Mitotic Counting and its Significance in Histopathological Grading of OSCC & Oral Epithelial Dysplasia

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Abstract

The development and continued growth of cancers involves altered rates of cell proliferation. Proliferation rates can provide useful information on prognosis and aggressiveness of individual cancers and can be used to guide treatment protocols in clinical practice. The mitotic rate is known to be of assessing proliferation and a powerful prognostic factor for malignancy and the staining technique should make it easier to identify mitoses, hence facilitating counting. Ever since the introduction of microscopes made the recognition of mitotic figures possible, counting mitotic figures has been applied as a diagnostic tool, especially in tumour pathology. Numerous selective stains like crystal violet, toluidine blue and giemsa which highlight chromatin patterns. Newer prognosticators like immunohistochemistry, flow cytometry, autoradiography, DNA ploidy measurements are now on the forefront. However, cost and time factors make them less feasible.

Introduction

Cancer is major disease burden worldwide. Approximately ten million people are diagnosed with cancer annually and more than six million die of the disease every year; currently over 22 million people in the world are cancer patients[1]. The survival curves of oral cancer have plateaued over the past two decades and remain among the worst of all cancer sites. It is therefore reasonable to hypothesize that early detection and/or treatment has played a role in the greater reduction of mortality over incidence.[2]

Oral potentially malignant disorders which are diagnosed as Oral epithelial dysplasia is significantly associated with an increased risk of oral cancer, with studies reporting transformation rates ranging from 6.6-36.4% after mean follow-up periods of 1.5-8.5 years.[2] The actual mechanism of malignant transformation is poorly understood and it is not inevitable that a dysplastic lesion will progress to cancer. At present, there are no molecular markers which enable us to distinguish lesions that may progress from those that will not. Potential markers include analysis of p53 mutations, and loss of heterozygosity, especially at chromosomes 3p and 9p, gross genomic aberrations, assessed by DNA ploidy status, also have promise as a predictor of malignant progression. But none of these markers have been evaluated in long term prospective studies.[3]

Despite the newest techniques with lights, rinses, and various tests, the gold standard in any unexplained finding, when an etiology cannot be found, is biopsy for microscopic confirmation.[4] Increased and abnormal mitoses indicate genetic damage. Thus identification and quantitation of mitotic cells forms an indivisible part of the histological grading systems used for prognostication of precancerous and cancerous lesions. Therefore, mitotic figure counting is widely used to assess in pathological diagnosis, the rate of proliferation and valuable in the assessment of prognosis.[5]

Mitotic Figure and Mitotic Index

Mitosis is a process in which two exactly identical daughter cells are formed from the division of a mother cell. The various phases of mitosis are prophase, metaphase, anaphase and telophase, some of which are seen in tissue sections. The various chromosomal arrangements in mitotic cells are referred as Mitotic figure. The abnormal excess of mitotic figures is commonly seen in oral epithelial dysplasia and oral squamous cell carcinoma. Defects of mitosis results in various nuclear abnormalities such as micronuclei, binucleation, broken egg appearance, pyknotic nuclei as well as increased number of mitotic figures.[5]

Usually, the proliferative activity of tissue is expressed as the mitotic index. Mitotic index is defined as the number of mitoses detected in a confined and well-defined microscopic area divided by a measure for that area. Classifications may fall into low, moderate, or high mitotic rate. To assess the proliferative activity of tumor cells, the mitotic count is related to the total number of analyzed nuclei.[6,7]

Mitotic Figures Counting

Counting of mitotic figures is the oldest way of assessing proliferation and has been applied as a diagnostic tool, especially in tumour pathology. The ease with which mitoses can be recognized without special equipment apart from a standard laboratory microscope and a well stained slide, has led to increasing popularity of this way of counting of mitotic figures up to the present. Strict morphological criteria should be applied for the recognition of mitotic figures. Mitoses can be defined as dark clots of chromosomes which can be often recognized by the presence of hairy extensions when focusing up and down, while the nuclear envelope is absent and the cytoplasm is basophilic rather than eosinophilic. These chromosomal clots can have the configuration of the metaphase, anaphase, or telophase.[8] The most cellular region of the tumor are selected for counting mitotic figures, preferably at the periphery of the tumor, avoiding regions showing necrosis, inflammation tissue folds or calcifications as much as possible.[9]

Use of mitosis counts[6]
- Distinction between benign and malignant tumor.
- For prognostic purpose.
- Estimation of proliferative activity.

