1.000).

3.4 Discussion

Overall, from the 166 cases that were found to be suitable for the study, 32 cases (19.3%) had undergone MT. This figure however does not represent the true incidence or prevalence rate of MT in OPMD patients as not all patients with OPMD from the centre were included in the study. Previous studies on a cohort of OPMD patients seen at a single institution in Newcastle upon Tyne have reported the incidence of MT in OPMD patients to be around 4.8% (Thomson *et al.*, 2017a; Thomson *et al.*, 2017b). However, this figure would not be a true representation of the population and may be an overestimation due to referral bias as this is a referral centre based (hospital-based) cohort.

3.4.1 Demographic and clinical parameters

In this study, the majority of patients were aged above 50 years at time of diagnosis with OPMD, however the age range of the cohort was rather wide; 23 to 93 years. It would appear that patients with non-transforming (NT) OPMD were younger than those that underwent malignant transformation (MT), with those older than 50 years of age at diagnosis being more likely to have MT, however the findings were not statistically significant for this study. This finding has been documented by several earlier studies and a recent review has mentioned that a moderate association with risk to malignant transformation in OPMD is found in patients aged more than 50 years old (Einhorn and Wersall, 1967; Banoczy and Sugar, 1972; Mehta *et al.*, 1972; Gupta *et al.*, 1980; Schepman *et al.*, 1998; Napier and Speight, 2008; Warnakulasuriya *et al.*, 2011; Speight *et al.*, 2018).

As OPMDs are chronic disorders, it is to be expected that the number of cases undergoing MT will increase over time in a given cohort, however, many studies have reported that the majority of cases that undergo MT do so within a span of 5 years from the date of initial presentation (Silverman *et al.*, 1984; Lind, 1987; Schepman *et al.*, 1998; Mehanna *et al.*, 2009). This was also the case for our study, with 71.9% of MT occurring within 5 years from date of initial presentation/diagnosis with the median time to MT being 41 months. There was one case in the molecular experiment cohort that developed OSCC after more than 10 years (128 months) of being diagnosed with an OPMD. This emphasizes the fact that though the risk of MT may diminish after 5 years, it does not disappear completely.

Although there were more male patients with OPMD, there was no statistically significant association between sex and clinical outcome in this cohort. Historically, females have been considered to have a higher risk for MT (Silverman *et al.*, 1984; Schepman *et al.*, 1998; Cowan *et al.*, 2001; Amagasa *et al.*, 2006; Speight, 2007; Napier and Speight, 2008; Speight *et al.*, 2018) however numerous studies have shown either a male predilection or no obvious difference between the sexes when it comes to MT (Einhorn and Wersall, 1967; Gupta *et al.*, 1980; Hsue *et al.*, 2007; Arduino *et al.*, 2009; Warnakulasuriya *et al.*, 2011; Ho *et al.*, 2012; Goodson *et al.*, 2015; Bates *et al.*, 2016).

The majority of OPMDs in this study were leukoplakias (85.5%), and due to the small numbers of other types of OPMD in this cohort, no statistically significant association between clinical outcome and type of OPMD was found. Although the literature is suggestive that erythroplakia has the highest risk of MT amongst OPMDs (Reichart and Philipsen, 2005; El-Naggar *et al.*, 2017), due to the relative rarity of erythroplakia, the true rate of MT is yet unknown.

There was a statistically significant association between site of index OPMD and clinical outcome for this cohort with MT being more frequently seen with lesions from the tongue, though on further sub-analysis (by combining sub-categories) this significance was not apparent. Association between OPMD on the tongue and increased malignant transformation is consistent with the findings of a recent meta-analysis on follow-up of oral dysplasia by Mehanna et al (2009) as well as a systematic review of malignant transformation in oral leukoplakia by Warnakulasuriya and Ariyawardana (2016) (Mehanna *et al.*, 2009; Kuribayashi *et al.*, 2015; Warnakulasuriya and Ariyawardana, 2016). All the tongue lesions in this cohort involved either the lateral or ventral or latero-ventral surfaces of the tongue.

Although the tongue and floor of mouth regions have been described as being “high-risk” areas for the development of OSCC because of prolonged exposure to carcinogens due to saliva pooling (Reibel, 2003; El-Naggar *et al.*, 2017), the location and subsequent progression to cancer of an OPMD may be related to the aetiologic factors that contributed to its development in the first instance. This is best illustrated by risk factor habits such as reverse smoking and betel-quid chewing; where betel-quid chewers will have an increased tendency to have lesions on the buccal mucosa whilst reverse smokers tend to have lesions on the palate (Gupta *et al.*, 1980; Warnakulasuriya and Ariyawardana, 2016; Speight *et al.*, 2018). As such, site of OPMD may be a prognostic indicator for clinical outcome, however due consideration should be given to risk factor habits and geographical variation. Unfortunately, risk factor habits such as tobacco usage and alcohol consumption could not be assessed in this cohort of patients due to incomplete medical records regarding these features.

3.4.2 Oral epithelial dysplasia (OED)

Presence and severity of OED in biopsy specimens is one of the most frequently used prognostic method to direct treatment and management of OPMDs worldwide

(Warnakulasuriya *et al.*, 2008; van der Waal, 2009; El-Naggar *et al.*, 2017; Speight *et al.*, 2018). Even though demographic and clinical parameters may be useful for clinical risk assessment, diagnostic biopsies and histopathological assessment are considered paramount to rule out malignancy and other pathologies that may mimic OPMDs as well as to provide a measure of cytological atypia and architectural changes that have occurred in the tissue to help with risk assessment. The measure most commonly used to communicate the level of cytological atypia and architectural disruption in oral epithelia is dysplasia grading. However, not all OED will undergo malignant change as shown by two recent systematic reviews on the topic of MT in OED, with MT observed in only around 10 – 12% of patients with OED (Mehanna *et al.*, 2009; Shariff and Zavras, 2015).

Intra- and inter-rater agreement has been shown to be a major issue associated with currently used OED grading systems (Abbey *et al.*, 1995; Fischer *et al.*, 2004; Warnakulasuriya *et al.*, 2008; Dost *et al.*, 2014). Some authors have suggested using a binary grading system to reduce intra- and inter-rater variation and improve prognostic strength (Kujan *et al.*, 2006; Kujan *et al.*, 2007; Nankivell *et al.*, 2013). The present study confirms the improvement of the inter-rater agreement when using the binary grading system using weighted kappa scoring, however the scores for both systems were similar in value in the larger cohort; with a high level of agreement between the pathologists (Landis and Koch, 1977). A reason as to why there was an improvement in the inter-rater agreement for the WHO 2017 OED grading system as compared to previous studies is possibly because of the reduced number of levels in the new WHO 2017 system compared to the previous WHO 2005 system (Kujan *et al.*, 2006; Kujan *et al.*, 2007; Nankivell *et al.*, 2013; El-Naggar *et al.*, 2017). One way to improve the prognostic ability of OED grading and reproducibility is through consensus grading exercises especially for cases that are difficult to classify as being either low- or high-grade (Kujan *et al.*, 2007; Warnakulasuriya *et al.*, 2008; Speight *et al.*, 2015; El-Naggar *et al.*, 2017).

Although the binary grading system was statistically significant as a prognostic indicator, the sensitivity and specificity levels for predicting which OPMD would undergo MT were found to be lacking with very low positive predictive values as well. One of the reasons why OED grading will almost always be found to be lacking is the fact that the grading is a snapshot of a supposedly “representative” portion of the oral

lesion. An incisional biopsy may not actually be representative of the true overall pathology due to poor or inadequate sampling. This is well illustrated by studies in which the authors describe “unexpected” OSCC in excision specimens of lesions where the incisional biopsies were diagnosed with either OED or non-dysplastic lesions (Giunta *et al.*, 1969; Pentenero *et al.*, 2003; Holmstrup *et al.*, 2007; Lee *et al.*, 2007; Thomson *et al.*, 2017a; Thomson *et al.*, 2017b). Such incidences where the results from the index biopsy is perceived to have “under-diagnosed” the case are most likely the result of non-representative sampling during the incisional/index diagnostic biopsies (Holmstrup *et al.*, 2007; Lee *et al.*, 2007; Chen *et al.*, 2016). Another possibility could be that the malignant transformation occurred during the period between incisional biopsy and surgical excision.

To overcome this problem, some clinicians advocate “field-mapping” or multiple incisional biopsies, whereby small samples are obtained from various regions of the lesional area (Lee *et al.*, 2007; Thomson and Hamadah, 2007; van der Waal, 2009). Although this may reduce the probability of inadequate or non-representative sampling it does not eliminate the possibility of missing the lesion that has the highest risk of MT. Other attempts have also been made in developing tools and methods to improve screening and detection of OPMDs such as using toluidine blue dye, brush biopsy sampling, fluorescence-based methods and also light reflectance/absorption methods (Lingen *et al.*, 2008; Rashid and Warnakulasuriya, 2015; Spivakovsky and Gerber, 2015; Lingen *et al.*, 2017; Yang *et al.*, 2018). However, none of these adjunct methods have been conclusively proven to improve detection of OPMDs or increase accuracy of biopsy sampling (Lingen *et al.*, 2008; van der Waal, 2009; Brocklehurst *et al.*, 2013; Macey *et al.*, 2015; Spivakovsky and Gerber, 2015; Lingen *et al.*, 2017; Yang *et al.*, 2018)

Lack of prognostic ability is also compounded by “field-change” in OPMD patients. The concept of “field-change” in OPMDs is well established and is possibly one of the reasons why not all OSCCs arise from the site of index OPMD. As such, an OED grade based on a biopsy at one site is not truly representative of the whole oral mucosa (Holmstrup *et al.*, 2007; van der Waal, 2009; Thomson *et al.*, 2017a). The chronicity of OPMDs is another factor that possibly reduces the prognostic utility of OED grading; lesions that have been diagnosed as having mild or low-grade dysplasia on index biopsies maybe considered as having a lower risk of transforming

at that particular point in time, but that does not mean that the lesion is static in nature, with some lesions regressing and others becoming more dysplastic or progressing to cancer.

Clinicians directly involved with patient management may view the binary grading system as being more “user-friendly” compared to the multi-tiered grading systems used traditionally. This is because of the difficulty faced by clinicians and pathologists in ascertaining the risk of malignant transformation related to those graded as being moderately dysplastic. The question always asked of the pathologist is whether these moderately dysplastic lesions should be observed or excised, a question that has no simple answer as was previously mentioned, even lesions with mild/low-grade OED can undergo MT; again, highlighting the shortcomings of OED grading in general.

Although far from being perfect, due to the lack of any other established prognostic factors for clinical outcome in OPMDs, OED grading in conjunction with demographic and clinical findings is still the most utilised method to direct clinical management of OPMD patients (Napier and Speight, 2008; Warnakulasuriya *et al.*, 2008; van der Waal, 2009; Macey *et al.*, 2015; El-Naggar *et al.*, 2017; Speight *et al.*, 2018). However, using a histo-morphological method such as OED grading is bound to be inadequate to predict malignant change in OPMDs due to the complexity of oral carcinogenesis. This underlies the importance of discovering additional methods and biomarkers to augment the prognostic power of OED grading through multivariate prognostic models.

3.4.3 Oral potentially malignant disorders (OPMD) associated leukocytes score (OPALS)

Although in this study there was no statistically significant relationship between OPALS and clinical outcome (n = 166), the true clinical significance of OPMD associated leukocytes (OPAL) is as yet unconfirmed; whether the leukocytic infiltrate influences malignant transformation in OPMDs or is a response to the atypical epithelium and is somewhat protective is still unclear.

Fitzpatrick et al (2014) in their study discovered that specimens with mild and moderate OED as well as OSCC specimens had more lichenoid features than cases with severe OED (Fitzpatrick *et al.*, 2014b). However, in our study, high-grade and

severe OED were more frequently associated with OPAL. This difference may be due to the fact that the current study only looked at one lichenoid-type feature, sub-epithelial inflammatory infiltrate, whilst Fitzpatrick et al (2014) looked at all lichenoid features (Fitzpatrick *et al.*, 2014b). Additionally, it was not clear whether there were differences that could act as confounding factors (such as site of OED) between the OED and OSCC sub-groups in the study by Fitzpatrick et al (2014); sub-groups that have differing clinico-demographic features may have an impact on the presence and intensity of lichenoid features (Fitzpatrick *et al.*, 2014b).

The recently published review on the hallmarks of cancer by Hanahan & Weinberg (2011) highlights the importance of inflammation as an enabling factor for carcinogenesis (Hanahan and Weinberg, 2011). The relationship between chronic inflammation and cancer is rather complex; with some studies showing that long-standing inflammatory conditions predisposes one to carcinogenic change as seen in colon cancer whilst other studies have suggested that inflammatory and immune responses may inhibit carcinogenesis (Balkwill and Mantovani, 2001; O'Byrne and Dalgleish, 2001; Mignogna *et al.*, 2004; Mantovani *et al.*, 2008; Colotta *et al.*, 2009). The role of inflammatory infiltrates in OPMD and OED is even more poorly understood. Results from our study would suggest that in oral carcinogenesis, the presence of sub-epithelial inflammation may not be an enabling factor but more of a host response to eliminate atypical cells with the inflammatory response increasing in tandem with the grade of OED. This however needs to be investigated further using molecular techniques to precisely identify the population of inflammatory cells in OPAL and assess their possible roles in either promotion or antagonism of oral carcinogenesis. There is possibly complex interplay between different inflammatory cells within the stroma and tumour micro-environment which may also be influenced temporally (Allavena *et al.*, 2008; Mantovani *et al.*, 2008; Hanahan and Weinberg, 2011; Feller *et al.*, 2013; Ferris, 2015).

3.4.4 High-risk human papillomavirus (HR-HPV) infection

There have been many studies that have attempted to evaluate the association between HPV and oral precursor lesions with a recent systematic review suggesting that there may be a causal association between HPV and OPMDs (Syrjanen *et al.*, 2011). However, many of the studies included in this systematic review had a high risk of bias with all of them being case-control studies (Syrjanen *et al.*, 2011).

There was a very low prevalence (4.6 %) of high-risk human papillomavirus (HR-HPV) positivity in this cohort with only three cases fulfilling the criteria to be confirmed as HPV-associated OED. This prevalence rate is quite low compared to the 25.3% published in a recent meta-analysis on prevalence of HPV 16/18 in OED (Jayaprakash *et al.*, 2011) and 37.1% published in a recent meta-analysis on detection of HPV in OPMDs (Syrjanen *et al.*, 2011) but comparable to the prevalence of 3.5% for oral oncogenic HPV in healthy individuals published by Kreimer et al (2010) (Kreimer *et al.*, 2010; Jayaprakash *et al.*, 2011; Syrjanen *et al.*, 2011).

The high values for prevalence in OPMD based on previous studies could be due to the use of polymerase chain reaction (PCR) as the sole method to detect HPV DNA. Of the 19 studies included in the systematic review by Syrjanen et al (2011) to assess prevalence of HPV in OPMDs, most used PCR as the sole detection/diagnostic method (Syrjanen *et al.*, 2011). PCR techniques though sensitive, lack specificity and may give a positive result even if the virus was not transcriptionally active (carrier/bystander) and should preferably be used in conjunction with other modalities (Smeets *et al.*, 2007; Thavaraj *et al.*, 2011). Transient infections as well as contamination could be another source of false positives seen when PCR techniques are employed.

At this point in time, there is no convincing evidence related to the role of HPV infection in OPMD and malignant transformation (Yang *et al.*, 2009; Lopes *et al.*, 2011; Lingen *et al.*, 2013; Nankivell *et al.*, 2014; El-Naggar *et al.*, 2017). Results from current studies are still inconclusive and further studies involving larger cohorts need to be performed to better understand the natural history of HPV-associated OPMD and OSCC (Yang *et al.*, 2009; Jayaprakash *et al.*, 2011; Lopes *et al.*, 2011; Lingen *et al.*, 2013; Chung *et al.*, 2014; Nankivell *et al.*, 2014; El-Naggar *et al.*, 2017; Fakhry *et al.*, 2017; Lerman *et al.*, 2017).

3.5 Conclusions

This chapter has highlighted the strengths and weaknesses of clinical and histopathological parameters as prognostic indicators for malignant transformation in OPMD. Clinical parameters were found to be mostly lacking as prognostic indicators for OPMD when larger cohorts were used for analysis. From the histopathological parameters, the binary OED grading system has shown some promise as a

prognostic instrument and is slightly superior to the traditional WHO 2017 three-tiered grading system, but it is still lacking in sensitivity and specificity. The relationship between OPAL and clinical outcome is as yet not very clear though it does appear that OPAL is more of a reaction to the severity of OED. Combining OED grading with clinical parameters may improve clinical prognostication and this will be discussed further in Chapter 7 of this thesis. HR-HPV associated OED is an infrequent finding in OPMDs and the association with MT is still unclear. By assessing and exploring the strengths and weaknesses of current methods (clinical and histological parameters) being employed in risk stratification and directing clinical management of OPMD patients, the findings from this chapter serve to illustrate the need for better prognostic indicators of clinical outcome in these patients.

Table 3.1 Demographic and clinical characteristics of cohort (n = 166)

Characteristics		Non-transforming (NT) n = 134	Malignant transforming (MT) n = 32	p-value
Age Mean [SD] (Range)		58.19 [12.79] (23 – 93)	61.31 [12.74] (35 – 92)	0.216 †
Age at diagnosis	≤ 50 years	38	5	0.180*
	> 50 years	96	27	
Time to last follow-up/ malignant transformation in months Median [IQR] (Range)		61.00 [93.00] (12 – 215)	41.00 [59.75] (7 – 128)	0.015α
Sex	Male	83	21	0.839*
	Female	51	11	
Type of OPMD	Leukoplakia	117	25	0.332**
	Erythroplakia	3	2	
	Erythroleukoplakia	14	5	
Site of index OPMD	Tongue	42	15	0.455**
	Floor of mouth	41	6	
	Buccal mucosa	34	6	
	Gingiva/Alveolar mucosa	7	2	
	Palate	10	3	
Site of index OPMD (binary)	Tongue	42	15	0.103*
	Other sites	92	17	

† Independent t-test; α Mann-Whitney U test; *Fisher's exact test; **Pearson's chi-square test (Exact method); SD = standard deviation; IQR = interquartile range

Table 3.2 Characteristics of cases chosen for molecular experiments (n = 65)

Characteristics		Non-transforming (NT) n = 35	Malignant transforming (MT) n = 30	p-value
Age [Mean (±SD)] (Range)		58.31 [13.57] (34 – 89)	61.97 [12.90] (35 – 92)	0.273 †
Age	≤ 50 years	13	4	0.046*
	> 50 years	22	26	
Time to last follow-up/ malignant transformation in months Median [IQR] (Range)		124.00 [112.00] (20 – 215)	32.50 [48.25] (7 – 128)	< 0.001α
Sex	Male	23	21	0.794*
	Female	12	9	
Type of OPMD	Leukoplakia	29	23	0.650**
	Erythroplakia	3	2	
	Erythroleukoplakia	3	5	
Site of index OPMD	Tongue	8	14	0.028**
	Floor of mouth	20	6	
	Buccal mucosa	5	5	
	Gingiva/Alveolar mucosa	1	2	
	Palate	1	3	
Site of index OPMD (binary)	Tongue	8	14	0.065*
	Other sites	27	16	

† Independent t-test; α Mann-Whitney U test; *Fisher's exact test; **Pearson's chi-square test (Exact method); SD = standard deviation; IQR = interquartile range

Table 3.3 Oral epithelial dysplasia grading of OPMD cases (n = 166)

Oral epithelial dysplasia (OED) grading system		**Non-transforming (NT) n = 134**	**Malignant transforming (MT) n = 32**	**p-value**
WHO 2017 OED grading	No OED	4	1	0.064**
	Mild OED	72	12	
	Moderate OED	33	6	
	Severe OED	25	13	
Binary OED grading	No OED	4	1	0.016**
	Low-grade	87	12	
	High-grade	43	19	

**Pearson's chi-square test (Exact method)

Table 3.4 Association of clinico-pathological features with binary OED grading (n = 166)

Features		No OED n = 5	Low-grade OED n = 99	High-grade OED n = 62	p-value
Age (years)	≤ 50 years	2	31	10	0.065*
	> 50 years	3	68	52	
Sex	Male	2	60	42	0.378*
	Female	3	39	20	
Type of OPMD	Leukoplakia	4	92	46	0.029**
	Erythroplakia	0	1	4	
	Erythroleukoplakia	1	6	12	
Site of index OPMD	Tongue	2	25	30	0.033**
	Floor of mouth	0	29	18	
	Buccal mucosa	2	31	7	
	Gingiva/Alveolar mucosa	1	5	3	
	Palate	0	9	4	
Site of index OPMD (binary)	Tongue	2	25	30	0.010*
	All other sites	3	74	32	

*Fisher's exact test; **Pearson's chi-square test (Exact method)

Table 3.5 Association of clinico-pathological features with WHO 2017 OED grading (n = 166)

Features		No OED n = 5	Mild OED n = 84	Moderate OED n = 39	Severe OED n = 38	p-value
Age (years)	≤ 50 years	2	28	7	6	0.095*
	> 50 years	3	56	32	32	
Sex	Male	2	50	26	26	0.529*
	Female	3	34	13	12	
Type of OPMD	Leukoplakia	4	78	34	26	0.017**
	Erythroplakia	0	1	0	4	
	Erythroleukoplakia	1	5	5	8	
Site of index OPMD	Tongue	2	18	19	18	0.020**
	Floor of mouth	0	23	12	12	
	Buccal mucosa	2	29	4	5	
	Gingiva/alveolar mucosa	1	5	1	2	
	Palate	0	9	3	1	
Site of index OPMD (binary)	Tongue	2	18	19	18	0.004*
	All other sites	3	66	20	20	

*Fisher's exact test; **Pearson's chi-square test (Exact/Monte Carlo method)

Table 3.6 Oral epithelial dysplasia grading of OPMD cases selected for downstream analysis (n = 65)

Oral epithelial dysplasia (OED) grading system		Non-transforming (NT) n = 35	Malignant transforming (MT) n = 30	p-value
WHO 2017 OED grading	Mild OED	18	11	0.490**
	Moderate OED	6	6	
	Severe OED	11	13	
Binary OED grading	Low-grade	23	11	0.026*
	High-grade	12	19	

*Fisher's exact test; **Pearson's chi-square test (Exact method)

Table 3.7 Evaluation of binary OED grading as a prognostic instrument (only cases with OED; n =161)

Statistic	Value based on 12.1% rate MT (Mehanna *et al.*, 2009)	Value based on 10.5% rate of MT (Shariff and Zavras, 2015)	Value based on 4.8% rate of MT (Thomson *et al.*, 2017a)
Sensitivity	61.3%		
Specificity	66.9%		
Accuracy	65.8%		
Positive predictive value	20.3%	17.8%	8.5%
Negative predictive value	92.9%	93.8%	97.3%

Table 3.8 Association of clinic-pathological features with OPALS (n = 166)

Features		OPALS negative n = 77	OPALS positive n = 89	p-value
Clinical outcome	Malignant transformation	18	14	0.240*
	No malignant transformation	59	75	
Age (years)	≤ 50 years	18	25	0.595*
	> 50 years	59	64	
Sex	Male	48	56	0.938*
	Female	29	33	
Type of OPMD	Leukoplakia	65	77	0.316**
	Erythroplakia	4	1	
	Erythroleukoplakia	8	11	
Site of index OPMD	Tongue	30	27	0.073**
	Floor of mouth	19	28	
	Buccal mucosa	14	26	
	Gingiva/alveolar mucosa	4	5	
	Palate	10	3	
WHO 2017 OED grading	No OED	2	3	0.001**
	Mild OED	50	34	
	Moderate OED	17	22	
	Severe OED	8	30	
Binary OED grading	No OED	2	3	0.004*
	Low-grade OED	56	43	
	High-grade OED	19	43	

*Fisher's exact test; **Pearson's chi-square test (Exact method)

Table 3.9 Association of clinic-pathological features with OPALS (n = 65)

Features		OPALS negative n = 27	OPALS positive n = 38	p-value
Clinical outcome	Malignant transformation	17	13	0.026*
	No malignant transformation	10	25	
Age (years)	≤ 50 years	7	10	1.000*
	> 50 years	20	28	
Sex	Male	18	26	1.000*
	Female	9	12	
Type of OPMD	Leukoplakia	20	32	0.233**
	Erythroplakia	4	1	
	Erythroleukoplakia	3	5	
Site of index OPMD	Tongue	8	14	0.724**
	Floor of mouth	11	15	
	Buccal mucosa	4	6	
	Gingiva/alveolar mucosa	1	2	
	Palate	3	1	
WHO 2017 OED grading	Mild OED	16	13	0.075**
	Moderate OED	5	7	
	Severe OED	6	18	
Binary OED grading	Low-grade OED	16	18	0.451*
	High-grade OED	11	20	
HR-HPV status	Negative	27	35	0.260*
	Positive	0	3	

*Fisher's exact test; **Pearson's chi-square test (Exact method)

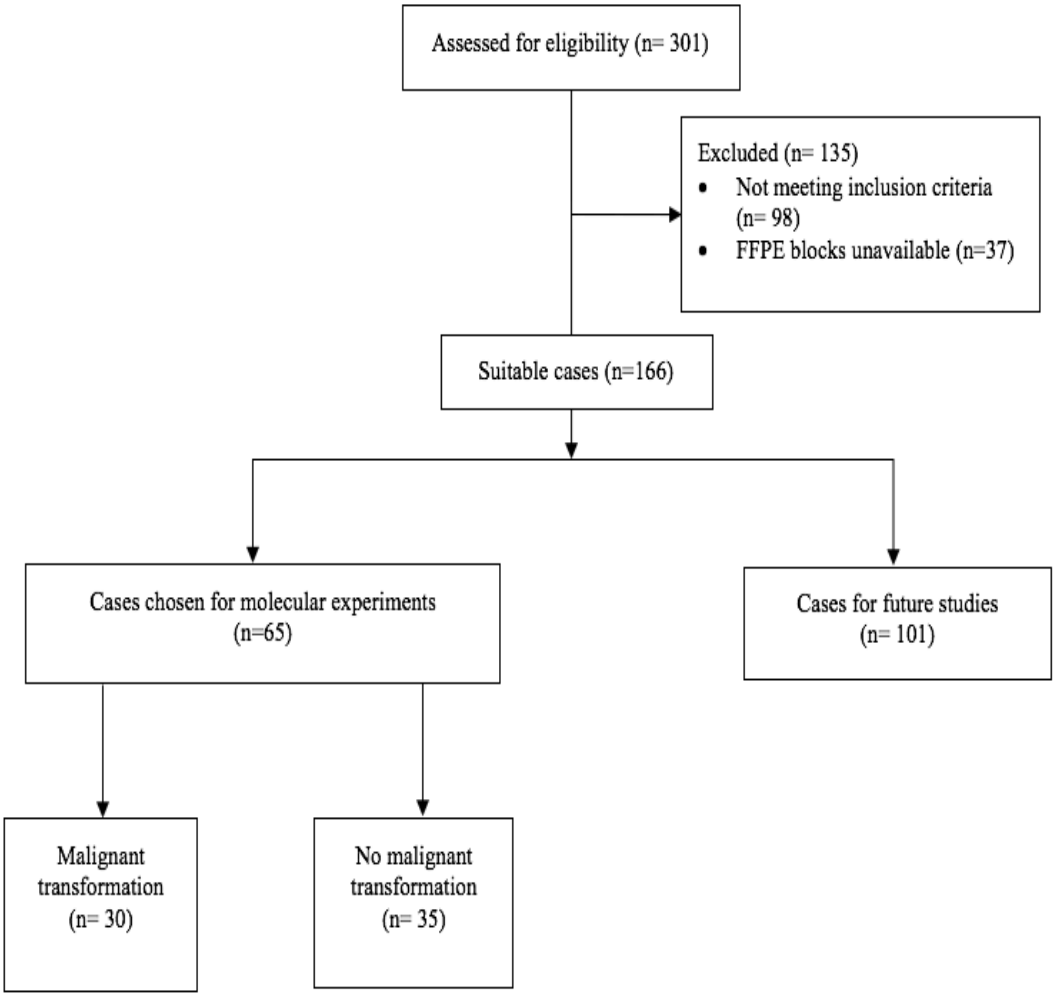


Figure 3.1 Flow chart outlining case selection for study.

Cases were chosen for molecular experiments based on the amount of FFPE tissue available.

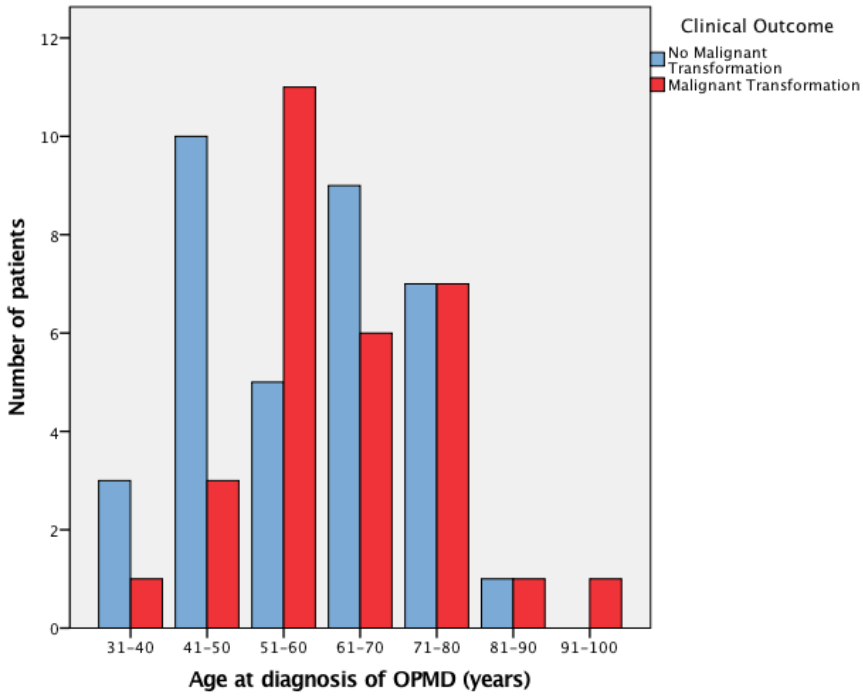
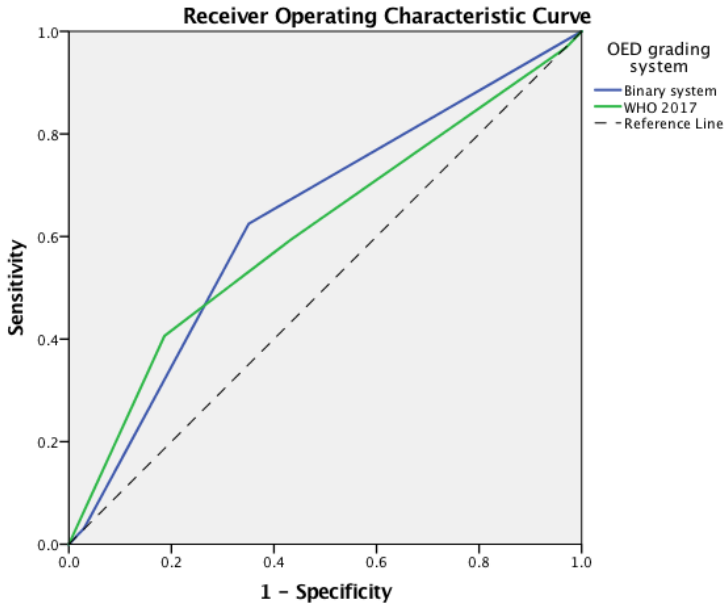


Figure 3.2 Bar chart showing age distribution of OPMD patients selected for molecular studies (n=65).

The patients with malignant transformation appear to be slightly older than the non-transforming group of patients.



OED grading system	Area under curve	Standard error	Asymptotic significance	Asymptotic 95% Confidence Interval	
				Lower Bound	Upper Bound
Binary OED grading	0.633	0.055	0.019	0.526	0.741
WHO 2017 OED grading	0.610	0.059	0.053	0.495	0.726

Figure 3.3 Receiver operating characteristics curves comparing efficacy of binary and WHO 2017 OED grading systems in prognosticating clinical outcome for the OPMD cohort (n = 166).

Only the binary grading system was statistically significant for prognosticating malignant transformation in OPMD (p = 0.019).

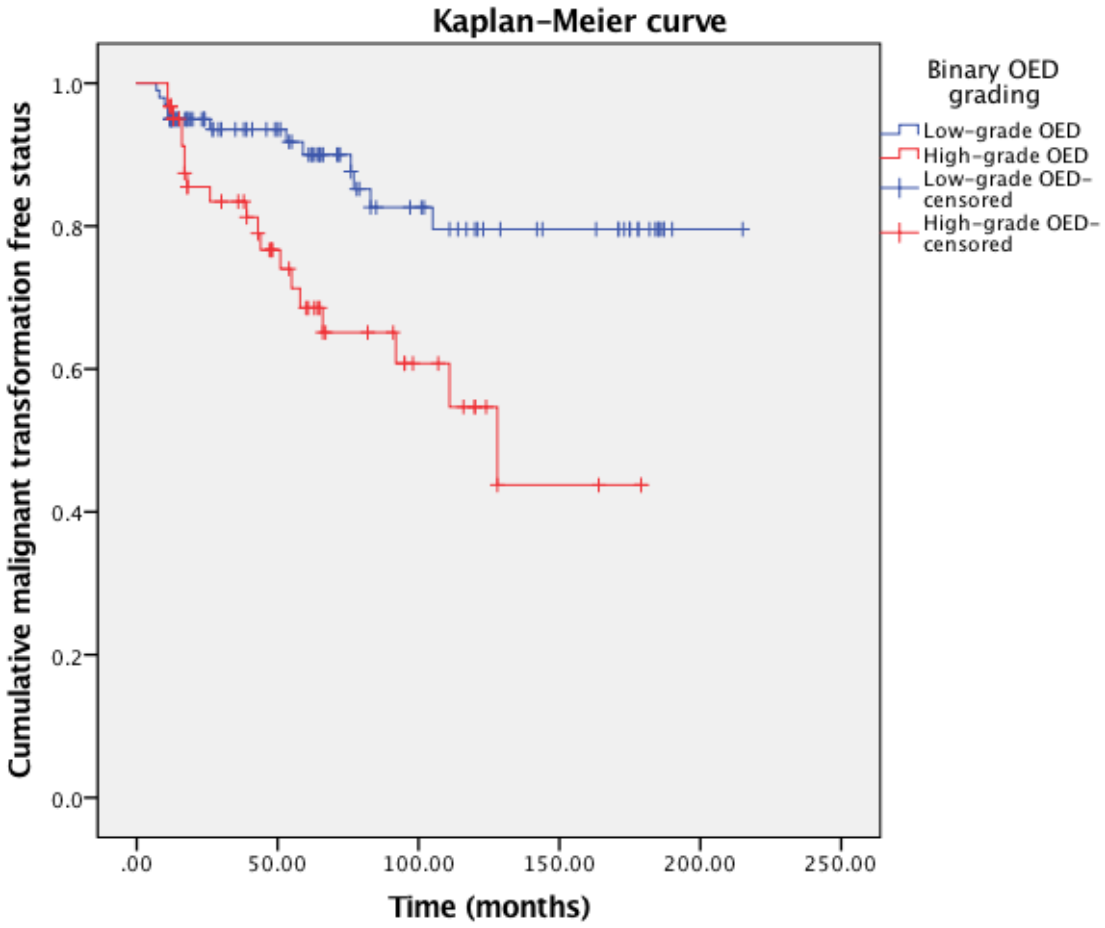


Figure 3.4 Kaplan-Meier curve for time to event analysis comparing malignant transformation in the OPMD cohort with OED stratified according to binary OED grading (n = 161).

There was statistically significant correlation between binary grading and clinical outcome with high-grade cases undergoing MT much more frequently and earlier than low-grade cases (Log rank test; p = 0.003; χ^2= 9.05; df = 1); Low-grade OED = blue line; High-grade OED = red line.

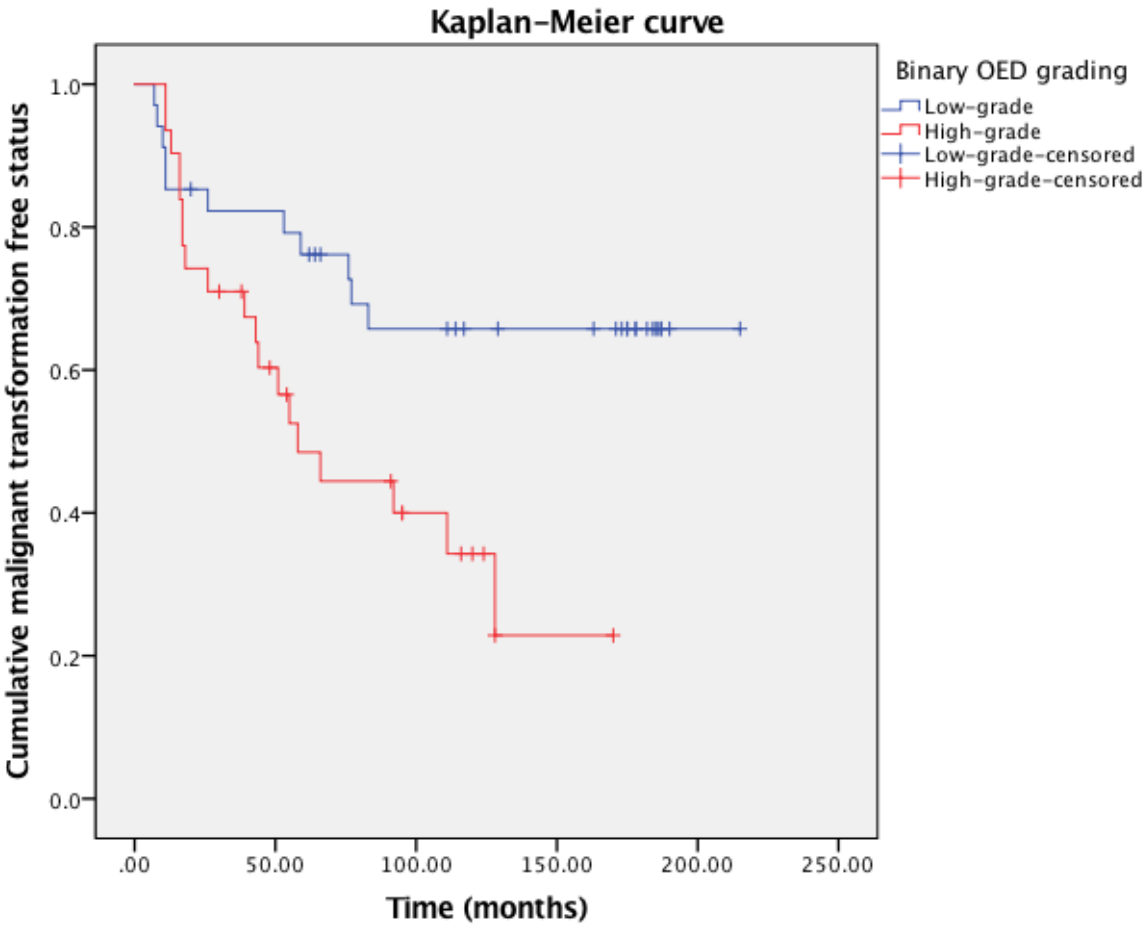


Figure 3.5 Kaplan-Meier curve for time to event analysis comparing malignant transformation in the OPMD cohort chosen for molecular assays stratified according to binary OED grading (n = 65).

