CORRELATION BETWEEN HISTOLOGICAL GRADE AND PLOIDY STATUS IN POTENTIALLY MALIGNANT DISORDERS OF THE ORAL MUCOSA

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CORRELATION BETWEEN HISTOLOGICAL GRADE AND PLOIDY STATUS IN POTENTIALLY MALIGNANT DISORDERS OF THE ORAL MUCOSA

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A research report submitted to the University of Pretoria in partial fulfilment of the requirements for the degree of

MChD (Oral Medicine and Periodontics)

October 2009

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DECLARATION

I, Eric Emil Langenegger, hereby declare that this dissertation is my own work. It is submitted in partial fulfilment of the requirements for the degree of Master of Dentistry in the field of Oral Medicine and Periodontics. It has not been submitted before for any degree or examination at this or at any other University.

E E Langenegger

October 2009

ACKNOWLEDGEMENTS

This study was made possible by the Department of Oral Pathology, University of Pretoria.

I would like to thank Professor André van Zyl, Professor Willie Van Heerden and Professor Sonja Boy for all their input and guidance during this study.

Mrs. Marlene Van Heerden for technical excellence in the laboratory, as well as the rest of the Oral Pathology Laboratory personnel.

Dr Eduard Langenegger and Dr Greg Pedro for their contributions and motivation during the writing of this research report.

CHAPTER 1

LITERATURE REVIEW

1.1 INTRODUCTION

The term “Potentially malignant disorders” (PMD) refers to a group of oral disorders some of which have an increased potential for malignant transformation (1). Histopathological grading of oral dysplastic lesions is currently the method of choice for evaluating the risk of oral cancer developing in PMD (2, 3). However this method of grading is unreliable, owing to the lack of a validated grading system and to the subjectivity of histopathological grading, with inter- and intra-observer variability (4).

DNA ploidy concerns the number of chromosomes in a cell. Determination of the DNA ploidy status of PMD may have value as an adjunct to the grading of oral epithelial dysplasias in the prediction of malignant transformation (5).

1.2 HISTORICAL BACKGROUND AND EPIDEMIOLOGY OF POTENTIALLY MALIGNANT DISORDERS

1.2.1 Historical background

Oral PMD are a group of disorders that include leukoplakia, erythroplakia, palatal lesions of reverse smoking, submucous fibrosis, actinic keratosis, lichen planus, discoid lupus erythematosus and two hereditary disorders,

dyskeratosis congenita and epidermolysis bullosa (1). Of these leukoplakia and erythroplakia are important. Reverse smoking is restricted to certain geographic areas and the other conditions listed contribute little to the global burden of PMD (6).

Over the past few decades, the definitions of PMD and leukoplakia have changed many times.

In 1967 the World Health Organization (WHO) established a centre for the study of precancerous lesions with the aim of characterising and defining these lesions for the purpose of epidemiological and clinical studies. The centre was established in order to achieve consistency in diagnosis, to determine an accurate prognosis and to make meaningful comparative assessments between different preventive or clinical management protocols to prevent malignant transformation of these disorders (7).

The classification of these disorders initially included two broad groups: precancerous lesions and precancerous conditions (7). “Precancerous lesions” referred to morphologically altered tissue in which oral squamous cell carcinoma (OSCC) was more likely to occur than in its apparently normal counterpart. “Precancerous conditions” referred to a generalised state with a significant increased risk for OSCC (7). This distinction implied that a precancerous lesion would develop into OSCC, while OSCC could arise at any site of the mouth and pharynx in a precancerous condition (7).

The first definition of leukoplakia in 1978 by the WHO Collaborating Centre for Oral Precancerous lesions was “a white patch or plaque that cannot be characterized, clinically or histopathologically, as any other disease” (7). It was emphasised by the Centre that this definition of leukoplakia should not carry a histological connotation and should only be used in a descriptive clinical sense. Similarly, the term “erythroplakia” was used analogously to the term “leukoplakia” for “lesions of the oral mucosa that present as bright red patches or plaques that cannot be characterized clinically or pathologically as any other condition” (7).

The definition of leukoplakia has changed many times since then. At an international seminar held in 1983, with the outcomes published in 1984 (8), the definition of “leukoplakia” was modified to exclude physical or chemical agents that could cause white lesions, with the exception of tobacco; thus the definition was revised to “leukoplakia is a whitish patch or plaque that cannot be characterized clinically or pathologically as any other disease and it is not associated with any physical or chemical causative agent except the use of tobacco” (8). At the same workshop etiological and clinical descriptions were also proposed. It became apparent that etiological factors of leukoplakia could influence the malignancy potential; therefore, etiological factors were categorised as either idiopathic, (having an unknown aetiology), or being associated with the use of tobacco. Clinically, leukoplakia was also described as being homogeneous or non-homogeneous. Homogeneous leukoplakia was defined as a whitish lesion with a smooth or corrugated surface and had

the lowest risk for malignant transformation, while non-homogeneous leukoplakia carried a higher risk for malignant transformation.

Non-homogeneous leukoplakia was subclassified as: erythro-leukoplakia, being a whitish lesion that included red areas; nodular leukoplakia, being a lesion slightly raised with rounded, red and/or whitish excrescences; or verrucous leukoplakia, being an exophytic lesion with irregular sharp or blunt projections (8).

The definition of “leukoplakia” was further modified in 1994 by Axell et al., at the International Collaborative Group on Oral White Lesions and this modification was published in 1996 (9). The changed definition of leukoplakia included diffuse white lesions and excluded any lesion definable as a separate entity, and was described as: “a predominantly white lesion of the oral mucosa that cannot be characterized as any other definable disease”. “Definable disease” was used instead of the words “clinical” or “pathological”, to exclude lesions with no malignant potential and implied that a biopsy was not necessary to diagnose leukoplakia, because this would place a restraint on epidemiological studies (9).

At a recent workshop coordinated by the WHO Collaborating Centre for Oral Cancer and Precancer in 2005, the definition of “leukoplakia” was amended to: “the term leukoplakia should be used to recognize white plaques of questionable risk having excluded (other) known diseases or disorders that

carry no increased risk for cancer" (6). This was the first time that the definition included the unknown malignant potential of leukoplakia. The consensus of the working group was that dividing leukoplakia into homogeneous and non-homogeneous leukoplakia was imprecise and of limited value, but that erythroleukoplakia should be recognised as having a higher risk status for malignant transformation (6).

A systematic approach for diagnosing leukoplakia was recommended at the 2005 Workshop that stated that histopathology evaluation must be performed before a definite diagnosis of leukoplakia can be made. If no other disorder is histologically confirmed, then leukoplakia is diagnosed with or without epithelial dysplasia (6).

A further recommendation was to refer to "precancerous lesions and conditions" with the encompassing term "potentially malignant disorders" (6, 10). PMD reflects on the disorder's widespread anatomical distribution, even in normal-appearing mucosa and the disorders having a "potential only" for malignant transformation.

Even though the clinical definitions of PMD and leukoplakia have changed many times, the clinical features used for predicting the malignant potential of PMD is still of limited value (11).

1.2.2 Epidemiology

Oral leukoplakia is the most frequently seen oral PMD and many studies have been performed on the epidemiology of leukoplakia (12-16). Epidemiological studies of oral leukoplakia are hampered by the low prevalence of these lesions within communities, the high costs of providing repeated oral examinations and a low participation rate at annual oral screenings. A low participation rate is especially true for high-risk groups for oral squamous cell carcinoma (OSCC) (12). Multiple epidemiological studies with large numbers of participants have been carried out on the incidence and prevalence of oral leukoplakia in India (13-16). The results of these studies, however, cannot be applied to other countries because the majority of the Indian population use more than one tobacco products concurrently; for example, cigarette smoking together with chewing tobacco and betel/tobacco quid (12, 14, 17).

Great variations are reported in studies on the incidence and prevalence of oral leukoplakia (11, 17). These variations are related to the differences between developed and developing countries, the use of specific tobacco habits of the study population, age ranges included in the various studies, and the methods of data collection (11). It should also be noted that the definition of “leukoplakia” has changed several times in the last three decades (6, 8, 9). For this reason, the specific definition used for “leukoplakia” in studies might also have an influence on the dissimilarities in the study results.

Studies on leukoplakia from the developed countries have reported an

estimated incidence of 409.2 for males and 70.0 for females per 100 000 person-years (19). In a systematic review of the world prevalence of leukoplakia from 26 studies, the estimated rate of leukoplakia ranged from 1.7% to 2.7% (17).

Leukoplakia may be located on any part of the oral mucosa, but is most common in the buccal mucosa, while involvement of the tongue, palate and floor of the mouth ranges between 1% (19) to 15% (20) of the intra-oral sites. It is also estimated that leukoplakia is three times more common in males than in females worldwide (12, 17, 18).

An important aspect in epidemiological studies is to obtain an estimate of the malignant transformation rate of leukoplakia to oral squamous cell carcinoma (OSCC). It is still uncertain how many OSCCs develop from PMD, compared to normal-appearing mucosa (21). Recent population-based studies reported a rate varying from 0.5% to 3.2% (21, 22). It should be noted that many studies on the malignant transformation rate of leukoplakia in the developed world are conducted in a hospital setting, most being referral clinics, and are likely to give an overestimation of malignant potential (17, 23).

