Analysis of DNA Content (Ploidy) and Potential New Biomarkers in Oral Malignant Lesions

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This thesis is dedicated to the memory of my mother, Hannia Zargoun.

I dedicate this work to my father, to my lovely husband Salem, my son "Abdulla", and daughters "Renad and Retal" and to

my brothers and sisters

Abstract

The purpose of this study was to evaluate the expression patterns of a range of cell cycle proteins (MCM2, Ki-67, geminin and cyclin D1) and assess the DNA content (ploidy) as diagnostic and prognostic markers in oral squamous cell carcinomas (OSCC). Immunohistochemical staining and DNA ploidy image cytometry analysis were performed on 86 cases. There were 47 primary OSCC that had not metastasised (NM), 39 that had metastasised (M) along with their matched positive cervical lymph nodes, and 17 normal oral mucosa (NOM) samples. Tissue microarrays (TMAs) were prepared with three cores from each of three areas: surface, middle and advancing front of each case. Sections were immunostained with MCM2, Ki-67, geminin and cyclin D1 antibodies.

The results revealed that there were no differences in protein expression in different areas of the tumours, nor between the metastatic and non-metastatic carcinomas. None of the cell cycle proteins showed significant differences in lymph node metastasis compared to the primary OSCCs, with the exception of Ki-67. MCM2 showed higher expression in nodal metastses, but this did not reach statistical significance. MCM2/Ki-67 and geminin/Ki-67 ratios were significantly different between metastatic and non-metastatic tumours.

Analysis of DNA ploidy showed that aneuploidy was detected in all (100%) cases of OSCC. Similarly, all lymph nodes samples (39 cases) were aneuploid. The results from this project suggest that dysregulation of cell cycle regulatory proteins was evident in the studied cohort. MCM2 expression, MCM2/Ki-67 and geminin/Ki-67 ratios may have prognostic significance in oral cancer. Aneuploidy is a common feature of oral carcinoma. The results also suggest that primary OSCC lesions may show heterogeneity. Greater incidence of abnormal DNA content was observed at the surface area of tumours.

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List of Abbreviations

5cER	Cells that exceed 5c in an image cytometry histogram
ACIS	Automated cellular imaging system
APC	Anaphase promoting complex
Cdc45	Cell division cycle 45 protein
Cdc6	Cell division cycle 6 protein
Cdc7-Dbf4	Dumb bell former 4-cell division cycle 7
CDK4	Cyclin-Dependent Kinase 4
CDK6	Cyclin-Dependent Kinase 6
CDKN2A	Cyclin-Dependent Kinase Inhibitor A2
Cdt1	DNA replication factor 1, cdc10-depdent transcript
CV	Coefficient of variation
DI	DNA index
DNA	Deoxyribonucleic acid
EF2	Transcription factors
EGFR	Epidermal growth factor receptor
ESACP	European society of analytical cellular pathology
G1	Gap 1 phase
G2	Gap 2 phase
GLM	General linear model
HNC	Head and neck cancer
Hox	Transcription factor of HOXb9 gene
HPV	Human papilloma virus
IOD	Integrated optical density
Ki-67	Ki-67 proliferation protein
LOH	Loss of heterozygosity
M	Mitosis phase
MCM	Minichromosome maintenance proteins 2-7
MCM 10	Minichromosome maintenance proteins 10

MCM 2	Minichromosome maintenance proteins 2
MCM 5	Minichromosome maintenance proteins 5
MMPs	Matrix metalloproteinases
NPV	Negative predicative value
OPC	Oropharyngeal cancer
ORC	Origin recognition complex
OSCC	Oral squamous cell carcinoma
P53	Tumour suppressor protein 53
PCNA	Proliferating cell nuclear antigen
PPV	Positive predictive value
pRb	Retinoblastoma protein
Pre-RC	Pre-replicative complex
RhoC	Homolog gene family member C
ROC	Receiver operating characteristic
RPA	Replication protein A
S	Synthesis phase
SCC	Squamous cell carcinoma
SD	Standard deviation
Six 3	Transcription factor of gene Six
TMA	Tissue microarray
TNF-α	Transforming growth factor alpha
TNM	Tumour node metastasis classification system
TSG	Tumour suppressor gene
WHO	World health organisation

Publications and presentations resulting from work presented in this thesis

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Poster presentations:

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CHAPTER 1: LITERATURE REVIEW

1. LITERATURE REVIEW

1.1 Oral cancer - overview

According to a recent international epidemiological study, cancer is one of the leading causes of death in the world (Ferlay et al., 2010) and head and neck cancer (HNC) is one of the most common cancers. HNC is the 15th most prevalent malignant neoplasm in the United Kingdom (Cancer Research UK, 2010). HNCs are located in the upper aerodigestive tract encompassing the nasal cavity, paranasal sinuses, pharynx, larynx and oral cavity (Argiris et al., 2008). According to the World Health Organisation (WHO) (Barnes, 2005) classification system, a wide range of neoplasms have been categorised in the head and neck region. However, squamous cell carcinoma (SCC) represents the most common epithelial malignancies in the region (Cooper et al., 2009). SCC in the oral cavity (oral cancer) is the sixth most common malignancy worldwide (Shah and Gil, 2009), representing about 3% of all malignancies (Speight et al., 1996). The Larynx and pharynx are the next most commonly affected sites for SCC in the head and neck region (Mehanna et al., 2010).

The annual incidence of oral and oropharyngeal squamous cell carcinoma (OSCC) accounts for nearly 92,000 new cases in Europe. According to Cancer Research UK, 6,539 people were diagnosed with oral cancer in 2010 and there is a high mortality rate, with approximately 2,056 deaths in 2011 (Cancer Research UK, 2010). In the last two to three decades the

incidence trends have not shown any noticeable improvement. The highest incidence across the UK was reported amongst Scottish people.

In the United States, approximately 22,000 new cases of oral cancer are reported each year (Neville et al., 2009). In recent decades a significant increase in the incidence of oral cancer, particularly tongue cancer, has been documented in young people in the United States as well as the United Kingdom (Sherin et al., 2008, Warnakulasuriya et al., 2007). In 2014, an estimated 42,440 new oral cavity cases were reported (including pharynx) and around 8,390 deaths registered in the United States (National Cancer Institute of U.S.A, 2014). It was recently reported that 263,900 cases were diagnosed as oral squamous cell carcinoma in 2008 around the world (Seethalakshmi, 2013). The incidence rate of oral squamous cell carcinoma is relatively high in most South Asian nations (Jemal et al., 2011). Trends in oral cavity cancer incidence have declined over the past decades across countries. This decrease is largely due to the decline in tobacco use (Chaturvedi et al., 2013). In contrast, oropharyngeal cancer (OPC) incidence rates have increased over time (Auluck et al., 2014). These contradictory incidence figures for oral and oropharyngeal cancers are most likely linked to human papillom virus (HPV) infection (Chaturvedi et al., 2013). HPV DNA is frequently detected in oropharyngeal tumours (Syrjänen, 2010, Marur et al., 2010). Most importantly the HPV type 16 (Moody and Laimins, 2010) is highly associated with oropharyngeal cancer (Westra, 2015). Consistent with this, a significant increase in the incidence

of HPV-associated OPC among men has been reported (Gillison et al., 2012).

1.2 Oral squamous cell carcinoma

Squamous Cell Carcinoma is an invasive epithelial neoplasm which is usually well differentiated, showing preponderance in males (2:1), and is common over the age of 60 years (Mehanna et al., 2010), with an annual incidence of about 8 per 100,000 people in the UK. According to a recent report, 6% of people affected with oral cancer are young adults (below 45 years of age) (Majchrzak et al., 2014). Oral squamous cell carcinoma (OSCC) comprises the vast majority (more than 90%) of all malignancies in the mouth and oropharynx (Cooper et al., 2009, Marur and Forastiere, 2008). However, oral (OSCC) and oropharyngeal (OPC) cancers have distinct clinical characteristics, and should be regarded as two separate entities (Auluck et al., 2014). Clinically, OSCC involves the tongue (anterior two third), lower lip, floor of the mouth, buccal mucosa, gingivae and hard palate. OPCs can be present in the tonsil area, soft palate, base of the tongue and the pharyngeal walls (Chaturvedi et al., 2013). Recent literature has reported that OSCC and OPC have different incidence rates, aetiology, and clinical outcome (Won et al., 2012, Auluck et al., 2014). The association of HPV, mainly type 16, is established as an etiologic cause of OPC, whereas its role in OSCC is uncertain (Chaturvedi et al., 2013). Higher HPV positivity has been reported in OPC compared to OSCC (Laco et al., 2011).

Patients with HPV-associated cancers have more favourable prognosis than patients with HPV-negative cancers (Ang et al., 2010, Won et al., 2012).

Disappointingly, in recent years there has been a marked increase in mortality and incidence of oral cancer. This has been documented as due to complexities of cancer growth pathways, late diagnosis, and recurrence. Overall mortality is about 50% (Johnson et al., 2011). This increases with metastasis (Noguti et al., 2012). Thus, the clinical need to prevent metastasis is crucial. To date, despite remarkable progression in cancer treatment, including surgical techniques and chemotherapy, the prognosis remains unchanged. However, the average 5-year survival rates remain at approximately 50% of affected people (Fung and Grandis, 2010, González-Moles et al., 2013), with more than 60% of patients presenting in stages III and IV (Lingen et al., 2008).

OSCC is characterised histologically by frank invasive growth across the basement membrane into the connective tissue (**Figure 1.1**). Keratin pearls and individual cell keratinisation are usually seen and are often accompanied by stromal reaction (Barnes, 2005). Furthermore, in advanced lesions, the malignant cells tend to invade deeply into adipose tissue, muscle, nerves, blood vessels and eventually bone.

Different histopathological classifications have been proposed over time in an attempt to increase the objectivity of histopathological parameters to predict the biological behaviour of cancer. A histopathologic grading system for OSCC was originally developed by Broders (Broders, 1941) in 1920.

Broder`s system was based on the resemblance between the neoplasm and normal epithelium cells. However, his system lacked correlation between histological grade and the prognosis of OSCC (Anneroth et al., 1987). In 1973, Jakobsson *et al.* (Jakobsson et al., 1973) introduced a multi-parameter classification system based on histopathologic characteristics of tumour cells, such as nuclear pleomorphism, mitoses, degree of keratinization and pattern and depth of invasion. The Bryne *et al.* (Bryne et al., 1992) grading system was proposed in 1992 to evaluate tumour cells at the deep invasive margins of OSCC. The World Health Organization (WHO) classification system, reviewed in 2005 (Barnes, 2005), is based on Broders' grading system, which takes into account the degree of cell differentiation. OSCC is graded into three categories: well, moderately and poorly differentiated (Barnes, 2005). Well differentiated squamous cell carcinoma resembles the normal epithelial tissues. In contrast, carcinoma cells that are difficult to recognise as epithelial (predominately immature cells) and show no keratin production are graded as poorly differentiated carcinomas. Between these two patterns tumours are graded as moderately differentiated (Barnes, 2005).

OSCC has a distinctive invasion pattern: a cohesive (expansive) pattern and non-cohesive (infiltrative) pattern, or combinations of both may be observed. The former pattern is characterised by large tumour islands and well defined margins. Non-cohesive growth is characterised by scattered individual cells or small irregular cords with ill-defined margins (Barnes, 2005).

Even though some OSCCs may arise from non-dysplastic epithelium (Warnakulasuriya et al., 2008), many are preceded by leukoplakia (white patch), erythroplakia (red patch) or speckled leukoplakia (mixed white/red). Erythroplakia, in particular, with epithelial alterations such as dysplasia, have an increased likelihood of progression to carcinoma (Harrison et al., 2009). The prevalence of oral premalignant lesions is estimated to be between 1 and 5% (Napier and Speight, 2008). The term epithelial dysplasia implies cytological changes (cellular atypia) and loss of the normal epithelial architecture.

Generally, dysplasia is graded into three categories: mild, moderate and severe. In mild dysplasia the architectural disturbances combined with cellular atypia are observed only in the lower third of the epithelium, moderate dysplasia is when such changes affect two thirds of the epithelial thickness. In severe epithelial dysplasia, loss of individual cell layer organisation and atypia involves more than two thirds of the epithelium. Carcinoma in-situ is a term used for the most severe dysplasia, in which changes of pronounced cytological atypia are seen throughout the full thickness of the epithelium, `top- to- bottom`, but invasion is not present (Warnakulasuriya et al., 2008).

Staging of oral cancer depends on the Tumour-Node-Metastasis (TNM) classification system, which was recently reviewed and updated in 2010 (Sobin et al., 2011). This system depends on the extent of the primary tumour (T1, T2, T3 and T4), lymph node involvement (N0, N1, N2 and N3)

and occurrence of distance metastasis (M0 and M1). The TNM staging system has been globally accepted and routinely used in clinical practice to facilitate treatment planning and is known as the clinical TNM classification (cTNM). Additionally, when this classification is carried out after histopathological assessment of tissue samples, it is known as the pathologic TNM classification (pTNM) (Takes et al., 2010). The possible TNM combinations are grouped into four main categories as follows: stages I, II, III and IV. In meta-analysis studies, the TNM system (T value) has shown a correlation between tumour thickness and nodal metastasis in OSCC (Huang et al., 2009), but it is not a very reliable factor (Akhter et al., 2011).

Lymph node metastatic tumours occure in 40% of OSCC patients (Noguti et al., 2012). Moreover, up to 25% of OSCC cases have evidence of distant metastasis (Woolgar, 2006). Patients with upper aerodigestive tract malignancy are more likely to develop second tumours in approximately 20% of cases (Hunter et al., 2005). This may be related to genetic alterations or epigenetic changes in the epithelium around the tumour, the so called “field of cancerization”, causing the subsequent recurrences (Leemans et al., 2010). Studies also suggest that the primary tumour of squamous cell carcinoma and the second tumour develop from separate genetic mutations in the same “field of cancerization” (Hunter et al., 2005). Unfortunately, tumours that develop in previously irradiated soft tissue tend to be more aggressive and have unpredictable survival (Woolgar, 2006).

Treatment of OSCC is based on the location and clinical stage of disease and consists of radical surgery, radiotherapy or a combination of both. Radiation therapy may be accompanied by chemotherapeutic agents. In small tumours, surgery alone is preferred, whilst for patients with larger lesions and lymph node metastasis, a combined therapy is required. In addition, new promising treatments, including gene therapy and photodynamic therapy, have been introduced (Agostinis et al., 2011, Shah and Gil, 2009).

Early diagnosis of OSCC is essential in terms of treatment and survival. In reality, clinical detection of early invasive carcinoma may be difficult. Nevertheless, traditional histological examination combined with biological and molecular markers can be useful and special attention should be paid to the deepest layers of the epithelium in suspicious lesions (Barnes, 2005). Early diagnosis can offer a significant change in clinical outcome and quality of life for patients with OSCC.

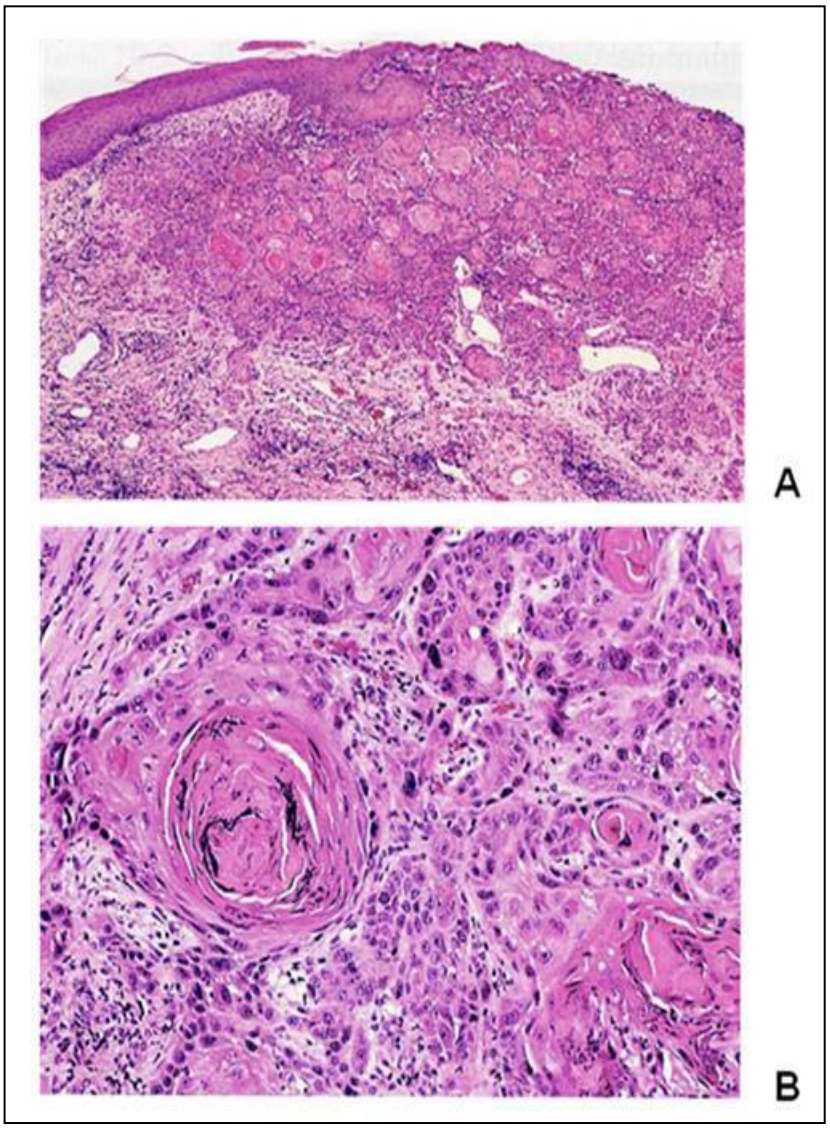

Figure 1.1: H&E section of a well-differentiated squamous cell carcinoma (SCC). A: Malignant squamous cells infiltrate through the basement membrane deep into connective tissue. B: High power view showing neoplastic epithelial cells and keratin pearl formation.

1.2.1 Pathogenesis and aetiology of OSCC

Several variants of OSCC are classified on the basis of the clinical presentation and the histological appearance. These variants are important in terms of difficulty in diagnosis. Several studies have suggested that oral squamous cell carcinomas are associated with, or are preceded by precancerous lesions (Pitiyage et al., 2009, Neville et al., 2009). Others found that OSCC may arise from clinically normal oral mucosa (Scully and Bagan, 2009a).

The causative agents of oral squamous cell carcinoma have not been fully defined and multiple factors may be involved. Nevertheless, the most prominent risk factors are tobacco use and alcohol abuse (La Vecchia et al., 2009). These associated risk factors were reported in approximately 70% to 90% of head and neck cancers (Brandizzi et al., 2008). Patients who combine both tobacco and alcohol consumption have a much higher risk of cancer development (Ansary-Moghaddam et al., 2009).

Human papilloma virus (HPV) has been shown to be another factor associated with carcinogenesis (Marur et al., 2010, Brandizzi et al., 2008, Ang and Sturgis, 2012). The viral genome consists of portions that are expressed ‘early’ in its lifespan (coding as E1-E7), a further two in the ‘late’ stage of its lifspan (coding as L1 and L2), and a non-coding long control region (LCR) gene (Ganguly and Parihar, 2009). The HPV genome may integrate into the host DNA and its amplification causes high expression of the oncoproteins E6 and E7 due to disruption of its E2 sequence; this leads

to inactivation of p53 (degraded by E6) and retinoblastoma protein (pRb) (degraded by E7) (Chaturvedi and Gillison, 2010). By this mechanism HPV can prevent apoptosis and increase DNA replication and cell proliferation (Pietsch and Murphy, 2008, Marur et al., 2010). Inactivation of pRb is associated with an increase of $p16^{INK4A}$ protein (Westra, 2014). Subsequently this will increase the chance of tumour formation in infected cells (**Figure 1.2**).

Oral cancer is not associated with HPV infection, however, the detection rate of HPV DNA in head and neck cancers varies widely between studies. It has been identified in 35 to 55% of OSCCs (Jalouli et al., 2012, Castillo et al., 2011). This variability in incidence of HPV can be attributed to the different detection methods (such as in-situ hybridisation or polymerase chain reaction) and the accuracy of the classification of anatomic sites. It may also be due to sample preparation, for example, whether formalin-fixed or frozen specimens were used (Syrjänen, 2010, Westra, 2015).

Betel nut chewing, ultraviolet radiation (sunlight), vitamin deficiency and candida infection have also been shown to be associated with an increased risk of OSCC (Scully and Bagan, 2009a, Muttagi et al., 2012).

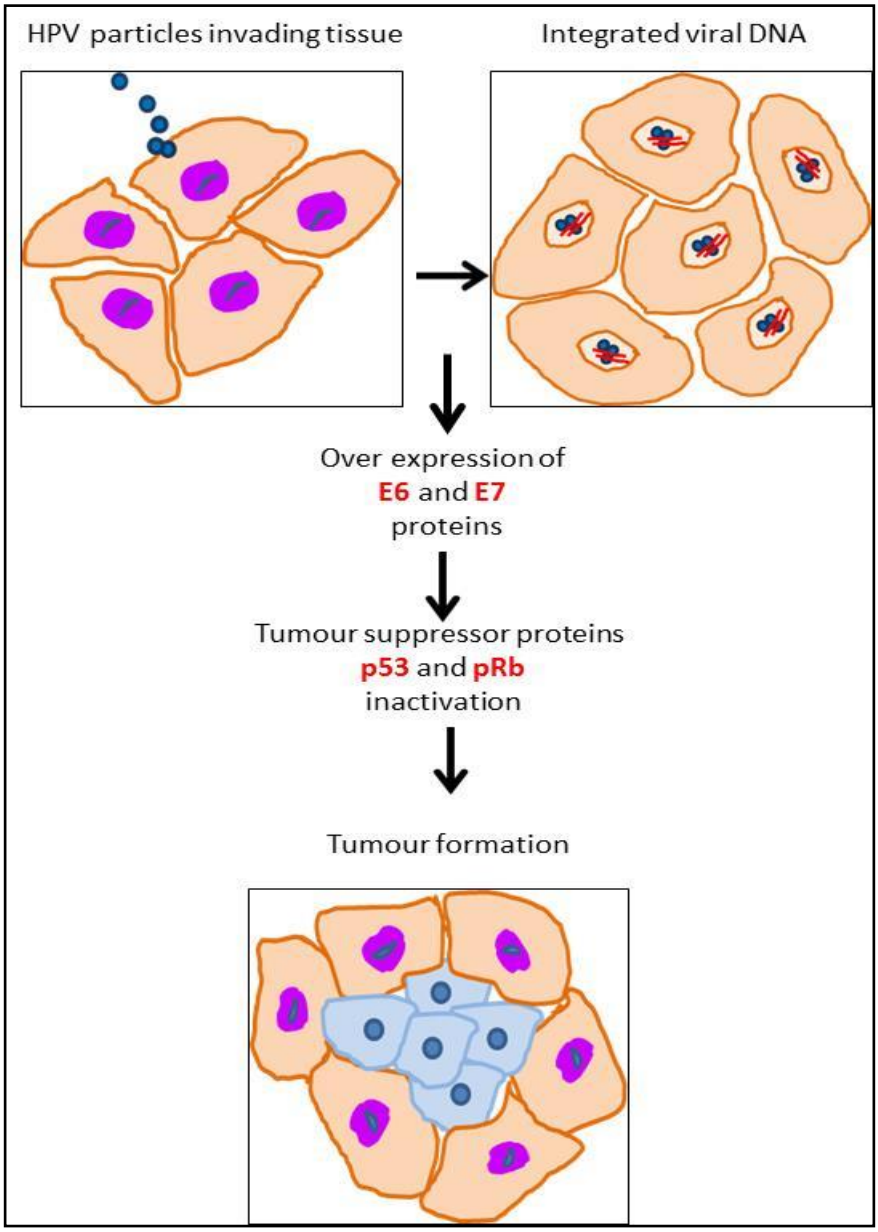

Figure 1.2: Diagram showing the mechanism by which the HPV genome invades and multiplies in the host cell. After E2 disruption, HPV circular episome allows HPV DNA integration, leading to higher expression of E6 and E7 proteins. This over-expression is able to inactivate tumour suppressor genes p53 and pRb, which promotes cell growth and possibly malignant change.

1.2.2 Genetic and molecular changes in oral cancer

Squamous cell carcinoma lesions arise as a consequence of multiple genetic alterations in tumour suppressor genes, which inhibit uncontrolled cell division, and oncogenes, which drive the malignancy, involving many chromosomes (Scully and Bagan, 2009a). Such genetic alterations are considered to be the hallmarks of human cancer. These include cell dysregulation, disruption to cell signalling, uncontrolled proliferation, cell immortality, apoptosis inhibition, metastasis and induction of angiogenesis (Hanahan and Weinberg, 2011). In 1996, Califano and colleagues (Califano et al., 1996) proposed a model for pathological genetic progression of head and neck carcinoma. They suggested that identification of loss of heterozygosity of chromosomes, such as 9q21, can be useful in carcinoma diagnosis.

Much research has been carried out in order to define these genetic alterations associated with carcinogenic events, but these events are still not clearly understood. Genetic changes have been suggested to be a key element in the variable susceptibility for oral carcinogenesis (Leemans et al., 2010). Molecular studies have shown that genetic alterations such as chromosomal loss at 1p, 3p, 4p, 5q, 8p, 10p ,11q, 13q and 18q with gains at 1q, 3q, 5p ,7q ,8q ,9q ,11q ,12q ,14q and 15q are frequently detected in OSCC, causing cell disturbance (Bauer et al., 2008, Leemans et al., 2010, Salahshourifar et al., 2014). In general, the accumulation of genetic imbalances such as up-regulation, down-regulation, deletion or mutation is

thought to drive the progression of cancer lesions by different mechanisms (Stadler et al., 2008). These genetic imbalances can stimulate different effects on cells under different conditions and modify interactions with neighbouring cells, as well as with stromal cells (Hunter et al., 2011).

Other molecular studies have indicated that tumour necrosis factor- alpha (TNF-α) and increased expression of interleukins (IL-4,-6,-8,-10) are strongly associated with an increased risk of OSCC (Brailo et al., 2012, Lippitz, 2013). These cytokines help tumour cells to inactivate tumour suppressor genes (p53), activate oncogenes (Ras), and escape apoptosis, leading to uncontrolled cell growth (Brailo et al., 2012).

Epidermal growth factor receptor (EGFR) is a receptor tyrosine kinase that regulates cell proliferation, differentiation and migration (Schneider and Wolf, 2009). It was claimed that over-expression of EGFR is associated with tumour growth, metastasis and poor prognosis (Mitsudomi and Yatabe, 2010, Normanno et al., 2006). Gene amplification of EGFR is seen most frequently in OSCC patients at 7p11.2 region (Sheu et al., 2009). Over-expression of EFGR was detected in 33% of areca-associated oral squamous cell carcinoma cases (Chiang et al., 2008). Combinations of anti-EGFR antibodies and radiotherapy have been introduced in clinical trials to increase the efficacy of cancer treatment (Leemans et al., 2010).

Activation of oncogenes (proto-oncogenes) is a fundamental step in carcinogenesis, and over-expression or dysregulation of these genes can lead to malignant transformation. For example, myc (c-myc, N-myc, L-myc)

and Ras (H-ras, K-ras, N-ras) genes are frequently associated with solid tumours (Garzon et al., 2009). Matrix metalloproteinases (MMPs), especially MMP-9, are also thought to play a role in cell proliferation, migration and invasion (Vilen et al., 2013). MMPs can induce the release of cytokines and cause degradation of the extracellular matrix, leading to changes in cell behaviour and promotion of tumour progression (Kessenbrock et al., 2010). Briefly, MMPs, integrins and the urokinase Plasminogen Activator (uPA) system are proposed to be involved in tumour progression and lymph node metastasis (Zhou et al., 2009).

Tumour suppressor genes (TSG) play a crucial role in normal growth control, including cell cycle, proliferation, differentiation and apoptosis; their dysfunction can result in uncontrolled growth and cancer development. One of the most extensively studied TSG, is the p53 gene, a gene involved in cell cycle regulation and programmed cell death (apoptosis). The p53 gene is mutated in about 50% of head and neck cancers. However, there is debate in the literature about the timing of its role in oral tumourigenesis. Some studies have stated that mutations of p53 appear to be late events in carcinogenesis and are therefore rare in precancerous lesions (Shahnavaz et al., 2000). In contrast, over-expression of p53 has also been reported in dysplasia (Campo-Trapero et al., 2008, De Oliveira et al., 2012).

Additionally, cell cycle checkpoint proteins such as ATM and ATR, play a vital role in DNA replication in the cell cycle by promoting senescence and apoptosis in cells with damaged DNA (Jackson and Bartek, 2009, Smith et

al., 2010). Impairments of DNA repair mechanisms have been observed in tumour cells and are associated with carcinogenesis (Cook, 2009, Truong and Wu, 2011). Other molecular events associated with oral cancer, for example, inactivation of $p16^{INK4A}$, pRb pathways and aberrations in the fragile histidine triad gene (FHIT), have also been identified (Hunter et al., 2005, Leemans et al., 2010).

In summary, it has been shown that the carcinogenesis process develops as multiple complicated genetic events. Therefore, a full understanding of the biologic basis of genetic alterations is essential for proper diagnostic and prognostic evaluation.

1.3 The cell cycle

The cell cycle is defined as a series of events that include nuclear DNA duplication and mitosis, which lead to cell division. The eukaryotic cell cycle traditionally consists of four phases (**Figure 1.3**): G1 (first gap phase) is the growth or synthesis phase and is the interval between the last mitosis and the initiation of DNA duplication. During this time the cell decides whether to start to proliferate or leave the cycle and enter into G0 (gap 0) or quiescent phase to allow more time for growth. The duration of the G1 phase is widely variable among different cells and is the longest phase in a typical mammalian cell. It has been estimated that in epithelial cells it may last from 4 to140 hours (Squier et al., 1976). Growth occurs in this phase.

According to some researchers, the G1 phase in a rapidly proliferating tissue such as gut lasts approximately 11 hours out of a total cell cycle time of approximately 24 hours (Bury and Cross, 2003). Most cells in this phase (G1) are regulated by two cell cycle checkpoints: a restriction checkpoint and a DNA damage checkpoint. If a cell escapes from these checkpoints with the presence of damaged DNA, cancer may develop.

S phase (DNA synthesis phase) is when DNA replication takes place and typically lasts for 8 hours (Bury and Cross, 2003). The G2 (second gap phase) is a period between the completion of DNA replication and mitosis (M phase which requires less than an hour). In this stage the cell provides the appropriate nutrients and proteins needed for mitosis. Cells in the G1 phase have a diploid DNA content and cells in the G2 phase are tetraploid

with double the normal DNA content. As previously stated, the cell cycle is regulated by many molecular events (checkpoints), which may detect and repair any damaged DNA and prevent cell division at any specific point (van den Heuvel, 2005). However, if repair is unsuccessful, cells may undergo apoptosis (Malumbres and Barbacid, 2009).

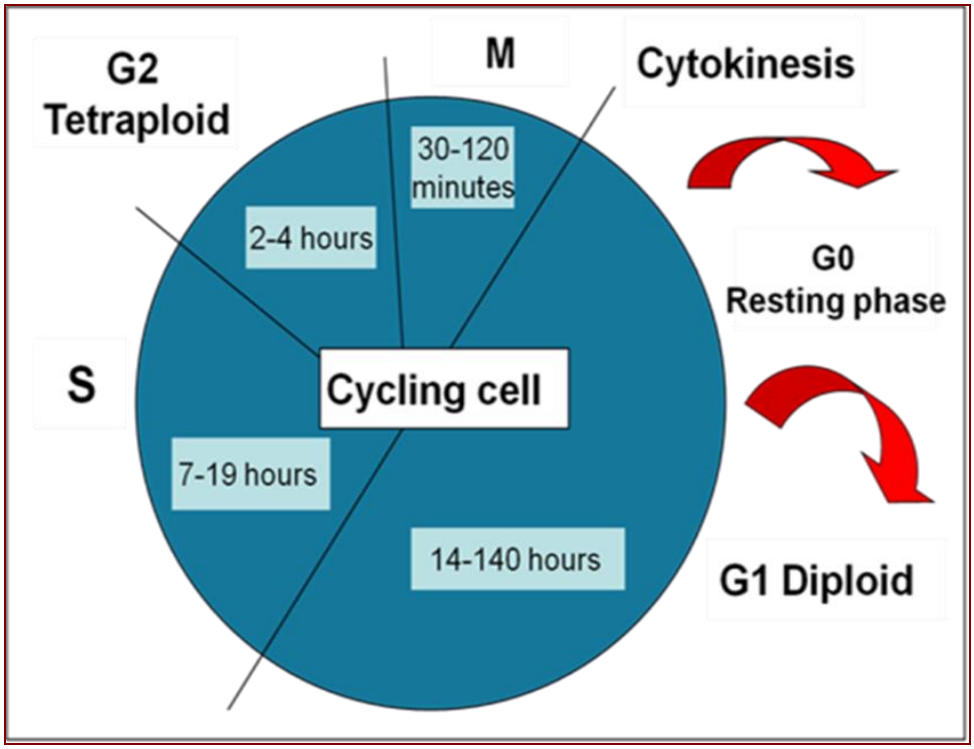

Figure 1.3: The four cell cycle phases: G1, S, G2, M. Cells in G0 are in a resting stage. G1 is the gap between mitosis and synthesis, while G2 is the gap between S phase and the cell division phase (M phase).

1.3.1 Initiation of DNA replication

Precise regulation of DNA replication in eukaryotic cells is crucial for the maintenance of genomic integrity. The genome is organised into pairs of chromosomes, which are precisely coordinated during S phase. The exact duplication of chromosomes is required in each proliferating cell. The complex series of events involved in the replication process initiates from hundreds of sites along the genome, called origins of replication (OR). In mammals, 30,000 to 50,000 origins are activated in each cell cycle (Symeonidou et al., 2012). This is tightly controlled by different proteins, such as cyclin, cyclin-dependent kinase (CDK) and the pRb pathway (Malumbres and Barbacid, 2009). Early studies provided significant advances in our understanding of the structure and function of budding yeast (*Saccharomyces cerevisa*). These advances led to the first comprehensive studies of the assets of chromosomal DNA replication in eukaryotic cells and helped identify molecular regulators of the cell cycle (Nieduszynski et al., 2006, Donaldson and Blow, 1999).

The initiation of DNA replication is an essential step during cell proliferation which is regulated by a series of multiprotein recruitments. It begins with the construction of a pre-replication complex (pre-RC) known as ‘‘licensing’’, which loads chromatin that is required for DNA synthesis (Karnani et al., 2010). This regulatory mechanism ensures that DNA replication is fired once per cell cycle by temporally separating the assembly of the pre-RC

(which occurs during G1 phase and late M phase in the cell cycle) from the initiation of DNA synthesis (**Figure 1.4**).

In eukaryotes, DNA replication is established by a sequence of events in a stepwise manner that begins with the origin recognition complex (ORC) binding to protein Cdc6 on chromatin which is required for the association of minichromosome maintenance (MCM) proteins (Aparicio, 2013). The ORC is constantly present throughout the cell cycle, providing an ideal location for the serial conjunction of the pre-replicative proteins (Newlon, 1997, Aparicio, 2013). In early G1 phase, ORC, MCM10, Cdc6, along with Cdt1, together load inactive MCM2-7 helicase complexes at origins. At this stage the MCM complex interacts with MCM10 to form the pre-replicative complex, resulting in the origin being licensed for replication in the subsequent S phase (Symeonidou et al., 2012). Nevertheless, to proceed to DNA replication cell cycle kinases need to be activated, including Dbf4-Dependent kinase (DDK) and cyclin-dependent kinase (CDK), which are sequentially activated during the G1/S phase transition (Aparicio, 2013). Cdc7 (cell division cycle 7) kinase is another key kinase that acts in association with Dbf4-Dependent kinase for full activity. Cdc7/Dbf4 complex kinase, Cdc7 and Cdk2 are required for MCM complex phosphorylation and recruit throughout the S phase to activate origins of replication (Labib, 2010). Next, the MCM complex is converted to an active stable helicase (**Figure 1.4**) and DNA replication starts with recruiting additional factors, including DNA polymerase α-primase and the replication protein A (single-strand specific DNA-binding protein), into the unwound ORC as a first step in forming

replication forks and initiation of DNA synthesis (Blow and Gillespie, 2008, Aparicio, 2013).

Once DNA replication is initiated, the pre-RCs are disassembled, Cdc6 is degraded, while Cdt1 is targeted for proteolytic degradation by geminin at the G1/S border. Cdt1 proteolysis suggests the importance for inactivating Cdt1 function during S phase and G2 to prevent re-replication (reviewed by (Aparicio, 2013, Truong and Wu, 2011). Inhibition of Cdt1, prevents pre-RC reassembly and loading of MCM2–7 complex in the same cell cycle. This is a very conserved mechanism among eukaryotic cells to prevent DNA re-replication in order to maintain genome stability (Labib, 2010).

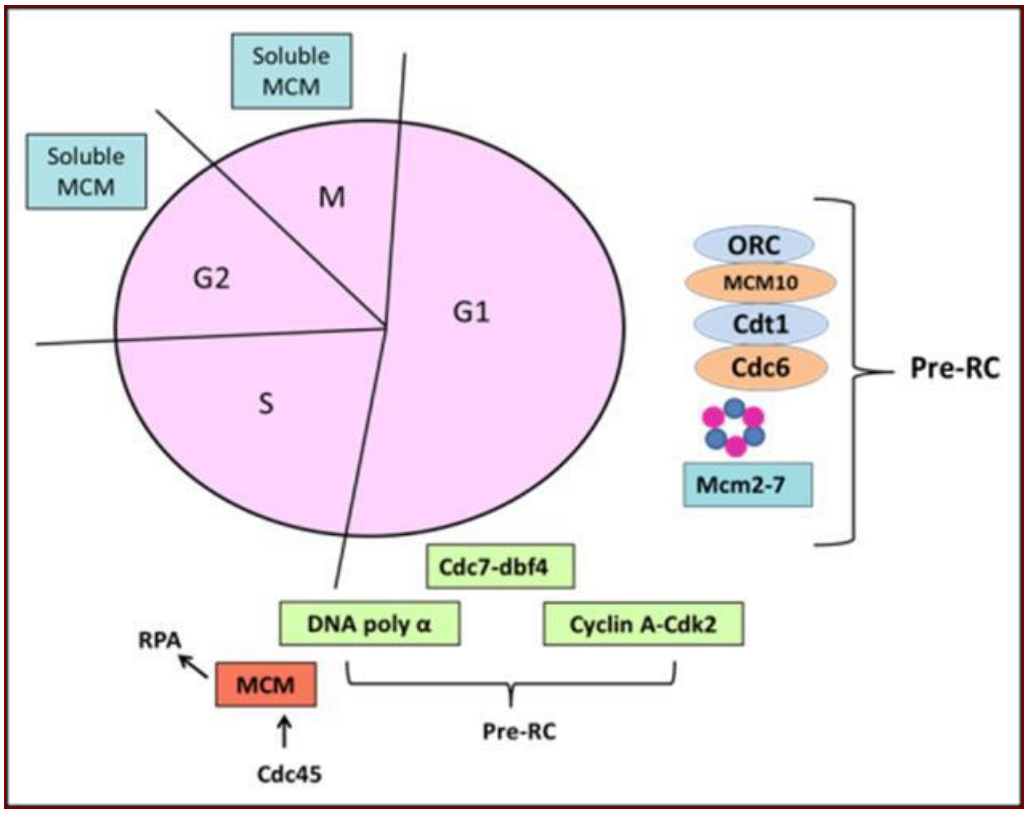

Figure 1.4: Sequence of events of DNA replication in the cell cycle. In G1 phase: ORC, cdt1 and cdt6 recruit MCM2-7 to replication origins to establish the pre-replication complex (pre-RC). In late G1, Cdc7-Dbf4 and other CDKS mediate MCM phosphorylation events (soluble state), followed by interactions of Cdc45 with the MCM2-7 complex to activate the DNA helicase. RPA and DNA polymerase α are then recruited to the replication origin and this initiates DNA synthesis.

1.3.2 Disorders of the cell cycle and oral cancer

OSCC pathogenesis is believed to be a multistep process in which several genetic alterations take place in the early stages of disease and disrupt cell growth control. Defects in the cell regulation system may lead to chromosomal missegregation, with loss and gain of chromosomes leading to aneuploidy. The literature suggests that continuous chromosomal missegregation could accelerate or induce tumourigenesis (Ricke et al., 2008, Baker et al., 2009). Some researchers believe that genetic instability, as a result of loss of heterozygosity (LOH) and expression of damaged or mutated suppressor genes, may be the first step in cancer development (Bester et al., 2011, Scully and Bagan, 2009b). Although, the exact mechanisms that mediate such structural chromosomal changes in different tumours are not fully described.

Alterations in cell cycle regulators such as CDKs, licensing proteins and their regulators (e.g. geminin) may contribute to the carcinogenesis process (Blow and Gillespie, 2008). Several investigators believe that mutations in proto-oncogenes and inactivation of tumour suppressor genes (eg. p53 and pRb) can trigger tumour growth (Baker et al., 2009, Scully and Bagan, 2009b, Oliveira and Ribeiro-Silva, 2011). Mutation of genes encoding CDK, cyclins, CDK-activating enzymes, CDK substrates, CKI and check point proteins, and over-expression of cell cycle regulating proteins are recognised in dysplastic and malignant lesions (Tsantoulis et al., 2007, Weaver et al., 2007, Pitiyage et al., 2009). Cell cycle aberration and

dysregulation of apoptosis as a result of oncogenic transformation have been suggested as useful therapeutic targets for oral cancer (Senderowicz, 2004, Mishra, 2010).

Furthermore, the minichromosome maintenance proteins (MCM) may be a valuable proliferation marker in tumour screening (Scott et al., 2006). Ki-67 and proliferating cell nuclear antigen (PCNA) are also useful markers of tumour proliferation, but no single cell cycle regulatory protein has yet emerged as an ideal biomarker.

1.4 Mini-Chromosome Maintenance proteins (MCM)

Minichromosome maintenance proteins (MCM) are considered to be essential for the initiation of DNA replication (*serving as DNA helicases)* in eukaryotic cells (described in Section 1.3.1) (Bochman and Schwacha, 2009). The MCM complex is a conserved protein family comprising six unique subunits, initially discovered via mutations in the yeast *Saccharomyces cerevisiae*. These proteins are composed of polypeptides, ranging from 600 to 1200 amino-acid residues (Costa and Onesti, 2009). They are considered to be ubiquitous regulators of chromosomal replication as MCM proteins exist in all eukaryotes (Lei, 2005). Analogous classes have been found in *Xenopus* and in human cells with significant conserved gene sequences (Remus and Diffley, 2009).

In all eukaryotic cells the replicative DNA helicases possess at least six related proteins (MCM2-7) that work together as a hexameric ring (Labib, 2010), which interact in dimeric complexes MCM5/3, MCM3/7, MCM7/4, MCM4/6, MCM6/2 and MCM2/5 (Vijayraghavan and Schwacha, 2012). The architecture model of MCM in *Saccharomyces cerevisiae* showed that the two subunits comprising dimeric complexes are neighbouring in the hexamer. The predicted structure also highlights a gap between MCM2 and MCM5. The MCMs are enzymes with a characteristic form of the AAA+ domain superfamily which is responsible for the catalytic activity. MCM proteins comprise two consensus ATP-binding motifs: ‘Walker A and B’. The Walker A motif, including the P-loop (phosphate-binding loop) of the active

site, contains the invariant lysine. Walker B motif is a hydrophobic amino acid, and best known to contribute to ATP hydrolysis rather than binding (Davey et al., 2003, Costa and Onesti, 2009, Bochman and Schwacha, 2009).

MCM proteins are present throughout the proliferative cell cycle and play a vital role in DNA licensing as part of the pre-RC (described in Section 1.3.1). They have been shown to be chromatin-bound around origins of replication during the G1 phase, displaced during replication in S phase, but remain in soluble form until the end of mitosis (**Figure 1.4**) (Kuipers et al., 2011). However, they are not expressed in differentiated somatic cells that have entered into the quiescent phase (G0) and senescent status (Tachibana et al., 2005).

The MCM complex is required to ensure that each initiation during DNA synthesis occurs only once during cell division within each cell cycle (Diffley, 2011). Among the MCM family, MCM2 has been widely studied in a range of malignancies. However, very few studies have evaluated the expression of other MCM members. Unlike other MCM subunits, MCM2 binds specifically to chromatin (Bochman and Schwacha, 2007). This protein may be involved in replication forks formation, and has been shown to regulate the replicative helicase activity of the complex (Samel et al., 2014).

