Original Article

Histologic and immunohistochemical evaluation of mirror image biopsies in oral squamous cell carcinoma

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ABSTRACT

Background and objective: The concept of field cancerization has frequently been quoted to explain the occurrence of multiple primary cancers in the head and neck region and recurrence following complete excision of the original tumor. The main objective is to study the occurrence of field changes in mirror image biopsy in relation to histopathological changes in the oral mucosa among oral cancer patients using hematoxylin and eosin and to study the expression pattern of cytokeratin, Ki-67 and p53 in oral squamous cell carcinoma and mirror image biopsy.

Materials and methods: A pilot study of 15 patients clinically diagnosed with carcinoma lesion and their corresponding mirror image sites were taken and stained using immunohistochemistry method for the expression of cytokeratin, Ki-67 and p53.

Results: Primary tumors showed strong positive staining for cytokeratin throughout both the epithelium and malignant epithelial islands but absence of staining for Ki-67 and p53.

Conclusion: CK may be useful in predicting epithelial differentiation and Ki-67 and p53 act as weak indicators of malignant disease progression in oral tissues.

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1. Introduction

The concept of field cancerization, proposed by P.J.Thomson,¹ has frequently been quoted to explain the occurrence of multiple primary cancers in the head and neck region and recurrence following complete excision of the original tumor. A second primary tumor that is detected within 3–6 months of detection of index tumor is called as “synchronous lesion”. Tumor that arise after 6 months are called as “metachronous tumors”. 41–49% of secondary primary tumors (SPTs) are synchronous tumors.

Recent molecular genetic studies provide evidence that the majority of, if not all, head and neck squamous cell carcinomas (HNSCCs) develop within a contiguous field of preneoplastic cells.² According to Brakhuus et al.,³ cells of a field show genetic alterations associated with the process of carcinogenesis. A subclone in a field gives rise to an invasive carcinoma. An important implication of this knowledge is that, after surgery of the initial carcinoma, part of the field may remain in the patient. A field with preneoplastic cells that share genetic alterations with cells of the excised tumor has been detected in the resection margins of at least 25% of patients, indicating that this frequently

occurs. Fields can be much larger than the actual carcinoma, sometimes having a diameter >7 cm. When a field remains after resection of the tumor, the risk for another carcinoma, designated as a second field tumor (SFT), is considerably greater.

Boffeta et al.^{4,5} found altered telomeres and unbalanced allelic loci present in both tumors and surrounding histologically normal tissues at distances at least 1 cm from the visible tumor margins. Although the extent of these genetic changes decreases as a function of the distance from the visible tumor margin, unbalanced loci are conserved between the surrounding tissues and the tumors, implying cellular clonal evolution. This is in accordance with the concept of “field cancerization”.

As very few literature is available on mirror image biopsies in patients with oral squamous cell carcinoma, the present study, a pilot study was designed to examine the changes that are occurring in the apparently (clinical) normal appearing mucosa opposite to carcinoma by using hematoxylin and eosin and immunohistochemical markers namely cytokeratin, Ki-67 and p53 for predicting the risk and detecting any changes that is suggestive of a strong possibility of development of OSCC.

2. Materials and methods

After obtaining institutional approval and obtaining informed consent from the patient, incisional biopsies from 15 patients

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clinically diagnosed with oral squamous cell carcinoma lesion and their corresponding mirror image site were taken in the Department of Oral Surgery, Saveetha Dental College and Hospitals, Chennai. Patients treated with chemotherapy or radiotherapy, with metastatic diseases and carcinoma occurring in the midline was excluded from the study. The blocks were sectioned and stained with hematoxylin and eosin. Subsequently, immunohistochemical staining for cytokeratin, Ki-67 and p53 (Biogenex, San Ramon and DAKO Corporation, Carpinteria, CA) was done.

The selected cases were further graded as well differentiated, moderately differentiated and poorly differentiated squamous cell carcinoma for the primary lesion of oral squamous cell carcinoma.

The mirror image biopsied tissue was analyzed histologically and any abnormal features including reactive change to chronic irritation, cellular atypia, dysplasia or malignancy recorded using standardized pathological criteria. Immunohistochemical staining of cytokeratin, Ki-67 and p53 was determined by three observers to eliminate inter observer bias. The grading was based on the intensity of the stain and the area of positive staining as follows:

–: Absence of staining.

+: Weak positive staining (cytokeratin)/average of 1–50 positive cells (p53 and Ki-67).

++: Moderate positive staining (cytokeratin)/average of 51–150 positive cells (p53 and Ki-67).

+++: Strong positive staining (cytokeratin)/average of >150 positive cells (p53 and Ki-67).

Location of immunohistochemical staining was observed as follows:

BL: Positive cell staining within basal layer of oral epithelium.

1/3: Positive cell staining with in lower third of oral epithelium.

2/3: Positive cell staining with in middle third of oral epithelium.

3/3: Positive cell staining with in upper third of oral epithelium.

Statistical analysis has been done by using Statistical Package for Social Science (SPSS package). Mean, median, standard deviation, chi-square test and Kruskal–Wallis test were used to test objectives in the study.