In the same pooled estimate on the prevalence of leukoplakia, it was estimated that the global transformation of oral leukoplakia is 1.36% per year (17). This figure is probably an overestimation of malignant transformation potential, and it has recently been proposed that a more realistic malignant

transformation of oral leukoplakia is estimated to be less than 0.5% per year globally (10). The floor of the mouth and lateral border of the tongue are associated with a disproportionately high rate of malignant transformation since these sites are possibly more exposed to carcinogens in pooled saliva than other areas in the mouth and also because the epithelium in these areas has a higher permeability (24). Reports of malignant transformation for both sites range from 13% (25) to 24% (20). These lesions are also associated with higher grades of dysplasia, with severe dysplasia reported in 13% of leukoplakias of the floor of the mouth and 5% of the lateral border of the tongue (20).

Tobacco is a major independent risk factor for the development of oral and oro-pharyngeal cancers (26), as well as oral leukoplakias (11, 23). Tobacco is consumed in a variety of different ways including cigarette- and cigar smoking and using smokeless tobacco (which includes chewing tobacco and snuff) (26). There is uncertainty as to the role of alcohol in the aetiology of PMD, but it probably has a synergistic effect with tobacco (15). Some leukoplakic lesions appear to be idiopathic in origin (11, 28).

1.3 FIELD CANCERIZATION

1.3.1 Concept of field cancerization

The concept of field cancerization was initially proposed in 1953 by Slaughter et al (29) in their study of oral cancer. It was not a clear concept, but it was

used to describe issues like multiple or recurrent carcinomas of the upper aero digestive tract and this concept was generally used in the context of the existence of “pre- neoplastic” processes at multiple sites. Field cancerization is now generally accepted as valid for OSCC as well and recently a genetic basis for this concept was described (30).

Field cancerization can be defined as the presence of one or more areas of oral epithelial cells (field lesions) that have the same genetic alterations (30). These alterations can be in genes that positively or negatively regulate aspects of cell proliferation, apoptosis, genome stability, angiogenesis, invasion and metastasis (31). These gene functions can be altered in different ways; as in tumour suppressor genes being inactivated by mutation, deletion or methylation - for example, the TP53 and pRB gene. Oncogenes can be activated by mutation or amplification – for example, the Ras or EFGR gene (32). There is ongoing research that focuses on identifying the critical genetic events and the order in which they occur during carcinogenesis.

A patch/field lesion is monoclonal in origin and there are currently multiple theories explaining this process of field lesion development (30). The patch/field carcinoma model has been proposed as a progression model for oral cancer (33) and will be briefly described.

Oral squamous epithelium is maintained throughout life by stem cells, defined as cells with the capacity to self-renew and to generate daughter cells that can

differentiate to cells that are found in the mature epithelial tissue (34). In the oral mucosa, stem cells are believed to be located in the basal layer of epithelium and have a slow proliferation rate (34).

The patch/field carcinoma model proposes that in the initial phase of carcinogenesis, a stem cell acquires one (or more) genetic alterations and forms a patch with genetically altered daughter cells (33). With subsequent genetic alterations, the stem cell escapes normal growth control and gains growth advantage, thereafter developing into an expanding clone. The critical hit that is important for the cells of a patch to leave their natural containment is still unknown. As the lesion grows it gradually becomes a field lesion and takes over the normal epithelium, without becoming invasive. As the lesion becomes larger, additional genetic hits give rise to various sub-clones within the field. As multiple clones develop within a field and genetic hits continue, the number of affected cells increases by virtue of the processes of clonal expansion and selection. Each time the daughter cells of the most dominant clone overtake the rest of the cells in the field in a wave-like fashion (35). Thus, large areas of normal mucosa are replaced by a cell population that is becoming increasingly more genetically aberrant but is of monoclonal origin. During the process of clonal selection, fields can become heterogeneous, because of the continuing accrual of genetic changes (36).

Details of the ultimate malignant transformation event have not yet been revealed, but the chance for malignant transformation to happen in a patient

will be proportional to the number of patches and fields and the number of additional hits. Two important steps in this model can be discerned: first, the conversion of a stem cell into an expanding patch of stem cells without proper growth control; and, two, the eventual transforming event, turning a field into an overt carcinoma with invasive growth and metastasis (30).

Field lesions do not show invasive growth and metastatic behavior, which is the hallmark characteristic of cancer, but appear to be at continuous risk for malignant transformation (37).

Some leukoplakias do contain cancer-associated genetic alterations like allelic loss at chromosome 3p, 9p and 17p and are fields by definition (37, 38). A field lesion can often extend beyond the visible potential malignant disorder lesion (30, 39). The detection and monitoring of field lesions may, therefore, have profound implications for cancer prevention and management after surgical removal of a tumour, since remaining field lesions can give rise to new cancers (30, 39).

1.3.2 Diagnostic aids for field detection

Research is currently ongoing into identifying diagnostic aids that could help to detect field lesions that are not clinically detectable. These aids could be used either as screening aids (defined as an application of a test or tests to people who are apparently free of disease in order to sort out those who probably have the disease from those who probably do not) or case-finding

tests in patients with PMD (defined as a diagnostic test or method that is applied to a patient who has abnormal signs or symptoms in order to establish a diagnosis and bring the patient to treatment) (40). Screening techniques are:

Conventional oral examination

Conventional oral examination, using normal (incandescent) light has long been the standard method for oral cancer screening, but has limited value in detecting PMD (41, 42), since some PMD may be present in the mucosa and appear clinically normal by conventional oral examination alone (39).

Oral cytology

Epithelial cells can be harvested from low-level suspicious lesions based on clinical features, using for example the brush biopsy technique (43, 44). If atypical cells are found, then a biopsy of the lesion can be carried out to provide a definitive diagnosis. This diagnostic aid seems to have a high sensitivity and specificity for the detection of PMD and might be of benefit in patients with multiple clinical or field lesions of the oral cavity (45).

Toluidine Blue

Toluidine blue (also known as tolonium chloride) is a vital dye that stains nucleic acids and possibly abnormal tissues (46). It has been used for decades in the secondary-care environment by specialists as an aid to identify mucosal abnormalities of the cervix and in the oral cavity, and also to demarcate the extent of a neoplastic lesion prior to excision (46). Toluidine

blue has high sensitivity but low specificity for detecting PMD. The determination of intensity of the stain for a positive diagnosis is subjective (47). The high false positivity and low specificity in staining of dysplasia probably outweigh the potential benefits for detecting PMD (45).

Narrow-emission tissue fluorescence

Fluorescence imaging involves the exposure of tissue to a specific wavelength of light, which results in the autofluorescence of cellular fluorophores after excitation (48). The presence of cellular alterations will change the concentrations of fluorophores, which will affect the scattering and absorption of light in the tissue, thus resulting in changes in color that can be observed visually (45). The VELscope® is a portable device that allows for direct visualization of the oral cavity and is being marketed for use in oral cancer screening. Under intense blue excitation light (400-460 nm), normal oral mucosa emits a pale green autofluorescence when viewed through the selective (narrow-band) filter of this instrument. Abnormal or suspicious tissue exhibits decreased levels of normal autofluorescence and appears dark by comparison to the surrounding healthy tissue. There are limited studies on fluorescence imaging, but it appears that the VELscope® may be useful in detecting PMD that cannot be seen by conventional oral examination (49) and also in detecting surgical margins of oral cancer in the operating theater (48).

1.4 HISTOPATHOLOGY OF POTENTIALLY MALIGNANT DISORDERS

1.4.1 Introduction

In an attempt to identify leukoplakia with a high risk of malignant transformation, risk factors like tobacco use and alcohol intake and also clinical parameters like location of the lesion (the lateral border of the tongue and floor of mouth) and non-homogeneous clinical presentation (erosive and hyperplastic) have been used, but the prediction potential of these is limited (3, 7, 50).

Because PMD have a variable clinical presentation, the standard practice is to perform a biopsy of the lesion and obtain a histological diagnosis (5). Histopathological grading of epithelial dysplasia remains one of the most clinically important predictors of malignant potential and is still accepted as the gold standard (2, 3).

Studies have found that between 5% to 25% of oral leukoplakias may have dysplasia (51, 52). Epithelial dysplasia is a histological diagnosis that can occur in PMD at various anatomical sites and is characterized by variations in cellular proliferation and maturation of the squamous epithelium of the mucosa (53). The cellular proliferation is caused by an alteration in cell kinetics (increased cell division) in the proliferative part of the epithelium, while disturbed maturation manifests in the form of irregular stratification and increased keratinization of individual cells beneath the keratin layer (53). It is

believed that the presence of oral epithelial dysplasia is associated with an increased risk for progression to OSCC. It may well be the morphological phenotypes of the different steps in the progression from normal to malignant tissue (54). The more severe the dysplasia, the greater the likelihood of progression to malignancy; however, non-dysplastic lesions may also transform (4). Treatment modalities of PMD with dysplasia are influenced by the grading of dysplasia, usually with surgical removal of moderately and severely dysplastic lesions (3).

1.4.2 WHO classification

Dysplasia is classified in certain grades and currently the 2005 WHO classification is accepted for general use (53).

The WHO criteria of 2005 are used to diagnose the presence of dysplasia based on architectural and cytological disturbances. Architectural disturbances are maturation irregularities and may include irregular epithelial stratification, loss of polarity of basal cells, basal cell hyperplasia, drop-shaped rete ridges, increased number of mitotic features, abnormally superficial mitosis, premature keratinization in single cells (dyskeratosis) and keratin pearls within rete ridges (53).