Several lines of evidence suggest that MCM2 function may be regulated by S phase regulating kinases (Cdc7/Dbf4) during DNA replication events (Chuang et al., 2009). The Cdc7/Dbf4 complex phosphorylates MCM2 at the

G1/S transition phase. Thus, phosphorylation of MCM2 is a critical step in initiation of DNA replication (Tsuji et al., 2006, Charych et al., 2008). Recent genetic evidence suggests that down-regulation of MCM2 subunits could lead to growth inhibition and cell cycle arrest in cancer cell lines (Magatti et al., 2012, Jin et al., 2014). MCM2 has been investigated in oral lesions and the results suggest that MCM2 may be used as a prognostic marker in OSCC (Torres-Rendon et al., 2009a, Kodani et al., 2003, Szelachowska et al., 2006, Gueiros et al., 2011). Therefore, it has also been proposed as a therapeutic target in human cancer (Liu et al., 2013).

1.4.1 Licensing factor and "Out of cell cycle" phases

Cell proliferation is controlled by initiation of DNA replication where chromatin is 'licensed' in the synthesis phase to regulate the growth process (Sun and Kong, 2010). Both *in vivo* and *in vitro* studies (Bochman and Schwacha, 2008, Moyer et al., 2006) have revealed that down-regulation of pre-replicative complex proteins such as Cdc6 and MCM is a common downstream mechanism for loss of proliferative ability in human cells. Down-regulation also appears to be a powerful growth regulatory mechanism that prevents cell proliferation. However, components of DNA replication licensing machinery are negatively regulated in senescent, non-proliferating quiescent, and differentiated cells (Blow and Gillespie, 2008). It has previously been mentioned that there is likely antagonism between proliferation and differentiation, hence raising the possibility that DNA

replication and differentiation cannot occur at the same time (Stoeber et al., 2001, Olson, 1992).

This orchestrated down-regulation mechanism is considered as an absolutely vital step in self-renewing and permanent human tissues, coinciding with the concept of antagonism between the cellular paths that regulate proliferation and differentiation (Myster and Duronio, 2000).

1.4.2 MCM as novel biomarkers of growth

Human cell proliferation proteins have been identified as essential to determine the growth fraction and proliferation in any given cell population. These proteins are indispensable through the active phase in the cell cycle, are present in all cell types, and are absent from resting or quiescent cells (Williams and Stoeber, 2007).

Stem cells of the proliferative compartment in human tissue have the capacity for unrestricted self-regeneration. These stem cells can produce highly differentiated descendants. In fact, there is an intermediate population of committed progenitors between the stem cell and its terminally differentiated progeny. This intermediate population can be characterised by limited proliferative ability and restricted differentiation capacity; these cells are identified as transit amplifying cells (Watt and Hogan, 2000). In addition, some human cell types (e.g. hepatocytes) have reversible withdrawal from the G0 phase. Other cells have irreversible (e.g. epithelial cells in surface

mucosa) withdrawal from cell division and proceed into differentiation (Williams and Stoeber, 2012a).

MCM origin licensing factors have recently been recognised as critical regulators of growth and differentiation. In addition, silencing of MCM2 may lead to cell growth inhibition and apoptosis in cancer cell lines (Liu et al., 2013). In normal human tissues, expression of MCM proteins is generally restricted to the basal proliferative compartment as epithelial cells undergo differentiation. Down-regulation of the MCM proteins is associated with transition from progenitor to terminally differentiated cells. The progenitor cells, which are located above the stem cell area, are known to express MCM licensing factors where persistent uncontrolled differentiation of cells occurs (Stoeber et al., 2001).

MCM proteins are essential for the initiation of DNA replication, serving as licensing components for the S phase of the cell cycle. The importance of MCM protein expression in proliferating cells, in all cell cycle phases, and their absence in the non-cycling phase (G0) reinforces their potential as cell proliferation markers (Williams and Stoeber, 2012a, Tamura et al., 2010). MCM2 expression levels have been recognised as powerful diagnostic and prognostic markers in a variety of human neoplasms, including bladder cancer (Saeb-Parsy et al., 2012), colorectal cancer (Wang et al., 2009) and laryngeal carcinoma (Lian et al., 2013). In this respect, recent studies demonstrate that MCMs have higher specificity and sensitivity to detect cells in the cell cycle than the conventional proliferation markers in a variety of

tissues (Giaginis et al., 2010). Thus, MCM proteins have an ability to ascertain diagnosis and/or prognosis of clinical significance in several types of human malignancy (Giaginis et al., 2009), especially in rapidly dividing cells (Wojnar et al., 2010).

1.4.3 Prognostic potential of MCM in cancer

Since MCM proteins have an essential role in the initiation of DNA replication, their expression may be associated with carcinogenesis (Das et al., 2013). The MCM proteins are highly expressed in malignant cells and possibly in pre-cancerous cells but not in differentiated somatic cells (Costa and Onesti, 2009, Karimi and Sadr, 2011). Numerous studies have reported that MCM2 nuclear expression is dysregulated in dysplastic epithelial cells (Torres-Rendon et al., 2009a) and in a large number of malignancies, including oesophageal (Tao et al., 2011), colorectal (Guzińska-Ustymowicz et al., 2009), laryngeal (Cai et al., 2012), ovarian (Levidou et al., 2012) and Hodgkin's lymphoma (Karimi et al., 2008). These alterations of expression were also more frequently seen in cells from malignant samples than those from normal human tissues (Toyokawa et al., 2011). For instance, expression of MCM2-7 proteins was much higher in HeLa cells and SV40-transformed human fibroblasts than in normal human fibroblast cells (Ishimi et al., 2003).

The first possibility why premalignant and malignant cells express licensing proteins is that it may reflect cell failure to exit the cell cycle. This could be the consequence of oncogene induced cell division that decreases the

number of cells in a quiescent state. In fact, oncogenes can either stimulate the re-licensing of replicated DNA, or can permit cells to enter S phase. The second possibility is that dysregulation of the licensing program may have a fundamental role in cancer growth (Blow and Gillespie, 2008). High expression of MCM proteins was found to be associated with more invasive tumours in oesophageal squamous cell carcinoma (Ahn and Chang, 2010). Interestingly, only MCM2 and MCM3 have recognisable nuclear localization sequences, providing nuclear targeting to the other MCM members (Karimi and Sadr, 2011). Likewise, antibodies against MCM, especially MCM2, demonstrated an association between over-expression and lymph node metastasis in tongue squamous cell carcinoma (Gueiros et al., 2011) and in colorectal carcinoma (Guzińska-Ustymowicz et al., 2009). Similarly, high MCM2 expression has been seen in areas of malignant transformation in recurrent pleomorphic adenoma (Soares et al., 2011). Nuclear MCM2 expression, recognised by immunohistochemistry, showed increased expression in dysplastic and malignant lesions of OSCC (Scott et al., 2006, Torres-Rendon et al., 2009a). Kodani *et al*. (Kodani et al., 2003) evaluated the immunoexpression of cell cycle proteins in 46 OSCC samples. Their results suggested there was no significant difference in MCM2 and Ki-67 labelling index (LI) in tumours with or without metastasis. Elevated LI of MCM2 was observed in moderately differentiated OSCCs compared to well differentiated OSCCs (Kodani et al., 2003). In a recent similar study, MCM2 immunoreactivity was shown to increase with histological grade of OSCC ($p=0.000$), with higher expression in poorly differentiated tumours (Shalash

et al., 2012). The authors also claimed that positive correlation between MCM2 LI and lymph node metastasis was significant (Shalash et al., 2012). A high LI of MCM2 identified in the lymph node metastasis lesions suggests that MCM2 may predict the proliferation rate of malignant tumours (Shalash et al., 2012, Guzińska-Ustymowicz et al., 2009). In 63 tongue squamous cell carcinomas, immunoexpression of proliferation markers (MCM2, Ki-67 and geminin) and their clinicopathological features were evaluated (Gueiros et al., 2011). Over-expression of MCM2 and Ki-67 was related to nodal metastasis and disease stage. A high immunoreactivity of geminin was associated with neural invasion (Gueiros et al., 2011). Moreover, association between MCM2 expression and histological grade of tumours was reported, indicating a wide spectrum of growth potential within each tumour grade (Dudderidge et al., 2005, Shalash et al., 2012). In OSCC, high levels of MCM proteins may indicate poor clinical outcome (Karimi and Sadr, 2011, Gonzalez et al., 2005). However, patients with low MCM2 LI have better survival rates than those with high MCM2 LI (Giaginis et al., 2010). Thus, inhibition of these proteins has been suggested as an anti-cancer drug target in various types of tumours (Costa and Onesti, 2009, Toyokawa et al., 2011, Zhang et al., 2015). Transient inhibition of the replication licensing system causes a transit cell cycle in normal cells. Likewise, inhibition can cause apoptosis in tumour cells (Shreeram and Blow, 2003, Liu et al., 2013). Positive correlation between the proliferation marker, MCM2, and tumour stage, has been demonstrated. Inhibition of the replication licensing biomarkers may provide a potential therapeutic target

against regulators of DNA replication (Gonzalez et al., 2005, Blow and Gillespie, 2008, Karimi and Sadr, 2011). Using existing proliferation markers along with licensing replication biomarkers may allow more accurate diagnosis and treatments of tumours. These observations suggest that MCM2 protein is likely to be a promising and reliable marker for cancer diagnosis and suggest further investigation of its prognostic value. Therefore, MCM2 will be the focus of this project rather than other MCM members.

In summary, the MCM proteins have been suggested to be more sensitive in detecting cells that are going through the cell cycle than existing proliferation markers such as Ki-67 and PCNA (Giaginis et al., 2010). This is discussed further in the next section.

1.4.4 Advantage of MCM proteins over conventional proliferation markers

Ki-67 protein is expressed in all phases of the cell cycle (G1, S, G2, and mitosis) and has been used to estimate the fraction of the tumour growth as a proliferation marker. The prognostic value of Ki-67 has been controversial with regard to its ability to differentiate tumours with a good or poor prognosis. The first reason for this controversy could be that Ki-67 shows some variation in its expression in G1 phase and may be absent in cells entering G1 from G0 (Bubán et al., 2009). Secondly, it has been shown that Ki-67 expression levels can be altered in nutritionally deprived cells (Verheijen et al., 1989). Claims have been made in the literature regarding

whether it is essential for cell proliferation or not (Brown and Gatter, 1990, Wojnar et al., 2010). Detection of Ki-67 by immunohistochemistry may therefore vary and show inconsistencies.

Proliferating cell nuclear antigen (PCNA) is a nuclear protein essential for cell division and is associated with DNA repair mechanisms. Its expression starts at the S phase of the cell cycle, but it has limited use as a proliferation marker (Myoung et al., 2006, Mahler et al., 2012). Moreover, immune staining of PCNA can be affected by a variety of factors, such as fixation time.

Notably, in normal tissue MCM2 is higher than Ki-67, and many studies have confirmed the superiority of MCM2 as a proliferation marker when compared to Ki-67 and PCNA, allowing better distinction between normal and neoplastic cells (Lei, 2005, Maiorano et al., 2006, Szelachowska et al., 2006). The most important advantage or characteristic of MCM2 is its ability to identify those cells that are licensed to enter the cell cycle, including licensed cells in G0 (Stoeber et al., 2001, Costa and Onesti, 2009, Gouvêa et al., 2013) (**Figure 1.5**). This property makes MCM2 an exceptional marker to assess the growth fraction in malignant tumours.

1.4.5 Ki-67 protein as a prognostic marker

Ki-67 is a non-histone protein that is expressed in proliferating cells. It was described in 1983, after mice were immunised with nuclei from cell line L 428 (Gerdes et al., 1983). Even though its exact biological role remains unclear, the expression of Ki-67 protein has been linked to cell proliferation and its nuclear antigen was detected in all active phases (**Figure 1.5**) of the cell cycle (G1, S, G2 and mitosis), but not in the G0 resting phase (Scholzen and Gerdes, 2000, Gerdes et al., 1984). This protein can be assessed by immunohistochemistry using monoclonal antibody (clone MIB-1) that reacts with different epitopes (Del Sordo and Sidoni, 2008). This simple technique has been used to estimate cell proliferation in fixed tissues (Cuevas et al., 1993).

Generally, the cellular expression of Ki-67 protein is not consistent, and its presence in early cell cycle phases has not been confirmed. It is widely accepted that its expression increases during S phase and the highest staining intensity is seen in metaphase (Scholzen and Gerdes, 2000). MIB-1 monoclonal antibody is the most frequently used proliferation marker in prognostic and diagnostic studies. These studies have shown that the Ki-67 labelling index can serve as a prognostic evaluation biomarker in some neoplasms (Tojo et al., 2005, Park et al., 2007, Tawfik et al., 2013). On the other hand, MIB-1 antibody alone did not correlate with disease outcome and therefore may not be beneficial as a prognostic indicator (Okamoto et al., 2002, Gonzalez-Moles et al., 2010, Massano et al., 2006, Shah et al.,

2009). Generally, the majority opinion is that Ki-67 antibody can differentiate cells in late G1, S, G2 and M phases (**Figure 1.5**) and be considered as a marker of the total growth fraction of proliferating cells.

1.4.6 MCM2/Ki-67 ratio

The MCM2/Ki-67 ratio represents the proportion of cells that are licensed to replicate (Shetty et al., 2005). The value of this ratio may go from 1 to infinity since MCM2 expression is always higher than Ki-67 (**Figure 1.5**). MCM protein expression also recognises non-cycling cells with proliferative potential (Stoeber et al., 2001, Eward et al., 2004, Wharton et al., 2004). A low MCM2/Ki-67 ratio, close to 1, means less cells are in the G1 phase, and cells in a given tumour population are actively proliferating (Torres-Rendon et al., 2009a). On the other hand, when this ratio increases, it means that a greater number of cells reside in a licensed state with proliferative capacity, but are not cycling (Eward et al., 2004, Wharton et al., 2004). Low values of this ratio have been associated with higher grade tumours and an unfavourable outcome (Dudderidge et al., 2005).

1.5 Geminin

Geminin was originally discovered in metazoans and first cloned in *Xenopus* (Xouri et al., 2004), but was also found in higher eukaryotes (Machida and Dutta, 2005). It is recognised by the anaphase promoting complex (APC) system and targets the protein for ubiquitin mediated proteolysis (McGarry and Kirschner, 1998). Its degradation was observed in transition from metaphase to anaphase, allowing DNA replication licensing in the cell cycle. Geminin expression is absent during the G1 phase, but at high level through the rest of the cell cycle until the onset of mitosis (**Figure 1.5**) (Williams and Stoeber, 2012b). Once the cell is licensed for DNA replication, geminin interacts with Cdt1 during S and G2 phase to inhibit DNA re-replication in the same round (Tamura et al., 2010), thus maintaining genome stability in normal cells. Inactivation of geminin expression has been found to induce re-initiation of DNA synthesis in normal and tumour cells in the presence of functional p53 (Melixetian et al., 2004).

Numerous lines of evidence have suggested that over-expression of geminin is associated with poor prognosis and it could be used as a reliable prognostic maker in different cancers (Moraes et al., 2012, Tamura et al., 2010, Torres-Rendon et al., 2009a). The biological function of geminin was thought to be an inhibitor of the cell cycle, but recent studies have suggested that geminin may not be involved in suppressing cell proliferation (Zhu and Pamphilis, 2009, Guo and Sun, 2013).

Geminin is characterised as a bi-functional molecule as it stimulates cell proliferation and also regulates the DNA replication licensing process to prevent over-replication in the cell cycle (Seo and Kroll, 2006). Its phosphorylation is CDK-dependent and previous studies suggested that geminin may be part of the checkpoint control that links CDK activity to the next round of replication (Nishitani and Lygerou, 2002). Additionally, geminin is responsible for the proliferating-differentiated transition process during nervous tissue development (Seo and Kroll, 2006).

Immunohistochemistry and immunoblotting studies have shown that, in contrast with carcinomas, geminin expression is very minimal or hardly detectable in normal tissue (Wohlschlegel et al., 2002, Torres-Rendon et al., 2009a). Geminin is over-expressed in tumours compared to normal and dysplastic tissues (Torres-Rendon et al., 2009a, Tamura et al., 2010).

Geminin physically interacts with the replication factor Cdt1 necessary for MCM loading. In high eukaryote cells, geminin inhibits Cdt1 protein during the S, G2 and late mitosis phase, however, this mechanism is currently unclear. Inhibition of Cdt1 protein prevents the abnormal assembly of pre-RC on chromatin (Klotz-Noack et al., 2012). Experimentally, high expression of Cdt1 can cause re-replication (Nishitani et al., 2004, Li and Blow, 2005). Interestingly, the absence of geminin and MCM expression may identify cells in the G0 phase that are out of cycle and not licensed to proliferate (Kingsbury et al., 2005).

With respect to Cdt1, geminin can bind to other proteins such as Six3 and Hox, which indirectly regulate cell cycle progression (proliferation) by displacing geminin from Cdt1 before pre-RC is formed (Li and Rosenfeld, 2004, Klotz-Noack et al., 2012). It was recently shown that geminin interacts with different proteins when bound on chromatin, including cyclins E, A1 and B1 (Nakuci et al., 2006). It was also shown that depletion or silencing of geminin in human cell lines induces DNA re-replication (Melixetian et al., 2004, Zhu and Pamphilis, 2009, Zhu et al., 2004).

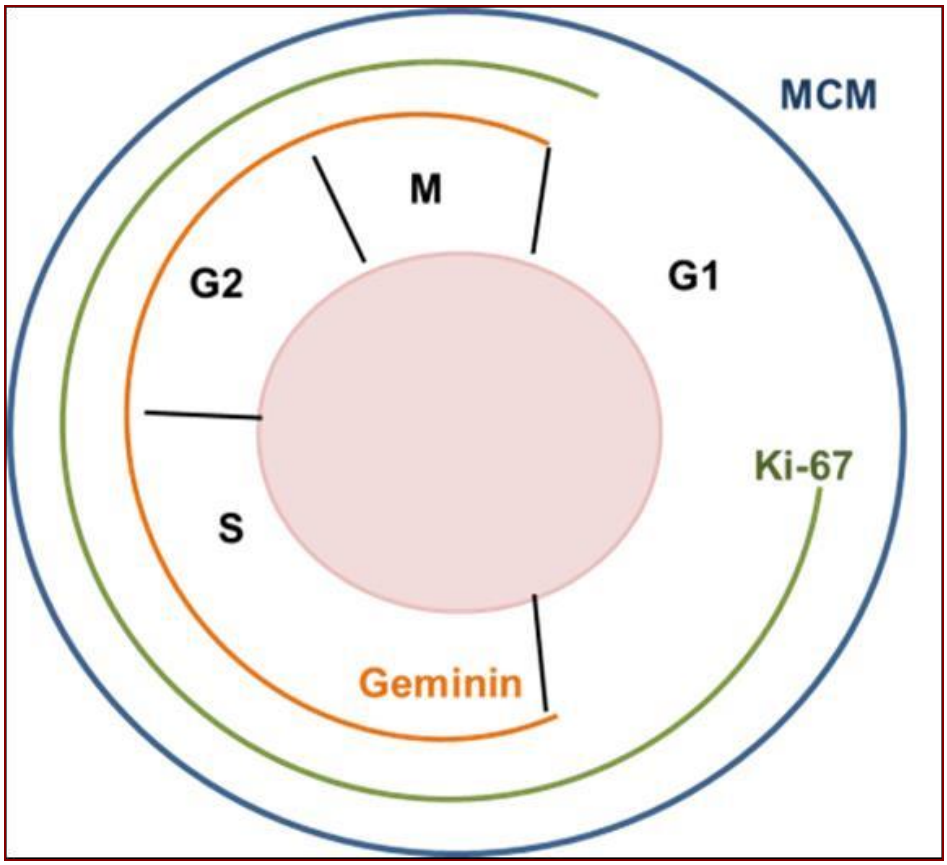

Figure 1.5: MCM2, Ki-67 and geminin expression during the cell cycle. MCM2 protein is present in all cell cycle phases, including G0. Ki-67 is expressed in all phases with the exception of early G1 phase. Geminin acts between G1/S transition stage and metaphase-to-anaphase transition.

1.5.1 Geminin/Ki-67 ratio

In several human tumours the presence of geminin is directly proportional to the cell proliferation index, quantified by Ki-67 expression (Wharton et al., 2004). Ki-67 is expressed in all active cell cycle phases (G1, G2, S and M) and absent in quiescent stages (G0, early G1) (**Figure 1.5**), while geminin is absent in the G1 phase (**Figure 1.5**). The geminin/Ki-67 ratio has therefore been suggested to estimate the length of the G1 phase (Wharton et al., 2004). As geminin identifies less positive cells than Ki-67, this ratio goes from 0 to 1 (e.g. 0.2, 0.3 0.4, 0.5 …etc.). The indication is that the higher the ratio (close to 1), the less cells in G1 and the higher the rate of proliferation.

Cells with an increased geminin/Ki-67 ratio, have a shorter G1 phase (Torres-Rendon et al., 2009a), which indicates an accelerated rate of proliferation and a worse clinical prognosis (Wharton et al., 2004, Shetty et al., 2005, Quaglia et al., 2006). Accordingly, the geminin/Ki-67 ratio has been proposed as a marker of prognosis in some neoplasms (Wharton et al., 2004).

1.6 $p16^{INK4A}$ (CDKN2A)

$p16^{INK4A}$ is the best characterised tumour suppressor protein of the INK-4/ARF family (Queiroz et al., 2010). In humans it is encoded by the CDKN2A gene (Cyclin-Dependent Kinase Inhibitor 2A), and is mapped on chromosome 9p21. The CDKN2A locus encodes for two overlapping, but structurally different proteins: $p16^{INK4A}$ and $p14^{ARF}$ (**Figure 1.6**).

The $p16^{INK4A}$ protein has a role in the regulation of the cell cycle. $p16^{INK4A}$ interacts with cyclin D1 to inhibit cyclin dependent kinase activity (CDK4 and CDK6), and subsequently prevents the phosphorylation of pRb protein during the G1 phase (**Figure 1.6**) (Chien et al., 2011, Nasser et al., 2011). Inhibition of phosphorylation of pRb prevents the cell cycle progressing into S phase and stops cell cycle progression (Queiroz et al., 2010, Pérez-Sayáns et al., 2011).

The consequence of these interactions is suggestive of its role as a tumour suppressor gene. Therefore, inactivation or reduction of $p16^{INK4A}$ encourages abnormal growth in tissues. In addition, inactivation of $p16^{INK4A}$ by genetic or epigenetic alterations, including promoter methylation and homozygous deletion or mutation, is frequently found in head and neck malignancies (Sailasree et al., 2008, Reed et al., 1996, Lang et al., 2002). Inactivation of $p16^{INK4A}$ is therefore defined as an early event in carcinogenesis (Ai et al., 2003, Karsai et al., 2007, Demokan et al., 2012).

Inactivation or elimination of $p16^{INK4A}$ by hypermethylation has been reported to increase proliferative activity and promote cell migration and invasion in head and neck cancer (Mariatos et al., 2000, Su et al., 2010). However, activation or an increase in expression suppresses angiogenesis by restoring wild-type $p16^{INK4A}$ gene and arrests cell growth in some other tumours (Harada et al., 1999). In contrast to its reduced expression in some SCC (see section 1.6.1), $p16^{INK4A}$ may be activated and increased in HPV-associated tumours due to inactivation of pRb by E7 protein. In addition, malignant tumours over-expressing $p16^{INK4A}$ have been suggested to have higher radiosensitivity (Romagosa et al., 2011).

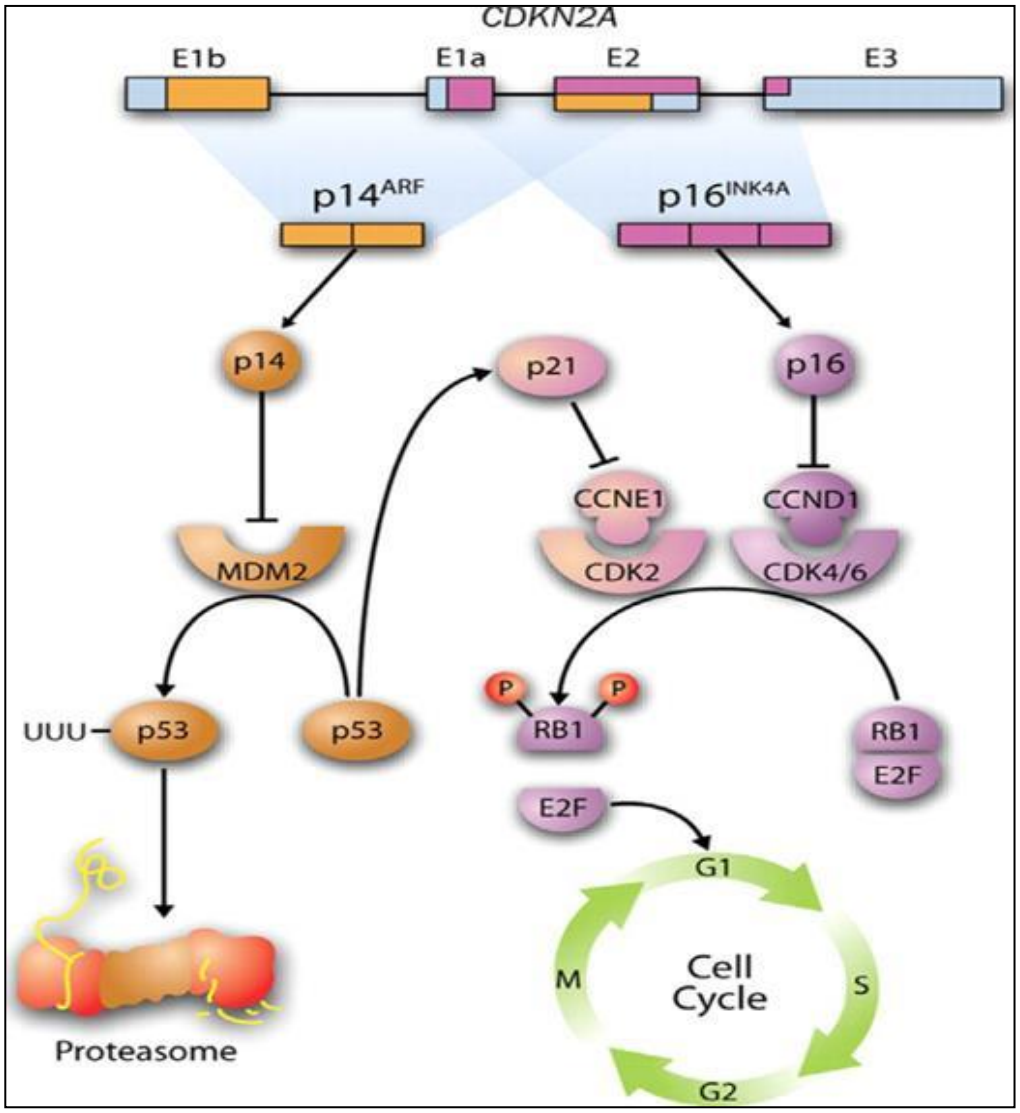

Figure 1.6: The CDKN2A locus structure and its protein pathways during cell cycle control. $p16^{INK4A}$ inhibits CDK4 and CDK6 activity, with consequent inhibition of pRb phosphorylation. (Adapted from Pérez-Sayáns et al., 2011).

1.6.1 Evaluation of $p16^{INK4A}$ expression in oral squamous cell carcinoma

Expression levels of $p16^{INK4A}$ have been extensively studied in oral squamous cell carcinoma (Abrahao et al., 2011, Gologan et al., 2005, Bradley et al., 2006, Nemes et al., 2006, Ohta et al., 2009, Buajeeb et al., 2009, Queiroz et al., 2010, Montebugnoli et al., 2010, Nasser et al., 2011, Chandarana et al., 2013). The $p16^{INK4A}$ pathway contributes to tumour induction and differentiation (Queiroz et al., 2010). Down-regulation of expression has been found to be highly associated with disease recurrence, metastasis and worse clinical outcome in HNC (Danahey et al., 1999, Suzuki et al., 2006, Kumar et al., 2008), while over-expression of $p16^{INK4A}$ protein might be an indicator of better prognosis in HPV-positive HNC patients (Lassen et al., 2009). On the other hand, others have reported that no association was found between expression of $p16^{INK4A}$ and the grading of OSCC (Abrahao et al., 2011, Reuschenbach et al., 2013).

Chen *et al.* (Chen et al., 1999) showed a significant association between immune positivity for $p16^{INK4A}$ and CDK4 in OSCC. In the same way, Karsai *et al.* (Karsai et al., 2007) reported that loss of $p16^{INK4A}$ expression seems to be associated with aberrant expression of p53. Further, using combinations of proliferation markers such as Ki-67 and cyclin D1 with p53 or $p16^{INK4A}$ has been suggested to improve prognostic predicative value in OSCC (Montebugnoli et al., 2010, Nasser et al., 2011). Immunohistochemical studies of $p16^{INK4A}$ in the oral cavity have reported both low (Pande et al.,

1998, Bradley et al., 2006, Isayeva et al., 2014) and high expression (Dong et al., 2012, Duncan et al., 2013, Reuschenbach et al., 2013, Lingen et al., 2013). Interestingly, over-expression of $p16^{INK4A}$ has been found to be associated with increased sensitivity to chemotherapy in OSCC (Suzuki et al., 2006). Silencing of $p16^{INK4A}$ is detected in approximately 70% of oral squamous cell carcinomas (Mascolo et al., 2012) that increase death risk (Barros-Angueira and Gandara-Vila, 2015). It is important to mention that HPV-associated HNCs have genetic alterations that are consistent with the functions of viral oncoproteins (E6 and E7). These cancers are characterised by wild-type p53 and wild-type $p16^{INK4A}$ (Isayeva et al., 2014). Conversely, p53 mutations and $p16^{INK4A}$ inactivation in HNCs have been shown to be mediated by tobacco and alcohol consumption (Ai et al., 2003). These observations support the possibility of at least two distinct pathways for tumour progression in the head and neck region: one driven mainly by the mutagenic effects of tobacco and/or alcohol mediated and the other driven by HPV infection (Westra, 2015).

Bova *et al*. (Bova et al., 1999) revealed that loss of $p16^{INK4A}$ expression was detected in 55 % of studied samples and 68% showed high expression of cyclin D1 protein. The results from this study suggested that loss of $p16^{INK4A}$ and increased expression of cyclin D1 was associated with a reduced five-year survival rate; this co-appearance of both proteins can be utilised as an independent prognostic marker in tongue carcinoma. These observations were consistent with other studies (Jayasurya et al., 2005, Pande et al., 1998) and the same correlation was reported.

More recently, studies have shown $p16^{INK4A}$ protein levels to be elevated in OSCC. This has been credited to HPV induced OSCC (Leemans et al., 2010), where inactivation of pRb by the HPV oncoprotein E7 results in increased $p16^{INK4A}$ expression (Romagosa et al., 2011). In recent published studies the prevalence of HPV positivity was recorded in approximately 6% of OSCC (Lingen et al., 2013, Krüger et al., 2014). Therefore, it is difficult to compare the results of different studies evaluating $p16^{INK4A}$ expression levels in OSCC, since results are variable and may depend on HPV status.

There is no wide agreement in the literature on the potential role of HPV in OSCC (Lingen et al., 2013, Samman et al., 2014). Studies have confirmed an association between high expression of $p16^{INK4A}$ and HPV in oropharyngeal cancers (OPC) (Jordan et al., 2012, Singhi and Westra, 2010, Westra, 2015). Hence, $p16^{INK4A}$ over-expression has been considered as an HPV surrogate marker in OPC (Seiwert, 2014, El-Naggar and Westra, 2012), but not in pre-malignant and malignant oral lesions (Nankivell et al., 2014). This over-expression can be simply determined by imunohistochemical means. The high sensitivity and low cost of this technique make it a more practical and alternative method to *in situ* hybridisation (ISH) or polymerase chain reaction (PCR) (Westra, 2014).

Since $p16^{INK4A}$ can also function as a tumour suppressor gene, its expression can be altered secondary to gene mutation or methylation (Romagosa et al., 2011). However, while most non HPV-associated carcinomas are $p16^{INK4A}$ negative, some can express $p16^{INK4A}$ by other

mechanisms (Hunt et al., 2014). Therefore, it is essential to detect strong and diffuse nuclear and cytoplasmic staining in more than 70% of cells for $p16^{INK4A}$ to be a surrogate marker of active HPV (Lewis et al., 2012, Hunt et al., 2014, Nankivell et al., 2014).

1.7 Cyclin D1

Cyclins are important proteins in the regulation of the cell cycle. The cyclin D family is composed of D1, D2 and D3, which drive cell cycle progression. In humans, D1 protein is encoded by the CCND1 gene (linked tightly to the bcl-1 oncogene) and is located on chromosome 11q13, also sometimes known as PRAD1 (benign parathyroid adenoma D1) (Donnellan and Chetty, 1998). The cyclin D proteins reach their maximum level during the G1 phase, and are thought to be responsible for the G1/S phase transition (**Figure 1.7**). Cyclins activate their cyclin dependent kinases (CDK4/CDK6) to phosphorylate the pRb protein (Pestell, 2013), which then releases transcription factors (EF2) necessary for the DNA replication initiation process. Cyclins, along with their partners, cyclin dependent kinases (CDKs), have been linked with proliferation and differentiation in different tissues (Agarwal et al., 2012) and are frequently altered in neoplasms (Kim and Diehl, 2009).

The amplification of cyclin D1 associated with loss of $p16^{INK4A}$ function has been widely seen in HNCs (Hall and Peters, 1996, Arora et al., 2012). Hanken *et al.* (Hanken et al., 2014) have recently shown that cyclin D1 over-expression and gene amplification correlated with decreased survival in OSCC patients. In most instances, high expression of cyclin D1 with or without gene (CCND1) amplification is frequently seen in primary tumours and cell lines (Huang et al., 2002, Miyamoto et al., 2003). In a study of 106 patients with laryngeal squamous cell carcinoma, increased cyclin D1

expression was associated with poor prognosis (Gibcus et al., 2008). Likewise, in OSCC, high expression of cyclin D1 was detected in 95% of studied samples and p53 positivity was seen in 65%. The results of this study have shown a positive correlation between increased cyclin D1 and p53 expression in OSCC samples (Swaminathan et al., 2012). High expression of cyclin D1 was also observed in previous studies (Kuo et al., 1999, Lam et al., 2000), and may be an independent prognostic marker for oral squamous cell carcinoma (Huang et al., 2012, Hanken et al., 2014). In a recent meta-analysis study of oral cancer, correlation between high expression of cyclin D1 and clinicopathological parameters, such as size of tumour, differentiation status, nodal metastasis, and clinical stage of disease has been reported (Zhao et al., 2014).

Molecular studies of cyclin D1 have highlighted possible new functions, for instance, it has a role in promoting cell adhesion and migration (**Figure 1.7**) by inhibition of Rho-associated (RhoA) protein kinase (Li et al., 2008). In contrast, cyclin D1 protein can indirectly inhibit cell migration by inhibiting epithelial/mesenchymal transition (Tobin et al., 2011).

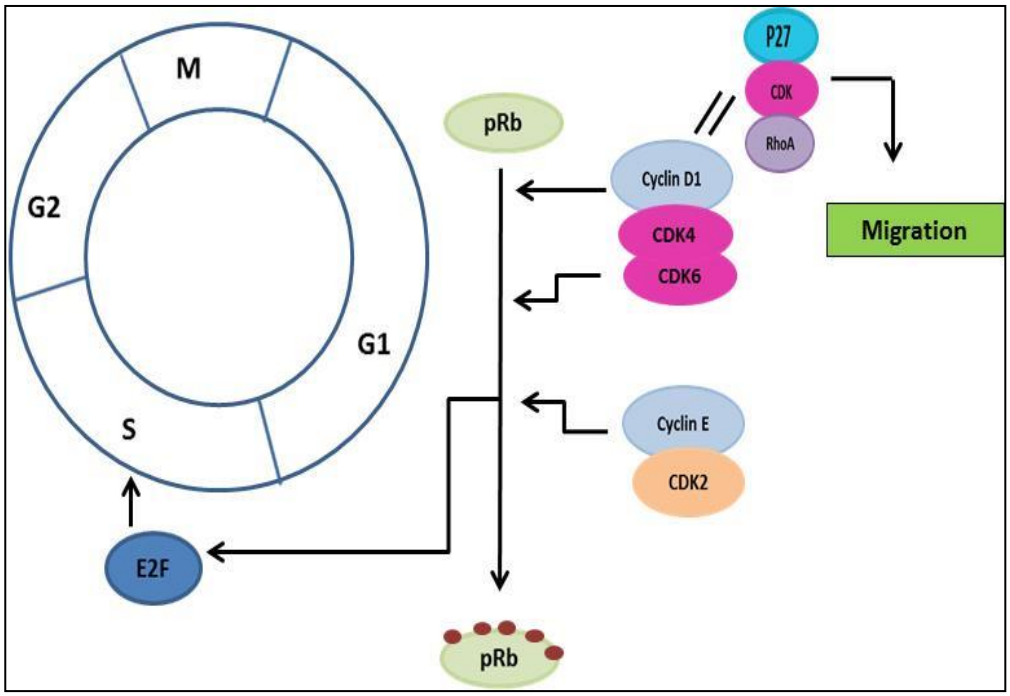

Figure 1.7: Presence of cyclin D1 in the cell cycle and its different protein interactions. In the G1 phase, cyclin D1 binds to cyclin dependent kinases (CDK4 and CDK6), with the help of cyclin E/DK2 complex, to phosphorylate tumour suppressor pRb protein. The phosphorylation of pRb is associated with release of the transcription factor E2F, which can then drive cell-cycle progression.

1.8 DNA content (ploidy)

DNA ploidy is a measure of the DNA content in a cell population. Normally, a non-dividing somatic cell contains 23 pairs of chromosomes (diploidy). During S phase, without subsequent cell division, the somatic cell will have double the amount of DNA (tetraploid or 4 copies).

The term polyploidy is applied when multiple copies of DNA exist. In cases of partial or total genetic damage, unequal distribution of chromosomes that causes chromosomal segregation is called aneuploidy (Sen, 2000). It has been shown that the best approach to evaluate potentially malignant lesions is to measure the DNA status (DNA ploidy). However, a variety of methods have been used to determine the ploidy status, including karyotyping, flow cytometry, image analysis and fluorescence *in situ* hybridisation. These measurements are performed by staining the nuclear material in the cell with stoichiometric stains (such as Feulgen) and comparing the intensity of staining to control cells by means of integrated optical density (IOD), which may be performed by flow cytometry (FCM) or image cytometry (ICM).

The method is validated by using internal control cells which must have a known DNA content. Normal cells, such as lymphocytes, are usually used (Haroske et al., 1997).

Estimation of the DNA index (DI) in populations of cells of interest by image cytometry can be performed on cell suspensions (cytospin) or directly on histological sections (Huang et al., 2008). In contrast, standard FCM can

only be achieved by suspending disaggregated cells and passing them through a flow cytometer. The advantage of FCM is that it can anlyse thousands of cells.

1.9 Aneuploidy

Aneuploidy is defined as numeric chromosomal aberrations in which the number of chromosomes is either more or less than the normal cell content (diploid). Caspersson (Caspersson et al., 1963) was the first investigator to describe abnormal genetic material in cancer cells (reviewed by (Shackney et al., 1989, Pihan and Doxsey, 1999)). Since then aneuploidy has become one of the main features of human cancer, particularly in solid tumours (Weaver et al., 2007). According to the European Society of Analytical and Cellular Pathology (ESACP), aneuploidy is defined as “those types of DNA distributions which are different at a statistically significant level from those of normal cell populations. DNA aneuploidy can either be seen as DNA stem-line aneuploidy, or can be indicated by rare events” (Haroske et al., 1997). Occurrence of rare events is defined by cells that exceed the 5c on an image cytometry histogram (Haroske et al., 1997).

According to Boveri‘s theory of malignancy, the consequence of chromosomal instability results from multipolar mitosis of an abnormal single somatic cell (centrosome), and may cause aneuploidy, a constant mark of cancers (Pathak and Multani, 2006).

Numerical chromosomal aberrations (aneuploidy) and structural chromosomal alterations can lead to cancer development (Teixeira et al., 2014). Indeed, some researchers believe that aneuploidy seems to be an early genetic change in cancer transformation (Visscher et al., 1996, Padilla-Nash et al., 2013, Michor et al., 2005). Therefore, the presence of aneuploidy in early stages of disease suggests that abnormal DNA content and its underlying cause may play a key role in tumour transformation and progression. However, it is still a long standing issue whether aneuploidy is essential for transformation and progression of cancer. Such genetic instability is frequently responsible for heterogeneity and progression of tumours (Lengauer et al., 1998). Abnormal DNA content (aneuploidy) is associated with poor prognosis, metastasis, and resistance to chemotherapy drugs (Gao et al., 2007, Choi et al., 2009, McClelland et al., 2009, Swanton et al., 2009). In contrast, diploid tumours often exhibit a better prognosis (Sinicrope et al., 2006, Hicks et al., 2006, Belien et al., 2009).

Several investigators have suggested that the presence of aneuploidy is the main characteristic of cancer cells and is associated with aggressive behaviour and poor prognosis (Bowen et al., 2009, Kristensen et al., 2003, Gawrychowski et al., 2003).

Laboratory experiments in human and animal cells suggest that aneuploidy appears to be required for malignant transformation (Schvartzman et al., 2010, Hanks et al., 2004, Gordon et al., 2012). In this concept, aneuploidy

has been proposed to promote and inhibit immortalisation, depending on which chromosomes are affected (Weaver and Cleveland, 2008). Moreover, defects in telomerase function, DNA damage response, chromosomal segregation and cell cycle regulation, may lead to chromosomal instability (reviewed by (Bakhoum and Compton, 2012)).

Interestingly, Fujiwara and colleagues (Fujiwara et al., 2005) found that cells with aneuploid DNA content (tetraploid) grown, *in vitro*, developed malignancies and genetic alterations in 10 out of 39 mice without carcinogen exposure. DNA aneuploidy in oral squamous cell carcinoma provided a good prognostic indicator for lymph node metastasis and recurrence (Seethalakshmi, 2013). In a recent study, it was reported that aneuploid oral dysplasia progressed to oral squamous cell carcinoma in 74% of cases compared to only 42% of diploid lesions (Torres-Rendon et al., 2009b). In contrast, higher incidence of aneuploidy (90%) was reported in malignant head and neck lesions (Abou-Elhamd and Habib, 2007).

Finally, it is important to mention that recent studies have shown no correlation between ploidy status and histopathological grade in either precancerous or cancerous lesions (Diwakar et al., 2005, Torres-Rendon et al., 2009b).

1.9.1 Causes of aneuploidy

In human tissue, many cell divisions occur every minute; accurate chromosome segregation with every cell division is crucial to maintain the normal karyotype. Unequal chromosome segregations can lead to cancer transformation. Importantly, some of the genes that regulate chromosome segregation have been found to be mutated in human cancer (Jiang et al., 2014). Aneuploidy arises through chromosomal instability caused by the continual loss and gain of whole chromosomes during cell division (Thompson and Compton, 2011). Such disruption has been implicated in numerical chromosome abnormality (aneuploidy) in cancer cells (Gordon et al., 2012, Holland and Cleveland, 2012).

There are a number of possible mechanisms (**Figure 1.8**) by which a human cell might gain or lose chromosomes during cell division. Some of these mechanisms are discussed briefly below.

1.9.1.1 Chromosomal missegregation

According to some authors, continual chromosomal missegregation is believed to contribute to or accelerate malignant transformation by facilitating the accumulation of chromosomes with oncogenes (growth promoting genes) and deletion of chromosomes with tumour suppressor genes. This would lead to heterogeneous DNA content of tumour cells. Aneuploidy could result from genetic changes, such as mutations at nucleotide level and alteration of apoptotic genes (Loeb and Loeb, 2000,

Sen, 2000, Panigrahi and Pati, 2009). Thus far, it has been debated whether aneuploidy contributes to malignant transformation rather than being a discrete event. Recent evidence strongly indicates that alterations in genes that control chromosomal segregation, DNA damage response, telomere function and cell cycle regulatory proteins are the main causes for chromosomal instability (Holland and Cleveland, 2009). There has been more attention directed towards the causes and consequences of chromosomal instability, since it is a common feature of many aneuploid cancer cells and generates tumour heterogeneity (Thompson and Compton, 2011, Chandhok and Pellman, 2009).

