Microscopic analysis of histological and immunohistochemical sections to differentiate normal, precancer and cancerous oral squamous epithelial tissues

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Analysis of histopathological sections by image analysis software and Immunohistochemical localization for investigation of different protein expression patterns can be used as a valued tool for detection of progress of malignancy in oral cancer. The present study has applied microscopic analysis for different sections of oral precancerous conditions and lesions such as Oral Submucous Fibrosis, leukoplakia, as well as Oral squamous cell carcinoma in comparison to normal. Epithelial thickness, cellular area and roundness were analysed by image analysis software. Potent biomarkers for carcinogenesis such as VEGF, MMP2 & MMP9, shows a strong positivity in both precancerous and cancerous tissue sections when estimated immunohistochemically. NQO1 expression was high in precancerous condition but decreased in OSCC revealing epithelial disintegration. Distribution of SOD was also observed as stress generated by ROS. Together all these can be used as a strong diagnostic approach for identification of different stages and severity of oral precancerous and cancerous conditions.

Keywords: Oral Submucous Fibrosis, leukoplakia, Oral squamous cell carcinoma, image analysis, immunohistochemistry

1. Introduction

Alteration of cellular morphology and altered tissue architecture as observed from histopathology presently contributes majorly in determination and confirmation of pathological states. However visual estimation lack reproducibility and may mislead diagnosis procedures. With evolution of computerized image processing and analysis system for image capture, storage and analysis using specialized software and hardware, this has been used as an investigative tool in pathological research. The Image Analysis first appeared as a readily available technique in 1963 with the introduction of the QTM A (Quantitative Television Microscope), designed by Metals Research Ltd., later to become part of Leica [1]. Since then, the techniques of image analysis have been applied in almost every field of science and technology.

Immunohistochemistry, since first reported in 1940’s [2], has become a crucial technique and has been used as a critical diagnostic tool in research as well as clinical investigations. It has apparent advantage over traditionally used special enzyme staining techniques that identify only a limited number of proteins, enzymes and tissue structures. Till date histopathology is used as a gold standard in quick determination of the tissue status and fast delivery of reports, but this fails in providing information about the severity and changes at the molecular level particularly in neoplastic conditions. With increasing number of tumor marker proteins being identified, these are used as molecular markers to determine the severity of necrosis [3]. These have increased the importance of immunohistochemistry in the field of cancer detection such as defining metastatic tumors [4]. Among such tumor markers VEGF (vascular endothelial growth factor), MMP2&9 (matrix metalloproteinases) are frequently used in pathological research to detect neoangiogenesis and metastasis [5,6]. On the other hand enzymes such as NQO1 (NADPH quinone oxidoreductases) and SOD (superoxide dismutase) are used as oxidative stress markers [7,8].

In Indian subcontinent a great number of patients reports with several oral pathological conditions following a diverse oral habits which mainly includes both smoking and chewing smokeless forms of tobacco. Such conditions mainly include oral leukoplakia, a precancerous sore (lesion) that develops on the tongue or the inside of the cheek; oral submucous fibrosis, a precancerous condition characterized progressive fibrosis of the submucosal tissues (lamina propria and deeper connective tissues), and an eventual inability to open the mouth is regarded as the most severe form; and finally oral squamous cell carcinoma [9]. Annual incidence of oral leukoplakia has been reported as 0.2–11.7% in different populations of India and about 2–12% of leukoplakia becomes malignant within several years [10]. Epidemiological data indicates that, the number of cases of OSF has risen rapidly in India from an estimated 250,000 cases in 1980 to 2 million cases in 1993 [11].

Currently one of the greatest challenges to oral oncobiologists is to determine and identify the degree of tissue damage or stages of various precancerous states of oral tissue and to detect the exact transition of a normal tissue to precancerous state. In this current scenario with improved Immunohistochemical techniques and morphometric analysis of histopathological images may go hand in hand to provide a better diagnosis and early detection of cancer.
2. Materials and methods

2.1 Selection of patients & Tissues

Oral biopsies from the site of lesion of clinically diagnosed cases of 4 oral leukoplakia patients, 4 patients suffering from oral submucous fibrosis, and 7 oral squamous cell carcinoma patients were included for this study and were recruited from patients at OPD of Dept of Oral & Maxillofacial pathology of Dr. R Ahmed Dental College & Hospital, Kolkata, India. Tissues from 3 patients who reported with some teeth problem were obtained. They were informed about the study and after a through oral examination and confirmed negative mucosal infection the tissues were obtained and were regarded as normal tissue. A written consent was obtained from each patients and the study was approved by an ethics review committee of the institute. All the patients had a history of use of panmasala & gutkha, smoking or alcohol intake. All of the cases were confirmed by performing histopathology.