There was statistically significant correlation between binary grading and clinical outcome with high-grade cases undergoing MT much more frequently and earlier than low-grade cases (Log rank test; p = 0.012; χ^2= 6.34; df = 1); Low-grade OED = blue line; High-grade OED = red line.

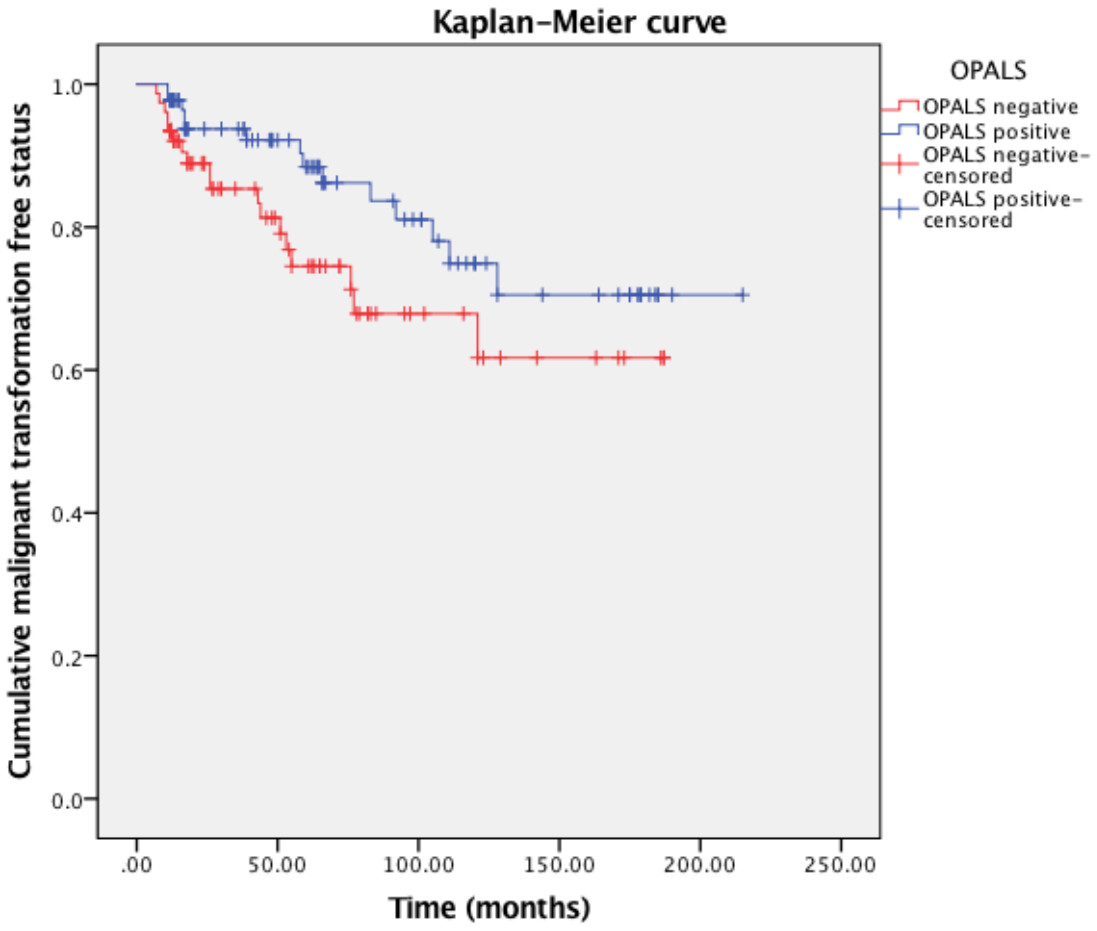


Figure 3.6 Kaplan-Meier curve for time to event analysis comparing malignant transformation in the OPMD cohort stratified according to OPALS (n = 166).

Although cases with a negative OPAL score appear to have poorer prognosis, the findings were not statistically significant (Log rank test; p = 0.084; χ^2= 2.99; df = 1); OPALS positive = blue line; OPALS negative = red line.

Chapter 4. DNA Ploidy Analysis

4.1 Introduction

It has been established that the majority of cancer cells exhibit abnormal genetic material and such variation in DNA content is also seen in some pre-malignant lesions (Grassel-Pietrusky *et al.*, 1982; Sen, 2000; Pihan and Doxsey, 2003; Duesberg *et al.*, 2004; Torres-Rendon *et al.*, 2009b; Duijf and Benezra, 2013). Abnormal or irregular DNA chromosomal complement exhibiting a karyotype that is not in multiples of a haploid set of chromosomes is known as aneuploidy. Aneuploidy is a marker and possibly the result of genomic instability. Genomic instability is considered an enabling characteristic that facilitates and drives carcinogenesis (Hanahan and Weinberg, 2011). Aneuploidy is most likely due to chromosomal instability, whereby defects in chromosomal segregation during mitotic cell division lead to variation in chromosomal copy numbers as well as chromosomal structure (Danielsen *et al.*, 2016). Ploidy analysis has been successfully used as a prognostic instrument for Barret's oesophagus (Dunn *et al.*, 2010), colorectal carcinoma (Hveem *et al.*, 2014) and a host of other cancers that have been reviewed recently by Danielsen et al (2016).

The utility and role of DNA ploidy status in OPMD and OED has been studied with great interest in recent years (Pentenero *et al.*, 2009; Torres-Rendon *et al.*, 2009b; Bradley *et al.*, 2010; Donadini *et al.*, 2010; Bremmer *et al.*, 2011; van Zyl *et al.*, 2012; Siebers *et al.*, 2013; Sperandio *et al.*, 2013). DNA ploidy analysis measures the total DNA content in nuclei and is not synonymous with conventional chromosomal ploidy analysis that measures chromosome numbers (Haroske *et al.*, 2001; Danielsen *et al.*, 2016). A recent meta-analysis suggested that aneuploidy may be of value as a biomarker for malignant transformation in OPMD, however this was a meta-analysis of only five retrospective studies (Alaizari *et al.*, 2018). DNA ploidy status has also been shown to be associated with the presence and severity of OED, with dysplastic lesions being more frequently aneuploid than non-dysplastic lesions and increasing severity of dysplasia being more frequently associated with an aneuploid status (Grassel-Pietrusky *et al.*, 1982; Saito *et al.*, 1995; Pentenero *et al.*, 2009; van Zyl *et*

al., 2012). A study by Islam et al (2010) found that lesions from the tongue have the highest frequency of aneuploidy compared to other oral sub-sites (Islam *et al.*, 2010).

DNA ploidy assessment for clinical specimens is most often performed using either image-based cytometry (IBC) or flow cytometry (Haroske *et al.*, 2001; Danielsen *et al.*, 2016). Flow cytometry requires fresh tissue samples (nuclei in suspensions) whilst IBC can be performed even using formalin-fixed paraffin-embedded (FFPE) material (Brouns *et al.*, 2012; Danielsen *et al.*, 2016). DNA ploidy status determination is based on DNA content measured using Feulgen-Schiff staining (stoichiometrically binds to DNA) in IBC or fluorescent dyes in flow cytometry (Caspersson, 1987; Carey, 1994; Biesterfeld *et al.*, 2011). Aside from the obvious advantage of being able to use FFPE material, IBC also allows users to specifically select areas of interest in the tissue prior to processing and smaller tissue samples can also be analysed (Danielsen *et al.*, 2016). IBC also allows users to sub-classify nuclei into different cell types based on morphology, allows quantitative measurement of nuclear morphology and has been shown to have better discriminatory value than flow-based cytometry (Bol *et al.*, 2003; Belien *et al.*, 2009; Dunn *et al.*, 2010; Danielsen *et al.*, 2016). In recent years, fluorescence *in situ* hybridisation (FISH) based methods using centromere probes to detect aneuploidies of specific chromosomes have been used as well (Rygiel *et al.*, 2007; Schramm *et al.*, 2011; Danielsen *et al.*, 2016).

In IBC, the amount of DNA within a cell is obtained by assessing the staining density of a dye (such as Feulgen's stain) that binds with DNA in a stoichiometric way (Caspersson, 1987; Carey, 1994; Biesterfeld *et al.*, 2011). Nuclear DNA content is thus expressed as integrated optical density (IOD) (Caspersson, 1987; Haroske *et al.*, 2001; Danielsen *et al.*, 2016). The amount of nuclear DNA from the target cells is compared to nuclear content in control/reference cells and scaled in units/multiples of 'c'. A value of '2c' is representative of the DNA content of diploid cells in the G0/G1 phases of cell cycle. The 2c value is usually established using control/reference cells such as lymphocytes or fibroblasts (depending on the target tissue) (Caspersson, 1987; Haroske *et al.*, 2001; Danielsen *et al.*, 2016). It must be emphasised that this is a scaling to represent DNA content and not chromosomal ploidy status.

DNA ploidy analysis output is usually in the form of a DNA content histogram that describes the frequency of nuclei containing differing amounts of DNA reflective of

the cell-cycle of diploid cells (Caspersson, 1987; Ross *et al.*, 2003; Darzynkiewicz, 2010; Biesterfeld *et al.*, 2011; Danielsen *et al.*, 2016). The resulting DNA ploidy histogram is interpreted as being either 'diploid', 'tetraploid' or 'aneuploid' depending on the distribution of the cells according to DNA content. A DNA ploidy histogram is interpreted as being diploid when a major peak is present at point 2c (IOD that corresponds to the DNA content of 46 chromosomes) whilst the corresponding peaks for G2/M and S phases of cell cycle (up to point 4c on the histogram) are minor and account for between 10 - 15% of the cells (Haroske *et al.*, 2001; Sperandio *et al.*, 2013; Danielsen *et al.*, 2016). A histogram may be interpreted as being tetraploid if there is a large/distinct peak at 4c (G2/M) together with a smaller population of cells at 8c or if ≥ 10% of target nuclei have DNA content of 4c. This would indicate that the specimen has a higher than usual number of cells with double the normal DNA content. The presence of a prominent separate peak differing from 2c and which cannot be accounted for by a 4c (G2) peak, is defined as an aneuploid peak (Haroske *et al.*, 2001; Danielsen *et al.*, 2016).

Scattering or spreading of DNA content around peaks in the histogram is due to variation in staining, instrumental errors and presence of debris (Haroske *et al.*, 2001; Danielsen *et al.*, 2016). This variation is expressed using the coefficient of variation (CV) and is measured on the 2c peak (Rabinovitch, 1994). Too large a CV would mean reduced resolution and an inability to detect abnormal peaks (Haroske *et al.*, 2001; Danielsen *et al.*, 2016). The ratio of the modal DNA content of target cell nuclei to reference nuclei (diploid) is known as the DNA index (DI); for normal tissue, DI for cells in G0/G1 would be 1.0, cells in G2/M would be 2.0 and for cells in S phase it would be between 1.0 and 2.0 (Shankey *et al.*, 1993; Haroske *et al.*, 2001; Danielsen *et al.*, 2016). Researchers would need to take into consideration all the mentioned factors when attempting to accurately interpret DNA ploidy histograms.

4.2 Aims

The aims of this chapter are:

1) To assess association between DNA ploidy status and clinical outcome in OPMD.
2) To assess association between DNA ploidy status and oral epithelial dysplasia.

4.3 Results

Of the 65 cases that were suitable for the study, ploidy analysis could not be carried out for 22 cases due to lack of FFPE material. Of the remaining cases, eight cases failed DNA ploidy analysis due to inadequate nuclei from the available material. DNA ploidy analysis was successfully performed on 35 cases.

4.3.1 Patient characteristics

Mean patient age at diagnosis of index lesion was 59.97 (SD ±13.00) with 80.0% of patients being more than 50 years of age at diagnosis. There was a male predominance in this cohort (65.7%). Leukoplakia was the most frequently encountered OPMD (80.0%) and the tongue was the most frequent site for index lesions (40.0%). There were 18 cases that underwent malignant transformation (MT) in this cohort versus 17 cases that did not undergo malignant change (NT). Patients aged > 50 years at age of diagnosis with OPMD were more likely to undergo MT ($p = 0.041$; $\chi^2 = 4.83$; $df = 1$). The cohort of patients with OPMD that underwent malignant transformation (MT) had a male preponderance although this finding was not statistically significant (Table 4.1). The tongue was the most commonly involved site that underwent MT, although not statistically significant for this cohort ($p > 0.05$) (Table 4.1). The non-transforming (NT) cases were followed-up for a median of more than 10 years whilst MT cases occurred within a median of 28.5 months of OPMD diagnosis in this cohort (Table 4.1). OPALS and the WHO 2017 OED grading system were not significantly associated with clinical outcome in this cohort of patients whilst the binary grading system was significantly associated with clinical outcome ($p = 0.006$; $\chi^2 = 8.58$; $df = 1$) (Table 4.1).

4.3.2 DNA ploidy and clinico-pathologic features

From the successfully analysed cases, 26 (74.3%) were diploid and 9 (25.7%) were aneuploid; there were no tetraploid cases. Examples of DNA ploidy histograms from this study are shown in Figure 4.1. The mean coefficient of variation (CV) of the diploid peaks for the samples in this study was 1.63 (SD ± 0.30; range 1.15 to 2.17). Of the 18 cases that underwent MT, 14 were scored as being diploid (77.8%) and four (22.2%) as aneuploid (Table 4.1 and Table 4.2). DNA ploidy status and clinical outcome were found to have no statistically significant relationship ($p > 0.05$) in this

cohort of patients (Table 4.1 and Table 4.2). There was no statistically significant association between any of the clinico-pathologic features and DNA ploidy status (Table 4.2). The OED grading and clinical outcome of these 35 patients according to DNA ploidy status are shown in Table 4.2.

Statistical analysis was repeated after excluding the two HR-HPV positive cases and the results showed that DNA ploidy status was not significantly associated with clinical outcome or OED grading (Table 4.3). Time to event analysis on this cohort of patients showed that DNA ploidy status was not significantly associated with clinical outcome for this cohort of patients (Figure 4.2; Log rank test; $p = 0.610$; $\chi^2 = 0.26$; $df = 1$). Sensitivity and specificity analysis of DNA ploidy in relation to clinical outcome showed that the sensitivity, positive predictive value and negative predictive value of this method were not as good as the binary OED grading system (Table 4.4).

4.4 Discussion

4.4.1 Patient characteristics

The age distribution of patients according to clinical outcome in this cohort was similar to other studies on DNA ploidy analysis of OPMD cases, with the group of patients that underwent MT having a mean age at diagnosis with OPMD that was higher than the mean age of NT cases (Torres-Rendon *et al.*, 2009b; Bradley *et al.*, 2010; Siebers *et al.*, 2013). However, the age difference between the two sub-groups (MT vs NT) was only statistically significant when patients were categorised as being either $\leq$ 50 years or > 50 years at age of diagnosis with OPMD. This finding is similar to previous studies that have looked at the clinical and demographic characteristics of OPMD patients that underwent MT (Einhorn and Wersall, 1967; Banoczy and Sugar, 1972; Mehta *et al.*, 1972; Gupta *et al.*, 1980; Schepman *et al.*, 1998; Napier and Speight, 2008; Warnakulasuriya *et al.*, 2011; Speight *et al.*, 2018).

The male predominance of this cohort is reflective of most studies on OPMD and OSCC, but sex was not significantly associated with clinical outcome in this cohort. At this point in time there is no consensus regarding the influence sex on clinical outcome in OPMD patients with some studies reporting that females having higher risk of MT, others reporting that males are more likely to undergo MT and others still reporting that there is no statistically significant difference (Einhorn and Wersall,

1967; Gupta *et al.*, 1980; Silverman *et al.*, 1984; Schepman *et al.*, 1998; Cowan *et al.*, 2001; Amagasa *et al.*, 2006; Hsue *et al.*, 2007; Arduino *et al.*, 2009; Warnakulasuriya *et al.*, 2011; Ho *et al.*, 2012; Goodson *et al.*, 2015; Bates *et al.*, 2016).

A number of studies on DNA ploidy and OPMD have reported some association between DNA ploidy status and OED grade with the majority of aneuploidy being seen in cases having severe OED (Saito *et al.*, 1995; Pentenero *et al.*, 2009; Bradley *et al.*, 2010; van Zyl *et al.*, 2012; Sperandio *et al.*, 2013). This trend was not present in our study, with no statistically significant association seen between OED grade and ploidy status.

The mean CV of the diploid peaks for the samples in this study was 1.63 (SD ± 0.30; range 1.15 to 2.17) which is well below the 5% diagnostic criteria cut-off. The low CV value meant a higher resolution and ability to discern minor aneuploid peaks (Rabinovitch, 1994; Danielsen *et al.*, 2016). DNA ploidy analysis for this study was performed at an established laboratory that provides DNA ploidy analysis as part of routine diagnostic pathology services and has been involved in published research on DNA ploidy analysis in OPMD (Sperandio *et al.*, 2013; Sperandio *et al.*, 2016). The ploidy histograms obtained were validated by an experienced pathologist, Professor Edward Odell (EWO), thus ensuring the quality of the experiment. The diagnostic criteria used for our study were the same as published previously (Haroske *et al.*, 2001; Sperandio *et al.*, 2013; Sperandio *et al.*, 2016).

4.4.2 DNA ploidy and clinical outcome

The relationship between DNA ploidy status and clinical outcome in OPMD patients has been studied with renewed interest in recent years (Grassel-Pietrusky *et al.*, 1982; Saito *et al.*, 1995; Pentenero *et al.*, 2009; Torres-Rendon *et al.*, 2009b; Bradley *et al.*, 2010; Donadini *et al.*, 2010; Bremmer *et al.*, 2011; van Zyl *et al.*, 2012; Siebers *et al.*, 2013; Sperandio *et al.*, 2013). Many of the studies on clinical outcome of OPMD have shown that DNA ploidy status has some value as a prognostic biomarker in such cases although most of the studies were retrospective in nature and involved cohorts from single centres (Torres-Rendon *et al.*, 2009b; Bradley *et al.*, 2010; Bremmer *et al.*, 2011; Siebers *et al.*, 2013; Sperandio *et al.*, 2013). A recently published meta-analysis by Alaizari et al (2018) assessing the utility of DNA ploidy as

a biomarker in OPMD cases concluded that aneuploidy was a good predictor of malignant change in OPMD (Alaizari *et al.*, 2018). However, the meta-analysis only included five retrospective studies, highlighting the limited availability of scientific evidence regarding the role of DNA ploidy analysis in prognosticating clinical outcome for OPMD patients (Alaizari *et al.*, 2018).

The current study failed to show any statistically significant correlation between DNA ploidy status and clinical outcome in OPMD. An important point to note is that not all lesions classified as being aneuploid underwent MT and conversely some lesions classified as being diploid did undergo MT; this is similar in some ways to OED grading whereby non-dysplastic or lesions with low-grade dysplasia may undergo MT whilst some lesions with high-grade OED will not undergo MT. However, it was demonstrated that IBC-DNA ploidy analysis was inferior to the binary OED grading system with regard to predicting malignant transformation in OPMD in this cohort.

Sperandio et al (2013) using a relatively large cohort of patients showed that DNA ploidy analysis was at least as good as the WHO OED grading as a prognostic marker of malignant change in OED (Sperandio *et al.*, 2013). The discrepancy between our study and the previous study could be due to the limiting factor of the small sample size in our study. However, even though the study by Sperandio et al (2013) had larger overall numbers, it had only 15 patients in whom MT occurred more than 6 months after index biopsy with the majority of lesions undergoing MT being diploid (53.3%) reflecting the inherently poor prognostic ability of DNA ploidy analysis (Sperandio *et al.*, 2013).

Previous studies assessing the efficacy of DNA ploidy in predicting MT in either OED or OPMD usually compared their findings with the traditional method of OED grading, the multi-tiered WHO system (hyperplasia, mild OED, moderate OED, severe OED and carcinoma in-situ) (Torres-Rendon *et al.*, 2009b; Bradley *et al.*, 2010; Bremmer *et al.*, 2011; Siebers *et al.*, 2013; Sperandio *et al.*, 2013). From Table 4.4, it becomes clear that the results for DNA ploidy (past and present studies) and the WHO 2017 grading system are comparable and not as good as the results from the current study using the binary OED grading system (Torres-Rendon *et al.*, 2009b; Bradley *et al.*, 2010; Bremmer *et al.*, 2011; Siebers *et al.*, 2013; Sperandio *et al.*, 2013). Results from Section 3.3.3 of this thesis as well as Table 4.4 clearly show that for this cohort the binary OED grading system performed better than the WHO 2017 OED grading

system as a prognostic instrument for OPMD patients. As such, it could be hypothesized that DNA ploidy analysis may be equivalent to the WHO 2005 OED grading system (Barnes *et al.*, 2005) but is inferior to the binary OED grading system for prognosticating clinical outcome in OPMD patients.

A sub-analysis after excluding HR-HPV positive cases was performed as active viral infections can change nuclear DNA content that is detectable by image-based cytometry (Haroske *et al.*, 2001). After excluding the HR-HPV positive cases, the association between DNA ploidy status and clinical outcome was still not statistically significant. The influence of HPV-associated OED has not been considered or raised in previous studies reporting on DNA ploidy assessment of oral lesions (Torres-Rendon *et al.*, 2009b; Bradley *et al.*, 2010; Bremmer *et al.*, 2011; Siebers *et al.*, 2013; Sperandio *et al.*, 2013). Other studies have indicated a rather high prevalence of HPV in the oral cavity with a recent meta-analysis arriving at a prevalence rate of 25.3% for HPV 16/18 presence in OED (Jayaprakash *et al.*, 2011) and another meta-analysis on detection of HPV in OPMD concluding that 37.1% of OPMD have detectable HPV (Syrjanen *et al.*, 2011). The natural history of HPV-associated OED is as yet not fully understood but it is imperative that future studies on OED/OPMD consider including assessment of HR-HPV infection as part of their study design to ensure clarity of any results obtained. Studies that have not assessed the influence of HPV infection on DNA ploidy analysis should then be read with caution.

A major limitation of this technique is the requirement for large amounts of epithelial tissue required to produce viable results unlike OED grading or immunohistochemistry-based biomarkers that require at most a few sections of FFPE tissue. From our cohort, IBC DNA ploidy analysis could not be performed on almost half the cases (46.2%) due to insufficient amounts of tissue available. Access to large amounts of tissue would be feasible for excision specimens, however, though most OPMDs would undergo incisional biopsies as part of the diagnostic process, surgical excisions are currently not the standard of care for all OPMDs (van der Waal, 2009; Balasundaram *et al.*, 2014; Lodi *et al.*, 2016). One of the main reasons why many of the samples in this study failed DNA ploidy analysis could be because they were small incisional biopsy specimens. As such, DNA ploidy analysis may not be a feasible biomarker for small incisional biopsies that are routinely available for most OPMD cases.

Another aspect of IBC-based DNA ploidy analysis that is not discussed frequently in the literature is the presence and influence of intra- and inter-observer variation in cell selection for IBC and interpretation of DNA ploidy histograms. The presence of such variability is similar to OED grading and its' influence cannot be under-estimated. It would be highly valuable for fellow researchers if studies could assess and report intra- and inter-observer variation using conventional methods.

It is also obvious that both DNA ploidy analysis as well as OED grading are not very good at prognosticating malignant transformation (poor positive predictive value) but are good at prognosticating lesions that are unlikely to undergo malignant change (good negative predictive values) (Sperandio *et al.*, 2013). There is some merit in suggestions that combining DNA ploidy analysis and OED grading will improve predictive values, but it would appear that the degree of improvement conferred would be minimal at best (Sperandio *et al.*, 2013). Though one may argue that some improvement is better than none, it would be best to investigate the benefit of doing additional DNA ploidy testing in augmenting the prognostic power of OED grading on larger cohorts of OPMD patients with known clinical outcomes before recommending it for routine clinical practice. It is acknowledged that the results from the current study should be interpreted with caution as the number of cases analysed were small.

4.5 Conclusions

Although previous studies have shown that DNA ploidy is a useful prognostic method for patients with OPMDs, similar results were not obtained from this study. However, the consistent finding of DNA ploidy analysis having low positive predictive values leaves much to be desired. The amount of tissue required for analysis is also a major limitation for clinical utility in OPMD patients availing only incisional biopsy samples. DNA ploidy analysis at this point in time does not seem to be much better than OED grading for predicting clinical outcome in OPMD patients and cannot be advocated for sole use in risk-stratification of patients with OPMD. Larger multicentre or prospective studies that include assessment of HR-HPV infection as part of the study design will be required before the true utility of this method as a prognostic biomarker for OPMD can be fully assessed.

Table 4.1 Characteristics of cases according to clinical outcome (n = 35)

Characteristics		Non-transforming (NT) n = 17	Malignant transforming (MT) n = 18	p-value
Age (years) Mean [SD] (Range)		56.41 [11.23] (34 -74)	63.33 [13.95] (35 – 92)	0.117†
Age	≤ 50 years	6	1	0.041*
	> 50 years	11	17	
Time to last follow-up/malignant transformation in months Median [IQR] (Range)		175.00 [40.00] (91 – 215)	28.50 [67.50] (7 – 128)	< 0.001α
Sex	Male	10	13	0.489*
	Female	7	5	
Type of OPMD	Leukoplakia	14	14	0.692**
	Erythroplakia	2	1	
	Erythroleukoplakia	1	3	
Site of OPMD	Tongue	4	10	0.105**
	Floor of mouth	9	2	
	Buccal mucosa	2	2	
	Gingiva/Alveolar mucosa	1	2	
	Palate	1	2	
Site of OPMD (binary)	Tongue	4	10	0.086*
	Others	13	8	
OPALS	OPALS negative	5	8	0.489*
	OPALS positive	12	10	
WHO 2017 OED grading	Mild	10	6	0.165**
	Moderate	4	3	
	Severe	3	9	
Binary OED grading	Low-grade	14	6	0.006*
	High-grade	3	12	
HR-HPV status	Negative	16	17	1.000*
	Positive	1	1	
DNA ploidy status	Diploid	12	14	0.711*
	Aneuploid	5	4	

† Independent t-test; α Mann-Whitney U test; *Fisher's exact test; **Pearson's chi-square test (Exact method); IQR = interquartile range; SD = standard deviation

Table 4.2 Characteristics of cases according to DNA ploidy status (n = 35)

Characteristics		Diploid n = 26	Aneuploid n = 9	p-value
Age (years) Mean [SD] (Range)		56.00 [14.34] (34 -92)	62.78 [8.00] (52 – 77)	0.461†
Age	≤ 50 years	7	0	0.153*
	> 50 years	19	9	
Time to last follow-up/malignant transformation in months Median [IQR] (Range)		100.00 [159.00] (7 – 187)	95.00 [105.50] (8-215)	0.806α
Sex	Male	17	6	1.000*
	Female	9	3	
Type of OPMD	Leukoplakia	22	6	0.623**
	Erythroplakia	2	1	
	Erythroleukoplakia	2	2	
Site of OPMD	Tongue	10	4	0.941**
	Floor of mouth	8	3	
	Buccal mucosa	3	1	
	Gingiva/Alveolar mucosa	2	1	
	Palate	3	0	
Site of OPMD (binary)	Tongue	10	4	0.086*
	Others	16	5	
OPALS	OPALS negative	11	3	0.431*
	OPALS positive	15	7	
WHO 2017 OED grading	Mild	14	2	0.311**
	Moderate	4	3	
	Severe	8	4	
Binary OED grading	Low-grade	16	4	0.451*
	High-grade	10	5	
HR-HPV status	Negative	24	9	1.000*
	Positive	2	0	
Clinical outcome	Non-transforming (NT)	12	5	0.711*
	Malignant transforming (MT)	14	4	

† Independent t-test; α Mann-Whitney U test; *Fisher's exact test; **Pearson's chi-square test (Exact method); IQR = interquartile range; SD = standard deviation

Table 4.3 Selected characteristics of HR-HPV negative cases according to DNA ploidy status (n = 33)

Characteristics		Diploid n = 24	Aneuploid n = 9	p-value
Clinical outcome	Non-transforming (NT)	11	5	0.708*
	Malignant transforming (MT)	13	4	
WHO 2017 OED grading	Mild	14	2	0.151**
	Moderate	3	3	
	Severe	7	4	
Binary OED grading	Low-grade	15	4	0.442*
	High-grade	9	5	

*Fisher's exact test; **Pearson's chi-square test (Exact method)

Table 4.4 Evaluation of DNA ploidy status (previous and current studies) as a prognostic instrument in OPMD (n = 35)

Statistic	Sperandio et al (2013) (MT > 6 months after index biopsy) **	IBC-DNA Ploidy	WHO 2017 grading (mild & moderate vs severe OED)	Binary grading
Sensitivity	46.7%	22.2%	50.0%	66.7%
Specificity	83.8%	70.6%	82.5%	82.4%
Accuracy	81.6%	45.7%	65.7%	74.3%
***Positive predictive value**	25.3%	8.1%	25.1%	30.8%
***Negative predictive value**	93.1%	88.6%	93.4%	95.5%

*Value based on 10.5% prevalence rate of MT in OED as all cases in this cohort had some level of OED (Shariff and Zavras, 2015). **Diploid and tetraploid results were combined.

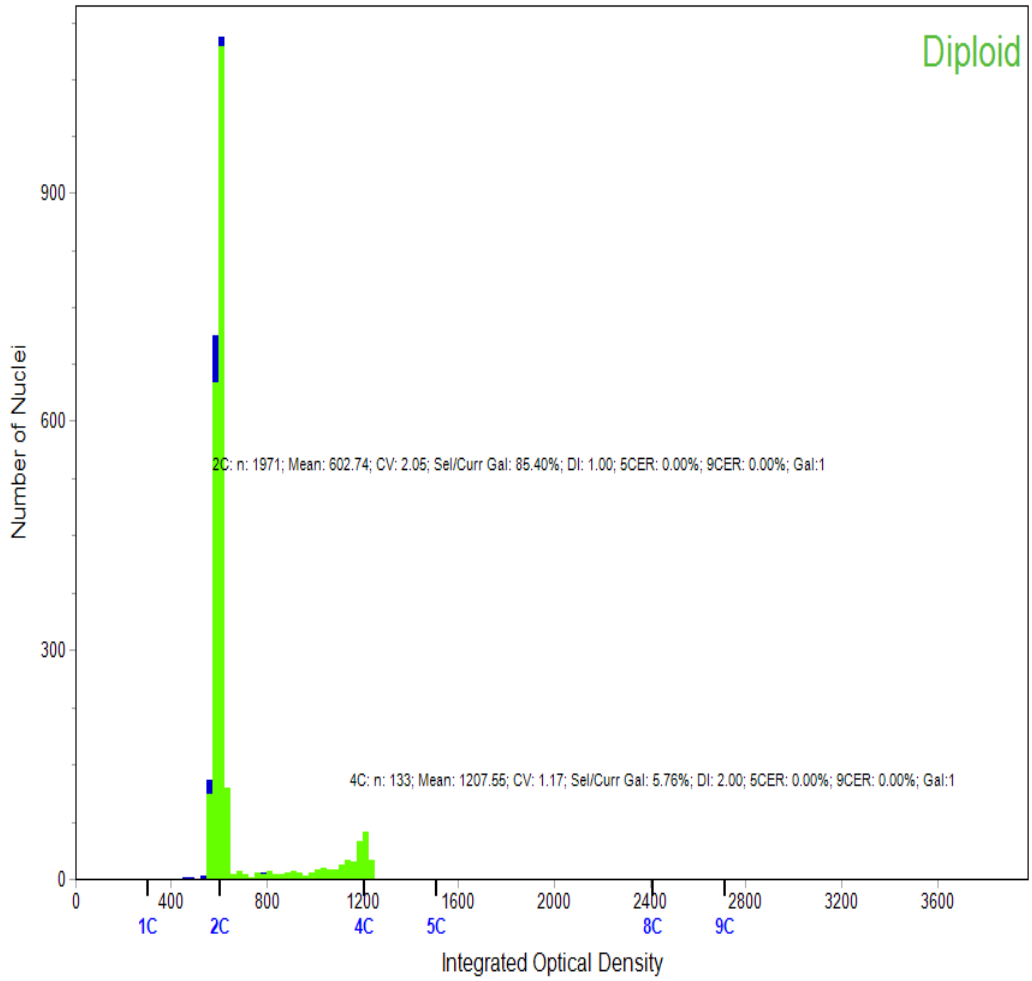


Figure 4.1 Example of IBC DNA ploidy histogram of a diploid case from this study.

X axes show integrated optical density (IOD) whilst Y axes show number of nuclei. Green: Epithelial cell nuclei; Blue: Internal control/reference nuclei (diploid nuclei). Summary: One 2c peak with 1971 nuclei and one 4c peak <10% of nuclei. No obvious 5c peak.

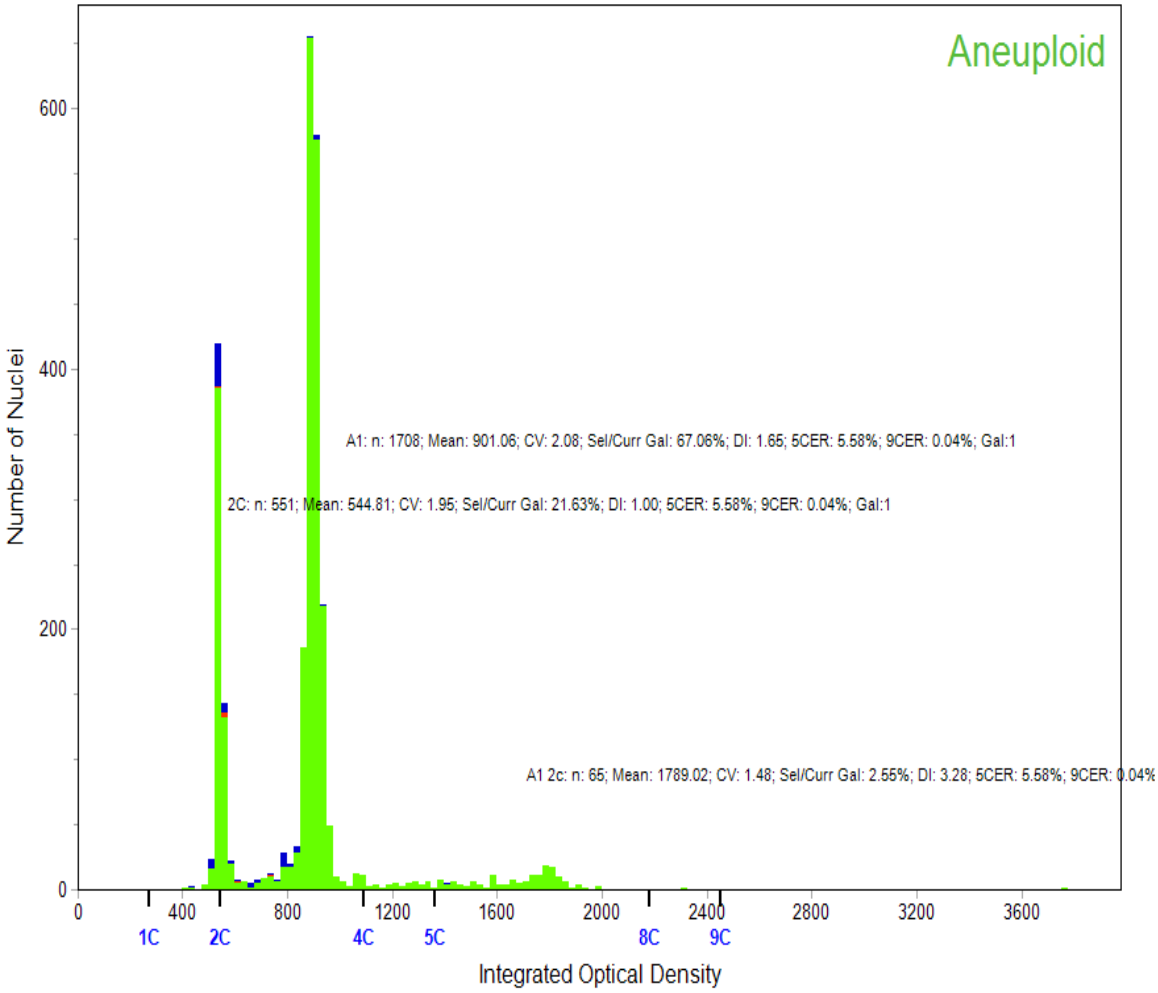


Figure 4.2 Example of IBC DNA ploidy histogram of an aneuploid case from this study.

X axes show integrated optical density (IOD) whilst Y axes show number of nuclei. Green: Epithelial cell nuclei; Blue & Red: Internal control/reference nuclei (diploid nuclei). Summary: Large aneuploid peak (A1) with 1708 nuclei (67.1% of total nuclei) and 5c peak composed of more than 1% of total nuclei.

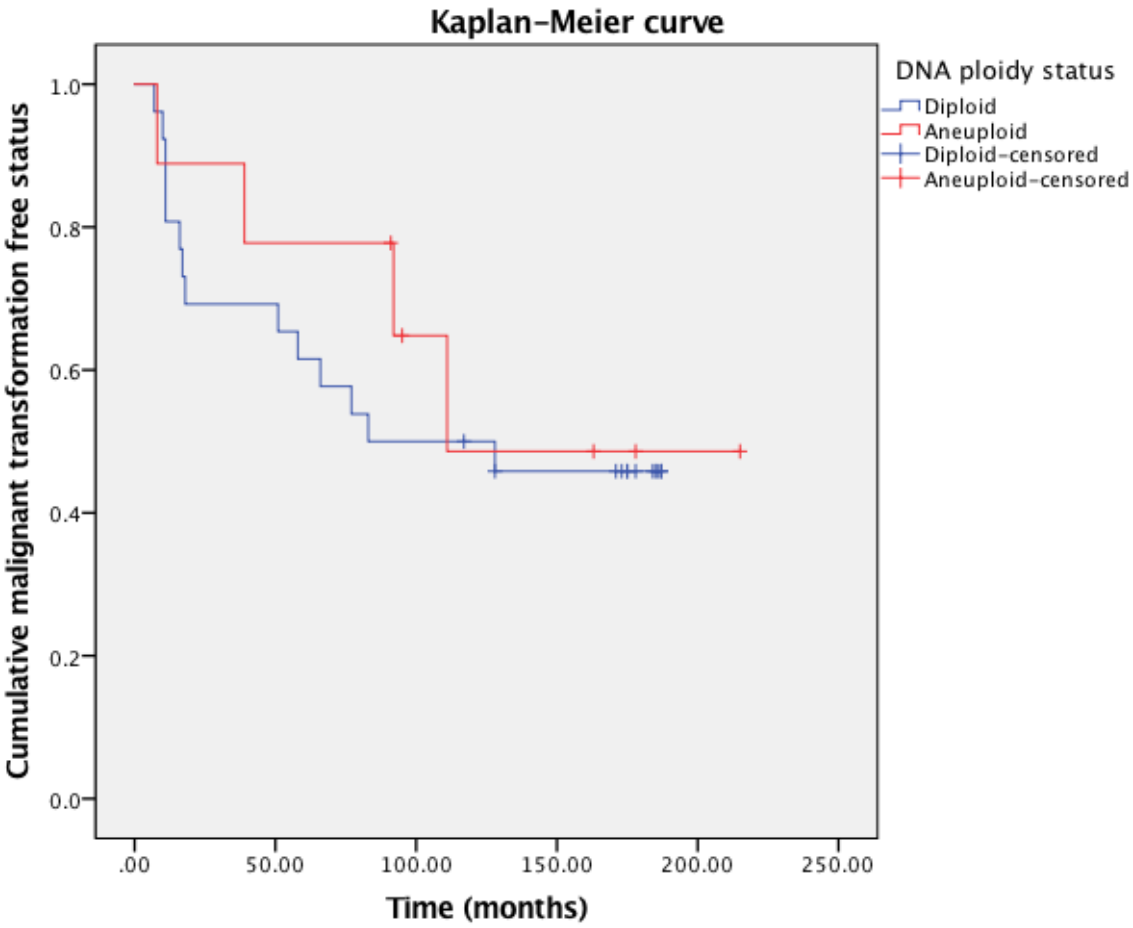


Figure 4.3 Kaplan-Meier curve for time to event analysis comparing malignant transformation in the OPMD cohort stratified according to the DNA ploidy status (n = 35).