Approaches may be followed when counting mitotic figures by light microscopy[10]
1. Number of mitoses expressed as the total number in a defined number (e.g. 10) high power fields.
2. Number of mitotic figures per unit area (e.g. $2\,mm^2$).
3. Number of mitoses per a certain number of tumour cells (e.g 1000).
4. Number of mitotic figures per area of tumour epithelium. The value of the standardized mitotic index (volume-corrected mitotic index or M/V index) was evaluated giving the results in mitotic figures per square mm of neoplastic epithelium. Volume corrected mitotic index (M/V) is estimated on a microscopic field at high magnification, expressing the number of mitoses per area of tumour epithelium (mm^2).

The first method is easier and faster, but is justifiably being criticized as imprecise and poorly reproducible where as the later two counting methods are accurate, but too time consuming and tedious. Mitosis count must be reproducible in order to assess the histological grade of malignancy.

Factors affecting the estimation of mitosis rate[10]
- Section thickness counting area and number of visual fields studied must also be comparable.
- In addition, number of cells in each visual field and thus the cell size and stroma quantity in tumor will.
- Another source of error in traditional mitosis counts was variation in tumor cellularity and in tumor cell size.

Factors considered while counting mitosis count[10]
1. Tumor sections that contain the most abundant mitosis be used since cell proliferation is subject to significant regional differences.
2. The interval between death and fixation of tumor tissue should not be prolonged

late or insufficient fixation of tumor especially in the centre of node that have not been sectioned results in lower mitosis rate.

3. Observer should not be restricted to single phase mitosis such as metaphase and anaphase, falsely low values will be obtained.

4. Do not involve the karhyorhectic and pyknotic nuclei.

Inspite of these limitations the frequency of mitosis is an important parameter in the grading of malignancy especially in soft tissue sarcomas, malignant melanomas, breast carcinomas malignant lymphomas etc. it is easy to count mitosis because all it is needed only mitosis per mm.[2,11]

Mitotic figures identification criteria given by Van Diest et. al.[5]

1. The nuclear membrane must be absent indicating the cells have passed the prophase.

2. Clear, hairy extension of nuclear material (condensed chromosome) must be present either clotted (beginning metaphase), in a plane (metaphase/ anaphase) or in separate clots (telophase).

3. Two parallel, clearly separate chromosome clots to be counted individually as if they are separate mitoses.

The mitosis counting should be done until all the cells in the field are scanned and this may take several minutes per field. Mitosis counting is a tedious, time consuming and boring job.

Stains for mitotic figures

Hematoxylin and Eosin (H&E) staining is used routinely in histopathology laboratories as it provides the pathologist/ researcher a very detailed view of the tissue. It clearly stains the cell structures including the cytoplasm, nucleus, and organelles and extra-cellular components. This information is often sufficient to allow a disease diagnosis based on the organization or disorganization of the cells and also shows any abnormalities or particular indicators in the actual cells (such as nuclear changes typically seen in cancer).

Reliability and reproducibility of mitotic index in H&E-stained slides are limited owing to several factors including[6]

• Selection bias of high power field (HPF) owing to subjective determination of the areas of highest mitotic activity and heterogeneity of mitotic activity in different areas of the tumor.

• Variation in sample size of tumor biopsy and resection samples and cellularity, both of which influence the number of evaluable cells.

• Distinguishing MFs in H&E-stained slides from similar chromatin changes, ie, in apoptotic cells or secondary to crush, distortion, or karyorrhectic debris, pyknosis, or necrosis, is a subjective task. Literature search revealed that numerous

selective stains such as crystal violet, malachite green with crystal violet, toludine blue, and Giemsa stain have been used for staining of mitotic figures. There are very few studies which are applied in cases of oral cavity.[5]

Crystal violet is a basic dye which has a high affinity for the highly acidic chromatin of mitotic cells. Mitotic cells are stained magenta and stand out distinctly against a light blue background of resting cells.[5] Giemsa's stain is a member of the Romanowsky group of stains. The stain functions by rearranging chromatin and inducing G bands producing a high quality stain for chromatin and the nuclear membrane. The staining methods are originally developed for blood films and bone marrow films, but cell smears, cellular imprints cytospin preparations of different origin and thin tissue sections work equally well.[12]

A selective stain for mitotic figures is the simple and cost effective technique to study mitotic cells in normal oral mucosa, oral epithelial dysplasia and oral squamous cell carcinoma where autoradiographic counting is not required. Although immuno- histochemistry is an advanced method in use, the cost and time factor makes it less feasible for many laboratories.[13]

Mitosis and its Significance in Histopathological grading of OSCC and oral epithelial dysplasia

For many years, TNM staging system has been used to clinically estimate response to therapy and survival. Many workers have devised histologic grading systems to predict the biologic behavior of oral carcinoma.