3. Results

Total of 30 biopsies, 15 each from oral squamous cell carcinoma and its corresponding mirror image site were selected. Mirror image biopsies (Figs. 1 and 2) for each case were assessed on the basis of hematoxylin and eosin staining and immunohistochemical staining. Primary tumors showed strong positive staining for cytokeratin throughout both the epithelium and malignant

Fig. 1. Mild dysplasia (H&E) 40× mirror image biopsy.

Fig. 2. Moderate dysplasia (H&E) 40× mirror image biopsy.

epithelial islands but absence of staining for Ki-67 and p53. For cytokeratin, out of 15 mirror image biopsies corresponding to OSCC, 7 cases showed strong positive staining and 8 cases showed moderate positive staining (Figs. 3 and 4). For Ki-67, out of

Fig. 3. Strong positivity for cytokeratin throughout the parakeratinized stratified squamous epithelium (mirror image biopsy) 40×.

Fig. 4. Moderate positivity for cytokeratin with negative basal cell layer and presence of chronic inflammatory cells (mirror image biopsy) 40×.

Fig. 5. Few cells in lower third of epithelium showing weak positivity for Ki-67 (mirror image biopsy) 40×.

Fig. 6. Mild positive staining for p53 in basal cell layer of epithelium (mirror image biopsy) 40×.

15 mirror image biopsies, 2 cases showed mild positive (average 1–50 positive cells) staining and 13 cases were negative (Fig. 5) and for p53, out of 15 mirror image biopsies, 1 case showed mild positive staining (Fig. 6) (average 1–50 positive cells) in basal cell layer.

80% of total OSCC patients were males and 20% were females. 80% of total OSCC patients had the habit of both smoking and tobacco chewing. 20% had the habit of tobacco chewing only. 67% of total OSCC patients had buccal mucosa as site for primary tumor.

4. Discussion

Recently, it has been found that even the clinically normal appearing mucosa in a patient harboring a cancerous lesion may have dysplasia on the contra lateral anatomic site.^{1,3} In the present study, an attempt was made to study histopathologic changes that are observed in mucosa opposite to oral cancers.

Biopsies from primary lesions and mirror image biopsies were routinely processed and stained with hematoxylin, eosin and panel of immunohistochemical markers like cytokeratin, Ki-67 and p53. Though 40% of our mirror image biopsies showed mild dysplasia and 13% showed moderate dysplasia which could be attributed to habits like tobacco chewing and smoking.

The field changes seen could also be explained by patch field carcinoma theory proposed by Brakhuys et al.,^{3,12} which is in accordance to our study. This theory assumes that a stem cell acquires one genetic alteration and forms a patch with genetically altered cells. This patch of genetically altered cells escapes growth control and expands in a lateral direction. Further mutations and genetic instability leads to development of carcinoma characterized by invasive growth and metastatic potential. Thus surrounding primary lesion of carcinoma, a field of altered epithelium exists which may be as large as 7 cm.³

Existence of genetically altered field is difficult to establish by routine histologic examination. This is because a genetically altered field may appear as normal histology. Thus histopathologic examination using certain IHC markers can be used to demonstrate field change in major cases of oral cancer.³ Our study assessed 15 mirror image biopsies corresponding to OSCC for staining intensity and staining location with regard to cytokeratin, Ki-67 and p53.

For cytokeratin AE1/AE3, out of 7 cases, showing strong positive staining (Table 1), one case showed more intense staining in stratum spinosum and stratum superficiale and less intense staining in stratum granulosum and basale. This may be because of same pattern of keratin expression in stratum superficiale and spinosum. On comparing H&E and immunohistochemistry results for CK, we found that out of 2 mirror image biopsies having features of moderate dysplasia, both showed moderate positive staining for cytokeratin throughout the epithelium. Basal layer showed moderate positive staining in both the cases. In three

Table 1
Location and intensity of cytokeratin, Ki-67 and p53.

No.	Cytokeratin (AE1/AE3)				Ki-67				p53			
	BL	1/3	2/3	3/3	BL	1/3	2/3	3/3	BL	1/3	2/3	3/3
1	++	++	++	++	–	–	–	–	–	–	–	–
2	++	++	++	++	+	–	–	–	–	–	–	–
3	+++	+++	+++	+++	–	–	–	–	–	–	–	–
4	–	++	++	++	–	–	–	–	–	–	–	–
5	+++	+++	+++	+++	–	–	–	–	–	–	–	–
6	++	++	++	++	–	–	–	–	–	–	–	–
7	–	++	++	++	–	–	–	–	–	–	–	–
8	+++	+++	+++	+++	–	–	–	–	–	–	–	–
9	+++	+++	+++	+++	–	–	–	–	–	–	–	–
10	–	+	+	++	–	–	–	–	–	–	–	–
11	++	++	++	++	–	–	–	–	–	–	–	–
12	+	+++	+	+++	–	–	–	–	–	–	–	–
13	+++	+++	+++	+++	–	–	–	–	–	–	–	–
14	+++	+++	+++	+++	+	+	–	–	–	–	–	–
15	++	++	++	++	–	–	–	–	+	–	–	–

BL: basal cell layer; 1/3: lower third of epithelium; 2/3: middle third of epithelium; 3/3: upper third of epithelium.

cases, basal cell layer was negative for CK and there was presence of moderate chronic inflammatory cell infiltrate.

