Pattern of Keratinization in Oral Squamous Cells during Carcinogenesis

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Abstract:
Background: Oral carcinogenesis is a multi-step process. Broadly, oral squamous cell carcinoma (OSCC) can be well-, moderately- or poorly-differentiated, and either keratinizing or non-keratinizing. Most cases are moderately to poorly-differentiated. Precursor lesions (dysplasia) can be categorized into mild, moderate, or severe (carcinoma in situ). In the present study, the pattern of keratin expression in oral squamous cells during carcinogenesis is vividly analysed.

Materials and Methods: Samples in the form of scraped and exfoliated cytosmear were collected from the affected sites of the clinically diagnosed 136 oral cancer patients and were immediately fixed in acetoalcohol (1:3). The wet fixed smears were stained by routine Papanicolaou’s staining protocol and Giemsa’s solution. Stained tissues were studied under the microscope.

Results: Cytological pleomorphism is a unique feature observed during carcinogenesis. There appears to be a spectrum of degrees of keratinization rather than distinct types, and the degree of keratinization is reflected in the degree of packing and orientation of keratin filaments. It is presumed that alteration in the architectural regularity of the cell membrane is an important aspect of keratinization which leads to cytological pleomorphism during oral carcinogenesis.

Conclusion: Pattern of keratinization along with cytological pleomorphism in exfoliated epithelial squamous cells has a practical utility in the diagnosis and early detection of oral cancer during carcinogenesis.

Keywords: Carcinogenesis, Keratinization, Keratins, Oral squamous cell carcinoma.

I. Introduction

Keratins are defined as intermediate filament forming proteins with specific physicochemical properties produced in any vertebrate epithelia [1]. These are primarily responsible for maintaining the structural integrity of keratinocytes [2]. These belong to multigene family of proteins constituting 85% of the total cellular protein in the cornified cells of the epidermis [3, 4]. In humans, there are around 30 keratin families divided into two groups, namely, acidic and basic keratins, which are arranged in pairs. They are expressed in a highly specific pattern related to the epithelial type and stage of cellular differentiation. The different types of keratin and their associated proteins serve as important markers of differentiation thus aiding in diagnosis of various pathological conditions including cancer [5-7]. The keratin proteins have a uniform mode of distribution among the various layers of epithelium which gives an indication of the disease process. Also, various disorders are associated with defects in the keratin and their associated proteins which may manifest in skin or oral cavity or both [8]. The keratins and keratin-associated proteins are useful as differentiation markers because their expression is both region specific and differentiation specific. Antibodies to keratin are considered as important tissue differentiation markers and therefore are an integral aid in diagnostic pathology. It has been reported that K8 is associated with transformation and increased malignant potential of oral squamous epithelial cells [9, 10]. In the present study, the pattern of keratin expression in the pleomorphic oral squamous cells during carcinogenesis is analysed.

II. Materials And Methods

Samples in the form of scraped and exfoliated cytosmear were collected during May 2007 to May 2009 from the affected sites of the clinically diagnosed 136 oral cancer patients, who have registered at the Out-patient Department (OPD) of Acharya Harihar Regional Cancer Centre, Cuttack, Odisha- the only Government Hospital for the treatment of cancer patient. Two smearing slides were prepared from each affected site. The
collected tissues in the form of smears on the slides were immediately fixed in acetoalcohol (1:3). Prior to the collection, the case history of the patients were collected through prepared questionnaire form. The wet fixed smears were stained by routine Papanicolaou’s staining protocol and Giemsa’s solution. Photomicrographs of the tissues were taken out as supporting evidence. The findings were statistically analyzed, interpreted and correlated with age, sex and addiction of the patients.