Broder initiated quantitative grading of oral carcinoma with a system originally based on the proportion of highly differentiated cells in the entire tumor, which is a simple and widely used system of grading of malignancies. Broder's grading system (1927)[14] classified squamous cell carcinoma as well, moderately and poor differentiated on the bases of degree of keratinization, nuclear polymorphism, pattern of invasion, host response and mitotic activity. This system has shown to have the best prognostic value when applied to the least differentiated tumor at the deep invasive front of oral carcinomas[14]. In various grading systems, mitosis is used as a parameter for grading the tumor. In some systems it is counted as individual unit where as in some others it counted as under high power field.

Table 1 : Shows histological malignancy grading system and mitosis by Anneroth and Hansen 1984.[14]

Histologic malignancy grading of tumor cell population				
Morphological Parameter	1	2	3	4
No. of mitoses* basal cell layer	Few (0-2)	Moderate number (3-4)	Numerous (5-6)	Extremely numerous (more than 6)

Table 2: Shows Bryne's Invasive Tumor Front Grading (ITF) System and relation of mitosis count (1992).[15]

Morphologic Feature	Grade 1	Grade 2	Grade 3	Grade 4
Number of mitoses (HPF)	0-1	2-3	4-5	>5

Table 3: Shows histological Grading of Oral Epithelial Dysplasia and relation of mitosis Smith and Pindborg (1969).[16]

Type of Change	Severity of Change		
Mitotic activity	Normal	Slight increase	Marked increase
Level of mitotic activity	Normal	Slight	Marked
Presence of bizarre mitosis	None	Slight	Marked

WHO System (1978)[17] in an attempt to standardize the criteria for oral precancer, in its report in 1978, it defined and listed out the 12 histologic characteristics that characterized the epithelial dysplasia as Mild, Moderate and Severe .

Kramer (1980)[18] suggested that an epithelium shows dysplasia, if it has following features in relation to mitotic figures.

• Increased number of mitotic figures: It is the increase in frequency of mitotic figures.

• Level of mitosis: It is the spread of mitotic activity into the higher levels of the epithelium with an increase in the total mitotic activity. In the normal epithelium, most mitosis is in the parabasal or suprabasal layer.

• Abnormal mitosis: It is the presence of mitotic figures in various forms other than normal in any layer of epithelium. Example, Tripolar mitotic figures.

Burkhardt and Maerker (1981)[16] listed six relevant histological and cytological parameters, based on which diagnosis and classification of epithelial dysplasia .

Table 4: Shows Burkhardt and Maerker (1981) criteria of grading dysplasia in relation to mitosis.

Degree	Characteristics
Low	• Basal cell hyperplasia • Basal cell polarity disrupted.
Medium	• Slight increase in rate of mitosis.
High	• Increase in rate of mitosis
Ca-in situ	• Characteristics of high degree dysplasia more marked. • Epithelial stratification lost • Stroma not yet invaded

Mahendra C Mahajan, VK Hazarey (2004)[16] have studied an assessment of oral epithelial dysplasia using criteria of `Smith & Pindborg Grading System' & `Ljubljana Grading System' in oral precancerous lesions Their study showed that, Ljubljana grading system provides more objectivity in evaluation of OED.

2005 WHO classification During the workshop coordinated by the WHO

Collaborating Centre for Oral Cancer and Precancer, the Working Group discussed the 2005 WHO classification and recommended its adaptation for wider use.[19]

Table 5 : Shows the criteria used for diagnosing oral epithelial dysplasia.

Architecture	Cytology
Irregular epithelial stratification	Abnormal variation in nuclear size (anisonucleosis)
Loss of polarity of basal cells	Abnormal variation in nuclear shape (nuclear pleomorphism)
Drop-shaped rete ridges	Abnormal variation in cell size (anisocytosis)
Increased number of mitotic figures	Abnormal variation in cell shape (cellular pleomorphism)
Abnormally superficial mitoses	Increases nuclear-cytoplasmic ratio
Premature keratinisation in single	Increases nuclear size cells (dyskeratosis)
Keratin pearls within rete ridges	• Atypical mitotic figures • Increased number and size of nucleoli • Hyperchromasia

Conclusion

Therefore, considering all the drawbacks, although notoriously considered unreliable, mitotic cell counting is the easiest, cheapest and fastest way of assessing proliferation. It can be reproducible when precisely standardized staining techniques and identification criteria are strictly followed.

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1 Biofilm forms in a periodontal Pocket.

2 An appropriately customized prescription tray places doctor prescribed solutions into the infected pocket. This technique may be combined with SRP or surgical therapies in necessary.

3 The goal of the Perio Protect Method is to help restore Oral Health and Assist with long-term management.