2.2 Histopathology

Briefly, biopsy specimens from the affected oral lesion were collected freed from blood by washing with normal saline. Formalin fixed tissues were embedded in paraffin and 5-7μm thin sections were obtained using a microtome and was collected on slides. Sections were stained with eosin and hematoxylin and the pathological changes were observed under light microscope under different magnification.

2.3 Immunohistochemistry

Paraffin embedded tissue sections of each of normal (N), leukoplakia (L), Oral submucous fibrosis (O) and oral squamous cell carcinoma (C), were cut at 4 μm and stained by the Novolink™ Max Polymer detection system (Novocastra™, UK ). After the sections were re-hydrated through a graded series of alcohol epitope retrieval was performed. Endogenous peroxidase was blocked by applying peroxidase block supplied by the Kit. This was followed by application of the protein block to reduce non-specific binding of primary and polymer. The sections were subsequently incubated with optimally diluted primary antibodies. Primay antibodies used were; mouse mAb NQO1 (Cell Signaling technology, USA), Monoclonal Anti-human VEGF165b Antibody (R&D systems, USA) (1:25), Human MMP-2 Affinity Purified pAb (R & D Systems, USA) (1:25), anti-human rabbit pAb SOD1 Cu/Zn antibody (Cell Signaling technology) (1:100), Human MMP-9 Polyclonal Ab (R & D Systems, USA) (1:25). Post primary block was used to enhance penetration of the subsequent polymer reagent. The polymer recognizes the primary antibody. Sections were further incubated with the substrate/chromogen 3’, 3’diaminobenzidine (DAB). Reaction with the peroxidase produces a visible brown precipitate at the antigen site. Sections were counterstained with hematoxylin and coverslipped. Slides were observed under LEICA DM 3000 microscope and results were interpreted for differential diagnosis of pathophysiological processes.

2.4 Measurement of cellular morphology

2.4.1. Selection of specific histological characters

Primarily the oral epithelium has been divided horizontally approximately in three equal zones, viz. lower, middle and upper, out of which the cells in the middle zone showed a defined morphology in all of the normal (N), leukoplakia (L), Oral submucous fibrosis (O) and oral squamous cell carcinoma (C), that can be compared with each other to examine any change. Further, morphometric features like cell area and cellular roundness were examined for each of the slides. A total of 310 normal epithelial cells from 3 normal individual slides, 259 leukoplakia cells from 4 patients, 350 oral submucous fibrosis cells from 4 individuals, and 1030 cells from 7 OSCC patients, were targeted for image processing and analysis.

2.4.2. Preparation of Images for analysis

For analysis of target features of normal (N), leukoplakia (L), Oral submucous fibrosis (O) and oral squamous cell carcinoma (C) grayscale 40X images were obtained from the Hematoxylin/ Eosin stained slides. At least 5 different fields of interest were taken and each region (200μm) was uploaded to the Leica QWin plus digital image processing and analysis software (Leica Microsystems Ltd, Switzerland). The grey levels were detected which defines the regions of the image that was measured by ‘Field’ and ‘Feature Measurements’. Detection selects these areas by comparing their brightness with either one or two threshold levels. There are three modes of detection which can be adjusted; Black, Grey and White. Once selected the values for these modes of detection can be set in a variety of ways. Filter was used for removing the noise in the images without harming edges. After selection of specified cells to be measured, the regions were delineated. Delineation sharpens the edges and boundaries of objects. Finally the output image is a defined as a binary image, which is detected by the software for measurement of various parameters. Each of the
cellular area and cellular roundness were measured. The roundness, which gives an idea of the shape is defined as $(\text{Perimeter})^2 \times 1000 / (4 \times \pi \times \text{Area})$.

2.4.3 Measurement of the epithelial thickness

Images 10X of Hematoxylin / Eosin stained section from each of normal mucosa, leukoplakia and oral submucous fibrosis were included for measurement of epithelial thickness. H & E stained OSCC slides did not reveal any defined epithelial layer so these were excluded. The epithelial thickness (in μm) was measured with the help of Leica QWin plus digital image processing and analysis software (Leica Microsystems Ltd, Switzerland)

2.5 Statistical analysis

Paired ‘t’ test was performed to observe the difference between different precancerous conditions and oral cancer with normal cellular dimensions. Resulting p values were noted and p<0.05 was considered as significant.