Ethical considerations

This study was approved by Institutional Ethical Committee of Utkal University, Bhubaneswar and necessary permission from the Director, AHRCC, Cuttack, Odisha, India was also obtained.
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Table 1 Degree of pathogenicity, age group, sex and addiction-wise individuals

<table>
<thead>
<tr>
<th>No</th>
<th>Group</th>
<th>Age group (in years)</th>
<th>No of samples</th>
<th>Chewers</th>
<th>Smokers</th>
<th>Alcoholics</th>
<th>Chewer-smokers</th>
<th>Non-addicted cancerous</th>
<th>Total</th>
</tr>
</thead>
<tbody>
<tr>
<td>M</td>
<td>F</td>
<td>M</td>
<td>F</td>
<td>M</td>
<td>F</td>
<td>M</td>
<td>M</td>
<td>F</td>
<td></td>
</tr>
<tr>
<td>1</td>
<td>Control</td>
<td>30-49</td>
<td>33 (40.2)</td>
<td>11 (20.4)</td>
<td>Nil</td>
<td>Nil</td>
<td>Nil</td>
<td>Nil</td>
<td>33 (40.2)</td>
</tr>
<tr>
<td></td>
<td></td>
<td>50-69</td>
<td>34 (41.5)</td>
<td>36 (66.6)</td>
<td>Nil</td>
<td>Nil</td>
<td>Nil</td>
<td>Nil</td>
<td>34 (41.5)</td>
</tr>
<tr>
<td></td>
<td></td>
<td>70-89</td>
<td>15 (18.3)</td>
<td>07 (13.0)</td>
<td>Nil</td>
<td>Nil</td>
<td>Nil</td>
<td>Nil</td>
<td>15 (18.3)</td>
</tr>
<tr>
<td>2</td>
<td>Pre-cancerous</td>
<td>30-49</td>
<td>08 (9.7)</td>
<td>05 (9.3)</td>
<td>0.2 (8.6)</td>
<td>03 (15.0)</td>
<td>Nil</td>
<td>01 (8.4)</td>
<td>03 (12.5)</td>
</tr>
<tr>
<td></td>
<td></td>
<td>50-69</td>
<td>18 (22.0)</td>
<td>14 (25.9)</td>
<td>0.3 (13.0)</td>
<td>07 (35.5)</td>
<td>03 (33.0)</td>
<td>01 (50.0)</td>
<td>02 (16.6)</td>
</tr>
<tr>
<td></td>
<td></td>
<td>70-89</td>
<td>06 (7.3)</td>
<td>04 (7.4)</td>
<td>0.4 (17.4)</td>
<td>02 (10.0)</td>
<td>01 (11.1)</td>
<td>Nil</td>
<td>01 (5.5 )</td>
</tr>
<tr>
<td>3</td>
<td>Cancerous</td>
<td>30-49</td>
<td>25 (30.5)</td>
<td>06 (11.1)</td>
<td>0.9 (39.1)</td>
<td>03 (15.0)</td>
<td>02 (22.2)</td>
<td>Nil</td>
<td>03 (25.0)</td>
</tr>
<tr>
<td></td>
<td></td>
<td>50-69</td>
<td>16 (19.5)</td>
<td>22 (40.7)</td>
<td>0.4 (17.4)</td>
<td>04 (20.0)</td>
<td>02 (22.2)</td>
<td>01 (50.0)</td>
<td>03 (25.0)</td>
</tr>
<tr>
<td></td>
<td></td>
<td>70-89</td>
<td>09 (11.0)</td>
<td>03 (5.6)</td>
<td>0.1 (4.4)</td>
<td>01 (5.0)</td>
<td>01 (11.1)</td>
<td>Nil</td>
<td>03 (25.0)</td>
</tr>
<tr>
<td>Total</td>
<td></td>
<td>30-89</td>
<td>164</td>
<td>108</td>
<td>23</td>
<td>20</td>
<td>09</td>
<td>02</td>
<td>12</td>
</tr>
</tbody>
</table>

M-Male   F-Female

rounded or both found in the same tissues and devoid of cytoplasm, in which the nuclear–cytoplasmic (N/C) ratio is 1:1 and so are non-keratinized. These NMSCs are poorly differentiated and observed in both carcinoma in situ and in frank malignant cases. Besides these, all other cells are highly keratinized, moderately differentiated and observed to be present at all stages of oral carcinogenesis (Figs. 3-9).