The analysis shows that DNA ploidy status did not have an obvious influence on clinical outcome for this cohort of patients. patients (Log rank test; $\chi^2 = 0.260$, df =1, p= 0.610); Diploid = blue line; Aneuploid = red line.

Chapter 5 . Loss of Heterozygosity Analysis

5.1 Introduction

Oral carcinogenesis is believed to be the result of a multi-step process of accumulated genetic alterations (Balmain *et al.*, 1993; Califano *et al.*, 1996; Forastiere *et al.*, 2001; Hanahan and Weinberg, 2011; Kasamatsu *et al.*, 2011; Leemans *et al.*, 2011; Leemans *et al.*, 2018) (Figure 1.2). The cancer-related genes can be altered via deletions, amplifications, rearrangements, translocations, and mutations (Scully *et al.*, 2000a; Kasamatsu *et al.*, 2011; Leemans *et al.*, 2011; Leemans *et al.*, 2018). Tumour suppressor genes and oncogenes are the two most widely studied cancer-related genes (Field, 1992; Scully, 1993; Ha *et al.*, 2009; Leemans *et al.*, 2011). Activation of oncogenes via alteration of proto-oncogenes may lead to uninhibited cellular proliferation and conversely, loss of function in tumour suppressor genes that are involved in negative regulation of cellular growth can also lead to uncontrolled cellular proliferation and growth (Scully, 1993; Scully *et al.*, 2000a; Hanahan and Weinberg, 2011; Leemans *et al.*, 2011).

Several studies have shown that allelic loss at chromosomal regions 3p, 4q, 8p, 8q, 9p, 11q, 13q and 17p occurs frequently in OSCCs with most studies placing a high degree of emphasis on chromosomal regions 3p, 9p and 17p (Hollstein *et al.*, 1991; Maestro *et al.*, 1993; Ah-See *et al.*, 1994; Nawroz *et al.*, 1994; van der Riet *et al.*, 1994; Wu *et al.*, 1994; Field *et al.*, 1995; Califano *et al.*, 1996; Reed *et al.*, 1996; Scully *et al.*, 2000a; Forastiere *et al.*, 2001; Beder *et al.*, 2003; Leemans *et al.*, 2011). Loss of genetic material at a tumour suppressor gene (TSG) chromosomal region is one way in which the activity of the relevant TSG could be altered and can be used as a proxy marker of TSG inactivation (Scully, 1993; Scully *et al.*, 2000a; Hunt, 2009; Kasamatsu *et al.*, 2011). Such TSG-associated deletions can be assessed using loss of heterozygosity analysis.

Loss of heterozygosity (LOH) can be defined as allelic loss from one chromosomal locus in a chromosomal pair and can be identified by assessing DNA polymorphism patterns between normal and abnormal tissue (Scully *et al.*, 2000a; Beder *et al.*, 2003; Kasamatsu *et al.*, 2011). Polymorphic DNA microsatellite markers are the most

frequently used method to identify allelic loss (Mao *et al.*, 1996; Zhang *et al.*, 1997; Tabor *et al.*, 2001; Zhang *et al.*, 2001; Bremmer *et al.*, 2008; Bremmer *et al.*, 2009; Hunt, 2009; Accurso *et al.*, 2011; Graveland *et al.*, 2013; William *et al.*, 2016).

Traditionally, LOH assessment was performed through gel electrophoresis where the intensity of the normal and abnormal bands is compared and by convention, LOH is said to have occurred when one band is less than 50% as intense as the other band (Mao *et al.*, 1996; Zhang *et al.*, 1997; Rosin *et al.*, 2000; Zhang *et al.*, 2001; Zhang *et al.*, 2012). However, currently most LOH analysis is performed using capillary electrophoresis, in which the relative amounts of PCR products for the two alleles is obtained from the respective peak heights in the electropherograms generated (Cawkwell *et al.*, 1993; Cawkwell *et al.*, 1994; Tabor *et al.*, 2001; Farrand *et al.*, 2002; Bremmer *et al.*, 2008; Bremmer *et al.*, 2009; Accurso *et al.*, 2011; William *et al.*, 2016). The ratio of peak heights in the normal tissue is compared to the ratio from tumour/dysplastic tissue to ascertain whether LOH has occurred. Similarly, the cut-off value by convention is 50% although some studies have used different cut-off values (Cawkwell *et al.*, 1993; Cawkwell *et al.*, 1994; Farrand *et al.*, 2002; Tabor *et al.*, 2002; Accurso *et al.*, 2011). A 50% cut-off allows for the presence of some normal cells in the test samples as there is a high probability that not all cells in the sample would harbour LOH (Tabor *et al.*, 2001; Farrand *et al.*, 2002; Tabor *et al.*, 2002).

Samples for LOH analysis are usually microdissected as contamination from normal tissue samples can mask allelic loss in the tumour/dysplasia tissue (Hunt, 2009). Although manual microdissection has been the conventional method for sample enrichment, LASER capture microdissection (LCM) which enables minimal physical manipulation of the tissue samples during microdissection has become more popular in recent years (Shumway *et al.*, 2008; Accurso *et al.*, 2011; Mallery *et al.*, 2014).

Deletion of several regions in chromosomal region 3p (3p13-p21.1, 3p21.3-p23 and 3p25) has been shown to occur frequently in OSCC, however the region of focus for most studies has been the 3p14.2 locus which contains the fragile histidine triad (*FHIT*) gene (Maestro *et al.*, 1993; Wu *et al.*, 1994; Virgilio *et al.*, 1996; Uzawa *et al.*, 1998; Scully *et al.*, 2000a; Forastiere *et al.*, 2001; Leemans *et al.*, 2011; Nikitakis *et al.*, 2018). Allelic loss at chromosomal region 9p is also frequently associated with OSCC specifically at locus 9p21 which is the site for the cyclin-dependent kinase inhibitor 2A (*CDKN2A*) gene (van der Riet *et al.*, 1994; Califano *et al.*, 1996; Reed *et*

al., 1996; Scully *et al.*, 2000a; Forastiere *et al.*, 2001; Leemans *et al.*, 2011). The *CDKN2A* gene is known to encode for two tumour suppressor proteins, $p16^{INK4a}$ and $p14^{ARF}$ that are involved in cell-cycle regulation (Scully *et al.*, 2000a; Forastiere *et al.*, 2001; Leemans *et al.*, 2011). The region of interest on chromosome 17p that is studied most frequently is the region associated with the *TP53* gene that encodes for the p53 tumour suppressor protein that is involved with cell-cycle regulation (Hollstein *et al.*, 1991; Somers *et al.*, 1992; Scully *et al.*, 2000a; Forastiere *et al.*, 2001; Leemans *et al.*, 2011). LOH involving this locus has been reportedly seen in about 50 – 60% of head and neck cancers (Hollstein *et al.*, 1991; Somers *et al.*, 1992; Burns *et al.*, 1993; Nawroz *et al.*, 1994; Forastiere *et al.*, 2001; Leemans *et al.*, 2011).

Many of the chromosomal aberrations observed in OSCCs such as LOH at 3p, 9p and 17p, have also been seen in OPMDs albeit in differing magnitudes (Califano *et al.*, 1996; Mao *et al.*, 1996; Zhang *et al.*, 1997; Califano *et al.*, 2000; Rosin *et al.*, 2000; Forastiere *et al.*, 2001; Zhang and Rosin, 2001; Braakhuis *et al.*, 2003; Braakhuis *et al.*, 2004a; Mithani *et al.*, 2007; Bremmer *et al.*, 2008; Bremmer *et al.*, 2009; Zhang *et al.*, 2012). Studies have reported the frequent occurrence of LOH at 3p14 and 9p21 in OPMDs, especially those that undergo malignant change (Mao *et al.*, 1996; Rosin *et al.*, 2000; Zhang *et al.*, 2012). Recent studies have shown that LOH analysis at these chromosomal regions can be used as prognostic biomarkers for clinical outcome of OPMDs (Rosin *et al.*, 2000; Zhang and Rosin, 2001; Zhang *et al.*, 2012; William *et al.*, 2016).

A recently concluded clinical trial that used LOH status for stratification of patients into different treatment arms also appears to have validated the ability of LOH as a prognostic biomarker for malignant transformation in OPMDs (William *et al.*, 2016). The findings of this study however have to be interpreted with caution as the clinical outcome for this study is biased due to the inclusion of patients with a previous history of oral cancer (58%) into the LOH positive group and conversely, the LOH "negative" group may have had some patient with LOH at 3p14 &/or 9p21 (William *et al.*, 2016). Furthermore, active intervention in the form of Erlotinib was administered to the patients in the treatment arm of LOH positive cases which may have influenced clinical outcome (William *et al.*, 2016). As such, although LOH has been proven to have some utility as a prognostic marker for malignant transformation in OPMD, adequate clinical validation is still pending.

5.2 Aims

The aims of this chapter are:

1) To compare two microdissection methods for LOH sample preparation.
2) To assess association between LOH and clinico-pathological features of OPMD patients.
3) To assess association between LOH and clinical outcome in OPMD.

5.3 Results

From the 65 cases that were suitable for this study, analysis could not be carried out for 15 cases due to lack of FFPE material and of the remaining cases, two failed LOH analysis. Thus, LOH analysis was successfully performed on 48 cases. For this section of the thesis, LOH refers to loss of heterozygosity at either one or more of the target loci (3p &/or 9p &/or 17p), whilst LOH (3p &/or 9p) refers to loss of heterozygosity at 3p and 9p either in isolation or in combination.

5.3.1 Patient characteristics

For the 48 cases analysed, mean patient age at diagnosis of index lesion was 61.25 (SD $\pm$13.10) with 77.1% of patients being more than 50 years of age at diagnosis. There was a male predominance in this cohort (62.5%). Leukoplakia was the most frequently encountered OPMD (79.2%) and the tongue was the most frequent site for index lesions (35.4%). There were 23 cases that underwent malignant transformation (MT) versus 25 cases that did not undergo malignant change in this cohort. Patients aged > 50 years at age of diagnosis with OPMD were more likely to undergo MT ($p = 0.039$; $\chi^2 = 5.06$; $df = 1$). The cohort of patients with OPMD that underwent malignant transformation (MT) had a male preponderance although this finding was not statistically significant (Table 5.1). The tongue was the most commonly involved site that underwent MT, and this was statistically significant ($p = 0.034$; $\chi^2 = 5.42$; $df = 1$) (Table 5.1). Binary OED grading was statistically significant with regard to clinical outcome ($p = 0.007$; $\chi^2 = 6.75$; $df = 1$), whereas the WHO 2017 grading was not ($p = 0.152$) (Table 5.1). The non-transforming (NT) cases were followed-up for a median of more than 10 years whilst MT cases occurred within a median of 39 months from OPMD diagnosis (Table 5.1).

5.3.2 Comparison between LASER capture microdissection (LCM) and manual microdissection methods for sample preparation

There were six cases included in the comparison; three non-transforming (NT) cases and three malignant transforming (MT) cases. There was no difference detected between the two methods in terms of the final LOH classification. The summarised results are displayed in table 5.2. The remainder of the samples were manually microdissected prior to LOH analysis.

5.3.3 Assessment of microsatellite markers for loss of heterozygosity (LOH) analysis

Twenty-four samples were initially assessed for LOH using nine microsatellite markers; three each per target loci. Three microsatellite markers performed relatively well with low failure rates, D3S1766 (8.3%), D9S171(16.7%) and TP53 (6.3%). There were relatively high failure rates with the other markers. D9S157 and D9S162 failed in all samples whilst four other microsatellites had high failure rates; D3S1293 (25.0%), D3S1029 (31.3%), CHRNB1 (22.9%) and D17S1866 (22.9%). The best microsatellite marker for each loci was chosen for the remaining samples; D3S1766, D9S171 and TP53. As such, all 48 samples were assessed for LOH using these three microsatellite markers.

5.3.4 Loss of heterozygosity (LOH) and clinical outcome

From the 48 analysed cases, 37 (77.1%) did not have loss of heterozygosity (LOH) at any of the loci tested whilst 11 (22.9%) had LOH at one or more loci. From the cases with LOH, eight (72.7%) had LOH at 3p &/or 9p. The summarised results are shown in Table 5.3. LOH at 3p &/or 9p &/or 17p (in combination or isolation) was found to be associated with clinical outcome in OPMDs and this was found to statistically significant when all 48 cases were analysed ($p = 0.016$; $\chi^2 = 6.57$; $df = 1$). Sub-group analysis on the HR-HPV negative cases showed that LOH status (at 3p &/or 9p &/or 17p) was still significantly associated with clinical outcome ($p = 0.015$; $\chi^2 = 6.70$; $df = 1$) (Table 5.4).

When statistical analysis was performed after excluding results from locus 17p, LOH (3p &/or 9p) status was significantly associated with clinical outcome ($p = 0.009$; $\chi^2 =$

7.45; df = 1) (Table 5.3). Sub-group analysis on the HR-HPV negative cases showed that LOH (3p &/or 9p) status was still significantly associated with clinical outcome (p = 0.015; χ^2= 6.70; df = 1) (Table 5.4). Electropherograms of two exemplary cases are displayed in Figure 5.1. The two cases that had LOH but did not undergo MT were followed up for more than 10 years.

Sensitivity and specificity analysis of LOH results in relation to clinical outcome showed that LOH has better specificity and positive predictive value than both OED grading systems in this 48-patient cohort (Table 5.5). Multivariate analysis was performed separately for LOH(3p/9p/17p) and LOH (3p &/or 9p), each time with inclusion of age at diagnosis, sex and binary OED grading. Cox proportional-hazards regression analysis (CRA) showed that both LOH(3p/9p/17p) and LOH (3p &/or 9p) were significantly associated with malignant transformation after adjustment for age at diagnosis, sex and binary OED grading (Table 5.6 and Table 5.7). Interestingly, binary OED grading was also significant in the multivariate model and its associated hazard ratios (HR) were equivalent to those of LOH status (Table 5.6 and Table 5.7). Time to event analysis showed that patients with LOH underwent MT more rapidly than those with no LOH (Figure 5.2 and Figure 5.3).

5.4 Discussion

It has been more than 20 years since the seminal study by Mao et al (1996) on the value of LOH analysis at 3p14 and 9p21 in cancer risk assessment of OPMDs (Mao *et al.*, 1996). Since then, several research groups have shown that LOH has promise as a prognostic biomarker for OPMDs (Zhang *et al.*, 1997; Rosin *et al.*, 2000; Bremmer *et al.*, 2009; Zhang *et al.*, 2012; Graveland *et al.*, 2013; William *et al.*, 2016) prompting its inclusion in the latest edition of the WHO reference text on head and neck tumours (El-Naggar *et al.*, 2017). The current study has also found a statistically significant association between LOH and clinical outcome in OPMD patients and supports this notion.

5.4.1 Patient characteristics

The overall clinical characteristics of this sub-cohort of patients for LOH analysis did not deviate much from the main cohort of patients for this study and have been described in Chapter 3. Age of patient (> 50 years), site of index OPMD (tongue) and

binary OED grading (high-grade) were the only clinico-pathologic features that were significantly associated with malignant transformation in this sub-cohort.

5.4.2 Sample preparation methods

The method employed for sample preparation in this study is an adaptation of the method described by one of the earliest studies on LOH in OPMDs by Mao et al (1996). Sample selection and preparation in OPMD samples is complicated by the amount of epithelial tissue in most biopsy samples which at times can be miniscule and also due to the close proximity of the epithelium to the underlying connective tissue. To overcome this problem, microdissection methods have been employed to obtain the relevant tissues of interest (Mao *et al.*, 1996; Zhang *et al.*, 1997; Partridge *et al.*, 1998; Partridge *et al.*, 2000; Rosin *et al.*, 2000; Zhang *et al.*, 2012).

Several recent studies on OPMD that involved LOH assessment have utilised LCM for sample preparation (Shumway *et al.*, 2008; Accurso *et al.*, 2011; Mallery *et al.*, 2014). Our experiment showed that for LOH analysis purposes, both LCM and manual microdissection produced similar results at each target locus suggesting that the manual microdissection method can be reliably used for sample preparation in LOH studies. This lack of difference is not surprising as the materials used for LOH analysis are not single cells but tissue and as such can be microdissected out manually without too much difficulty. The use of LCM for sample preparation is attractive due to its “non-contact” tissue manipulation technique that minimises contamination from tissues that are not of interest. However, LCM is also not without its disadvantages as it requires the availability of specialised hardware that is usually only available at research laboratories and not readily available at most diagnostic pathology laboratories. Although our results suggest that manual microdissection is just as good as LCM for LOH analysis, this would need to be verified using a much larger cohort.

5.4.3 Loss of heterozygosity and clinical outcome

The findings from this study were in agreement with most of the published literature, confirming that LOH is an acceptable prognostic biomarker for clinical outcome in OPMD (Mao *et al.*, 1996; Rosin *et al.*, 2000; Bremmer *et al.*, 2009; Graveland *et al.*, 2013; William *et al.*, 2016). Only 22.9% of the cases in this cohort showed LOH

compared to previous studies that have shown LOH in the majority of their patient cohorts (Rosin *et al.*, 2000; Zhang *et al.*, 2012). This was possibly due to the fact that only one microsatellite marker per target region was employed for all the samples in this study which could have reduced the detection of allelic loss that may have occurred in those cases that underwent malignant transformation (MT). However, it is conceivable that this approach may have reduced the number of false positives that could have occurred. In the past, most studies have used multiple markers that flank known polymorphisms that are near a gene of interest (Zhang *et al.*, 1997; Rosin *et al.*, 2000; Bremmer *et al.*, 2009; Zhang *et al.*, 2012; Graveland *et al.*, 2013; William *et al.*, 2016).

Although the usage of multiple markers was attempted in this study, the majority of markers that were flanking the genes of interest underperformed when compared to the markers that were specific for the three genes of interest (*FHIT, CDKN2A* and *TP53*). Although this may have reduced the sensitivity of the assay, by reducing the number of microsatellites that were acting as surrogate markers for a specific gene (flanking the region), true allelic loss of the relevant genes was able to be identified. This in turn, reduced the number of false positives that may have occurred in cases where there may have been LOH at the region around the gene but did not truly affect the gene itself; resulting in increased specificity of the assay (92%). This is best exemplified by results from a previous study by Zhang et al (2012) that had numerous false positive results, with only 22 cases undergoing MT from 252 showing LOH at their target loci (specificity of 13.2%) (Zhang *et al.*, 2012). However, the same study showed 100% sensitivity with all cases that underwent malignant change having LOH at one or more of the target loci (Zhang *et al.*, 2012).

Two recent studies have shown that LOH solely at 9p had a strong association with malignant change in OED/OPMD (Zhang *et al.*, 2012; Graveland *et al.*, 2013). Zhang et al (2012) in their prospective study suggested that allelic loss at 3p could be a bystander/passenger change instead of being an active participant in malignant change of OPMDs (Zhang *et al.*, 2012). The authors also developed a new algorithm based on allelic loss at 9p and additional allelic loss at 4q and 17p which is yet to be validated (Zhang *et al.*, 2012). However, other studies have shown that assessment of LOH at both 3p and 9p has some prognostic value, especially as a negative predictor in cases of retention at both regions (Mao *et al.*, 1996; Roz *et al.*, 1996;

Rosin *et al.*, 2000; Zhang *et al.*, 2012; William *et al.*, 2016). This, together with the fact that allelic change at 3p and 9p has been shown to occur frequently in both OSCC and OPMD, suggests that it is rather premature to designate LOH at chromosome 3p as a “passenger alteration” (Zhang *et al.*, 2012) and as such it would be best to include both loci with regard to risk-stratification of OPMD patients (Califano *et al.*, 1996; Mao *et al.*, 1996; Zhang *et al.*, 1997; Califano *et al.*, 2000; Rosin *et al.*, 2000; Forastiere *et al.*, 2001; Zhang and Rosin, 2001; Braakhuis *et al.*, 2003; Braakhuis *et al.*, 2004a; Mithani *et al.*, 2007; Bremmer *et al.*, 2008; Bremmer *et al.*, 2009; Zhang *et al.*, 2012).

Despite the pending validation of LOH as a prognostic biomarker for OPMDs, a recently completed clinical trial used LOH to stratify OED patients in a chemo-prevention study (William *et al.*, 2016). The authors concluded that although the intervention method (Erlotinib) did not reduce the cancer-free survival in high-risk patients, LOH analysis was validated as a biomarker for the development of OSCC (William *et al.*, 2016). This statement by the authors is peculiar as it would appear that the authors acknowledge using an un-validated method to stratify patients in a clinical trial. Aside from that, as mentioned earlier in this chapter, the findings of this clinical trial should not be considered a true validation of LOH as a prognostic biomarker for OED due to active intervention (Erlotinib) in the treatment arm as well as the irregular patient stratification methods employed (William *et al.*, 2016). Additionally, similar to our findings their study also showed that OED grading performed just as well as LOH status upon multivariate analysis (William *et al.*, 2016).

Both cases with HPV-associated OED in this study were shown to have retention of heterozygosity. A sub-group analysis after excluding these cases was performed as the natural history of HPV-associated oral epithelial dysplasia is still unclear (McCord *et al.*, 2013; Woo *et al.*, 2013; El-Naggar *et al.*, 2017; Lerman *et al.*, 2017). The results from the sub-group analysis confirmed that LOH had a statistically significant association with clinical outcome. In view of the possibility that HPV-associated OED may have a different carcinogenic pathway that traditional OED, it would be prudent to screen all cases of OED for high-risk HPV prior to including them in studies investigating prognostic or predictive biomarkers in OED/OPMD. Such cases may not contain allelic loss at the 3p, 9p or 17p sites during progression from OPMD to carcinoma as the pathogenesis may be entirely different. A study by Braakhuis et al

(2004) showed that LOH at chromosomal regions 3p, 9p and 17p is far less frequently observed in HPV-associated HNSCC compared to HPV negative HNSCC (Braakhuis *et al.*, 2004b).

Although LOH appears to be a prognostic biomarker for OPMD, a major drawback with this technique is the need for relatively large amounts of tissue to produce valid results compared to histopathological methods (OED grading). This requirement poses a major problem especially when analysis involves incisional biopsies from the oral cavity that tend to be rather small and tissue may need to be conserved for routine histopathological assessment and diagnosis.

Overall, LOH analysis does appear to show promise as a prognostic biomarker for clinical outcome in OPMDs although further investigation and validation is required before clinical recommendations can be put forth.

5.5 Conclusions

The results from the current and previous studies have highlighted the advantages and shortcomings of LOH analysis as a prognostic biomarker of malignant transformation in OPMD. LOH at 3p &/or 9p appears to perform better than LOH at 3p &/or 9p &/or 17p as a prognostic marker for OPMDs. Although LOH status appears to have good specificity, the sensitivity and positive predictive values are still lacking. However, LOH analysis does merit further investigation preferably in larger multicentre or prospective studies that include assessment of HR-HPV infection as part of the study design. At this point in time, it would be ill-advised to use LOH as a sole risk-stratification method for clinical practice or clinical trials; combined use with other clinico-pathological features would be more appropriate in such instances.

Table 5.1 Characteristics of cases according to clinical outcome (n = 48)

Characteristics		Non-transforming n = 25	Malignant transforming n = 23	p-value
Age (years) Mean [SD] (Range)		59.36 [13.19] (34 -89)	63.30 [12.98] (35 – 92)	0.302†
Age	≤ 50 years	9	2	0.039*
	> 50 years	16	21	
Time to last follow-up/malignant transformation in months Median [IQR](Range)		173.00 [72.00] (60 – 215)	39.00 [55.00] (7 – 128)	< 0.001α
Sex	Male	14	16	0.502*
	Female	11	7	
Type of OPMD	Leukoplakia	20	18	0.886**
	Erythroplakia	3	2	
	Erythroleukoplakia	2	3	
Site of OPMD	Tongue	5	12	0.015**
	Floor of mouth	14	3	
	Buccal mucosa	4	3	
	Gingiva/Alveolar mucosa	1	2	
	Palate	1	3	
Site of OPMD (binary)	Tongue	5	12	0.034*
	Other sites	20	11	
WHO 2017 OED grading	Mild	15	9	0.152**
	Moderate	5	3	
	Severe	5	11	
Binary OED grading	Low-grade	20	9	0.007*
	High-grade	5	14	
HR-HPV status	Negative	24	22	1.000*
	Positive	1	1	

† Independent t-test; α Mann-Whitney U test; *Fisher's exact test; **Pearson's chi-square test (Exact method); IQR = interquartile range; SD = standard deviation

Table 5.2 Results from loss of heterozygosity (LOH) analysis using different sample preparation methods

Sample	Targets		
	3p	9p	17p
LCM1	No LOH	No LOH	No LOH
MMD1	No LOH	No LOH	No LOH
LCM2	No LOH	No LOH	No LOH
MMD2	No LOH	No LOH	No LOH
LCM3	No LOH	NI	No LOH
MMD3	No LOH	NI	No LOH
LCM4	No LOH	No LOH	No LOH
MMD4	No LOH	No LOH	No LOH
LCM5	NI	No LOH	No LOH
MMD5	NI	No LOH	No LOH
LCM6	No LOH	No LOH	No LOH
MMD6	No LOH	No LOH	No LOH

* LCM = LASER capture microdissection samples; MMD = manual microdissection samples; NI = non-informative

Table 5.3 Association of loss of heterozygosity (LOH) and clinical outcome in all cases (n = 48)

LOH		Non-transforming (NT) (n = 25)	Malignant transforming (MT) (n = 23)	p-value
LOH (3p &/or 9p &/or 17p)	No LOH	23	14	0.016 *
	LOH	2	9	
LOH (3p &/or 9p)	No LOH	24	15	0.009 *
	LOH	1	8	

*Fisher's exact test

Table 5.4 Association of loss of heterozygosity (LOH) and clinical outcome in HR-HPV negative cases (n = 46)

LOH		Non-transforming (NT) (n = 24)	Malignant transforming (MT) (n = 22)	p-value
LOH (3p &/or 9p &/or 17p)	No LOH	22	13	0.015 *
	LOH	2	9	
LOH (3p &/or 9p)	No LOH	23	14	0.009 *
	LOH	1	8	

*Fisher's exact test

Table 5.5 Comparison and evaluation of LOH as a prognostic instrument in OPMD against OED grading (n = 48)

Statistic	LOH (3p &/or 9p &/or 17p)	LOH (3p &/or 9p)	WHO 2017 grading (mild & moderate vs severe OED)	Binary OED grading	Zhang et al (2012)
Sensitivity	39.1%	34.8%	47.8%	60.9%	100%
Specificity	92.0%	96.0%	80.0%	80.0%	13.2%
Accuracy	66.7%	66.7%	64.6%	69.0%	19.9%
***Positive predictive value**	36.4%	50.5%	21.9%	26.3%	11.9%
***Negative predictive value**	92.8%	92.6	92.9%	94.6%	100%

*Value based on 10.5% prevalence rate of MT in OED as all cases in this cohort had some level of OED (Shariff and Zavras, 2015)

Table 5.6 Cox proportional-hazards regression analysis (CRA) assessing association between malignant transformation and LOH (3p &/or 9p &/or 17p) status (n = 48)

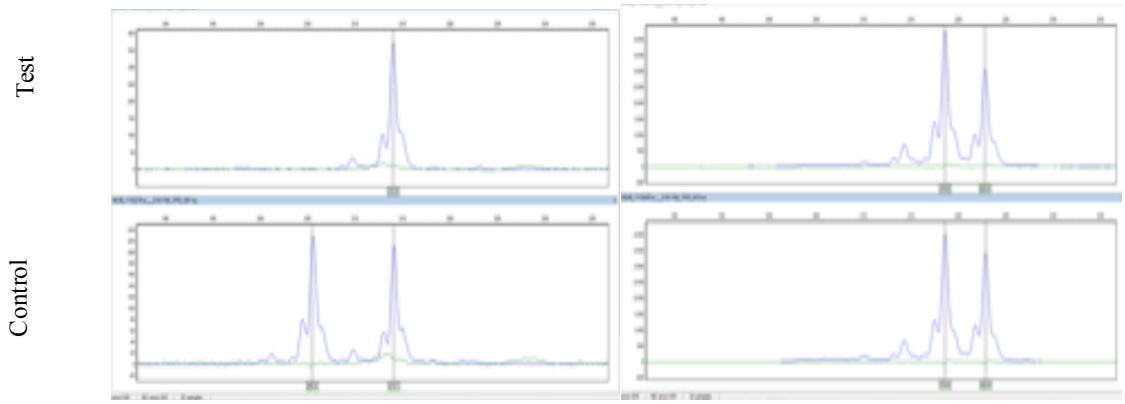
Variable		Univariate		Multivariate	
		HR (95% CI)	p - value	HR (95% CI)	p - value
LOH (3p &/or 9p &/or 17p)	No LOH	1.00		1.00	
	LOH	2.88 (1.24, 6.72)	0.014	2.49 (1.06, 5.89)	0.037
Binary OED grading	Low-grade	1.00		1.00	
	High-grade	3.31 (1.40, 7.82)	0.006	3.01 (1.26, 7.21)	0.013

LOH = Loss of heterozygosity; HR = Hazard ratio; CI = Confidence interval. Variables included for multivariate analysis: age at diagnosis, sex, LOH and binary OED grading.

Table 5.7 Cox proportional-hazards regression analysis (CRA) assessing association between malignant transformation and LOH (3p &/or 9p) status (n = 48)

Variable		Univariate		Multivariate	
		HR (95% CI)	p - value	HR (95% CI)	p - value
LOH (3p &/or 9p)	No LOH	1.00		1.00	
	LOH	3.51 (1.46, 8.42)	0.005	3.02 (1.24, 7.33)	0.015
Binary OED grading	Low-grade	1.00		1.00	
	High-grade	3.31 (1.40, 7.82)	0.006	2.98 (1.24, 7.13)	0.014

LOH = Loss of heterozygosity; HR = Hazard ratio; CI = Confidence interval. Variables included for multivariate analysis: age at diagnosis, sex, LOH and binary OED grading.



a) LOH present

b) No LOH detected

Figure 5.1 Exemplary electropherograms from LOH analysis.

A case with LOH at the target site with only one peak in the test tissue (oral epithelial tissue) in contrast to two peaks in the control tissue (connective tissue). This case was confirmed to have undergone malignant transformation.

A case without LOH at the target site with two peaks in both the test and control tissues. Although the second peak in the test tissue is slightly shorter than in the control, it is still above the 50% threshold for LOH. This case did not have malignant transformation (at census date).

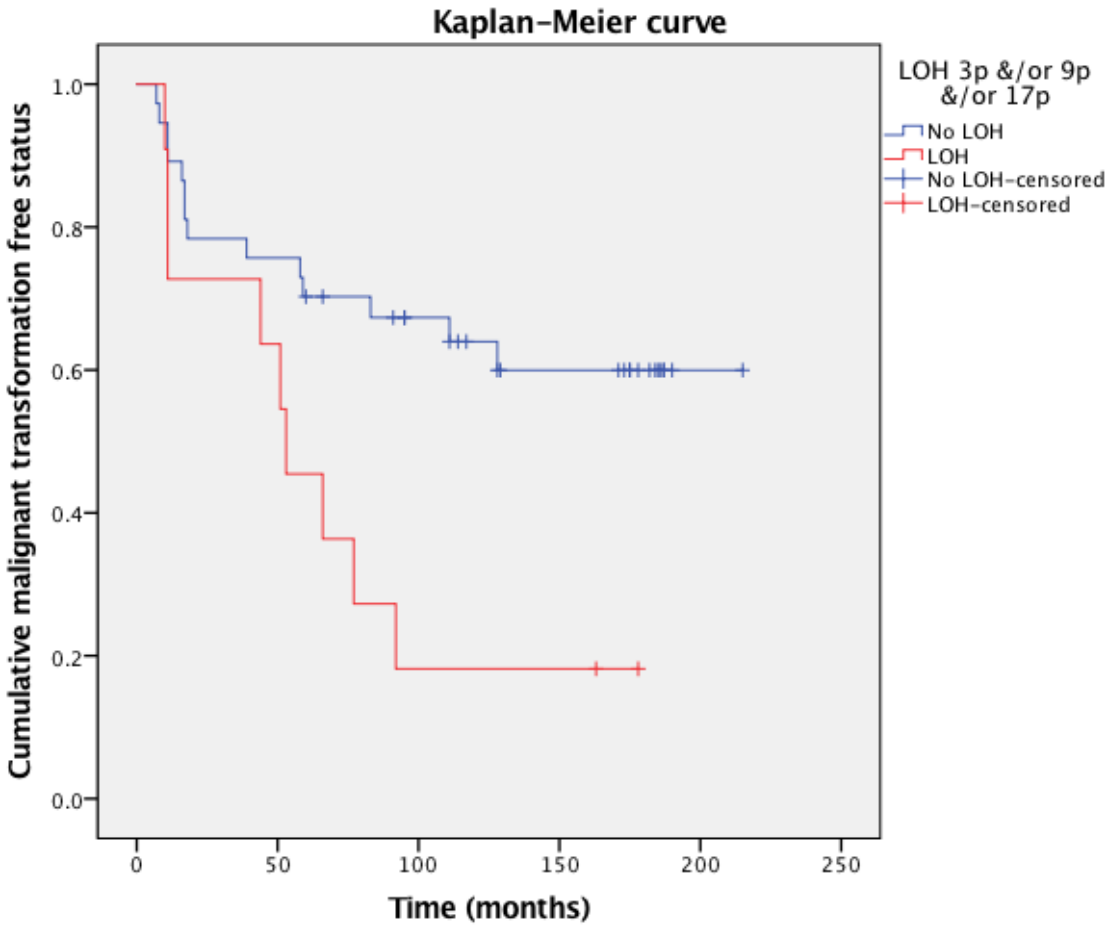


Figure 5.2 Kaplan-Meier time to event analysis comparing malignant transformation in the OPMD cohort stratified according to LOH (3p &/or 9p &/or 17p) status.

The analysis shows that patients with LOH underwent malignant change of OPMDs more rapidly than those with no LOH (Log rank test; $\chi^2 = 6.66$, df =1, p = 0.010); No LOH = blue line; LOH = red line.

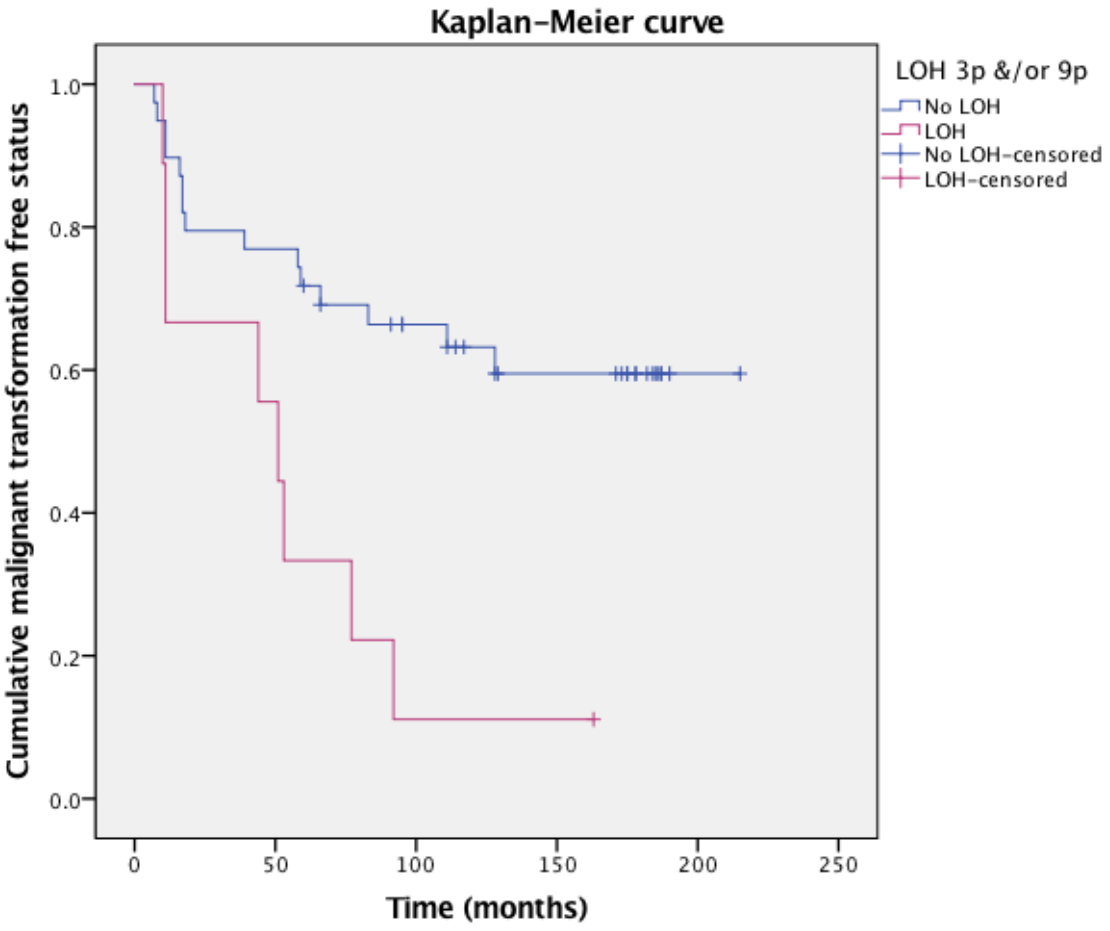


Figure 5.3 Kaplan-Meier time to event analysis comparing malignant transformation in the OPMD cohort stratified according to LOH (3p &/or 9p) status.