3. Results

3.1 Image analysis and morphometric measurements

To examine the quantitative differences among the morphological features of normal, OSF, leukoplakia and OSCC, digital image analysis was adopted as described in materials and methods. Segmented sample images before and after thresholding are shown in Fig.1. OSF cells shows a deviation from the normal cellular morphology, a specific increase in roundness can be observed. The leukoplakia cells show a significant increase in cellular size; however the cellular roundness did not revealed deviation from the normal cells. In both OSF and leukoplakia conditions the degree of deviation is specific to the severity of the disease. A maximum divergence was observed in OSCC cases where both the roundness and cellular area was affected and showed a high degree of variation of cellular morphology.
The mean cell area, the cell roundness and the epithelial thickness were determined quantitatively for the normal, OSF, Leukoplakia and OSCC as described in materials and methods and are presented in Table 1. The mean cell area for each of the normal, OSF, Leukoplakia and OSCC differed among themselves. The cellular area in case of leukoplakia increased significantly compared to that of the normal (P=0.004) and OSF (P=0.001). Again, the mean cell roundness also differed significantly between leukoplakia and OSCC cells (P=0.02). Epithelial thinning was observed in OSF compared to normal which was supported by morphometric measurements (P=0.016). The decrease in epithelial thickness could be one of the reasons for which a burning sensation is being felt by almost all the patients suffering from OSF. An increase in the mean epithelial thickness was observed in leukoplakia which might be due to frequent keratinization observed in this type of lesion.
Table 1: Morphometric measurements in H & E stained histopathological samples from Normal (N), Leukoplakia (L), Oral submucous fibrosis (O) and Oral squamous cell carcinoma (C) tissues.

<table>
<thead>
<tr>
<th>Sample type</th>
<th>Area (µm²) (mean±SD)</th>
<th>Roundness (mean±SD)</th>
<th>Epithelial thickness (µm) (mean±SD)</th>
</tr>
</thead>
<tbody>
<tr>
<td>Normal (N) (n=310)</td>
<td>720.1±37.21</td>
<td>1.94±0.14</td>
<td>339.0±126.85</td>
</tr>
<tr>
<td>Leukoplakia (L) (n=259)</td>
<td>2439.75±697.6</td>
<td>1.74±0.18</td>
<td>512.0±162.6</td>
</tr>
<tr>
<td>OSF (O) (n=350)</td>
<td>1045.2±516.2</td>
<td>2.9±0.77</td>
<td>174.2±50.34</td>
</tr>
<tr>
<td>OSCC (C) (n=1030)</td>
<td>1932.005±955.5</td>
<td>2.32±0.49</td>
<td>ND</td>
</tr>
</tbody>
</table>

P-values

| N vs L              | 0.004*                | 0.14                  | 0.19                                |
| N vs O              | 0.42                  | 0.32                  | 0.016*                              |
| N vs C              | 0.7                   | 0.24                  | ND                                  |
| L vs O              | 0.001*                | 0.12                  | 0.005*                              |
| L vs C              | 0.33                  | 0.02*                 | ND                                  |
| O vs C              | 0.46                  | 0.26                  | ND                                  |

*Significant P-values (P<0.05 is considered significant). ND, not determined.

Quantitation of cell roundness versus cell area for the four cell types is shown in the graph (Fig. 2). Distinct clustering of each cell types was observed suggesting that the cell roundness/cell area could be a distinguishing morphometric parameter in oral precancer and cancer.

Figure 2. Plot of cell roundness versus cell area for Normal, leukoplakia, OSf and OSCC cell types.

3.2 Immunolocalization of VEGF, NQO1, MMP2, MMP9 and SOD1 (Cu-Zn)

The immunolocalization of various molecular markers were tested to understand the expression and distribution of these proteins in the diseased conditions selected in the present study and the normal tissue was used as negative control (Fig. 3).
3.3. Oral leukoplakia

Immunolocalization of VEGF was mostly observed in the basal and parabasal layers of the epithelium and around the blood vessels in the connective tissue. Strongly positive NQO1 expression was observed throughout the thickness of stratified squamous epithelium sparing the surface keratinized layer. MMP9 was found to be distributed in a diffused pattern throughout the connective tissue and to some extent at the basal layer of the epithelium. Weakly positive immunolocalization of MMP2 observed in the superficial parakeratin layer the stratified squamous epithelium diffuse non-specific expression on the connective tissue and deeper muscle layers. Cu-Zn SOD observed specifically in the superficial layers of lamina propria.