1.9.1.2 Mitotic checkpoint defects

A large number of mitosis genes have been mutated in human tumour cells. Not surprisingly, altered or mutated checkpoint genes could lead to chromosome missegregation, and subsequently, aneuploidy and tumour growth (Hanks et al., 2004, Holland and Cleveland, 2009, Snape et al., 2011). Over-expression of proteins that regulate spindle checkpoints, such as Mad2 (mitotic arrest deficient 2) and Kinetochore protein (encoded by NDC80 gene), have been observed in human cancer and are often associated with a poor prognosis (Holland and Cleveland, 2009). Nevertheless, the frequency of such mutations or defects is rare in human tumours (Thompson et al., 2010, Holland and Cleveland, 2012).

1.9.1.3 Chromosome cohesion defects

In an attempt to identify other pathways that stimulate chromosomal instability, researchers have suggested that mutations in genes that control sister chromatid cohesion may participate in aneuploidy (Holland and Cleveland, 2009, Chandhok and Pellman, 2009). Inactivation in the STAG2 gene results in chromatid cohesion or attachment defects and leads to aneuploidy in human cancer (Solomon et al., 2011).

1.9.1.4 Merotelic attachments

Merotelic attachment is another mechanism that probably contributes to chromosome missegregation. By this mechanism inappropriate attachment of kinetochores to spindle microtubules may occur when a single kinetochore binds to microtubules that arise from both poles of the spindle (Thompson and Compton, 2011). Centrosome amplification is likely to increase the incidence of merotelic attachments, and strongly associates with chromosomal instability (Nigg, 2006, Gordon et al., 2012). Extra centrosomes are reported to be common in many aneuploid human cancers (Nigg, 2002).

Another popular hypothesis suggested as a cause of aneuploidy proposes that replication of genetic content in the absence or failure of cell division (Endoreduplication) can result in tetraploidy or polyploidy (Fujiwara et al., 2005, Zimmet and Ravid, 2000, Holland and Cleveland, 2009). However, tetraploid cells can arise by several pathways, including cytokinesis failure,

mitotic slippage, and cell fusion (Ganem et al., 2007). In this way, it has been proposed that Aurora B dependent kinase acts as a No Cut pathway “abscission inhibitor” to ensure that all chromosomes have been pulled out from the midzone during cell mitosis (Holland and Cleveland, 2009). However, there is strong evidence suggesting that tetraploidy can trigger malignant transformation (Ganem et al., 2007). This evidence came from an *in-vitro* study which showed that tetraploid p53 mouse cells promote malignant transformation after mutagen exposure (Fujiwara et al., 2005).

1.9.2 Aneuploidy as a cause of tumourigenesis

Hansemann (Hansemann, 1890) and Boveri (Boveri, 1914) proposed that aneuploidy was the cause of malignant development, and others have suggested that it is a necessary step for carcinogenesis (Knauss and Klein, 2012). However, the aneuploidy hypothesis has been underestimated by many researchers since the discovery of genes, and demonstration of mutagenic agents such as X-rays and alkylating agents (Pihan and Doxsey, 1999). Consistent with this concept, other studies revealed that chemical carcinogens such as dimethylbenzanthracene, cytosine arabinoside and methylcholanthrene have been shown to impede mitosis in embryonic cells. Such disturbance during cell division leads to asymmetric cell mitosis and causes so called preneoplastic aneuploidy. Cells with the preneoplastic phenotype may result in immortal cell aneuploidy (Duesberg et al., 2000).

In general, aneuploidy roles are largely unknown in some tissues. However, according to some genetic concepts, aneuploidy might be a driver or a passenger (context dependent) in the tumourigenesis transformation. Aneuploidy may depend on particular interactions of the karyotype with the microenvironment in different human tissues (reviewed by (Gordon et al., 2012)). Recently, data has shown that aneuploidy has a role in promotion of and/or inhibition of carcinogenesis in certain circumstances (Weaver et al., 2007, Weaver and Cleveland, 2008). These findings show that a high rate of abnormal DNA content (aneuploidy) in chemically and genetically induced tumours can act as an inhibitor rather than a motivator of tumour formation

(Weaver et al., 2007), depending on the specific genetic changes in distinct environments. The effects of aneuploidy on tumour cell fate are influenced by the gain or loss of specific chromosomes (Williams et al., 2008). These different effects refer to three possible relevant factors: firstly, the presence of specific combinations of gain or loss of chromosomes in aneuploid cells; secondly, the stability of aneuploid cells as additional chromosomal missegregations occurred; thirdly, further mutations in oncogenes and tumour suppressor genes in a given cell (Weaver and Cleveland, 2008).

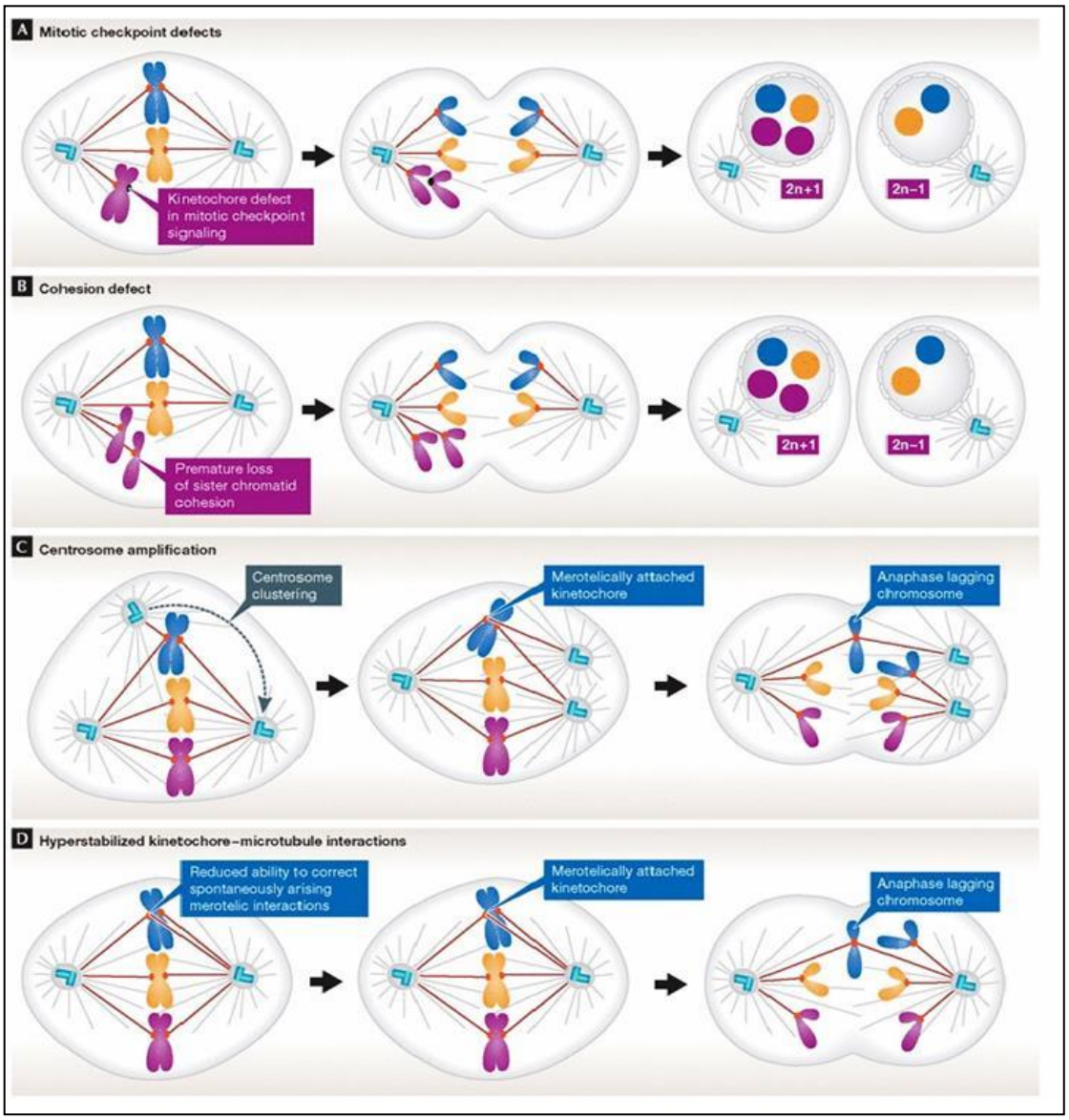

Figure 1.8: Possible pathways by which the human cell may develop aneuploidy. (A) Mitotic checkpoint signalling defect. (B) Chromosome attachment defects. (C) Amplification of centrosome resulting in multipolar mitotic spindle (Adapted from Holland and Cleveland, 2012).

1.10 DNA image cytometry (ICM)

A variety of techniques have been used to detect alterations in DNA content (aneuploidy) in tumour cell populations. These include flow cytometry, spectral karyotyping, fluorescence in situ hybridisation and image cytometry. Flow cytometry (FCM) has been the most popular method for evaluation of DNA ploidy in tumour cells. However, FCM has some limitations, and therefore DNA image cytometry (ICM) has become a more popular technique (Massimo, 2013). Different systems have been introduced in this field, the semi-automated cell analysis system (CAS) (Khan et al., 1994), and more recently the automated cellular imaging system (ACIS) (Huang et al., 2005, Huang et al., 2008, Santos-Silva et al., 2011, Gouvêa et al., 2013). Some OSCC studies have been carried out using various techniques of DNA image cytometry (Santos-Silva et al., 2011, Torres-Rendon et al., 2009b).

DNA image cytometry (ICM) is widely used as a diagnostic and prognostic method for quantification of DNA content by means of integrated optical density (IOD). It can be performed using different systems (Huang et al., 2005) incorporating a microscope with a motorised scanning stage attached to a digital camera. This microscope is connected to a computer with a software program able to scan and categorise the nuclei of interest according to the IOD, and to translate the results into DNA histograms.

The principle behind DNA image cytometry for evaluation of tumour cells is that normal human cells have diploid DNA content. The criteria to distinguish the cell cycle stages on the DNA histogram are as follows: cells with normal DNA content (diploid cell) have 2n DNA content; this is decoded as 2c (copies of the chromosomes) on the histogram. The nuclei at 2c on the histogram belong to a G0/G1 phase representing a diploid peak (**Figure 1.9**). Cells with double DNA content located in the 4c area (tetraploid cells) represent cells in the G2 phase (**Figure 1.9**), thus the tetroploid peak is positioned in the duplication region of the G0/G1 phase fraction (Böcking and Nguyen, 2004). Cells with polyploid DNA content represent the multiploids of a 2c peak, so these cells are located at 4c, 8c…etc. Additionally, cells between 2c and 4c are in S phase (**Figure 1.9**). Aneuploidy cells with abnormal DNA content are observed at 3c and 5c areas on the histogram (Baak and Janssen, 2004). When using an image cytometry system, a 2c (normal diploid) peak is generated from selected normal cells as an internal control. When analysing tumour tissue, lymphocytes are usually selected as the normal cell population.

In summary, DNA image cytometry systems are capable of identifying cells or stem-lines with aneuploid content. They also provide information about the polyploidisation of euploid or aneuploid DNA stem-lines (Böcking and Nguyen, 2004). The pre-established statistical parameters can be calculated to increase the cell nuclei data, and therefore improve the sample evaluation.

Recently, the evaluation of DNA content by automated image cytometry has been shown to be a valuable diagnostic tool in cytological diagnosis, and in oesophageal and oral cancer screening (Meng et al., 2013, Zhao et al., 2012, Bradley et al., 2010).

In the present study, ploidy analysis was undertaken using an automated cell imaging system (ACIS III, Automated Cellular Imaging System; Dako, Glostrup, Denmark) on tissue sections to increase the analysis sensitivity, with high-fidelity DNA histograms generated by the system.

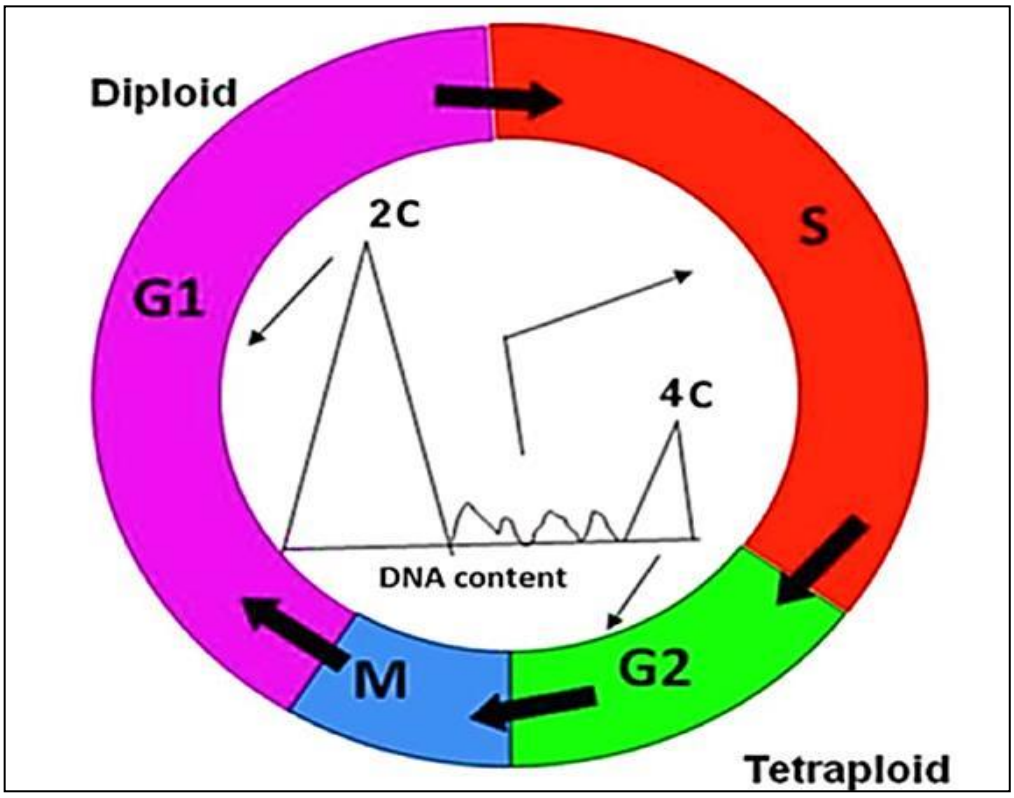

Figure 1.9: DNA content represented in the cell cycle. The nuclei that belong to a G1 peak (2c) do not represent a duplication region. Cells in G2/M phase (tetraploid cells) are located in the duplication region (4c) of the G0/G1 fraction.

1.10.1 DNA index

According to the European Society of Analytical and Cellular Pathology (ESACP), the DNA index is defined as the quotient of the modal value of a DNA stemline divided by the modal value of a G0/G1 peak of reference cells (Haroske et al., 2001). A DNA index of 1 means that the DNA content of analysed cells does not differ from that of the reference diploid cells (control cells). Normal diploid cells have double strands of chromosomes and a DNA index of 1. This means that tumour cells with a DNA index of 1 are the same as reference diploid cells in G1 phase (Huang et al., 2005).

According to some authors, cells with a DI value between 0.9 and 1.05 are considered as diploid samples, however, others regard cells as diploid when the DI is less than 1.1 (Huang et al., 2005, Gschwendtner et al., 1999). This may vary according to the study, author, and DNA image cytometry system used (Huang et al., 2005).

1.10.2 Coefficient of variation (CV)

One of the most used statistical parameters in DNA cytometry analysis is the coefficient of variation (CV). The CV is the quotient of the standard deviation of the IOD values divided by the mean of the IOD values of the reference cells in the G0/G1 phase fraction as defined by the European Society of Analytic Cellular Pathology (ESACP). It is given as a percentage, and should typically be less than 5% (Baak and Janssen, 2004). However, others have considered that less than 10% is acceptable (Santos-Silva et

al., 2011). The ESACP formula is CV=SD/Mean ×100 (Haroske et al., 2001, Haroske et al., 1997).

In fact, the CV value indicates the width of the reference peak (G0/G1 peak). The narrower the reference peak, the lower the CV and hence the greater the accuracy and reliablility of the analysis. Therefore, in practice the CV parameter indicates the quality of the analysis but does not provide any discriminatory information. Nevertheless, the CV is an important parameter to identify the aneuploidy and estimation of S phase.

1.10.3 Cells that exceed 5c

Besides the DNA index, cells with a DNA content of more than 5c (5cER) provide another DNA cytometry parameter. Some investigators suggest that if only one single cell exceeds 5c it would be considered as aneuploid (Baak and Janssen, 2004). Cells with 5cER are known as non-proliferating cells with abnormal nuclear content (aneuploid) and a large chromosome population (Haroske et al., 2001).

Normal cell populations do not show a 5cER, but when it is present in more than 1% of the gross nuclei analysed it is considered as an indicator of aneuploidy. Thus, a 5cER of more than 1% is regarded as a criterion to classify a lesion as aneuploid (Kristensen et al., 2003, Palmeira et al., 2010, Torres-Rendon et al., 2009b, Gockel et al., 2006, Pektaș et al., 2006, Santos-Silva et al., 2011).

1.10.4 DNA image cytometry versus flow cytometry

Image and flow cytometry have both been commonly used for DNA analysis; in both techniques stoichiometric staining of the nuclei is needed to quantify the DNA content. ICM generally measures the DNA content by light optical density (LOD). Samples are prepared by either cytospin preparations of extracted and dispersed cell nuclei or by examination of whole tissue sections, in which the nuclei are stained with Feulgen stain. FCM measures the DNA content of single tumour cells by cell dispersion. The nuclei are stained with fluorescent dyes, and the cells are passed through a flow cytometer. Using FCM, it is not possible to visualise or check the nuclei before analysis.

DNA image cytometry has some advantages over flow cytometry, as the latter has some limitations. Lack of standardisation in assays is the most important drawback of flow cytometry (Jahan-Tigh et al., 2012). Also, it is not possible to choose individual cells from specific areas of tissue, or exclude any artefacts from the measurements. A small number of cells with abnormal DNA content (aneuploid) can be undetected in the presence of a large number of cells with a diploid content. Cells of interest that need to be analysed can be easily mixed with tumour stromal cells (Jahan-Tigh et al., 2012, Rygiel et al., 2008). Therefore, accuracy of the results can be compromised and be less reliable compared with ICM.

In contrast to FCM, DNA image cytometry allows direct visual detection and selection of cells of interest. The standardisation of DNA measurements is a

well-defined algorithm and it has been reported as having well established diagnostic validity and reproducibility (Böcking and Nguyen, 2004). Therefore, DNA imaging systems are more accurate for the detection of DNA ploidy abnormalities than flow cytometry (Dunn et al., 2010, Belien et al., 2009).

1.10.5 DNA cytometry and diagnosis of tumours

The potential prognostic value of DNA ploidy analysis using ICM from paraffin embedded oral precancerous samples was reignited by J.Sudbø (Sudbø et al., 2001, Sudbø et al., 2004, Sudbø and Reith, 2005). His promising work raised the theory of aneuploidy as an early stage in oral cancer transformation. Unfortunately, these studies have been retracted from the literature due to fictional data (Couzin and Schirber, 2006, Curfman et al., 2006). Nonetheless, his work suggested that aneuploidy was a good prognostic indicator in oral epithelial dysplasia.

Many studies have analysed different solid tumours using DNA image cytometry, for instance, in oesophageal lesions (Fang et al., 2004, Gockel et al., 2006, Dunn et al., 2010), ovarian and urothelial tumours (Flezar et al., 2003, Baak and Janssen, 2004), oral premalignant lesions (Torres-Rendon et al., 2009b, Brouns et al., 2012, Van Zyl et al., 2012), gastric cancer and lung cancer (D'Urso et al., 2010). More recent studies have shown the predictive value of DNA image cytometry (ICM) as an adjunct method in cytological analysis, such as in malignant effusions and cervical cancer lesions (Meng et al., 2013, Demirel et al., 2013). Results from these studies

indicated that ICM was considerably more predictive and sensitive than flow cytometry. It can also predict late relapse in Barrett's oesophagus; patients with residual aneuploid tumours have a higher chance to develop cancer than those who become diploid. DNA aneuploidy by image cytometry is considered to be a good prognostic marker in patients with Barrett's oesophagus (Dunn et al., 2010).

DNA abnormalities detected by image cytometry can predict recurrence and stage of progression. In oral epithelial dysplastic lesions, 74% of studied samples with an abnormal DNA content (aneuploidy) showed malignant progression, compared to only 42% of the diploid lesions. 52.6% of patients with aneuploid content progressed to carcinoma within the first 5 years. In contrast, 25.3% of the diploid lesions progressed within the first 5 years (Torres-Rendon et al., 2009b). Similarly, in urothelial carcinomas, abnormal DNA content and a high S phase fraction appeared to predict malignant progression (Baak and Janssen, 2004).

According to some authors, DNA cytometry should be combined with tumour proliferation markers to improve the prognostic evaluation of some cancers (Zhu et al., 2005, D'Urso et al., 2010).

1.10.6 DNA cytometry in oral squamous cell carcinoma

Measurement of DNA content has been evaluated extensively using DNA FCM and ICM (Abou-Elhamd and Habib, 2007, Fleskens et al., 2010, Torres-Rendon et al., 2009b, Santos-Silva et al., 2011, El-Deftar et al., 2012, Diwakar et al., 2005). Although good correlation between prognosis and DNA status has been shown, no correlation between genomic content and histological differentiation has been found (Das et al., 2005, Torres-Rendon et al., 2009b, El-Deftar et al., 2012). However, it has been shown that there is a clear association between severity of aneuploidy and histological grade of dysplastic lesions (Yu et al., 2007). Significant correction between ploidy status (aneuploidy) and lymph node involvement has been detected (Das et al., 2005, Seethalakshmi, 2013). In addition, multiple genetic alterations, such as losses or gains of chromosomes, have been correlated with disease outcome in head and neck tumours (Gollin, 2014). It is also clinically relevant that aneuploid cases are more sensitive to radiotherapy than diploid tumours (Xiaoyan et al., 2005).

On the other hand, Saiz-Bustillo (Saiz-Bustillo et al., 2005) and co-workers reported that there is no significant correlation between tumour free survival and DNA content, measured by flow cytometry . Some studies have shown that oral squamous cell carcinoma cases are frequently diploid by DNA image cytometry (Pektaș et al., 2006, Torres-Rendon et al., 2009b) or flow cytometry, despite the fact that the vast majority of patients had advanced disease (El-Deftar et al., 2012). The incidence of DNA aneuploidy evaluated

by FCM among primary OSCC ranges from about 20% to 90%. Donadini *et al* (Donadini et al., 2010) reported that the incidence of abnormal DNA content (aneuploidy) in OSCC was 92%. In addition, El-Deftar *et al.* (El-Deftar et al., 2012) examined the DNA status in primary OSCC and matched metastatic lymph nodes; aneuploidy was recorded in only 19% of cases, and all metastatic lesions were diploid. These variations in values may relate to tissue material (paraffin embedded or frozen) used or to resolution of the FCM system.

In 2005, Diwakar *et al.* (Diwakar, et al., 2005) published a paper in which they noticed a high incidence of heterogeneity of ploidy in samples of OSCC (using ICM on cytospins of dispersed nuclei to study ploidy). The authors suggested that this may be due to sampling error and contamination, or increased genomic instability in tumour clones, although the cause of this instability is still unclear (Gordon et al., 2012, Holland and Cleveland, 2009). Other authors have started to investigate ploidy status along with the cytomorphometric pattern of tumour cells in tissue and smear samples (Pektaş et al., 2006).

Diwakar *et al.* (Diwakar et al., 2005) reported an aneuploid rate of 52% in 42 OSCC cases. Twenty-two were uniformly aneuploid, one uniformly tetraploid and three uniformly diploid, but 16 cases were heterogeneous in their DNA content. This low percentage of aneuploid lesions may be due to sampling error as a result of the heterogeneity of tumour contents. Generally, heterogeneity amongst malignant cells with significant biological

differences within the same tumour is not uncommon (Sipos et al., 2014). Multiple samples analysed for DNA ploidy using an image system are recommended. Additionally, the recent study by Torres-Rendon *et al.* (Torres-Rendon et al., 2009b) found 11 aneuploid lesions (52%) out of 22 OSCC. This data suggested that DNA abnormalities are not essential for malignant progression. In this regard, Santos-Sliva *et al.* (Santos-Silva et al., 2011) studied two groups of patients affected by OSCC of the tongue. In the group of young patients (less than 40 years) they found 86.5% of the samples were aneuploid compared to only 64.3% of the older group. This rate of aneuploidy is higher than shown in other studies (Diwakar et al., 2005, Torres-Rendon et al., 2009b). However, these studies used dispersed cell nuclei in cytospin preparations, while Santos-Silva *et al*.(Santos-Silva et al., 2011) and colleagues analysed tissue sections with an ACIS system as used in the present study. This suggests that using tissue sections and an automated system (ACIS) allows better selection of relevant tissues, without contamination from stromal and diploid normal cells.

Image cytometry has been reported to be a sensitive technique to examine DNA aneuploidy of OSCC (Yu et al., 2007, Torres-Rendon et al., 2009b, Santos-Silva et al., 2011) and this technique can predict recurrence and clinical outcome (Böcking et al., 2010).

1.11 Summary

Early diagnosis of OSCC can improve the patient's quality of life and decrease mortality rates. So far, no single predictive marker can assess OSCC. Several markers have been suggested to have prognostic value in cancerous lesions, including minichromosome maintenance proteins (MCM2), geminin, cyclin and other cell cycle regulatory biomarkers. These could help in understanding the cell cycle kinetics of OSCC, and may be useful markers in lesion metastasis. There is a strong suggestion in the literature that aneuploidy is associated with malignant transformation and is a hallmark of solid malignant tumours. DNA image cytometry has been shown to be a valuable method for measurement of gross genomic damage in single cells or stemlines. Determination of DNA content could also be a predicative marker of tumour metastasis in OSCC lesions.

Assessment of nuclear content (DNA ploidy) as a surrogate measure of gross genomic aberrations in malignant populations has provided promising data. Confirmatory studies are needed for further evaluation of the efficiency of DNA image cytometry as a predictive adjunctive method.

CHAPTER 2: AIMS AND OBJECTIVES

2. AIMS AND OBJECTIVES

2.1 Hypotheses

- Alterations in cell cycle regulatory proteins can be found in oral squamous cell carcinoma and may predict prognosis.
- DNA aneuploidy may indicate high risk of progression of oral cancer.
- DNA image cytometry analysis of oral cancer may be a useful diagnostic and prognostic tool.

2.2 Aims and objectives

The aims of this project were to:

1. Determine the expression patterns of a range of potential biomarkers in oral cancers (squamous cell carcinoma) including cell cycle regulatory proteins (e.g. MCM2 and cyclins).

2. Evaluate DNA content (ploidy) as a diagnostic and prognostic marker in these lesions.

3. Investigate the relationship between gross genomic aberrations (abnormal DNA content) and expression of cell cycle proteins.

4. Assess heterogeneity of lesions by means of cell cycle proteins expression and DNA ploidy status.

Specific objectives were to:

1. Determine the expression of potential biomarkers in oral cancer using immunocytochemistry. Analyse the expression of Minichromosome maintenance (MCM2) protein and other biomarkers including cell cycle proteins and proliferation markers in the same cases, and correlate expression to stage of disease (including lymph node metastases), conventional histological grade, and to grading by DNA status (diploid or aneuploid).

2. Determine the ploidy status of oral cancers using an automated system of image cytometry. Ploidy will be correlated to the histological grade, and outcome in terms of metastatic disease.

3. Determine heterogeneity of ploidy status in lesions of oral cancer using samples from different regions of squamous cell carcinomas (surface and advancing front). For each region the DNA content and expression of biomarkers will be investigated.

CHAPTER 3: MATERIALS AND METHODS

3. MATERIALS AND METHODS

3.1 Health and Safety and Ethics

All tissue samples, materials and chemicals were handled according to university health and safety guidelines. Any chemical reagents producing toxic fumes were processed in a fume cupboard. The requirements and precautions recommended in Control of Substances Hazardous to Health were followed before starting any laboratory work.

Ethical approval for the project was obtained from South Sheffield Research Ethics Committee (08/S0709/70).

3.2 Tissue Specimens

The archive of pathology specimens in the Unit of Oral and Maxillofacial Pathology, School of Clinical Dentistry, University of Sheffield, was searched for specimens which had been histologically diagnosed as oral squamous cell carcinoma. Pathology records were reviewed to identify cases that had metastasised and cases that had not. Metastatic cases were defined as histologically proven metastastic lesions in a cervical lymph node. Only cases which had been treated by primary surgery were included, cases that had received radiotherapy before surgical removal were excluded. The patient database was also searched for clinicopathological and demographic information, including age, gender and location. Disease stage was defined in

accordance with the TNM classification by the International Union Against Cancer (UICC) (Sobin and Wittekind, 2002). All sections and blocks were reviewed to confirm the diagnosis and to ensure that sufficient material was available for study. The grade of each tumour (differentiation) was retrieved from the histological reports and was confirmed on review.

A total of one hundred and forty two cases were included in the study divided as follow:

- 17 cases of normal oral mucosa (NOM)
- 47 cases of OSCC that had not metastasised (OSCC NM)
- 39 cases of OSCC that had metastasised (OSCC M)
- 39 cases of matched metastatic lesions from cervical lymph nodes (OSCC LN)

3.3 Tissue Microarrays

For immunocytochemical studies, tissue specimens were prepared into tissue microarrays. This enabled more rapid analysis of markers associated with disease diagnosis and prognosis.

Tissue microarrays of specimens were constructed using a Beecher manual micro-array system (The L.S. Starrett Company, USA), with array punches 1.0 mm in diameter (Beecher instrument Inc., Sun Prairie, Wisconsin, USA) (**Figure 3.1**). This technique involves removing a core of tissue from the specimen (donor block) and transferring it into a new paraffin block (recipient

block). A recipient block may include up to a hundred or more cores, enabling staining and examination of many samples at the same time.

For each sample a haematoxylin and eosin stained section was examined and representative areas of the tumour were selected and marked on the glass slide using a cytology slide marker (Nikon). Care was taken to select representative areas of tumour with minimal stroma, and avoiding areas of necrosis or ulcer. The marked slide was then superimposed on the paraffin block to identify and mark the sites on the donor block from where the TMA cores were to be taken. For each OSCC primary tumour sample, 3 areas were sampled:

- The surface
- The centre of the lesion
- The advancing front

Whenever possible three cores were taken from each of the three areas, giving a maximum of 9 samples per tumour. Some tumours were too small to obtain cores from all three areas, in which case two areas (6 cores) were sampled – the surface and the advancing front. For the metastatic lesions three representative cores were taken from each tumour. Each TMA block contained 48 to 60 cores (**Figure 3.1**). In each block, one core of a non-relevant sample (epithelial tissue) was placed in the bottom right corner for orientation purposes.

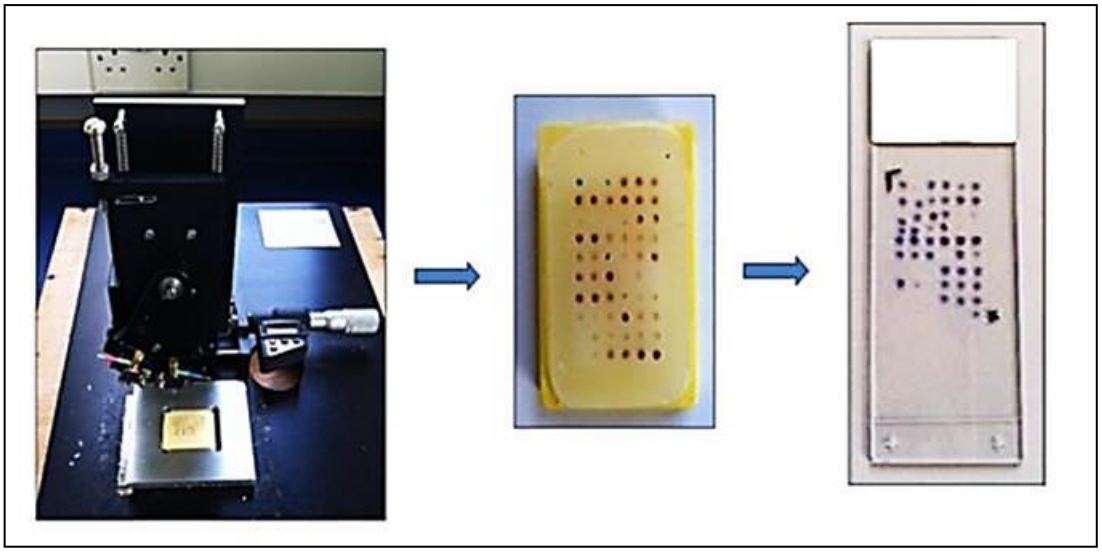

Figure 3.1: Construction of Tissue Micro Arrays (TMA). Areas of interest were identified on an H&E stained slide and then punched from the donor block using a Beecher manual micro-array system. Cores were then inserted into a recipient paraffin wax block (Centre). Each block contained up to 60 representative cores of the tissue specimens.

3.4 Immunohistochemistry

3.4.1 Antibodies used in the IHC

3.4.1.1 MCM2

A rabbit polyclonal antibody raised against human MCM2 was provided by Sigma (Sigma-Aldrich, USA). MCM2, the presence of which has been determined with human homolog BM28, a protein of 250 amino acids that encodes the ATPase active site (Costa and Onesti, 2009). Its IgG1 subclass reacts with human MCM nuclear antigen. It has been reported that MCM2 is expressed throughout the G1 phase of the cell cycle and is gradually lost as S phase proceeds. However, its expression is very low in G0 phase.

3.4.1.2 Ki-67

Anti-human (clone MIB-1) monoclonal mouse antibody was obtained from Dako (Glostrup, Denmark). The Ki-67 nuclear antigen is differentially expressed as isotype IgG1 during all active phases of the cell cycle (late G1, S, M and G2), but absent in non-cycling cells (G0 phase).

Immunohistochemical and flow cytometric analysis has shown that MIB-1 reacts with Ki-67 antigen and is a valuable indicator of the growth fraction of cells undergoing active division in normal and neoplastic conditions. Thus, MIB-1 antibody has been recognised as the ideal monoclonal antibody for detecting the Ki-67 antigen in routinely formalin-fixed paraffin embedded specimens (Kubbutat et al., 1994).

3.4.1.3 Geminin

Polyclonal rabbit anti-human geminin (clone ab12147) was provided by Abcam (Cambridge, UK). Geminin has a role in the governance of licensing in the G2 phase and is negatively regulated during S phase to prevent DNA re-replication. This antibody has been previously detected in western blots of cell lysates from a human embryonic kidney cell line. Immunogen affinity purified antibody detected a band at 33 kDa (Hochegger et al., 2007); this band can be blocked by the immunising peptide.

3.4.1.4 Cyclin D1

Monoclonal rabbit anti-human cyclin D1 (clone EP12) was obtained from Dako (Glostrup, Denmark). This antibody reacts with cyclin D1 antigen encoded by the bcl-1 gene located on chromosome 11q13. Cyclin D1 is involved in normal cell cycle regulation and tumour transformation in mammalian cells. During the cell cycle cyclin D1 protein is responsible for progression from the G1 phase to the S phase by pRb phosphorylation. The corresponding antigen has been found useful for demonstration of cycling cells in mantle cell lymphomas (Donnellan and Chetty, 1998).

3.4.1.5 $p16^{INK4A}$

Purified anti-$p16^{INK4A}$ mouse monoclonal antibody (obtained from Abcam, Cambridge, UK) is isotype IgG2b and is encoded by the gene CDKN2A. CDKN2A homozygous deletions or mutations are frequently seen in a variety

of malignant tumours, particularly melanoma and it is known to be an important tumour suppressor gene. This protein expression strongly correlates with infection by human papilloma virus (HPV) (Lewis et al., 2012).

3.4.2 Immunohistochemical staining procedures

Four-micrometre sections of the TMA paraffin blocks were cut and mounted on coated slides (Menzel Gläser, Germany) and used for IHC. Details of the antibodies used in IHC are listed in **Table 3.1**. Sections were dewaxed in 2 changes of xylene (5 minutes each) and rehydrated in absolute alcohol (100% alcohol x 2, 5 minutes each). To suppress endogenous peroxidase activity, sections were treated with freshly prepared hydrogen peroxide in methanol (3% Met OH) for 30 minutes at room temperature. Then, a heat-mediated antigen retrieval protocol was carried out using citrate buffer (pH 6.0) (Appendix 1) in a steamer, for 30 minutes for MCM2, Ki-67, geminin and $p16^{INK4A}$. For cyclin D1 protein, Tris-EDTA buffer (pH 9.0) was used for 30 minutes (Appendix 1). Slides were then allowed to cool down and briefly washed with phosphate buffered saline (PBS) (Appendix 1) using a stirrer to ensure adequate washing.

To prevent false positive and high background staining, sections were blocked by incubating for 60 minutes, with normal horse serum for mouse monoclonal antibodies, or goat serum for rabbit polyclonal antibodies. Primary antibodies were diluted and incubated as detailed in **Table 3.1**. Next, slides were washed with PBS buffer for 5 minutes. The visualisation system was the ABC Standard kit (Vector laboratories, US) that includes incubation with

secondary biotinylated antibody and with an appropriate substrate Vectastain for 30 minutes each at room temperature. Two washes of PBS were applied for 5 minutes each between secondary and Vectastain steps. Positive staining was detected after 6 minutes incubation with Vector Novared (Vector Laboratories, US). Finally, the slides were rinsed twice with distilled water to stop the hydrogen peroxide reaction, and then counterstained with Mayer`s haematoxylin (Appendix 1) before being dehydrated and mounted with low viscosity DPX mounting media. In order to ensure the specificity of antibodies and that there was no contamination, negative controls without primary antibody were run during the standardisation process. Sections of normal oral mucosa were used as positive controls and underwent the same procedure for MCM2, Ki-67, geminin and cyclin D1. Two sections of oropharyngeal carcinoma known to be positive for HPV were used as a positive control for $p16^{INK4A}$.

Antibodies	Clone	Supplier	Clonality	Dilutions & incubation time
MCM2	BM28	Sigma	Rabbit polyclonal anti-human	1:1000, overnight at 4°C
Ki-67	MIB-1	Dako	Monoclonal mouse anti-human	1:75, overnight at 4°C
Geminin	ab12147	Abcam	Rabbit polyclonal anti-human	1:300, overnight at 4°C
Cyclin D1	EP12	Dako	Rabbit monoclonal anti-human	1:100, overnight at 4°C
$p16^{INK4A}$	2D9A12	Abcam	Mouse monoclonal antibody	1:500, overnight at 4°C

Table 3.1: Details of the antibodies used in immunohistochemistry.

3.4.3 Protein expression analysis

3.4.3.1 Traditional cell counting method

After the staining of the TMA sections the levels of expression of each protein were anaylsed by cell counting to determine a labelling index (LI). The number of positively and negatively stained epithelial cells were counted in eight to ten non-overlapping microscope fields using a x40 objective to give a final magnification of x400. Immunostaining for all cell cycle proteins was evaluated blindly without any knowledge of the clinical data.

3.4.3.2 Determination of cell count

The cumulative mean method was used (Williams, 1977) to estimate the total number of cells that needed to be counted to produce a consistent result. Malignant epithelial cells were counted in random fields and the number of positive and negative cells were recorded for each field in turn. After the second field was counted, the average of fields 1 and 2 was determined. This process continued for each field in turn and the cell counts in each subsequent field were included to produce a cumulative mean. The number of cell counts required to produce three consecutive running means within +/-5% of the final average is considered to be the minimum number of cells required to achieve a reliable result (Williams, 1977). The number of tumour cells that needed to be counted can then be determined. A total of 800 to 1500 cells need to be counted. Whenever possible the maximum number of cells was

counted (1500). However, in some cases the specimens were too small to count the maximum and fewer cells were counted (800).

3.4.3.3 Cell Counting

High power fields (x400 magnification) were identified using a stratified random sampling method. The first field was selected randomly and subsequent fields selected by moving the microscope stage one field horizontally or vertically as required. Usually ten fields were required to ensure that 800 to 1500 nuclei were counted and analysed. An eyepiece graticule was used to delineate the counting area and all cells that were inside the square of the graticule were counted. The positive, the negative and total number of cells were counted using a manual cell counter and recorded on an excel spread sheet. Positive expression was considered as nuclei with light to dark brown staining, negative cells showed counterstain only, as any shade of blue colour. The labelling index (LI) was calculated by dividing the total number of positive cells by the total number of cells counted per case and multiplied by 100.

3.5 DNA image cytometry

3.5.1 Preparation for Feulgen-Schiff staining

TMAs were not used for DNA analysis; instead, sections from paraffin blocks of each case were taken before the preparation of the TMAs. Seven micrometre thick sections were cut and mounted on coated slides (X-tra adhesive, Surgipath, USA). Sections were placed in slide boxes to dry and stored in a dust-free environment at room temperature. Sections were deparaffinised with three washes of xylene for 5 minutes each, and rehydrated with different concentrations of alcohol for 3 minutes (absolute alcohol, 95% and 70%) followed by distilled water (2 minutes).

3.5.2 Feulgen-Schiff staining

The slides were placed in a Coplin jar containing 5N hydrochloride acid (HCL) (Appendix 1) for 1 hour at room temperature in a fume hood; the jar was completely sealed with Parafilm during the incubation. Subsequently, the slides were rinsed with distilled water for 2 minutes, and immersed in blue Feulgen stain for 1 hour (Scy Tek Laboratories Kit, USA). Afterwards, the slides were washed in three changes of distilled water for 2 minutes. Next, they were placed in 3 changes of rinse solution (Scy Tek Laboratories Kit, USA) for 5 minutes followed by 3 washes of distilled water (2 minutes each). Finally, the slides were dehydrated with increasing concentrations of ethanol (70%, 95% and 100%) and fixed with xylene. A cover slip was mounted with low viscosity DPX on each slide.

3.5.3 Image cytometry for DNA analysis

The intensity of Feulgen staining was measured using an Automated Cellular Imaging System, ACIS III (Dako, Glostrup, Denmark). This machine consists of a microscope unit (4, 10, 20, 40 and 60X objectives) with an automated X-Y stage, a digital JAI camera 3-chip, 30 frames per second, connected to a CPU with Dual Xeon Intel, LCD monitor and label printer (InfoGlyph label) (**Figure 3.2**). For optimal function, a calibration kit provided by the manufacturer was run daily in the system.

Briefly, ACIS III uses advanced colour detection software that is pre-programmed for various parameters of analysis including microscope objective, stain colour and/or intensity of cellular objects. There are two main steps for ploidy anaylsis using ASCI III.

Firstly, in the accessioning step (**Figure 3.3**), each stained slide is labelled with a unique barcode to create a unique patch that is used by the ACIS III database for reference and to track information and to analyse results, then labels are printed and stuck onto the slides.

Secondly, in the analysis step, each slide is scanned by the automatic microscope to create a virtual image of the whole section (**Figure 3.4**). Based on morphometric characteristics of whole cells as measured by the software application, such as size, shape and integrated optical density (IOD), the application is then used to capture each field for recording data such as colour and chromogen intensity.

Figure 3.2: Automated Cellular Imaging System ACIS III (Dako, Denmark).

This process had to be repeated twice for the same slide in each group (except OSCC lymph node metastasis) and recorded with a different barcode and different patch name to be able to analyse different areas in the same sample (surface and advancing front). This was because the ACIS III does not provide multiple area measurements in a single section (same slide).

The software algorithms built into the system were then used to allow the computer to automatically select, first, control cells, and then potential malignant cells. The system has different cutting levels (**Figure 3.4**) for tumour cell collection whereby sample level L5 has the most aggressive cutting algorithm and only selects separated cells. Once the slides were scanned the histological reconstructions were reviewed and the imaging system identified and the cells that had been automatically selected were outlined with a thin blue line (**Figure 3.4**). The histological reconstruction is a high resolution image that allows complete section to be reviewed for accurate selection of representative areas. The operator then reviews the image and confirms visually that the selected cells meet the criteria for control cells or malignant cells. Unwanted cells may be rejected, for example, incomplete sections of nuclei, overlapping nuclei or obvious cell debris can be selected and rejected. Once the control cells have been checked and selected, and the control peak (2c) assigned, tumour nuclei in the target area are collected and presented as montage images (**Figure 3.5**).

At least three hundred qualified malignant epithelial nuclei were selected and approximately 50 control nuclei (lymphocytes) in the same tissue were obtained at sample level 3 (L3). Cells can also be accepted or rejected by the operator from the montage window (**Figure 3.5**). The operator can also do further analysis by navigating between individual nuclei and their exact location on the histogram. The results are generated automatically by ACIS as a report, and displayed as a histogram, which contains assessment parameters such as DNA index, coefficient of variation (CV) and the proportion of nuclei that exceed 5C (5cER) (**Figure 3.5**).