Expression of keratin in the aforesaid pleomorphic cells in non-keratinized squamous epithelia of the oral cavity is a remarkable feature during carcinogenesis. The atypical pleomorphic cells observed to be thicker, denser and hyperkeratinized in patients habituated with any form of tobacco and alcohol than the non-addicted cancerous patients. Similarly, the exfoliated mucosal cells collected from occasionally habituated normal individuals are observed to be keratinized so far as non-habituated normal individuals are concerned in our study. The degree of keratinization is also observed to be varying from cell to cell in different sites of the same individual is probably due to the habit of keeping tobacco (khaini, gutkha, snuffls etc.), chewing of paan (betel) and paan masala at specific site of the oral cavity. Not only that smoking of tobacco (in the form of bidi and cigarette) and drinking of alcohol may induce the process of keratinization in different sites of the oral cavity demands further study.
### Table 2 Cellular differentiations of the control and cancer affected individuals with respect to sites and sex

<table>
<thead>
<tr>
<th>No</th>
<th>Oral Site</th>
<th>Control Group</th>
<th>Cancer affected Group</th>
<th>Total</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td></td>
<td>WDSC</td>
<td>MDSC</td>
<td>PDSC</td>
</tr>
<tr>
<td></td>
<td></td>
<td>M</td>
<td>F</td>
<td>M</td>
</tr>
<tr>
<td>1</td>
<td>Lip</td>
<td>05</td>
<td>06</td>
<td>Nil</td>
</tr>
<tr>
<td></td>
<td></td>
<td>(6.1)</td>
<td>(11.1)</td>
<td></td>
</tr>
<tr>
<td>2</td>
<td>Tongue</td>
<td>11</td>
<td>07</td>
<td>Nil</td>
</tr>
<tr>
<td></td>
<td></td>
<td>(13.4)</td>
<td>(12.9)</td>
<td></td>
</tr>
<tr>
<td>3</td>
<td>Alveolus and gingiva</td>
<td>16</td>
<td>06</td>
<td>Nil</td>
</tr>
<tr>
<td></td>
<td></td>
<td>(12.5)</td>
<td>(11.1)</td>
<td></td>
</tr>
<tr>
<td>4</td>
<td>Floor of the mouth</td>
<td>07</td>
<td>06</td>
<td>Nil</td>
</tr>
<tr>
<td></td>
<td></td>
<td>(8.5)</td>
<td>(11.1)</td>
<td></td>
</tr>
<tr>
<td>5</td>
<td>Palate</td>
<td>06</td>
<td>03</td>
<td>Nil</td>
</tr>
<tr>
<td></td>
<td></td>
<td>(7.3)</td>
<td>(5.6)</td>
<td></td>
</tr>
<tr>
<td>6</td>
<td>Buccal mucosa</td>
<td>37</td>
<td>26</td>
<td>Nil</td>
</tr>
<tr>
<td></td>
<td></td>
<td>(45.2)</td>
<td>(48.2)</td>
<td></td>
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<tr>
<td>7</td>
<td>Total</td>
<td>82</td>
<td>54</td>
<td>Nil</td>
</tr>
<tr>
<td></td>
<td></td>
<td>(100)</td>
<td>(100)</td>
<td></td>
</tr>
</tbody>
</table>