The analysis shows that patients with LOH (3p &/or 9p) underwent malignant change of OPMDs more rapidly than those with no LOH (Log rank test; $\chi^2 = 9.11$, df =1, p = 0.003); No LOH (3p &/or 9p) = blue line; LOH (3p &/or 9p) = purple line

Chapter 6. Differential Gene Expression

6.1 Introduction

The previous chapters detailed the assessment of two different molecular methods (DNA ploidy and loss of heterozygosity analysis) in prognosticating the clinical outcome of patients with OPMD. This chapter will detail the differential gene expression (DGE) experiments between OPMD cases that underwent malignant transformation (MT) and those that did not. Two different gene expression profiling platforms were used for this purpose; RNA sequencing (RNASeq) and NanoString nCounter platform.

6.1.1 Whole transcriptome sequencing

Whole transcriptome sequencing is a major advancement in studying and understanding gene expression as it allows researchers to obtain a comprehensive view of the transcriptional profile at a given moment in time. A widely used method for profiling the whole transcriptome in a "snapshot" manner is RNASeq. As it captures the whole transcriptome, RNASeq is able to detect both known and novel transcripts and is suitable for assessing genes that are differentially expressed between different disease states. As such, RNASeq is suitable for preliminary DGE experiments prior to more targeted gene expression assays as well as being an excellent technique for discovering novel differentially expressed genes. In this study, RNASeq has been used as a discovery platform to identify both known and novel transcripts of genes that may be involved in the malignant transformation of OPMD.

6.1.2 NanoString nCounter Platform

FFPE tissue contains ample genetic material, however formalin fixation is known to adversely affect the quality of nucleic acids extracted hampering downstream applications such as gene expression microarrays (von Ahlfen *et al.*, 2007). NanoString nCounter platform allows detection of up to 800 targets in a single reaction using probe-based technology to provide accurate gene expression analysis using total RNA from FFPE material without need for amplification (Geiss *et al.*, 2008; Reis *et al.*, 2011). Recent studies have shown that the performance of NanoString

technology using FFPE samples is comparable to qPCR and superior to microarray technology especially with regard to low-abundance transcripts (Geiss *et al.*, 2008; Reis *et al.*, 2011; Balko *et al.*, 2012; Scott *et al.*, 2013; Saba *et al.*, 2015; Veldman-Jones *et al.*, 2015a; Veldman-Jones *et al.*, 2015b).

This technology also allows researchers to customise the panel of target genes to be studied. For the first part of the NanoString experiment the PanCancer Pathways Panel was used. This is a pre-designed panel containing 770 genes from 13 canonical cancer pathways and internal reference genes. The following is the list of cancer pathways that are included in the panel:

- NOTCH signalling pathway
- WNT signalling pathway
- Hedgehog signalling pathway
- Chromatin modification
- Transcriptional regulation
- DNA damage control
- Transforming growth factor beta (TGF-β) signalling pathway
- Mitogen activated protein kinase (MAPK) signalling pathway
- Janus kinase (JAK) – signal transducer and activation of transcription protein (STAT) signalling pathway
- Phosphatidylinositol-4,5-bisphosphate 3-kinase (PI3K) signalling pathway
- Ras signalling pathway
- Cell Cycle
- Apoptosis

The second part of the NanoString experiment involved assessing differential gene expression between the two sub-groups (MT vs NT) using a customised panel of target genes (Customised CodeSet). Four OED cell line samples were also included to compare and contrast the gene expression profiles between cell line and FFPE tissue (clinical) samples. The design of the Customised CodeSet was based on the results from RNASeq analysis and NanoString PanCancer Pathways Panel Plus analysis as well as a systematic search through relevant scientific literature.

6.2 Aims

The aims of this chapter are:

1) To examine the differential gene expression profile between formalin-fixed paraffin-embedded clinical OPMD samples and OED cell line samples.
2) To identify differentially expressed genes between OPMD that undergo malignant transformation and those that do not undergo malignant transformation.
3) To discover a gene-signature that characterises OPMDs with a high risk of undergoing malignant transformation.

6.3 Results

6.3.1 Whole transcriptome sequencing

Twenty cases (10 malignant transforming vs 10 non-transforming) from the cohort of 65 patients were chosen for total RNA sequencing (RNASeq). The demographic, clinical and histopathological characteristics of the cases are shown in Table 6.1. None of the clinical or pathological features were significantly correlated with clinical outcome. The cohort was composed predominantly of males and patients aged more than 50 years at diagnosis.

Results from the quality assessment of the reads from an exemplary sample (sample code: W1) are shown in Figure 6.1 and Figure 6.2. Figure 6.3 shows the summary of the quality assessment parameters for all the samples. All samples passed the quality control assessments to proceed for downstream analysis. During quality assessment, DNA base contents were found irregularly distributed in the first 13-14 bp of reads, which in fact is commonly observed across next generation sequencing (NGS) datasets and originates from random hexamer priming (Figure 6.2) (Hansen *et al.*, 2010).

Bioinformatic analysis of RNASeq outputs revealed 41 genes that were significantly differentially expressed between MT and NT cases. The log2 fold change for the statistically significant differentially expressed genes ranged from -2.63 to 2.48, with 27 genes being downregulated and 14 genes being upregulated in MT cases compared to NT (baseline) cases. The visual distribution of differentially expressed

genes is shown in Figure 6.4 and the list of statistically significant differentially expressed genes is shown in Table 6.2.

6.3.2 NanoString nCounter platform

These experiments involved two stages:

- Stage 1: Differential gene expression experiment between MT and NT groups using the PanCancer Pathways Panel set of target genes. An additional ten customised probes (9 target genes and 1 housekeeping gene) targeting specific mRNA were supplemented to the 770 gene PanCancer Pathways Panel (Appendix D). The additional targets were chosen from significant differentially expressed genes from the RNASeq experiment as described in Section 2.7.4. The final panel was composed of 739 targets and 41 housekeeping genes. This set of target probes will be referred to as the "PanCancer Pathways Panel Plus" for the remainder of the chapter.

- Stage 2: Differential gene expression experiment between MT and NT group using a customised list of target genes (Appendix F) that will be referred to as the "Customised CodeSet Panel" for the remainder of the chapter

Forty-eight cases (25 NT vs 23 MT) were analysed for DGE studies using the PanCancer Pathways Panel Plus. The demographic, clinical and histopathological characteristics of the cases are shown in Table 6.3. The cohort was composed predominantly of males and patients aged more than 50 years at diagnosis.

All raw data passed the QC parameter measures. Prior to DGE analysis, the raw data were normalised using the geNorm algorithm that chooses only the most stable housekeeping genes in the analysed dataset (Vandesompele *et al.*, 2002). Raw data normalisation and DGE analysis were performed twice: once with all cases (n = 48) and a second run with the two HR-HPV positive cases excluded (n = 46). Principal component analysis (PCA) was used to get an overall view of any obvious clustering in the data as well as outliers. The results are shown in Figure 6.5. PCA was unable to separate the malignant transforming and non-transforming OPMD cases. One sample (H5) was shown to be an outlier during this analysis. However, upon review of the case, it was decided that the sample should still be included for downstream analysis.

DGE analysis of all 48 cases revealed that 14 genes were significantly differentially expressed when the FDR value was set at the 5% level and 19 gene were differentially expressed when the FDR value was set at the 10%. DGE results from the experiment are shown in Table 6.4. When DGE analysis was repeated after excluding the two HR-HPV positive cases, seven genes were differentially expressed when the FDR value was set at the 5% level and 14 gene were differentially expressed when the FDR value was set at the 10%. DGE results are shown in Table 6.5.

The seven genes that were statistically significant at the 5% level were similar in both analyses (Tables 6.4 and 6.5). A False Discovery Rate (FDR) of < 0.1 was chosen for this part of the NanoString experiment as this was an exploratory experiment to find genes with altered expression between OPMD that undergo malignant transformation and those that do not. Additionally, unlike the RNASeq experiment that looked at the whole transcriptome, the PanCancer Pathways Panel only looked at 739 target genes. Setting the FDR rate too low can cause exclusion of key genes with biological relevance. A Kyoto Encyclopaedia of Genes and Genomes (KEGG) overview of all 13 pathways rendered using Pathview is shown in Figure 6.6. The most frequently encountered cancer-associated pathways were the PI3K/AKT, MAPK, RAS, JAK-STAT and Wnt signalling pathways (Tables 6.4 and 6.5). The top five KEGG pathways are shown in Appendix H.

The Customised CodeSet Panel experiment was performed using RNA extracted from 44 FFPE samples (24 NT vs 20 MT) and four OED cell lines. The 44 cases were chosen from the earlier 48 cases that were used for the PanCancer Pathways Panel Plus. All cases were HR-HPV negative. The demographic, clinical and histopathological characteristics of the clinical cases are shown in Table 6.6. All raw data from the Customised CodeSet Panel experiment passed the QC measures. Principal component analysis (PCA) showed an obvious separation between the cell line samples and FFPE samples highlighting the different gene expression profile between the two sample types (Figure 6.7). PCA also showed considerable overlap between MT and NT cases (Figure 6.8). Raw data normalisation and DGE analysis were performed twice; once with all cases (OED cell lines and FFPE tissue samples; n = 48) and a second run with only the FFPE cases (n = 44).

DGE analysis between OED cell lines against clinical cases revealed a high number of statistically significant differentially expressed genes (Table 6.7). Sub-analysis between OED cell lines and clinical cases with differing outcomes (NT or MT) also showed a high number of statistically significant differentially expressed genes (Table 6.8).

There were five genes that were statistically significant differentially expressed between MT and NT cases (Table 6.9). The dichotomised log2 normalised gene expression of the five genes were then fitted into a Cox regression (proportional hazards regression) model to produce a gene-signature using the relevant coefficients from the regression model. The results of the analysis are shown in Table 6.10. Only three genes were retained after multivariate analysis; *NOTCH1*, cyclin E1 (*CCNE1*), and *TP63*. Gene-signature scores were then generated for each case. The concordance index (Harrell's C-index) for the scores in relation to clinical outcome was 0.82 (CI: 0.69, 0.95) (Figure 6.9). The gene-signature scores were then dichotomised into low-risk and high-risk using the median of the scores. The dichotomised gene-signature score was significantly associated with clinical outcome of OPMD ($p < 0.001$; $\chi^2 = 17.65$; df = 1). Sensitivity and specificity analysis of the dichotomised gene-signature score in relation to clinical outcome showed that the gene-signature score had better sensitivity, specificity, negative predictive value and positive predictive values than the OED grading systems in this 44-patient cohort (Table 6.11). Time to event analysis showed that patients that were scored as high-risk underwent MT more rapidly than those with low-risk scores (Figure 6.10).

6.4 Discussion

Archived FFPE tissues are an invaluable resource that can be successfully used for molecular-based assays despite the degradation that often accompanies fixation and embedding of tissues in paraffin wax (von Ahlfen *et al.*, 2007; Geiss *et al.*, 2008; Mittempergher *et al.*, 2011; Reis *et al.*, 2011; Eikrem *et al.*, 2016; Wimmer *et al.*, 2018). The current study adds to the increasing body of work on utilisation of FFPE material for gene expression studies. Of note, compared to the previous molecular methods used in this study (DNA ploidy and LOH analysis), there was adequate RNA for the DGE experiment and none of the samples failed QC assessments. The amount of tissue required to obtain the necessary amount of RNA for the assays was also lower than that required for DNA ploidy and LOH analysis (Table 2.1). The

overall clinical characteristics of this sub-cohort of patients did not deviate much from the main cohort of patients for this study and have been described in Chapter 3.

6.4.1 Whole transcriptome sequencing

Gene expression analysis is based on the premise that valuable information regarding the function and activity of a cell can be gleaned from the expression levels and activity of genes. Assessment of RNA transcripts is believed to be representative of gene expression activity (Crick, 1970). RNASeq captures a "snapshot" of the whole transcriptome at a given point in time and has the advantage of detecting novel gene transcripts (Byron *et al.*, 2016). The main disadvantage of RNASeq would be the complex data analysis involved (Byron *et al.*, 2016). Collaborative work with bioinformaticians is routinely required for the data pre-processing and analysis to ensure results are quality-assured.

For this study, RNASeq was used as a discovery platform to identify known and novel transcripts that were differentially expressed between MT and NT cases. The current study is the first to assess DGE between OPMDs with differing clinical outcomes using whole transcriptome profiling in the form of RNASeq as well as subsequent targeted transcriptome profiling. The one previous study that similarly assessed DGE in OPMD (MT vs NT) used microarray technology that assesses only known transcripts and does not capture the whole transcriptome (Saintigny *et al.*, 2011; Byron *et al.*, 2016). Interestingly, the list of statistically significant genes from this study do not overlap with the gene list from the study by Saintigny et al (2011). This could possibly be due to one of three reasons: the molecular heterogeneity of OPMDs, the different gene expression analysis platforms employed and the fact that the patient cohort in the Saintigny et al (2011) study were enrolled in a chemo-preventive trial for their leukoplakia (Saintigny *et al.*, 2011). Their proposed gene-expression based prognostic model for OPMD has yet to be replicated or validated by other groups (Saintigny *et al.*, 2011).

A recent study by Conway et al (2015) also employed RNASeq to assess DGE in "normal", OED and OSCC tissues however all three tissue states ("normal", OED and OSCC) were obtained from the same excision specimen (Conway *et al.*, 2015). Due to the well-recognised theory of field change in OPMD patients, it is understood that histologically "normal" tissue may not be molecularly "normal" and free from

molecular change (Slaughter *et al.*, 1953; Braakhuis *et al.*, 2003) which introduces a confounder to the results obtained by Conway et al (2015). This confounding problem of normal epithelial tissue affects the majority of published gene expression studies involving OPMD and OSCC (Yu *et al.*, 2008). Such studies may only provide an approximation of the molecular events that take place during malignant transformation of OPMD (Ha *et al.*, 2003; Banerjee *et al.*, 2005; Odani *et al.*, 2006; Kondoh *et al.*, 2007; Yu *et al.*, 2008; Conway *et al.*, 2015).

One of the limitations of this part of the study is the relatively small number of cases included compared to the study by Saintigny et al (2011) that had an 86-patient cohort (Saintigny *et al.*, 2011). Another limitation is that gene expression studies only allow a snapshot of the transcriptomic profile at that given point in time to be taken and as such is a very simplistic and static representation of a dynamic temporal process. Furthermore, an OPMD that was categorised as being a non-transforming case may eventually undergo MT. However, RNASeq analysis for this study was to serve only as an initial broad overview of the transcriptomic differences between OPMD cases that undergo MT and those that do not, which was successfully performed.

6.4.2 NanoString nCounter platform

The current study provides additional evidence to the clinical utility of the NanoString nCounter platform in providing robust gene expression outputs using RNA from FFPE tissue (Geiss *et al.*, 2008; Reis *et al.*, 2011; Scott *et al.*, 2013; Saba *et al.*, 2015; Veldman-Jones *et al.*, 2015a; Veldman-Jones *et al.*, 2015b). Though relatively new, the NanoString nCounter assay has been shown by several studies to be sensitive and reproducible, with sensitivity and accuracy levels that are better than microarrays and similar to real-time PCR (qPCR) (Geiss *et al.*, 2008; Reis *et al.*, 2011; Scott *et al.*, 2013; Saba *et al.*, 2015; Veldman-Jones *et al.*, 2015a; Veldman-Jones *et al.*, 2015b). A recent study by Veldman-Jones et al (2015) that evaluated the robustness of the nCounter platform in analysing clinical samples showed that the platform has high sensitivity of target detection and good reproducibility even with low RNA amounts making it suitable for clinical utility (Veldman-Jones *et al.*, 2015a). There are two main advantages of NanoString compared to conventional gene expression analysis methods such as qPCR and microarrays. In the nCounter platform, transcript levels are measured from un-amplified total RNA unlike other

platforms thus reducing errors/biases that may be introduced through increased sample manipulation and enzymatic reactions (Geiss *et al.*, 2008; Reis *et al.*, 2011). Another advantage of NanoString is that it can be multiplexed to measure up 800 target genes in one sample unlike qPCR based methods that usually measures the expression of a single gene at a time (Geiss *et al.*, 2008; Reis *et al.*, 2011; Scott *et al.*, 2013; Saba *et al.*, 2015; Veldman-Jones *et al.*, 2015a; Veldman-Jones *et al.*, 2015b).

Results from the PanCancer Pathways Panel Plus showed that there was no single cancer pathway that was highly significant in the malignant transformation for this OPMD cohort. The most commonly implicated cancer-related pathways in this cohort appear to be the PI3K, RAS, MAPK, JAK-STAT and Wnt signalling pathways (Table 6.4, Table 6.5 and Appendix H) which are also commonly implicated in head and neck SCC (HNSCC) (Kalyankrishna and Grandis, 2006; Molinolo *et al.*, 2009; Rothenberg and Ellisen, 2012; Iglesias-Bartolome *et al.*, 2013; Makarev *et al.*, 2017).

A recent pathway analysis study by Makarev et al (2017) showed that even though OSCCs may have a huge variety of distinct genetic alterations, these changes appear to involve only a few well-known pathways; Wnt, PI3K, RAS/MAPK, JAK-STAT and TGF-β (Makarev *et al.*, 2017). They also found that the usual cancer-related pathways were not similarly affected in the majority of OPMDs, with many cancer-related pathways being downregulated and the pro-apoptotic pathways being slightly upregulated compare to normal tissue. This would fit in with the existing body of evidence that the majority (~ 90%) of OPMDs do not undergo MT (Mehanna *et al.*, 2009; Shariff and Zavras, 2015). Of note, they did find a sub-set of the OPMDs that had differentially activated pathways that were more similar to OSCCs. These findings suggest that although the majority of OPMD exhibit signalling pathway activity that is less aggressive than OSSC, a subset with dysregulation of these cancer-related pathways does exist. However, this also is suggestive that OSCC possibly arises from changes in different molecular pathways with no single prominent signalling pathway. The findings from our current study appears to be similar whereby the differentially expressed genes between MT and NT are implicated in those cancer-driving pathways. The pathway analysis performed on OPMD by Makarev et al (2017) however was based only on one gene expression dataset which was obtained from patients in a chemo-preventive clinical trial

(Saintigny *et al.*, 2011), and as such the results need to be interpreted with caution (Saintigny *et al.*, 2011; Makarev *et al.*, 2017).

The T-cell leukaemia homeobox 1 (*TLX1*) gene was the only gene that was significantly differentially expressed in both the RNASeq and PanCancer Pathways Panel Plus experiments. *TLX1* belongs to the homeobox gene family of transcription factors involved in the early stages of embryonic development (McGinnis and Krumlauf, 1992; Garcia-Fernandez, 2005). *TLX1* has been linked to splenic development, immortalisation of haematopoietic precursor cells and formation of neuronal circuity of central as well as peripheral nervous systems (Dear *et al.*, 1995; Logan *et al.*, 1998; Zhang *et al.*, 1999). *TLX1* has also been linked to the development of T-cell acute lymphoblastic leukaemia (Hatano *et al.*, 1991). At this point in time, there have been no studies linking *TLX1* to OPMD/OSCC. From this study, *TLX1* appears to be downregulated in OPMD that undergo MT. None of the additional genes supplemented to the PanCancer Pathways Panel (Appendix D) were found to be statistically significant upon DGE analysis.

From the Customised CodeSet Panel experiment, 22 genes were significantly differentially expressed between OED cell lines and FFPE tissue samples (clinical cases). This high number of differentially expressed genes in a targeted CodeSet made up of only 38 genes (excluding housekeeping genes) was to be expected due to two main reasons. Firstly, cell line RNA was obtained from fresh tissue and would not be as degraded as RNA from FFPE tissue (von Ahlfen *et al.*, 2007). Secondly, OED cell lines are not as complex as human tissue samples and were composed of only epithelial tissue whilst the FFPE samples were composed of epithelium as well as underlying connective tissue. As such, although cell lines are invaluable for *in-vitro* experiments, the findings from the current study serve to remind us that OED cell lines are too simplistic and do not adequately represent the *in-vivo* environment of oral epithelial dysplasia. When comparing the list of differentially expressed genes separately between cell lines vs MT cases as well as cell line vs NT cases, the list of significant genes was similar, once again highlighting the vastly similar gene expression profile between NT and MT cases.

After excluding the cell line samples, only five genes were significantly differentially expressed between the NT and MT cases using the Customised CodeSet Panel: *NOTCH1*, cyclin E1(*CCNE1*) and *TLX1* were down-regulated whilst integrin subunit

beta 8 (*ITGB8*) and *TP63* genes were up-regulated. *NOTCH1* encodes for one of the proteins involved in the Notch signalling pathway and the association between *NOTCH1* and oncogenesis was initially identified in T-cell acute lymphoblastic leukaemia (Ellisen *et al.*, 1991). Since then, *NOTCH1* has been implicated in several different types of cancer (Reedijk *et al.*, 2005; Westhoff *et al.*, 2009; Hanlon *et al.*, 2010; Ranganathan *et al.*, 2011; Viatour *et al.*, 2011; Yoshida *et al.*, 2013; Yap *et al.*, 2015). Findings regarding the role of *NOTCH1* in OSCC have been conflicting as some earlier studies have indicated that *NOTCH1* is oncogenic whilst more recent studies tend to favour the hypothesis that *NOTCH1* behaves as a tumour suppressor gene in OSCC (Leethanakul *et al.*, 2000; Zeng *et al.*, 2005; Agrawal *et al.*, 2011; Stransky *et al.*, 2011; Zhang *et al.*, 2011; Lee *et al.*, 2012; Sakamoto *et al.*, 2012; Pickering *et al.*, 2013; Yoshida *et al.*, 2013; Song *et al.*, 2014; Yap *et al.*, 2015). Findings from this study show that expression of *NOTCH1* is downregulated in OPMD that undergo MT, consistent with findings from studies suggesting that *NOTCH1* has a tumour suppressive role in oral carcinogenesis (Agrawal *et al.*, 2011; Sakamoto *et al.*, 2012; Pickering *et al.*, 2013; Song *et al.*, 2014; Yap *et al.*, 2015). Interestingly, both Notch1 and *TLX1* have been implicated in the pathogenesis of T-cell acute lymphoblastic leukaemia (Ellisen *et al.*, 1991; Hatano *et al.*, 1991; Rakowski *et al.*, 2011).

Expression of *CCNE1* was found to be downregulated in this study. *CCNE1* encodes cyclin E1, a protein that belongs to the cyclin family that function as regulators of cyclin-dependent kinases (CDKs). *CCNE1* is particularly involved in the G1 to S phase transition in cells. *CCNE1* has been implicated in several different types of cancers such as ovarian, breast, hepatocellular and oesophageal cancers (Kohzato *et al.*, 2001; Sui *et al.*, 2001; Moroy and Geisen, 2004; Scaltriti *et al.*, 2011; Cancer Genome Atlas Research Network, 2017). At this point in time, there have been no studies linking CCNE1 to OPMD/OSCC.

The *ITGB8* gene is a member of the integrin family and encodes a membrane protein. In this study *ITGB8* expression was upregulated in OPMD cases that underwent malignant transformation. Integrins are cell surface receptors involved with cellular adhesion and believed to mediate cell-to-cell as well as cell-to-extracellular matrix interactions. *ITGB8* has recently been implicated in several different types of cancers such as ovarian, hepatic, lung and laryngeal cancer (Ni *et*

al., 2012; Xu and Wu, 2012; Mertens-Walker *et al.*, 2015; Wala *et al.*, 2015; Wang *et al.*, 2015; Cui *et al.*, 2018). At this point in time, there have been no studies linking *ITGB8* to OPMD/OSCC.

The *TP63* gene encodes for the p63 protein a member of the p53 family of transcription factors. An isoform of p63, ΔNp63 has been reported to be overexpressed in OPMDs that transformed to OSCC by several studies (Chen *et al.*, 2005; Saintigny *et al.*, 2009; Matsubara *et al.*, 2011; Varun *et al.*, 2014). Findings from this study appear to support the notion that expression of *TP63* is increased in cases of OPMD that undergo MT. However, the evidence is still inconclusive as to its role in oral carcinogenesis as well as being a prognostic biomarker for OPMDs (Smith *et al.*, 2009; Lingen *et al.*, 2011; Nankivell and Mehanna, 2011).

A novel way to generate a prognostic gene-signature for OPMD patients utilising a tiered approach from whole transcriptome to targeted transcriptome assessment was performed in the current study. The gene-signature developed from this study appears to have good potential in discriminating OPMD that may undergo MT and those that do not, with very high specificity and negative predictive values. A major issue with developing gene-signatures is clinical validation (Koscielny, 2010; Brulard and Chibon, 2013; Chibon, 2013). An earlier prognostic gene-signature developed by Saintigny et al (2011) for oral leukoplakia, though initially promising, remains un-validated and as such has not been translated into clinical practice (Saintigny *et al.*, 2011). The optimum method for validation of a new gene-signature would be for it to be validated by an independent research team on an independent patient cohort (Koscielny, 2010; Brulard and Chibon, 2013; Chibon, 2013). Lack of independence between the training and test/validation cohorts can lead to an over-estimation of the prognostic ability of a gene-signature. The lack of published validation for a gene-signature may also not be due to a lack of trying, as some researchers may hesitate to publish findings that fail to validate the developed gene-signature.

Another barrier for successful validation of a prognostic gene-signature is the presence of inter- and intra-tumour heterogeneity in OSCC as well as heterogeneity in OPMD (Hirsch *et al.*, 1983; Perou *et al.*, 2000; Chung *et al.*, 2004; Diwakar *et al.*, 2005; Russnes *et al.*, 2011; Marusyk *et al.*, 2012; Fisher *et al.*, 2013; De Cecco *et al.*, 2015; Gomes *et al.*, 2015; Gay *et al.*, 2016; Mroz and Rocco, 2016). As such, although promising, the newly developed gene-signature needs to be validated on a

separate larger cohort of patients to ascertain clinical utility as a prognostic indicator of clinical outcome in OPMD.

6.5 Conclusions

This chapter has highlighted the potential use of gene expression as a prognostic biomarker for OPMD. A stratified method to refine a suitable prognostic gene-signature was detailed ranging from whole transcriptome sequencing to specifically targeted transcriptome assessment. The next generation sequencing platforms used in this experiment have shown good results using FFPE material with all samples being successfully analysed. Aside from developing a prognostic gene-signature, several new candidate genes with possible roles in oral carcinogenesis such as TLX1, CCNE1 and ITGB8 have been identified. Further investigation of these genes may provide more clarity to the natural history of OMPD as well as the aetiopathogenesis of OSCC. Importantly, the gene-signature that has been developed needs to be validated in an external cohort to ascertain its' prognostic utility.

Table 6.1 Characteristics of cases selected for RNASeq experiment (n=20)

Characteristic		Non-transforming (NT) n=10	Malignant transforming (MT) n=10	p-value
Age [Mean (± SD)]		55.7 (±14.86)	60.0 (±12.41)	0.491 †
Time to last follow-up/malignant transformation in months [Median (IQR)]		28.00 (46.25)	17.00 (42.75)	0.684α
Sex	Male	9	7	0.582*
	Female	1	3	
Site of OPMD	Tongue	4	3	1.000*
	Other sites	6	7	
WHO 2017 OED grading	Mild	3	3	0.635**
	Moderate	1	3	
	Severe	6	4	
Binary OED grading	Low-grade	3	3	1.000*
	High-grade	7	7	

† Independent t-test; α Mann-Whitney U test; *Fisher's Exact test; **Pearson's Chi-square test; SD = Standard deviation; IQR = Interquartile range

Table 6.2 Significant differentially expressed genes from RNASeq experiment (n=20)

Ensembl gene ID	HGNC symbol	Gene biotype	Log2 fold Change	FDR [BH p-value]
ENSG00000196805	*SPRR2B*	Protein coding	2.48	0.015
ENSG00000283029	NA	Non-coding	2.44	0.015
ENSG00000115844	*DLX2*	Protein coding	2.30	0.015
ENSG00000229035	*SPRR2C*	Unprocessed pseudogene	2.28	0.015
ENSG00000223802	*CERS1*	Protein coding	2.19	0.044
ENSG00000166165	*CKB*	Protein coding	2.11	0.015
ENSG00000137869	*CYP19A1*	Protein coding	2.10	0.046
ENSG00000235852	NA	Antisense	2.00	0.019
ENSG00000186648	*LRRC16B*	Protein coding	1.79	0.030
ENSG00000276368	*HIST1H2AJ*	Protein coding	1.78	0.037
ENSG00000123416	*TUBA1B*	Protein coding	1.72	0.025
ENSG00000137331	*IER3*	Protein coding	1.61	0.046
ENSG00000066248	*NGEF*	Protein coding	1.58	0.031
ENSG00000127824	*TUBA4A*	Protein coding	1.48	0.015
ENSG00000162836	*ACP6*	Protein coding	-1.05	0.031
ENSG00000164808	*SPIDR*	Protein coding	-1.32	0.025
ENSG00000117335	*CD46*	Protein coding	-1.43	0.031
ENSG00000111670	*GNPTAB*	Protein coding	-1.44	0.037
ENSG00000135338	*LCA5*	Protein coding	-1.45	0.035
ENSG00000166432	*ZMAT1*	Protein coding	-1.47	0.020
ENSG00000181804	*SLC9A9*	Protein coding	-1.67	0.026
ENSG00000204789	*ZNF204P*	Processed pseudogene	-1.78	0.037
ENSG00000165186	*PTCHD1*	Protein coding	-1.79	0.036
ENSG00000112773	*FAM46A*	Protein coding	-1.80	0.037
ENSG00000139292	*LGR5*	Protein coding	-1.82	0.046

Ensembl gene ID	HGNC symbol	Gene biotype	Log2 fold Change	FDR [BH p-value]
ENSG00000185499	*MUC1*	Protein coding	-1.83	0.026
ENSG00000214290	*COLCA2*	Protein coding	-1.83	0.016
ENSG00000267395	*DM1-AS*	Antisense	-1.86	0.033
ENSG00000196724	*ZNF418*	Protein coding	-1.91	0.019
ENSG00000177707	*NECTIN3*	Protein coding	-2.01	0.026
ENSG00000115648	*MLPH*	Protein coding	-2.03	0.019
ENSG00000279387	NA	NA	-2.12	0.019
ENSG00000180347	*CCDC129*	Protein coding	-2.19	0.020
ENSG00000235902	NA	Antisense	-2.21	0.024
ENSG00000115112	*TFCP2L1*	Protein coding	-2.22	0.015
ENSG00000116039	*ATP6V1B1*	Protein coding	-2.31	0.025
ENSG00000177685	*CRACR2B*	Protein coding	-2.33	0.015
ENSG00000134398	*ERN2*	Protein coding	-2.41	0.015
ENSG00000167165	*UGT1A6*	Protein coding	-2.44	0.015
ENSG00000107807	*TLX1*	Protein coding	-2.46	0.016
ENSG00000181143	*MUC16*	Protein coding	-2.63	0.015

*FDR = False discovery rate; BH = Benjamini-Hochberg. False Discovery Rate calculated using Benjamini-Hochberg method and significance set at the 5% level ($p < 0.05$). HGNC = Human Genome Organisation Gene Nomenclature Committee. NA = not available

Table 6.3 Characteristics of cases for PanCancer Pathways Panel Plus experiment (n=48)

Characteristics		Non-transforming (NT) n = 25	Malignant transforming (MT) n = 23	p-value
Age at diagnosis in years Mean [SD] (Range)		59.36 [13.19] (34 - 89)	63.30 [12.98] (35 - 92)	0.302†
Age at diagnosis	≤ 50 years	9	2	0.039*
	> 50 years	16	21	
Time to last follow-up/malignant transformation in months Median [IQR] (Range)		173.00 [72.00] (60 – 215)	39.00 [55.00] (7 – 128)	< 0.001α
Sex	Male	14	16	0.502*
	Female	11	7	
Type of OPMD	Leukoplakia	20	18	0.886*
	Erythroplakia	3	2	
	Erythroleukoplakia	2	3	
Site of OPMD (binary)	Tongue	5	12	0.034*
	Other sites	20	11	
OPALS	OPALS negative	7	10	0.367*
	OPALS positive	18	13	
WHO 2017 OED grading	Mild	15	9	0.152*
	Moderate	5	3	
	Severe	5	11	
Binary OED grading	Low-grade	20	9	0.009*
	High-grade	5	14	
HR-HPV status	Negative	24	22	1.000*
	Positive	1	1	

† Independent t-test; α Mann-Whitney U test; *Pearson's chi-square test (Exact method); IQR = interquartile range; SD = standard deviation

Table 6.4 Differentially expressed genes for PanCancer Pathways Panel Plus experiment (n =48)

Gene	Log2 fold change	p-value	FDR [BH p-value]	Associated cancer pathway sets
ITGB8	0.711	7.29E-06	0.005	PI3K
IGF1R	0.401	2.24E-05	0.008	PI3K, Ras,
NTRK2	0.886	3.71E-05	0.009	MAPK
CCNE1	-0.936	4.91E-05	0.009	Cell Cycle - Apoptosis, PI3K
IL22RA1	-0.896	6.87E-05	0.010	JAK-STAT
FZD7	0.698	0.000117	0.014	Wnt
GATA1	-0.969	0.000259	0.026	Driver gene
ARID1B	0.224	0.000318	0.026	Driver gene
PLCB1	0.782	0.000332	0.026	Wnt
PLA2G4E	-1.200	0.000348	0.026	MAPK, Ras
PBX1	0.603	0.000387	0.026	Transcriptional misregulation
MECOM	0.691	0.000546	0.034	MAPK
SOX9	1.380	0.00081	0.046	Driver gene
DUSP5	-1.040	0.000886	0.047	MAPK
COL4A5	0.727	0.00107	0.053	PI3K
AMH	-0.844	0.00132	0.060	TGF-beta
CACNA2D1	0.853	0.00137	0.060	MAPK
TLX1	-1.970	0.00154	0.063	Transcriptional misregulation
SMAD9	0.828	0.00249	0.097	TGF-beta

* FDR = False discovery rate; BH = Benjamini-Hochberg. FDR calculated using Benjamini-Hochberg method and significance set at the 10% level ($p < 0.10$). ** 'Log2 fold-change' estimates a gene's differential expression; a negative value would mean down-regulation and a positive value would mean up-regulation in MT cases versus a baseline of NT cases, holding all other variables in the analysis constant.

Table 6.5 Significant differentially expressed genes in HR-HPV negative cases for PanCancer Pathways Panel Plus experiment (n =46)

Gene	Log2 fold change	P-value	FDR [BH p-value]	Associated cancer pathway sets
ITGB8	0.700	1.85E-05	0.012	PI3K
IGF1R	0.406	3.72E-05	0.012	PI3K, Ras
NTRK2	0.908	4.77E-05	0.012	MAPK
CCNE1	-0.935	9.56E-05	0.018	Cell Cycle - Apoptosis, PI3K
IL22RA1	-0.827	0.000224	0.031	JAK-STAT
GATA1	-0.99	0.000252	0.031	Driver gene
FZD7	0.678	0.000297	0.031	Wnt
PBX1	0.6	0.000666	0.056	Transcriptional misregulation
MECOM	0.7	0.000682	0.056	MAPK
PLA2G4E	-1.14	0.000788	0.058	MAPK, Ras
PLCB1	0.74	0.000857	0.058	Wnt
ARID1B	0.207	0.00102	0.063	Driver gene
TLX1	-2.01	0.00134	0.077	Transcriptional misregulation
COL4A5	0.717	0.00188	0.099	PI3K

* FDR = False discovery rate; BH = Benjamini-Hochberg; FDR calculated using Benjamini-Hochberg method and significance set at the 10% level ($p < 0.10$). ** 'Log2 fold-change' estimates a gene's differential expression; a negative value would mean down-regulation and a positive value would mean up-regulation in MT cases versus a baseline of NT cases, holding all other variables in the analysis constant.

Table 6.6 Characteristics of cases selected for NanoString Customised CodeSet Panel experiment (n = 44)

Characteristic		Non-transforming (NT) n = 24	Malignant transforming (MT) n = 20	P-value
Age at diagnosis in years Mean [SD] (Range)		59.67 [13.38] (34 - 89)	61.30 [12.58] (35 – 92)	0.681†
Age at diagnosis	≤ 50 years	9	2	0.078*
	> 50 years	15	18	
Time to last follow-up/ malignant trans-formation in months Median [IQR] (Range)		174.00 [65.00] (66 – 215)	28.50 [47.75] (7 – 111)	< 0.001α
Sex	Male	14	13	0.760*
	Female	10	7	
Site of OPMD	Tongue	4	11	0.011*
	Other sites	20	9	
WHO 2017 OED grading	Mild	15	8	0.140**
	Moderate	5	3	
	Severe	4	9	
Binary OED grading	Low-grade	20	8	0.005*
	High-grade	4	12	

† Independent t-test; α Mann-Whitney U test; *Fisher's Exact test; **Pearson's Chi-square test; SD = Standard deviation; IQR = Interquartile range

Table 6.7 Significant differentially expressed genes between clinical cases and OED cell lines using Customised CodeSet Panel with OED cell lines being the baseline (n =48)

Gene name	Log2 fold change	FDR [BH p-value]
NTRK2	10.4	7.65E-27
BCL2	5.19	1.49E-17
CACNA2D1	8.36	1.49E-17
PLA2G4E	8.56	5.90E-17
Notch1	3.58	1.11E-12
SPRR2B	8.55	4.08E-11
LGR5	5.85	4.42E-11
TP63	6.37	3.27E-10
PAX9	4.52	1.37E-07
FHIT	3.67	1.52E-07
DUSP5	3.31	7.64E-07
CTSL	-1.97	9.84E-07
DSPP	2.88	1.51E-06
CERS1	3.24	2.06E-05
TLX1	5.23	0.000156
NECTIN3	-1.46	0.000156
IBSP	1.9	0.000453
BIRC5	-1.6	0.00321
CDKN2A	2.36	0.00321
TP53	1.06	0.00436
CHEK2	-1.21	0.0056
PDPN	-1.33	0.00607

*FDR = False discovery rate; BH = Benjamini-Hochberg; FDR calculated using Benjamini-Hochberg method and significance set at the 5% level ($p < 0.05$). ** 'Log2 fold-change' estimates a gene's differential expression; a negative value would mean down-regulation and a positive value would mean up-regulation in MT cases versus a baseline of NT cases, holding all other variables in the analysis constant.