Cytological features include abnormal variation in nuclear size (anisonucleosis), abnormal variations in nuclear shape (nuclear pleomorphism), abnormal variation in cell size (anisocytosis), abnormal

variation in cell shape (cellular pleomorphism), increased nuclear-cytoplasmic ratio, increased nuclear size, atypical mitotic figures, increased number and size of nucleoli, and hyperchromatism (5).

The 2005 WHO classification of dysplasia is widely used to grade dysplasia into hyperplasia, mild, moderate and severe dysplasia. The more prominent or numerous the features of dysplasia are in a given biopsy, the more severe the grade of dysplasia (53).

Hyperplasia is the first grade and describes an increase in cell numbers. This increase can occur in the spinous layer, and lead to hyperplasia or acanthosis in the basal/ parabasal cell layer. For this reason, this hyperplasia is termed “basal cell hyperplasia”. The architecture shows regular stratification and there is no cellular atypia present (53).

Mild dysplasia is characterized by architectural disturbances limited to the lower third of the epithelium, accompanied by minimal cytological atypia, and defines the minimum criteria of minimal dysplasia (53).

Moderate dysplasia is characterized by architectural disturbances and cytological atypia extending into the middle third of the epithelium; but if the atypia is pronounced then the lesion should be categorized as severe dysplasia despite it not extending into the upper third of the epithelium. On the contrary, a lesion with mild atypical features extending into the middle third

of the epithelium may justifiably be graded as mild dysplasia (53).

Epithelial architectural disturbances together with cytological atypia extending through more than two-thirds of the epithelial width will be graded as severe dysplasia, though as mentioned above, architectural disturbances extending into the middle third of the epithelium with cytological atypia of sufficient severity may be upgraded from moderate to severe dysplasia (53).

Clearly this grading system is very subjective, the histological and cytological features may not be, indeed seldom are uniform throughout a PMD of any size, and moreover there is uncertainty about the significance of subdividing epithelial dysplasia into mild, moderate and severe grades (23, 55).

These caveats notwithstanding, it is generally accepted that the more severe the dysplasia, the greater the risk of progression to OSCC (5). OSCC arising from dysplastic lesions usually develops within two to five years of the diagnosis of dysplasia, but can occur much later (3, 11, 50, 56, 57). The risk of cancer in moderately and severely dysplastic PMD has been shown to be at least double that of mild dysplasia or hyperplasia (57, 58). The risk of malignant transformation of severe dysplasia has been reported as being between 12% and 36% (50, 57); moderate dysplasia between 3% and 15% (9, 11) and mild dysplasia 5% (11).

It has always been a challenge for the pathologist to assess the degree of

dysplasia in PMD and current histopathological grading of oral dysplastic lesions is notoriously unreliable, mainly owing to the lack of well-defined criteria (4). Grading is hampered by the arbitrary separation into distinct categories of what is in fact a process of continuous progression towards malignant transformation, without any naturally defined stages (59). It is an attempt to impose discrete categories on what is in effect a continuous scale and, therefore, any grading scheme is by definition artificial (60). The ultimate diagnosis of a particular grade of dysplasia depends on the significance that the observer attaches to each of the architectural and cytological characteristics of the tissue, and therefore is inescapably subjective (59, 61-63). When evaluating agreement on architectural and cytological features that are used to grade dysplasia, the highest degree of agreement was found for increased number of mitotic figures, drop-shaped rete ridges, increased nuclear size and abnormal variations in cell shape, while disagreement was found for irregular epithelial stratification, loss of polarity of basal cells, abnormal variation in nuclear size, atypical mitotic figures and hyperchromatism (59). There appears to be satisfactory agreement on the distinction between mild and severe dysplasia; but assessment of moderate dysplasia remains highly subjective (54).

1.4.3 Binary grading system

Considering the problems of reliably distinguishing between the different grades of epithelial dysplasia, especially moderate dysplasia, the 2005 WHO Working Group considered collapsing the four grades into two when reporting

the presence or absence of epithelial dysplasia: “no/questionable/mild” implying low risk, and “moderate or severe” – implying high risk (5).

If mild dysplasia is used as the cut-off point when deciding whether or not to remove a lesion surgically, near misses of grading are not particularly relevant in the context, and intra- and inter-observer variability would be significantly reduced by having only two grades (54). The Working Group was of the view that reducing the number of choices for degree of dysplasia from three to two may increase the likelihood of agreement between pathologists (5).

This two-class classification was tested (54) and grading was based on the same morphological criteria used by the WHO classification of 2005 (architectural and cytology changes), but grading was either into low risk or high risk, based on the scoring of these features.

It was shown that better agreement was reached in grading lesions as low- or high risk and also that high-risk lesions tended to have a significantly greater malignant transformation rate during follow-up periods than low-risk lesions. This shows that the binary system has the potential to help clinicians to make more appropriate and more consistent treatment decisions when dealing with oral epithelial dysplasia (54).

1.5 DNA PLOIDY

1.5.1 Introduction

An ovum or a sperm, containing 23 single chromosomes, is termed haploid. Normally, a non-dividing somatic cell contains in its nucleus a diploid amount of DNA in 23 pairs (46) chromosomes. Just before cell division, the DNA is doubled and in mitosis, the 23 pairs of chromosomes are evenly distributed to two daughter cells. If the chromosomes are not uniformly distributed to the daughter cells or parts of any chromosomes become detached, the chromosome segregation during mitosis is termed “unbalanced”, and the situation created is termed “aneuploid” (70).

1.5.2 Significance of DNA ploidy

Many authors believe that aneuploidy is a cause rather than the result of malignant transformation, and is therefore important in the process of carcinogenesis (71, 72). It has been shown that in some PMDs the epithelial cells exhibit changes from a diploid pattern to an aneuploid pattern preceding malignant transformation, which means that DNA alterations take place before transformation is apparent (68) and is seen as a surrogate marker of gross genetic damage (70). In many cancers including OSCC, genetically stable diploid cells are replaced by genetically unstable aneuploid cells (23, 71-73). In some studies of malignancies such an aneuploid tumour population appears to be an important prognostic factor (74), while other studies have not found DNA ploidy status to have any prognostic value (75).

In 2008 Warnakulasuriya concluded that assessment of gross genomic aberration (DNA aneuploidy) may be the key to improved methods of diagnosis and grading of PMD (5).

In oral leukoplakia, aneuploid populations have been reported with or without correlation to the grade of epithelial dysplasia (67, 76, 77). Identification of aneuploidy could possible aid in diagnosing PMD with aggressive biological behavior and, therefore, would permit early effective treatment, but the clinical and pathological significance of DNA ploidy and the predictive potential for malignant transformation of oral leukoplakia are still not clear (67, 78).

If any correlation between DNA ploidy and the histological grading of dysplasia can be demonstrated, this might be used as an adjunctive aid to consensus between pathologists in diagnosing the grade of epithelial dysplasia (5). This correlation could be especially useful if implemented with the new binary grading system of epithelial dysplasia, since it has been proven that the binary grading system reduces inter- and intra-observer variability for the pathologist and possibly could help clinicians to make more appropriate treatment decisions when dealing with OED (54).

The rationale for the treatment of PMD is mainly to prevent malignant transformation (10) and includes surgical (79, 80) and chemopreventative therapy (81-83). Currently, there is no scientific evidence that treatment, of whatever modality, truly prevents the possible future development of

squamous cell carcinoma (84). It is still uncertain how to identify lesions with a high risk of malignant transformation (85).

1.5.3 Aneuploidy

It has been stated that carcinogens are believed to be aneuploidogens (70, 86). Chemical carcinogens are believed to generate aneuploidy by chemically or physically altering one or more of the chromosomes, or by altering one of the many proteins of the spindle apparatus resulting in chromosome loss or gain during cell division in the germ or somatic cells (70, 86, 87). Examples of these aneuploidogens include polycyclic hydrocarbons in cigarette smoke, which may disrupt microtubules by binding to tubulin proteins and induce chromosome non-disjunction (86, 88, 89), and physical carcinogens, such as X-rays or alpha-rays which can also generate aneuploidy by fragmenting chromosomes (70) or by damaging the spindle apparatus (90).

Genetic material gain is better tolerated than loss; therefore, hyperploidy is preferable to hypoploidy (91). Late-stage cancer cells typically have 60 to 90 chromosomes and this number may arise by a gradual, stepwise increase in the level of aneuploidy/hyperploidy (92). The stepwise progression of aneuploidy usually produces cells of less and less viability, which should inhibit progression to cancer; but decades of genetic instability may eventually produce a viable cell with the level of aneuploidy necessary for malignancy (70).

A two-step model of carcinogenesis termed initiation and promotion that corresponds to two levels of aneuploidisation has been proposed. (93). The initial step in carcinogenesis is the production of a non-cancerous, aneuploid cell. In this cell, the level of aneuploidy is below the threshold for cancer. In the promotion step, the threshold of aneuploidy for cancer is reached or exceeded by a gradual, stepwise increase in the level of aneuploidy (92, 94).

1.5.4 Detection of DNA ploidy

DNA ploidy is normally determined by flow cytometry (95) or image cytometry (96).

Flow cytometry is an accurate high-speed technique to determine the DNA ploidy of individual cells in a suspension (97). The nuclei are stained with fluorescent dye and sent through a flow channel, where the fluorescent dye is excited by a laser beam or mercury lamp light. DNA content measurement is based upon the proportional binding of DNA-specific dyes to nuclear DNA, which allows for an accurate measurement of dye fluorescence and results in a DNA content histogram of the entire cell population. The measured fluorescence is proportional to the DNA content of the cell (98).