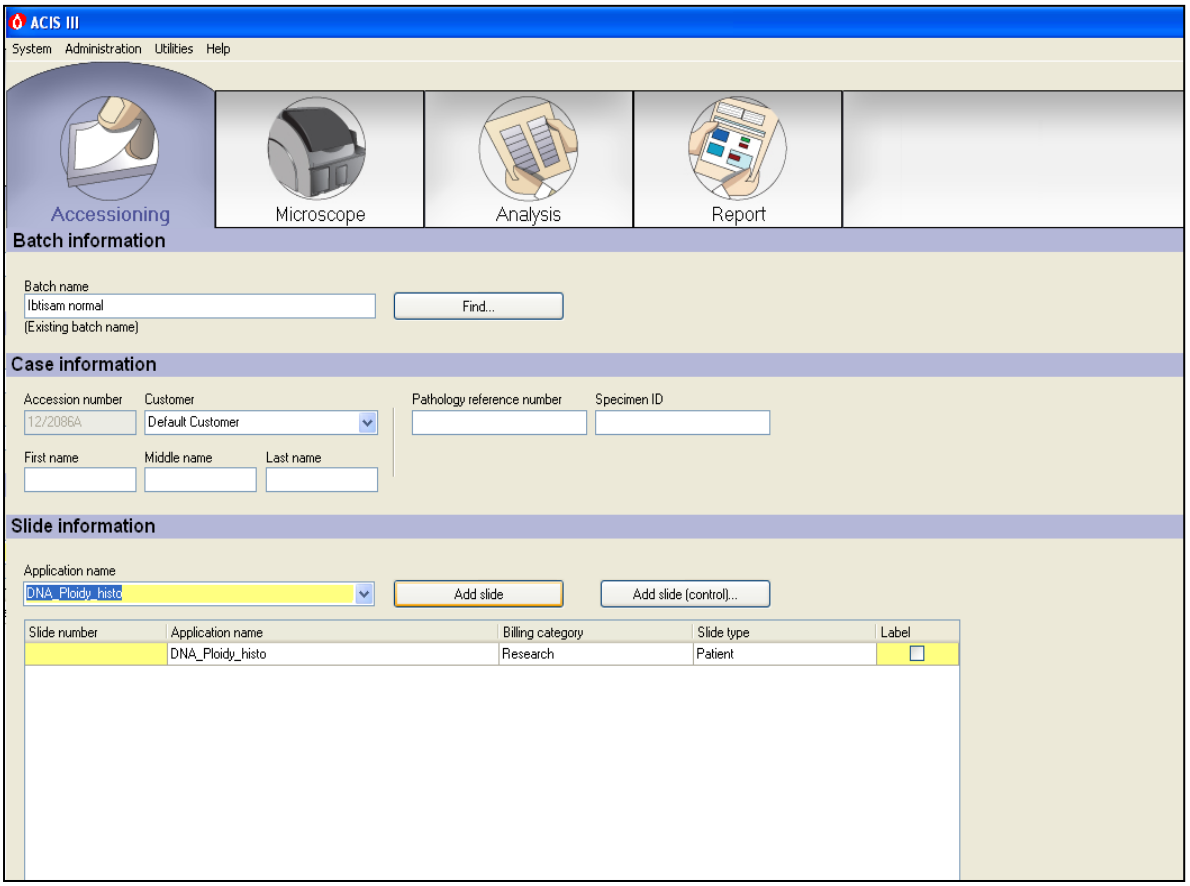

Figure 3.3: Screen shot of ACIS III showing the first step of ploidy analysis (Accessioning). Firstly, assign a batch name, then give slide an accession number (labelled with a unique barcode). Next, select application name and add slide. Finally, labels print, and stuck onto the slide.

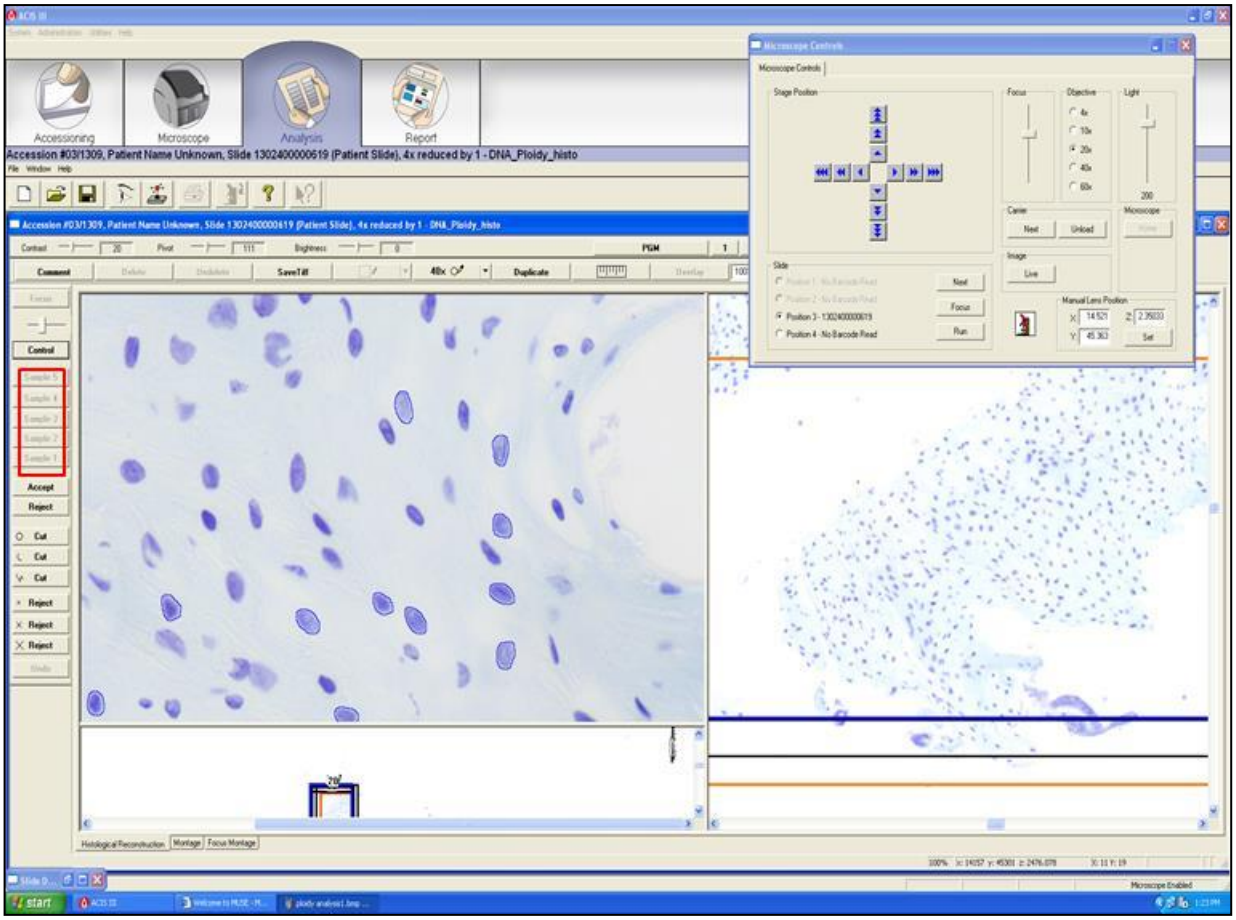

Figure 3.4: Screen shot of ACIS III showing the second step of ploidy analysis. Different cutting levels from L1 to L5 (red rectangle), where sample level L5 has the most aggressive cutting algorithm. On the left side is the high power view of the scanned image, which can be seen on the right.

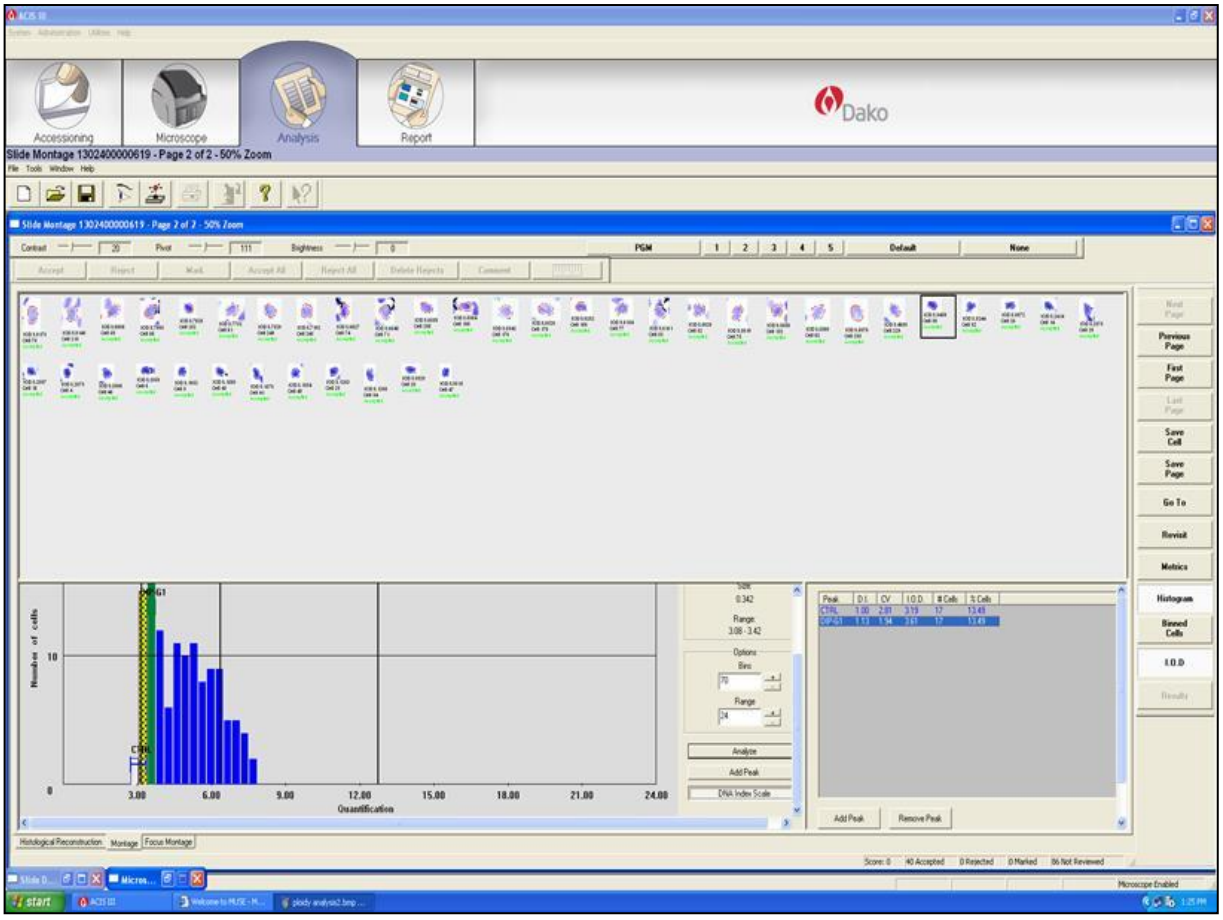

Figure 3.5: ACIS III montage window of microscopically edited Feulgen-stained nuclei (top). Once the DNA content histogram has been generated on the basis of integrated optical density (IOD), this montage window allows the operator to undertake further analyses (accepting or rejecting undesired cells).

3.5.4 Criteria for classification of DNA histograms

DNA histograms were analysed by one observer (IZ). DNA histograms were evaluated according to the criteria proposed by Huang *et al* (Huang et al., 2005) and Yu *et al.* (Yu et al., 2007) and previously used in our department (Santos-Sliva et al., 2011). The DNA index and coefficient of variation (CV) parameters were calculated for each sample. The CV was maintained at less than 5% to ensure the consistency of the cell selection.

A diploid specimen was defined as only one peak at 2c (which presented at 0.3 intervals on the ACIS histogram) during the G0/G1phase, and if the number of nuclei with a DNA content of more than 5c did not exceed 1% of the total (**Figure 3.6**). Aneuploidy was defined if separate aneuploid peaks were identified, located within 0.3, 0.6, 0.9….etc. intervals, or the number of cells exceeding 5c (pentaploid range) was more than 1% (**Figure 3.7**). Ploidy was also classified according to the value of the DNA index as follows:

- Diploid (DI= 0.9 – 1.10)
- Mild aneuploid (DI = 1.11 – 1.30)
- Moderate aneuploid (DI = 1.31 – 1.80)
- Severe aneuploid (DI = > 1.81)

A lesion was characterised as tetraploid if the DI was between 1.81 and 2.20, which was considered to represent a subtype of severe aneuploidy. In the case of two peaks, the DI of the most prominent peak was considered (**Figure 3.7**).

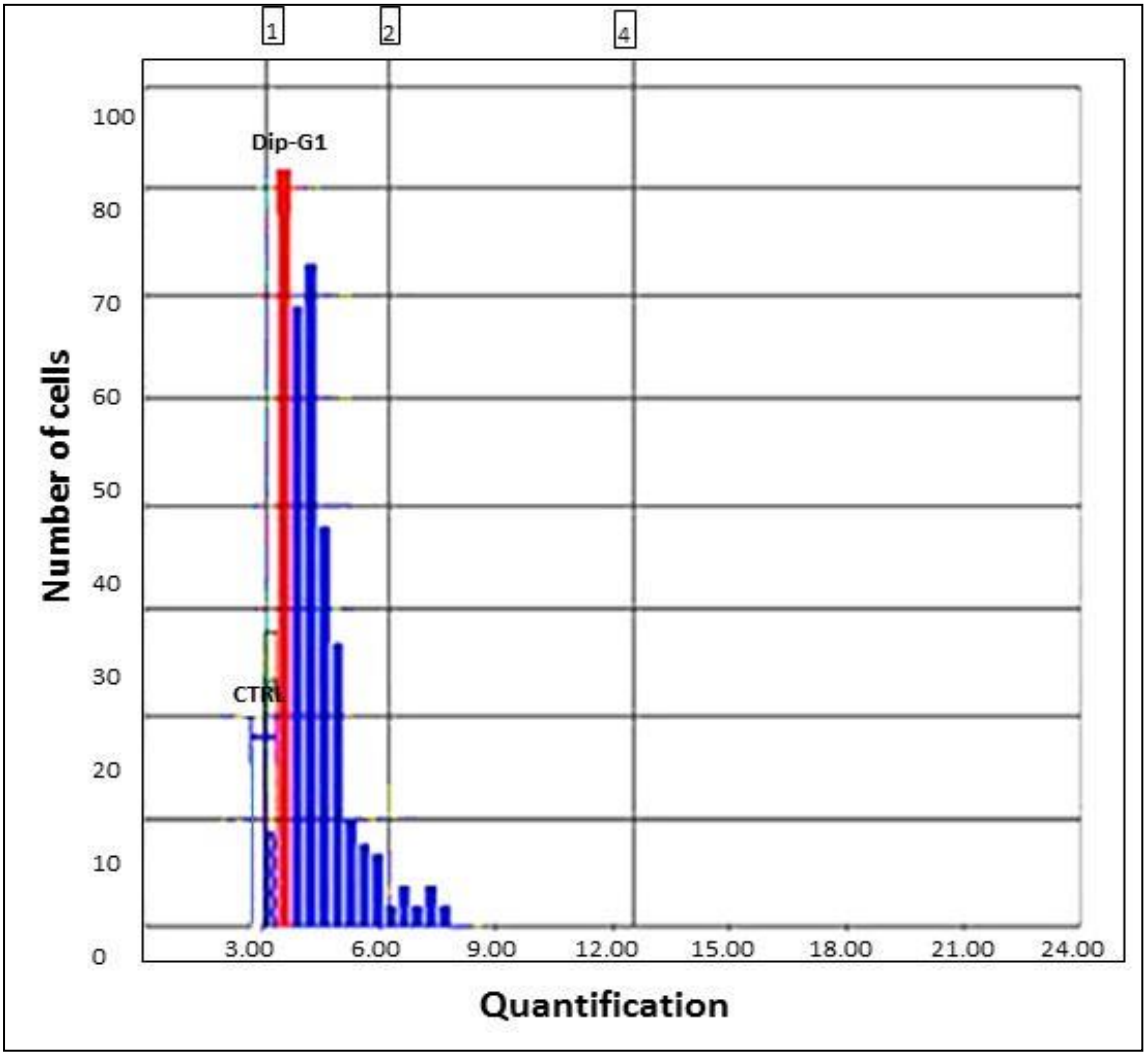

Figure 3.6: An example of a diploid histogram generated by ACIS, with a single peak in c2 (represented as 3.00 interval in the histogram) and no cells that exceed 5c. CTRL indicates the control G1 peak and Dip-G1 indicates the diploid G1 peak.

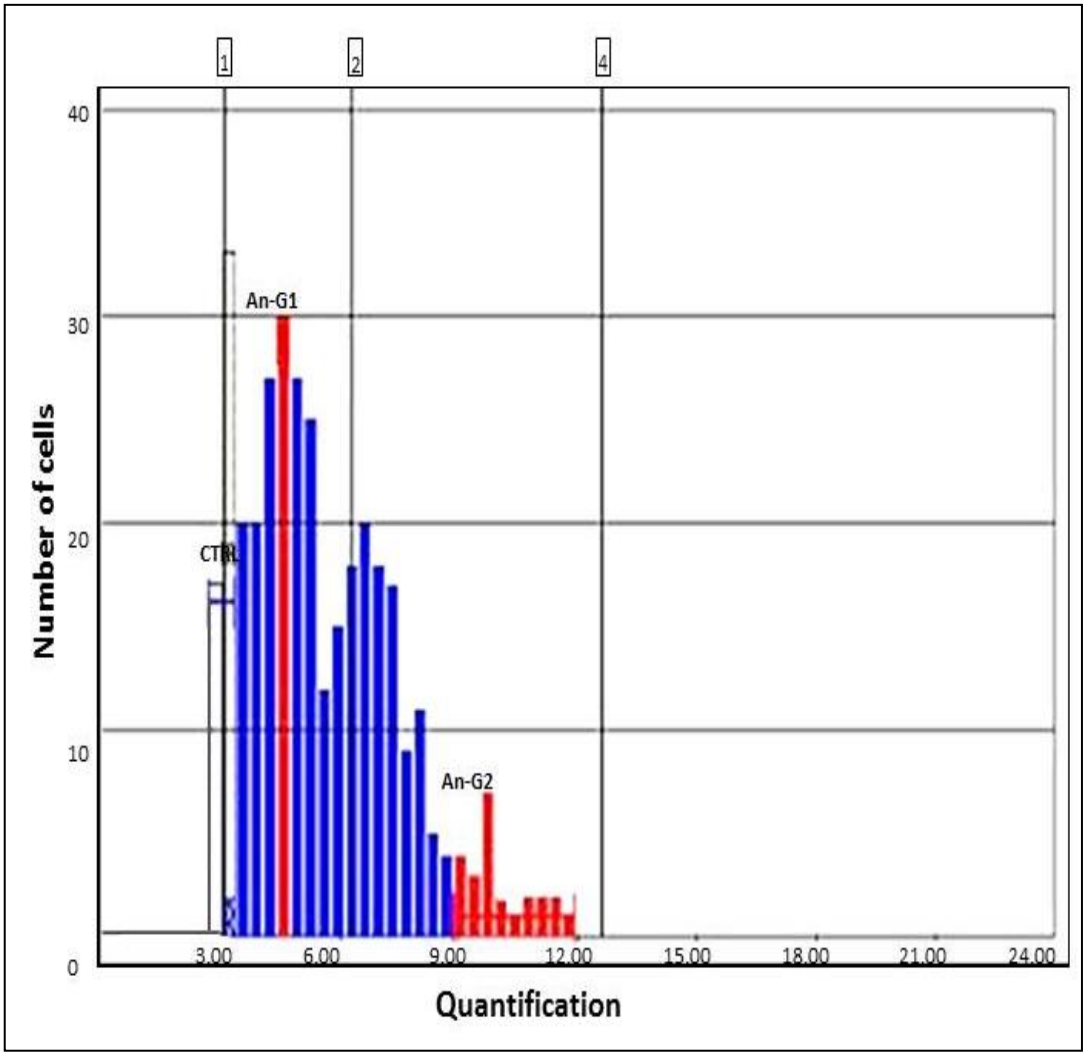

Figure 3.7: Example of a moderate aneuploid histogram with two prominent peaks. In this case DI = 1.5 and more than 1% of cells exceed 5c. CTRL indicates the control G1 peak, An-G1 indicates the aneuploid G1 peak and An-G2 indicates the aneuploid G2 peak.

3.6 Statistical analysis

Statistical analysis of all data in this study was undertaken using SPSS statistical software (Version 21.0).

The Two-tailed Kolmogorov-Smirnov test was used to evaluate the distribution of data in each group (Appendix 2). Cross tabulation tables were created to show the age, gender and site distribution among groups. The labelling index (LI) of MCM2, Ki-67, geminin and cyclin D1 was normally distributed in all analysed groups (NOM, OSCC NM, OSCC M and OSCC LN). Therefore, these parameters were analysed using parametric tests.

For the ploidy analysis, the DI and 5cER parameters were normally distributed in most of the groups with the exception of the DI in the OSCC LN and 5cER in OSCC M. Nominal data such as ploidy status were analysed by non-parametric tests. Non-parametric tests are often more powerful in detecting differences in given populations, therefore data were treated as not normally distributed.

3.6.1 Evaluation of protein expression in NOM, OSCC NM, OSCC M and OSCC LN

ANOVA and t-test were used to compare the mean LI of MCM2, Ki-67, geminin and cyclin D1 protein between groups (NOM, OSCC NM, OSCC M). Paired t-test was used to evaluate differences in proteins expression between OSCC M and their matched OSCC LN.

Bar charts and scatter plots were generated to demonstrate the protein distribution among the groups. A p value less than 0.05 was considered statistically significant.

3.6.2 Evaluation of DNA image cytometry and ploidy status in OSCC NM, OSCC M and OSCC LN

Mann-Whitney and Wilcoxon Signed tests were used to compare the DI, 5cER and ploidy status among groups. Receiver operator characteristic (ROC) curves were plotted using the DNA index and 5cER values for each sample. Sensitivity, specificity, positive predictive value and negative predictive value were calculated to evaluate the DNA image cytometry as a prognostic method.

3.6.3 Correlation of protein expression and ploidy status

The Spearman rank correlation coefficient test was used to identify any correlation between protein expression (MCM2, Ki-67, geminin and cyclin D1) and ploidy status in OSCC lesions.

CHAPTER 4: EXPRESSION OF CELL CYCLE PROTEINS IN OSCC AND MATCHED LYMPH NODE METASTASES

4. EXPRESSION OF CELL CYCLE PROTEINS IN OSCC AND MATCHED LYMPH NODE METASTASES

4.1 Introduction

The molecular mechanisms that control entry to the cell cycle are highly conserved processes. The precise events include the formation of the pre-replication complex (pre-RC) by the origin recognition complex (ORC) in early G1 phase (Symeonidou et al., 2012). The ORC is required for the recruitment of cdc6, MCM2-7 and Cdt1 at the replication origin (Section 1.3.1 and **Figure 1.4**). Subsequently, CDK and Dbf 4-dependent kinase are recruited to initiate the replication process. These, along with geminin (Cdt1 inhibitor), impede loading of the MCM2–7 helicase at origin (Aparicio, 2013) and prevent another cell cycle round. Once mitosis is complete, the licensing proteins ensure the genomic stability in the cell cycle (Labib, 2010).

Expression of minichromosome maintenance proteins (MCM2-7) is required for DNA replication and cell cycle initiation (Symeonidou et al., 2012). MCM proteins are expressed throughout the cell cycle phases, including cells leaving the quiescent phase (G0) to enter into early G1 phase (licensed to proliferate). Notably, this characteristic is not found in other proliferation markers (Ki-67 or geminin), therefore, MCM2 in particular has been proposed as a sensitive prognostic marker that may indicate the presence of an increased growth fraction in malignant lesions. MCMs are emerging as

powerful cancer diagnostic tools (Torres-Rendon et al., 2009a, Kodani et al., 2003, Gueiros et al., 2011, Szelachowska et al., 2006, Shalash et al., 2012). Due to its predominance as a potential proliferation marker in normal and malignant lesions, MCM2 will be the focus of this project.

Ki-67 protein is expressed in all cell cycle phases except early G1 (Gerdes et al., 1984). It has been suggested that evaluation of this protein along with MCM2 and geminin may provide additional information about the proliferation rate in epithelial cells (Sections 1.4 and 1.5). The MCM2/Ki-67 ratio has recently been used as a prognostic parameter to estimate the population of cells that are licensed to proliferate (Shetty et al., 2005, Torres-Rendon et al., 2009a). A high ratio indicates that many cells are in cycle, and could indicate a poor prognosis (Dudderidge et al., 2005).

Geminin is a proliferation marker expressed only during the S-G2-M transition (Section 1.5 and **Figure 1.5**). It is believed to regulate the cell cycle initiation by preventing the second pre-RC assembly once replication has taken place (Guo and Sun, 2013). Geminin expression may be altered in cancer cells as it has been found to be over-expressed in several cancer cell lines (Xouri et al., 2004). The geminin/Ki-67 ratio estimates the relative length of G1, where a short G1 phase will approximate to a ratio of 1, cells with a prolonged G1 will approximate to a ratio of 0 (Wharton et al., 2004). The higher the ratio, the faster the rate of cell division, and the worse the prognosis (Torres-Rendon et al., 2009a).

$p16^{INK4A}$ is a tumour suppressor gene that plays an important role in cell cycle regulation during the G1 phase. Down-regulation or inactivation of this protein in human cancer may help to evaluate tumour progression, recurrence and survival (Dong et al., 2012, Kumar et al., 2008, Chen et al., 2012). High expression of $p16^{INK4A}$ is seen in HPV-associated oropharyngeal cancer (Westra, 2015). $p16^{INK4A}$ over-expression has been considered as an HPV surrogate marker in OPC (Seiwert, 2014). However, high HPV positivity has been reported in OPC compared to OSCC (Laco et al., 2011). Therefore, OSCC and OPC cancers should be regarded as two entities (Auluck et al., 2014). Expression of $p16^{INK4A}$ was tested in this study to ensure that this cohort is confined to non-HPV associated OSCC.

The cyclin D1 is a key regulatory protein required for transition from G1 to S phase by binding to cyclin-dependent kinases (CDK4 and CDK6). Its over-expression has attracted significant attention because of its role in tumourgenesis. Cyclin D1 may be a valuable prognostic indicator for oral squamous cell carcinoma (Huang et al., 2012, Hanken et al., 2014), however, the exact role remains controversial. In addition, high levels of cyclin D1 were suggested to be associated with lymph node metastasis in OSCC (Huang et al., 2012).

Generally, OSCC is characterised by a high rate of recurrence and metastasis to the regional lymph nodes. Nodal metastasis is considered to be a significant prognostic indicator (Noguti et al., 2012). However, tumour size, grade of differentiation and disease stage are also important factors which affect cervical metastasis and prognosis.

In summary, analysis of these bio-markers may help to evaluate the cell cycle phase distribution in OSCC NM, OSCC M and OSCC LN, and may provide additional prognostic information.

The purpose of this study was to look at the heterogeneity observed in the oral squamous cell carcinoma lesions and to evaluate the expression levels of these proteins in OSCC NM, OSCC M and OSCC LN. Additionally, I sought to determine if altered expression could be used as a prognostic indicator of tumour progression and predict nodal metastasis in OSCC.

4.2 Materials and methods

Four micrometre (4µm) TMA sections were cut and immunostained with antibodies to MCM2, Ki-67, geminin, cyclin D1 and $p16^{INK4A}$ according to the protocol and methodology explained in Section 3.4.2. Dilutions and incubation times are summarised in Chapter 3 (**Table 3.1**). Oropharyngeal carcinoma sections were used as a posititive control to ensure antibody specificity for $p16^{INK4A}$. Immunostaining of $p16^{INK4A}$ was considered positive if there was strong and diffuse nuclear and cytoplasmic staining in more than 70% of tumour cells (Lewis et al., 2012, Nankivell et al., 2014). Protein expression was analysed and labelling index (LI) was calculated in each area (surface, middle and front) for each case as previously described in section 3.4.3. The final analysis was based on all the TMA cores counted for each case and LI for the total was calculated.

4.2.1 Statistical analysis

Statistical analysis was described in Chapter 3, Section 3.6. Parametric ANOVA and t-tests were used to evaluate the differences in the nuclear LI of MCM2, Ki-67, geminin and cyclin D1 in the NOM, OSCC NM, OSCC M and LN metastasis groups. Mean and standard deviations were calculated for the LI of all proteins. The general linear model (GLM) of repeated measures analyses was used to evaluate the differences in the LI of all proteins (MCM2, Ki-67, geminin and cyclin D1) in every group (e.g. NOM, OSCC NM, OSCC M, and OSCC LN).

Bar-charts were generated to observe the differences in protein expression between groups. Scatter plots were prepared to illustrate the association between proteins (MCM2, Ki-67 and geminin).

4.3 Results

4.3.1 Patient information

A total of one hundred and forty two samples were included in this study. In the OSCC NM group (n=47), TMA immunostaining was available in 40 samples for MCM2, 44 samples for Ki-67, 47 samples for geminin, 47 samples for $p16^{INK4A}$ and 47 samples for cyclin D1. In the OSCC M group (n=39), immunostaining was available in 37 samples for MCM2, 39 samples for Ki-67, 39 samples for geminin, 39 samples for $p16^{INK4A}$ and 39 samples for cyclin D1. In the LN metastasis group (n=39), 32 samples for MCM2, 26

samples for Ki-67, 33 samples for geminin, 32 samples for cyclin D1 and 35 samples for $p16^{INK4A}$ were available for analysis.

There were some missing cases due to missing TMA cores which were lost during the sectioning or during the immunostaining procedure. Additionally, some TMA cores did not show immuno reactivity and were therefore excluded from analysis. However, in all positive cases, LI was recorded from 6 to 9 TMA cores for each case.

Demographic details for all 142 samples are shown in **Table 4.1** and **Table 4.2**. These include age, gender (**Table 4.1**) and lesion site (**Table 4.2**). The OSCC differentiation status was retrieved from the histological reports and is shown in **Table 4.3**. The OSCC NM and OSCC M were histopathologically graded by an expert pathologist (PMS). From the 47 OSCC NM samples analysed for protein expression, 21 were well differentiated, 20 were moderately differentiated and 6 were poorly differentiated (**Table 4.3**). In OSCC M samples 13 were well differentiated, 20 were moderately differentiated and 6 were poorly differentiated (**Table 4.3**). In the metastatic samples (OSCC LN) 15 cases were well differentiated, 21 were moderate and 3 cases were poorly differentiated (**Table 4.3**). Tumour size (pT classification) and clinical stage of disease are shown in **Table 4.3**.

The mean age of NOM was 51.55 years with a standard deviation of 15.71 (range; 24.50-74.50). In the OSCC NM group the mean age of patients was

63.86 years (SD=12.58, range; 34.50-64.50) and was 61.67 (SD=12.55, range; 34.50-84.50) in the OSCC M group.

Representative examples of immunoexpression of MCM2, Ki-67, geminin and cyclin D1 in NOM, OSCC NM, OSCC M and OSCC LN samples are shown in **Figure 4.1**, **Figure 4.2**, **Figure 4.3** and **Figure 4.4**. $p16^{INK4A}$ negative and positive controls are shown in **Figure 4.5**.

	Groups			
	NOM	**OSCC NM**	**OSCC M**	**Total**
Age (by decades)				
20-29	2 (12)	0	0	2
30-39	1 (6)	1	2	4
40-49	5 (29)	7	3	15
50-59	4 (24)	7	13	24
60-69	2 (12)	15	10	27
70-79	3 (17)	13	8	24
80-89	0	4	3	7
Total	17	**47**	**39**	**103**
Gender (%)				
Male	8 (47)	32 (70)	26 (67)	66
Female	9 (53)	15 (30)	13 (33)	37
Total	**17**	**47**	**39**	**103**

NOM= Normal oral mucosa.
OSCC M= Oral squamous carcinoma that had metastases.
OSCC NM= Oral squamous carcinoma that did not metastasise.

Table 4.1: Demographic data (age and gender) for all groups .

Groups	Lesion sites										Total
	Tongue	Floor of mouth	Palatal mucosa	Hard palate	Soft palate	Alveolar mucosa	Buccal mucosa	Tonsil area	Pharyngeal wall	Retromolar area	
NOM	6	1	1	0	1	3	3	2	0	0	17
OSCC M	17	8	1	0	1	6	6	0	0	0	39
OSCC NM	16	8	1	1	2	8	7	1	2	1	47
Total	39	17	3	1	4	17	16	3	2	1	103

NOM= Normal oral mucosa
OSCC M = Oral squamous carcinoma that had metastases.
OSCC NM= Oral squamous carcinoma that did not metastasise.

Table 4.2: Site of lesions from all patients in all groups.

	Groups			Total
	OSCC NM n (%)	**OSCC M n (%)**	**OSCC LN n (%)**	
Tumour grad				
Well	21 (45)	13 (33)	15 (38)	49
Moderate	20 (43)	20 (51)	21 (54)	61
Poor	6 (13)	6 (15)	3 (8)	15
Total	47	39	39	125
Tumour stage (TNM)				
I	15 (32)	0	-	15
II	15 (32)	0	-	15
III	17 (36)	8 (21)	-	25
IV	0	27 (69)	-	27
NA	-	4 (10)	-	4
Total	47	39		86
Tumour size stage				
pT1	15 (32)	7 (18)		22
pT2	15 (32)	10 (26)		25
pT3	17 (36)	4 (10)		21
pT4	0	18 (46)		18
Total	47	39		86
Extra capsular spread				
Yes	-	-	16 (41)	16
No	-	-	20 (51)	20
NA	-	-	3 (8)	3
Total			39	39

OSCC NM= Oral squamous carcinoma that did not metastasise.
OSCC M = Oral squamous carcinoma that had metastases.
OSCC LN= Lymph node metastases.
NA= Not available.

Table 4.3: Distribution of OSCCs according to their clinicopathological parameters in all groups.

4.3.2 $p16^{INK4A}$ expression

Sections of oropharyngeal carcinoma were used as a positive control of $p16^{INK4A}$ and showed strong nuclear and cytoplasmic staining in the malignant epithelial cells. Negative control sections showed no staining (**Figure 4.5**). The $p16^{INK4A}$ staining confirmed that all cases (NOM, OSCC M, OSCC NM and OSCC LN) were negative and therefore this cohort was not HPV associated OSCC. A second observer (PMS) examined all the cases for $p16^{INK4A}$ and found 100% agreement.

4.3.3 Expression of proteins in normal oral mucosa

The NOM samples showed positive nuclear staining of MCM2, Ki-67, geminin and cyclin D1 mainly in the basal and suprabasal cell layers (**Figure 4.1**). This group had the lowest LI expression (60.09) of MCM2, Ki-67 and geminin when compared with OSCC M & OSCC NM groups (**Table 4.4**).

4.3.4 MCM2 expression

Significant differences (p=0.007, ANOVA test) in MCM2 LI values were found among analysed groups (NOM, OSCC NM and OSCC M) (**Table 4.4**). The mean MCM2 LI values were slightly higher in the OSCC M than OSCC NM samples (**Figure 4.6**), but this did not reach statistical significance. Although there were no significant differences, the MCM2 LI was 73.58 in the OSCC M group, and 69.96 in OSCC NM group (**Table 4.4**). The positive nuclear staining of MCM2 showed variable expression in tumour areas;

however, whilst its expression was greater at the invasive front, this was not statistically significant in the OSCC NM and OSCC M groups (p=0.6, p= 0.1 respectively, ANOVA test) (**Table 4.5** and **Figure 4.7**).

The mean MCM2 LI expression showed higher expression in the lymph nodes metastases than in the matched primary OSCC lesions, but the difference did not reach significance (p=0.8, Paired t-test) (**Table 4.6** and **Figure 4.7**).

4.3.5 Ki-67 expression

The mean LI of Ki-67 showed high expression in the NOM samples. There was no difference in Ki-67 expression between OSCC NM and OSCC M (**Table 4.4** and **Figure 4.8**). The mean LIs were 64.61 and 54.23 respectively. There were no differences in Ki-67 expression when the different areas of tumours were compared (**Table 4.5** and **Figure 4.9**). However, Ki-67 expression was higher in OSCC primary lesions (p=0.03, Paired t-test) than in the LN metastases (**Table 4.6** and **Figure 4.8**).

4.3.6 Geminin expression

Overall, the mean geminin LI showed no differences in expression among the analysed groups (NOM, OSCC NM and OSCC M). The mean LI was 27.34 in NOM, 34.23 in OSCC NM group and 31.16 in OSCC M (**Table 4.4**). There were no differences between the different areas of tumour in OSCC M and OSCC NM groups (**Table 4.5**). Differences in mean geminin expression were seen when primary tumours and nodal metastases (OSCC

LN) were compared (p=0.21, Paired t-test), but without reaching statistical significance (**Table 4.6** and **Figure 4.10**).

4.3.7 Cyclin D1 expression

Overall, the mean LIs in NOM, OSCC NM and in OSCC M were 58.99, 55.35 and 56.84 respectively (**Table 4.4** and **Figure 4.12**). However, p values did not reach statistical significance. Cyclin D1 LI expression was slightly higher at the middle and invasive front than the surface when different parts of tumour were compared in OSCC M and OSCC NM groups (**Figure 4.13**); this was statistically significant (p=0.01, ANOVA test) (**Table 4.5**). No significant difference was seen between the primary tumours and the nodal metastases (OSCC LN) (p=0.27, Paired t-test) (**Table 4.6** and **Figure 4.12**).

4.3.8 MCM2/Ki-67 and geminin/Ki-67 ratios

The MCM2/Ki-67 ratio was higher than the geminin/Ki-67 ratio in all samples. There were no significant differences between the LIs of the MCM2 and Ki-67 LIs (determined by the MCM2/Ki-67 ratios) (**Table 4.4** and **Figure 4.16**). The difference between geminin and Ki-67 (demonstrated by the geminin/Ki-67 ratio) increased significantly (p=0.02, t-test) when comparing OSCC NM to OSCC M samples (**Table 4.4** and **Figure 4.17**). The MCM2/Ki-67 and geminin/Ki-67 ratios showed no intra-tumour differences in their LIs in OSCC NM and OSCCM (**Table 4.5**). Both ratios were proportionally lower in the nodal metastases than in the primary

lesions, and the difference was statistically significant (p=0.05, p=0.01 respectively) (**Table 4.6**).

4.3.9 MCM2, Ki-67, geminin and cyclin D1 as a proliferation marker in normal and oral SCC lesions (OSCC M & OSCC NM)

Even though no differences were seen in the mean LIs of MCM2, Ki-67, geminin and cyclin D1 expression between OSCC NM and OSCC M samples (**Table 4.4**), the mean MCM2 LI gradually increased from NOM through OSCC NM to OSCC M (p=0.007, ANOVA test). The proteins LIs were increased in the nodal metastases compared to the primary lesions (**Table 4.6**). However, Ki-67 and geminin showed an increase from NOM to OSCC NM, but not in OSCC M. Cyclin D1 LI showed a tendency towards decreased expression from normal to OSCCs, although this was not statistically significant (**Table 4.4**).

There was a positive linear association between MCM2 and Ki-67 in all analysed groups (**Figure 4.14**). Equally, there was also a strong positive linear association between geminin and Ki-67 in all analysed samples (**Figure 4.15**).

	Labelling index (LI) Mean (Standard deviation)						
	MCM2	**Ki-67**	**Geminin**	**Cyclin D1**	**Stat. GLM**	**MCM2/Ki-67 ratio**	**Geminin/Ki-67 ratio**
NOM (n=17)	60.09 (16.46)	47.16 (21.43)	27.34 (5.97)	58.99 (6.55)	p<0.001	1.55 (0.67)	0.58 (0.23)
OSCC NM (n=47)	69.96 (16.28)	64.61 (28.31)	34.23 (14.11)	55.35 (17.96)	p<0.001	1.35 (1.00)	0.66 (0.27)
OSCC M (n=39)	73.58 (16.12)	54.23 (21.39)	31.16 (15.94)	56.84 (12.97)	p<0.001	1.58 (0.84)	0.68 (0.30)
ANOVA test	p=0.007	NS	NS	NS		NS	NS
p* value	*NS	*NS	*NS	*NS		*NS	p*=0.02

p*=t-test (OSCC NM vs OSCC M).
NOM= Normal oral mucosa.
OSCC NM= Oral squamous carcinoma that did not metastasise.
OSCC M = Oral squamous carcinoma that had metastases.
OSCC LN= Lymph node metastases.
Stat GLM= General linear model.
NS= Not significant.

Table 4.4: Mean and standard deviation of MCM2, Ki-67, geminin and cyclin D1 Labelling index (LI) values, along with MCM2/Ki-67 and geminin/Ki-67 ratios for all groups.

	Labelling index (LI) Mean (Standard deviation)					
	MCM2	**Ki-67**	**Geminin**	**Cyclin D1**	**MCM2/Ki-67 ratio**	**Geminin/Ki-67 ratio**
OSCC NM						
Surface	70.25 (14.33)	58.45 (32.61)	30.43 (16.63)	53.10 (20.42)	1.08 (0.53)	0.48 (0.33)
Middle	70.25 (14.33)	18.85 (30.39)	28.29 (25.90)	59.24 (21.46)	1.46 (0.83)	0.66 (0.28)
Front	72.47 (21.13)	50.93 (28.79)	31.23 (18.74)	57.35 (21.23)	2.73 (7.60)	0.75 (1.12)
ANOVA test	p=0.6	p=0.7	p=0.1	p=0.1	p=0.2	p=0.5
OSCC M						
Surface	62.05 (32.17)	35.51 (37.39)	32.53 (19.30)	50.79 (15.43)	1.35 (1.13)	0.59 (0.30)
Middle	61.43 (30.74)	35.51 (37.39)	24.47 (16.96)	60.42 (16.36)	1.04 (0.71)	0.41 (0.23)
Front	72.84 (23.63)	37.94 (28.74)	28.26 (22.56)	57.98 (17.56)	5.40 (20.29)	1.29 (4.04)
ANOVA test	p=0.1	p=0.6	p=0.2	p=0.01	p=0.3	p=0.6

OSCC NM= Oral squamous carcinoma that did not metastasise.
OSCC M = Oral squamous carcinoma that had metastases.

Table 4.5: Mean and standard deviation of MCM2, Ki-67, geminin and cyclin D1 Labelling index (LI) values, along with MCM2/Ki-67 and geminin/Ki-67 ratios for tumour areas in all groups.

	Labelling index (LI) Mean (Standard deviation)						
	MCM2	**Ki-67**	**Geminin**	**Cyclin D1**	**Stat. GLM**	**MCM2/Ki-67 ratio**	**Geminin/Ki-67 ratio**
OSCC M (n=39)	73.57 (16.20)	54.22 (21.38)	31.16 (15.94)	56.84 (12.97)	p<0.001	1.58 (0.84)	0.68 (0.30)
OSCC LN (n=39)	79.88 (12.66)	49.66 (36.04)	20.75 (21.90)	59.20 (20.81)	p<0.001	1.27 (0.43)	0.31 (0.30)
Paired t-test	p=0.84	p=0.03	p=0.21	p=0.27		p=0.05	p=0.01

OSCC M = Oral squamous carcinoma that had metastasise.
OSCC LN= Lymph node metastases.
Stat GLM= General linear model.

Table 4.6: Mean and standard deviation of MCM2, Ki-67, geminin and cyclin D1 Labelling index (LI) values, along with MCM2/Ki-67 and geminin/Ki-67 ratios in primary tumours (OSCC M) and their lymph node metastasis (OSCC LN).