M- Male  F- Female

### IV. Discussion

The process by which the structural protein is made tough and insoluble is known as keratinization, a process that results in a wide range of products and whose nature is far from being fully understood. In the oral cavity the lining mucous membrane becomes keratinized to varying degrees in different areas of the mouth. It has been observed that the oral epithelium of the human, has a surface which varies in its degree of keratinization from none at all, as in the cheek, through partial or parakeratinization, as found sometimes on the gingiva and parts of the palate, to full or orthokeratinization, as seen in most parts of the hard palate and overlying the gingival where it is attached to bone. The degree of keratinization-that is, the thickness of the keratin layer and whether para- or orthokeratinization occur is affected not only by genetic but also by the systemic factors. Nanci has reported that oral epithelium is classified into three types based on their morphology and specific pattern of differentiation: keratinized stratified squamous epithelium (masticatory mucosa distributed in hard palate and gingiva), non-keratinized stratified squamous epithelium (buccal mucosa, labial mucosa) and specialized mucosa (dorsal surface of the tongue) [13].

Keratinization, also termed as cornification, is a process of cytodifferentiation which the keratinocytes undergo when proceeding from their post germinative state (stratum basale) to finally differentiated, hardened cell filled with protein, constituting a structurally and functionally distinct keratin containing surface layer such as stratum corneum [9]. SCC is characterized by squamous differentiation (often seen as keratinization, sometimes with keratin pearl formation) and invasive growth with disruption of the basement membrane.

![Fig.1 Normal oral squamous cells (Papanicolaou’s stain).](image-url)
Fig. 2 Normal oral squamous cells (Giemsa stain).

Fig. 3 Micronucleated cells during leukoplakia.

Fig. 4 Micronucleated cells during erythroplakia.

Fig. 5 Keratinized tadpole cells (KTCs).
Fig. 6 Keratinized spindle cells (KSCs).

Fig. 7 Well differentiated oral squamous cells (Hypo- and hyperkeratosis in PKSCs).

Fig. 8 Moderately differentiated oral squamous cells (Hyperkeratinized pleomorphic squamous cells, viz. KSC, LKFC, SKRCs)

Fig. 9 Poorly differentiated NMSCs at carcinoma in situ.
SCC is graded into well-, moderately-, and poorly-differentiated. Well-differentiated SCC closely resembles normal squamous mucosa whereas moderately-differentiated SCC displays nuclear pleomorphism, mitoses (including atypical forms), and usually less keratinization. In poorly-differentiated SCC, immature cells predominate, with numerous typical and atypical mitoses, minimal keratinization, and sometimes necrosis. Most SCCs are moderately-differentiated [14-16]. Contrary to these reported facts, in our study, not only in well-differentiated (PKSCs) but also in moderately differentiated squamous cells (viz. KSC, LKFC, SKRCs), both hypo- and hyper-keratinized cells are observed. These cells are mostly observed during premalignant stage and rarely during malignant stage of oral carcinogenesis. However, the poorly differentiated NKSCs are observed to be non-keratinized and are restricted to carcinoma in situ and malignant stages only.

During keratinization the water content of the cells decreases dramatically in spite of the fluid environment in the mouth. Keratin in the mouth remains relatively translucent as compared with skin, probably because it is kept moist and because the cornified layers are normally compact. In the mouth if the layers of keratin become broken up or if abnormal keratinization occurs there is a loss of translucency and the area appears white, perhaps because of the spaces in the cornified layer.

Keratin expression patterns are characteristic for distinct stages during cellular epithelial differentiation from embryonal to adult and of the internal maturation program during development [17]. Epithelial tumors including metastasis most widely retain their keratin patterns of their normal origin; thus the determination of the keratin patterns of tumors is widely exploited for cell and tumor typing. Therefore, keratins have evolved to be one of the most potent epithelial differentiation and tumor markers in cell biology, embryology, and surgical pathology [18]. Specific antibodies against several keratins are routinely used for immunohistochemical typing of carcinoma in tumor diagnostics. Keratins can be used as differentiation markers in normal oral epithelia [10].