When a cell is in the premitotic growth phase 2 *(G_2)* or mitotic phase *(M)* of the cell cycle *(Figure 1)*, the cell has replicated its genome; thus it will contain twice as much DNA as a cell in the presynthetic growth phase 1 *(G_1)* (98). Consequently, a cell in the *G_2M* phase of the cell cycle will bind twice as much

DNA dye as a cell in the G_1 phase and, thus, will yield twice as much fluorescence. Cells in the early-, mid- or late synthesis phase *(S)* of the cell cycle will accumulate in between the G_1 population and the G_2M population in the DNA histogram. Non-cycling cells (G_0 phase) have the same DNA content as G_1 cells and a fluorescent signal identical to G_1 cells *(Figure 2 and 3)*. Theoretically, signals from cells with the same DNA content will accumulate in the same channel of the DNA histogram. However, due to small differences in chromatin compactness between individual cells (which affects the binding of a DNA strain), minor instrumental errors and preparation artifacts give a Gaussian (normal) distribution. A measurement of histogram quality is the width of the G_0G_1 peak expressed as the coefficient of variation (CV) or normalized standard deviation. A low CV is indicative of a good DNA histogram resolution (98).

Figure 1

Cell cycle stages (G_O, G_1, S, G_2 and M). During the G_0/G_1 stages the cell will contain a diploid amount of DNA and during the G_2/M stages the cell will contain a tetraploid amount of DNA (replicated genome).

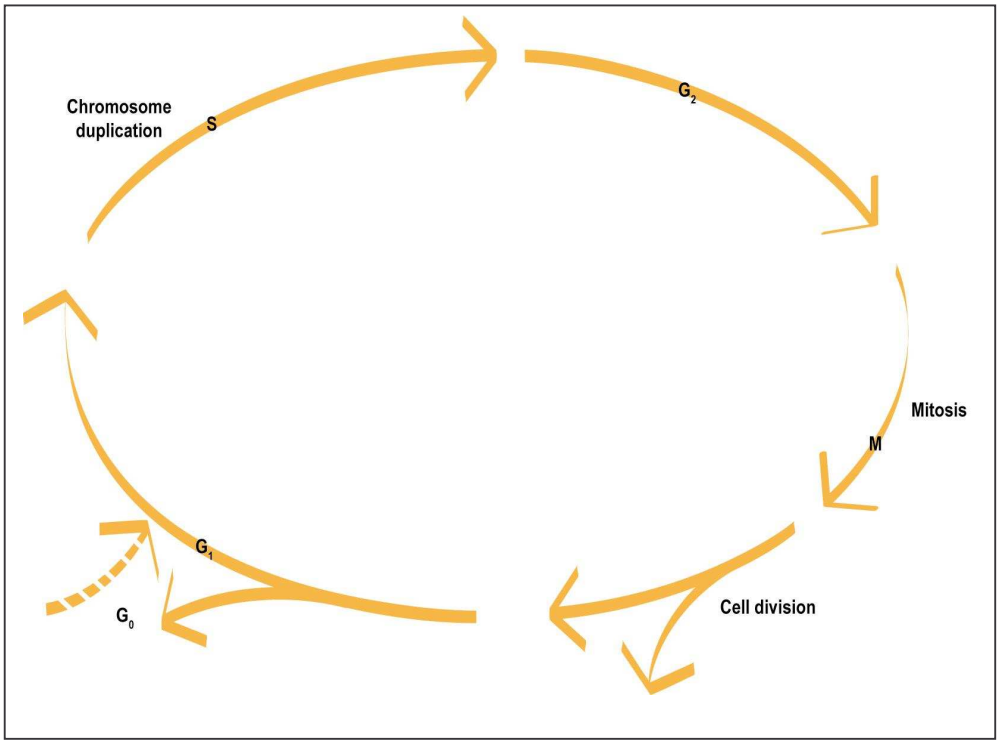


Figure 2

Representative DNA histogram of diploid oral dysplasia. Diploid oral dysplasia is characterized by a prominent peak representing the cells in the G_0/G_1-phase of the cell cycle. The much smaller peak with exactly double the DNA content of the first one contains cells in the G_2-phase and mitosis. The counts plotted between both peaks reflect cells in S-phase characterized by intermediate DNA values. The width of the G_0/G_1 peak is expressed as the coefficient of variation (CV).

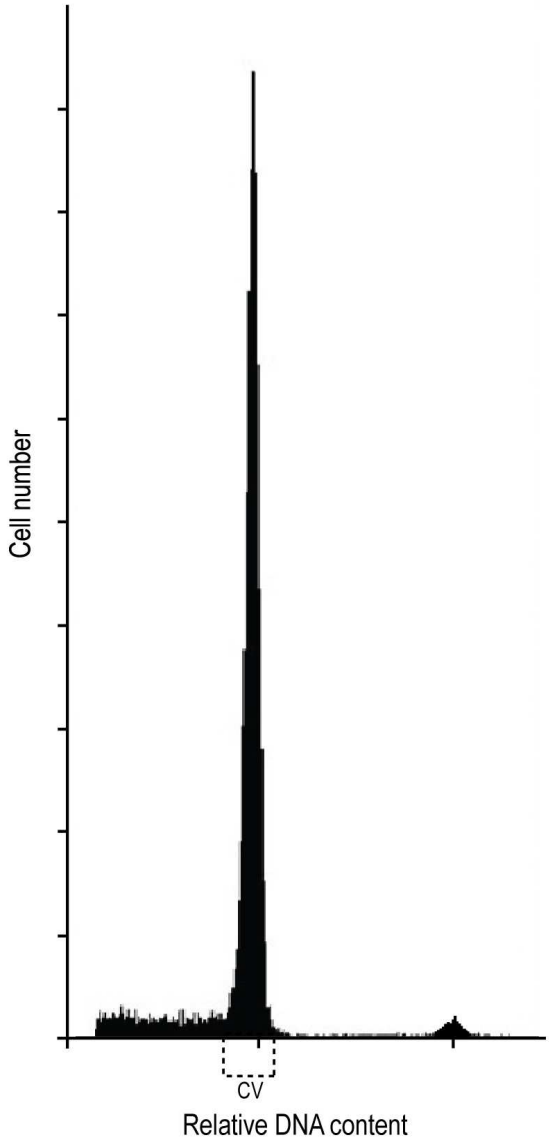
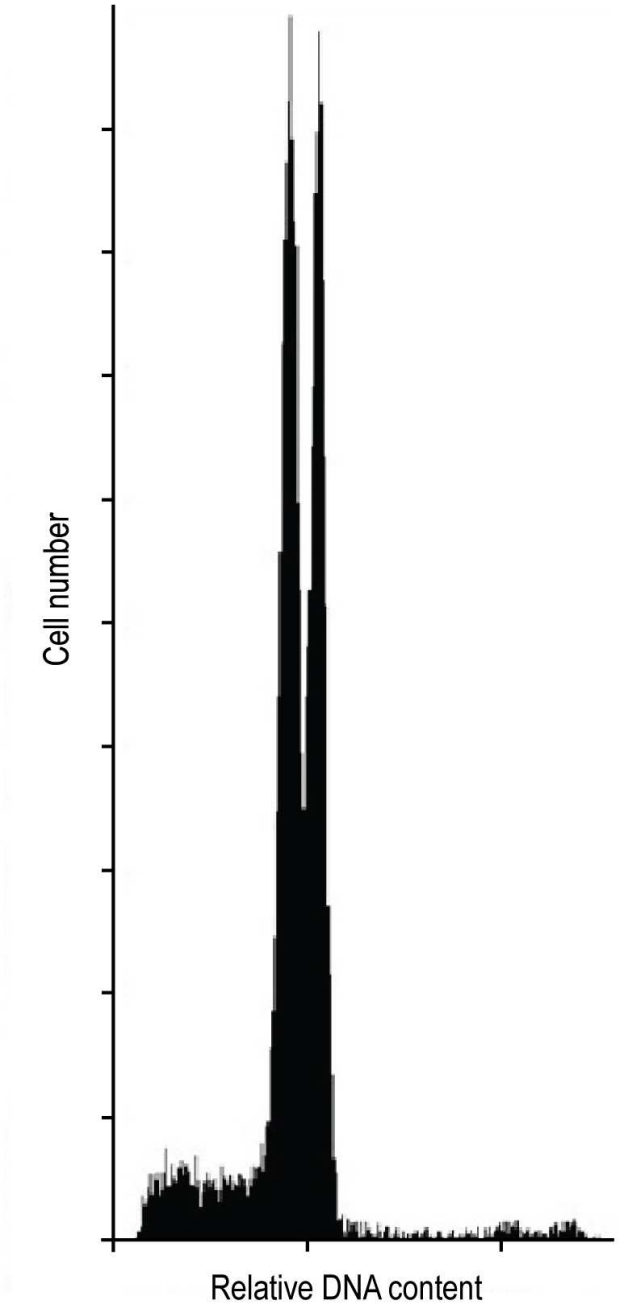


Figure 3

Representative histogram of aneuploid oral epithelial dysplasia. Aneuploid oral epithelial dysplasia shows additional cell lines with abnormal DNA content.