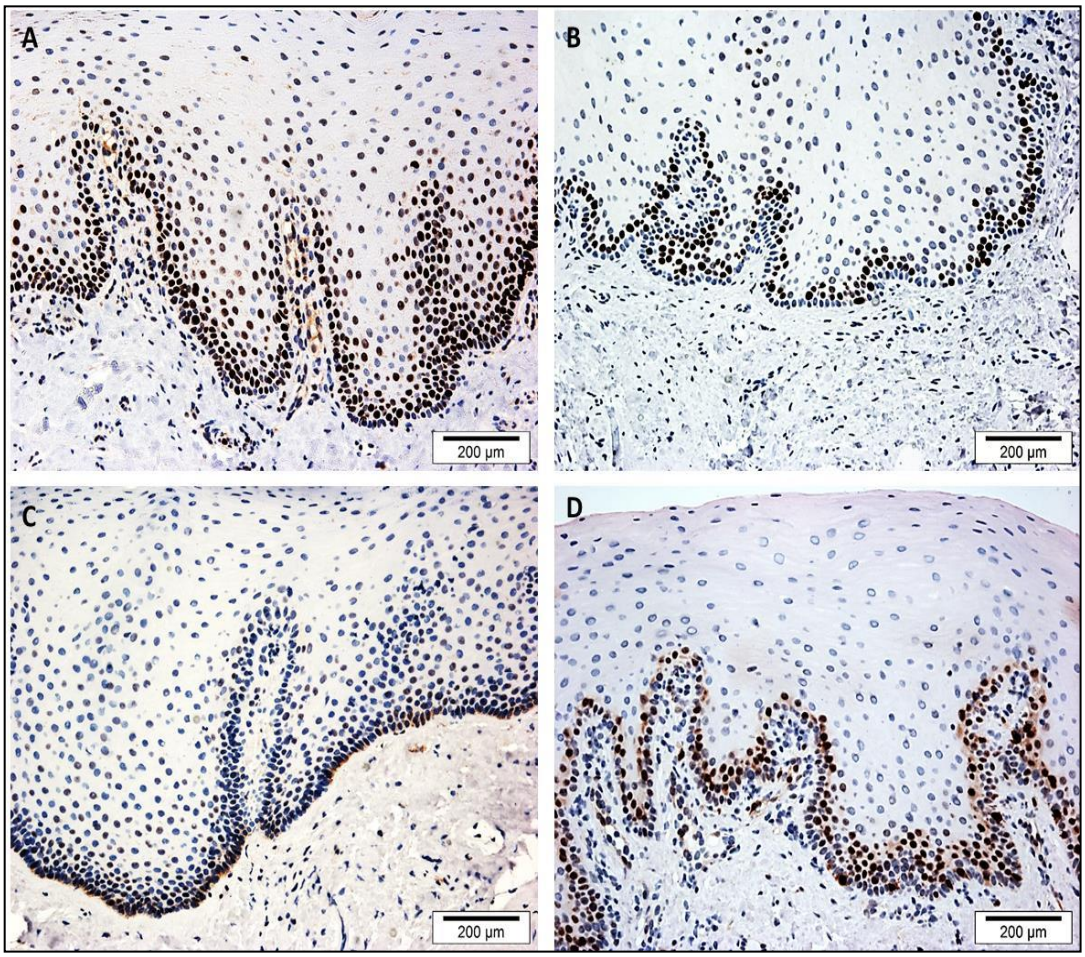

Figure 4.1: Representative immuno-staining illustrating the distribution of MCM2 (A), Ki-67 (B), geminin (C) and cyclin D1 (D) in NOM samples. The MCM2, Ki-67, geminin and cyclin D1 showed similar patterns of expression in NOM samples, mainly in basal and suprabasal cell layers. However, Ki-67, geminin and cyclin D1 were expressed in fewer cells than MCM2. All images are at ×200 magnification.

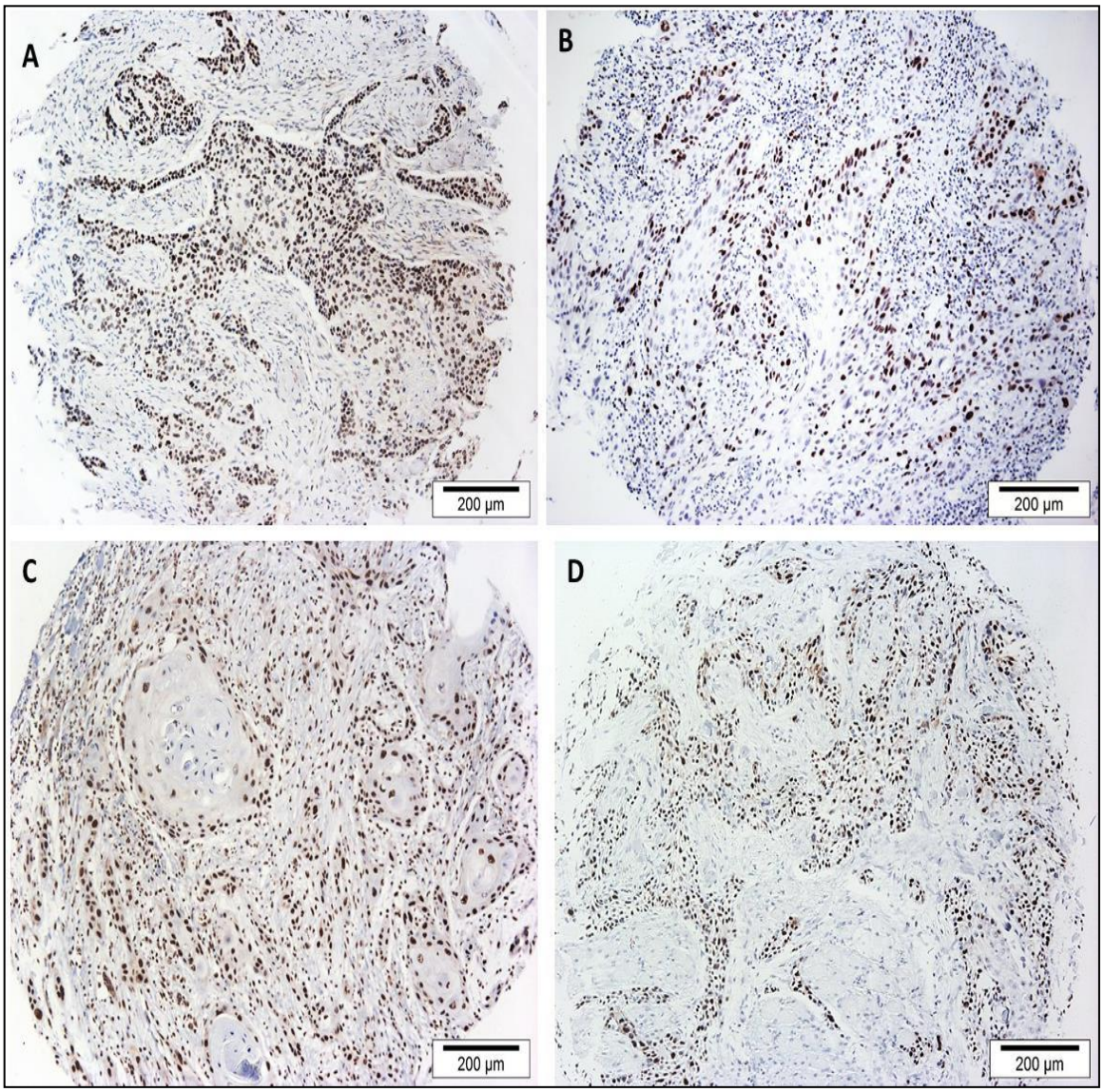

Figure 4.2: Representative immuno-staining of MCM2 (A), Ki-67 (B), geminin (C) and cyclin D1 (D) proteins in samples of OSCC that did not metastasise. All images are at ×100 magnification.

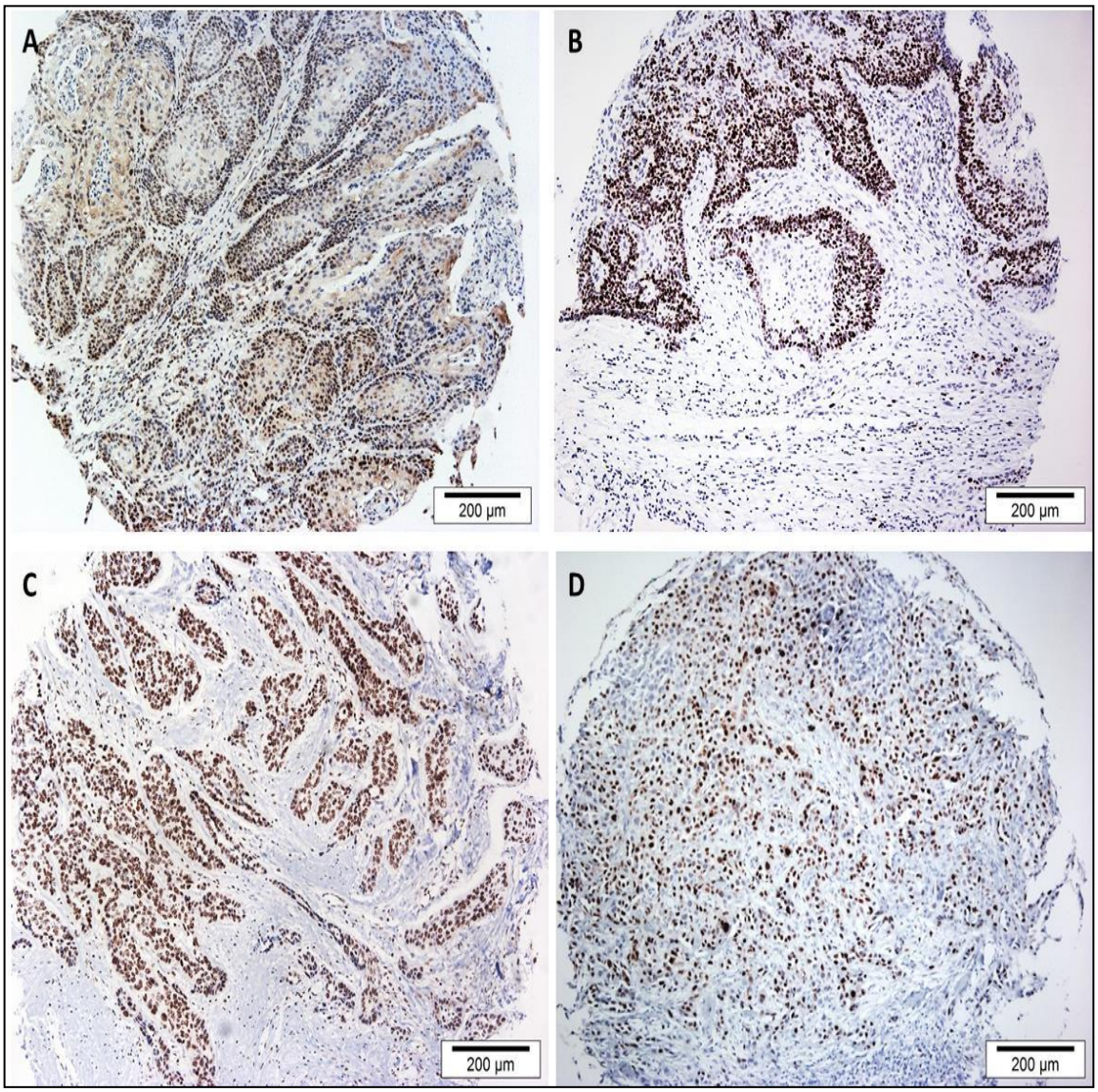

Figure 4.3: Representative immuno-staining of MCM2 (A), Ki-67 (B), geminin (C) and cyclin D1 (D) proteins in samples of OSCC that did metastasise. All images are at ×100 magnification.

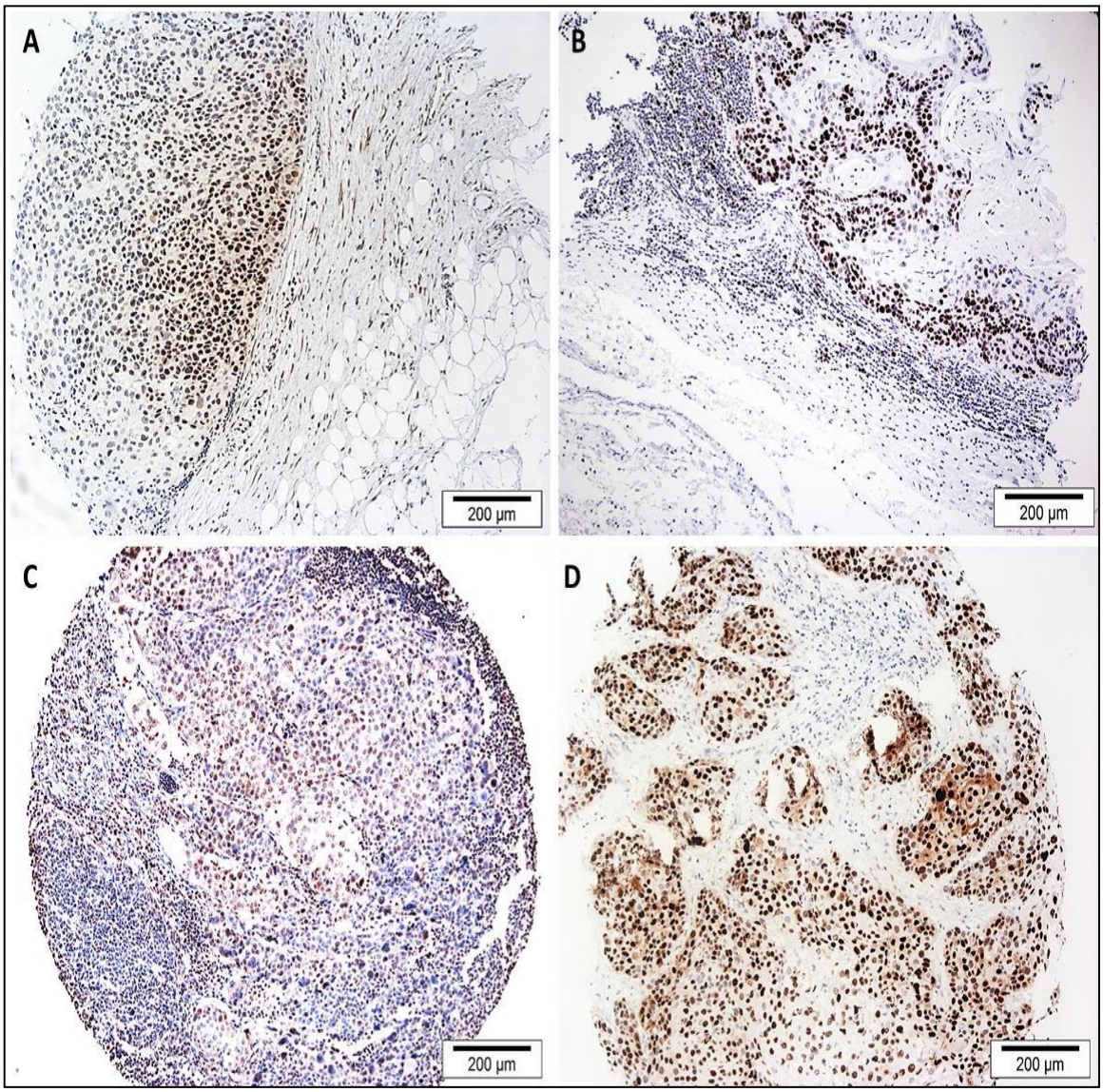

Figure 4.4: Representative immuno-staining of MCM2 (A), Ki-67 (B), geminin (C) and cyclin D1 (D) proteins in samples of lymph node metastases. All images are at ×100 magnification.

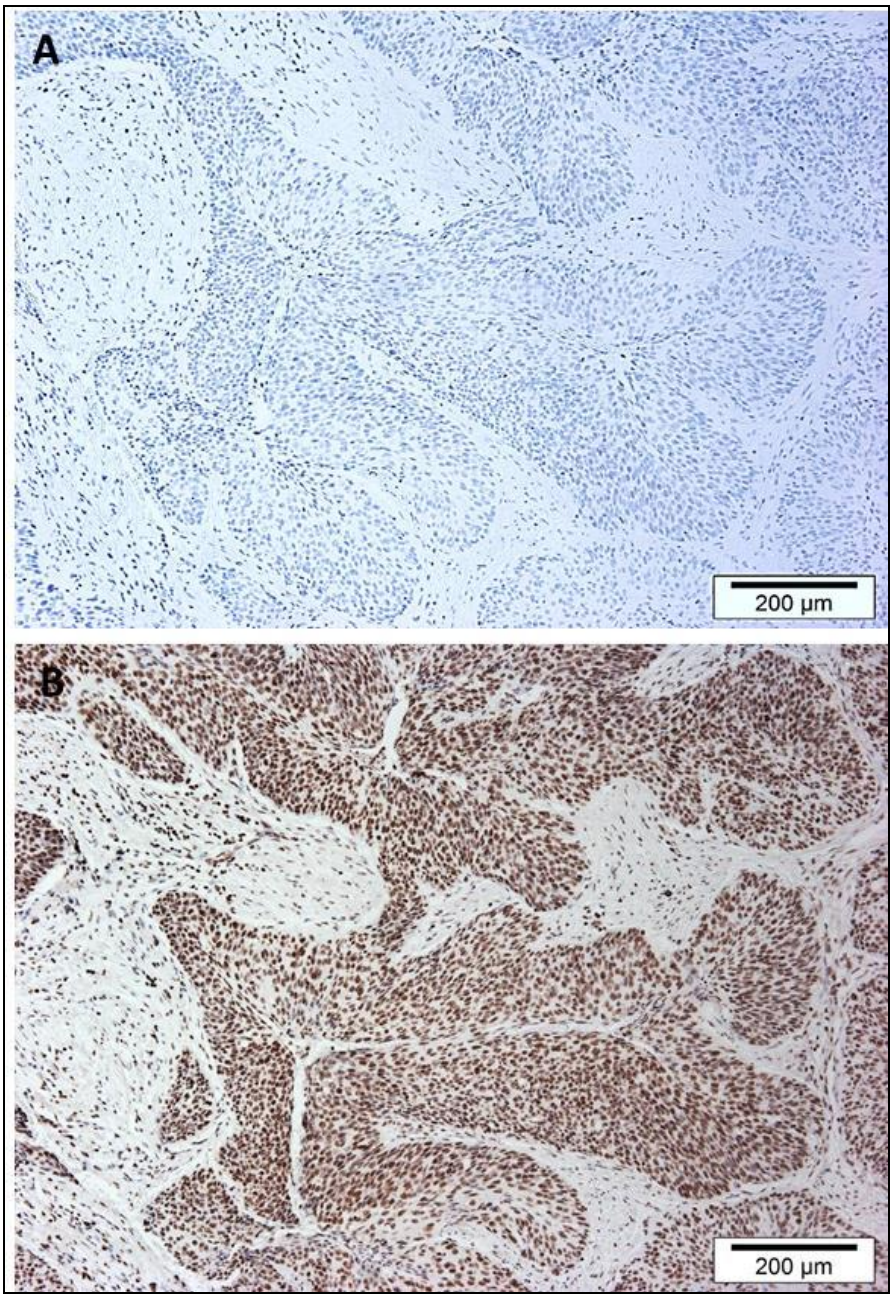

Figure 4.5: Oropharyngeal carcinoma sections showing negative (A) and positive (B) controls for $p16^{INK4A}$. Images are at ×100 magnification.

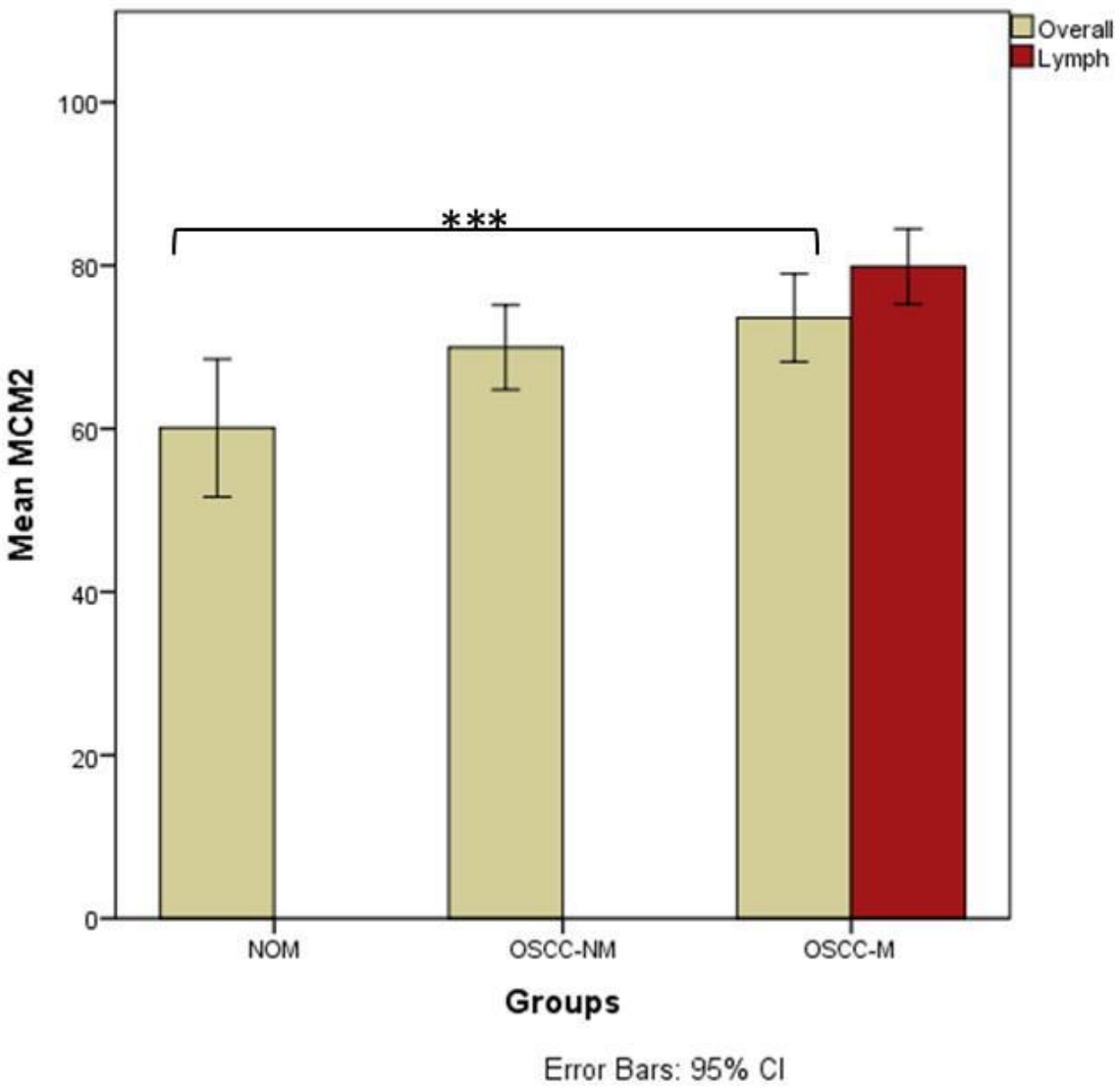

Figure 4.6: Histogram showing the mean LI of MCM2 expression in NOM, OSCC NM, OSCC M and LN samples. There was a significant difference between groups (***p=0.007, ANOVA, **Table 4.4**). There was no significant difference between the primary OSCC M and the lymph node metastasis (red bar).

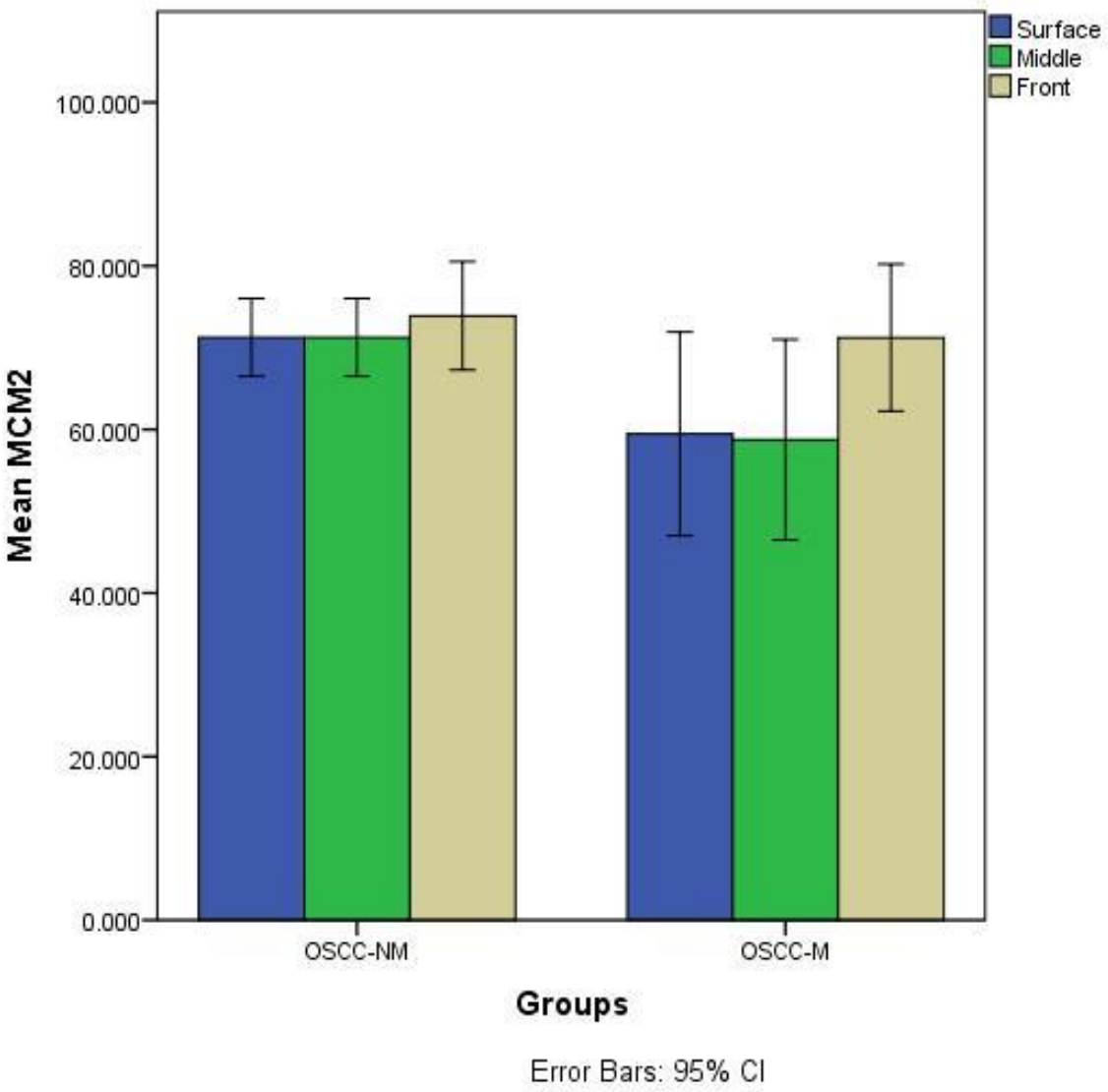

Figure 4.7: Histogram showing the mean LIs of MCM2 at different areas (surface, middle and invasive front) of tumours in OSCC M and OSCC NM groups. There were no significant differences between areas in OSCC M or OSCC NM.

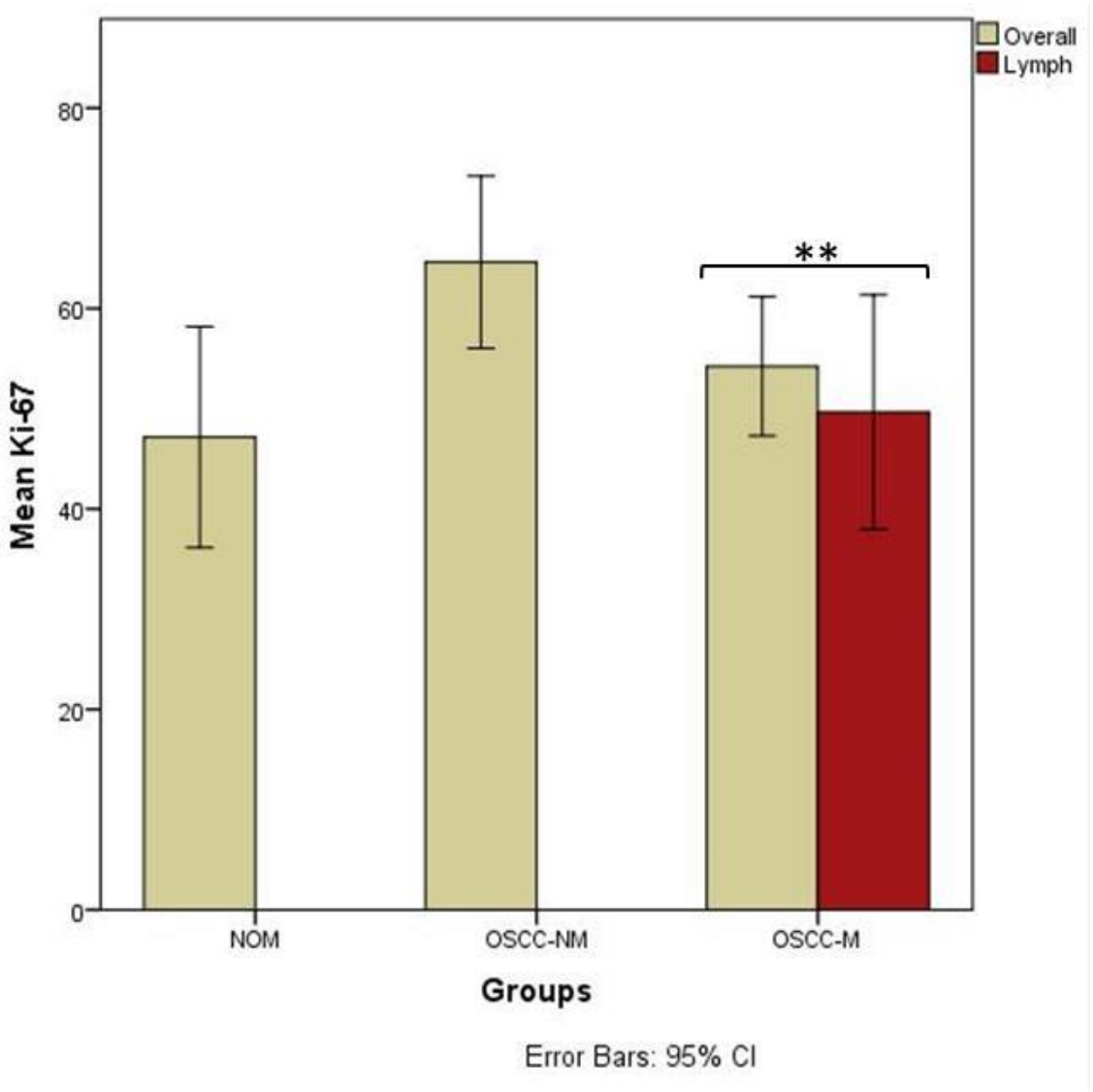

Figure 4.8: Histogram showing the mean LI of Ki-67 expression in NOM, OSCC NM, OSCC M and LN samples. A significant difference (**p=0.03, Paired t-test, **Table 4.6**) was seen between the primary tumours and metastatic lymph node tumours.

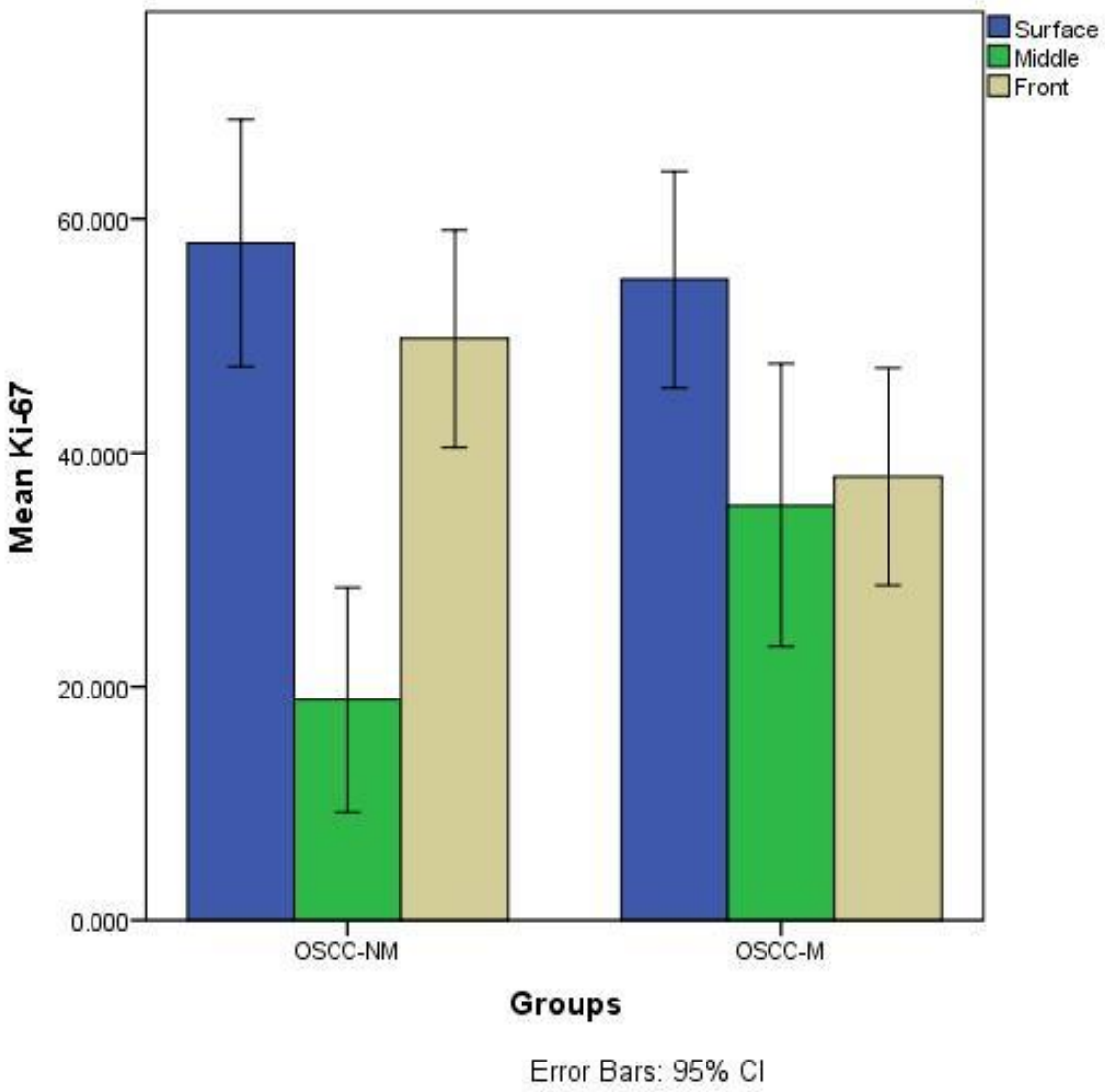

Figure 4.9: Histogram showing the mean LIs of Ki-67 at different areas (surface, middle and invasive front) of tumours in OSCC M and OSCC NM groups. No significant differences were seen between areas.

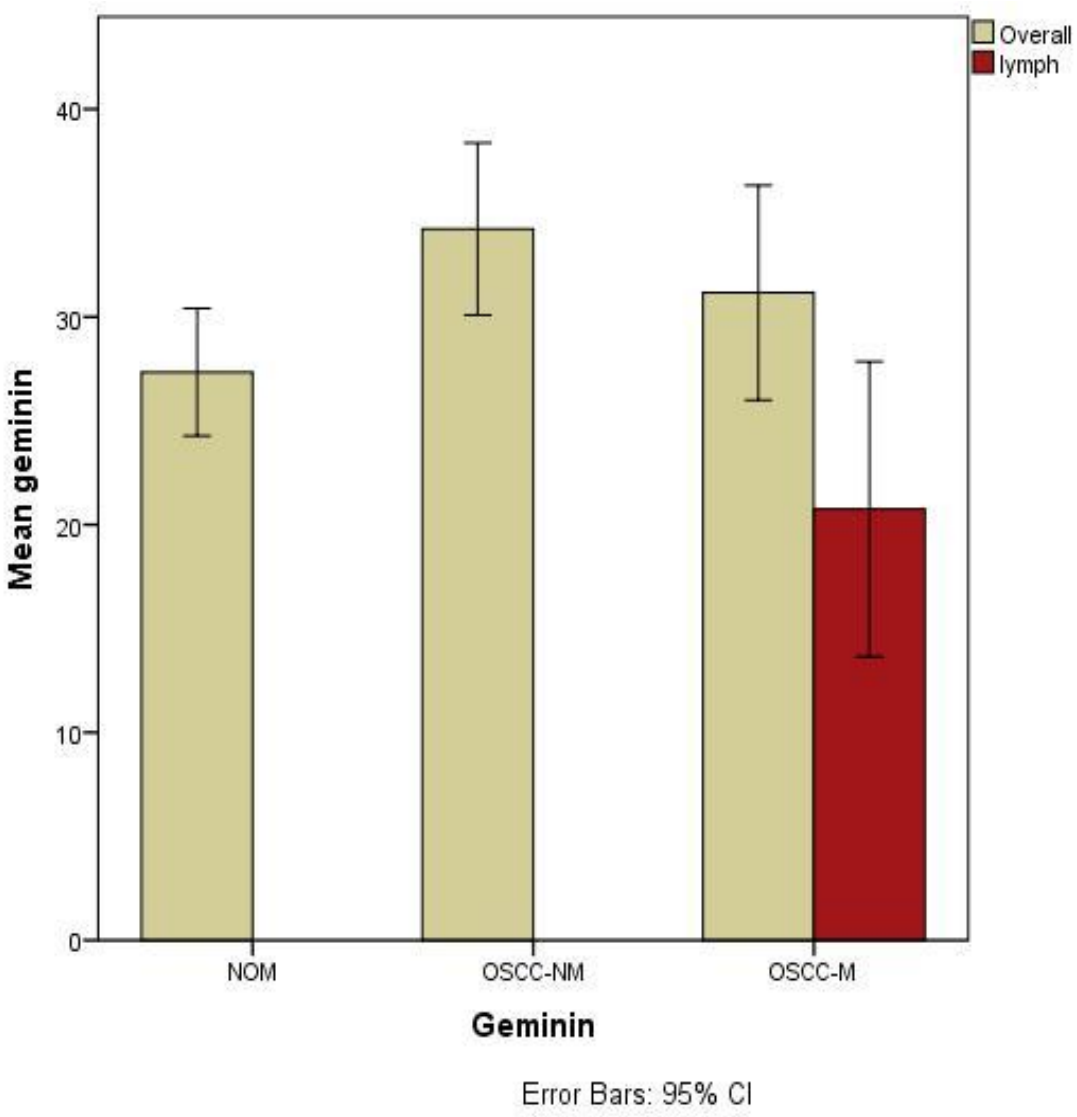

Figure 4.10: Histogram showing the mean LI of geminin expression in NOM, OSCC NM, OSCC M and LN samples. Differences between OSCC M and OSCC NM were not significant. Similarly, differences between the primary OSCC M and their nodal metastases were not statistically significant.

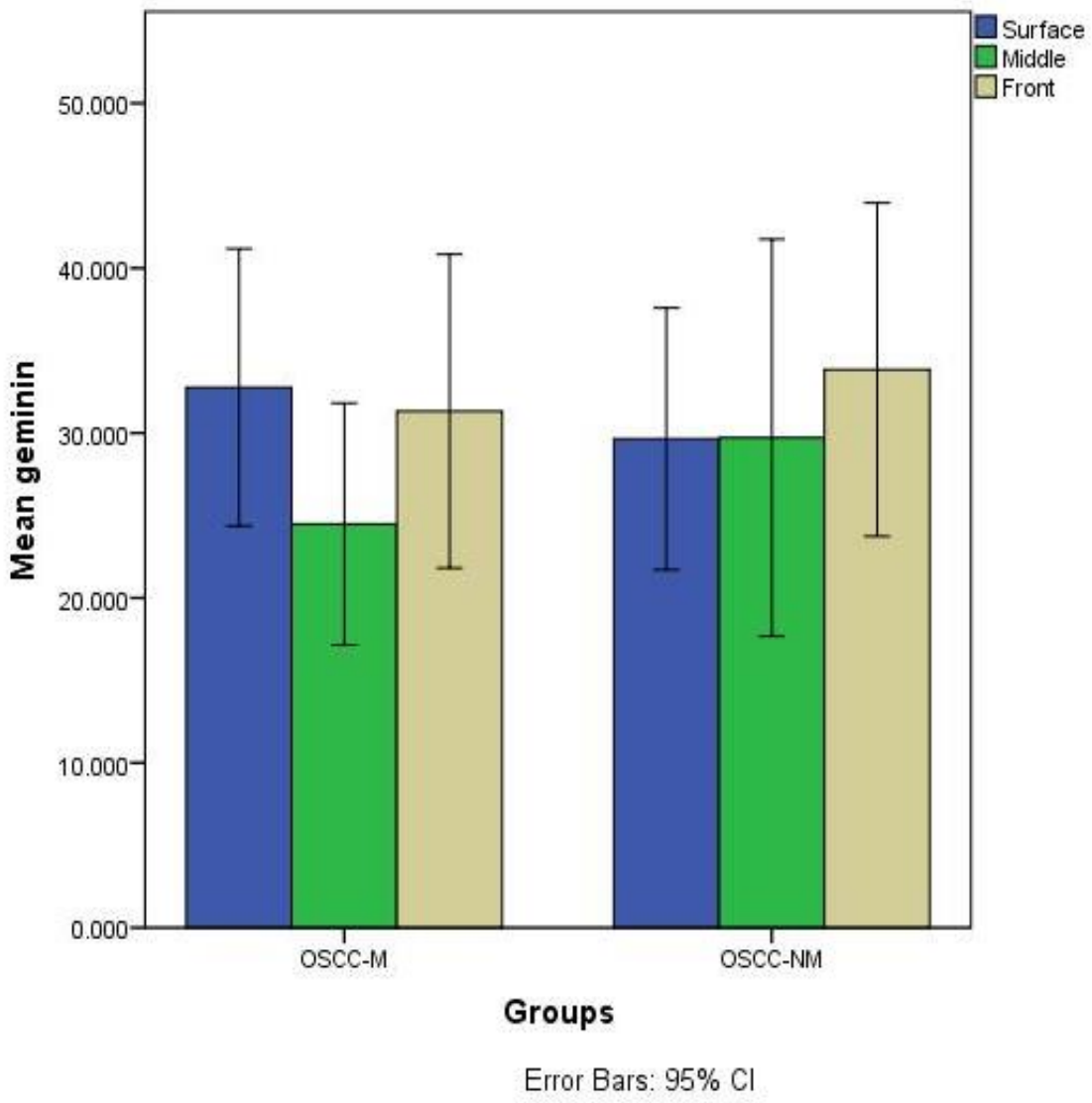

Figure 4.11: Histogram showing the mean LIs of geminin at different areas (surface, middle and invasive front) of tumours in OSCC M and OSCC NM. There were no significant differences between areas in OSCC M or OSCC NM.

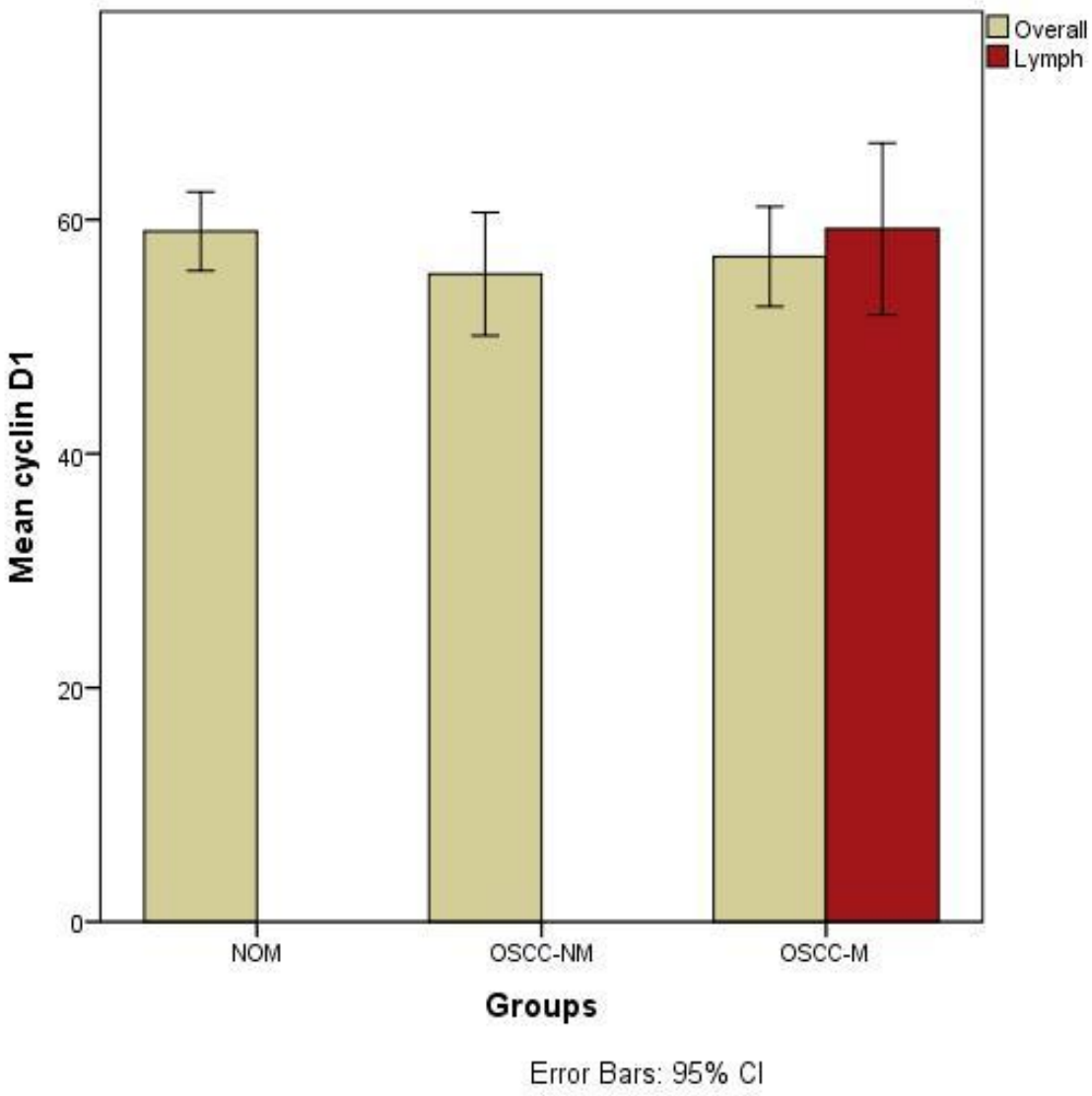

Figure 4.12: Histogram showing the mean LI of cyclin D1 expression in NOM, OSCC NM, OSCC M and LN samples. Differences between OSCC M and OSCC NM were not significant. Similarly, differences between the primary OSCC M and their nodal metastases were not statistically significant.