Endoscopically, the lesions may be discrete or diffuse, smooth or irregular, flat or exophytic. Precursor lesions may present as a small flat patch or as a large warty plaque. The surface may be brown to red (erythroplakia) or present with circumscribed whitish plaques (leukoplakia). White patches may be ulcerated. Leukoplakia, in contrast to erythroplakia, tends to be well demarcated and seems to have a lower risk of malignant transformation. The lesions are commonly diffuse, with a thickened appearance. Malignant transformation of the mucosal lining is a genetic process resulting from accumulation of multiple genetic alterations that dictate the frequency and pace of progression to invasive carcinoma. It is generally accepted that most sporadic tumors are the result of a multi-step process of accumulated genetic alterations. These alterations affect epithelial cell behaviour by way of loss of chromosomal heterozygosity (LoH) which in turn leads to a series of events progressing to the ultimate stage of invasive squamous cell carcinoma. The corresponding genetic alterations are reflected in clinical and microscopic pathology from hyperplasia through invasiveness [19]. LoH studies indicate that the earliest alterations appear to target specific genes located on chromosomes 3p14, 9p21 (CDKN2A), and 17p13 (TP53). Alterations that tend to occur in association with higher grades of dysplasia and SCC include cyclin D1 amplification, PTEN inactivation, and LOH at 13q21, 14q32, 6p, 8, 4q27, and 10q23 [14]. Keratinization of tumors and the high expression of microRNAs (miRNAs) were the major factors related to the poor prognosis of patients. Interestingly, a majority of the keratinized tumors expressed high levels of miR-21 [20]. The potential to identify accurately and prospectively the subset of dysplastic lesions which are likely to progress through dysplasia to cancer are of premier scientific and clinical significance. Thorough examination and appropriate selection of biopsy sites, coupled with expert histopathologic evaluation, are required for diagnosis of oral leukoplakia and erythroplakia [21]. Analysis of correlations between biomarkers, stages of dysplasia and their progression to OSCC is complex and it requires incorporation of multiple variables [22].

In our study, it has been revealed that the keratinizing SCC, unlike the non-keratinizing counterpart presents with pleomorphic, highly keratinized squamous cells. These cells vary greatly in size and shape and tend to occur singly with few small groups or syncytial arrangements. Squamous cells with bizarre shapes, such as spindle, strap, fibre, round, caudate tadpole, etc., present a characteristic picture on the routine Papanicolaou’s stain test. The changes can be confidently identified as true pleomorphism and not due to smearing artifact. The exaggerated cell to cell variability both in cell size and more importantly, cell shape, distinguishes keratinizing SCC from keratinizing dysplastic lesions. Similar variability in the size and shape of the nuclei are also seen. Nuclei exhibit pycnotic and prominent hyperchromasias, so called "ink-dot black", and at times can appear "ghost like" because of the abundant keratinization. Another feature observed due to the abundant keratinization is keratinized blebs of cytoplasm extending from the body of the cell. This feature leads to the pleomorphic appearance.

Keratins play a major functional role in the integrity and mechanical stability of both the single epithelial cells and via cell to cell contacts of that of the epithelial tissues [23]. In humans, keratins are encoded in 54 genes and these genes are clustered on 22 chromosomes. The acidic type I keratin genes are clustered on chromosome 17 and the basic type II keratin genes are clustered on chromosome 12 [24-26]. The expression of specific keratin genes is regulated by the differentiation of epithelial cells within the stratifying squamous
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epithelium [27]. Mutations in most of these genes are now associated with specific tissue fragility disorders which may manifest both in skin and mucosa depending on the expression pattern [10].

The production of specific types of cytokeratins by different cell types as well as in individual cells of the oral epithelium reflects the degree of cellular differentiation and maturation [28, 29]. Keratins and certain keratin associated proteins are useful as markers of differentiation because their expression is both region and differentiation specific [30,31]. Certain basic keratins are minor constituents of the cytoskeleton that are expressed in the suprabasal cells of various stratified soft-keratinizing epithelia [32]. K76 is produced in the upper layers of the stratified epithelium of the hard palate and gingiva [33]. K78 is produced in the cells of the epithelial covering of the human tongue while K80 is produced in the suprabasal cells of the epithelium of the human tongue [34]. Kanojia et al. have reported that there was an increased level of K5/ K6a and aberrant expression of K8 in dysplastic papillomatous lesions and carcinoma tissues of lingual epithelium. K6 is a marker of cell proliferation and its over expression has been observed in hyperproliferative disorder [35]. K26 is exclusively specific to taste bud anlagen in developing oral mucosa [36]. K24 is sometimes expressed in the epithelium of tongue. Myoepithelial cells were found to express K17 [37].