3.4. Oral submucous fibrosis

Moderately positive immunolocalisation of VEGF in the lower half of the atrophic stratified squamous epithelium with diffuse immunolocalised in the juxta epithelial connective tissue. Strongly positive NQO1 expression was observed throughout the thickness of stratified squamous epithelium. A generalized strong positive extracellular MMP9 expression in the epithelial layer was observed. A diffused and weak localization of MMP2 in the atrophic stratified squamous epithelium was observed. Weakly diffused positive bands of Cu-Zn SOD in the lamina propria region were observed.

3.5. Oral squamous cell carcinoma

Strong expression of VEGF was observed in the neoplastic epithelial islands and adjoining connective tissue but sparing the keratosis pearls. A moderately positive NQO1 expression throughout the thickness of the surface epithelium and neoplastic epithelial islands were present in the connective tissue. Mild localization of MMP9 in the surface
4. Discussion

As an early sign of damage to oral mucosa, tobacco smokers and chewers often develop pre-cancerous lesions such as leukoplakia and submucosal fibrosis. The epithelial cells at this stage go specific morphological variation indicative of severeness and tendency to transform to malignancy. So an intervention to changes of the epithelial cells is important for specific diagnosis. Recently we have developed a novel artificial neural network based CAD technique to identify the progressive stages of the oral precancerous condition as OSF based on microscopic images of TEM [12] and light microscopy [13]. Utilization of image analysis in measurement of epithelial thickness has also been used as a powerful technique to determine the degree of healing after management of OSF using specific treatment modality [14]. In this paper, we have presented and proposed possible role of utilization of computer aided image analysis of histopathological images in combination with immunohistochemical techniques for localization and qualitative expression of some tumor marker proteins for helping the doctors in tracking the severity and transition of oral pre-cancer to malignancy.

Analysis of morphological features in terms of cellular area, roundness and epithelial thickness revealed distinct difference among leukoplakia, OSF, OSCC and normal. The normal cellular features were within physiological limits reflecting normal cell division and maturation of the epithelium. Leukoplakia could be distinguished from others by the increase in cellular area and decrease in cellular roundness. In case of OSF, on the other hand, significant thinning of epithelial layer (as measured by epithelial thickness) was observed. The use of quantitative histological methods in determining the squamous epithelial structures thus makes it possible to evaluate differences which are not obvious on qualitative analysis.

Angiogenesis and ECM degradation are two hallmarks of carcinogenesis, tumor development and metastasis. VEGF regarded as one of the major contributing factor in angiogenesis and MMPs (a family of zinc-containing endopeptidases) that degrade various components of the ECM are the major components of these two hallmarks. VEGF is a potent mitogen and chemoattractant for endothelial cells and induces the release of MMP-2, MMP-9 which in turn regulates the angiogenic switch that can occur very early in some cancers, even before malignant progression, with increased vessel density seen in precancerous lesions [15]. This study mainly has focused on identification of this angiogenic switch so that an early detection is possible. On the other hand NAD(P)H: quinone oxidoreductase 1 (NQO1), originally called DT-diaphorase, is a cytosolic enzyme detected mainly in epithelial cells and it catalyzes the two-electron reduction of quinone compounds and prevents the generation of semiquinone free radicals and reactive oxygen species, thus protecting cells from oxidative damage other than its implication in protection against oxidative stress [16] and carcinogenesis, including stabilization of the p53 [17] a tumor suppressor. So a high expression of NQO1 has been observed in various tumor progressions indicative of increased oxidative stress.

A gradual increase of VEGF, MMP2 and MMP9 and a decrease in NQO1 expression in different tissues of leukoplakia, OSF and OSCC respectively is clearly indicative of cancer progression in these precancerous lesions and conditions. The observed reduction in NQO1 expression in OSCC might be due to the epithelial disintegration.

So to conclude, in this study we propose significant variations of cellular morphologies in different cases of leukoplakia, OSF and OSCC compared to normal along with variation in expression patterns of tumor marker proteins in different oral pathological conditions is clearly indicative that this can be used as a powerful tool to study cancer progression.

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References