Flow cytometry (FCM) is a fast procedure and large cell numbers (10 000-30 000) can be measured in 3 to15 minutes, thereby providing statistically sound results. The coefficient of variation (CV) is low for fresh specimens (between 1-4%), although it is usually higher on paraffin-embedded material (3-15%) (95). The disadvantages of this procedure are that if the numbers of aneuploid cells are too small, false negative (diploid) samples can be obtained and there is also a lack of visual control and artifact reflection due to an automated process (95).

Image cytometry (ICM) selects single tumour cells only (96). ICM specimens are made by centrifuging the single-cell suspension on a glass slide and the nuclei are then stained. An image cytometer usually consists of a microscope and camera. Optical density filters must be used to calibrate the image cytometer. The corrected grey-values then provide an accurate measure for the optical density and the DNA content: the darker the nucleus, the more DNA there is. The number of cells measured by ICM is often small (100-300) and the operator time of one DNA-ICM ploidy determination is often 45-60 minutes, which makes ICM expensive. Interactive operator-guided selection of nuclei for measurement with ICM has the advantage over FCM of analyzing epithelial tumour-cell nuclei only (excluding damaged nuclei and cell clumps), but it carries the risk of selection bias and lack of reproducibility (96).

1.6 CORRELATION BETWEEN HISTOLOGICAL GRADING AND DNA PLOIDY

There are only a few studies that have reported on the relationship between DNA ploidy and histological grading (76, 97, 99). There was great variability in methodologies and methods used in these studies for the grading of dysplasia. Small populations of samples were studied; histopathological grading of epithelial dysplasia was not according to the WHO 2005 criteria, and most often, ICM was used to determine DNA ploidy.

This study used high-resolution FCM evaluation of a large number of tissue blocks with different grades of epithelial dysplasia graded according to the binary system.

CHAPTER 2

AIMS AND OBJECTIVE

To determine the correlation between DNA ploidy as determined by high-resolution flow cytometry and histopathological grading of PMD.

HYPOTHESIS

The hypothesis of this study was that there is a correlation between DNA ploidy and the histological grading of oral epithelial dysplasia.

The null hypothesis was that there is no correlation between DNA ploidy and the histological grading of oral epithelial dysplasia.

CHAPTER 3

MATERIALS AND METHODS

Study description: A longitudinal cross-sectional study of an archival cohort of oral tissue blocks with epithelial dysplasia.

Ethical approval to conduct this study was obtained from the Faculty of Health Sciences Research Ethics Committee, University of Pretoria.

3.1 INCLUSION CRITERIA AND CASE SELECTION

The tissue blocks of all cases diagnosed with epithelial dysplasia or early invasive/infiltrating squamous cell carcinoma on the floor of the mouth and lateral and ventral surfaces of the tongue during the period 2000-2008 were retrieved from the archives of the Department of Oral Pathology and Oral Biology of the School of Dentistry, Faculty of Health Sciences, University of Pretoria.

The age and gender of each patient, the anatomical location of the biopsy and the clinical description and histological diagnosis of the intra-oral lesions, were noted on a data sheet.

The haematoxylin and eosin-stained sections of all cases were examined by two senior specialists in Oral Pathology for agreement on the diagnosis and grading of dysplasia. Grading of epithelial dysplasia was done according to

the 2005 WHO classification of epithelial dysplasia and was then further defined as low risk or high risk, according to the workshop coordinated by the WHO Collaborating Centre for Oral Cancer and Precancer, 2005 (5).

Cases where there was disagreement on the histologic grading of the dysplasia, ulceration with extensive inflammation or an amount of tissue inadequate for flow cytometric evaluation were excluded from the study.

The aim was to retrieve at least 30 tissue blocks for each of mild, moderate and severe dysplasia for flow cytometric analysis. Only one tissue block was selected per case for mild and moderate dysplasia. Multiple tissue blocks per case were selected for severe dysplasia – some from the tongue and floor of mouth of cases diagnosed as early invasive squamous cell carcinoma.

3.2 FLOW CYTOMETRY

Sections were prepared according to the modified Hedly method. Four to six 40 µm sections were cut from a paraffin block, wrapped in 50µm nylon mesh, placed in a histology cassette, manually de-waxed and hydrated in distilled water. After being washed in distilled water overnight the enwrapped sections were digested in subtilisin Carlsberg solution at 37 ºC in a centrifuge tube for 120 min and stirred for 20 min thereafter. The Carlsberg solution was then filtered through a 50µm nylon mesh and DAPI (4'6 diamidino 2 phenyl-indole) (Research Organics, Cleveland. OH, USA) staining solution was added. Flow cytometry was carried out using a PAS II flow cytometer equipped with a high-

pressure 100W mercury lamp (Partec, Münster, Germany). DNA histograms of at least 10 000 cells were plotted. The diploid cell populations were used as an internal reference standard for the identification of aneuploid clones.

Tissue samples were categorized as diploid or aneuploid. The mean coefficient of variation of DNA content during the diploid peak and DNA distribution pattern were determined. A lesion was classified as diploid if there was only one peak (2c) at the G_0 or G_1 phase. A lesion was defined as aneuploid if there were aneuploid peaks.

3.3 OUTCOME MEASUREMENTS

The primary outcome of this study was to be statistical correlation of DNA ploidy with histopathological grading of PMD.

DNA ploidy as a diagnostic tool and adjunct to histopathology was evaluated in terms of sensitivity, specificity, positive predictive value and negative predictive value.

3.4 SAMPLE SIZE AND STATISTICAL CONSIDERATIONS

Thirty tissue blocks were used for each of mild, moderate and severe dysplasia for flow cytometric analysis. Categorical data was analyzed using the Chi-square test. Results were considered significant at a p-value of <0.05 and confidence intervals and relative risks are reported. Statistical analysis was performed by a clinical epidemiologist.

CHAPTER 4

RESULTS

4.1 GENERAL CLINICAL INFORMATION

The mean age of the study group was 55 years, ranging from 28 years to 83 years. Clinical data is summarized in *Table 1*.

Table 1: *Clinical data for block histological grade*

Grade	**Cases**	**Blocks**	**Ages (range)**	**M:F**	**Anatomical Location**	
					Floor of Mouth	**Ventrum of tongue**
Mild	39	39	51.1 years (28-79) Unknown=1	0.8:1 (17:22)	18	21
Moderate	35	35	57 years (32-83) Unknown=2	0.7:1 (14:21)	15	20
Severe	27	40	55.5 years (43-78)	2.9:1 (20:7)	15	25
Total	101	114	55 years (28-83)	1.02:1 (51:50)	48	66

4.2 HISTOLOGICAL GRADING SECTIONS AND DNA PLOIDY

Figure 4

Severe epithelial dysplasia demonstrated by cytological atypia extending through more than two-thirds of the epithelial width and DNA aneuploidy (insert) seen as an additional peak caused by cells with abnormal DNA content.

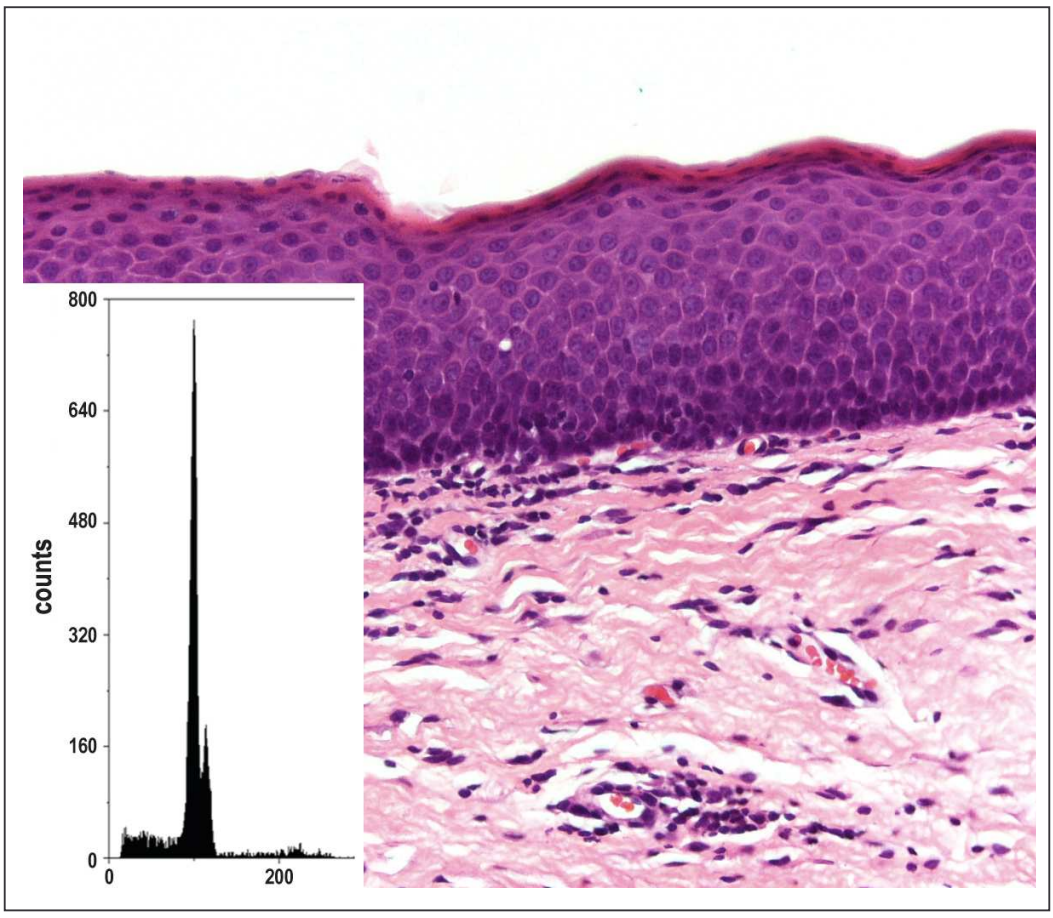


Figure 5

Moderate epithelial dysplasia demonstrated by cytological atypia extending to the middle third of the epithelial width and DNA aneuploidy (insert) seen as an additional peak due to the presence of cells with abnormal DNA content.