Table 6.8 Significant differentially expressed genes between OED cell lines and clinical cases with differing outcomes (MT & NT) using the Customised CodeSet Panel with OED cell lines being the baseline (n =48)

NT vs Cell Lines			MT vs Cell Lines		
Gene name	**Log2 fold change**	**FDR [BH p-value]**	**Gene name**	**Log2 fold change**	**FDR [BH p-value]**
NTRK2	10.2	2.80E-26	***NTRK2***	10.7	6.87E-27
BCL2	5.22	5.98E-17	***CACNA2D1***	8.61	3.33E-17
PLA2G4E	8.8	5.98E-17	***BCL2***	5.16	1.23E-16
CACNA2D1	8.15	7.43E-17	***PLA2G4E***	8.26	7.37E-16
NOTCH1	3.86	8.09E-15	***NOTCH1***	3.24	3.89E-12
SPRR2B	8.41	1.83E-10	***SPRR2B***	8.73	9.42E-11
TP63	6.08	1.91E-09	***TP63***	6.71	1.90E-10
DUSP5	3.63	7.72E-08	***PAX9***	4.57	3.38E-07
PAX9	4.49	3.27E-07	***CTSL***	-2.1	6.69E-07
CTSL	-1.87	4.37E-06	***DUSP5***	2.92	7.03E-06
NECTIN3	-1.29	0.000813	***NECTIN3***	-1.66	2.83E-05
TP53	1.21	0.00177	***BIRC5***	-1.64	0.00439
PDPN	-1.51	0.00269	***CHEK2***	-1.3	0.00527
BIRC5	-1.57	0.0054	***TP53***	0.886	0.0205
CHEK2	-1.14	0.0111	***PDPN***	-1.1	0.0269

*FDR = False discovery rate; BH = Benjamini-Hochberg; FDR calculated using Benjamini-Hochberg method and significance set at the 5% level ($p < 0.05$). ** 'Log2 fold-change' estimates a gene's differential expression; a negative value would mean down-regulation and a positive value would mean up-regulation in MT cases versus a baseline of NT cases, holding all other variables in the analysis constant.

Table 6.9 Significant differentially expressed genes using Customised CodeSet Panel (n = 44)

Gene name	Log2 fold change	p-value	FDR (BH p-value)
NOTCH1	-0.63	8.71 e-05	0.0027
CCNE1	-0.824	0.000813	0.0065
TLX1	-2.24	0.000864	0.0059
ITGB8	0.528	0.00102	0.0059
TP63	0.621	0.00409	0.0188

*FDR = False discovery rate; BH = Benjamini-Hochberg; FDR calculated using Benjamini-Hochberg method and significance set at the 5% level ($p < 0.05$). ** 'Log2 fold-change' estimates a gene's differential expression; a negative value would mean down-regulation and a positive value would mean up-regulation in MT cases versus a baseline of NT cases, holding all other variables in the analysis constant.

Table 6.10 Cox regression analysis (CRA) assessing association between malignant transformation and significant differentially expressed genes between MT and NT cases (n = 44)

Variable	HR (95% CI)	p-value	β-coefficient
NOTCH1	0.27 (0.09, 0.81)	0.020	-1.317
CCNE1	0.12 (0.04, 0.36)	< 0.001	-2.158
TP63	4.22 (1.44, 12.37)	0.009	1.441

HR = Hazard ratio; CI = Confidence interval. Variables included for multivariate analysis: Dichotomised (low-expression; high-expression) log2 normalised gene expression for *NOTCH1*, *CCNE1*, *TLX1*, *ITGB8* and *TP63*.

Table 6.11 Comparison and evaluation of dichotomised gene-signature score as a prognostic instrument in OPMD against OED grading (n = 44)

Statistic	Dichotomised gene-signature score	WHO 2017 grading (mild & moderate vs severe OED)	Binary OED grading
Sensitivity	70.0%	45.0%	60.0%
Specificity	91.7%	83.3%	83.3%
Accuracy	81.8%	65.9%	72.7%
***Positive predictive value**	49.7%	24.1%	29.7%
***Negative predictive value**	96.3%	92.8%	94.7%

*Value based on 10.5% prevalence rate of MT in OED as all cases in this cohort had some level of OED (Shariff and Zavras, 2015)

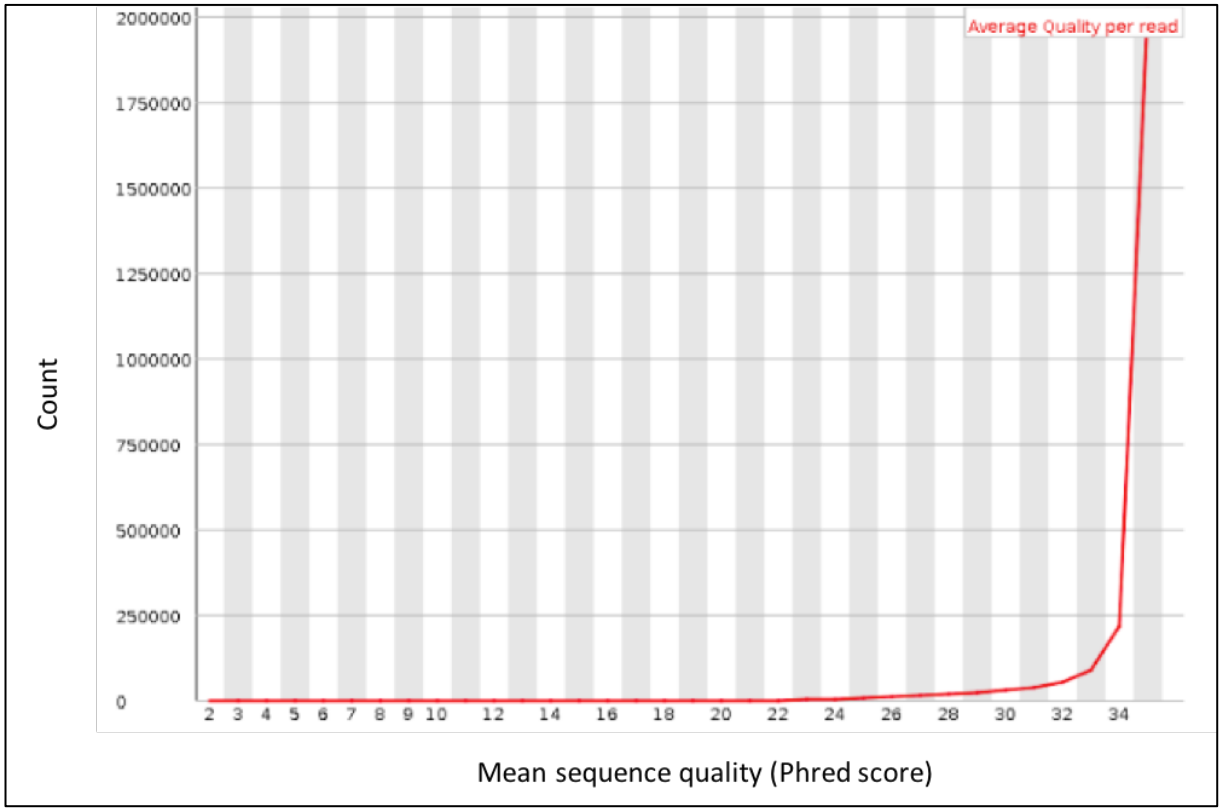


Figure 6.1 Quality score distribution over all sequences with accuracy for sample W1 measured by Phred quality score.

Per sequence quality score shows that most reads for this sample have a high Phred score of > 30.

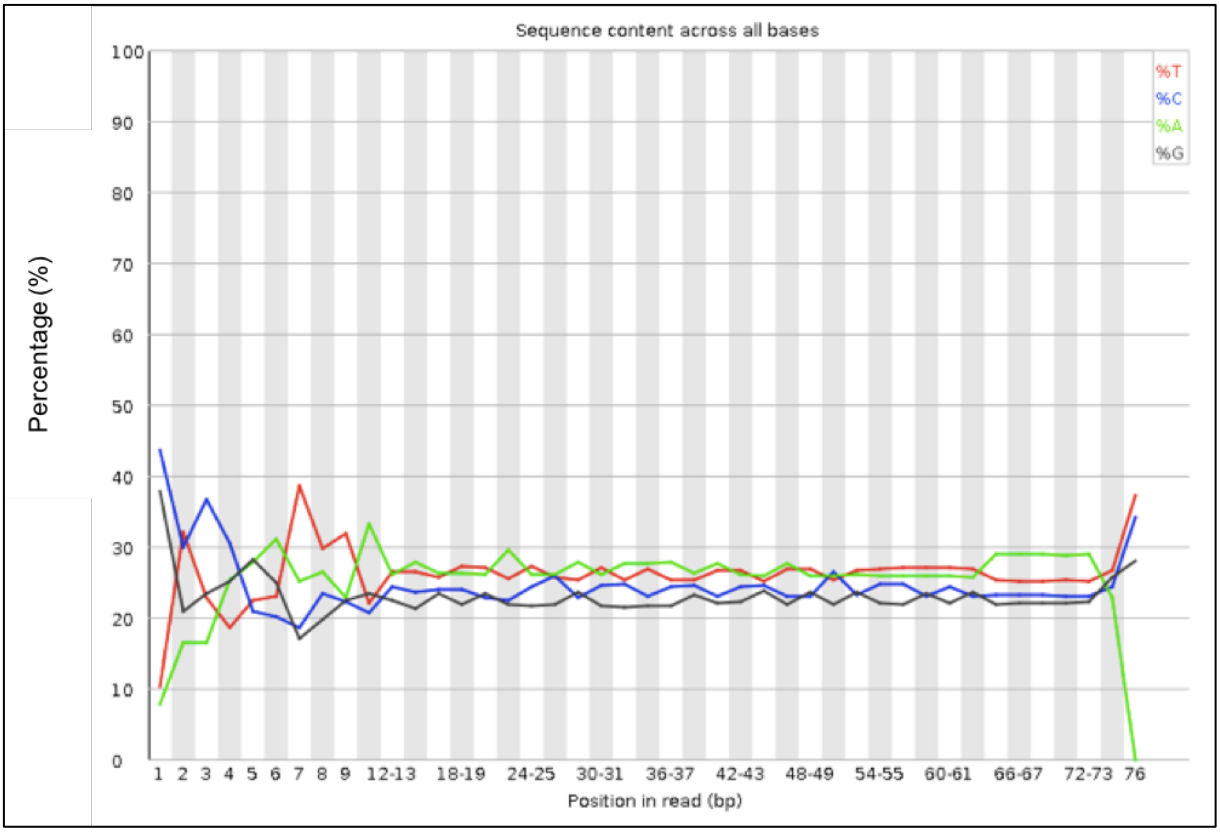


Figure 6.2 Percentage of bases in across all reads for sample W1.

Non-normal distribution is observed on the first 12-13bp. This type of DNA base distribution is commonly seen in next-generation sequencing outputs and originates from the random hexamer priming (Hansen *et al.*, 2010).

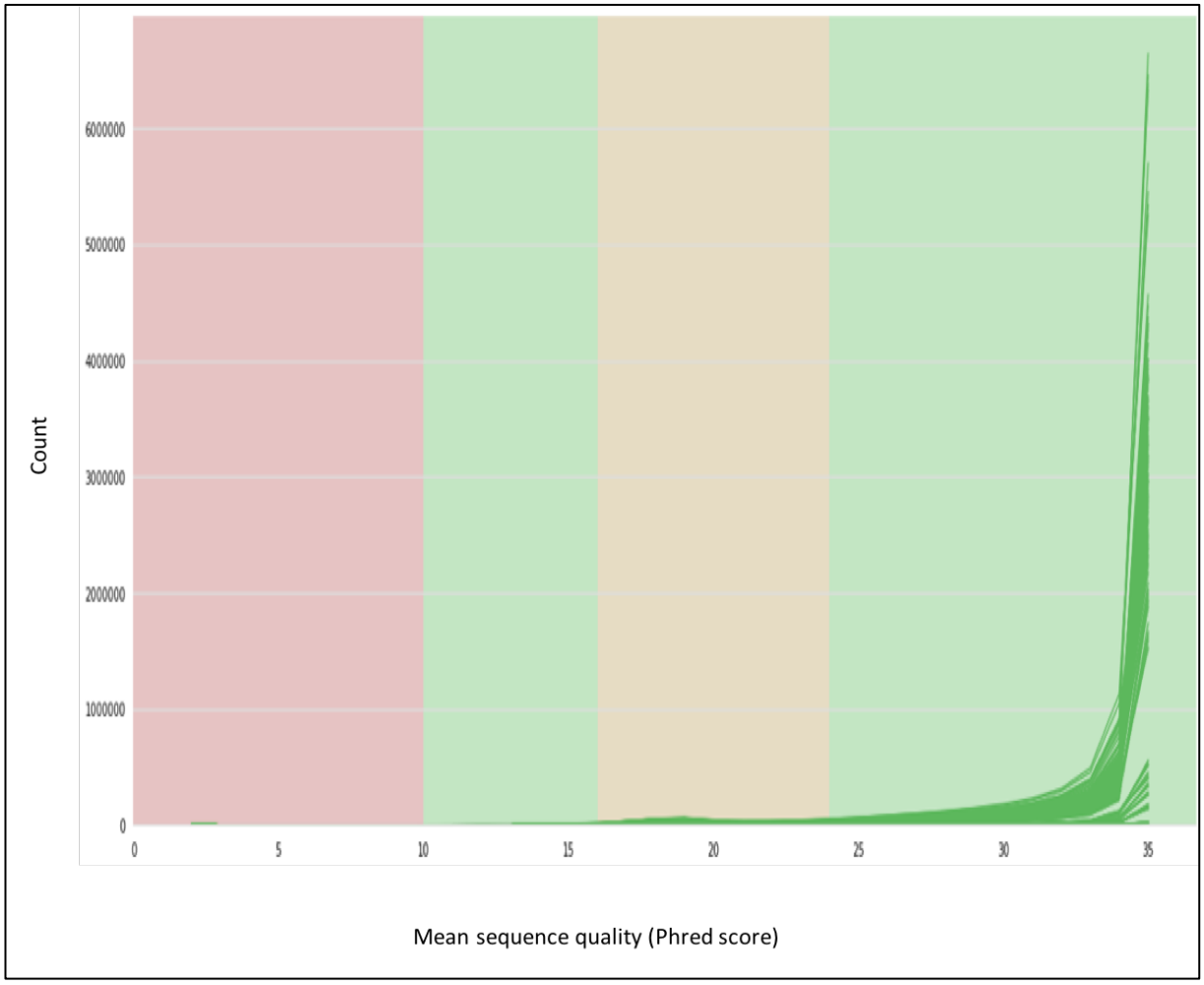


Figure 6.3 Summary of quality assessment of raw reads from all 20 samples for RNASeq experiment.

Per sequence quality score shows that all samples have reads with a high Phred score of > 30. There were no low-quality reads from all samples.

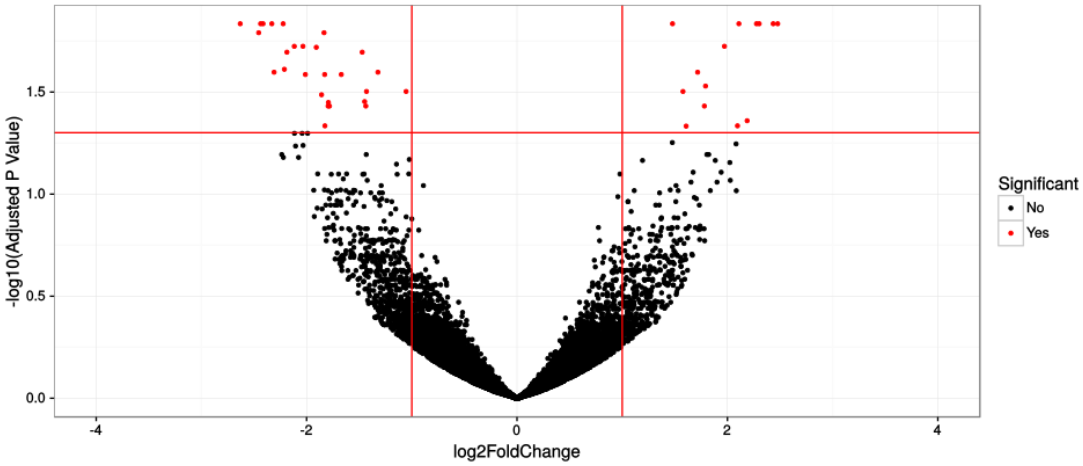


Figure 6.4 Volcano plot showing log2 fold change and significance of all genes expressed.

The red dots represent statistically significant differentially expressed genes between MT and NT sub-groups from the RNASeq experiment.

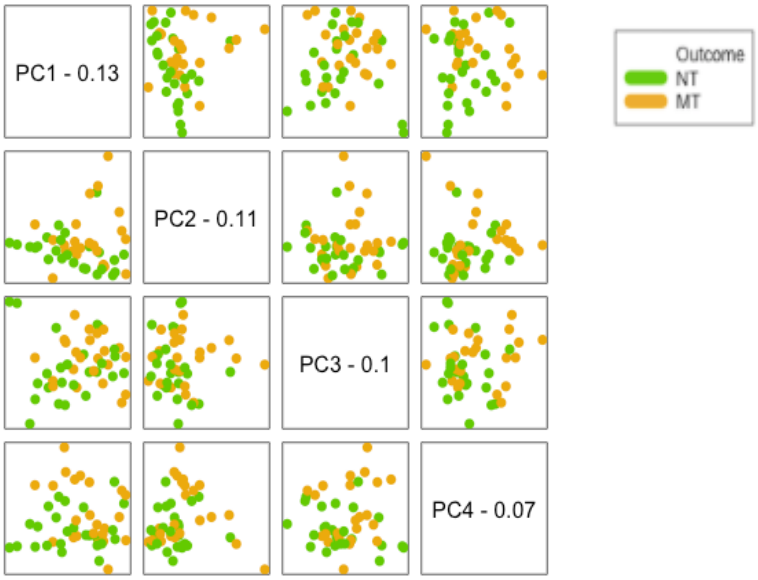


Figure 6.5 Principal component analysis scatter plot showing first four principal components (n = 48) from the PanCancer Pathways Panel Plus.

There appears to be no clear separation between MT and NT cases. MT = malignant transforming; NT = non-transforming.

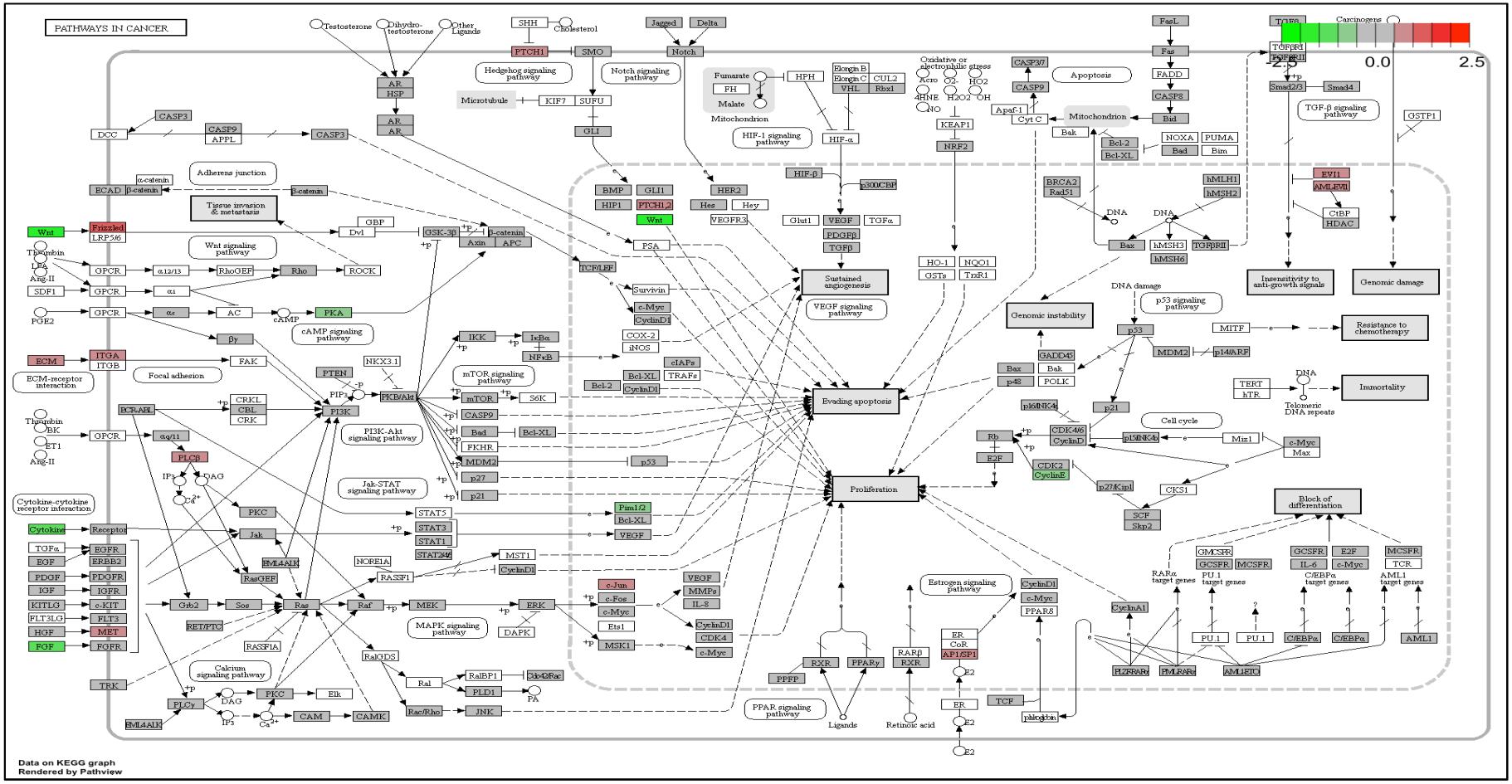


Figure 6.6 Kyoto Encyclopaedia of Genes and Genomes (KEGG) pathway overview for all 13 cancer pathways.

Pathway nodes: white = no genes in the panel that map to them; grey = have corresponding genes in the panel, however no significant differential expression; green = downregulation in MT cases; red = upregulation in MT cases.

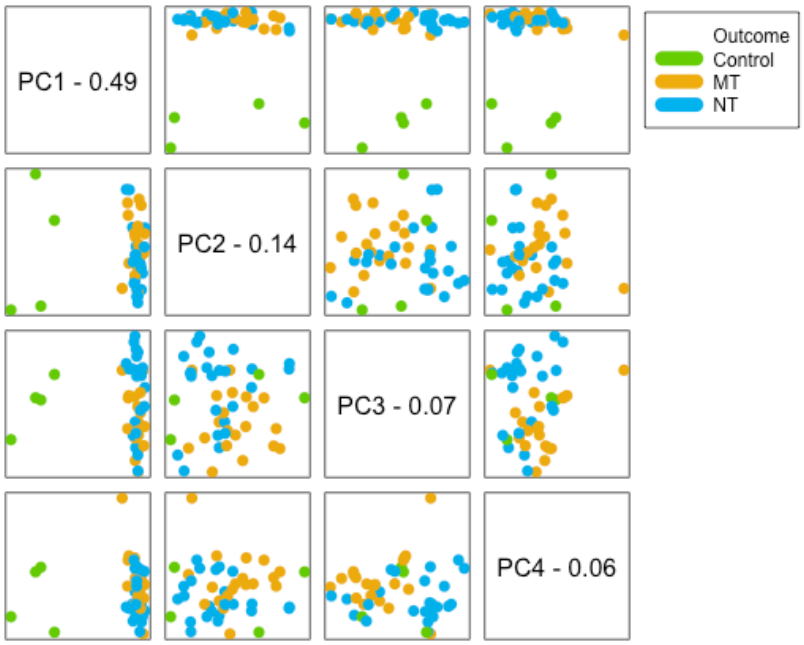


Figure 6.7 Principal component analysis scatter plot showing first four principal components for all cases (n = 48) from the Customised CodeSet Panel.

There is obvious separation between the OED cell line samples and FFPE samples. Control = OED cell lines; MT = malignant transforming; NT = non-transforming.

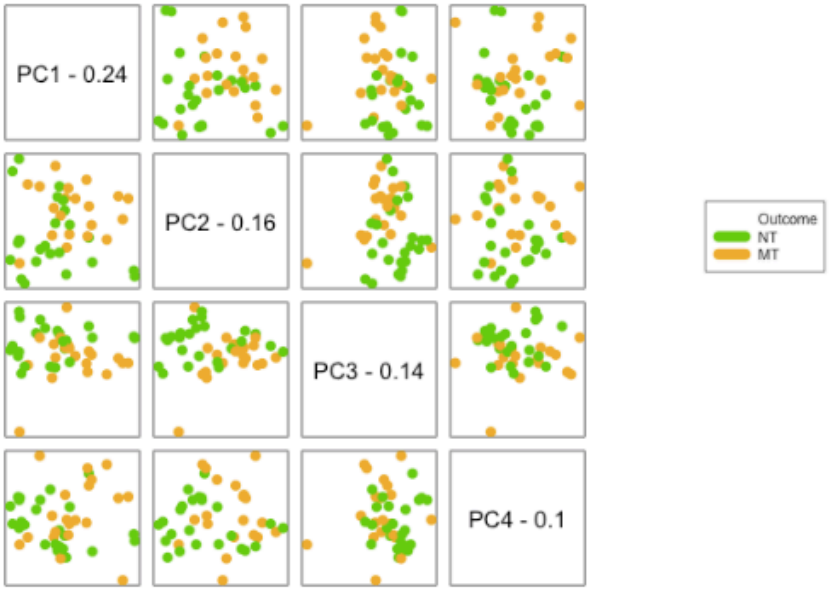


Figure 6.8 Principal component analysis scatter plot showing first four principal components for FFPE cases (n = 44) from the Customised CodeSet Panel.

There is considerable overlap between the MT and NT cases. MT = malignant transforming; NT = non-transforming.

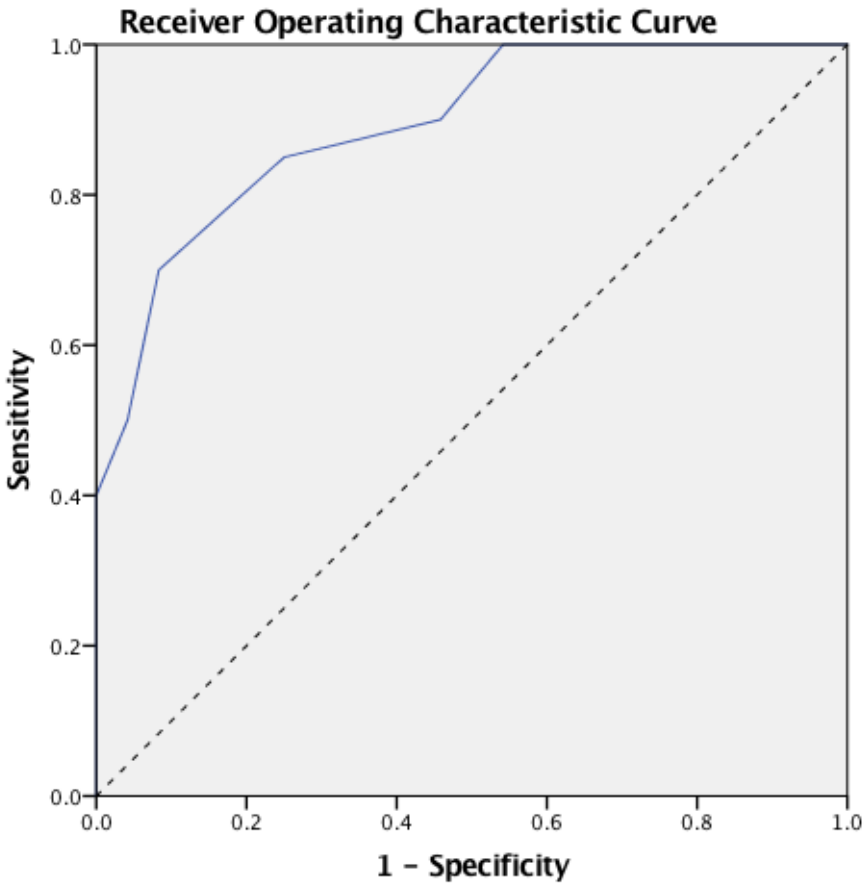


Figure 6.9 Receiver operating characteristic (ROC) curve for the gene-signature derived scores in relation to prognosticating clinical outcome.

The gene-signature has a concordance index of 0.82 (CI: 0.69, 0.95) and area under the curve of 0.893.

Source of curve	Area under curve	Standard error	Asymptotic significance	Asymptotic 95% Confidence Interval	
				Lower Bound	Upper Bound
Gene-signature derived scores	0.893	0.047	< 0.001	0.800	0.985

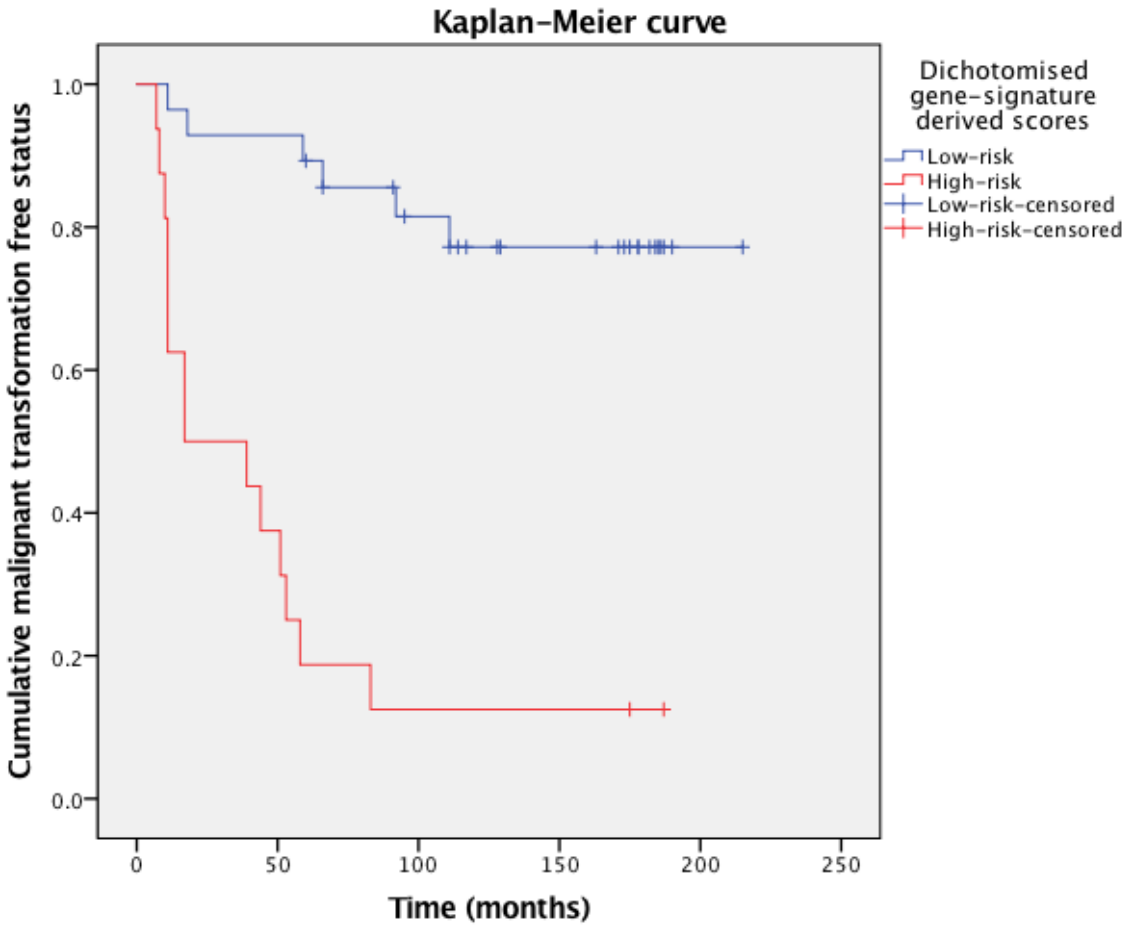


Figure 6.10 Kaplan-Meier time to event analysis comparing malignant transformation in the OPMD cohort stratified according to the dichotomised gene-signature derived scores.

The analysis shows that patients with a high-risk score underwent malignant change of OPMDs more rapidly than those with a low-risk score (Log rank test; $\chi^2 = 27.56$, df =1, $p < 0.001$). Concordance index = 0.76 (CI: 0.66, 0.86). Low-risk score = blue line; High-risk score = red line

Chapter 7. Construction of Multivariate Prognostic Model

7.1 Introduction

Despite being extensively studied, a robust prognostic biomarker or model for OPMD patients has yet to be successfully developed and validated with most biomarkers having sensitivity and specificity that are not markedly better than OED grading (Smith *et al.*, 2009; Nankivell and Mehanna, 2011; Nikitakis *et al.*, 2018). Instead of relying on single biomarkers, prognostic models composed of several variables may prove to be more accurate in risk-stratifying OPMD patients (Moons *et al.*, 2009; Royston *et al.*, 2009; Collins *et al.*, 2011; Steyerberg *et al.*, 2013; Moons *et al.*, 2015). Recently, Sperandio et al (2013) showed that combining OED grading with a molecular parameter (DNA ploidy analysis) may improve prognostication for OPMD patients, though not by very much (Sperandio *et al.*, 2013).

The prognostic ability of several factors (demographic, clinical, histopathology and molecular parameters) in OPMD patients were evaluated in the previous chapters. In this study, when used in isolation, the performance of most conventional methods such as clinical and histopathological findings did not show very good prognostic utility in identifying OPMD patients with a higher risk of undergoing MT (Chapter 3). In this chapter, the construction of prognostic models composed of two or more variables using data obtained from the 44-patient cohort that was analysed using the Customised CodeSet Panel will be described and discussed.

7.2 Aims

The aims of this chapter are:

1) To develop novel prognostic models for patients with OPMD.
2) To evaluate the prognostic utility of the models developed against a model based on conventional clinico-pathological parameters.
3) To assess the performance of the best prognostic model on an external validation cohort of OPMD patients.

7.3 Results

Prognostic model building was performed using the 44-patient cohort that had complete data for the statistically significant parameters from earlier assessments described in Chapters 3 to 6:

- Age at diagnosis of OPMD ($\leq$ 50 vs > 50 years of age)
- Site of index OPMD (tongue vs others)
- Binary OED grading (low-grade vs high-grade)
- LOH status
    - LOH at 3p &/or 9p &/or 17p
    - LOH at 3p &/or 9p
- Gene-signature score (obtained from results of Customised CodeSet Panel experiment described in Chapter 6)

7.3.1 Univariate analysis

Preliminary assessment of the variables using either the Fisher's exact test or Pearson's chi squared test showed that all the above listed variables were statistically significant except for age at diagnosis (Tables 7.1 and 7.2). Time to event analysis using the log-rank test however showed that all the variables were statistically significant (Figures 7.1 to 7.6). Subsequent univariate analysis of the variables using Cox (proportional-hazards regression) analysis (CRA) showed that all the variables except age at diagnosis were statistically significant (Table 7.3). Univariate analysis of LOH results showed that results from LOH analysis assessing only two chromosomal regions (3p &/or 9p) outperformed analysis that included assessment of three chromosomal regions (3p &/or 9p &/or 17p) (Table 7.2 and Table 7.3).

7.3.2 Multivariate analysis

Several prognostic models were constructed using the earlier mentioned variables. A conventional model, Model 1, utilising only demographic, clinical, and histopathological parameters showed that only binary OED grading was statistically significant using CRA, with a concordance index of 0.63 (CI: 0.52, 0.74) in relation to clinical outcome (Table 7.4). A second prognostic model (Model 2) utilising four variables; age at diagnosis, site of OPMD, binary OED grading and LOH at

chromosomal regions 3p &/or 9p was then assessed using CRA. The analysis produced a prognostic model with a concordance index of 0.67 (CI: 0.56, 0.79) in relation to clinical outcome (Table 7.5). A comparison of the prognostic ability of Models 1 and 2 using the receiver operating characteristic (ROC) curve is shown in Figure 7.7. The performance of Model 2 appears to be slightly better than Model 1 (Figure 7.7).

A third prognostic model (Model 3) that included age at diagnosis, site of OPMD, binary OED grading, LOH (3p &/or 9p) and the dichotomised gene-signature based score was assessed using CRA. The results are shown in Table 7.6. The resultant prognostic model (Model 3) was composed of age at diagnosis, site of OPMD, binary OED grading and the dichotomised gene-signature based scores; LOH (3p &/or 9p) was not retained in the model. This model had a concordance index of 0.85 (CI: 0.72, 0.98) in relation to clinical outcome; higher than the concordance index from the other two models. Assessment of the prognostic utility of Model 3 visualised using the ROC curve showed that Model 3 had a much better performance than both Models 1 and 2 with a much larger area under the curve (Figure 7.8).

Model 3 derived scores were then dichotomised for further analysis. Time to event analysis showed cases that were categorised as being high-risk were more likely to have undergone malignant transformation (Figure 7.9). Sensitivity and specificity analysis comparing the dichotomised scores (low-risk vs high-risk for MT) of the three models showed that Model 3 performs better than Models 1 and 2 in prognosticating clinical outcome for the training cohort (Table 7.7).

7.3.3 Validation of prognostic model on an external cohort of OPMD patients

A validation cohort of similar size (23 NT vs 21 MT) to the training cohort was identified through a systematic search of the Royal Victoria Infirmary Cellular Pathology Database. The search identified 359 cases that fulfilled the inclusion and exclusion criteria of which 28 cases (7.8%) underwent MT. Subsequently, 44 cases (21 MT vs 23 NT) were selected as the validation cohort based upon completeness of medical records and availability of adequate FFPE tissue. For NT cases, the duration of follow-up was also taken into consideration, with patients having the longest follow-ups being preferentially selected. Binary OED grading, HR-HPV testing and the NanoString Customised CodeSet Panel experiment were carried out

as described in Chapter 2. The characteristics of the validation cohort are shown in Table 7.8. Age at diagnosis (binary), site of index OPMD, binary OED grade and gene-signature score were all statistically significant in this cohort (Table 7.8). There were no HR-HPV positive cases in the validation cohort.

This cohort was used to validate the best prognostic model (Model 3). Model 3 had an area under the curve (AUC) of 0.902 and a concordance index of 0.76 (CI: 0.67, 093) in relation to clinical outcome in the validation cohort. Assessment of the prognostic utility of Model 3 in this cohort visualised using the ROC curve is shown in Figure 7.10. Model 3 appears to have better prognostic ability than binary OED grading in this cohort of patients as well. Interestingly, the AUC for the binary OED grade in both the training and validation cohorts were quite similar (Figures 7.8 and 7.10). The performance of Model 3 was almost similar in both the training and validation cohorts.

7.4 Discussion

The development, validation and reporting of multivariate prognostic models is a challenge due to the extensive work that is involved from inception to reporting. A major issue with reports on prognostic/predictive models is the lack of detail provided regarding all the different areas of model development and validation (Mallett *et al.*, 2010; Collins *et al.*, 2011; Bouwmeester *et al.*, 2012; Collins *et al.*, 2013; Collins *et al.*, 2014; Moons *et al.*, 2014). Adequate review and assessment of such models can only be undertaken if reports are able to provide sufficient information on all aspects of the model construction and validation. The multivariate prognostic models constructed in this study were guided by the recommendations in the "Transparent Reporting of a multivariable prediction model for Individual Prognosis or Diagnosis (TRIPOD) Statement" checklist that was developed and published recently in several medical journals (Collins *et al.*, 2015; Moons *et al.*, 2015).