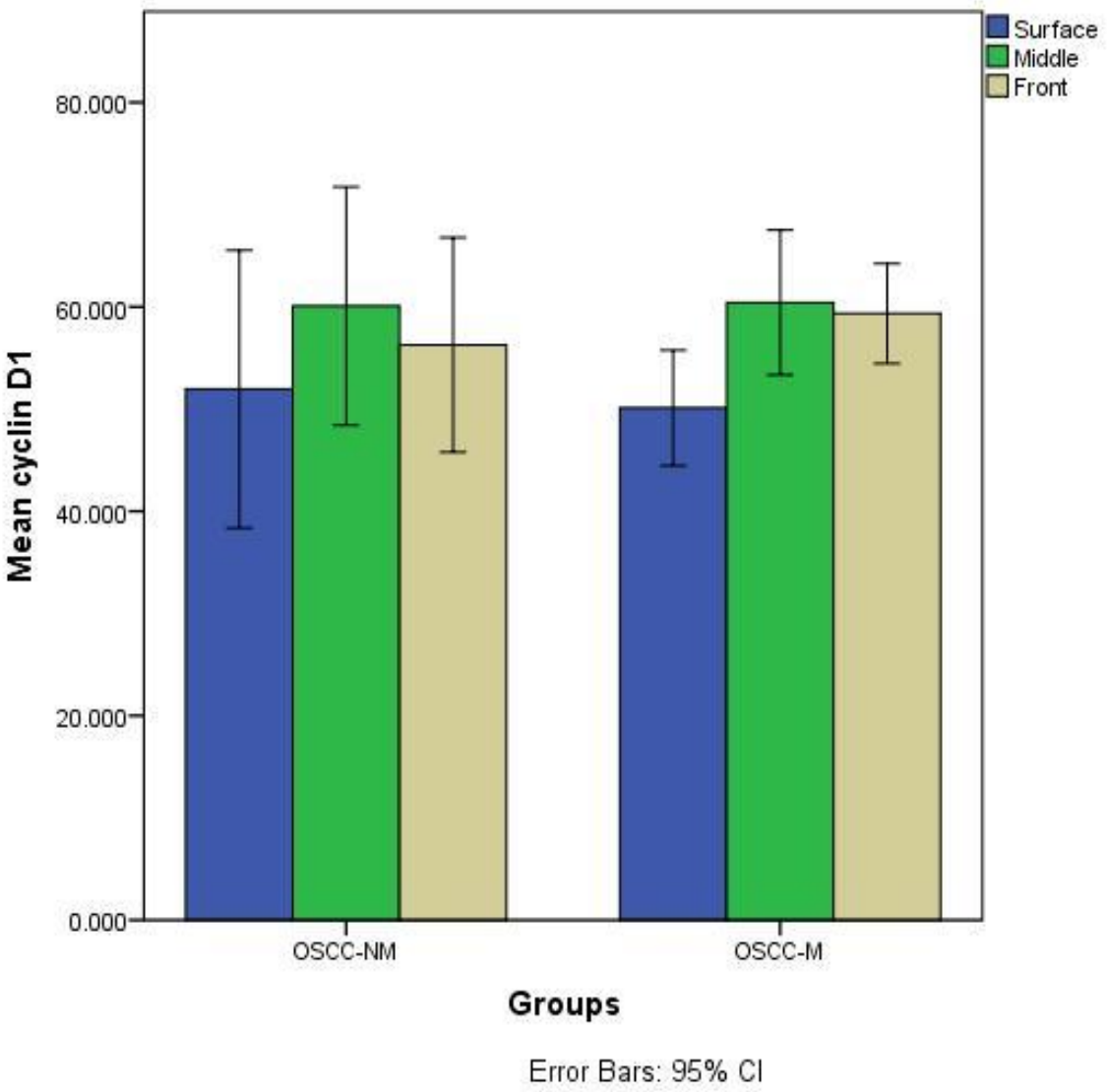

Figure 4.13: Histogram showing the mean LIs of cyclin D1 at different areas (surface, middle and invasive front) of tumours in OSCC M and OSCC NM groups. There were no significant differences between areas in analysed groups.

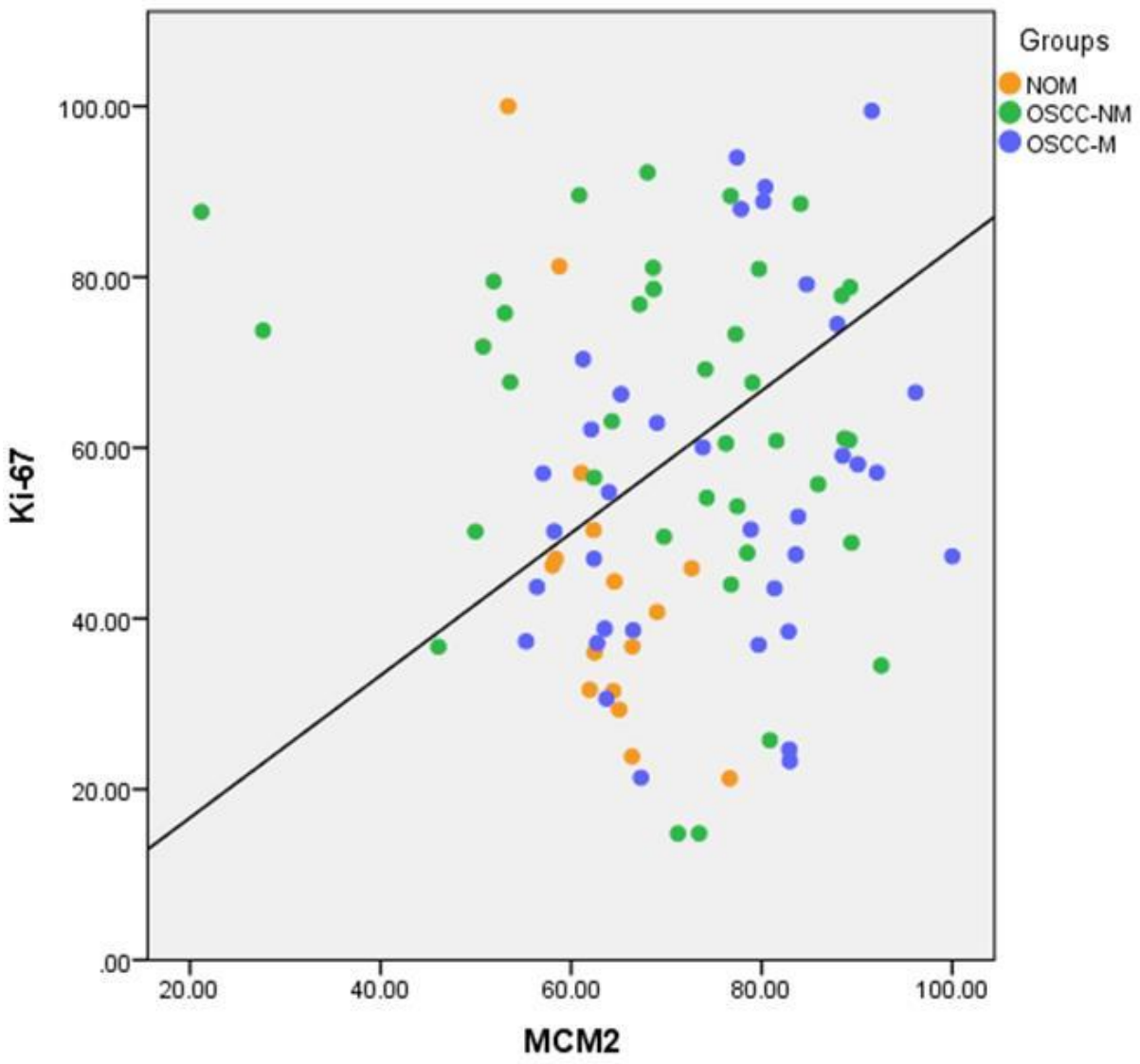

Figure 4.14: Scatter plot displaying the positive association between LI value of MCM2 and Ki-67 in NOM, OSCC M and OSCC NM groups.

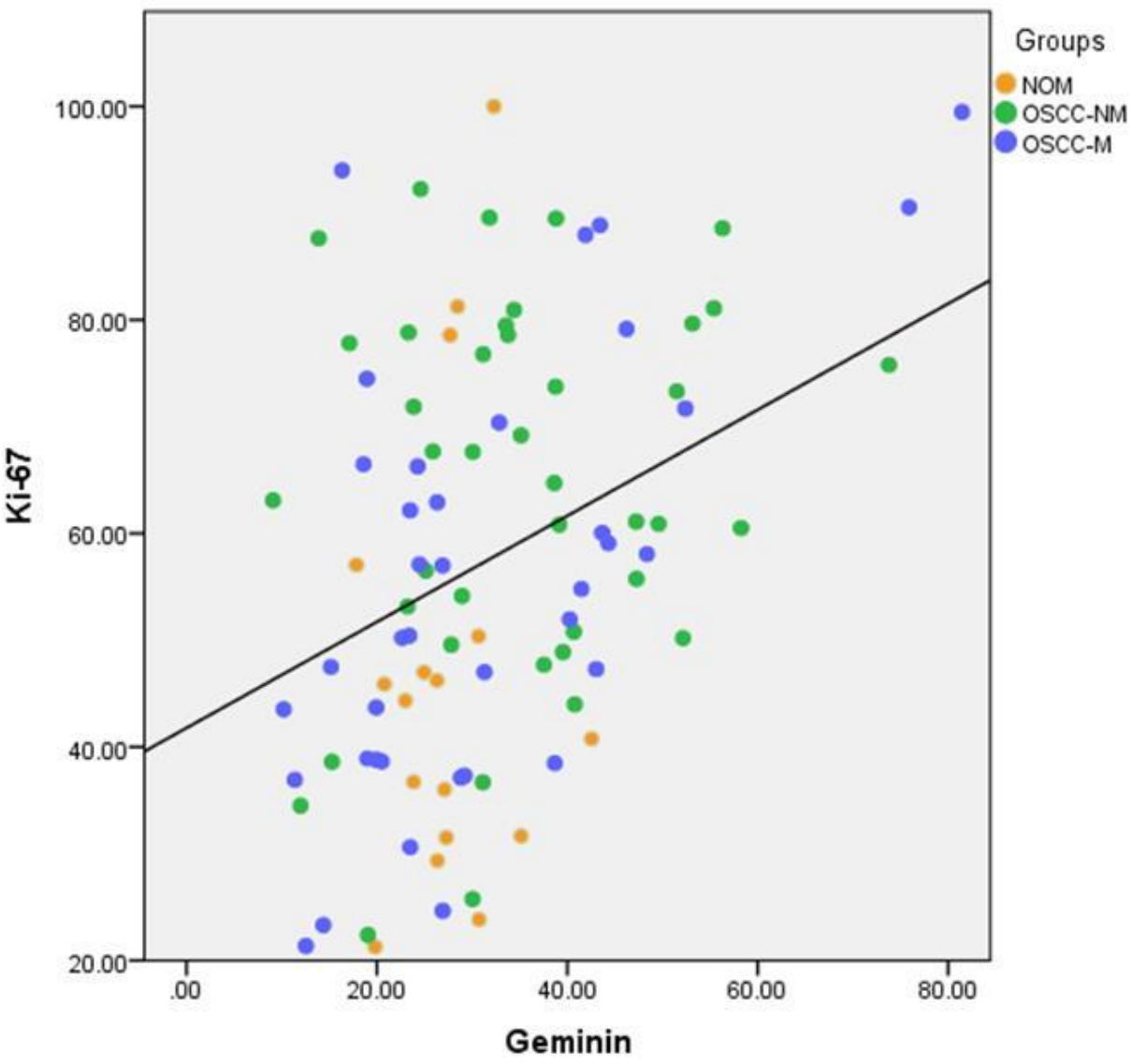

Figure 4.15: Scatter plot displaying the positive association between LI value of Ki-67 and geminin in NOM, OSCC M and OSCC NM groups.

4.3.10 Protein expression and clinicopathological features

The histopathological differentiation of OSCC was described in Section 4.3.1. In the OSCC NM group there were no significant differences between the proteins LIs and ratios in well, moderately or poorly differentiated oral SCC (**Table 4.7**). Likewise, in OSCC M samples there were also no significant differences in protein expression associated with histological differentiation (**Table 4.7**), except for MCM2 expression, which showed significant differences (p=0.01, ANOVA test) associated with higher expression in moderately differentiated tumours in the OSCC LN samples (**Table 4.7**)

The results showed that sixteen out of 39 cases (41%) of the nodal metastasis samples showed extra capsular spread, while 20 (51%) showed no spread (**Table 4.3**). The majority of samples in OSCC M group were stage IV (27/39), whereas the majority of OSCC NM samples were classified as stage III. Mean LI expression of MCM2, Ki-67, geminin and cyclin D1 did not show any significant association with clinicopathological features in either the metastatic or non-metastatic lesions (Appendix 2).

	Labelling index (LI) Mean (Standard deviation)					
	MCM2	**Ki-67**	**Geminin**	**Cyclin D1**	**MCM2/Ki67 ratio**	**Geminin/Ki67 ratio**
OSCC NM						
Well (n=21)	68.76 (16.77)	61.20 (21.27)	34.68 (16.82)	52.72 (17.54)	1.33 (0.98)	0.77 (0.23)
Moderate (n=20)	68.65 (16.17)	70.12 (35.58)	32.89 (12.10)	55.92 (19.27)	1.25 (1.04)	0.56 (0.22)
Poor (n=6)	85.35 (4.23)	56.25 (22.44)	37.09 (11.17)	62.60 (15.14)	1.93 (1.06)	0.73 (0.61)
ANOVA	p=0.82	p=0.40	p=0.69	p=0.58	p=0.29	p=0.20
Correlation	p=0.25	p=0.33	p=0.39	p=0.87	p=0.77	p=0.20
OSCC M						
Well (n=13)	79.68 (10.24)	52.65 (23.71)	34.90 (23.55)	58.43 (14.88)	1.84 (0.77)	0.73 (0.24)
Moderate (n=20)	69.31 (18.51)	55.89 (20.82)	28.79 (9.77)	54.94 (13.01)	1.318 (0.35)	0.47 (0.17)
Poor (n=6)	74.86 (16.27)	52.09 (21.33)	30.92 (13.36)	59.42 (8.71)	1.70 (0.99)	0.66 (0.23)
ANOVA	NS	NS	NS	NS	NS	NS
Correlation	p=0.70	p=0.55	p=0.58	p=0.57	p=0.66	p=0.22
OSCC LN						
Well (n=15)	78.78 (11.64)	57.75 (30.83)	23.89 (16.83)	61.45 (22.92)	1.31 (0.45)	0.36 (0.24)
Moderate (n=21)	83.85 (11.33)	46.47 (40.08)	15.97 (21.78)	59.50 (19.44)	1.23 (0.42)	0.23 (0.32)
Poor (n=3)	57.67 (2.86)	31.45 (29.52)	38.50 (39.98)	40.92 (15.58)	1.30 (0.50)	0.45 (0.56)
ANOVA	p=0.01	p=0.36	p=0.87	p=0.28	p=0.99	p=0.69
Correlation	p=0.84	p=0.90	p=0.42	p=0.50	p=1.00	p=0.52

OSCC NM= Oral squamous carcinoma that did not metastasise.
OSCC M = Oral squamous carcinoma that had metastases.
OSCC LN= Lymph node metastases
NS= Not significant.

Table 4.7: Mean and standard deviation of proteins labelling index according to the differentiation grad of OSCCs (NM, M & LN).

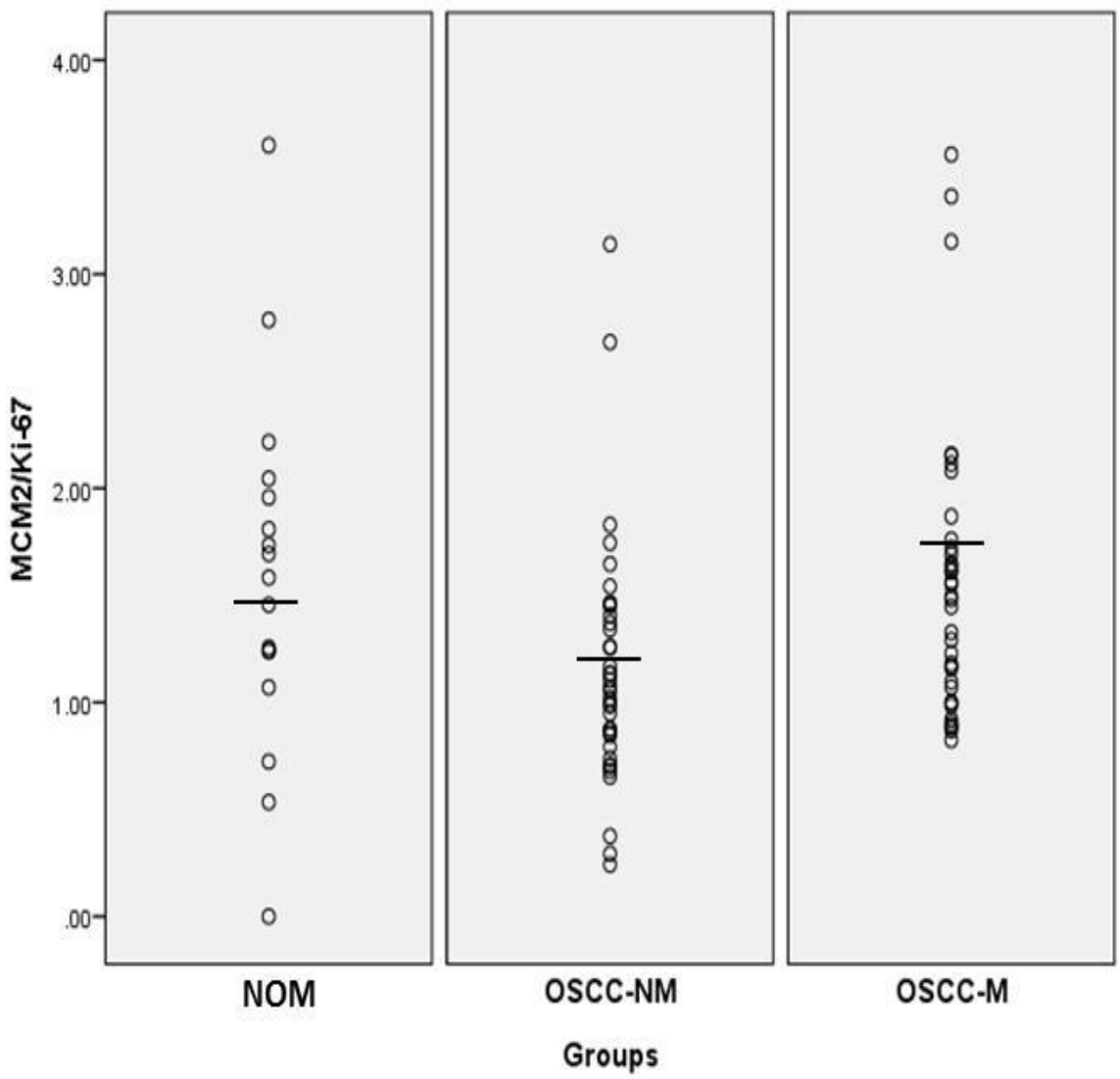

Figure 4.16: Scatter graph showing the MCM2/Ki-67 ratios for the NOM, OSCC NM and OSCC M groups. The horizontal bars indicate the mean values in each group.

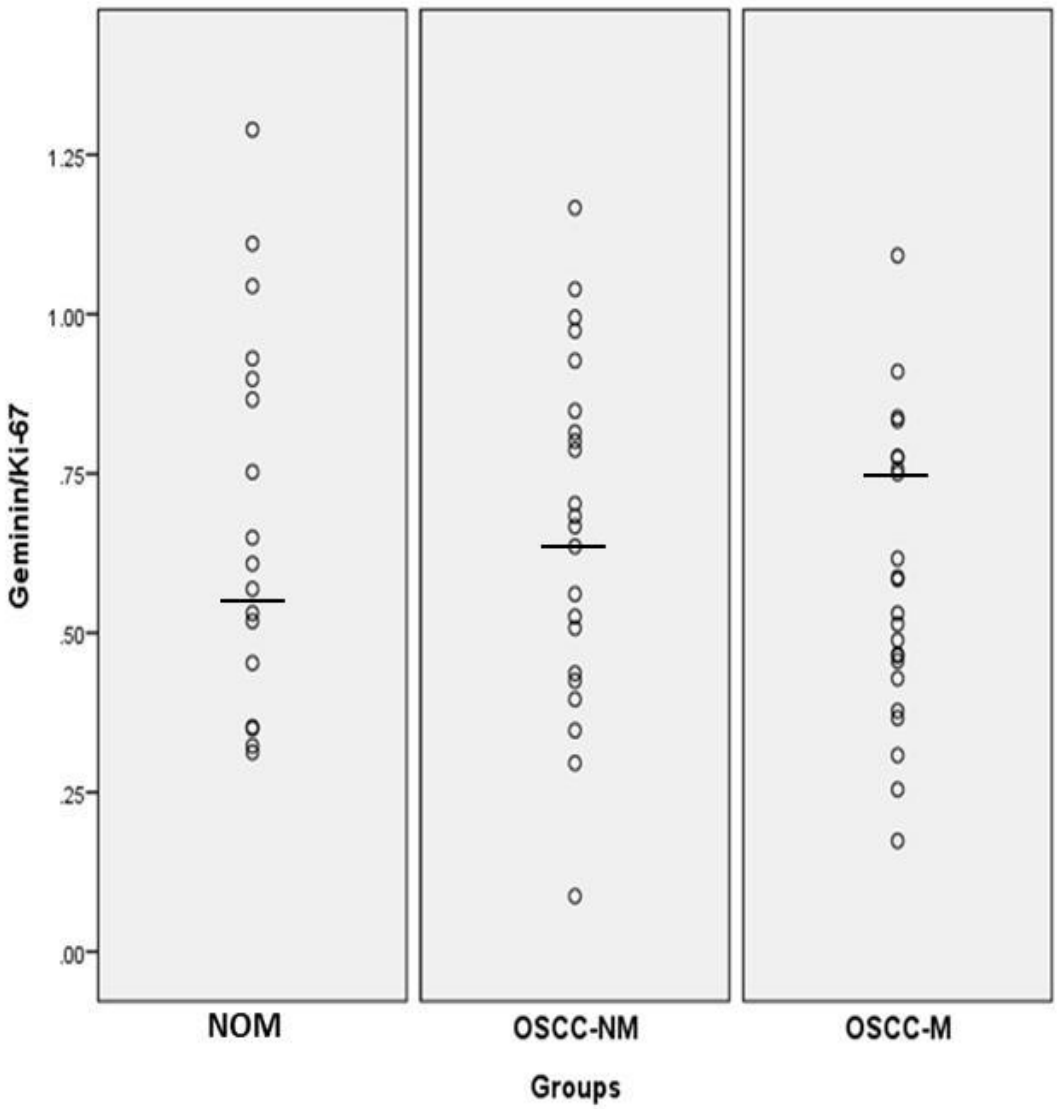

Figure 4.17: Scatter graph showing the geminin/Ki-67 ratios for the NOM, OSCC NM and OSCC M groups. Differences were significant between OSCC NM and OSCC M (p=0.02, Paired t-test, **Table 4.4**). The horizontal bars indicate the mean values in each group.

4.4 Discussion and conclusions

4.4.1 DNA replication licensing proteins in metastatic and non-metastatic oral squamous cell carcinoma and their corresponding lymph node metastatic lesions

During the tumourigenesis process, dysregulation of cell cycle regulatory proteins is a pivotal event. Several studies have described the importance of pre-replication proteins as prognostic markers in oral and other human neoplasms (Going et al., 2002, Wharton et al., 2004, Kodani et al., 2003, Gonzalez et al., 2005, Eward et al., 2004, Dudderidge et al., 2005, Quaglia et al., 2006, Scott et al., 2006, Torres-Rendon et al., 2009a, Santos-Silva et al., 2011, Gouvêa et al., 2013, Shalash et al., 2012).

To our knowledge, this is the first time that heterogeneity of cell cycle protein expression has been determined in the same tumour with or without metastasis. This study reports the pattern of expression of these biomarkers in OSCCs. The aim was to identify markers associated with metastatic lesions, and to evaluate their value to predict lymph node metastasis. Overall, the mean LI for MCM2 was consistently higher than Ki-67, due to MCM2 being expressed during the whole cell cycle, including the G1 phase where Ki-67 is absent. Low labelling index in normal tissue has also been observed by others (Shomori et al., 2010, Torres-Rendon et al., 2009a). The OSCCs (NM & M) expressed MCM2 with variable levels when different areas of tumour were compared. Although, the advancing front showed

higher mean LIs, the difference was not significant. This would support the concept that there is greater cell proliferation at the advancing front of tumours, but further studies are needed to confirm this and to determine the degree of heterogeneity in tumours.

The LI of MCM2 was higher in OSCC M than in OSCC NM, but this difference did not reach statistical significance, possibly because of the small sample size. This is in agreement with a previous study conducted by Kodani *et al*. (Kodani et al., 2003), in which neither MCM2 nor Ki-67 LIs were different in OSCC with or without metastasis. Since MCM2 expression increased from normal through OSCC NM to OSCC M ($p=0.007$, ANOVA test), this indicates the presence of a high number of cells licensed to proliferate. This in turn suggests that MCM2 may provide useful information about the growth fraction in normal and malignant cells. Further studies with a larger number of samples are needed to confirm this finding.

The results of the present study indicated no significant difference in the cell cycle regulatory proteins between primary and matched lymph node metastsis, other than for Ki-67, with higher expression in primary tumours. A significant decrease in Ki-67 in the nodal metastases has been reported in breast cancer (Tawfik et al., 2013). The prognostic impact of MCM2 expression has been assessed in primary oral carcinomas and their lymph node metastases. MCM2 LI showed greater mean protein LI in nodal metastases, however this was not statistically significant. Low Ki-67 and high MCM2 LIs in the nodal metastases may suggest that a majority of metastatic cells are in G0 phase, but are licensed to proliferate. This is in

keeping with the idea that nodal metastases are often better differentiated than the primary tumours. It may also suggest that high MCM2 LIs may reflect a cell proliferation capacity of OSCC in nodal metastases. This has also been observed in preivous reports evaluating MCM2 in OSCC (Shalash et al., 2012) and in colorectal cancer (Guzińska-Ustymowicz et al., 2009).

In this study, geminin expression showed no significant difference between OSCC NM and OSCC M tumours. High expression of geminin in nodal metastases was observed, but did not reach significant levels. This relatively high expression may indicate that large numbers of tumour cells successfully progressed through the S-G2-M phases of the cell cycle. In accordance with previous reports, geminin LI values were smaller than the Ki-67 mean LIs in all analysed samples (Torres-Rendon et al., 2009a, Tamura et al., 2010).

Surprisingly, Ki-67 showed lower mean LI value in OSCC M than OSCC NM. This decrease may be due to some tumours seeming to exhibit relatively high expression of geminin and relatively low expression of Ki-67 for unknown mechanisms (Yamazaki et al., 2010). Additionally, Ki-67 protein tended to show patchy staining in OSCC, which has been reported in previous studies (Birajdar et al., 2014). This may be another reason for divergent results. The statistical analysis revealed that Ki-67 did not show any correlation with histological differentiation of OSCC (NM or M); this was in accordance with a previous report (Deyhimi et al., 2013).

Cyclin D1 is a protein that controls the progression from G1 to S phase and controls the cellular proliferation rate. Alterations in cyclin D1 may result in loss of cell cycle regulation. High expression of cyclin D1 is a common event in squamous cell carcinoma (Hanken et al., 2014, Swaminathan et al., 2012). Cyclin D1 results revealed no significant differences between OSCC NM and OSCC M. Similar to the findings presented in this study, no correlations between cyclin D1 overexpression and clinicopathological factors have previously been reported (Freier et al., 2003, Wang et al., 2006, Kaminagakura et al., 2011). However, this is contradictory to a more recent finding by Zhao *et al.* (Zhao et al., 2014), who reported significant correlations with differentiation status, tumour size, lymph node metastasis and clinical stage of disease in OSCC.

Histologically poorly differentiated carcinoma has been strongly correlated with a high expression of MCM2 and Ki-67 in OSCC (Shalash et al., 2012, Birajdar et al., 2014). However, in the current study there was no significant difference in protein expression between well, moderately or poorly differentiated tumours in OSCCs (NM & M). This finding is consistent with another previous study (Torres-Rendon et al., 2009). Positive correlation was only observed with MCM2 expression and moderately differentiated carcinoma in OSCC LN ($p=0.01$). These findings are in accordance with Kodani *et al*. (Kodani et al., 2003), who found a positive correlation between high MCM2 LI and moderate OSCC. More cells may have been in a proliferative phase and hence showed an increase in MCM LI.

No significant correlation was found between clinical parameters (such as, age, tumour size and stage) and cell cycle licensing proteins either in OSCC NM or in OSCC M. This is compatible with previous studies (Naderi et al., 2014, Gouvêa et al., 2013). Further similar studies are recommended to investigate the heterogeneity of tumour cells in primary and metastatic lesions.

In the case of the present study, instead of performing immunohistochemistry on tissue sections, the TMA technique was used so all staining could be achieved on only 15 slides of TMA sections. This was to save laboratory processing time and reagent cost, as well as to save valuable biological material. All TMAs were treated the same way to ensure consistent results. However, cores for the same case on the same TMA did not react similarly. Some tumour cores were lost and some did not show immune-reactivity within the same TMAs section It has been reported that about 10-15% of cores are expected to be lost during technical processing (Ilyas et al., 2013). Therefore, the TMA method appears to have some limitations. According to published data, there is no concordance in results obtained from whole tissue sections and TMA techniques (Kim et al., 2011); this could be a possible explanation for some unexpected results in this chapter.

The findings in this chapter require confirmation from further analysis; using a larger number of samples of OSCC lesions. It may also be advisable to undertake staining on carefully prepared sections of individual cases since

the TMA cores showed variable staining and many sections were lost or were not interpretable.

4.4.2 Conclusions

The findings in this study suggest that:

- It is possible to obtain cell cycle data by assessing the MCM2, Ki-67, geminin and cyclin D1 protein expression by analysis of immune-staining in tissue microarrays.
- There was no difference in cell cycle protein expression, in different areas of the tumours, nor between tumours which metastasised (M) or did not metastasise (NM).
- There were no significant differences between primary lesions and their lymph node metastases for all cell cycle proteins, except for Ki-67.
- An increase in MCM2 expression in OSCC LN suggests that this protein is a marker of proliferative capacity and that might be more useful for histological and prognostic evaluation.
- This is the first time the MCM2/Ki-67 and geminin/Ki-67 ratios have been evaluated in primary and matched nodal metastases; the difference was significant but needs further confirmation.
- The geminin/Ki-67 ratio was significantly different between metastatic and non-metastatic OSCC.
- No correlation was noted between the immunohistochemical findings and clinicopathological features.

CHAPTER 5: DNA PLOIDY ANALYSIS AS A PREDICTOR OF METASTASIS IN ORAL CANCER

5. DNA PLOIDY ANALYSIS AS A PREDICTOR OF METASTASIS IN ORAL CANCER

5.1 Introduction

Despite recent improvements in diagnostic and therapeutic approaches to oral squamous cell carcinoma (OSCC), the survival rate has remained virtually unchanged for decades. Clinical and histopathological parameters are the most frequently used predictors of lesion progression, but to date these have lacked reliability. Thus objective methods are needed to distinguish lesions that may metastasise from those that may not. Research carried out over recent years has shown that most malignant neoplasms are clonal, although they may exhibit widespread heterogeneity with respect to phenotypic diversity in individual tumours (Marusyk and Polyak, 2010).

Aneuploidy, as a measure of abnormal nuclear DNA content, is known to be an indicator of numerical chromosomal and DNA aberrations, and its emergence is often a crucial step in tumour transformation (associated with malignant and premalignant lesions). The literature shows that aneuploidy is seen in solid tumours (Holland and Cleveland, 2012) and is associated with a high risk of recurrence (Janisson-Dargaud et al., 2008, Baak and Janssen, 2004). Clinical studies have shown that up to 40% of patients with oral squamous cell carcinoma will develop lymph node metastases (Alkaisi et al., 2014) and the prognosis is unfavourable (Kowalski and Sanabria, 2007, Ganly et al., 2012). In practice, metastasis to lymph nodes is probably the

most important prognostic factor (Brannan et al., 2011). However, the incidence of aneuploidy has been shown to be higher in lymph node metastases than in primary lesions (Hemmer et al., 1997). This is consistent with previous findings that have shown that aneuploidy was found in 71% of metastatic tumours compared with 61% of primary lesions (Frankfurt et al., 1984).

Nuclear DNA status can be detected using robust and sensitive techniques, including flow cytometry (FCM) or image cytometry (ICM) (Brouns et al., 2012). The latter has several advantages over FCM as it allows for visual control and selection of interesting cells or areas of tumour (Bremmer et al., 2011). The DNA content of the tumour cell population can be measured by comparing integrated optical density (IOD) of control cells (usually lymphocytes) with nuclei of interest (Section 1.10). In addition, ICM provides two established parameters: the DNA index and the percentage of cells that exceed 5c (5cER), that may be used as quantitative measures of DNA alterations (Section 1.10.1 and 1.10.3).

The prognostic importance of DNA ploidy in oral cancer remains uncertain (discussed in Chapter 1, Section 1.10.5). Therefore, the aim of this part of the project was to determine if DNA ploidy can be used as a useful prognostic marker in OSCC, and if it can predict which tumours may metastasise.

5.2 Material and methods

5.2.1 Patient and tissue selection

A total of 125 tumour samples were studied. The primary lesions in both groups (OSCC NM & OSCC M) were analysed twice for two different areas of each tumour (surface and advancing front). The inclusion criteria for ploidy analysis included adequate numbers of neoplastic epithelial cells (300 nuclei) and inflammatory cells (to be used as controls) in the same section.

5.2.2 Tissue preparation for DNA image cytometry

Paraffin sections (7µm) were cut and slides were stained with Feulgen-Schiff stain (ScyTek Laboratories Kit, USA), as previously described in Section 3.5.2.

5.2.3 Image cytometry for DNA analysis

The Automated Cellular Imaging System, ACIS III (Dako, Glostrup, Denmark) was used to analyse Feulgen stained sections. Details of the equipment and the scanning process are described in Section 3.5.3.

5.2.4 Criteria for classification of DNA content

The criteria for diploid and aneuploid lesions are described in detail in Section 3.5.4 and are summarised in **Table 5.1**. These criteria are adapted

from a recent study in our laboratories (Santos-Silva et al., 2011). In addition, all analyses were performed blind to the histopathological features.

Ploidy status	DI of peak on the histogram	5cER %
Diploid	0.9 – 1.10	<1%
Mild aneuploid	1.11 – 1.30	>1%
Moderate aneuploid	1.31 – 1.80	>1%
Severe aneuploid	> 1.81	>1%
DI = DNA index. 5cER= Number of cells that exceed 5c.		

Table 5.1: Criteria used for ploidy status determination in the histograms of OSCC lesions. For diploid samples, the two characteristics must be identified. In the aneuploid samples, at least one of the two criteria should be present.

5.2.5 Statistical analysis

All Statistical analyses were performed using SPSS (v 21) software. Non-parametric tests were used to compare the DI, 5cER and ploidy status between groups (described in Section 3.6.2). Spearman correlation tests were used to determine whether primary lesions with metastases and OSCC differentiation correlated with ploidy status. The ability of ploidy to be used as a test to differentiate lesions that metastasised from those that did not was determined by calculation of sensitivity, specificity, predictive values and receiver operating characteristic curves (ROC) (Section 3.6).

5.3 Results

5.3.1 Patient data and ploidy

The ploidy status of each case was taken as the ploidy value of the most severely aneuploid area (surface or advancing front).

In the OSCC group that did not metastasise (OSCC NM, median age= 64.50, range= 34.50-84.50), thirty two (68%) were males, of which 21 cases showed moderate aneuploidy and 11 exhibited severe aneuploidy (**Table 5.2**). Fifteen cases were female (32%), 2 showed mild aneuploidy, 4 moderate aneuploidy and 9 cases were severe (**Table 5.2**). The most commonly affected site was the tongue with 16 cases (34%); 11 were moderate, 2 exhibited severe aneuploidy and 3 cases showed mild aneuploidy. Floor of mouth and alveolar mucosa ranked second with 8 cases each. Seven (15%) aneuploid cases were found in the buccal mucosa (**Table 5.3**).

In the OSCC group that had metastasised (OSCC M, median age= 64.50, range= 34.50-84.50) the incidence of moderate and severe aneuploidy was higher in males (**Table 5.2**). The majority of cases were located in the tongue and 10 cases (59%) showed moderate aneuploidy. Severe aneuploidy was seen in 7 cases (41%). Floor of mouth (8 cases) was the second most affected site, although only 2 cases showed severe aneuploidy (**Table 5.3**). The next most common sites were alveolar and buccal mucosa, with 6 cases each, of which 3 cases exhibited severe aneuploidy.

The quality of DNA histogram is assessed from the width of G1 peak, which is measured by the coefficient of variation (CV) across the peak.The coefficient of variance (CV) (explained in Section 1.10.2) was less than 5% in all cases, which complied with the recommendations of the ESACP guidelines (Haroske et al., 1997, Haroske et al., 2001). The median CV of the OSCC M group was 3.40 and in the OSCC NM group was 3.32 (**Figure 5.1** and **Figure 5.2**).

	OSCC NM			OSCC M			OSCC LN	
Median age	64.50			64.50			54.50	
Gender	**Male**	**Female**		**Male**	**Female**		**Male**	**Female**
Total	32	15		26	13		26	13
DNA status								
Diploid	0	0		0	0		0	0
Mild aneuploidy	0	2		0	0		0	0
Moderate aneuploidy	21	4		13	8		13	8
Severe aneuploidy	11	9		13	5		13	5
Total	47			39			39	

OSCC NM = Oral squamous cell carcinoma that had not metastasised.
OSCC M = Oral squamous cell carcinoma that had metastasised.
OSCC LN= metastasis in the lymph node.

Table 5.2: Patient data and number of cases showing severe aneuploidy in each of the three patient groups.

	OSCC NM				OSCC M			
	DNA status No (%)				DNA status No (%)			
Biopsy site	**No**	**Mild**	**Moderate**	**Severe**	**No**	**Mild**	**Moderate**	**Severe**
Tongue	16	3 (19)	11 (69)	2 (13)	17	0	10 (59)	7 (41)
Floor of mouth	8	2 (25)	3 (38)	3 (37)	8	0	6 (75)	2 (25)
Palatal mucosa	1	1 (100)	0	0	1	0	0	1 (100)
Hard palate	1	0	0	1 (100)	0	0	0	0
Soft palate	2	1 (50)	0	1 (50)	1	0	1 (100)	0
Alveolar mucosa	8	2 (25)	3 (38)	3 (37)	6	0	3 (50)	3 (50)
Buccal mucosa	7	0	4 (57)	3 (43)	6	0	3 (50)	3 (50)
Tonsil area	1	0	1 (100)	0	0	0	0	0
Pharyngeal wall	2	1 (50)	0	1 (50)	0	0	0	0
Retromolar area	1	0	1 (100)	0	0	0	0	0
Total	47	10	23	14	39	0	23	16
p Value	p=0.4				p=0.7			

OSCC NM = Oral squamous cell carcinoma that had not metastasised.
OSCC M = Oral squamous cell carcinoma that had metastasised.
P values= Chi-Square test.

Table 5.3: Site of lesions and incidence of severe aneuploidy in all analysed samples.

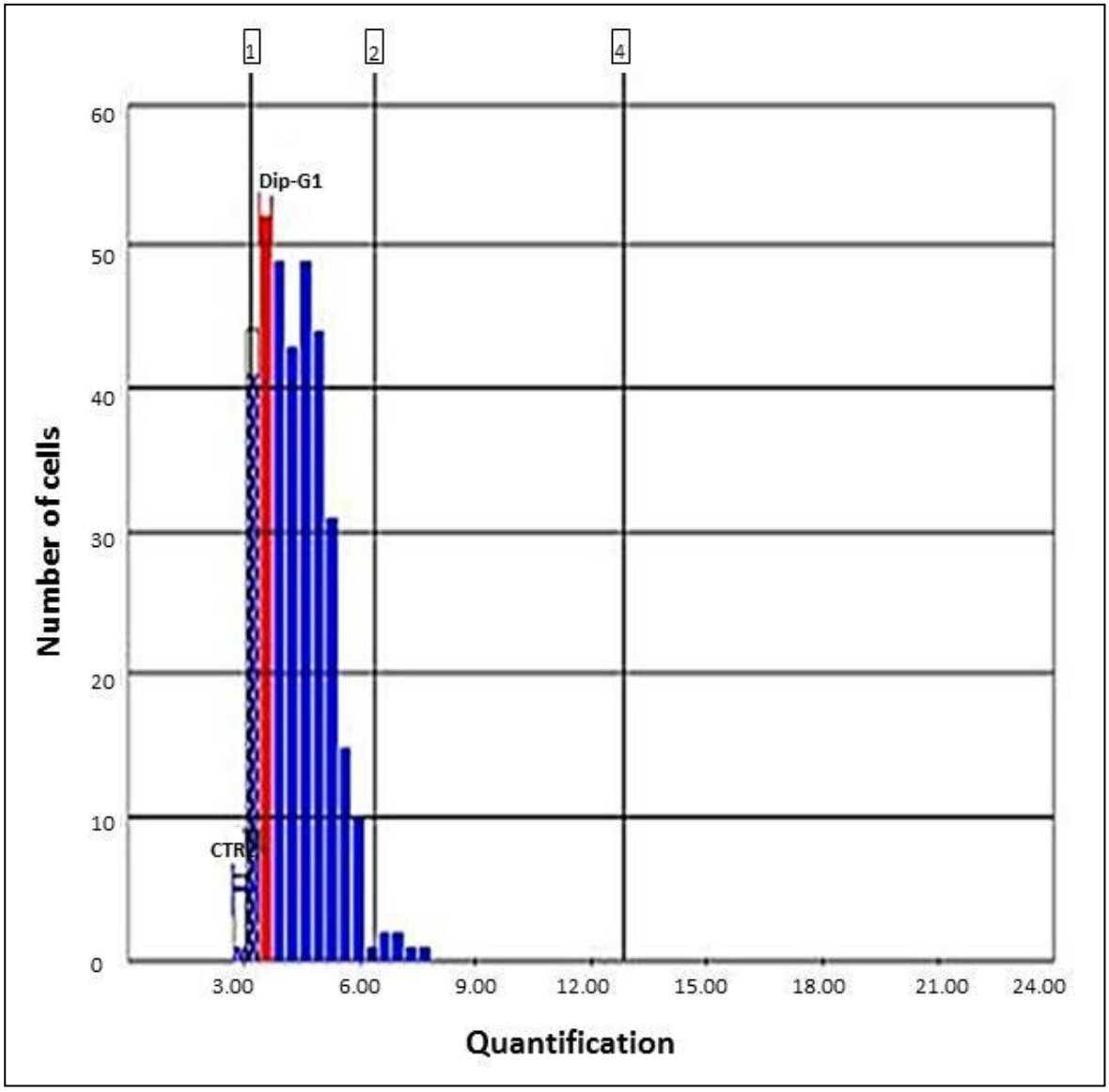

Figure 5.1: DNA ploidy pattern of diploid histogram generated by ACIS III, this case had DI of 1.1, CV of 2.7% and 5cER of 0. CTRL indicates the control G1 peak and Dip-G1 indicates the G1 peak.