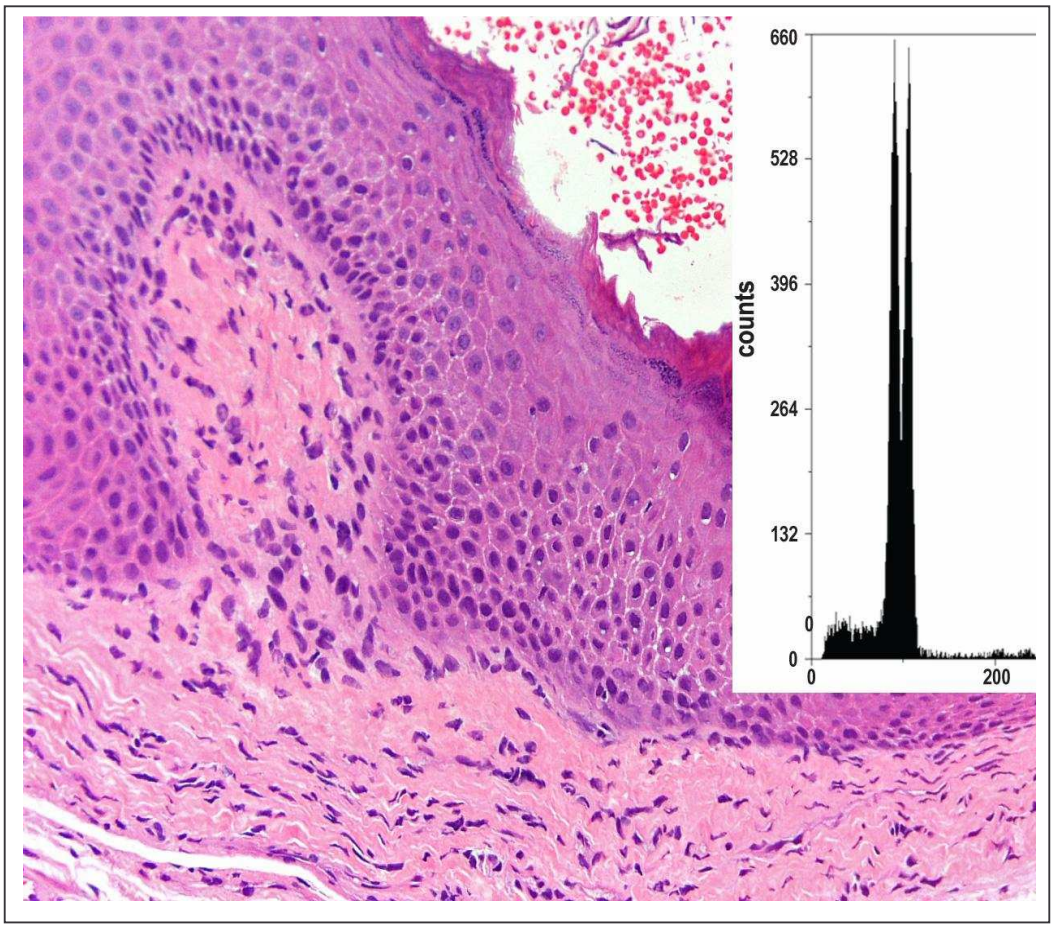


Figure 6

Mild epithelial dysplasia demonstrated by architectural disturbances (drop-shaped rete ridges and basal cell hyperplasia) limited to the lower third of epithelium and aneuploidy (insert) demonstrated by an additional peak.

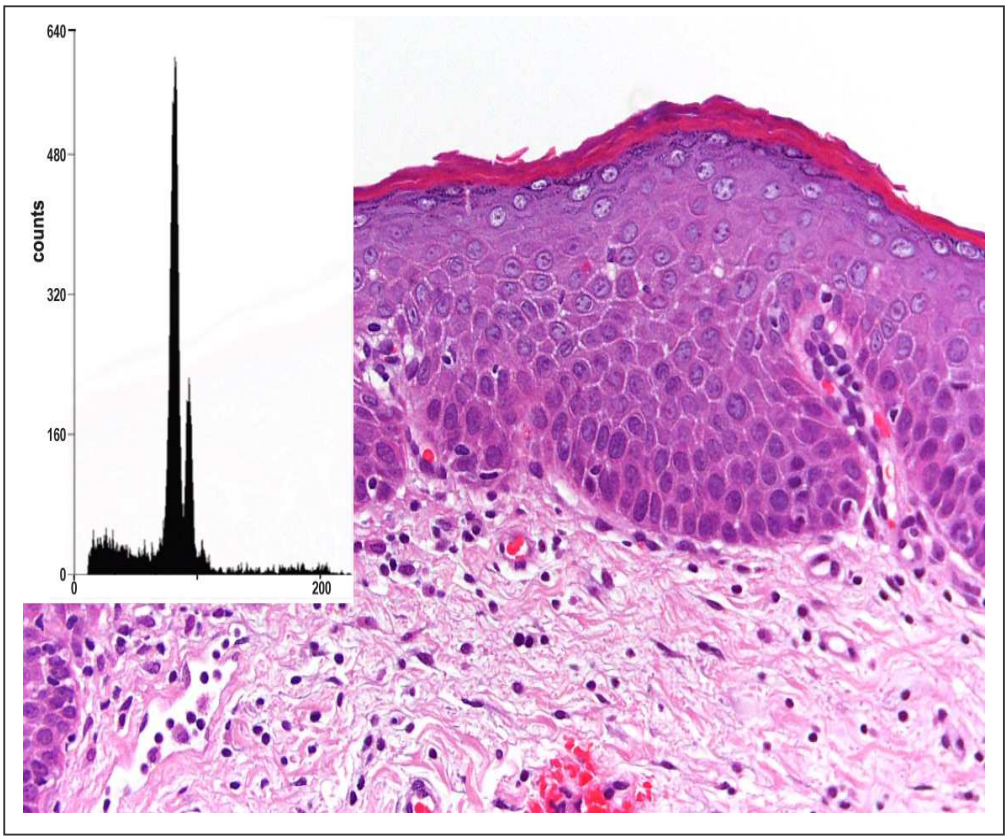


Figure 7

Severe epithelial dysplasia with cytological atypia extending through more than two-thirds of epithelial width and diploid DNA histogram (insert) characterized by a prominent 2n peak representing the cells in the G1/G0-phase of cell cycle.

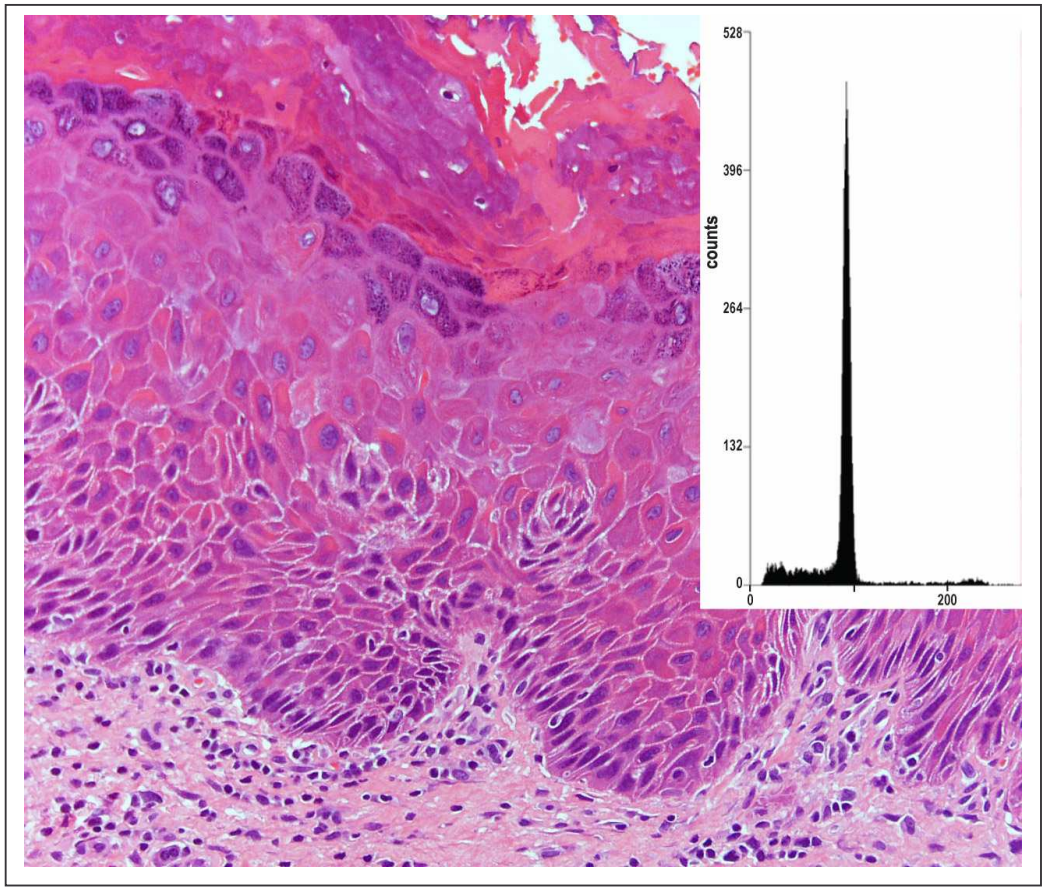


Figure 8

Moderate epithelial dysplasia with cytological atypia and architectural disturbances extending to the middle third of epithelium with diploid DNA histogram (insert) characterized by a prominent 2n peak representing the cells in the G1/G0-phase of cell cycle.