Currently, risk-stratification of OPMD patients in clinical practice is usually based on a combination of clinico-demographic and histopathological features (Reibel, 2003; Barnes *et al.*, 2005; van der Waal, 2009; van der Waal, 2010; El-Naggar *et al.*, 2017; Speight *et al.*, 2018). However, the prognostic utility of these features has been found to be lacking and at times inconsistent (Mincer *et al.*, 1972; Banoczy and Csiba, 1976; Pindborg *et al.*, 1977; Silverman *et al.*, 1984; Lumerman *et al.*, 1995; Lee *et al.*,

2000; Cowan *et al.*, 2001; Holmstrup *et al.*, 2006; Mehanna *et al.*, 2009; Warnakulasuriya *et al.*, 2011; Sperandio *et al.*, 2013; Dost *et al.*, 2014). In this study, when considering clinico-pathological parameters, only three were found to be statistically significant using statistical analysis; age at diagnosis of OPMD (< 50 years of age), site of index OPMD (tongue vs others) and OED grading using the binary system. These three variables have previously been shown to have an association with MT in OPMDs.

An increased risk of MT amongst older patients has been reported by several studies and is possibly due to increasing cumulative genetic/molecular damage and longer period of exposure to risk factors that predispose to carcinogenesis (Banoczy and Sugar, 1972; Mehta *et al.*, 1972; Schepman *et al.*, 1998; Napier and Speight, 2008; Warnakulasuriya *et al.*, 2011). Tongue lesions have been linked to a higher risk of MT compared to other oral sites in a recent meta-analysis by Mehanna et al (2009), however, several other studies have shown that oral sub-sites are not significantly associated with MT (Banoczy, 1977; Silverman *et al.*, 1984; Schepman *et al.*, 1998; Holmstrup *et al.*, 2006; Mehanna *et al.*, 2009; Liu *et al.*, 2011; Brouns *et al.*, 2014). Though imperfect, the presence and severity of OED has long been considered a prognostic indicator for clinical outcome in OPMD (Mincer *et al.*, 1972; Banoczy and Csiba, 1976; Pindborg *et al.*, 1977; Silverman *et al.*, 1984; Lumerman *et al.*, 1995; Lee *et al.*, 2000; Cowan *et al.*, 2001; Holmstrup *et al.*, 2006; Mehanna *et al.*, 2009; Warnakulasuriya *et al.*, 2011; Sperandio *et al.*, 2013; Dost *et al.*, 2014).

However, when these three variables were fitted together using a CRA model, only the binary OED grading of cases was found to be statistically significant. This suggests that of all the clinico-pathological parameters, OED grading is the most useful prognostic indicator for MT in OPMD. This is consistent with the findings of most studies that have indicated that OED grading is currently the “gold-standard” for prognosticating clinical outcome in OPMD cases (Warnakulasuriya, 2001; Barnes *et al.*, 2005; Warnakulasuriya *et al.*, 2008; van der Waal, 2009; van der Waal, 2010; Sperandio *et al.*, 2013; El-Naggar *et al.*, 2017; Speight *et al.*, 2018). Interestingly, the prognostic ability of the binary OED grading appears to be rather consistent with the values for the area under the ROC curves being almost similar in both the training and validation cohorts highlighting the highly dependable nature of this grading system.

One limitation of the current study was the inability to assess the influence/prognostic utility of habit-related factors, specifically tobacco smoking history in this cohort of patients. Although the addition of data on habit-related factors may have improved the prognostic utility of the clinico-pathological prognostic model, such information was not available for this cohort of patients and as such the impact of smoking history could not be assessed. One point to consider with regard to smoking history is the fact that in most instances, smoking history related information is self-reported and the veracity of self-reported smoking history in HNSCC and OPMDs is ambiguous (Murray *et al.*, 2002; Hald *et al.*, 2003; Connor Gorber *et al.*, 2009; Warren *et al.*, 2012; Morales *et al.*, 2013; Alberg *et al.*, 2015; Khariwala *et al.*, 2015). It is also worth noting that previous studies have shown that a non-smoking status is more often associated with MT in OPMDs suggesting that self-reported smoking history on its own may not be a very useful prognostic indicator of MT in OPMD (Reibel, 2003; Liu *et al.*, 2010; Liu *et al.*, 2011; Ho *et al.*, 2012; Rock *et al.*, 2018).

To compensate for the lack of prognostic utility of existing risk-stratification methods, several different molecular assays were assessed in this study with the intent to augment the prognostic ability of existing methods. Although a multitude of biomarkers in OPMDs have been studied, there has been no breakthrough with most biomarkers showing either inconsistent results or prognostic power that is not much better than OED grading (Smith *et al.*, 2009; Lingen *et al.*, 2011; Nankivell and Mehanna, 2011; Zhang *et al.*, 2012; Sperandio *et al.*, 2013; Nikitakis *et al.*, 2018). LOH and DNA ploidy analysis are currently the two most promising biomarkers (Pitiyage *et al.*, 2009; Smith *et al.*, 2009; Lingen *et al.*, 2011; Zhang *et al.*, 2012; Sperandio *et al.*, 2013; Nikitakis *et al.*, 2018; Speight *et al.*, 2018). In the current study, LOH at chromosomal region 3p &/or 9p and the gene signature derived from a customised panel of genes were both found to be statistically significant as prognostic indicators of MT in OPMD, whilst DNA ploidy status was not statistically significant.

A slight increase in the prognostic ability of Model 2 compared to Model 1 was seen due to the addition of LOH (3p &/or 9p) as another variable. Although LOH (3p &/or 9p) has been shown to have some prognostic utility for OPMDs in this study, sole use of LOH analysis for risk-stratification as well as treatment direction would be ill-advised as its prognostic power has yet to be demonstrated to be superior to OED

grading in OPMDs (Rosin *et al.*, 2000; Zhang *et al.*, 2012). Even in this study, the binary OED grading system appears to perform either as well or better than LOH analysis as a prognostic instrument, depending on the parameters being assessed. The current study is the first to compare the prognostic utility of LOH analysis and the binary OED grading in OPMDs.

The landmark study by Zhang et al (2012) that purportedly validated LOH profile as an independent predictor of disease progression in OED only assessed the utility of LOH profiles in non-dysplastic and low-grade (mild/moderate) OED cases thus their findings should not be generalised for all OPMD cases (Zhang *et al.*, 2012). Another point to consider regarding the prognostic models constructed in the study by Zhang et al (2012) that highlighted the potential value of LOH profiles in distinguishing high-risk and low-risk cases, is that their endpoint/event of interest was the progression of the cases to either severe OED, carcinoma *in-situ* or OSCC and not just malignant transformation of cases. The data for sub-analysis utilising only development of OSCC as the endpoint/event of interest was not presented in their manuscript (Zhang *et al.*, 2012). Unlike the current study, OED grading was also found to be non-significant in their univariate analysis of variables. This could be due to one of two reasons; because only low-grade/non-dysplastic cases were included in the study or due to the usage of the WHO 2005 version of OED grading that has more than two categories.

Although the performance of Model 2 was better than Model 1, it was still inferior to the gene-signature derived from the NanoString Customised CodeSet Panel experiment detailed in Chapter 6. The gene-signature that was developed appears to have good discriminatory value in determining OPMD that underwent MT and those that did not. Model 3 that was constructed with the addition of the gene-signature to the previous demographic, clinical and histopathological parameters showed much better prognostic utility than the other prognostic models highlighting the potential of gene expression-based methods in prognosticating clinical outcome.

Our findings are somewhat similar to the findings reported by Saintigny et al (2011) where the authors showed that gene-expression based methods were superior to clinical and histological variables in determining clinical outcome in OPMD patients (Saintigny *et al.*, 2011). In their study, Saintigny et al (2011) compared microarray derived gene-expression based models against a model that contained only age,

histology (dysplasia vs hyperplasia) and two biomarkers (ΔNp63 and podoplanin) (Saintigny *et al.*, 2011). The two models with microarray data in their study showed much better performance compared to the model without any microarray data. Their final model, which combined the microarray data with clinical and pathological covariates, showed a slight improvement compared to the model with only microarray data (Saintigny *et al.*, 2011). Interestingly, only nine transcripts were similar between the two models with microarray data, highlighting the rather unstable methodology employed in constructing their prognostic model. This could also be one of the reasons why their prognostic model has yet to be validated. Aside from that, other major differences between the current study and the study by Saintigny et al (2011) are the type of tissue material used, the platform utilised to obtain the gene-expression data as well as the methodology used to arrive at the final gene-expression profile/signature.

In the current study, FFPE tissue was successfully used for all the assays whilst Saintigny et al (2011) used fresh frozen tissue. Although fresh tissue may provide better quality RNA for gene expression studies compared to archived FFPE tissue, current next generation sequencing platforms have been shown to produce good results even when RNA derived from FFPE tissue is used despite the degradation that often accompanies formalin fixation and paraffin embedding of tissues (von Ahlfen *et al.*, 2007; Geiss *et al.*, 2008; Mittempergher *et al.*, 2011; Carey *et al.*, 2015; Conway *et al.*, 2015; Eikrem *et al.*, 2016; Wimmer *et al.*, 2018). The current study also adds to the increasing body of work on utilisation of FFPE material for gene expression studies. Another point to consider is that the patients in the Saintigny et al (2011) study were enrolled in a clinical trial in which some of the patients received active intervention in the form of drugs that may have influenced clinical outcome and gene expression (Saintigny *et al.*, 2011). The stepwise method employed in the current study, from whole transcriptome sequencing to a final customised list of target genes, covered a larger number of transcripts/genes compared to the microarray method employed by Saintigny et al (2011). The clinical and histological parameters included in the model construction for the current study are also more detailed compared to their study.

Even though the current study has demonstrated the value of a molecularly-driven multivariate prognostic model over traditional risk-stratification methods for OPMD patients, molecular based methods are not without their drawbacks. The existence of

inter- and intra-tumour heterogeneity in OSCC as well as heterogeneity in OPMD pose a major problem when it comes to gene-expression studies (Hirsch *et al.*, 1983; Perou *et al.*, 2000; Chung *et al.*, 2004; Diwakar *et al.*, 2005; Russnes *et al.*, 2011; Marusyk *et al.*, 2012; Fisher *et al.*, 2013; De Cecco *et al.*, 2015; Gomes *et al.*, 2015; Gay *et al.*, 2016; Mroz and Rocco, 2016). Another major issue is that the material used for gene-expression based assays only provide a snapshot of the transcriptome that may not be truly representative of the underlying dynamic pathological process (Hirsch *et al.*, 1983; Perou *et al.*, 2000; Chung *et al.*, 2004; Diwakar *et al.*, 2005; Russnes *et al.*, 2011; Marusyk *et al.*, 2012; Fisher *et al.*, 2013; De Cecco *et al.*, 2015; Gomes *et al.*, 2015; Gay *et al.*, 2016; Mroz and Rocco, 2016). The same can also be inferred for LOH analysis as information regarding LOH at one point in time may not be adequate to prognosticate clinical outcome over prolonged time-frames and as such, these molecular assays may need to be repeated periodically in high-risk patients.

A major limitation of the current study is the sample size and the almost equal number of MT and NT cases that is not truly representative of the population where MT occurs in approximately 10% of cases with OED (Shariff and Zavras, 2015). However, this study was designed to be a proof-of-principle study to explore the possibility of using FFPE derived material to construct a molecularly-driven multivariate prognostic model for OPMD patients that is superior to traditional methods of risk-stratification in these patients. As such, it is acknowledged that this study is but a first step in the development of a final gene-expression based prognostic model for OPMD patients.

The issues mentioned earlier highlight the importance and need for clinical validation of the prognostic model using an independent patient cohort and preferably by an independent research team (Koscielny, 2010; Brulard and Chibon, 2013; Chibon, 2013). Although the findings from this study have successfully shown that the prognostic model developed is indeed superior to conventional risk-stratification methods in a validation cohort, the validation cohort was obtained in a retrospective manner and the size of the cohort was also rather small. Going forward, it may be wiser to assess the prognostic model in a prospective clinical trial setting.

7.5 Conclusions

A prognostic model for OPMD patients composed of clinical, histopathological and molecular parameters was constructed and compared against conventional risk-stratification methods. The gene-signature developed has good prognostic utility even as a stand-alone biomarker for OPMD patients with its' prognostic ability being augmented with the addition of clinical and histopathological parameters. The molecular methods included in the final model were performed using FFPE material, once again highlighting that FFPE-derived material is more than adequate for molecular based assays. The current prognostic model however needs to be assessed in a larger external cohort to validate its prognostic efficacy.

Table 7.1 Demographic, clinical and histopathological characteristics of cases selected for prognostic modelling (n = 44)

Characteristic		Non-transforming (NT) n = 24	Malignant transforming (MT) n = 20	p-value
Age at diagnosis in years **Mean [SD] (Range)**		59.67 [13.38] (34 - 89)	61.30 [12.58] (35 – 92)	0.681†
Age at diagnosis	≤ 50 years	9	2	0.078*
	> 50 years	15	18	
Sex	Male	14	13	0.760*
	Female	10	7	
Site of OPMD	Tongue	4	11	0.011*
	Other sites	20	9	
WHO 2017 OED grading	Mild	15	8	0.140**
	Moderate	5	3	
	Severe	4	9	
Binary OED grading	Low-grade	20	8	0.005*
	High-grade	4	12	

† Independent t-test; *Fisher's Exact test; **Pearson's Chi-square test; SD = Standard deviation

Table 7.2 Results from molecular parameters for training cohort (n = 44)

Characteristic		Non-transforming (NT) n = 24	Malignant transforming (MT) n = 20	p-value
LOH (3p &/or 9p &/or 17p)	No LOH	22	12	0.027*
	LOH	2	8	
LOH (3p &/or 9p)	No LOH	23	13	0.015*
	LOH	1	7	
Dichotomised gene-signature based score	Low-risk	22	6	< 0.001*
	High-risk	2	14	

*Fisher's Exact test

Table 7.3 Univariate analysis of selected variables using Cox proportional-hazards regression analysis (CRA) (n = 44)

Variable	HR (95% CI)	p-value
Age at diagnosis (> 50 years)	4.02 (0.93, 17.38)	0.063
Binary OED grading (High-grade)	3.58 (1.43, 8.96)	0.007
Site of index OPMD (Tongue)	2.86 (1.18, 6.93)	0.020
LOH (3p &/or 9p &/or 17p)	3.00 (1.22, 7.42)	0.017
LOH (3p &/or 9p)	3.74 (1.47, 9.57)	0.006
Dichotomised gene-signature based score	9.36 (3.47, 25.27)	< 0.001

HR = Hazard ratio; CI = Confidence interval

Table 7.4 Results from Cox proportional-hazards regression analysis (CRA) utilising only demographic, clinical and histopathological parameters. (Model 1; n = 44)

Variable	β-coefficient	HR (95% CI)	p-value
Age at diagnosis (> 50 years)	NA	NA	> 0.05
Site of index OPMD (Tongue)	NA	NA	> 0.05
Binary OED grading (High-grade)	1.275	3.58 (1.43, 8.96)	0.007

HR = Hazard ratio; CI = Confidence interval; NA = not applicable. Variables included for multivariate analysis: age at diagnosis, site of OPMD and binary OED grading. Concordance index of 0.63 (CI: 0.52, 0.74)

Table 7.5 Results from Cox proportional-hazards regression analysis (CRA) utilising age at diagnosis, site of OPMD, binary OED grading and LOH at chromosomal regions 3p &/or 9p. (Model 2; n = 44)

Variable	β-coefficient	HR (95% CI)	p-value
Age at diagnosis (> 50 years)	NA	NA	> 0.05
Site of index OPMD (Tongue)	NA	NA	> 0.05
Binary OED grading (High-grade)	1.066	2.90 (1.11, 7.57)	0.029
LOH at 3p &/or 9p	1.008	2.74 (1.03, 7.57)	0.043

HR = Hazard ratio; CI = Confidence interval; NA = not applicable. Variables included for multivariate analysis: age at diagnosis, site of OPMD, binary OED grading and LOH at chromosomal regions 3p &/or 9p. Concordance index of 0.67 (CI: 0.56, 0.79).

Table 7.6 Cox proportional-hazards regression analysis (CRA) utilising age at diagnosis, site of OPMD, binary OED grading, LOH at chromosomal region 3p &/or 9p and dichotomised gene-signature based scores. (Model 3; n = 44)

Variable	β-coefficient	HR (95% CI)	p-value
Age at diagnosis (> 50 years)	1.650	5.21 (1.14, 23.84)	0.0334
Site of index OPMD (Tongue)	0.959	2.61 (1.02, 6.67)	0.0453
Binary OED grading (High-grade)	1.005	2.73 (1.00, 7.45)	0.0496
Dichotomised gene-signature based score (High-risk)	2.506	12.26 (4.09, 36.76)	< 0.0001
LOH at 3p &/or 9p	NA	NA	> 0.05

HR = Hazard ratio; CI = Confidence interval; NA = not applicable. Variables included for multivariate analysis: age at diagnosis, site of OPMD, binary OED grading, LOH at chromosomal region 3p &/or 9p and dichotomised gene-signature based scores. Concordance index of 0.85 (CI: 0.72, 0.98).

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Table 7.7 Comparison between Models 1, 2 and 3 as prognostic instruments for clinical outcome in training cohort of OPMD patients (n = 44)

Statistic	Dichotomised Model 1	Dichotomised Model 2	Dichotomised Model 3
Sensitivity	60.0%	70.0%	90.0%
Specificity	83.3%	79.2%	83.3%
Accuracy	72.7%	75.0%	86.4%
***Positive predictive value**	29.7%	28.3%	38.7%
***Negative predictive value**	94.7%	95.7%	98.6%

*Value based on 10.5% prevalence rate of MT in OED as all cases in this cohort had some level of OED (Shariff and Zavras, 2015). Model scores were dichotomised according to median into low-risk and high-risk for MT.

Table 7.8 Demographic, clinical and histopathological characteristics of the validation cohort (n = 44)

Characteristic		Non-transforming (NT) n = 23	Malignant transforming (MT) n = 21	p-value
Age at diagnosis in years **Mean [SD] (Range)**		55.39 [13.00] (33 - 82)	61.90 [11.12] (43 – 81)	0.083†
Age at diagnosis	≤ 50 years	10	2	0.017*
	> 50 years	13	19	
Sex	Male	14	12	1.000*
	Female	9	9	
Site of OPMD	Tongue	6	11	0.011*
	Other sites	17	10	
Binary OED grading	Low-grade	21	10	0.002*
	High-grade	2	11	
WHO 2017 OED grading	Mild	18	7	0.001**
	Moderate	5	6	
	Severe	0	8	
Dichotomised gene-signature based score	Low-risk	20	9	0.004*
	High-risk	3	12	
LOH (3p &/or 9p)	No LOH	23	17	0.044*
	LOH	0	4	

† Independent t-test; *Fisher's Exact test; **Pearson's Chi-square test; SD = standard deviation

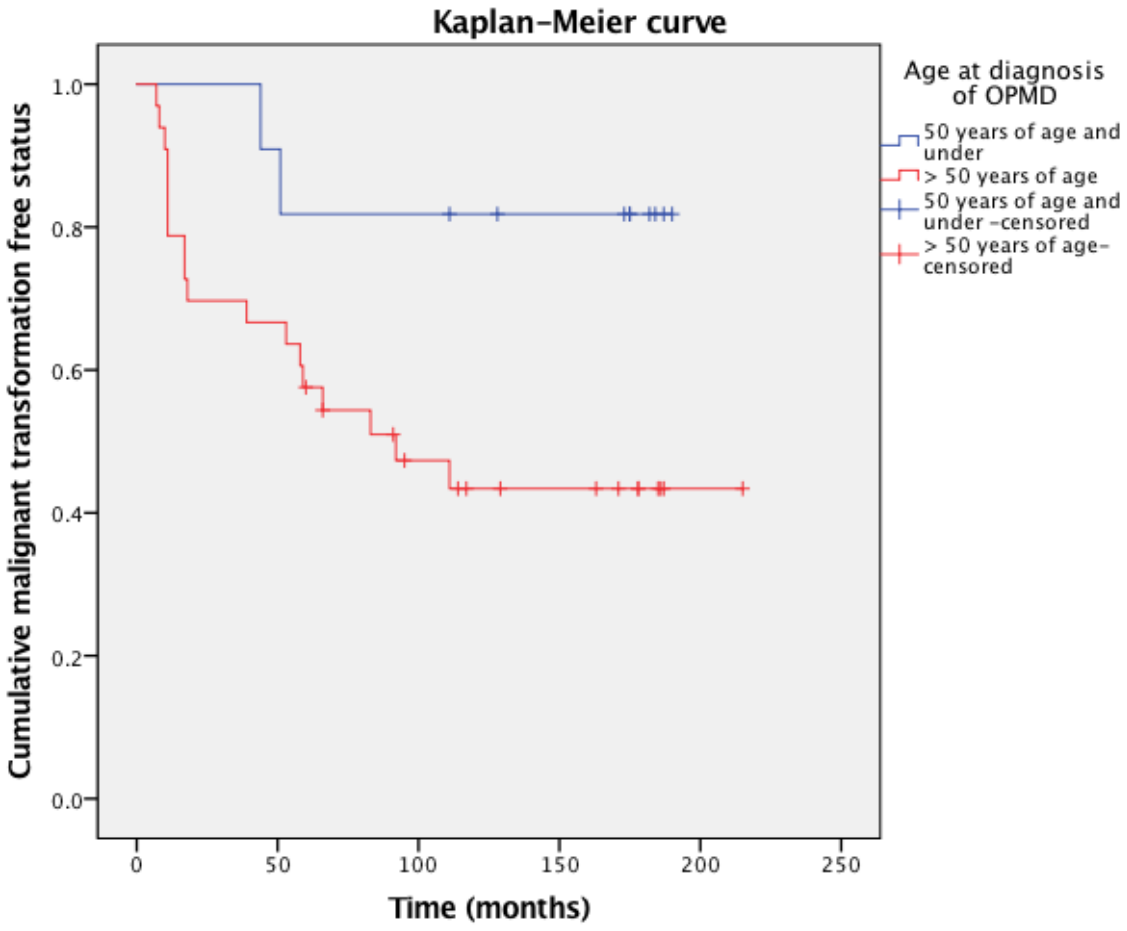


Figure 7.1 Kaplan-Meier time to event analysis comparing MT in the OPMD cohort stratified according to age at diagnosis.

The analysis shows that patients aged > 50 years at diagnosis of OPMD underwent malignant change of OPMDs more rapidly than those aged ≤ 50 years. (Log rank test; $\chi^2 = 4.13$, df =1, p = 0.042). Patients aged ≤ 50 years at diagnosis of index OPMD = blue line; Patients aged > 50 years at diagnosis of index OPMD = red line.

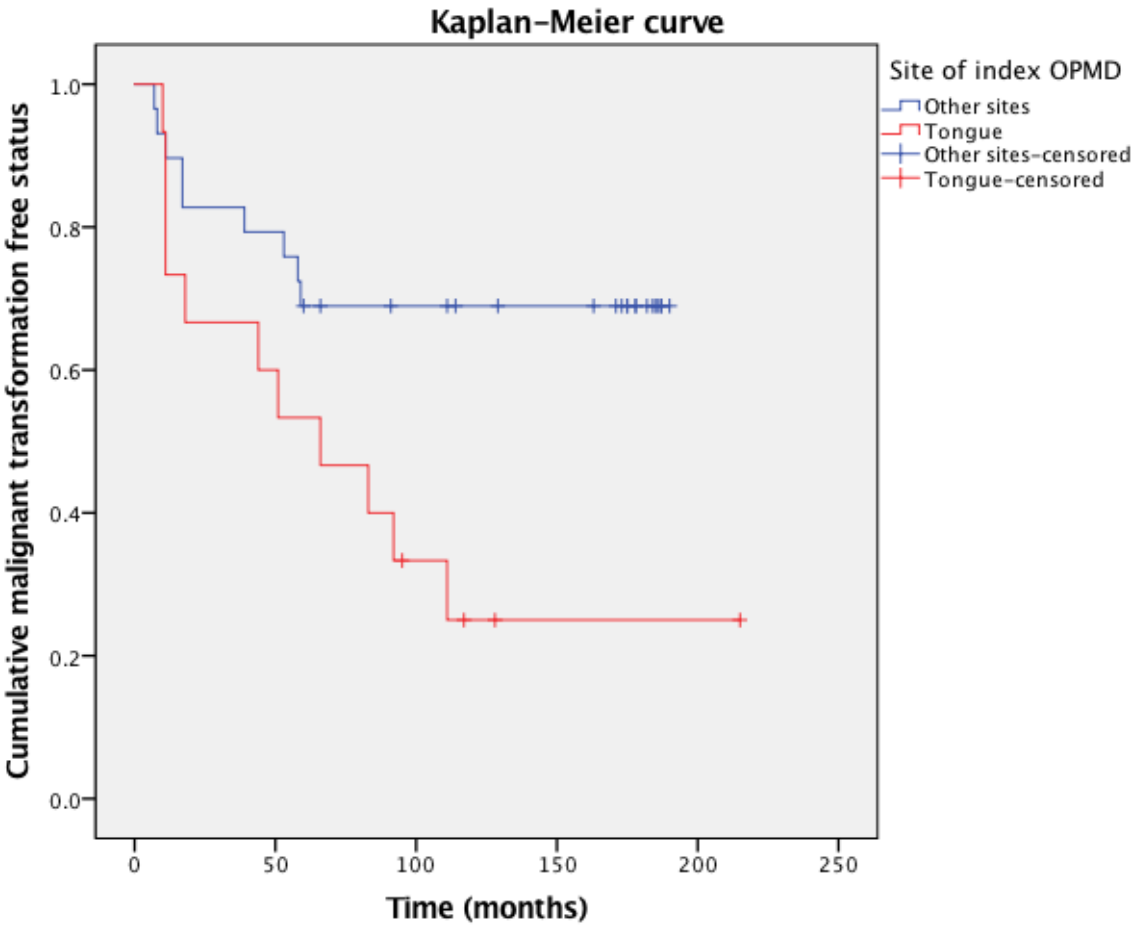


Figure 7.2 Kaplan-Meier time to event analysis comparing MT in the OPMD cohort stratified according to site of index OPMD.

The analysis shows that patients with OPMD of the tongue underwent malignant change of OPMDs more rapidly than those with OPMD at other sites (Log rank test; $\chi^2 = 6.03$, df =1, p = 0.014). OPMD at other oral sites = blue line; OPMD of the tongue = red line.

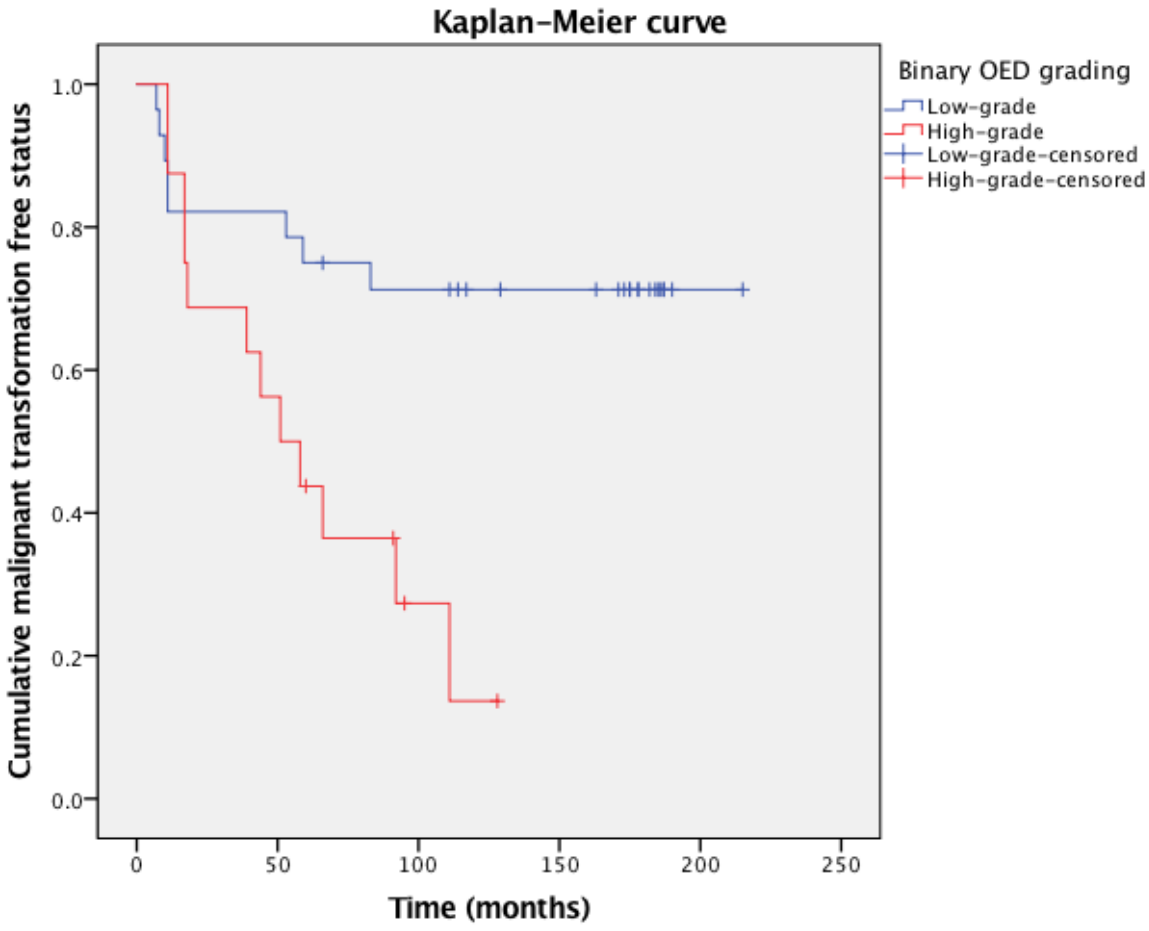


Figure 7.3 Kaplan-Meier time to event analysis comparing MT in the OPMD cohort stratified according to binary OED grading.

The analysis shows that patients with a high-grade OED underwent malignant change of OPMDs more rapidly than those with low-grade OED (Log rank test; χ^2 = 8.52, df =1, p = 0.004). Low-grade OED = blue line; High-grade OED = red line.

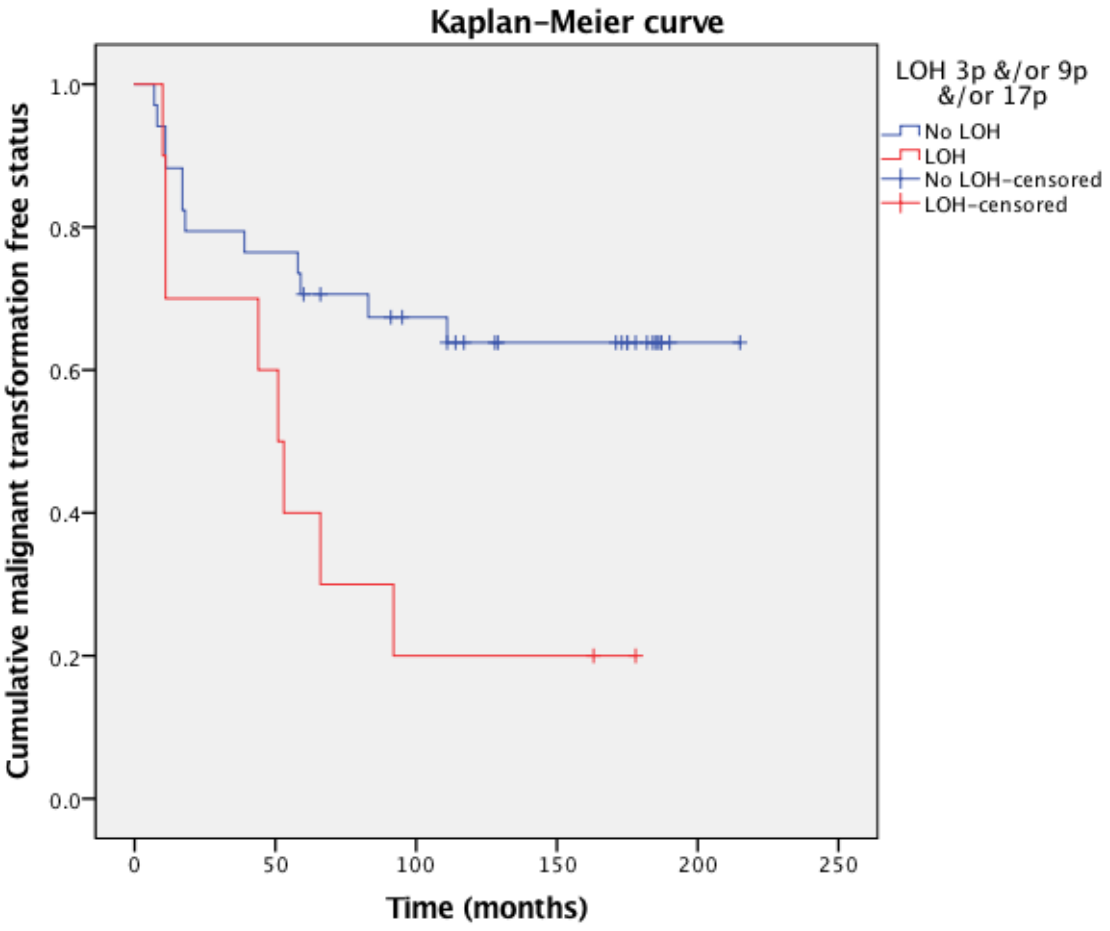


Figure 7.4 Kaplan-Meier time to event analysis comparing malignant transformation in the OPMD cohort stratified according to LOH (3p &/or 9p &/or 17p) analysis results.

The analysis shows that patients with LOH at 3p &/or 9p &/or 17p underwent malignant change of OPMDs more rapidly than those with no LOH (Log rank test; χ^2 = 6.39, df =1, p = 0.011). No LOH = blue line; LOH at 3p &/or 9p &/or 17p = red line

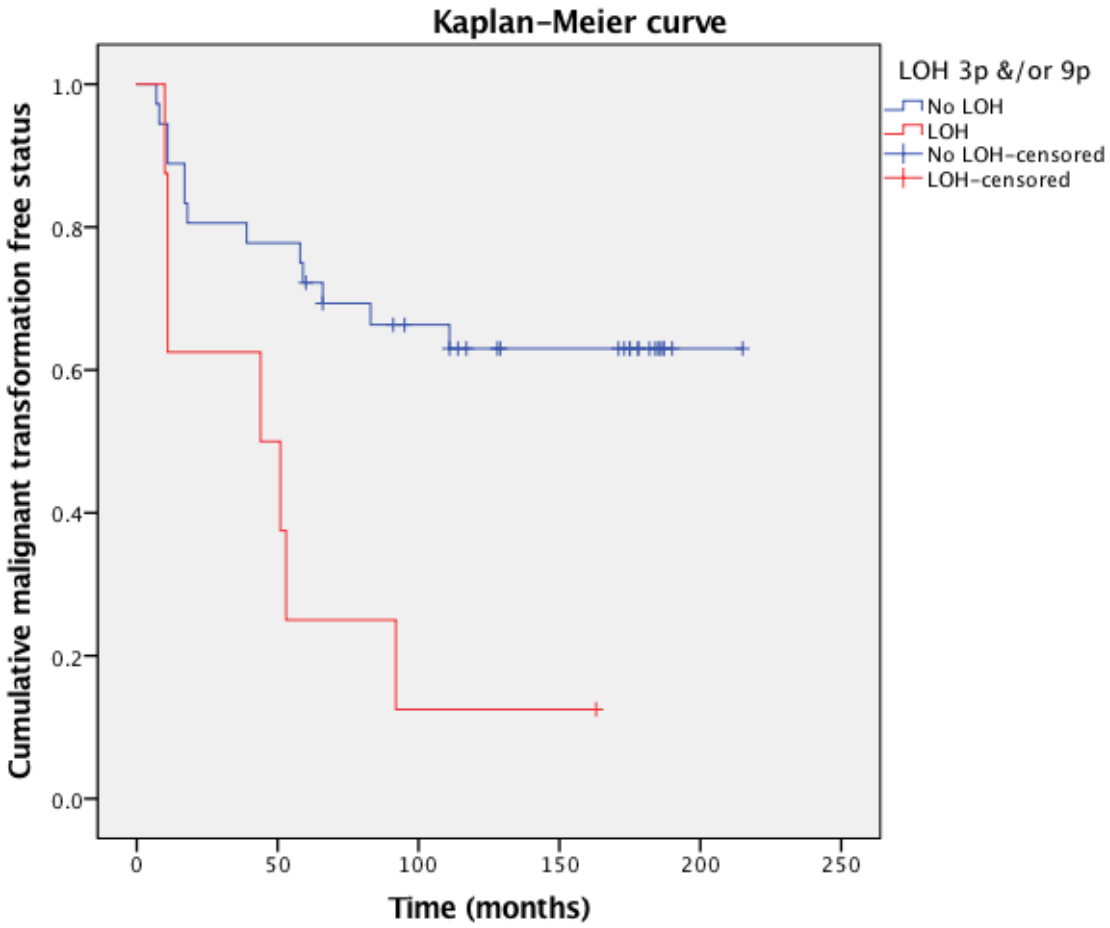


Figure 7.5 Kaplan-Meier time to event analysis comparing malignant transformation in the OPMD cohort stratified according to LOH (3p &/or 9p) analysis results.