There are around 30 families of keratin proteins divided into two groups namely acidic and basic which are arranged in pairs and different studies have suggested that this coexpression seems to be more pronounced in poorly differentiated squamous cell carcinomas. Stratified-epithelial keratins, in particular K5 and K6, are useful as general markers for squamous cell carcinomas in histologically uncertain, poorly differentiated, or metastatic tumor cases [38, 39]. K8/18 serve as markers for simple epithelial differentiation, K1/10 are markers for keratinized epithelium, and K4/13 can be used as markers for non-keratinized epithelium. K6/16 is considered as hyperproliferative marker which are expressed in sites of high epidermal keratinocyte turnover and in pathological hyperproliferative conditions affecting the skin [40]. In the oral cavity, there is increased expression of K1/10, K4/13 in well-differentiated oral squamous cell carcinomas. Less well-differentiated oral squamous cell carcinoma express neither of these keratin pairs to any significant extent but cells in the basal cell layer of oral epithelium do express K19 and K14 and reflects its proliferation potential, while cells in the suprabasal instead produce K1 and K10. When K19 is produced by suprabasal cells of the oral mucosa, this indicates alteration in cell behaviour and probable premalignant changes [41]. Moderated and poorly differentiated oral squamous cell carcinoma may express K8 and sometimes K7 and 18. Also K5 and K6 are considered as useful markers for squamous cell carcinomas in histologically uncertain, poorly differentiated or metastatic tumor cases [33, 38]. It has been reported that K8 is associated with transformation and increased malignant potential of oral squamous epithelial cells [34]. Recently, it has also been demonstrated that the expression of K8 and K18 is an independent prognostic marker in squamous cell carcinomas of the oral cavity and indicates a decreased overall and progression-free survival [42].

V. Conclusion

Different studies suggest that conversion rate of precancer to cancer varies significantly and alteration predominantly begin after the dysplastic changes during oral carcinogenesis. Malignant transformation of the mucosal lining is a genetic process resulting from accumulation of multiple genetic alterations that dictates the frequency and pace of progression to invasive carcinoma. Site specific expression of keratins in oral mucosa is nothing but the ultimate result of genetic alteration in the respective site. Accumulation and pattern of keratin expression in oral squamous cells are essentially of the same kind in both normal and malignant cells. At the onset of keratinization, the cytoplasm usually increases in volume, relatively to the nucleus. Lobed and fragmented nuclei, as well as amitotic nuclear division, are probably indications of attempts at readjustment of the normal nuclear-cyttoplasmic relationship which lead to cytological pleomorphism. Pattern of keratinization in the pleomorphic oral squamous cells has a practical utility in the diagnosis and early detection during oral carcinogenesis. Although, the pattern of keratinization during oral carcinogenesis is less understood, the impact of various etiologic factors like tobacco and alcohol and their role in the process of keratinization needs further study.

Acknowledgements

Authors are thankful to the Head, P.G. Dept. of Zoology, Utkal University, Vani Vihar, Bhubaneshwar, Odisha for providing laboratory and library facilities; to the Director, Acharya Harihar Regional Cancer Center (AHRCC), Cuttack, Odisha for permitting us to collect samples from the oral cancer patients and also for providing library and laboratory facilities and one of us (AM) is grateful to the University Grants Commission (UGC), New Delhi for awarding UGC Meritorious Research Fellowship to carry out the research work.

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