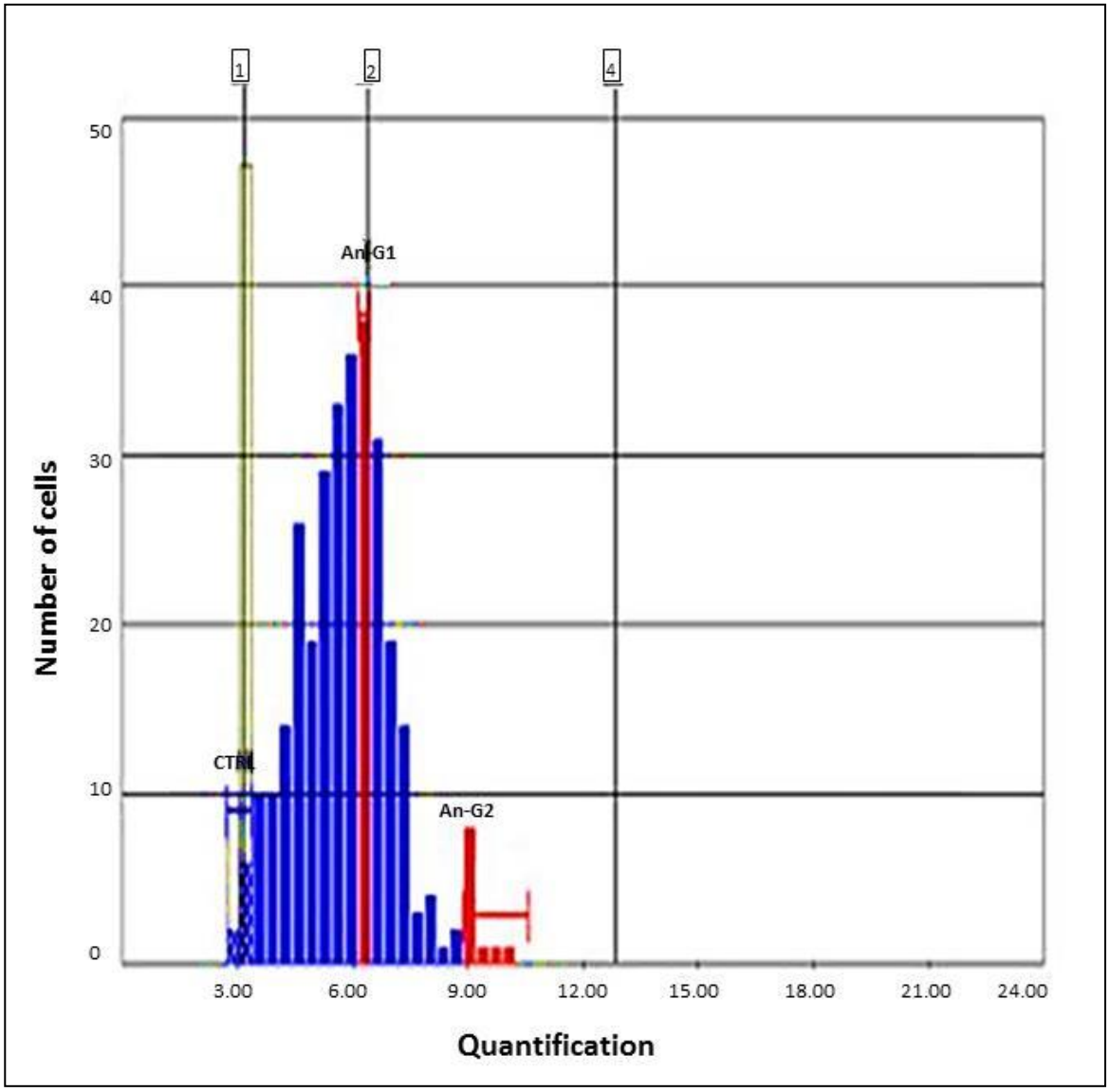

Figure 5.2: DNA ploidy pattern of severe aneuploid histogram with a tetraploid area. This case had DI of 2.0, CV of 1.5% and 5cER of 3.01%. CTRL indicates the control G1 peak, An-G1 represents the aneuploid G1 peak and An-G2 represents the aneuploid G2 peak.

5.3.2 Ploidy status, DI and 5cER

The ploidy data is summarised in **Table 5.4**. All cases of OSCC (n=86) showed abnormal DNA content in at least one of the analysed areas. In the OSCC group that had not metastasised (n=47), severe aneuploidy was observed in 20 cases (43%), moderate aneuploidy in 25 (53%), and mild aneuploidy was seen in 2 samples (4%) (**Table 5.4**).

In the OSCC samples that had metastasised 46% (18/39) showed severe aneuploidy and 54% (21/39) were moderate (**Table 5.4**). There were no significant differences in ploidy status between OSCC that had metastasised and those that had not (p=0.5, Chi-Square test) (**Table 5.4**). The lymph node metastasis samples (OSCC LN) exhibited moderate (21/39) or severe aneuploidy (18/39) and no cases were mild or diploid (**Table 5.4**). There was no significant difference (p=0.2, Wilcoxon Signed Rank test) in the incidence of the DNA abnormality between the primary tumour lesions and lymph node metastases (**Table 5.4** and **Figure 5.3**).

Most of the studies using image cytometry for ploidy analysis consider a DI of 1.0 as the default value for normal tissue. No upper limit has been established definitively; whilst some authors use a cut-off point at 1.1 as the upper limit for normal tissue (Huang et al., 2005, Fang et al., 2004, Gschwendtner et al., 1999), other researchers have used cut off points at 1.15 or 1.2 (Baak and Janssen, 2004, Furuya et al., 2000). It is worth mentioning that these studies employed different image cytometry systems

and different types of tumour tissue, and therefore, both cut off points were used to determine which of these had better prognostic results.

The median DI for the OSCC NM group was 1.63 (**Table 5.4**). The differences in DI between the OSCC M group and their metastases were significant (p=0.05, Wilcoxon Signed Rank test); a median DI of 1.61 was seen in primary lesions compared to a DI of 1.70 in the matched lymph node metastases (**Table 5.4**).

The 5cER cut-off point was selected at 1% as it has been shown to be an indicator of malignancy in image cytometry in several studies (Diwakar et al., 2005; Gockel et al., 2006; Pektaş et al., 2006). There was a significant difference (p=0.01, Wilcoxon Signed Rank test) in 5cER values between the primary lesions (OSCC M) and their metastases, where the lymph node lesions had higher values (**Table 5.4**).

5.3.3 Heterogeneity in the OSCC tumours

In the OSCC NM group there was a high incidence of moderate (23/47; 49%) and severe aneuploidy (14/47; 30%) at the surface of the lesion; no diploid areas were detected. At the advancing front, moderate aneuploidy was detected in 30 cases (64%), while severe aneuploidy was seen in 11 cases (23%) (**Table 5.5**).

In the OSCC M group, although the same pattern of heterogeneity was observed, this group showed four tumour areas with diploid DNA content at the advancing front area. Severe aneuploidy was more frequently seen at

the surface of the lesions than at the invasive front, with 16 cases (41%) and 7 cases (18%) respectively.

OSCC M showed more severe aneuploidy at the surface (41%) than the OSCC NM group (30%). The difference was significant (p=0.009, Chi-Square test) (**Table 5.5** and **Figure 5.4**). Overall, tumours showed heterogeneity within the same lesion; the surface cells showed more severe aneuploidy than the advancing front (**Figure 5.5**).

5.3.4 Comparison of primary lesions (OSCC M) with metastases

The ploidy status of OSCC primary lesions and their corresponding metastasis were compared. In 23 cases the degree of aneupoidy was the same in both lesions. In 8 of the 39 cases (21%) the changes were more severe in the lymph node metastases than in the primary lesion, and in a further 8 the changes were less severe (**Table 5.6**).

Groups	Mild aneuploid n (%)	Moderate aneuploid n (%)	Severe aneuploid n (%)	DI Median (25%-75%)	5cER Median (25%-75%)
OSCC NM (n=47)	2 (4)	25 (53)	20 (43)	1.63 (1.44-1.91)	5.06 (2.60-10.79)
OSCC M (n=39)	0	21 (54)	18 (46)	1.61 (1.50-1.79)	3.36 (1.00-7.80)
Chi-Square test	p=0.5			p=0.82	p=0.17
OSCC LN (n=39)	0	21 (54)	18 (46)	1.70 (1.5-2.20)	9.90 (0.83-15.70)
Wilcoxon Signed Rank Test	p=0.2			**p=0.05**	**p=0.01**

OSCC NM = Oral squamous cell carcinoma that had not metastasis.
OSCC M = Oral squamous cell carcinoma that had metastasis.
OSCC LN = Lymph node metastasis.
DI = DNA index.
5cER= Number of cells that exceed 5c.

Table 5.4: Results of ploidy status, DI and 5cER in OSCC NM, OSCC M and OSCC LN.

Groups	Ploidy status n (%)				
	Diploid	**Mild aneuploid**	**Moderate aneuploid**	**Severe aneuploid**	**Total**
OSCC NM (surface)	0	10 (21)	23 (49)	14 (30)	47
OSCC NM (advancing front)	0	6 (13)	30 (64)	11 (23)	47
OSCC M (surface)	0	0	23 (59)	16 (41)	39
OSCC M (advancing front)	4 (10)	4 (10)	24 (62)	7 (18)	39
P value indicating the difference between surface cells in OSCC M and OSCCNM					**p=0.009**
OSCC NM = Oral squamous cell carcinoma that had not metastasised. OSCC M = Oral squamous cell carcinoma that had metastasised. OSCC LN = Lymph node metastasis.					

Table 5.5: Ploidy results of OSCC M and OSCC NM at different areas of the tumours (surface and advancing front).

Case No	Lesion Site	primary OSCCs	lymph node metastasis
1	Tongue	Severe aneuploid	Severe aneuploid
2	Alveolar mucosa	Moderate aneuploid	Severe aneuploid
3	Buccal mucosa	Severe aneuploid	Sever aneuploid
4	Tongue	Severe aneuploid	Severe aneuploid
5	Tongue	Moderate aneuploid	Moderate aneuploid
6	Tongue	Moderate aneuploid	Moderate aneuploid
7	Floor of mouth	Moderate aneuploid	Moderate aneuploid
8	Buccal mucosa	Severe aneuploid	Severe aneuploid
9	Tongue	Severe aneuploid	Severe aneuploid
10	Alveolar mucosa	Severe aneuploid	Severe aneuploid
11	Floor of mouth	Moderate aneuploid	Moderate aneuploid
12	Tongue	Severe aneuploid	Moderate aneuploid
13	Alveolar mucosa	Severe aneuploid	Severe aneuploid
14	Alveolar mucosa	Severe aneuploid	Severe aneuploid
15	Soft palate	Moderate aneuploid	Moderate aneuploid
16	Floor of mouth	Moderate aneuploid	Severe aneuploid
17	Palatal mucosa	Severe aneuploid	Moderate aneuploid
18	Buccal mucosa	Moderate aneuploid	Moderate aneuploid
19	Buccal mucosa	Moderate aneuploid	Severe aneuploid
20	Tongue	Moderate aneuploid	Moderate aneuploid
21	Floor of mouth	Moderate aneuploid	Moderate aneuploid
22	Tongue	Moderate aneuploid	Severe aneuploid
23	Buccal mucosa	Severe aneuploid	Moderate aneuploid
24	Tongue	Severe aneuploid	Severe aneuploid
25	Tongue	Moderate aneuploid	Moderate aneuploid
26	Tongue	Severe aneuploid	Moderate aneuploid
27	Tongue	Moderate aneuploid	Severe aneuploid
28	Tongue	Moderate aneuploid	Severe aneuploid
29	Tongue	Severe aneuploid	Moderate aneuploid
30	Tongue	Moderate aneuploid	Moderate aneuploid
31	Tongue	Severe aneuploid	Severe aneuploid
32	Floor of mouth	Moderate aneuploid	Moderate aneuploid
33	Alveolar mucosa	Severe aneuploid	Moderate aneuploid
34	Floor of mouth	Moderate aneuploid	Moderate aneuploid
35	Floor of mouth	Severe aneuploid	Moderate aneuploid
36	Alveolar mucosa	Moderate aneuploid	Moderate aneuploid
37	Buccal mucosa	Moderate aneuploid	Severe aneuploid
38	Floor of mouth	Severe aneuploid	Moderate aneuploid

Case No	Lesion Site	primary OSCCs	lymph node metastasis
39	Tongue	Moderate aneuploid	Severe aneuploid

Table 5.6: Ploidy status and site of the lesions in OSCC that had metastasised. Comparing the primary lesion to its corresponding metastasis, there were 8 cases in which the changes were more severe in the metastases (Red) and 8 cases where they were less severe (Green).

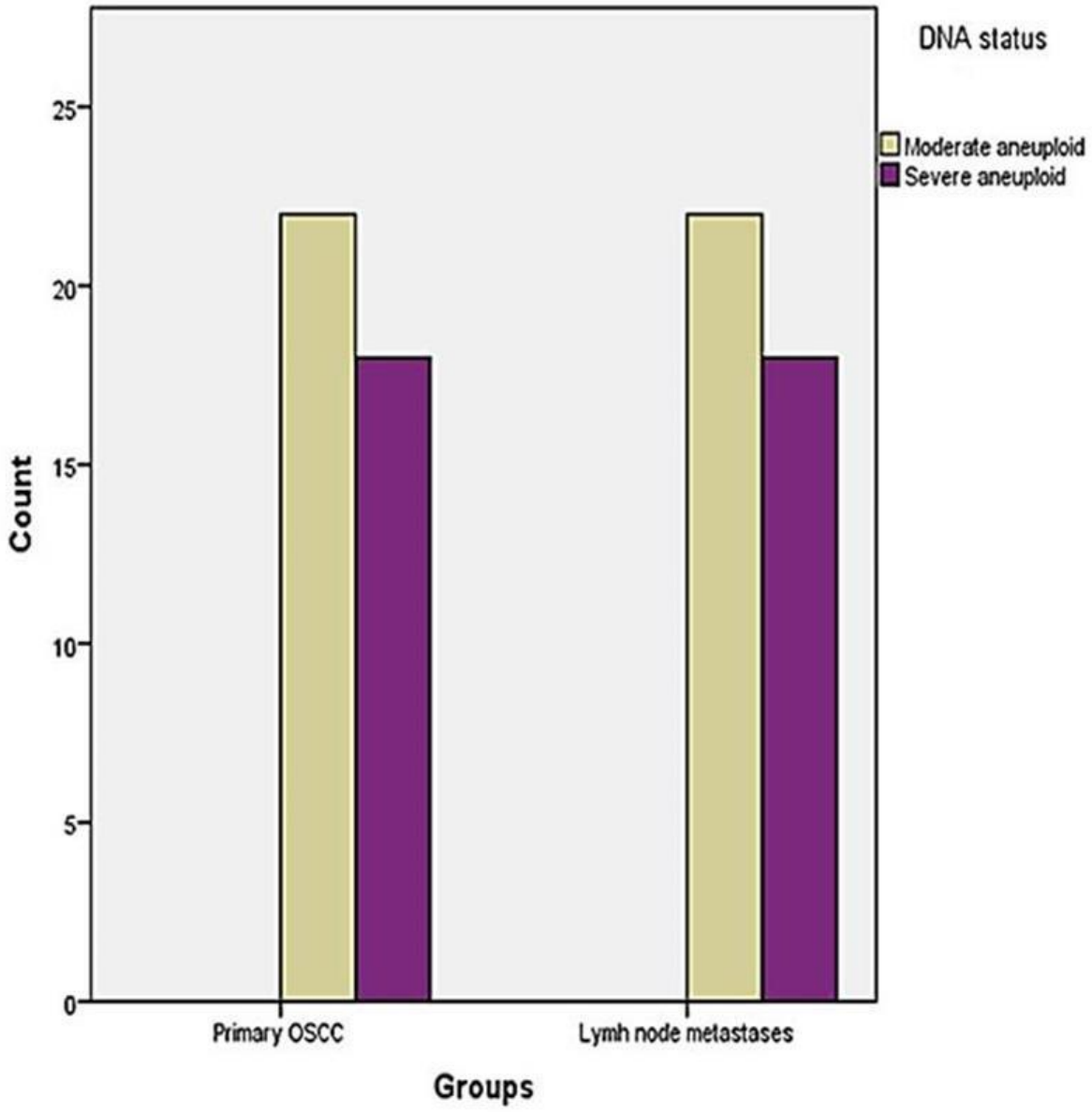

Figure 5.3: Bar-chart showing the incidence of aneuploidy in the lymph node metastases when compared to the matched OSCC (p=0.2, Wilcoxon Signed Rank test).

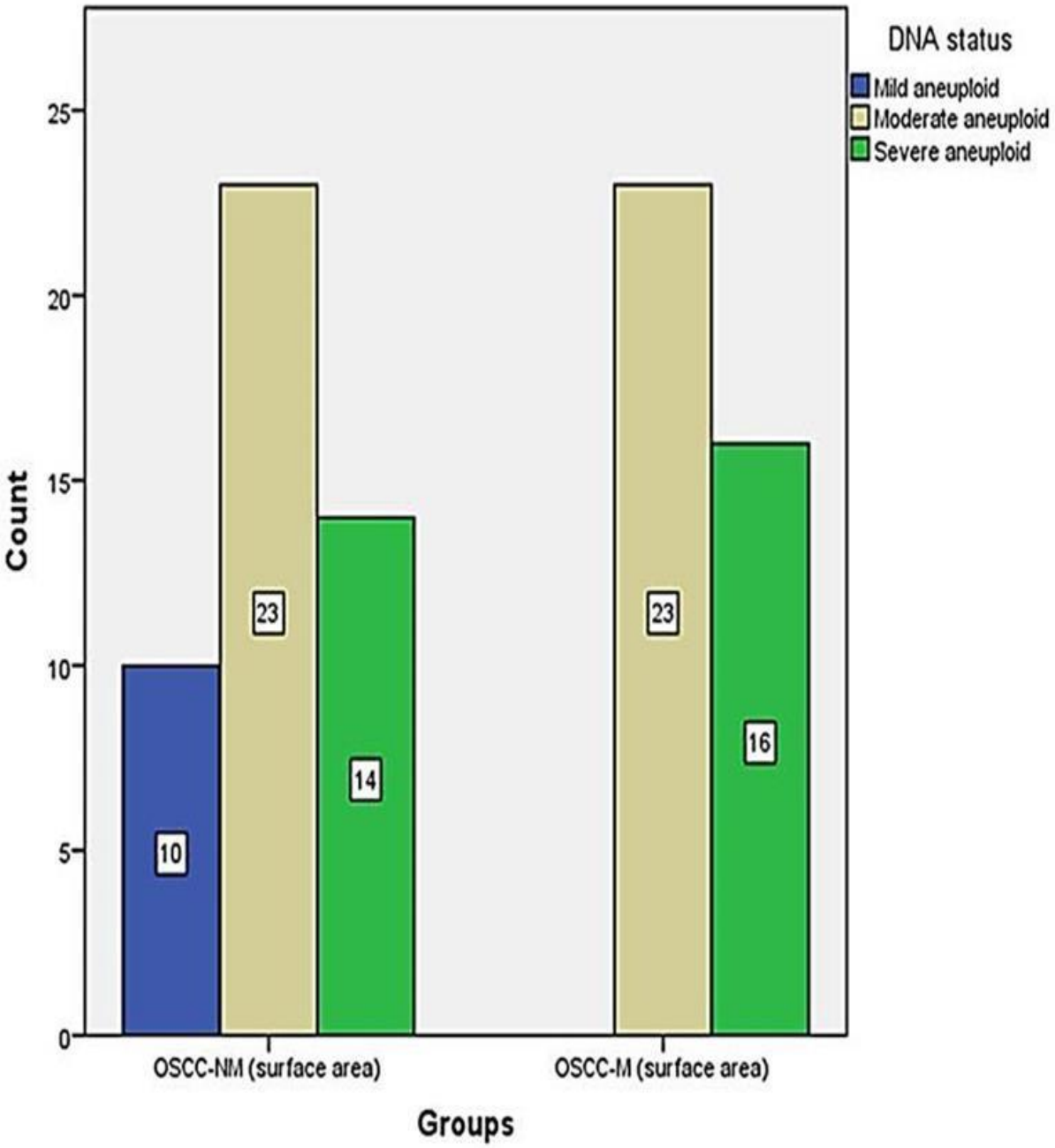

Figure 5.4: The incidence of severe aneuploidy at the surface area in OSCC with metastases (M) compared to OSCC without (NM). Differences among groups were highly significant (p=0.009, Chi-Square test).

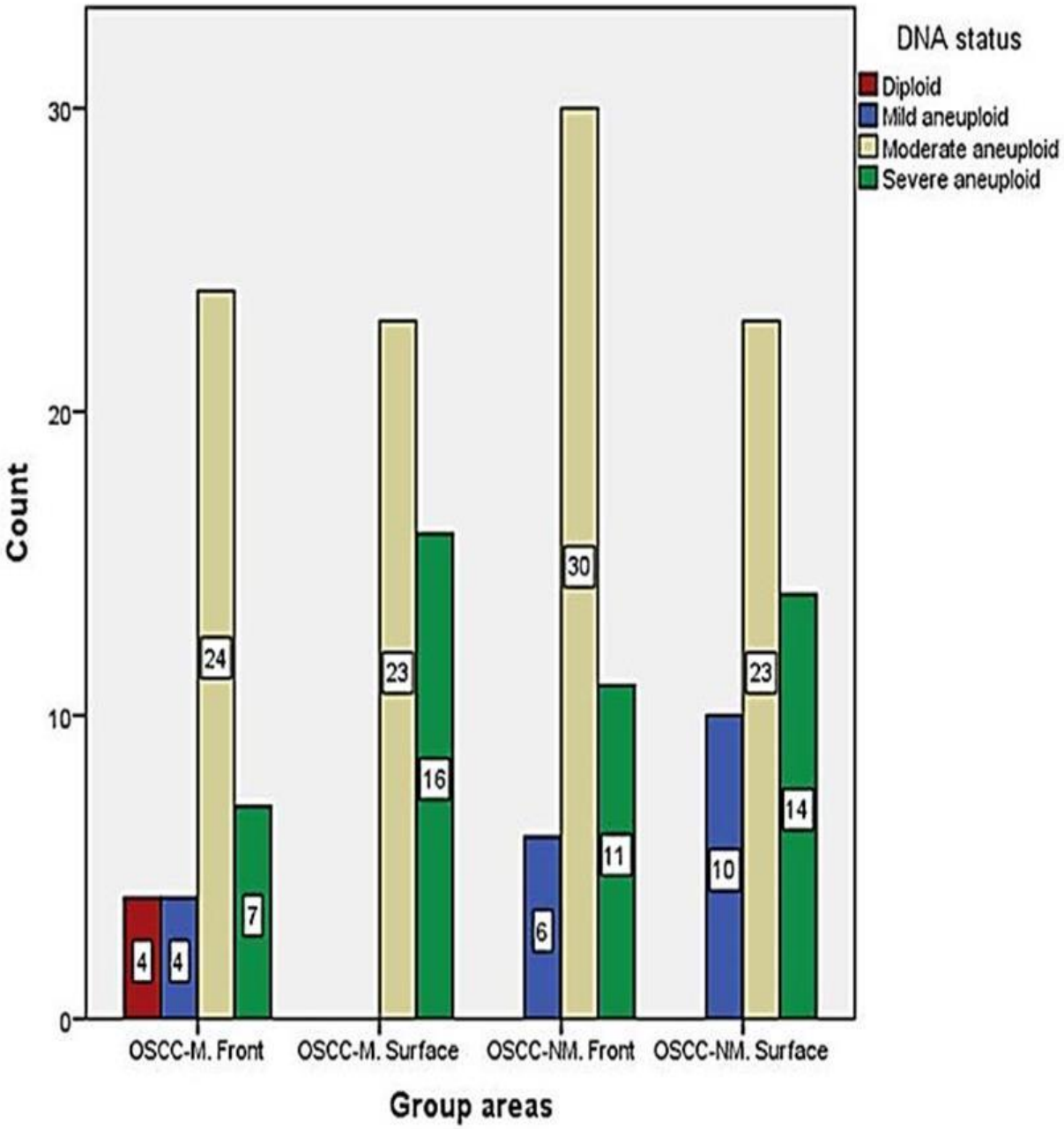

Figure 5.5: Bar-chart showing the incidence of aneuploidy in OSCC M and OSCC NM.

5.3.5 Correlation of clinical and histopathological grading with ploidy status

In the OSCC NM group, 21 cases were well differentiated OSCC, of which 13 showed moderate aneuploidy and 8 were severe (**Table 5.7**). Among the moderately differentiated OSCC the majority were moderate (n=11) or severely aneuploid (n=7) compared to only 2 cases (10%) which showed mild aneuploidy. Five of the six (83%) poorly differentiated OSCC samples showed severe aneuploidy (**Table 5.7** and **Figure 5.6**).

In the OSCC M group, all OSCC lesions showed moderate (21/39) or severe (18/39) aneuploidy. There was an almost even distribution of moderate and severe aneuploidy regardless of differentiation (**Table 5.7** and **Figure 5.7**). In the OSCC LN category one of 3 poorly differentiated carcinomas showed severe aneuploid (**Table 5.7** and **Figure 5.8**), but overall the results showed no significant correlations between DNA status and histological differentiation.

Correlation of the clinical and pathological parameters was performed by grouping T1 with T2 tumours, and T3 with T4 tumours in order to avoid dispersing data. Similarly, patients with clinical stages I and II were considered as a single group, as were stage III and IV patients, for comparing to DNA status (**Table 5.8**). Among OSCC NM samples, small or less advanced tumours (T1 and T2, stage I and II) tended to show more severe aneuploidy than the large tumours but this was not significant. In OSCC M specimens, larger or advanced stage tumours showed more

severe aneuploidy, but without significance (**Table 5.9**). Nine out of 16 cases with extra-capsular spread showed severe aneuploidy and 7 were moderate (**Table 5.9**).

Generally, none of the clinical parameters (gender, tumour site, histopathological grade, and clinical stage) showed any significant correlation to DNA content in either primary OSCC or metastatic LN lesions.

OSCC differentiation	DNA status n (%)			
	Mild aneuploid	Moderate aneuploid	Severe aneuploid	Total
OSCC NM				
Well	0	13 (62)	8 (38)	21
Moderate	2 (10)	11 (55)	7 (35)	20
Poor	0	1 (17)	5 (83)	6
Total (%)	2 (4)	25 (53)	20 (42)	47
Spearman correlation	p=0.31			
OSCC M				
Well	0	7 (54)	6 (46)	13
Moderate	0	11 (55)	9 (45)	20
Poor	0	3 (50)	3 (50)	6
Total	0	21 (54)	18 (46)	39
Spearman correlation	p=0.36			
OSCC LN				
Well	0	6 (40)	9 (60)	15
Moderate	0	13 (62)	8 (38)	21
Poor	0	2 (67)	1 (33)	3
Total (%)	0	21 (54)	18 (46)	39
Spearman correlation	p=0.36			

OSCC NM = Oral squamous cell carcinoma that had not metastasised.
OSCC M = Oral squamous cell carcinoma that had metastasised.
OSCC LN = Lymph node metastasis.

Table 5.7: Descriptive data showing the DNA ploidy status according to the differentiation of the tumours.

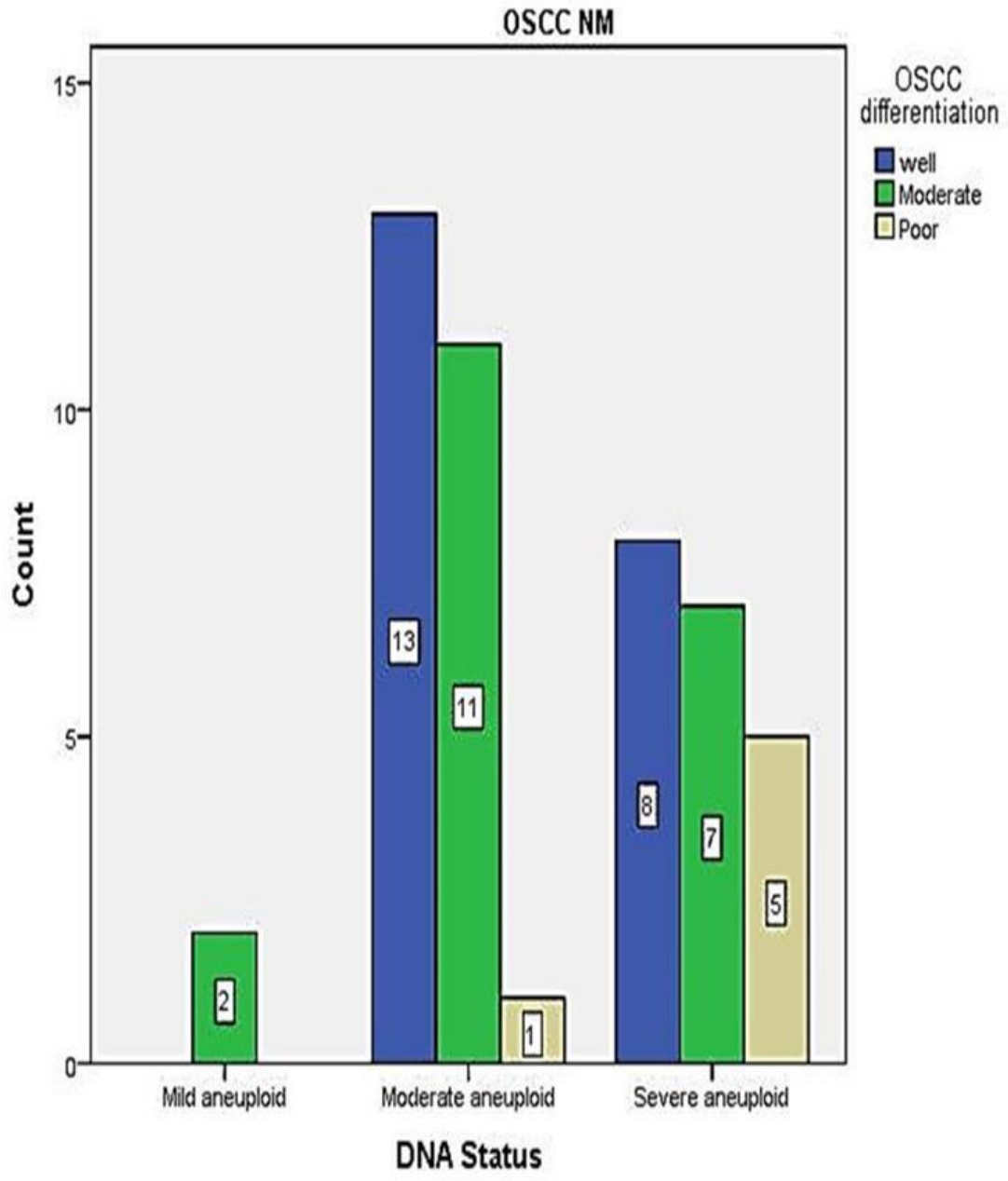

Figure 5.6: Bar-chart illustrating the distribution of the ploidy status of OSCC NM group according to their differentiation.

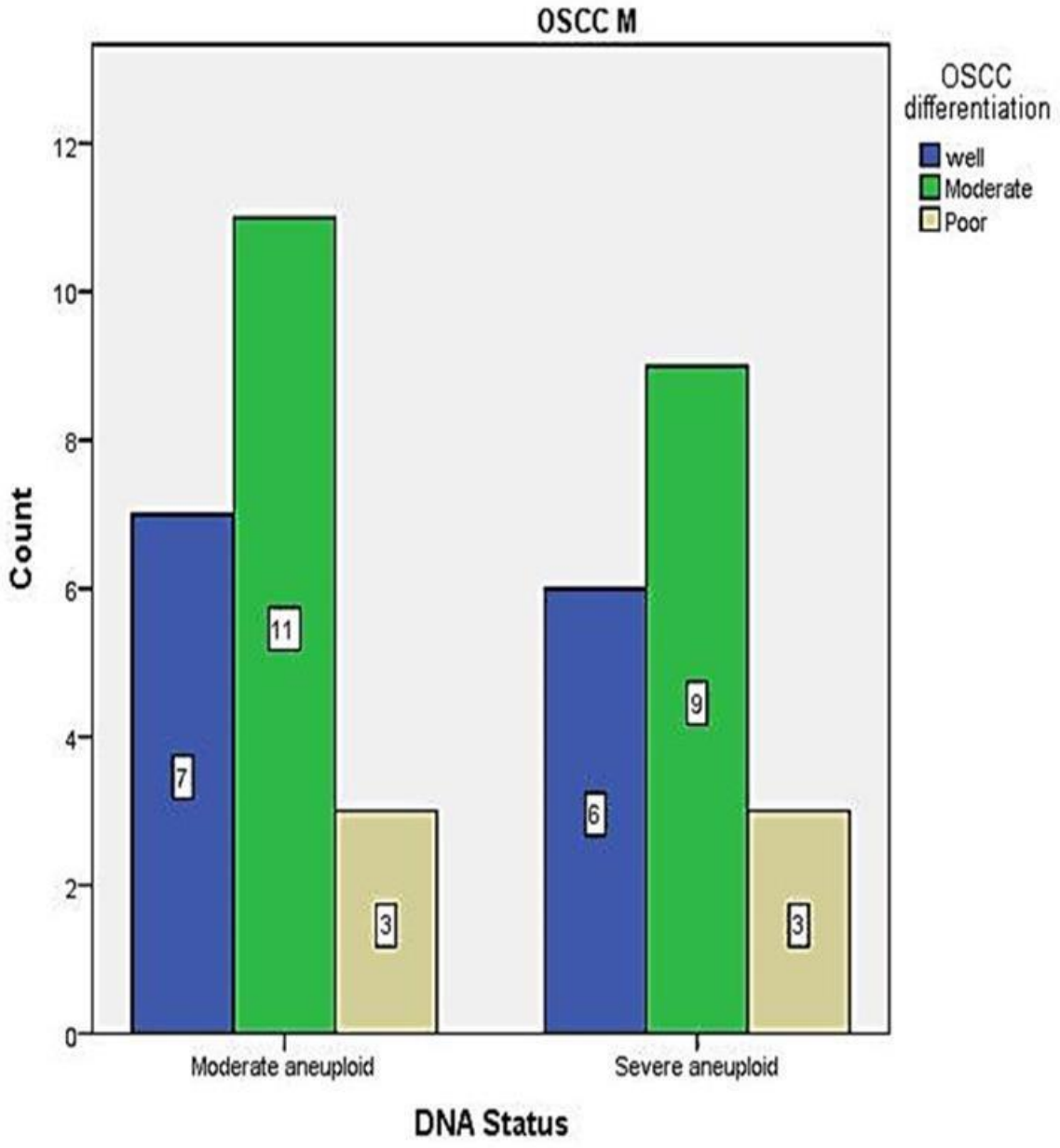

Figure 5.7: Bar-chart illustrating the distribution of the ploidy status of OSCC M group according to their differentiation.

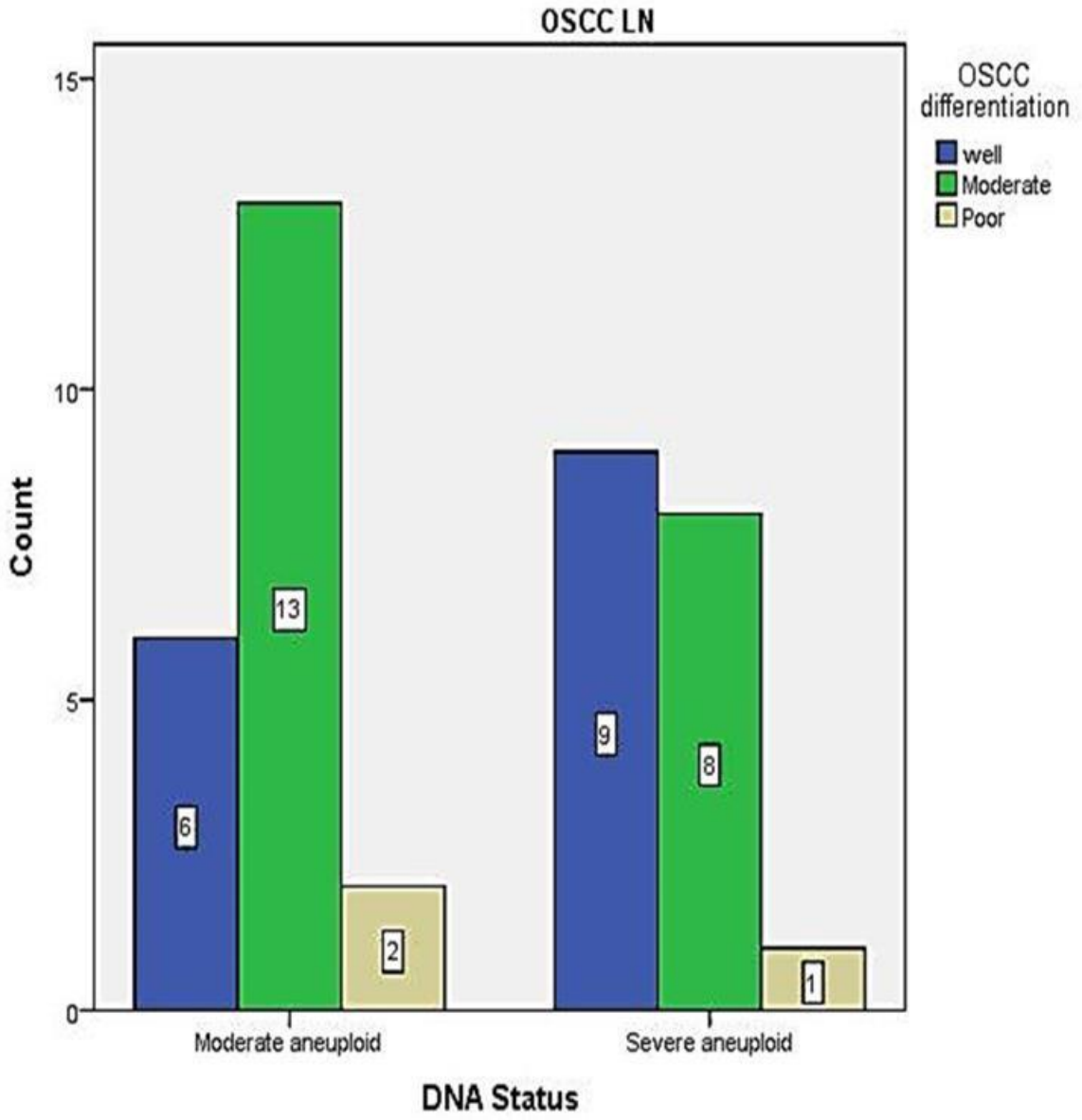

Figure 5.8: The distribution of the ploidy status of OSCC nodal metastases according to their differentiation.

	DNA status (OSCC NM)				
	Mild	Moderate	severe	Total	p value
Gender					
Male	0	21	11	32	p=0.74
female	2	4	9	15	
Tumour size (pT)					
T1+T2	0	16	14	30	p=0.29
T3+T4	2	9	6	17	
Clinical Stage					
I+II	0	16	14	30	p=0.64
III+IV	2	9	6	17	
OSCC NM = Oral squamous cell carcinoma that had not metastasised.					

Table 5.8: Correlation between clinical parameters in OSCC NM. No correlation was detected.

	DNA status (OSCC M)				
	Mild	Moderate	severe	Total	p value
Gender					
Male	0	13	13	26	p=0.73
female	0	8	5	13	
Tumour size (pT)					
T1+T2	0	8	9	17	p=0.52
T3+T4	0	13	9	22	
Stage					
I+II	0	0	0	0	p=0.29
II+IV	0	19	16	35	
Extra-capsule spread					
Yes	0	7	9	16	p=0.50
No	0	12	8	20	
OSCC M = Oral squamous cell carcinoma that had metastasised.					

Table 5.9: Correlation between clinical parameters in OSCC M. No significant correlation was detected.

5.3.6 Sensitivity, specificity and predictive values (PPV & NPV)

To evaluate DNA image cytometry analysis as a prognostic test, sensitivity (true positives correctly identified) and specificity (true negatives correctly identified) were calculated. The ploidy status of OSCC was evaluated as either diploid or aneuploid as a marker of progression, and showed a sensitivity and specificity of 100% and 0% respectively (**Table 5.10**). This was expected, as all OSCC cases showed aneuploidy. Similarly, using a cut off value of >1.1 or >1.2 for the DI, resulted in a high sensitivity and low specificity. Additionally, the 5cER parameter had sensitivity of 26% and specificity of 15%. To assess the predictive ability of DNA image analysis, positive and negative predictive values are measured, depending on the prevalence of the disease. Positive predictive value (PPV) is defined as: true positives divided by the total number of population tested positively, multiplied by 100. Whereas, negative predictive value (NPV) is defined as: true negatives divided by the total number of population tested negatively, multiplied by 100. The PPV ranged from 45% to 59% and NPV from 0% to 58% (**Table 5.10**). A low PPV indicates a high false positive rate, and tests with a high PPV are preferred.

In order to visualise the efficacy of ploidy as a prognostic test in the oral squamous cell carcinoma lesions, DI and 5cER values were plotted in receiver operating characteristic curves (ROC curve) (**Figure 5.9** and **Figure 5.10**). For diagnostic accuracy of ROC, the area under the curve was determined for each tested variable. A value of 1 under the ROC curve

represents a perfect test and an area of 0.5 or less represents a worthless test. The area under the ROC curve using the DI values was 0.514 (**Figure 5.9**) and for 5cER values was 0.415 (**Figure 5.10**). In this statistical test the DI was the parameter with the largest area under the curve, suggesting that this was the most effective. However, the two curves had areas nearer to 0.5 than to 1 meaning neither was very useful as a discriminating test. It is not surprising that this analysis has limitations (Halligan et al., 2015), as it does not take into consideration the prevalence of the disease to be tested. Nonetheless, it is still a good test to visualise the sensitivity and specificity values of any diagnostic or prognostic analysis.

	Sensitivity	**specificity**	**PPV**	**NPV**
Ploidy status	100%	0%	45%	0%
DI> 1.1	100%	0%	45%	0%
DI> 1.2	100%	0%	45%	0%
5cER >1%	26%	15%	59%	58%

DI = DNA index
5cER= Number of cells that exceed 5c
PPV= Positive predictive value
NPV= Negative predictive value

Table 5.10: Sensitivity, specificity, PPV and NPV results for progression of OSCC. Prognostic parameters in DNA image cytometry analysis were: Ploidy status (diploid or aneuploid), DI> 1.1, DI> 1.2, 5cER >1%.