The analysis shows that patients with LOH at 3p &/or 9p underwent malignant change of OPMDs more rapidly than those with no LOH (Log rank test; $\chi^2 = 8.91$, df =1, p = 0.003). No LOH = blue line; LOH at 3p &/or 9p= red line

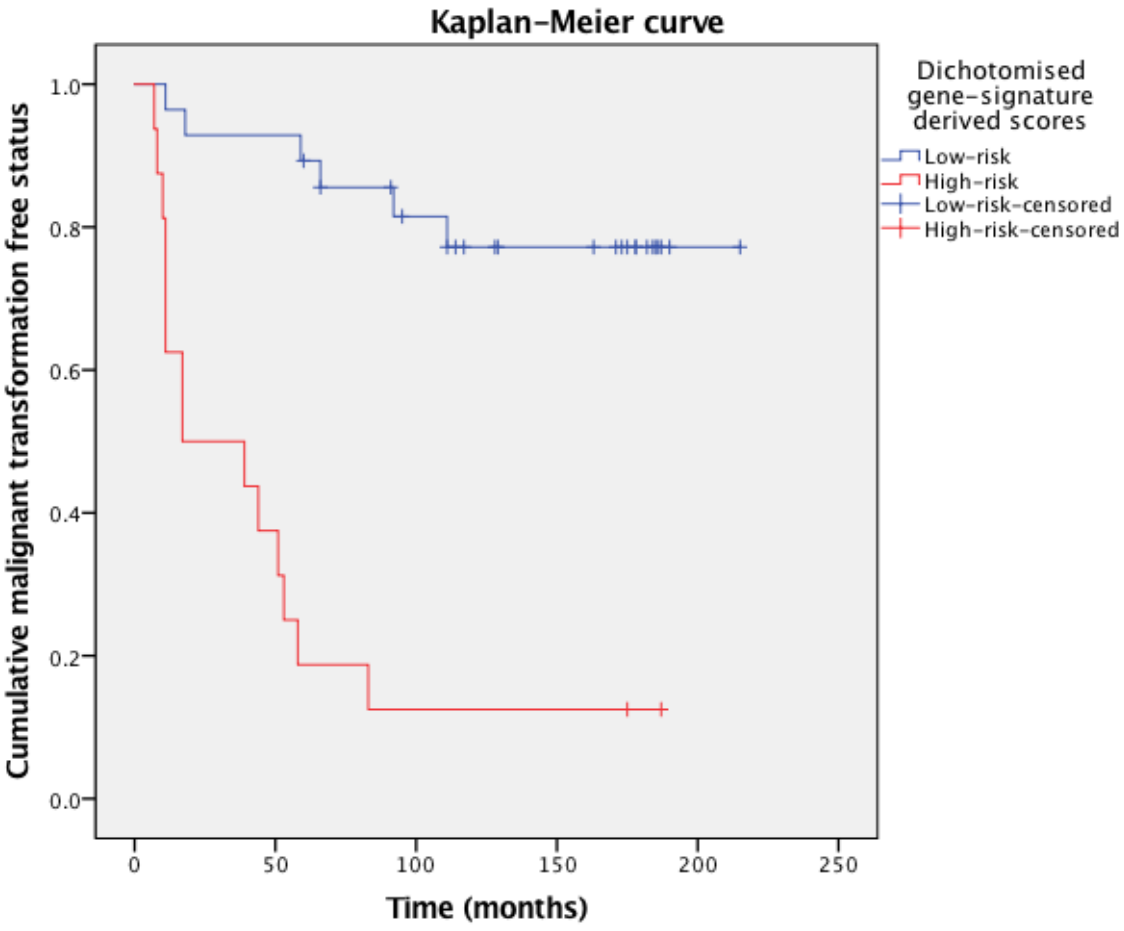
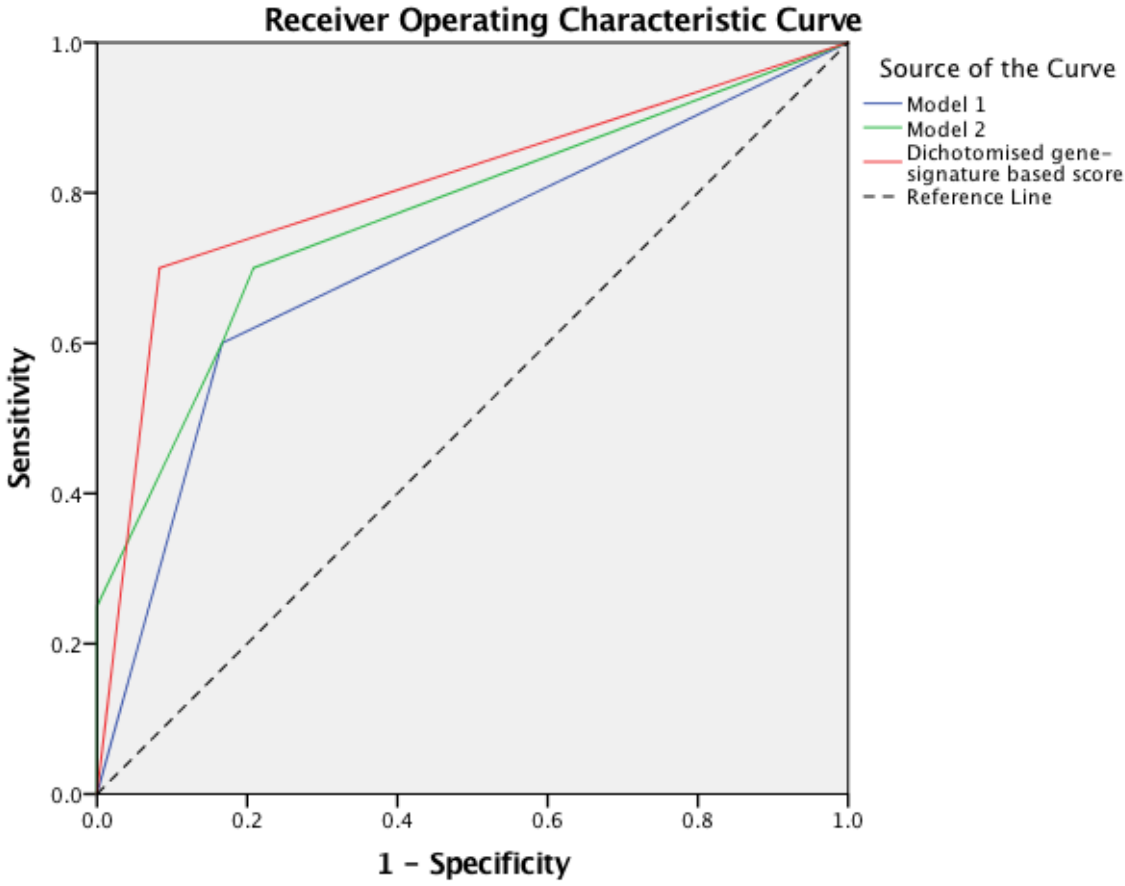


Figure 7.6 Kaplan-Meier time to event analysis comparing malignant transformation in the OPMD cohort stratified according to the dichotomised gene-signature score.

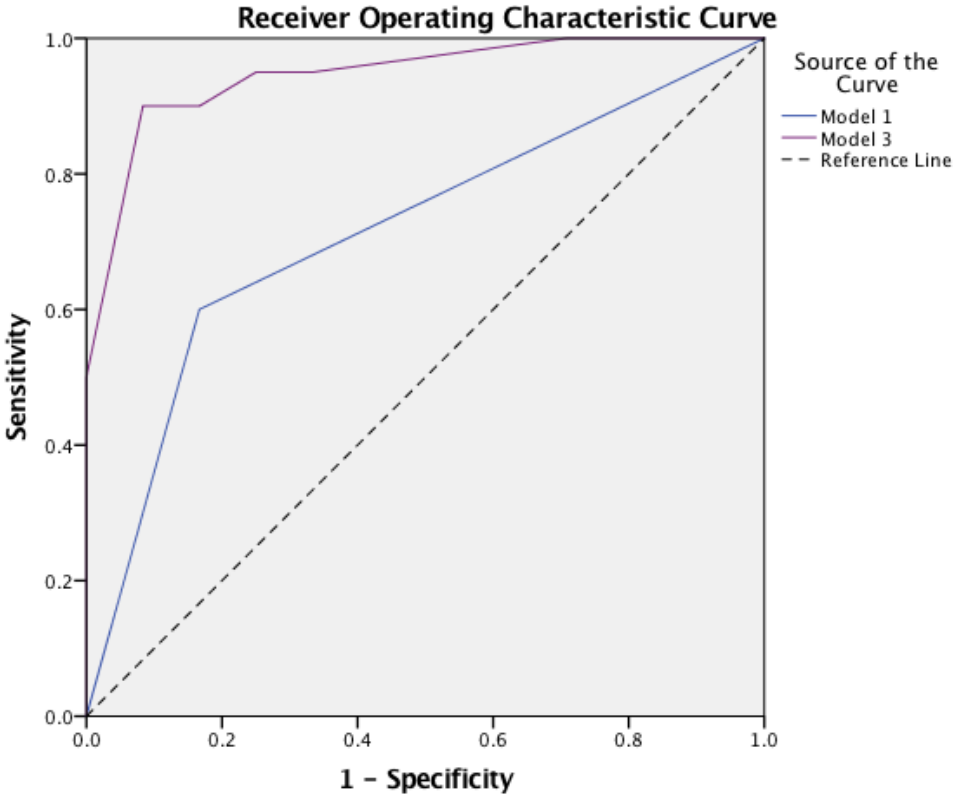
The analysis shows that patients with a high-risk score underwent malignant change of OPMDs more rapidly than those with a low-risk score (Log rank test; $\chi^2 = 27.56$, df =1, $p < 0.001$). Concordance index = 0.76 (CI: 0.66, 0.86). Low-risk score = blue line; High-risk score = red line



Source of curve	Area under curve	Standard error	Asymptotic significance	Asymptotic 95% Confidence Interval	
				Lower Bound	Upper Bound
Model 1	0.717	0.081	0.014	0.559	0.875
Model 2	0.771	0.074	0.002	0.629	0.916
Dichotomised gene-signature based score	0.808	0.071	< 0.001	0.669	0.947

Figure 7.7 Receiver operating characteristic (ROC) curves comparing efficacy of Models 1 and 2 in prognosticating clinical outcome for the OPMD cohort.

Model 2 which has the addition of a molecular parameter (LOH) outperformed Model 1. However, both Model 1 and 2 were still inferior to the dichotomised gene-signature based score in prognosticating clinical outcome. Blue line: Model 1; Green line: Model 2; Red line: Dichotomised gene-signature based score



Source of curve	Area under curve	Standard error	Asymptotic significance	Asymptotic 95% Confidence Interval	
				Lower Bound	Upper Bound
Model 1	0.717	0.081	0.014	0.559	0.875
Model 3	0.947	0.033	< 0.001	0.882	1.000

Figure 7.8 Receiver operating characteristic (ROC) curves comparing efficacy of Models 1 and 3 in prognosticating clinical outcome for the OPMD training cohort.

Model 3 was found to outperform Model 1 as a prognostic model for clinical outcome in the training cohort of OPMD patients. Model 3 had a concordance index of 0.85 (CI: 0.72, 0.98) in relation to clinical outcome. Blue line: Model 1; Purple line: Model 3

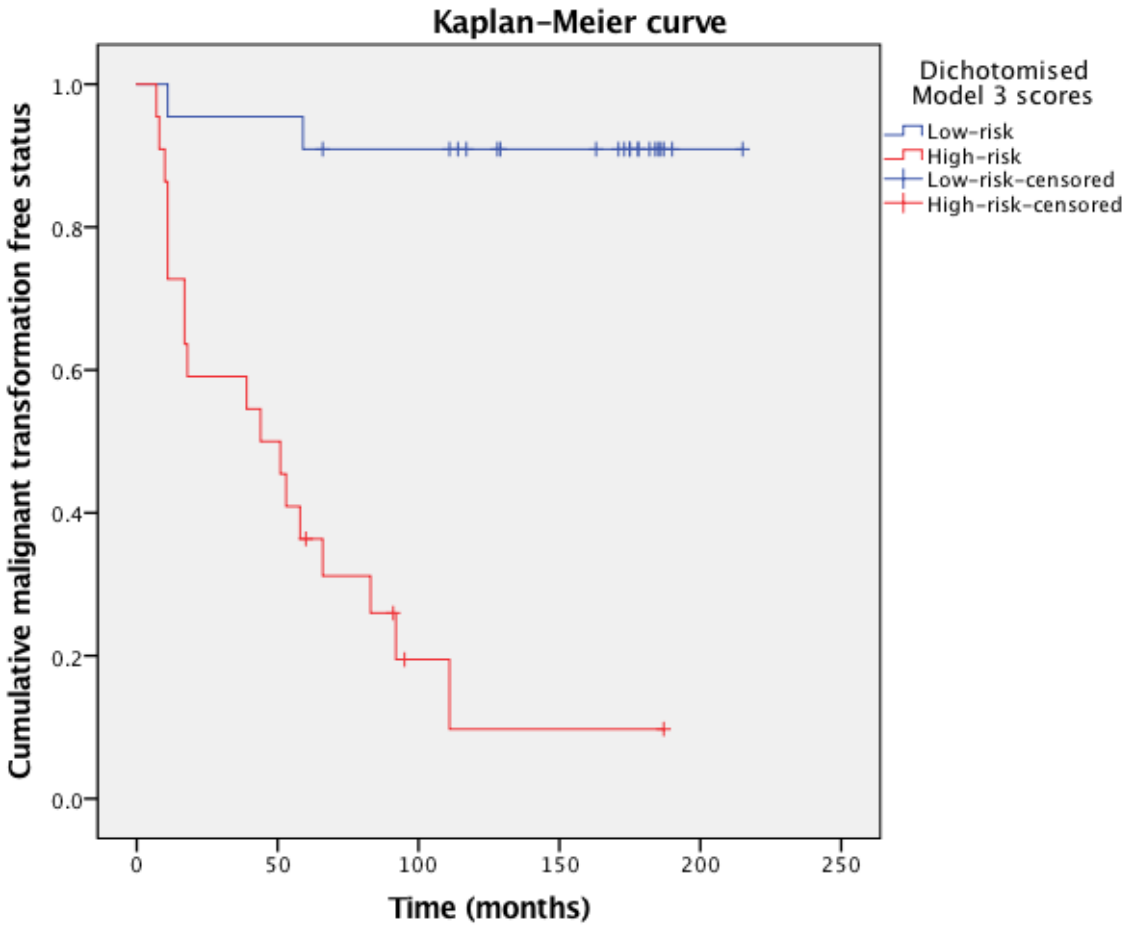
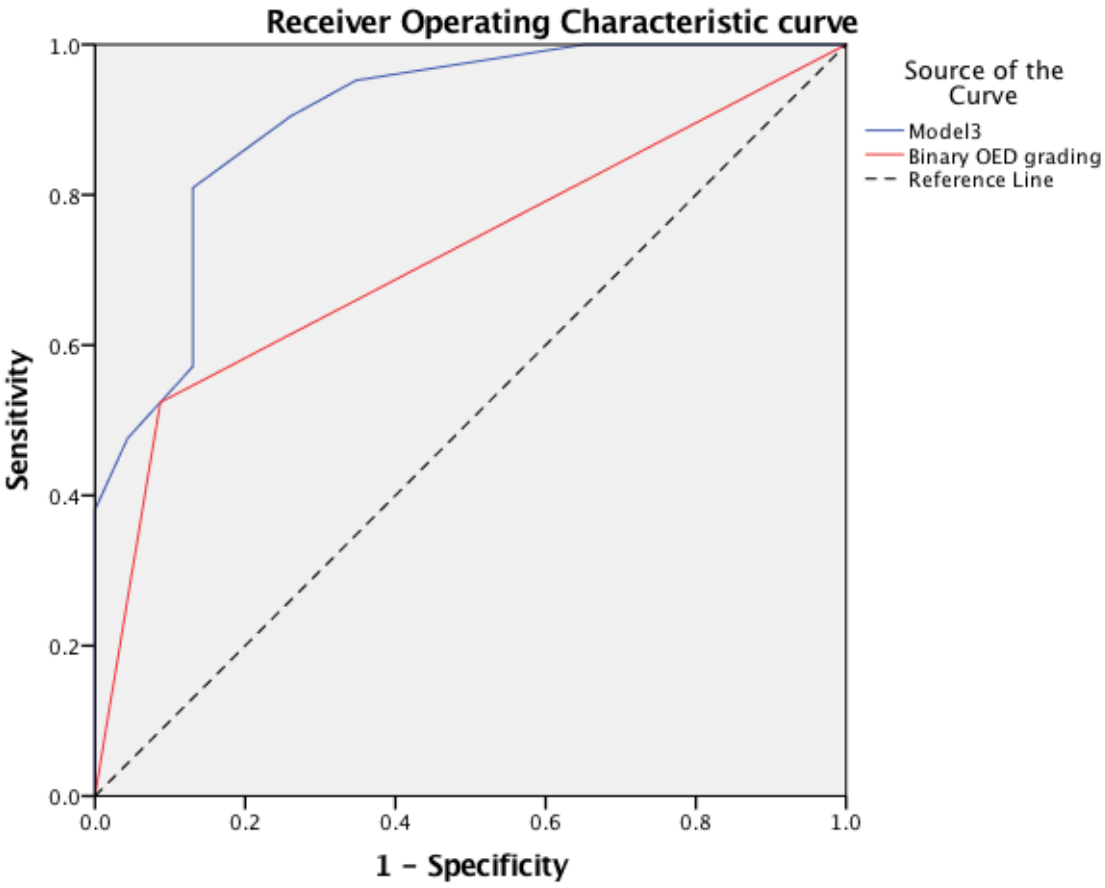


Figure 7.9 Kaplan-Meier time to event analysis comparing malignant transformation in the OPMD training cohort stratified according to dichotomised Model 3 scores.

The analysis shows that patients with a high-risk score underwent malignant change of OPMDs more rapidly than those with a low-risk score (Log rank test; $\chi^2 = 26.35$, df =1, $p < 0.0001$). Low-risk score = blue line; High-risk score = red line



Source of curve	Area under curve	Standard error	Asymptotic significance	Asymptotic 95% Confidence Interval	
				Lower Bound	Upper Bound
Model 3	0.902	0.045	< 0.0001	0.813	0.990
Binary OED grading	0.718	0.080	0.013	0.562	0.875

Figure 7.10 Receiver operating characteristic (ROC) curves assessing efficacy of Model 3 in prognosticating clinical outcome in the validation cohort.

Model 3 was found to outperform binary OED grading in prognosticating clinical outcome for the validation cohort with a concordance index of 0.76 (CI: 0.67, 093) in relation to clinical outcome.

Chapter 8. Discussion

8.1 Introduction

Delayed detection of OSCC is thought to be one of the major contributors to the mortality and morbidity associated with OSCC (Barnes *et al.*, 2005; Warnakulasuriya, 2009; Goodson and Thomson, 2011). Early detection of OSCC is thought to be feasible because a proportion of OSCC are preceded by clinically identifiable lesions termed 'oral potentially malignant disorders' (OPMDs), however the precise number of OSCC arising from OPMDs is as yet unclear (Warnakulasuriya *et al.*, 2007; van der Waal, 2009; El-Naggar *et al.*, 2017; Speight *et al.*, 2018). The majority of OPMDs do not undergo MT and at this point in time there exists no validated method to determine the clinical behaviour of OPMDs to a high degree of accuracy (Napier and Speight, 2008; Mehanna *et al.*, 2009; Shariff and Zavras, 2015; Warnakulasuriya and Ariyawardana, 2016; El-Naggar *et al.*, 2017).

The current study was undertaken to address the need for an objective method to risk-stratify OPMD patients using FFPE tissue to improve their management as well as clinical outcome. The prognostic classifier developed from this study may pave the way for more personalised management protocols for individual patients with OPMDs to improve clinical outcome. This chapter will focus on the key findings from the current study. The limitations and future directions of the research will also be discussed.

8.2 Key Findings

8.2.1 Demographic and clinical features are weakly associated with clinical outcome in OPMD patients

In the current study, demographic and clinical features were largely found to have a minor association with clinical outcome of OPMD patients. Although age at diagnosis (> 50 years) and site of index OPMD were two features that were found to have some prognostic value in the smaller sub-cohort selected for molecular experiments (65 patients), the strength of the association appears to be diminished when assessed in the larger overall cohort of 166 patients (Tables 3.1 and 3.2). Numerous

studies have looked at the association of demographic and clinical features with clinical outcome in OPMD patients with varying results (Gupta *et al.*, 1980; Schepman *et al.*, 1998; Amagasa *et al.*, 2006; Hsue *et al.*, 2007; Napier and Speight, 2008; Arduino *et al.*, 2009; Mehanna *et al.*, 2009; van der Waal, 2009; Warnakulasuriya *et al.*, 2011; Ho *et al.*, 2012; Bates *et al.*, 2016; Warnakulasuriya and Ariyawardana, 2016; Speight *et al.*, 2018). The variation in results between studies could largely be due to geographic variation, with different populations having differing risk factor habits such as smoking, reverse-smoking and betel-quid chewing that may influence the incidence and clinical outcome of OPMDs (Gupta *et al.*, 1980; Warnakulasuriya and Ariyawardana, 2016; El-Naggar *et al.*, 2017; Speight *et al.*, 2018).

One of the limitations of the current study was the inability to assess the association of risk factor habits in MT of OPMDs for this group of patients due to incomplete medical records regarding these features. However, the true value of such habits can only be assessed if objective assessment of the exposure to the said risk factor habits can be performed. This is not usually possible as most data on risk factor habits are obtained through clinical history taking reliant on self-reporting which may not accurately represent the true exposure to the risk factor habit (Murray *et al.*, 2002; Hald *et al.*, 2003; Connor Gorber *et al.*, 2009; Warren *et al.*, 2012; Morales *et al.*, 2013; Alberg *et al.*, 2015; Khariwala *et al.*, 2015). As such, self-reported risk factor history on its own may not be a very useful prognostic indicator of MT in OPMD (Reibel, 2003; Liu *et al.*, 2010; Liu *et al.*, 2011; Ho *et al.*, 2012; Rock *et al.*, 2018).

The lack of association between clinico-demographic features and clinical outcome highlights the importance of including other parameters such as OED grading when risk-stratifying OPMD patients or formulating management strategies for these patients. Although experienced clinicians may defer biopsies for well-recognised clinical entities such as nicotinic stomatitis or median rhomboid glossitis, the majority of white, red or mixed red-white oral lesions should be biopsied for histopathological assessment; risk-stratification based solely on demographic and clinical features is not recommended (Epstein *et al.*, 2012; Forman *et al.*, 2015; National Institute for Health and Care Excellence, 2015a).

8.2.2 The binary OED grading system is a good prognostic marker for clinical outcome in OPMD

Oral epithelial dysplasia (OED) grading is currently considered the "gold-standard" for prognosticating clinical outcome in OPMD cases and its' presence and severity is frequently used to direct treatment and management of OPMDs (Warnakulasuriya, 2001; Barnes *et al.*, 2005; Warnakulasuriya *et al.*, 2008; van der Waal, 2009; van der Waal, 2010; Sperandio *et al.*, 2013; El-Naggar *et al.*, 2017; Speight *et al.*, 2018). Two different grading systems were described in the recent edition of the WHO text on head and neck pathology; the three-tiered traditional grading system and the binary grading system (El-Naggar *et al.*, 2017).

The findings from the current study show that the binary grading system is a better indicator for clinical outcome in OPMD cases compared to the three-tiered WHO 2017 system (Tables 3.3 and 3.6). Not only did it have better prognostic utility, it also showed improved inter-observer variability compared to the WHO 2017 grading system. One major advantage of the binary OED grading system over the three-tiered grading systems from a clinician's perspective may well be the lack of uncertainty caused by the "moderate OED" grading in traditional grading systems that cause much ambiguity with regard to patient management. This grading system also appears to have good consistency with regard to its prognostic ability as it was shown to give similar values when assessed in both the training and validation cohorts unlike the WHO 2017 grading system that was statistically significant in the validation cohort but not in the training cohort.

However, although the presence and severity of OED is believed to be associated with clinical outcome of OPMDs, only between 10 – 12 % of OED undergo MT and conversely non-dysplastic lesions have been reported to undergo malignant change, demonstrating its' lack of prognostic utility (Mattsson *et al.*, 2002; Hsue *et al.*, 2007; van der Meij *et al.*, 2007; Mehanna *et al.*, 2009; Bagan *et al.*, 2011; Shariff and Zavras, 2015). OED grading by itself will possibly never have excellent prognostic utility as it is a snapshot of a supposedly "representative" portion of an oral lesion; a lesion may evolve over time and also the biopsy specimen may not actually be representative of the overall pathology. Despite being superior to the WHO 2017 system in this cohort, the sensitivity and specificity levels for the binary grading

system were still found to be lacking and it requires further validation using larger cohorts before it can be recommended for routine clinical usage.

8.2.3 Presence of OPMD associated leukocytes (OPAL) does not correlate with clinical outcome

Data from the current study suggests that presence of OPAL are not significantly associated with clinical outcome in OPMD patients. Importantly however, the presence of OED in cases with increased leukocytic infiltrate may be overlooked as atypia due to the inflammatory component and these lesions can also mimic chronic inflammatory lesions such as oral lichen planus to the untrained eye resulting in possible mismanagement of such cases (Krutchkoff and Eisenberg, 1985; Muller, 2011; Gillenwater *et al.*, 2014; Muller, 2017; Muller, 2018). It is as yet unclear as to whether the presence of increased leukocytic infiltrate is a response to atypia in the epithelium or as an inducer of MT in OPMDs or a precursor to invasive carcinoma. Further exploration using molecular methods to accurately identify the sub-population of leukocytes present in OPMDs will need to be performed before such questions can be answered.

8.2.4 Prognostic utility of DNA ploidy status in OPMD patients using incisional biopsy specimens is limited

There was no obvious association between DNA ploidy status and clinical outcome in this cohort of patients and a large number of samples also failed DNA ploidy analysis due to inadequate tissue. This poses a major problem for OPMD cases as the majority of initial incisional biopsy specimens that are provided to obtain a “working diagnosis” are rather small in size. Although larger excision specimens may be available at centres where lesions are excised, this is not commonplace as surgical excisions are currently not the standard of care for all OPMDs (van der Waal, 2009; Balasundaram *et al.*, 2014; Lodi *et al.*, 2016). As such, DNA ploidy analysis may not be a feasible biomarker for small incisional biopsies that are routinely available for most OPMD cases. In this cohort, binary OED grading performed much better than DNA ploidy status in discriminating cases with differing clinical outcome (Tables 4.1 and 4.4). However, it is acknowledged that our results should be interpreted with caution as the number of cases analysed were small.

8.2.5 Loss of heterozygosity at chromosomal regions 3p &/or 9p shows promise as a prognostic biomarker for clinical outcome in OPMD

Loss of heterozygosity (LOH) analysis was assessed at three chromosomal regions in the current study; 3p, 9p and 17p. Both the sample preparation methods showed comparable results, though the number of cases assessed was limited. Manual microdissection is a much more feasible and cost-effective sample preparation method as it does not require specialised hardware and software. However, LCM has the advantage of minimising contamination due to its “non-contact” tissue manipulation technique.

LOH status was found to have significant association with clinical outcome in this cohort of patients. Further statistical analysis showed that LOH at 3p &/or 9p has better utility than LOH at chromosomal regions 3p &/or 9p &/or 17p in terms of prognostic ability. In agreement with most studies, LOH analysis does appear to show promise as a prognostic biomarker for clinical outcome in OPMDs (Mao *et al.*, 1996; Rosin *et al.*, 2000; Bremmer *et al.*, 2009; Graveland *et al.*, 2013; William *et al.*, 2016). Interestingly, when sensitivity and specificity analysis was performed and LOH status was compared to binary OED grading, it was found that LOH status had better specificity whilst binary OED grading had better sensitivity, thus hinting at the possibility that a combination of both parameters may improve prognostic strength. This was proven when prognostic Model 2 that was developed by combining LOH with other conventional risk-stratification methods did show an increase in prognostic power compared to Model 1 (conventional risk-stratification method) (Figure 7.7). However, the improvement observed was only marginal and still inferior to the developed gene-signature.

8.2.6 Several novel candidate genes with possible roles in malignant transformation of OPMDs were identified

A number of novel candidate genes associated with oral carcinogenesis were identified using two different gene expression analysis platforms (RNASeq and NanoString nCounter). Although the final gene-signature developed only contained one novel gene (*CCNE1),* the other candidate genes identified are worth exploring further, especially *TLX1.* The T-cell leukaemia homeobox 1 (*TLX1*) gene was the only gene that was significantly differentially expressed between malignant

transforming and non-transforming OPMDs in both gene expression analysis platforms (Tables 6.2 and 6.4). Preliminary work to explore TLX1 protein expression in OPMD in archived FFPE tissue using immunohistochemistry (IHC) was undertaken following the results of the gene expression assays. The results showed that TLX1 protein expression was seen in oral epithelial tissue as well as amongst the leukocytic infiltrate in the connective tissue. Though down-regulation of TLX1 protein expression was seen in OPMDs that underwent MT, the findings were not statistically significant (data not shown).

8.2.7 The developed gene-signature was superior to other clinico-pathological and molecular parameters in prognosticating clinical outcome

A novel method utilising a tiered approach from whole transcriptome to targeted transcriptome assessment to generate a prognostic gene-signature for OPMD patients was used in the current study. The gene-signature composed of *NOTCH1*, *CCNE1* and *TP63* showed promising results in being able to discriminate OPMD with differing clinical outcome in the current study. Addition of the gene-signature to conventional risk-stratification methods served to create a highly useful prognostic model (Model 3) which was validated on an external cohort of OPMD patients. Although the gene-signature may have strong prognostic utility on its own, at this point in time it would be best to take into consideration clinico-pathological features and include the gene-signature as an additional parameter as the gene-signature has yet to be adequately validated.

8.2.8 Archived FFPE material can be successfully used for molecular assays

Several molecular techniques were explored using archived FFPE tissue in the current study. It was shown that FFPE-based material, though degraded, can still prove useful for molecular-based assays. Adequate RNA was successfully obtained from FFPE material and the amount of tissue required to obtain valid results from gene expression studies was much lower than that required for DNA ploidy and LOH analysis (Table 2.1). It is also interesting to note that OED grading uses much less FFPE tissue than both DNA ploidy analysis and LOH analysis but provides almost equal prognostic information to these molecular techniques (Tables 4.4 and 5.5).

8.2.9 Molecular assay driven prognostic model for OPMD patients performed better than conventional risk-stratification methods

Construction of the multivariate prognostic model in the current study was guided by the recommendations described in the "Transparent Reporting of a multivariable prediction model for Individual Prognosis or Diagnosis (TRIPOD) Statement" as well as the recommendations described in the "Reporting Recommendations for Tumour Marker Prognostic Studies (REMARK)" studies (McShane *et al.*, 2005; Collins *et al.*, 2015; Moons *et al.*, 2015). The two prognostic models that had the addition of molecular parameters (Model 2 and Model 3) performed better than conventional risk-stratification methods based on clinico-demographic and histopathological parameters (Model 1). The final model (Model 3) also showed good results in an external validation cohort.

Despite having shown a marked increase in prognostic utility over conventional risk-stratification methods, there are several limitations to molecularly-driven prognostic classifiers. Heterogeneity of OPMDs pose a great problem for molecular analysis as does the temporal nature of OPMDs; different areas of a lesion can have a different molecular profile and lesions can evolve over time (Hirsch *et al.*, 1983; Perou *et al.*, 2000; Chung *et al.*, 2004; Diwakar *et al.*, 2005; Napier and Speight, 2008; Liu *et al.*, 2011; Russnes *et al.*, 2011; Marusyk *et al.*, 2012; Fisher *et al.*, 2013; De Cecco *et al.*, 2015; Gomes *et al.*, 2015; Gay *et al.*, 2016; Mroz and Rocco, 2016). However, this problem also affects histopathological and clinical parameters, necessitating the need for repeat biopsies and analysis over time. In situations where multiple biopsies may be necessary, minimally invasive biopsy techniques such as brush biopsies may prove to be useful (Lingen *et al.*, 2008).

One limitation of the current study was the small sample size that was not truly representative of the population, wherein MT occurs in approximately only 10-12% of cases with OED. However, the prevalence rate was taken into consideration for the statistical analysis and the final prognostic classifier was still found to be superior to conventional risk-stratification methods. Even though validation using an external cohort was performed, the size of the validation cohort was also rather small and further validation using a larger cohort will need to be performed to ascertain the true worth of this prognostic model.

8.3 Future Work

Numerous parameters were assessed during the development of the prognostic classifier resulting in many possible avenues for further research. The consistent results shown by the binary OED grading regardless of the sample size is a good reason to encourage other pathologists to begin concurrent usage of this grading system together with the WHO 2017 system to enable comparisons to be made using larger cohorts from multiple centres. Another avenue for further validation would be to utilise both grading systems for future clinical trials. Routine sole usage of only the binary OED grading system however is not encouraged as it does require further assessment and validation in clinical practice.

As there were very few cases of HR-HPV associated OED encountered in the current study, the role of HR-HPV in clinical outcome could not be elucidated. Due to the possible difference in aetiopathogenesis between HR-HPV associated OED and conventional OED, the transcriptomic profile of the two types of OED may be entirely different. As such, future work looking at differential gene expression between HR-HPV associated OED and conventional OED with differing clinical outcome should be performed in a step-wise manner, using both whole transcriptome profiling as well as targeted transcriptome profiling.

The role and relationship of sub-epithelial leukocytic infiltrate with the overlying epithelium as well as clinical outcome in OPMD has yet to be fully understood. There are numerous areas that require further investigation, from sub-typing the inflammatory infiltrate to looking at the influence of OPAL in different types of OPMD. Hanahan & Weinberg (2011) recently highlighted the importance of inflammation as an enabling factor for carcinogenesis and whether this holds true for malignant transformation of OPMD is yet inconclusive (Hanahan and Weinberg, 2011). If sub-epithelial inflammation does indeed facilitate malignant transformation, it does not explain the relatively low malignant transformation rates seen in inflammatory lesions such as oral lichen planus (Fitzpatrick *et al.*, 2014a; Aghbari *et al.*, 2017; Giuliani *et al.*, 2018). It is obvious that the relationship between OPAL and malignant transformation is not something that is so simplistic as to be dependent on only the intensity of the inflammatory infiltrate.

Further exploration of DNA ploidy analysis is warranted as the technique may prove to be more useful when assessed in a larger cohort using larger biopsy specimens. As the parameter assessed is different from both OED grading and LOH analysis, addition of DNA ploidy status and LOH status to conventional risk-stratification that uses clinico-pathological features may provide an added dimension to risk-stratification as well as increased prognostic strength of the model.

The work from the current study has shown that a reduced microsatellite panel for LOH analysis may actually reduce the number of false positives. The possible utility of a reduced panel will need to be assessed in a larger cohort that is more representative of the OPMD patient population. Combined use with DNA ploidy as mentioned earlier is of course an avenue that should be explored further.

The differential gene expression (DGE) experiments performed yielded several novel candidate genes that may potentially be involved in oral carcinogenesis. Further work to investigate these candidates have already begun starting with *TLX1* using immunohistochemistry (IHC) to assess *TLX1* protein expression. Further DGE experiments should also be undertaken to better understand the transcriptomic profile in various sub-populations of OPMD. For example, one could evaluate the DGE between low-grade OED and high-grade OED cases that undergo malignant transformation. Another example would be to assess the DGE in the different types of OPMDs which was not possible in our cohort as the vast majority of cases were oral leukoplakias. Future collaborative studies with centres in other parts of the world such as South Asia and East Asia that have high rates of oral submucous fibrosis may provide a way forward to obtain adequate samples from other types of OPMDs. As the molecular assays being utilised have been shown to perform well with FFPE tissue, global collaborations can easily be carried out as most pathology laboratories worldwide have vast numbers of archived FFPE material.

The gene-signature as well as prognostic classifier that were developed should be assessed in a larger cohort of OPMD patients with known clinical outcomes and adequate follow-up. This once again can be facilitated through a collaborative approach. In summary, though the findings from this study look promising, there is a need to validate it using a larger, preferably multicentre cohort that would be more representative of the population.

8.4 Conclusions

a) Age at diagnosis (> 50 years) and site of index OPMD (tongue) appear to be the clinical features more likely to be associated with malignant transformation. However, clinical features were not as good at prognosticating clinical outcome for OPMD patients as the binary OED grading system.
b) The binary OED grading system has better prognostic utility than the three-tiered WHO 2017 grading system for discriminating OPMD cases with differing clinical outcomes. It also appears to have better clinical utility than both DNA ploidy and LOH status in terms of prognosticating clinical outcome for OPMD patients.
c) There was no clear association between OPMD associated leukocytes (OPAL) and clinical outcome.
d) DNA ploidy status was not significantly associated with clinical outcome of OPMD cases in this cohort. As DNA ploidy requires a fairly large amount of tissue for successful analysis, it may not be suitable for small incisional biopsy specimens.
e) Loss of heterozygosity (LOH) was proven to have significant prognostic utility in discriminating OPMD cases with differing clinical outcomes. However, its' prognostic ability is not very much better than binary OED grading. LOH at chromosomal regions 3p &/or 9p has better utility than LOH at chromosomal regions 3p &/or 9p &/or 17p in terms of prognostic ability. Both manual microdissection and LASER capture microdissection methods are suitable for LOH analysis.
f) The gene-signature that was developed had better prognostic utility than conventional prognostic features such as demographic, clinical and histopathological parameters. It also performed better than DNA ploidy and LOH analysis.
g) Several novel candidate genes with possible roles in malignant transformation of OPMD as well as oral carcinogenesis have been identified using RNA derived from FFPE tissue.
h) FFPE tissue can be successfully used for molecular assays.
i) A prognostic classifier composed of demographic (age at diagnosis), clinical (site of index OPMD), histopathological (binary OED grading) and molecular (dichotomised gene-signature derived score) parameters outperformed

conventional prognostic classifiers in terms of discriminating OPMD cases with differing clinical outcomes.

Chapter 9. References

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Appendix A. Reporting recommendations for tumour marker prognostic studies (REMARK) checklist

The Reporting Recommendations for Tumour Marker Prognostic Studies (REMARK) Checklist

	Item to be reported	Page no.
INTRODUCTION		
1	State the marker examined, the study objectives, and any pre-specified hypotheses.	
MATERIALS AND METHODS		
Patients		
2	Describe the characteristics (e.g., disease stage or co-morbidities) of the study patients, including their source and inclusion and exclusion criteria.	
3	Describe treatments received and how chosen (e.g., randomized or rule-based).	
Specimen characteristics		
4	Describe type of biological material used (including control samples) and methods of preservation and storage.	
Assay methods		
5	Specify the assay method used and provide (or reference) a detailed protocol, including specific reagents or kits used, quality control procedures, reproducibility assessments, quantitation methods, and scoring and reporting protocols. Specify whether and how assays were performed blinded to the study endpoint.	
Study design		
6	State the method of case selection, including whether prospective or retrospective and whether stratification or matching (e.g., by stage of disease or age) was used. Specify the time period from which cases were taken, the end of the follow-up period, and the median follow-up time.	
7	Precisely define all clinical endpoints examined.	
8	List all candidate variables initially examined or considered for inclusion in models.	
9	Give rationale for sample size; if the study was designed to detect a specified effect size, give the target power and effect size.	
Statistical analysis methods		
10	Specify all statistical methods, including details of any variable selection procedures and other model-building issues, how model assumptions were verified, and how missing data were handled.	
11	Clarify how marker values were handled in the analyses; if relevant, describe methods used for cutpoint determination.	
RESULTS		
Data		
12	Describe the flow of patients through the study, including the number of patients included in each stage of the analysis (a diagram may be helpful) and reasons for dropout. Specifically, both overall and for each subgroup extensively examined report the numbers of patients and the number of events.	
13	Report distributions of basic demographic characteristics (at least age and sex), standard (disease-specific) prognostic variables, and tumor marker, including numbers of missing values.	
Analysis and presentation		
14	Show the relation of the marker to standard prognostic variables.	

15	Present univariable analyses showing the relation between the marker and outcome, with the estimated effect (e.g., hazard ratio and survival probability). Preferably provide similar analyses for all other variables being analyzed. For the effect of a tumor marker on a time-to-event outcome, a Kaplan-Meier plot is recommended.	
16	For key multivariable analyses, report estimated effects (e.g., hazard ratio) with confidence intervals for the marker and, at least for the final model, all other variables in the model.	
17	Among reported results, provide estimated effects with confidence intervals from an analysis in which the marker and standard prognostic variables are included, regardless of their statistical significance.	
18	If done, report results of further investigations, such as checking assumptions, sensitivity analyses, and internal validation.	
DISCUSSION		
19	Interpret the results in the context of the pre-specified hypotheses and other relevant studies; include a discussion of limitations of the study.	
20	Discuss implications for future research and clinical value.	