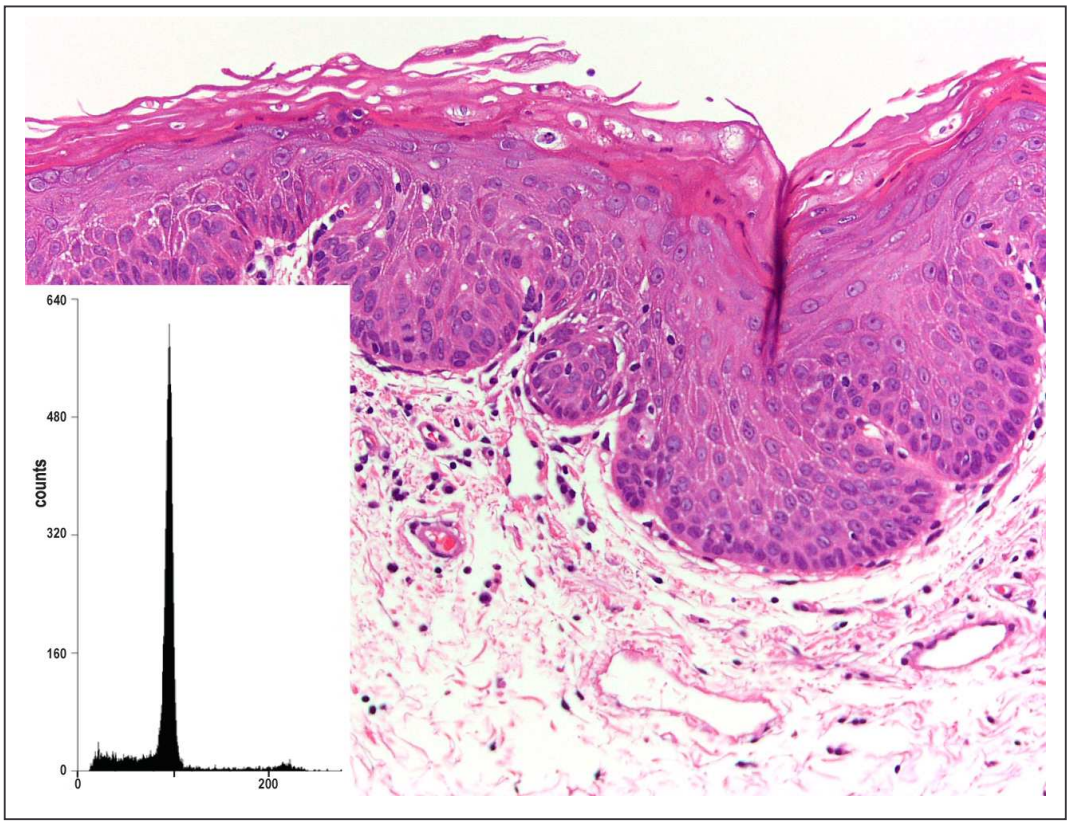
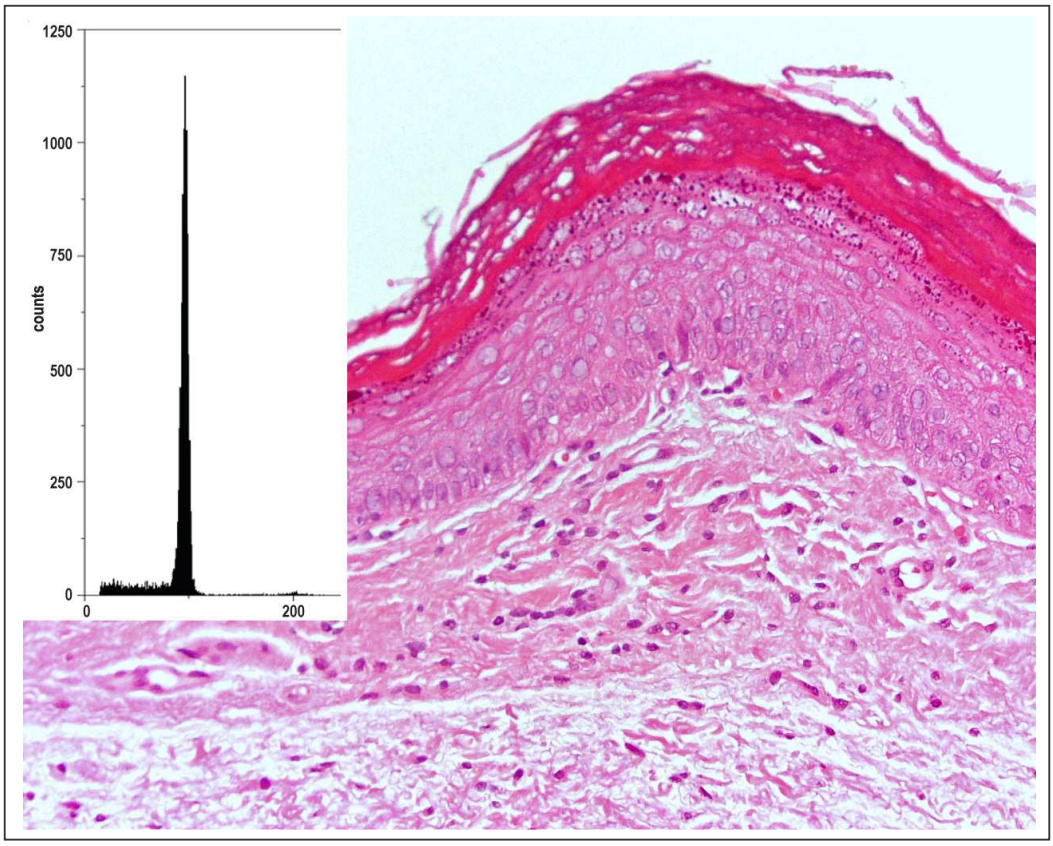


Figure 9

Mild epithelial dysplasia demonstrated by architectural disturbances (basal cell hyperplasia) limited to the lower third of epithelium with diploid DNA histogram (insert).



4.3 CORRELATION BETWEEN HISTOLOGICAL GRADING OF EPITHELIAL DYSPLASIA AND DNA PLOIDY

There was a statistically significant correlation between the WHO 2005 histological grading of epithelial dysplasia and DNA ploidy (p=0.00) *(Table 2).*

Table 2: *2005 WHO grading of epithelial dysplasia and DNA ploidy*

	Mild (n=39)	Moderate (n=35)	Severe (n=40)
Aneuploid (n=39)	5	11	23
Diploid (n=75)	34	24	17

There was a statistically significant correlation between the binary grading system for epithelial dysplasia and DNA ploidy (p=0.001) *(Table 3).*

Table 3: *Binary grading system of epithelial dysplasia and DNA ploidy*

	Low risk (n=39)	High risk (n=75)
Aneuploid (n=39)	5	34
Diploid (n=75)	34	41

The null hypothesis of this study was that there is no correlation between DNA ploidy and the histological grade of epithelial dysplasia. The results of this study found that the null hypothesis must be rejected.

4.4 DIAGNOSTIC TEST EVALUATION OF DNA PLOIDY

A diagnostic test was performed to evaluate the performance of DNA aneuploidy as a candidate diagnostic test against the histological grading of epithelial dysplasia as the gold standard.

The diagnostic test evaluation for DNA aneuploidy to differentiate between WHO 2005 mild and moderate dysplasia grades demonstrated a specificity of 87.18% (CI=95%). Other test evaluation markers are tabulated in *Table 4*.

Table 4: *Diagnostic test evaluation for DNA aneuploidy and WHO 2005 mild/moderate epithelial dysplasia grading*

Parameter	**Estimate (CI=95%)**
Sensitivity	31.43%
Specificity	87.18%
Positive predictive value	68.75%
Negative predictive value	58.62%

The diagnostic test evaluation for DNA aneuploidy to differentiate between WHO 2005 moderate and severe dysplasia grading demonstrated a specificity of 68.57% (CI=95%). Other test evaluation markers are tabulated in *Table 5*.

Table 5: *Diagnostic test evaluation for DNA aneuploidy and WHO 2005 moderate/severe epithelial dysplasia grading.*

Parameter	**Estimate (CI=95%)**
Sensitivity	57.5%
Specificity	68.57%
Positive predictive value	67.65%
Negative predictive value	58.54%

The diagnostic test evaluation for DNA aneuploidy to differentiate between low- and high-risk of the binary grading system of epithelial dysplasia demonstrated a specificity of 87.18% (CI=95%). Other test evaluation markers are tabulated in *Table 6*.