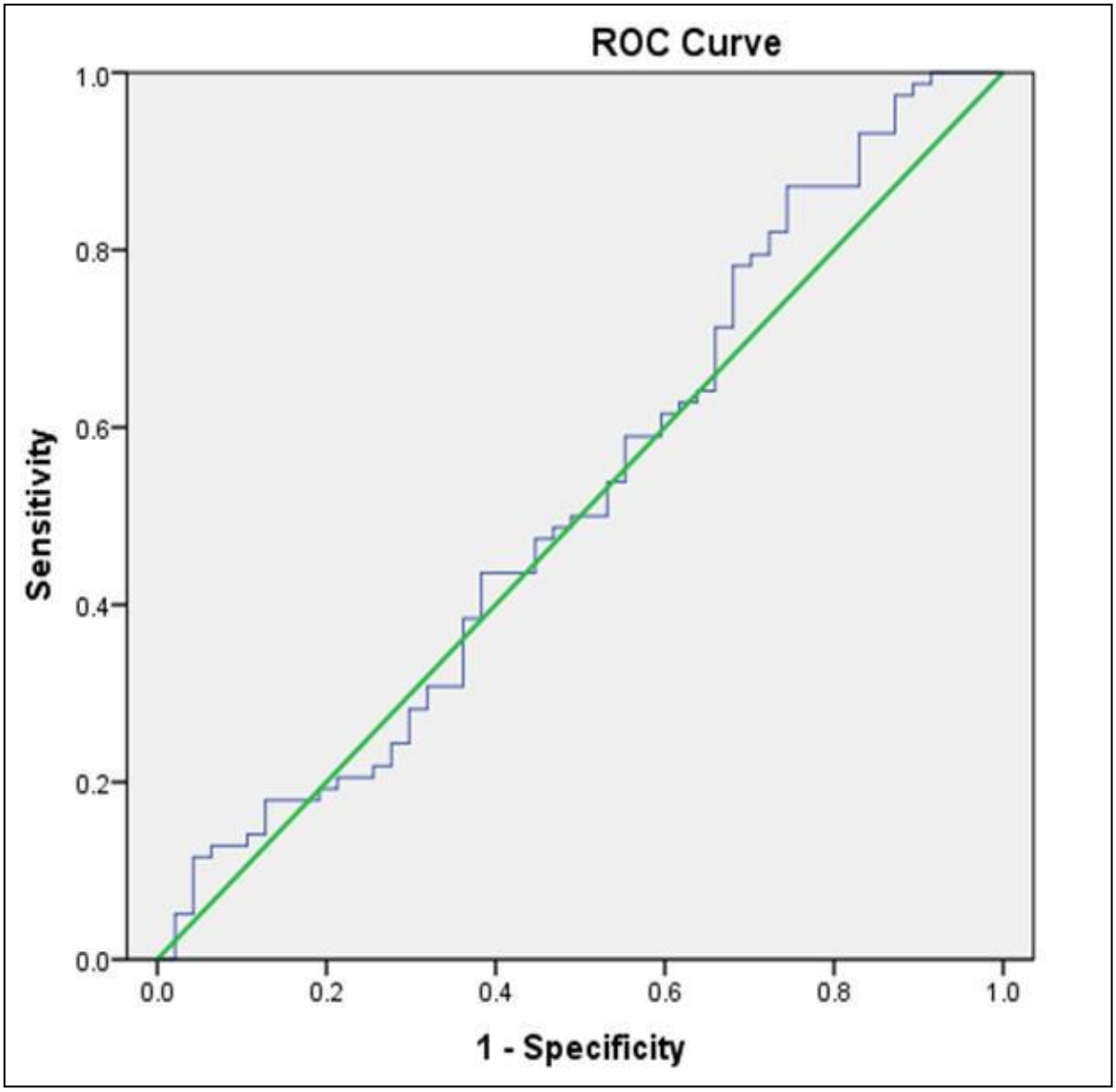

Figure 5.9: DI index value of the OSCC M and OSCC NM plotted in the ROC curve. The area under the curve was 0.514.

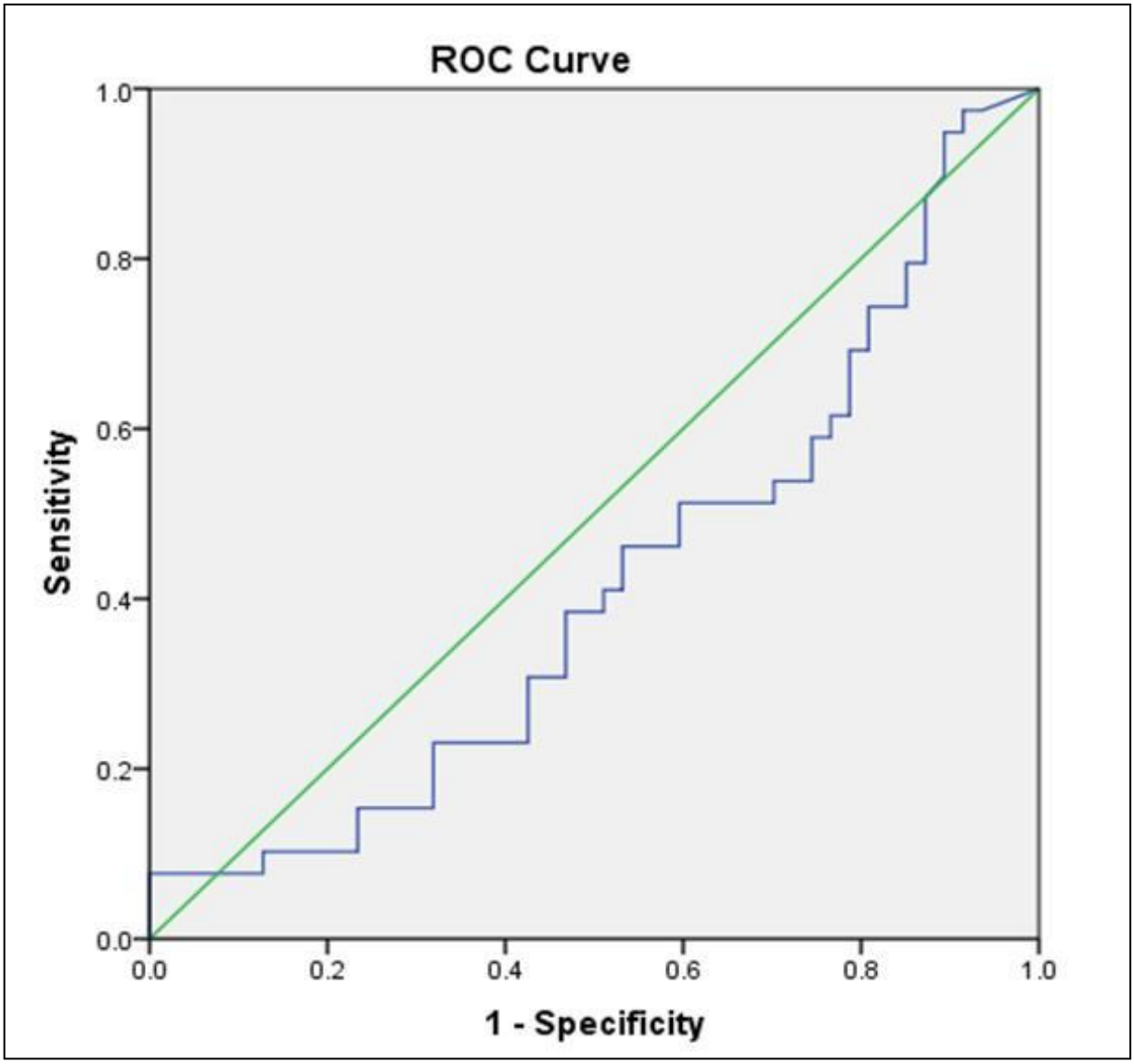

Figure 5.10: 5cER index value of the OSCC M and OSCC NM plotted in the ROC curve. The area under the curve was 0.415.

5.4 Discussion

5.4.1 Ploidy status in oral carcinomas and nodal metastases

Aneuploidy indicates the presence of abnormal genetic content and is associated with worsening clinical prognosis as the DNA index increases. The association between DNA ploidy status of primary tumours and their nodal metastases and disease free survival is frequently debated. The aim of this study was to explore the DNA ploidy status by image cytometry in metastatic and non-metastatic OSCC; in addition, to look at tumour heterogeneity with regard to ploidy status. High-fidelity DNA histograms generated by ACIS III, incorporate two important parameters, DI and 5cER, that may indicate poor clinical outcome (Yu et al., 2007, Haroske et al., 1997).

In the present study, all OSCC cases showed abnormal DNA content in at least one area of each tumour. Only four areas were found to be diploid. A low rate of aneuploidy incidence in OSCC has previously been observed (El-Deftar et al., 2012, Torres-Rendon et al., 2009b), but in agreement with our results, a high incidence of aneuploidy has been reported by others (Abou-Elhamd and Habib, 2007, Santos-Silva et al., 2011). This variation may be due to differences in the ploidy system, cell nuclei preparation and techniques used in the various studies. In the present study ICM was used, which has the advantage that tumour areas can be accurately selected, ensuring representative sampling and removal of stromal contamination.

Additionally, DNA ploidy is known to be heterogeneous within OSCC and the rate of abnormal content (aneuploidy) is higher when multiple samples are analysed in each tumour (Baretton et al., 1995, Diwakar et al., 2005). In the current study, tumours showed heterogeneity within the same lesion. More severe aneuploidy was seen in the surface cells of OSCC lesions (34%) than cells at the advancing front (21%). Interestingly, there was a highly significant difference in ploidy severity at the surface between the OSCC M (40%) and OSCC NM groups (30%), suggesting an increase in aberrant nuclear DNA content in tumours that metastasise. One explanation for this observation could be that tumours behave differently at different stages, depending on ploidy status. Diploid cells detected at the front area may reflect a decrease in aneuploidy with tumour progression. It may be that tumours need to be more severe at the surface to provide a good environment for growth at the start of the lesion and to allow more genetic alterations. Additionally, probably tumour cells may shift down their ploidy alterations to endorse other changes at the advancing front. As this is the first time that both sites have been compared, further analysis with a larger number of samples is needed.

The results also revealed that the lymph node metastases were more severely aneuploid in some cases (n=8) compared to the matched primary lesions (**Table 5.6**). This suggests that more malignant and aggressive cells in primary lesions have an ability to migrate into lymph nodes. In contrast, other studies did not find any association between incidence of aneuploidy and high rates of metastasis (Saiz-Bustillo et al., 2005).

5.4.2 High DI and 5cER are associated with nodal metastasis

The DI values (**Table 5.4**) were significantly higher in the lymph node metastatic lesions than the matched primary lesions (p=0.05). Similarly, in other cytometric studies increases in the DNA index indicated an increased risk of disease progression (Isharwal et al., 2008, Hoofnagle Jr et al., 1995). This was also the case for 5cER values (p=0.01), which were higher in the metastatic lesions. This could indicate that metastatic cells with high levels of aneuploidy could be a characteristic of progression of disease.

Taken together, our findings of increased aneuploidy in metastatic OSCC and in lymph nodes suggest that aberrant DNA content is associated with aggressive tumours and that DNA ploidy analysis may be a useful prognostic indicator. However, the results indicate that confirmatory studies on quantitative evaluation of DI and 5cER are needed if these parameters are to have any prognostic value.

5.4.3 DNA image cytometry as a prognostic test in OSCC

Even though the specificity of aneuploidy to predict disease progression was 0% the sensitivity was high (100%) and the positive predictive value was 45%. Similarly, other studies which have evaluated aneuploidy in patients with cervical cancer have shown high sensitivity (92%) and low specificity (54%) values (Tong et al., 2009).

When the DNA index and 5cER values were evaluated as independent prognostic factors, the 5cER showed 26% sensitivity and 15% specificity (**Table 5.10**).

This suggests that DNA ploidy may not be a good tool to evaluate tumour prograssion; however, it may be useful if considered along with histopathological findings and other clinical and molecular prognostic factors.

5.4.4 Correlation of ploidy status with tumour differentiation and clinicopathological parameters

In the present cohort, OSCC differentiation and ploidy status were not associated (**Table 5.7**). This was in agreement with finding of other studies (Diwakar et al., 2005, Torres-Rendon et al., 2009b, Santos-Silva et al., 2011) which also reported no correlation between ploidy and differentiation in OSCC. In line with previous studies, no significant association was found between DNA ploidy of either primary or metastatic samples with age, lesion site, tumour size and tumour stage (El-Deftar et al., 2012, Das et al., 2005, Santos-Silva et al., 2011). However, the association between DNA ploidy status and clinicopathological factors is controversial. In accordance with previous studies (Bueno et al., 1998), incidence of moderate and severe aneuploidy was more frequent in men and advanced stages (III & IV) in OSCC M samples.

5.4.5 DNA ploidy imaging system

DNA image cytometry has become a common method for evaluation of DNA ploidy in tumour cells. It has been reported that image cytometry is more accurate than flow cytometry (Dunn et al., 2010, Belien et al., 2009) since the former system allows visual selection of tumour areas and cells of interest, and artefacts or areas which are not relevant can be discarded either semi-automatically or manually. In addtion, DNA ICM has well established diagnostic validity and reproducibility (Böcking et al., 2010, Böcking and Nguyen, 2004). The principles of DNA analysis consist of measuring of the DNA content in a large number of individual cells and construction of a histogram representing the distribution of the cell population. Even though image cytometry provides a more precise analysis, it has been reported that image cytometry systems may vary and some could be more accurate than others (Huang et al., 2005, Bremmer et al., 2011).

In this study, the Dako Automated Cell Imaging System (ACIS III) was used to undertake image cytometry on tissue sections, although we identified a number of technical limitations with this system. This is a semi-automatic system where the operator helps with the selection of the cells to be analysed. However, according to our experience with the ACIS III, too many sample cells (not originally selected by the system) need to be selected manually by the operator. The only option for selecting cells is to draw around the nuclei of each cell that was not originally pre-selected by the software. This is laborious and lacks accuracy. Additionally, ACIS III does

not provide multiple area measurements in a single section (on the same slide), and each area has to be rescanned and measured again. This is also very laborious and increases the operator time for every ploidy analysis. While the system may currently have flaws, future improvements in the analysis software and cheaper components will address these limitations.

5.4.6 Conclusions

- DNA ploidy is a common feature of OSCC as aneuploidy was found in all 86 cases (100%) and in 168 of 172 tumour areas analysed (98%).
- Primary OSCC lesions show heterogeneity, suggesting that cancers are polyclonal.
- Aneuploidy may only be useful as a tool to evaluate prognosis when used along with histopathological analysis and other clinical and molecular factors.
- A dichotomous division of diploid/aneuploid may not be accurate enough for use as a marker, and calculation of DI and 5cER provides a more sensitive parameter.
- There is no association between DNA status and histological differentiation.
- Even though it is unlikely that the ploidy status alone can accurately predicate OSCC progression, further studies are needed to determine the ideal test parameters and conditions under which it should be used, as well as the cost-effectiveness.

CHAPTER 6: CORRELATION OF CELL CYCLE REGULATORY PROTEIN EXPRESSION WITH PLOIDY STATUS

6. CORRELATION OF CELL CYCLE REGULATORY PROTEIN EXPRESSION WITH PLOIDY STATUS

6.1 Introduction

MCM2 is a key regulatory protein of the cell cycle and is recruited as part of the replicative helicase to initiate eukaryotic DNA synthesis. In fact, it has been reported that down-regulation of MCM proteins leads to a loss of proliferative capacity in human cells (Zhang et al., 2015). Nuclear expression of this protein is seen not only in cycling cells, but also in cells which are licensed or enabled to proliferate (Torres-Rendon et al., 2009a). This property makes MCM proteins ideal as diagnostic proliferation markers in various tissues (Williams and Stoeber, 2012a, Tamura et al., 2010)

Ki-67 protein identifies cells in active phases (late G1-S-G2-M) of the cell cycle, but not cells in early G1 or quiescent (G0) phases. Thus, evaluation of this antigen may serve as a marker of growth (Dudderidge et al., 2005, Gonzalez-Moles et al., 2010). At the same time, Ki-67 is not co-expressed with other G1 markers, suggesting that MCM2 protein is a superior proliferation marker over Ki-67. When combined with other cell cycle proteins it may help to define cells with different cell cycle kinetics.

Geminin has been identified as a novel proliferation marker. It is mainly present in the S-G2-M transition stage of the cell cycle. Geminin helps to regulate cell cycle entry and functions by down regulating MCM complex

recruitment and preventing re-replication in mammalian cells. When geminin is evaluated along with Ki-67, the geminin/Ki-67 ratio gives information about the length of the G1 phase (Section 1.5.1). The geminin/Ki-67 ratio has been proposed as a prognostic marker, as shown in oligodendroglial tumours (Wharton et al., 2004). The higher the geminin/Ki-67 ratio, the poorer the prognosis.

The MCM2/Ki-67 ratio defines the proportion of cells that are licensed to proliferate. The difference between MCM2 and Ki-67 is important as it may help to identify the cell kinetics of a lesion (Quaglia et al., 2006). The higher the ratio, the greater the proportion of cells that reside in a licensed proliferative state (Wharton et al., 2004, Shetty et al., 2005).

Cyclin D1 is a crucial cell cycle regulator of the G1 phase. Various studies have been conducted to evaluate cyclin D1 in premalignant and malignant lesions. Studies have shown that cyclin D1 over-expression may be involved in the early stages of tumorigenesis (Wilkey et al., 2009, Shintani et al., 2002).

DNA ploidy abnormalities are considered important indicators of genomic instability and correlate with worse prognosis in a variety of cancers (Blanco et al., 2013, Santos-Silva et al., 2011, Torres-Rendon et al., 2009b). Dysregulation of the cell cycle, particularly during cell division (mitosis) and DNA synthesis events, is believed to generate aneuploidy and can promote tumorigenesis (Weaver and Cleveland, 2008).

Interestingly, a recent study carried out by Torres-Rendon *et al*. (Torres-Rendon et al., 2009b) showed that aneuploid oral epithelial dysplasia lesions have a higher risk of malignant progression than cases with normal DNA content (diploid). Other studies showed aneuploidy in approximately 87% of OSCC (Santos-Silva et al., 2011). Aneuploidy has been proposed to help identify cases that develop cancer in Barrett`s oesophagus (Bird–Lieberman et al., 2012). Therefore, it is important to investigate possible relations with prognostic biomarkers within aneuploid lesions.

As mentioned in Chapter 1 (Section 1.10.1), the nuclear DNA index (DI) has been established as an important parameter of DNA image cytometry. The upper limit of DI for a diploid sample among DNA image cytometry studies has usually been 1.1 (Huang et al., 2005, Gschwendtner et al., 1999, Pentenero et al., 2009, Santos-Silva et al., 2011). A DI of 1.2 has also been used as the upper limit in diploid cases (Furuya et al., 2000, Fang et al., 2004). The proportion of cells that exceed 5c (5cER) in image cytometry analysis has been proposed as a reliable parameter of malignant progression when the value is more than 1% of the total number of analysed nuclei (Gockel et al., 2006, Pektaș et al., 2006).

This chapter explores whether there is any correlation of cell cycle regulatory proteins (MCM2, Ki-67, geminin and cyclin D1) with ploidy status in non-metastatic and metastatic oral squamous cell carcinomas.

6.2 Material and methods

A total of 125 paraffin blocks were analysed for both ploidy content and expression of the cell cycle regulatory proteins, MCM2, Ki-67, geminin and cyclin D1. Forty-seven were oral squamous cell carcinomas that had not metastasised (OSCC NM) and 39 cases were primary oral squamous cell carcinoma that had metastasised (OSCC M), with their matched lymph node metastases (39 samples).

The cases that were used for the correlation analyses were the biopsy samples that had been analysed for DNA ploidy and also had sections available for immunohistochemistry staining (**Table 6.1**). Quantitative analysis of protein expression was previously explained in Chapter 3, section 3.4.3. The DNA analysis by image cytometry of the paraffin sections was also described in sections 3.5.1, 3.5.2, 3.5.3 and.3.5.4

Spearman's rank correlation coefficient was the statistical test used to evaluate the strength of the correlations. All correlation coefficient values are in Appendix 2.

	OSCC NM	OSCC M	OSCC LN	Total
MCM2 - Ploidy analysis	40	37	31	108
Ki-67 - Ploidy analysis	44	39	36	119
Geminin - Ploidy analysis	47	39	36	122
Cyclin D1 - Ploidy analysis	47	38	31	116
MCM2/Ki-67 ratio - Ploidy analysis	39	37	26	102
Geminin/Ki-67 ratio - Ploidy analysis	22	23	27	72

OSCC NM = Oral squamous cell carcinoma that had not metastasised.
OSCC M = Oral squamous cell carcinoma that had metastasised.
OSCC LN = Lymph node metastasis.

Table 6.1: Number of cases that were available for the correlation analyses between DNA ploidy and immunostaining of the different proteins.

6.3 Results

6.3.1 Correlation between MCM2, Ki-67, geminin and cyclin D1 expression and ploidy status in OSCC NM

6.3.1.1 MCM2- Ploidy analysis

There was no correlation between MCM2 and ploidy status in all analysed groups. However, whilst the LI mean showed a significant correlation with DI (p=0.03) (**Table 6.2**), in contrast, the 5cER, showed no significant correlation in this group.

6.3.1.2 Ki-67- Ploidy analysis

There was no evident correlation between Ki-67 and ploidy status, and either, DI or 5cER in OSCC NM (**Table 6.2**).

6.3.1.3 Geminin- Ploidy analysis

Geminin showed no significant correlation with ploidy status. Likewise, the DI and 5cER were not significant (**Table 6.2**).

6.3.1.4 Cyclin D1 - Ploidy analysis

This protein showed no correlation with ploidy status, DI or 5cER (**Table 6.2**) .

6.3.1.5 MCM2/Ki-67 ratio - Ploidy analysis

The MCM2/Ki-67 value showed no correlation with ploidy statu in the OSCC NM group (**Table 6.2**).

6.3.1.6 Geminin/Ki-67 ratio - Ploidy analysis

There was no correlation between the geminin/Ki-67 ratio and the ploidy analysis in the OSCC NM group (**Table 6.2**).

6.3.2 Correlation between MCM2, Ki-67, geminin and cyclin D1 expression and ploidy status in OSCC M

With the exception of cyclin D1, which showed significant correlation with the DI value (**Table 6.2**), there was no correlation between the expression of cell cycle proteins (MCM2, Ki-67, geminin) and ploidy status in this group. Similarly, there was no correlation between the MCM2/Ki-67 and geminin/Ki-67 ratios and the ploidy status, DNA index or 5cER values (**Table 6.2**).

6.3.3 Correlation between MCM2, Ki-67, geminin and cyclin D1 expression and ploidy status in OSCC LN

No significant correlation between cell cycle proteins and ploidy status, DI or 5cER was found in this cohort, except for Ki-67 LI, whcih was significantly correlated with ploidy status (p=0.04) (**Table 6.2**).

Protein / DNA status	OSCC NM	OSCC M	OSCC LN
	Spearman's rank correlation test		
MCM2/ Ploidy status	0.23	0.10	0.42
MCM2/ DI	**0.03**	0.20	0.79
MCM2/ 5cER >1%	0.42	0.27	0.80
Ki-67/ Ploidy status	0.09	0.71	**0.04**
Ki-67/ DI	0.69	0.70	0.10
Ki-67/ 5cER >1%	0.23	0.84	0.92
Geminin/ Ploidy status	0.40	0.97	0.29
Geminin/ DI	0.95	0.19	0.09
Geminin/ 5cER >1%	0.67	0.55	0.09
Cyclin D1/ Ploidy status	0.65	0.24	0.19
Cyclin D1/ DI	0.64	**0.02**	0.41
Cyclin D1/ 5cER >1%	0.78	0.16	0.80
MCM2/Ki-67/ Ploidy status	0.05	0.78	0.07
MCM2/Ki-67/DI	0.24	0.33	0.13
MCM2/Ki-67/ 5cER >1%	0.35	0.68	0.55
Geminin/Ki-67/ Ploidy status	0.17	0.59	0.12
Geminin/Ki-67/ DI	0.68	0.19	0.09
Geminin/Ki-67/ 5cER >1%	0.10	0.61	0.09

OSCC NM = Oral squamous cell carcinoma that had not metastasised.
OSCC M = Oral squamous cell carcinoma that had metastasised.
OSCC LN = Lymph node metastasis.
Ploidy status= Aneuploid or diploid.
DI= DNA index.
5cER= Abnormal cells that exceed 5c with a cut-off point at 1%.

Table 6.2: Correlation of DNA content with MCM2, Ki-67, geminin, cyclin D1 and MCM2/Ki-67 and geminin/Ki-67 ratios. P value of less than 0.05 was considered significant.

	MCM2 mean±SD	Ki-67 mean±SD	Geminin mean±SD	Cyclin D1 mean±SD	MCM2/Ki67 mean±SD	Geminin/Ki67 mean±SD
OSCCNM						
DI>1.2	69.96±16.28	64.61±28.3	34.23±14.11	55.35±17.96	1.35±1.00	0.66±0.27
5cER>1%	70.39±16.84	62.29±18.51	34.98±14.08	54.89±18.22	1.33±0.87	0.69±0.25
OSCC M						
DI>1.2	73.58±16.21	54.23±21.39	31.16±15.94	56.84±12.97	1.55±0.67	0.58±0.23
5cER>1%	74.79±17.08	53.72±21.09	29.68±14.67	57.53±13.41	1.57±0.66	0.56±0.22
OSCC LN						
DI>1.2	79.88±12.66	49.66±36.04	20.75±21.90	59.20±20.81	1.27±0.43	0.31±0.30
5cER>1%	80.17±12.42	49.93±36.59	23.72±23.03	60.33±18.78	01.31±0.45	0.36±0.32

SD= Standard deviation.
OSCC NM = Oral squamous cell carcinoma that had not metastasised.
OSCC M = Oral squamous cell carcinoma that had metastasised.
OSCC LN = Lymph node metastasis.
DI= DNA index.
5cER >1%= Abnormal cells that exceed 5c.

Table 6.3: DNA index and the 5cER mean and standard deviation of MCM2, Ki-67, geminin and cyclin D1 LIs in OSCC NM and OSCC M.

6.4 Discussion and conclusions

The Spearman`s rank correlation coefficient is a non-parametric measure of correlation that is the most suitable to assess the relation between two variables (Altman, 1990). All analysed samples in OSCC were aneuploid and all had DI of 1.2 or more. Thus, correlation with different cut off points between groups was not applicable.

6.4.1 High MCM2 expression is not correlated to ploidy status in OSCC NM and OSCC M

In Chapter 4, it was shown that the OSCC M samples had slightly higher MCM2 LI values than the OSCC NM samples but this was not statistically significant (**Table 4.4**). However, in this chapter`s results the ploidy status of OSCC samples (NM & M) did not correlate with the MCM2 LI value (**Table 6.2**), although, the mean LI values of cases with DI>1.2 and 5cER >1% were much higher in the OSCC M group (**Table 6.3**).

The increase in expression of MCM2 in the OSCC M group may represent cells with an increased and steady proliferation rate but not necessarily with aberrant DNA content.

6.4.2 Low Ki-67 expression is correlated with ploidy status in OSCC LN

According to the Spearman rank correlation coefficient, Ki-67 expression showed a significant correlation (**Table 6.2**) with ploidy status (p=0.04). The

results in Chapter 4 showed that the mean Ki-67 LI value in the OSCC LN samples was significantly different (p=0.03) to the LI value in the primary tumours (OSCC M) (**Table 4.5**). One possible explanation for the lower Ki-67 value in the aneuploid OSCC LN samples may be that aneuploid cases have a population of cells that are not in a proliferative state but are in the exit of G0 - early G1 phase.

6.4.3 Low geminin expression is not correlated with ploidy status in OSCC M

There was no significant correlation of low geminin and ploidy status (p=0.97) in the OSCC M samples (**Table 6.2**). This lack of significance was also observed between OSCC NM and OSCC M groups (Chapter 4, **Table 4.4**).There was a tendency for geminin to be lower in OSCC M samples and in cases with a high DI value and 5cER (**Table 6.3**). Moreover, the OSCC M and OSCC LN cases did not show any significant correlation.

6.4.4 There is no correlation of high cyclin D1 expression with ploidy status

Even though there was no significant correlation between OSCC NM and OSCC M samples (Chapter 4, **Table 4.4**), this study demonstrated a significant correlation (p=0.02) of high cyclin D1 with abnormal DNA content as shown by the DI parameter (**Table 6.2**).

6.4.5 MCM2/Ki-67 and geminin/Ki-67 ratios are not correlated with ploidy status

In Chapter 4, geminin/Ki-67 ratio was significanly different when OSCC NM and OSCC M groups were cmpared (**Table 4.4**). Also, it was shown that differences between OSCC M and OSCC LN in MCM2/Ki-67 and geminin/Ki-67 ratios were statistically sgnificant (p=0.05, p=0.01, t-test, respectively). Reults from this Chapter showed no correlation (**Table 6.2**) between ploidy status and MCM2/Ki-67 or geminin/Ki-67 ratios in any of the OSCC samples (**Table 6.3**).

6.5 Conclusions

- MCM2 LI correlated with abnormal DNA content as shown by DI in OSCC NM samples.
- Ki-67 did not correlate with ploidy status or 5cER>1% values. However, Low Ki-67 LI correlated with aneuploidy in the OSCC LN group.
- Cyclin D1 LI correlated with ploidy status in OSCC M, but not with DI or 5cER values in OSCC NM, OSCC M or OSCC LN.
- MCM2/Ki-67 and geminin/Ki-67 ratios did not correlate with abnormal DNA content in any of the analysed groups.
- In the OSCC LN group there was no correlation between aneuploidy with expression of the cell cycle regulatory proteins, MCM2, geminin and cyclin D1.
- Ki-67 expression correlated with ploidy status in the OSCC LN group.

CHAPTER 7: FINAL DISCUSSION

7. FINAL DISCUSSION

7.1 Overview

The final chapter of this dissertation discusses the findings which have emerged from the data and statistical analyses presented in the previous chapters. This project employed two different analytical methods that used paraffin embedded tissue blocks of oral squamous cell carcinoma lesions. The first method was immunohistochemical analysis of cell cycle regulatory proteins MCM2, Ki-67, geminin and cyclin D1. The objectives outlined in Chapter 4 of this thesis were met by performing an accurate quantification of positive and negative tumour cells in the TMA sections. It was considered that quantitative measures would usefully supplement and extend the qualitative analysis.The second method was DNA ploidy analysis by image cytometry.

The following section provides a brief overview of the current literature, discusses shortcomings, and finally, suggestions for future work.

7.2 MCM2, Ki-67, geminin and cyclin D1 expression in OSCC with and without metastasis and in lymph node metastases

Squamous cell carcinoma is a common malignant tumour in the oral cavity characterised by complex genetic alterations, which may cause cell

dysregulation. Alterations in cell cycle proteins, including licensing proteins and regulators, may contribute to the development and progression of OSCC. Cancer heterogeneity in the same tumour and between primary lesions and their nodal metastases has long been recognised (Greaves and Maley, 2012, Marte, 2013). The heterogeneity in tumour cells can be influenced by changes in the tumour microenvironment and the genomic divergence in the tumour itself. These changes are likely to be the reason for the intra and inter-tumour heterogeneity observed in cancer patients (Junttila and de Sauvage, 2013).

This dissertation has investigated the heterogeneity of protein expression within the tumour. One of the findings to emerge from this study is that there were no differences in cell cycle protein expression in different areas of the tumours, nor between tumours which metastasised (M) or did not metastasise (NM). OSCC M had a higher mean of MCM2 LI than in OSCC NM, although this was not statistically significant. This may be attributable to the small sample size in this study and limitation of statistical power. In addition, MCM2 showed an increase from normal through OSCC NM to OSCC M, indicating the presence of a high number of cells licensed to proliferate.

Overall, the present results showed greater proportion of cells that express MCM2 than Ki-67 or geminin in the NOM, OSCC NM and OSCC M groups. Moreover, MCM2 expression increased from normal through OSCC NM to OSCC M. This pattern of expression in NOM demonstrates that cells are in the G0 or in G0-G1 phase (established by MCM2 expression). These results

are in line with previous reports (Torres-Rendon et al., 2009a, Gouvêa et al., 2013).

The geminin/Ki-67, but not MCM2/Ki-67 ratio for OSCC M was significantly associated with nodal metastases. This finding could be investigated in future projects to determine if the geminin/Ki-67 ratio may be a reliable marker of metastasis in OSCC.

Another finding in the current study was that there was no difference in the cell cycle regulatory proteins between primary and matched lymph node metastases, except for Ki-67, which had higher expression in primary tumours. Higher expression of Ki-67 in the primary lesions compared with in the lymph nodes may reflect a lower proliferative capacity of metastatic tumour cells (Tawfik et al., 2013). In contrast, MCM2 expression was greater in the nodal metastasis cells, but with no statistical significance. Low Ki-67 and high MCM2 LIs in the nodal metastases may suggest that a large population of metastatic cells are in G0 phase but are licensed to proliferate. High MCM2 LIs may reflect a cell proliferation capacity of OSCC in nodal metastases and supports the idea that metastatic lesions may be better differentiated (Park et al., 2007). However, no study has yet explored the proliferative activity of OSCC in lymph nodes.

No significant associations between LI of MCM2, Ki-67, geminin and cyclin D1 and the TNM stages, lymph node metastases and differentiation grade were found.

7.3 DNA ploidy by image cytometry and prognosis of oral squamous cell carcinoma

The main goal of the current study was to evaluate DNA content (ploidy) as a diagnostic and prognostic marker in oral cancers (squamous cell carcinoma). DNA image cytometry has become a common method for evaluation of DNA ploidy in tumour cells. Sensitivity, specificity, positive and negative predictive values were calculated for this diagnostic method.

In OSCC that had metastasised, all cases (100%) showed an aneuploid DNA content. This result differed from previous studies which had recorded lower aneuploidy incidence in OSCC (Abou-Elhamd and Habib, 2007, Santos-Silva et al., 2011). Such variation may be attributable to differences in methodology used in each study and possibly the tumour biology of our analysed samples. Likewise, all lymph node metastasis lesions (39 samples) showed DNA abnormalities with varying degrees of severity. The ploidy parameters, DI and 5cER values, were significantly higher in the nodal metastasis samples than in the primary carcinomas. This suggests an aberrant or increase in the nuclear DNA content in metastatic tumour cells. In contrast, other studies did not find any association between high aneuploid incidence and high metastasis rates (Saiz-Bustillo et al., 2005). The sensitivity of aneuploidy to predict metastasis was very good (100%), however, specificity was 0% since all OSCC cases were aneuploid. Overall, these results indicate that DNA aneuploidy is a necessary feature for

metastasis progression in OSCC, but is not specific to lesions which progress.

Thirty-one of the 39 cases which metastasised (**Table 5.6**) showed the same or more severe aneuploidy in their matched nodal metastasis. Taken together, these results suggest that there are aggressive primary cells that have the ability to migrate into the lymph nodes and cause tumour formation. The ability to metastasise may only be induced late in progression by a change in the tumour microenvironment allowing the clone to grow more (Chaffer and Weinberg, 2011).

Most studies (Torres-Rendon et al., 2009b, Santos-Silva et al., 2011, Gouvêa et al., 2013) in the field of DNA image cytometry analysis, have only focussed on the invasive front area. The present study has for the first time investigated heterogeneity of OSCC tumours in terms of DNA ploidy analysis; surface and invasive front areas were also compared. Tumour heterogeneity has previously been investigated, by examining multiple samples from OSCC at the invasive front area only (Diwakar et al., 2005). The most striking result to emerge from the data is that the surface more often showed severe aneuploidy (34%) than the invasive front (21%). Moreover, the incidence of abnormal DNA content was greater at the surface area in the OSCC M (40%) than OSCC NM (30%) (**Table 5.5**). This result may be related to the cellular heterogeneity found in malignant lesions, which is a common feature of all cancers (Greaves and Maley, 2012). This genetic heterogeneity may be due to biological differences among malignant cells developed within the same clone (Marusyk and

Polyak, 2010). Another possible scenario for this may be that prestressing caused by aneuploidy leads to an increase in genomic imbalances (Torres et al., 2008), necessary for tumour formation in early stages. Subsequent malignant cells may then become less aneuploid as the tumour progresses but this has not yet been investigated. Understanding the cellular consequences of aneuploidy could offer a new insight into cancer therapy.

7.4 Correlation of MCM2, Ki-67, geminin and cyclin D1 expression with ploidy status in OSCCs and their matched lymph node metastasis

To meet the objectives outlined in Chapter 6, expression of MCM2, Ki-67, geminin, cyclin D1, MCM2/Ki-67 and geminin/Ki-67 ratios were correlated with the DNA ploidy results.

This study showed a significant correlation of low Ki-67 expression with abnormal DNA content in OSCC LN (**Table 6.2**). These results suggest that aneuploid metastatic tumours could have a population of cells that are not proliferating (in G0 or early G1) but are licensed to proliferate (demonstrated by MCM2 expression). In the OSCC group that had metastasis , MCM2, Ki-67 and geminin, showed no correlation with ploidy status, DI or the 5cER values, Whereas cyclin D1 correlated with DI value.

7.5 Shortcomings

1- The main limitation of this study was that the number of samples was relatively small.

2- In this project, the first biopsy showing OSCC was retrieved for every patient that progressed to metastasis. The time of malignant nodal metastasis was not assessed at this stage.

3- The TMA method appeared to have some limitations. Some cores were lost during immunohistochemistry processing and others did not show immune-reactivity within the same TMA section.

4- One technical limitation was the ACIS III software, as this was only semi-automated. This system required too many sample cells to be selected by the operator, manually drawing around the cell nuclei of interest. This was very laborious and subjective. Nonetheless, the procedures for ploidy analysis were followed exactly according to standard protocols previously published and used in our laboratories.

5- It is possible that, for some cases, the cells analysed for DNA ploidy may not be from the same area where the immunohistochemistry results were quantified. This was because for the immunostaining quantification, three representative samples from the surface, middle and advancing front were selected by the operator.

7.6 Future work

1- Additional studies on OSCC evaluating MCM2, Ki-67 expression, along with their ratios, could be of great value to correlate with differentiation status, tumour size and disease outcome in term of disease free and overall survival.

2- Evaluation of Ki-67 and MCM2 levels in metastatic tumour cells with a large sample size may identify subgroups of patients with low Ki-67 and high MCM2 expression in lymph nodes. Future work may include genomic comparisons between patient groups and control groups which may help in selection of treatment options.

3- This is the first time that MCM2/Ki-67 and geminin/Ki-67 ratios have been calculated in OSCC with and without nodal metastases, but the differences in values were relatively small. However, as the sample size was small, these findings need to be confirmed in future similar studies.

4- One of the most interesting findings of this work was the heterogeneity of cell cycle proteins. While this has been previously reported it remains highly complex and poorly understood. Further studies are recommended to investigate the relationship between tumour microenvironment and heterogeneity of cell cycle proteins within primary and metastatic tumours.

5- In this thesis I reported for the first time a substantial difference in DNA status at the surface compared to the advancing front of OSCC. This finding should be confirmed in a large cohort of oral cancer and may indicate an increased risk of metastasis. Future studies could investigate the

differences between surface and advancing front cells. These cells could be isolated using laser capture microdissection (LCM) and analysed by single cell sequencing. This may identify important differences that could lead to new treatment options.

7.7 General conclusions

The conclusions that can be drawn from the present study are that altered expression of replication licensing proteins are found in oral squamous cell carcinoma. Therefore, the pre-replication MCM2 protein may serve as a diagnostic and prognostic tool for OSCC.

DNA ploidy alone may not be a good diagnostic tool to evaluate OSCC progression in the oral cavity. However, aneuploidy is a common feature of OSCC and a highly sensitive parameter. Primary lesions showed heterogeneity in support of previous literature. Further studies on this may identify novel treatments. Finally, this is the first report showing differences in DNA status between the surface and advancing front within OSCC. This is a very interesting finding that requires further study.

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APPENDIX 1

Solution and Reagents

1.1 Buffers used in immunohistochemistry methodology

Phosphate buffered saline (PBS)	75.2g K2HPO4 13.2G NaH2PO4 72.0G NaCL 800ml distal water
Sodium citrate buffer pH 6	Sodium citrate 2.94g Distilled water 1L 1M HCL (add it until pH reached)
Tris / EDTA pH 9.0	1.21x5 Tris 0.37X5 EDTA 5 litres distal water
Mayer`s haematoxylin	1g Haematoxylin 50g potassium alum 0.2g sodium iodate 50g chloral hydrate 1g citric acid
3% Hydrogen peroxide	3ml H2O2 in 100ml distal water
APES solution	2ml APES in 100ml acetone
0.1% Calcium Chloride (pH 7.8)	1g Calcium chloride 1 litre Distal water
0.1M Sodium hydroxide	4g NaOH 1 litre

1.2 Solutions used in Feulgen-Schiff stain for ploidy analysis

5N HCL (1000 ml)	431 ml HCL 569 ml deionised water
• **0.05 HCL (900 ML)**	ml 5N HCL 1 ml deionised water
Feulgen stain kit	Scy Tek Lab , USA

Appendix 2

Detailed Data from the Each Chapter

Chapter 2

	MCM2	Ki67	Geminin	Cyclin D1	MCM2/Ki67 ratio	geminin/Ki67 ratio
NOM	0.50	0.65	0.83	0.73	0.98	0.47
OSCC NM	0.52	0.49	0.22	0.91	0.85	0.99
OSCC M	0.94	0.14	0.92	0.930	0.93	0.44
OSCC LN	0.13	0.42	0.0034	0.20	0.40	0.90

OSCC NM = Oral squamous cell carcinoma that had not metastasised.
OSCC M = Oral squamous cell carcinoma that had metastasised.
OSCC LN = Lymph node metastases.

	DI	5cER	CV
OSCC NM	0.311	0.215	0.990
OSCC M	0.060	0.018	0.162
OSCC LN	0.051	0.301	0.015

*The CV value was not used for any statistical analysis.
DI= DNA index.
5cER= Cells that exceed 5c.
CV= Coefficient of variance.

Tow-tailed Kolmogorov-Smirnov test values from the analysed LIs, DI, 5cER results. P values that are less than 0.05 are not normally distributed.

Chapter 4

Correlation of protein expression with clinicopathological features. Correlation was not available between proteins expression and stage of disease in the OSCC M group, as all cases were stage III and IV.

	OSCC NM		
	Gender	**Tumour size**	**Disease stage**
MCM2	p=0.83	p=0.83	p=0.83
Ki-67	p=0.41	p=0.89	p=0.89
Geminin	p=0.16	p=0.17	p=0.17
Cyclin D1	p=0.25	p=0.52	p=0.52
MCM2/Ki-67	p=0.80	p=0.32	p=0.32
Geminin/Ki-67	p=0.25	p=0.46	p=0.46
Gender: Male and female. Tumour size: (T1&T2) and (T3 &T4). *Correlation was performed by t-test.			

	OSCC M		
	Gender	**Tumour size**	**Disease stage**
MCM2	p=0.58	p=0.61	NA
Ki-67	p=0.58	p=0.57	NA
Geminin	p=0.48	p=0.38	NA
Cyclin D1	p=0.93	p=0.66	NA
MCM2/Ki-67	p=0.31	p=0.11	NA
Geminin/Ki-67	p=0.53	p=0.83	NA

Gender: Male and female.
Tumour size: (T1&T2) and (T3 &T4).
*Correlation was performed by t-test.
NA: Not applicable.

	OSCC LN			
	Gender	**Tumour size**	**Disease Stage**	**Extra capsular spread**
MCM2	p=0.65	p=0.82	p=0.83	p=0.32
Ki-67	p=0.09	p=0.80	p=0.89	p=0.70
Geminin	p=0.71	p=0.92	p=0.17	p=0.98
Cyclin D1	p=0.28	p=0.40	p=0.52	p=0.54
MCM2/Ki-67	p=0.36	p=0.94	p=0.32	p=0.53
Geminin/Ki-67	p=0.74	p=0.41	p=0.46	p=0.52

Gender: Male and female.
Tumour size: (T1&T2) and (T3 &T4).
Stage: (I &II) and (III &IV).
Extra capsular spread: Yes and no.
*Correlation was performed by t-test.

Chapter 5

Contingency tables of the ploidy status data that was analysed for the sensitivity, specificity, PPV and NPV calculation.

	Groups		Total
	OSCC M	**OSCC NM**	
Aneuploidy	39 (TP)	47(FP)	86
Diploid	0 (FN)	0 (TN)	0
	Groups		
	OSCC M	**OSCC NM**	
DI> 1.2	39 (TP)	47 (FP)	86
DI<1.2	0 (FN)	0 (TN)	0
Total	39	47	86
TP= True positive. FP= False positive. TN= True negative. FN= False negative.			

	Groups		Total
	OSCC M	**OSCC NM**	
5cER >1	10 (TP)	7(FP)	17
5cER <1	29 (FN)	40 (TN)	69
Total	39	47	86
TP= True positive. FP= False positive. TN= True negative. FN= False negative.			

Chapter 6

Correlation Coefficient values of DNA content (DI and 5cER) with MCM2, Ki-67, geminin, cyclin D1 and MCM2/Ki-67 and geminin/Ki-67 ratios.

	Groups		
	OSCC-M	**OSCC-NM**	**OSCC LN**
	DNA Status		
	Correlation Coefficient		
Total.MCM2	0.268	0.194	-0.150
Total.Ki-67	0.059	-0.254	-0.335*
Total. Geminin	0.005	0.124	0.180
Total.CD1	0.195	0.066	-0.238
Total.MCM2/Ki-67	0.046	0.316	0.358
Total. Geminin/Ki-67	-0.118	0.300	0.305

		Groups		
		OSCC M	**OSCC NM**	**OSCC LN**
		Correlation Coefficient		
Total.MCM2	DI	0.219	0.336*	-0.049
	5CER >1%	0.188	0.133	0.045
Total.Ki-67	DI	-0.067	-0.065	-0.267
	5CER >1%	0.034	-0.196	-0.016
Total. Geminin	DI	-0.221	0.010	0.269
	5CER >1%	-0.102	0.069	0.272
Total.CD1	DI	0.377*	0.077	-0.148
	5CER >1%	0.238	-0.044	0.045
Total.MCM2/Ki-67	DI	0.164	0.193	0.303
	5CER >1%	0.071	0.152	0.122
Total. Geminin/Ki-67	DI	-0.289	-0.100	0.318
	5CER >1%	-0.113	0.387	0.323