Appendix B. Transparent reporting of a multivariable prediction model for individual prognosis or diagnosis (TRIPOD) statement checklist

TRIPOD Checklist: Prediction Model Development and Validation

Section/Topic	Item		Checklist Item	Page
Title and abstract				
Title	1	D;V	Identify the study as developing and/or validating a multivariable prediction model, the target population, and the outcome to be predicted.	
Abstract	2	D;V	Provide a summary of objectives, study design, setting, participants, sample size, predictors, outcome, statistical analysis, results, and conclusions.	
Introduction				
Background and objectives	3a	D;V	Explain the medical context (including whether diagnostic or prognostic) and rationale for developing or validating the multivariable prediction model, including references to existing models.	
	3b	D;V	Specify the objectives, including whether the study describes the development or validation of the model or both.	
Methods				
Source of data	4a	D;V	Describe the study design or source of data (e.g., randomized trial, cohort, or registry data), separately for the development and validation data sets, if applicable.	
	4b	D;V	Specify the key study dates, including start of accrual; end of accrual; and, if applicable, end of follow-up.	
Participants	5a	D;V	Specify key elements of the study setting (e.g., primary care, secondary care, general population) including number and location of centres.	
	5b	D;V	Describe eligibility criteria for participants.	
	5c	D;V	Give details of treatments received, if relevant.	
Outcome	6a	D;V	Clearly define the outcome that is predicted by the prediction model, including how and when assessed.	
	6b	D;V	Report any actions to blind assessment of the outcome to be predicted.	
Predictors	7a	D;V	Clearly define all predictors used in developing or validating the multivariable prediction model, including how and when they were measured.	
	7b	D;V	Report any actions to blind assessment of predictors for the outcome and other predictors.	
Sample size	8	D;V	Explain how the study size was arrived at.	
Missing data	9	D;V	Describe how missing data were handled (e.g., complete-case analysis, single imputation, multiple imputation) with details of any imputation method.	
Statistical analysis methods	10a	D	Describe how predictors were handled in the analyses.	
	10b	D	Specify type of model, all model-building procedures (including any predictor selection), and method for internal validation.	
	10c	V	For validation, describe how the predictions were calculated.	
	10d	D;V	Specify all measures used to assess model performance and, if relevant, to compare multiple models.	
	10e	V	Describe any model updating (e.g., recalibration) arising from the validation, if done.	
Risk groups	11	D;V	Provide details on how risk groups were created, if done.	
Development vs. validation	12	V	For validation, identify any differences from the development data in setting, eligibility criteria, outcome, and predictors.	
Results				
Participants	13a	D;V	Describe the flow of participants through the study, including the number of participants with and without the outcome and, if applicable, a summary of the follow-up time. A diagram may be helpful.	
	13b	D;V	Describe the characteristics of the participants (basic demographics, clinical features, available predictors), including the number of participants with missing data for predictors and outcome.	
	13c	V	For validation, show a comparison with the development data of the distribution of important variables (demographics, predictors and outcome).	
Model development	14a	D	Specify the number of participants and outcome events in each analysis.	
	14b	D	If done, report the unadjusted association between each candidate predictor and outcome.	

Model specification	15a	D	Present the full prediction model to allow predictions for individuals (i.e., all regression coefficients, and model intercept or baseline survival at a given time point).	
	15b	D	Explain how to the use the prediction model.	
Model performance	16	D;V	Report performance measures (with CIs) for the prediction model.	
Model-updating	17	V	If done, report the results from any model updating (i.e., model specification, model performance).	
Discussion				
Limitations	18	D;V	Discuss any limitations of the study (such as nonrepresentative sample, few events per predictor, missing data).	
Interpretation	19a	V	For validation, discuss the results with reference to performance in the development data, and any other validation data.	
	19b	D;V	Give an overall interpretation of the results, considering objectives, limitations, results from similar studies, and other relevant evidence.	
Implications	20	D;V	Discuss the potential clinical use of the model and implications for future research.	
Other information				
Supplementary information	21	D;V	Provide information about the availability of supplementary resources, such as study protocol, Web calculator, and data sets.	
Funding	22	D;V	Give the source of funding and the role of the funders for the present study.	

*Items relevant only to the development of a prediction model are denoted by D, items relating solely to a validation of a prediction model are denoted by V, and items relating to both are denoted D;V.

Appendix C. Protocol for RNA extraction from formalin-fixed paraffin-embedded tissue

Protocol for RNA extraction and purification using the QIAGEN RNeasy FFPE kit following the manufacturer's protocol (QIAGEN, Manchester, UK).

i. 160µl (1 - 2 FFPE sections) or 320µl (> 2 FFPE sections) of Deparaffinization Solution (QIAGEN, Germany), was added to the sample and vortexed vigorously for 10s and centrifuged briefly to bring the sample to the bottom of the tube.
ii. The sample was then incubated at 56°C for 3 minutes and allowed to cool to room temperature.
iii. 150µl (1 - 2 FFPE sections) or 240µl (> 2 FFPE sections) Buffer PKD was added and mixed by vortexing.
iv. The samples were then centrifuged for 1 minutes at 11,000 x g.
v. 10µl proteinase K was then added to the lower (clear) phase and mixed gently by pipetting up and down.
vi. The samples were incubated at 56°C for 15 minutes, and then at 80°C for 15 minutes in heating blocks with a shaking function.
vii. After incubation, the lower (uncoloured) phase was transferred into a new 2ml microcentrifuge tube and left to incubate on ice for 3 minutes.
viii. The samples were then centrifuged for 15 minutes at 20,000 x g.
ix. The supernatant was then transferred to a new 2ml microcentrifuge tube
x. DNase Booster Buffer equivalent to a tenth of the total sample volume and 10µl DNase I stock solution was then added to the sample and mixed by inverting the tube. The tube was then centrifuged briefly to collect residual liquid from the sides of the tube.
xi. The sample was then incubated at room temperature for 15 minutes.
xii. After incubation, 320µl (1 - 2 FFPE sections) or 500µl (> 2 FFPE sections) of Buffer RBC was added to the sample and the lysate was mixed thoroughly.
xiii. 720µl (1 - 2 FFPE sections) or 1200µl (> 2 FFPE sections) of ethanol (100%) was added to the sample and mixed well by pipetting.
xiv. 700µl of the sample was then transferred to a RNeasy MinElute spin column placed in a 2ml collection tube and centrifuged for 15 s at ≥8000 x g. The flow-

through was discarded. This step was repeated until the entire sample has passed through the RNeasy MinElute spin column.

xv. 500µl Buffer RPE was then added to the RNeasy MinElute spin column and centrifuged for 15 s at ≥8000 x g. The flow-through was discarded.

xvi. 500µl Buffer RPE was then added to the RNeasy MinElute spin column and centrifuged for 2 minutes at ≥8000 x g to wash the sample. After centrifugation, the RNeasy MinElute spin column was carefully removed from the collection tube so that the column did not contact the flow-through. The collection tube with the flow-through was then discarded.

xvii. The RNeasy MinElute spin column was then placed in a new 2ml collection tube. The tube was the centrifuged at full speed for 5 minutes with lid of the spin column being left open (to dry the spin column membrane as residual ethanol may interfere with downstream reactions). The collection tube with the flow-through was then discarded.

xviii. The RNeasy MinElute spin column was then placed into new 1.5ml collection tube.

xix. 16µl of RNase-free water was then placed directly onto the spin column membrane and the tubes were centrifuged for 1 minute at full speed to elute the RNA.

Appendix D. Additional target genes for PanCancer Pathways Panel Plus

List of additional targets for PanCancer Pathways Panel Plus

Ensembl gene ID	HGNC symbol	Log2 fold change	FDR [BH p-value]
ENSG00000196805	*SPRR2B*	2.478102	0.014621
ENSG00000115844	*DLX2*	2.301966	0.014621
ENSG00000223802	*CERS1*	2.187502	0.043672
ENSG00000139292	*LGR5*	-1.83	0.046
ENSG00000166165	*CKB*	2.11	0.015
ENSG00000177707	*NECTIN3*	-2.01	0.036
ENSG00000066248	*NGEF*	1.58	0.031
ENSG00000115112	*TFCP2L1*	-2.22	0.015
ENSG00000111640	§*GAPDH*	NA	NA
ENSG00000198807	**PAX9*	NA	NA

§*GAPDH* was included as an additional housekeeping gene **PAX9* was included based on previous work performed by the Newcastle University Oral Cancer Research Group (OCRG). FDR = False discovery rate; BH = Benjamini-Hochberg; HGNC = Human Genome Organisation Gene Nomenclature Committee; NA = not available

Appendix E. Laboratory workflow for PanCancer Pathways Panel Plus

Summarised laboratory workflow for the NanoString nCounter assay using the PanCancer Pathways Panel Plus is listed below:

i. Aliquots of Reporter CodeSet, Capture ProbeSet, and Plus reagents were removed from the freezer to thaw at room temperature. Reagents were inverted several times to mix well and then spun down.
ii. 28 µl of Reporter Plus reagent was added to the thawed Reporter CodeSet in the supplied 12-well strip tubes. Reagents were inverted several times to mix well and then spun down.
iii. A master mix was created by adding 70µl of hybridization buffer to the tubes containing the Reporter CodeSet and Reporter Plus reagents. The tube with master mix was inverted several times to mix well and then spun down
iv. The hybridisation tubes were labelled.
v. 10µl of master mix (made up of 3µl Reporter CodeSet, 2µl Reporter Plus and 5µl hybridisation buffer) was added to each tube.
vi. 5µl of sample was added to each tube.
vii. When necessary, RNase-free water was added to bring the volume of each assay to 15 µl.
viii. 14 µl of Capture Plus reagent was added to the thawed aliquot of Capture ProbeSet. Reagents were inverted several times to mix well and then spun down.
ix. 3 µl of Capture ProbeSet & Capture Plus reagent mix was added to each tube. Tube caps were closed, and reagents were mixed by inverting the tubes several times and flicking with a finger (to ensure complete mixing).
x. The tubes were briefly spun down and placed in a pre-heated 65°C thermal cycler.
xi. Reactions were left to incubate for at least 16 hours.
xii. Samples were then processed the following day on the “nCounter MAX/FLEX Prep Station” (NanoString Technologies, Seattle, USA). The nCounter MAX/FLEX Prep Station is a multi-channel pipetting robot.

xiii. Sample cartridges were removed from -20°C storage and allowed to equilibrate to room temperature. Prep plates were removed from -4°C storage and centrifuged at 2000xg for 2 minutes and then allowed to equilibrate to room temperature. The last row of the plates was checked to confirm presence of magnetic beads.

xiv. The prep plates were placed on the prep deck with the green labels facing forwards. The lids were discarded, and tips were loaded.

xv. The foil from the cartridge was removed and the cartridge was placed under the electrode fixture and electrodes were lowered into the cartridge.

xvi. Empty NanoString strip tubes were placed into the heater block.

xvii. Sample tubes were un-capped and placed into the metal tube holder. The heater lid was then closed and processing on the nCounter MAX/FLEX Prep Station was initiated (hybridised samples were purified and immobilised on the MAX/FLEX Prep Station).

xviii. After completing the run on the nCounter MAX/FLEX Prep Station, the cartridges were covered to prevent evaporation of liquids.

xix. The Reporter Library File (RLF) was then uploaded to the nCounter MAX/FLEX Digital Analyzer

xx. The Sample Cartridges (each containing 12 samples) were then transferred from the nCounter MAX/FLEX Prep Station to the “nCounter MAX/FLEX Digital Analyzer” (NanoString Technologies, Seattle, USA) to obtain the target probe counts. The nCounter MAX/FLEX Digital Analyzer is a multi-channel epifluorescence scanner.

xxi. The probe counts were tabulated in a comma separated value (CSV) format for data analysis.

Appendix F. Gene list for Customised CodeSet Panel

List of genes for NanoString Customised CodeSet Panel experiment

Ensembl Gene ID	HGNC gene symbol	Designation
ENSG00000107807	*TLX1*	TARGET
ENSG00000148053	*NTRK2*	TARGET
ENSG00000198807	*PAX9*	TARGET
ENSG00000146648	*EGFR*	TARGET
ENSG00000100985	*MMP9*	TARGET
ENSG00000196611	*MMP1*	TARGET
ENSG00000087245	*MMP2*	TARGET
ENSG00000089685	*BIRC5*	TARGET
ENSG00000162493	*PDPN*	TARGET
ENSG00000148400	*NOTCH1*	TARGET
ENSG00000141510	*TP53*	TARGET
ENSG00000147889	*CDKN2A*	TARGET
ENSG00000189283	*FHIT*	TARGET
ENSG00000078900	*TP73*	TARGET

Ensembl Gene ID	HGNC gene symbol	Designation
ENSG00000073282	*TP63*	TARGET
ENSG00000120217	*PDL1*	TARGET
ENSG00000073111	*MCM2*	TARGET
ENSG00000135047	*CTSL*	TARGET
ENSG00000122512	*PMS2*	TARGET
ENSG00000076242	*MLH1*	TARGET
ENSG00000095002	*MSH2*	TARGET
ENSG00000116062	*MSH6*	TARGET
ENSG00000183765	*CHEK2*	TARGET
ENSG00000029559	*IBSP*	TARGET
ENSG00000152591	*DSPP*	TARGET
ENSG00000115844	*DLX2*	TARGET
ENSG00000223802	*CERS1*	TARGET
ENSG00000139292	*LGR5*	TARGET
ENSG00000196805	*SPRR2B*	TARGET
ENSG00000177707	*NECTIN3*	TARGET
ENSG00000188089	*PLA2G4E*	TARGET

Ensembl Gene ID	HGNC gene symbol	Designation
ENSG00000105855	*ITGB8*	TARGET
ENSG00000188153	*COL4A5*	TARGET
ENSG00000138166	*DUSP5*	TARGET
ENSG00000171791	*BCL2*	TARGET
ENSG00000125398	*SOX9*	TARGET
ENSG00000105173	*CCNE1*	TARGET
ENSG00000153956	*CACNA2D1*	TARGET
ENSG00000167553	*TUBA6*	HOUSEKEEPING
ENSG00000075624	*ACTB*	HOUSEKEEPING
ENSG00000111640	*GAPDH*	HOUSEKEEPING
ENSG00000073578	*SDHA*	HOUSEKEEPING

HGNC = Human Genome Organisation Gene Nomenclature Committee

Appendix G. Laboratory workflow for Customised CodeSet Panel

Summarised laboratory workflow for the Customised CodeSet Panel gene expression assay is listed in below:

i. Aliquots of Reporter CodeSet, and Capture ProbeSet were removed from the freezer to thaw at room temperature. Reagents were inverted several times to mix well and then spun down.
ii. A master mix was created by adding 70µL of hybridization buffer to the Reporter CodeSet in the supplied 12-well strip tubes. The tubes with master mix was inverted several times to mix well and then spun down
iii. The hybridisation tubes were labelled.
iv. 8µl of master mix (made up of 3µl Reporter CodeSet and 5µl hybridisation buffer) was added to each tube.
v. 5µl of sample was added to each tube.
vi. When necessary, RNase-free water was added to bring the volume of each assay to 13µl.
vii. Capture ProbeSet tube was inverted several times to mix well and then spun down.
viii. 2 µl of Capture ProbeSet was added to each tube. Tube caps were closed, and reagents were mixed by inverting the tubes several times and flicking with a finger (to ensure complete mixing).
ix. The tubes were briefly spun down and placed in a pre-heated 65°C thermal cycler.
x. Reactions were left to incubate for at least 16 hours.
xi. Samples were then processed the following day on the “nCounter MAX/FLEX Prep Station” (NanoString Technologies, Seattle, USA). The nCounter MAX/FLEX Prep Station is a multi-channel pipetting robot.
xii. Sample cartridges were removed from -20°C storage and allowed to equilibrate to room temperature. Prep plates were removed from -4°C storage and centrifuged at 2000xg for 2 minutes and then allowed to equilibrate to room temperature. The last row of the plates was checked to confirm presence of magnetic beads.

xiii. The prep plates were placed on the prep deck with the green labels facing forwards. The lids were discarded, and tips were loaded.

xiv. The foil from the cartridge was removed and the cartridge was placed under the electrode fixture and electrodes were lowered into the cartridge.

xv. Empty NanoString strip tubes were placed into the heater block.

xvi. Sample tubes were un-capped and placed into the metal tube holder. The heater lid was then closed and processing on the nCounter MAX/FLEX Prep Station was initiated (hybridised samples were purified and immobilised on the MAX/FLEX Prep Station).

xvii. After completing the run on the nCounter MAX/FLEX Prep Station, the cartridges were covered to prevent evaporation of liquids.

xviii. The Reporter Library File (RLF) was then uploaded to the nCounter MAX/FLEX Digital Analyzer

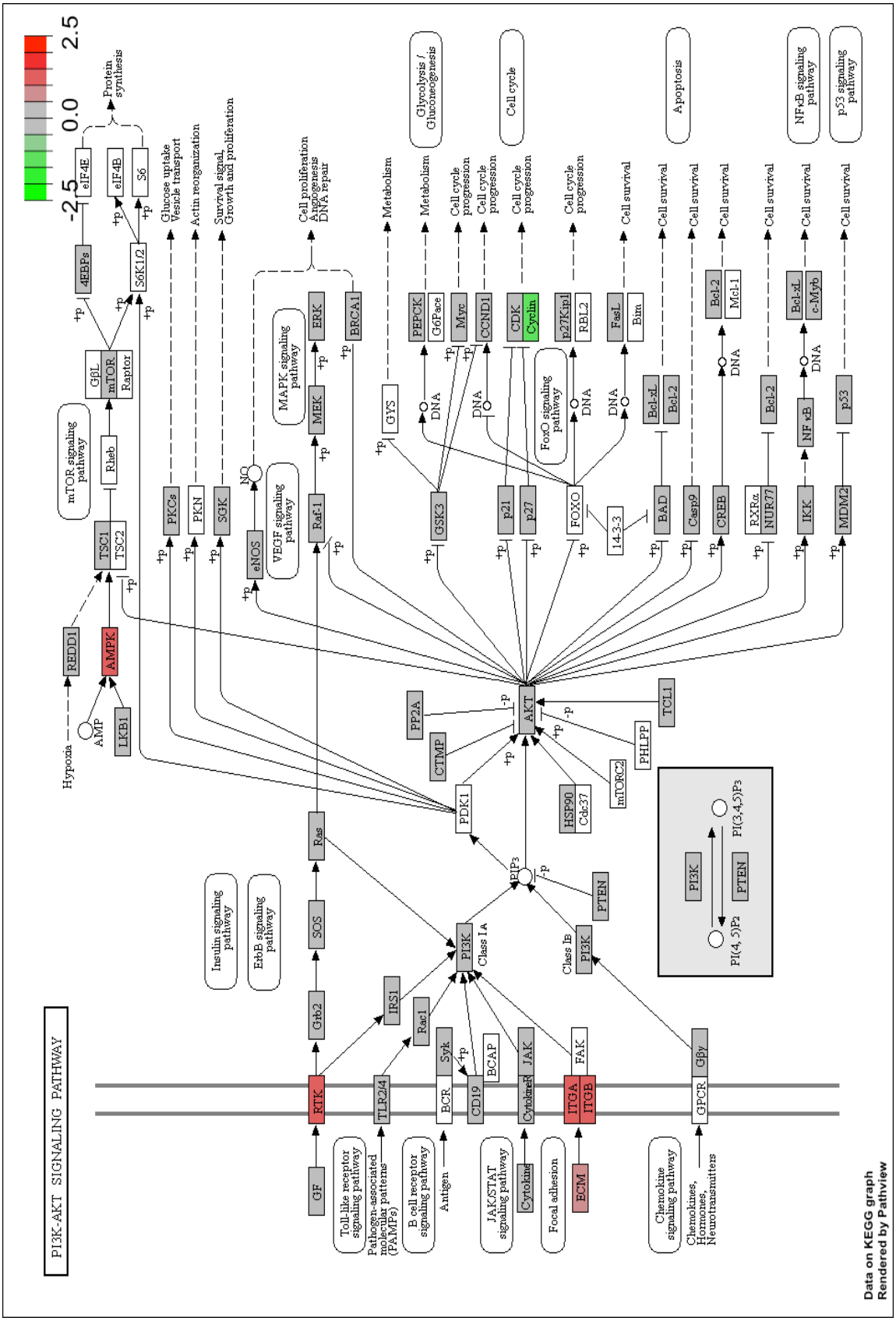
xix. The Sample Cartridges (each containing 12 samples) were then transferred from the nCounter MAX/FLEX Prep Station to the “nCounter MAX/FLEX Digital Analyzer” (NanoString Technologies, Seattle,USA) to obtain the target probe counts. The nCounter MAX/FLEX Digital Analyzer is a multi-channel epifluorescence scanner.

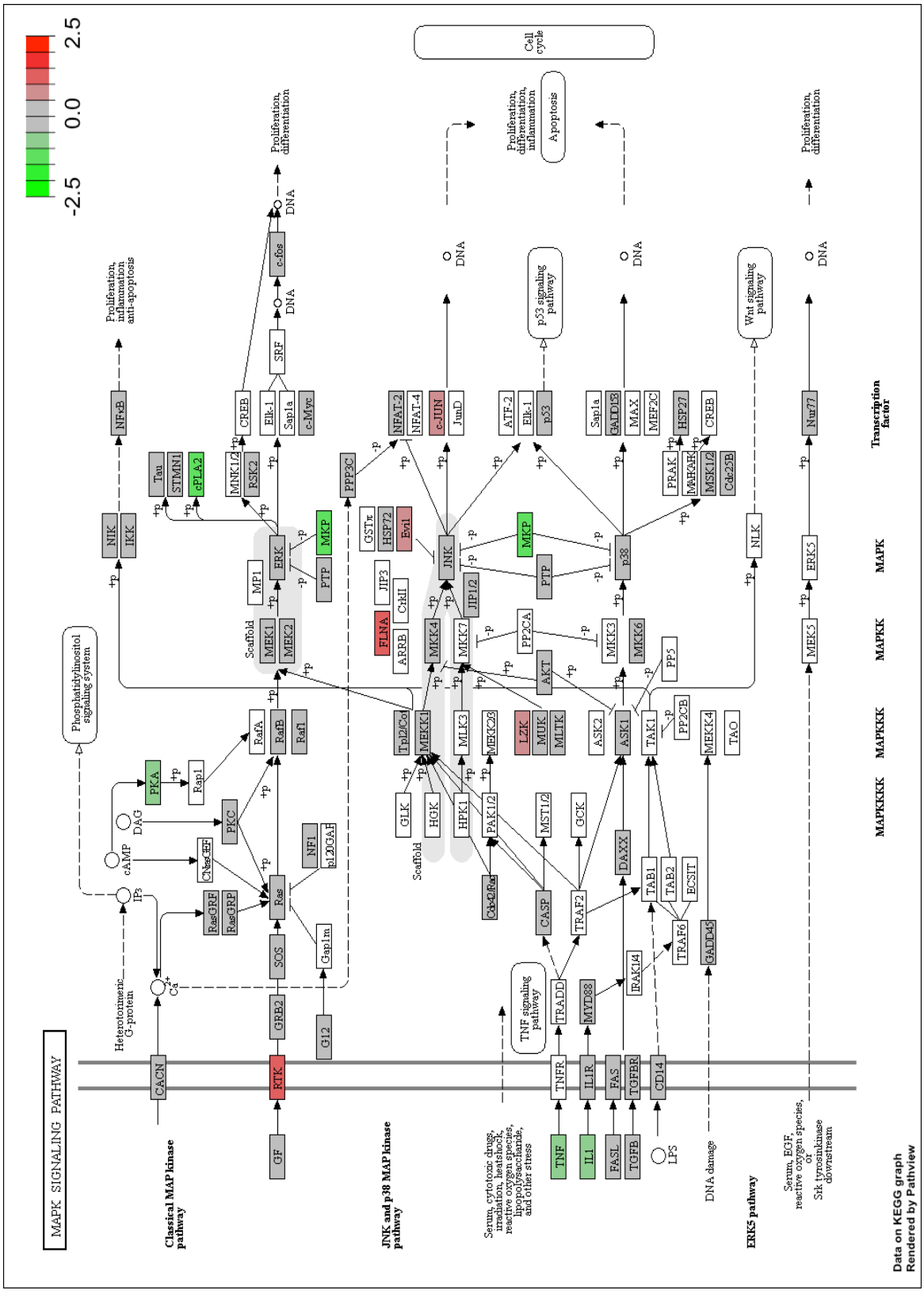
xx. The probe counts were tabulated in a comma separated value (CSV) format for data analysis.

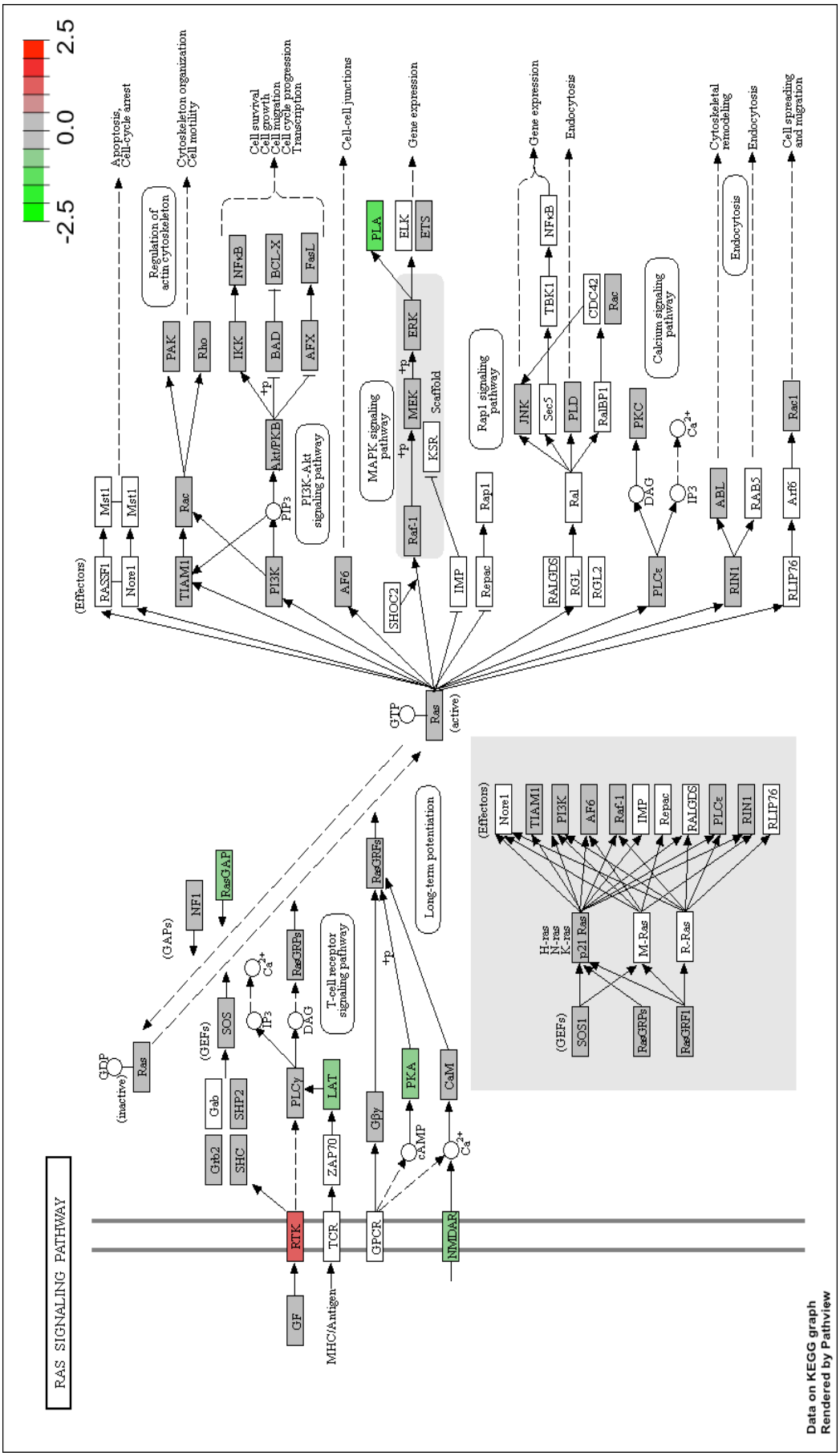
Appendix H. Associated Kyoto Encylopaedia of Genes and Genomes (KEGG) pathways

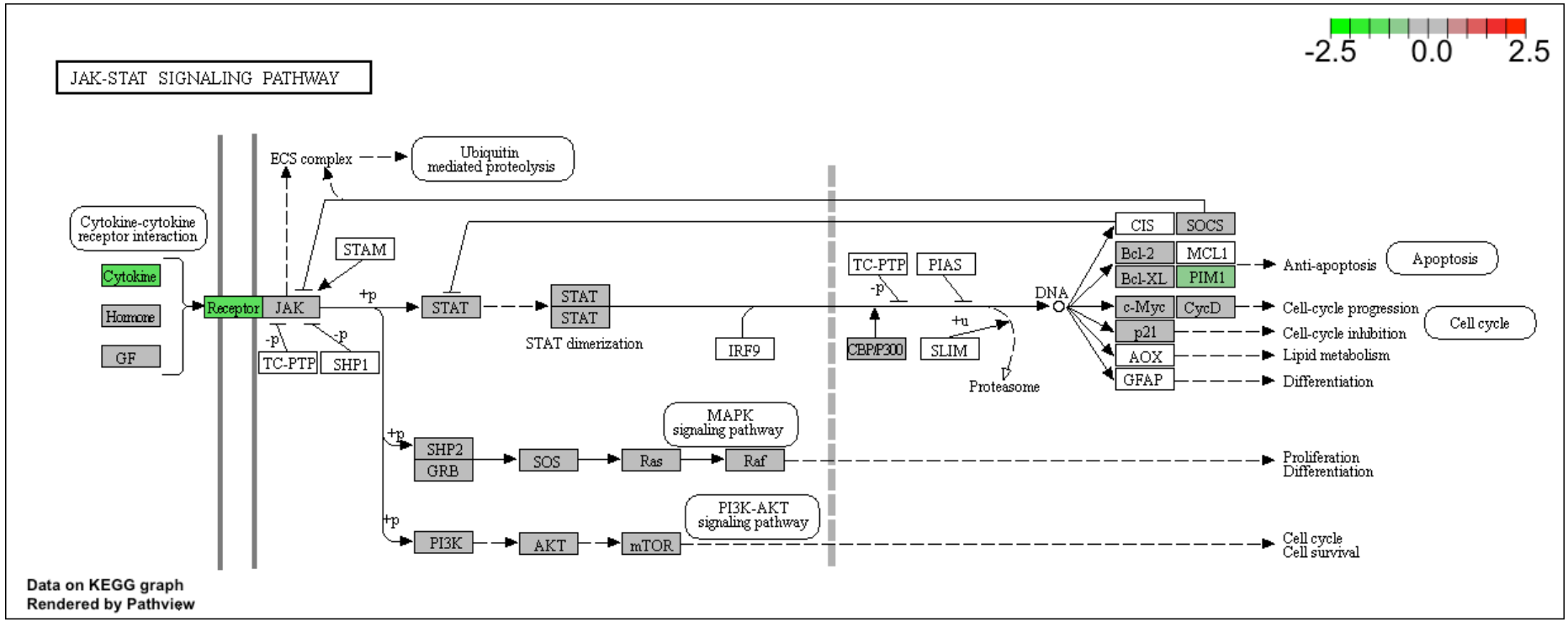
Kyoto Encyclopaedia of Genes and Genomes (KEGG) pathways of top five cancer-related pathways from PanCancer Pathways Panel Plus experiment (n = 48).

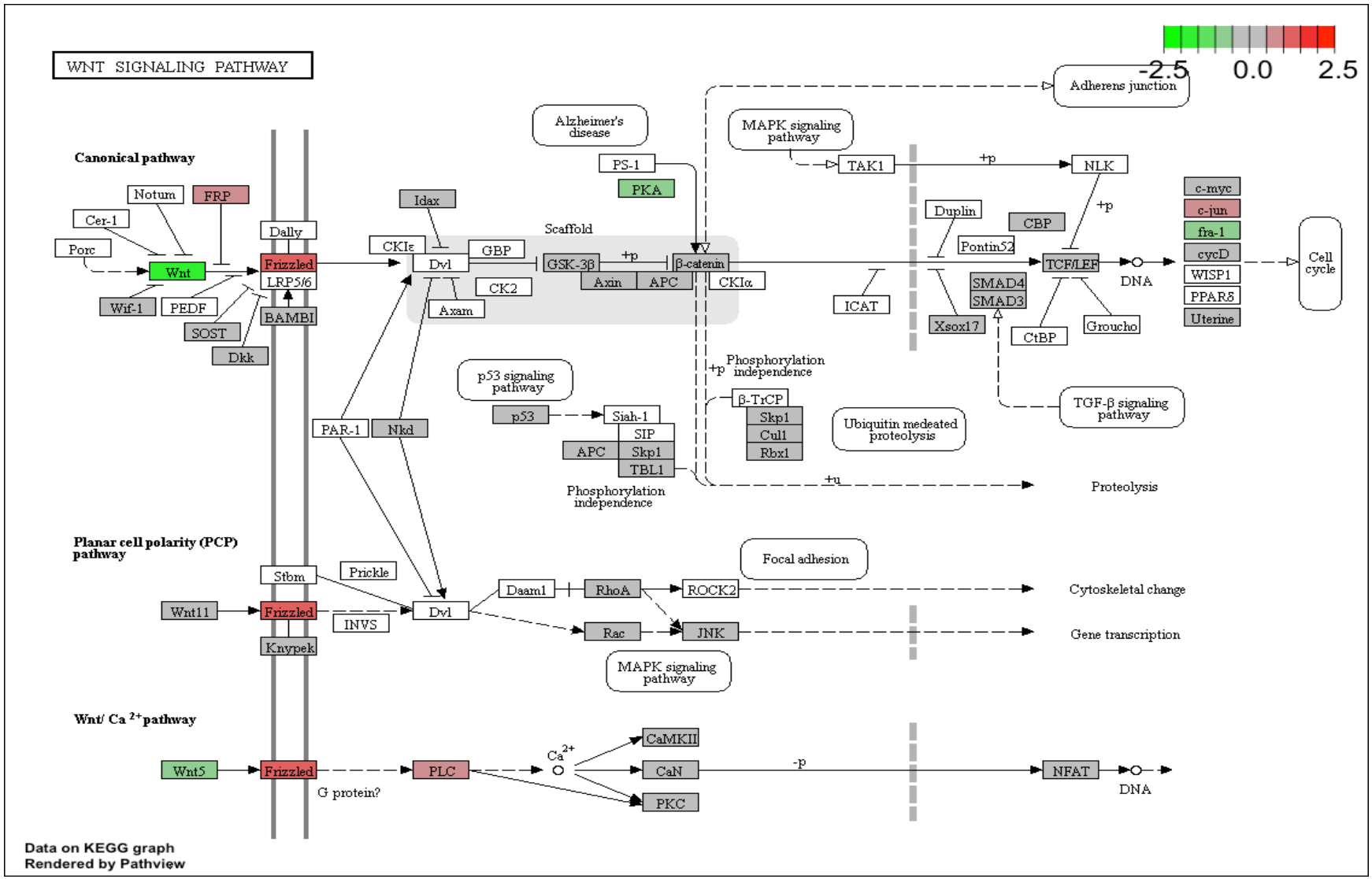
For each KEGG pathway, genes within the panel are mapped to the pathway and differential expression information is overlaid on the protein-based KEGG pathway image. Pathway nodes shown in white have no genes in the panel that map to them. Pathway nodes in grey have corresponding genes in the panel, however no significant differential expression is observed. Nodes in green denote downregulation in malignant transforming cases relative to non-transforming cases, whereas nodes in red denote upregulation in malignant transforming cases relative to non-transforming cases.


PI3K-AKT SIGNALING PATHWAY
-2.5
0.0
2.5
Data on KEGG graph
Rendered by Pathview


MAPK SIGNALING PATHWAY
-2.5
0.0
2.5
Classical MAP kinase pathway
JNK and p38 MAP kinase pathway
ERK5 pathway
MAPKKKK
MAPKKK
MAPKK
MAPK
Transcription factor
Data on KEGG graph
Rendered by Pathview


RAS SIGNALING PATHWAY
-2.5
0.0
2.5
Data on KEGG graph
Rendered by Pathview


-2.5
0.0
2.5
JAK-STAT SIGNALING PATHWAY
ECS complex
Ubiquitin mediated proteolysis
Cytokine-cytokine receptor interaction
Cytokine
Hormone
GF
Receptor
JAK
STAM
+p
-p
TC-PTP
SHP1
STAT
STAT
STAT
STAT dimerization
IRF9
TC-PTP
PIAS
-p
+u
CBP/P300
SLIM
Proteasome
DNA
CIS
SOCS
Bcl-2
MCL1
Bcl-XL
PIM1
c-Myc
CycD
p21
AOX
GFAP
Anti-apoptosis
Apoptosis
Cell-cycle progression
Cell-cycle inhibition
Cell cycle
Lipid metabolism
Differentiation
MAPK signaling pathway
+p
SHP2
GRB
SOS
Ras
Raf
Proliferation
Differentiation
PI3K-AKT signaling pathway
+p
PI3K
AKT
mTOR
Cell cycle
Cell survival
Data on KEGG graph
Rendered by Pathview


WNT SIGNALING PATHWAY
-2.5
0.0
2.5
Canonical pathway
Notum
FRP
Cer-1
Dally
Porc
Wnt
Frizzled
LRP5/6
Wif-1
PEDF
SOST
BAMBI
Dkk
Idax
CKIε
GBP
Dvl
Axam
CK2
Scaffold
GSK-3β
+p
β-catenin
Axin
APC
CKIα
Alzheimer's disease
PS-1
PKA
MAPK signaling pathway
TAK1
+p
NLK
Adherens junction
Duplin
CBP
Pontin52
+p
TCF/LEF
DNA
c-myc
c-jun
fra-1
cycD
WISP1
PPARδ
Uterine
Cell cycle
ICAT
SMAD4
SMAD3
Xsox17
CtBP
Groucho
TGF-β signaling pathway
+p
Phosphorylation independence
β-TrCP
Skp1
Cul1
Rbx1
Ubiquitin medeated proteolysis
p53 signaling pathway
p53
Siah-1
SIP
APC
Skp1
TBL1
Phosphorylation independence
+u
Proteolysis
PAR-1
Nkd
Planar cell polarity (PCP) pathway
Stbm
Prickle
Wnt11
Frizzled
INVS
Knypek
Dvl
Daam1
RhoA
ROCK2
Focal adhesion
Cytoskeletal change
Rac
JNK
Gene transcription
MAPK signaling pathway
Wnt/ Ca 2+pathway
Wnt5
Frizzled
G protein?
PLC
Ca2+
CaMKII
CaN
PKC
-p
NFAT
DNA
Data on KEGG graph
Rendered by Pathview