This is in accordance with the work done by Farrar et al.,⁶ in which AE1/AE3 immunostaining was negative in the majority of cases of mild, moderate and severe dysplasia except in severe dysplasia where 50% of cases showed a positively stained basal layer. This phenomenon of negative basal layer staining is difficult to explain. Archival material was used in the present study and therefore its possible that antigenicity may have been lost over time, making immunostaining with certain antibodies less effective.³ Vaidya et al.⁸ demonstrated a reduction in CK 5 expression in cases of mild and moderate oral dysplasia, indicating an increased potential for progression to malignancy. In the present study, cases of dysplasia that showed no AE1/AE3 staining in the basal layer may be lesions that are more likely to progress to malignancy.

Also, in our study, negative basal cell layer CK expression in three cases of mild dysplasia could be attributed to absence of CK 19 expression which is expressed in basal cell layer of oral mucosa in normal conditions. It has been suggested that CK 19 expression, particularly in those oral sites where it is not usually seen, is related to inflammation.⁷ All our three respective cases showed moderate chronic inflammatory cell infiltrate. Ogden et al. and Nie et al.^{8–10} showed suprabasal expression of CK 19 supportive of a field change and can be used as a candidate marker for diagnosis of oral precancerous lesions and determination of the differentiation level of oral squamous cell carcinoma.

So, AE1/AE3 antibody proved helpful in detecting malignant changes in clinically normal appearing mucosa (mirror image biopsy).

For Ki-67 (Table 1), one case (normal mirror image biopsy) showed mild positive staining (average 1–50 positive cells) in basal cell layer and lower one third of epithelium and one case (moderate dysplasia) showed mild positive staining in basal layer only. This was in accordance with work done by Farrar et al.,⁶ in which they showed that Ki-67 staining in the normal tissue category was mostly limited to the basal layer, however some Ki-67 positive cells were seen in the lower third of the epithelium in three cases. This may be explained by field cancerization hypothesis or may indicate a proliferative response to chemical stress.

So, Ki-67 appears to be weak indicator of malignant disease progression in oral tissues. Ki-67 antibodies may be useful in the development of a quantitative system for more accurate classification of oral lesions.

For p53 (Table 1), out of the total number of 15 mirror image biopsies, only 1 case showed mild positive staining with mild positivity (average 1–50 positive cells) in basal cell layer. Similarly, on comparing H&E and immunohistochemistry results, we found that 1 mirror image biopsy having features of moderate dysplasia, showed mild positive staining for p53 in basal cell layer. These findings correlated with the previous studies in the literature. Bongers et al.¹¹ did not find any expression of p53 protein in adjacent, clinicopathological normal-looking mucosa in all cases ($n = 44$). In eighteen cases, primary tumors showed p53 positivity. Primary tumors in our study did not show any positive staining for p53. The absence of antibody reactivity does not rule out genetic alterations of p53. Frameshift mutations as well as nonsense mutations, that may result in the production of truncated forms of p53, can lead to altered p53 undetectable by the monoclonal antibodies.¹¹

The underlying mechanism by which p53 protein is stabilized is of great importance when interpreting immunocytochemical staining using p53 antibodies. Although there is strong correlation between p53 gene mutation and the resultant stabilization/accumulation of the p53 protein within cells, other factors may

also bring about this effect. Infection with certain types of human papillomavirus can result in stabilization and inactivation of wild-type p53 protein. Cellular proteins such as mdm-2 participate in the autoregulation of p53 function, and overexpression of mdm-2 can cause cellular transformation due to the associated inactivation of p53.⁶

Recently, a 40% discrepancy between single strand conformational polymorphism analysis for the detection of p53 mutations in exons 5–9, the most common sites for p53 abnormalities, and immunohistochemistry, has been shown. However, the now known discrepancy between molecular genetic and antibody-based studies caution against drawing a definitive conclusion about the value of p53 over expression by using a single detection modality. The approximately positive predictive value of p53 detection in tumors by immunohistochemistry is 63%. It is still an option that detecting p53 mutation at the genomic level has some value in predicting the development of a secondary primary tumor.¹³

Thus, p53 also appears to be a weak indicator of malignant disease progression in oral tissues. The value of p53 antibodies in such a quantitative system is limited due to variability in the expression, detection and accurate interpretation of results.

Within limitations of the study, the results achieved from our pilot study showed that field changes are observed in oral mucosa opposite to the primary lesion of oral cancer as more than half of a cohort of 15 patients presenting with OSCC in our study displayed mild dysplasia in their mirror British English stained sections to verify any changes in the epithelium. This tissue instability has potential implications not only in relation to the risk of second primary tumor development, but also in terms of the interpretation of dysplasia identified at tumor resection margins. CK may be useful in predicting epithelial differentiation and Ki-67 and p53 act as weak indicators of malignant disease progression in oral tissues.

Conflicts of interest

The authors have none to declare.

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