Table 6: *Diagnostic test evaluation for DNA aneuploidy and binary grading system (low and high risk) of epithelial dysplasia.*

Parameter	**Estimate (CI=95%)**
Sensitivity	45.33%
Specificity	87.18%
Positive predictive value	87.18%
Negative predictive value	45.33%

CHAPTER 5

DISCUSSION

The aim of this study was to identify any correlation between the histological grading of epithelial dysplasia and the ploidy status in the evaluation of potentially malignant disorders.

In this study a statistically significant correlation between DNA ploidy and histological grading of oral epithelial dysplasia was observed. This correlation was found with both the WHO 2005 classification and with the binary grading system for epithelial dysplasia. The correlation was found regardless of the fact that paraffin-embedded tissue blocks were used instead of fresh tissue. According to our knowledge, this study included the greatest number of tissue blocks per epithelial dysplasia grade up to date. Our results are in line with those from some previous studies that have reported a correlation between ploidy and the degree of epithelial dysplasia (67, 99).

We also performed a diagnostic test evaluation for the sensitivity, specificity, positive predictive value and negative predictive value of DNA aneuploidy against the WHO 2005 classification and the binary grading system of epithelial dysplasia. With regard to the WHO 2005 classification of epithelial dysplasia, a diagnostic test evaluation for DNA ploidy was done for two separate levels of dysplasia grade – severe/moderate and moderate/mild epithelial dysplasia.

Concerning DNA ploidy and WHO 2005 grading at the level between mild/moderate dysplasia, DNA aneuploidy had a sensitivity of 31.43% and a specificity of 87.18% for moderate dysplasia, if moderate dysplasia with DNA aneuploidy was regarded as the true positive result. The positive predictive value was 68.75% and the negative predictive value was 58.62%. These results indicate that DNA aneuploidy can be used as an adjunct to distinguish mild dysplasia from moderate dysplasia if the WHO 2005 classification is used to grade epithelial dysplasia.

Concerning DNA ploidy and WHO 2005 grading at the level between moderate/severe epithelial dysplasia, DNA aneuploidy had a sensitivity of 57.5% and a specificity of 68.57% for severe dysplasia. The positive predictive value was 67.65% and the negative predictive value was 58.54%. These results indicate a limited potential for DNA aneuploidy to distinguish between moderate and severe dysplasia.

When the diagnostic test for DNA ploidy was applied to the binary grading system of epithelial dysplasia, DNA aneuploidy had a sensitivity of 45.33% for high-risk dysplasia and a specificity of 87.18%. Concerning the predictive value, the positive prediction value was 87.18% and the negative predictive value 45.33%. These results indicate a great potential for DNA aneuploidy to distinguish between low- and high-risk epithelial dysplasia and DNA aneuploidy can be applied to supplement the clinical advantages of the binary

grading system.

Clinically, the results of the study suggest that epithelial dysplasia should be histologically graded by the pathologist as high risk or low risk according to the binary grading system and then the dysplastic epithelium should be subjected to flow cytometric examination. If the dysplasia is graded as high risk and flow cytometric analysis reveals aneuploidy, then there is an 87.18% chance that it is a true high-risk lesion. DNA ploidy may also aid in compensating for intra- and inter-observer variability in the grading of epithelial dysplasia; and aneuploidy may aid in directing the management of the lesion, possibly towards more aggressive treatment of DNA aneuploid lesions.

The coefficient of variation (CV) of flow cytometric analysis ranged between 2.45% and 6.4%, with a mean of 3.8%. The accepted CV value for paraffin-embedded material should be between 4% and 6% (100). Thus, the quality of flow cytometric analysis was according to the accepted standard. DAPI staining might have contributed to the relatively low CV obtained for the paraffin-embedded material (101). Other studies have also found a statistically significant correlation between DNA ploidy and the histological grading of epithelial dysplasia (67, 99). In a study by Saito et al. (67), flow cytometry was used to analyze the DNA ploidy of 39 cases of mild and severe epithelial dysplasia. Aneuploidy was found in 4/22 of mild dysplastic lesions, while 11/17 severe dysplastic lesions were aneuploid. The authors concluded that

aneuploidy was significantly more frequent in severely dysplastic lesions than in mildly dysplastic lesions.

In an earlier study in 1982 by Grässel-Pietrusky et al. (99), DNA ploidy was studied in 11 leukoplakic lesions of the oral mucosa. Four cases displayed no dysplasia, three showed mild dysplasia, one showed moderate dysplasia, and three lesions showed severe dysplasia. Aneuploidy was detected in only two of the three severe dysplasias and the authors concluded that advanced degrees of epithelial dysplasia were detectable by FCM, although the number of cases in this particular study was too small to draw conclusions.

Seoane et al. and Kahn et al. found no correlation between DNA ploidy and the grade of epithelial dysplasia (76, 97). In the study by Seoane et al. (97), flow cytometric analysis was performed on a total of 41 leukoplakias. The diagnosis of oral leukoplakia was according to the WHO 1978 criteria (102). Thirty-one cases were classified as benign leukoplakias, since they did not transform to OSCC within two years, while 10 leukoplakias were classified as malignant leukoplakias, since they had undergone malignant transformation in a five-year follow-up period. In the benign leukoplakia group of 31 leukoplakias, there were three aneuploid patterns (9.7%) and 20 diploid patterns (90.3%), without a statistically significant difference between ploidy and the presence or absence of epithelial dysplasia. In the group of 10 leukoplakias that underwent malignant transformation, only one leukoplakia was aneuploid, without a statistical significant correlation with histological

grading. The authors concluded that DNA analysis was not of value as a diagnostic aid for the differentiation of dysplastic leukoplakias from the non-dysplastic ones. It should be noted that the aim of this study was not to study different grades of epithelial dysplasia but rather their malignant potential.

In a study by Kahn et al. (76) flow cytometry was performed on 36 leukoplakias which included nine cases of mild, nine moderate and six severe epithelial dysplasias. Aneuploid cell populations were detected in six of the 24 dysplastic lesions, with a specific breakdown of mild=2, moderate=3, and severe=1. The authors concluded that DNA analysis did not prove to be an aid in separating different grades of dysplastic architecture of PMD.

In our study DNA aneuploidy was observed in five cases of mild dysplasia/low grade. The same results have been reported in other studies concerning PMD (67, 76, 97). It has been suggested that atypical phenotypic changes may occur at a later stage than the genetic changes in PMD and that aneuploidy might indicate a high risk for malignant transformation, especially when no phenotypic changes are present (103).

In our study 34 of 75 “high risk” lesions had DNA aneuploidy. It is known that recognition of aneuploid cell clones is essentially dependent upon the number of aneuploid cells in the tissue examined. An explanation for this is that if only a small proportion of heteroploid cell clones are evaluated, the clones may be hidden in the base of the peak gradient and thus escape FCM detection (99).

The clinical data of the study was retrieved from the histology request forms in the Faculty of Health Sciences' Department of Oral Pathology at the University of Pretoria. It was aimed to select one tissue block per case, with at least 30 tissue blocks of each grade of dysplasia. With mild and moderate dysplasia, only one tissue block per case was selected. Owing to the limited number of available cases with severe dysplasia, multiple blocks with severe dysplasia were selected per case. Ten tissue blocks diagnosed with severe dysplasia were selected in four patient cases. Blocks with severe dysplasia were also selected from cases of early invasive squamous cell carcinoma with severe dysplasia adjacent to the infiltrating tumour (17 severe dysplasia tissue blocks were selected from 10 cases). The selection method of severe dysplasia biased our clinical data to the study of age, gender and site of lesions. The ages of three cases were unknown.

This study included only tissue blocks from the floor of the mouth and lateral and ventral surfaces of the tongue. The inclusion of these sites was done to increase the likelihood of having a representative study of a sufficient number of tissue blocks of all the grades of dysplasia, since it is known that lesions at these sites are associated with a higher grade of dysplasia and with malignant transformation (20). The definition and diagnosis of early invasive squamous cell carcinoma is unclear and subjective, but historically the definition emphasizes the loss of the epithelial basement membrane and infiltration of epithelial cells into the lamina propria (5).

Within the limitations of the study, the ages of the patients from whom tissue was studied ranged from 28 to 83 years, with a mean of 55 years. The distribution corresponds with reports from elsewhere in the world, since leukoplakia is usually found in middle-aged and elderly persons in the fourth to seventh decades of life (11, 12, 18).

The male to female ratio of cases was 1.02:1. In other studies oral PMDs were reported to be three times more common in males than in females (12, 17, 18).

Another limitation of our study was that we did not follow up the clinical status of the patients with PMD whose tissues were included in the study, to determine whether or not the diploid and aneuploid cell populations had become malignant. Thus we could not evaluate DNA ploidy in predicting the malignant potential of PMD, so the possible clinical application of DNA ploidy status of PMD to predict future malignant transformation remains unknown.

To determine the value of ploidy in predicting malignant transformation in the cases of PMD included in this study, long-term follow-up of the subjects would be well worthwhile.

CHAPTER 6

CONCLUSION

In this study DNA ploidy correlated significantly with degree of epithelial dysplasia. DNA ploidy determination by high-resolution flow cytometry has a specificity of 87.18% in differentiating between low- and high-risk PMD as established by the binary grading system for oral epithelial dysplasia. DNA ploidy can be used to help the pathologist to arrive at a correct histopathological grading of epithelial dysplasia.

There is a need for further research to assess the value of DNA ploidy as a screening tool to detect high-risk PMD.

CHAPTER 7

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