Is oral squamous cell carcinoma unique in terms of intra- and inter-tumoral heterogeneity?

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Abstract
Tumoral heterogeneity has become one of the important issues in cancer research and carries outmost weightage in designing personalized cancer medicine. Oral squamous cell carcinoma (OSCC) is present in unique microenvironment of oral cavity attributed to site-specific diversified tissue compositions, microbial flora, diversified carcinogenic attacks, potentially malignant disorders, epithelial turnover rate, saliva, and closeness to external environment. Such microenvironment has potential to modulate the pathogenesis of many oral pathologies including OSCC. We believe that such factors and microenvironment are unique for OSCC and cannot be found in other carcinomas of the body. Methodologies such as multiregional sequencing, single-cell genomics, genomic landscape of bulk tumor sequencing, and so on, will help in understanding OSCC uniqueness in terms of heterogeneity. If explored, it will enable us to know the attributes of heterogeneity and their magnitude in OSCC, which will benefit in designing predictable biomarker and effective therapeutic strategy.

Keywords
Oral squamous cell carcinoma, oral cancer, heterogeneity, carcinogenesis

Introduction
Tumoral heterogeneity has become one of the important issues in cancer research and carries outmost weightage in designing personalized cancer medicine. Heterogeneity is reflected at various levels such as cellular morphology, gene expression, metabolism, motility, angiogenesis, proliferation, and immunology. Both heritable and nonheritable causes have been proposed as a source for intra-tumor as well as inter-tumoral heterogeneity.¹ Research into understanding and characterizing heterogeneity can allow for a better understanding of the causes and progression of the disease. In turn, this has a potential to guide the creation of more refined treatment strategies that incorporate knowledge of heterogeneity to yield higher efficacy.²

Oral squamous cell carcinoma (OSCC) is the most common malignancy of the oral cavity, which arises from lining epithelium. OSCC is present in a unique microenvironment attributed to the site-specific diversified tissue compositions, microbial flora, site-specific variations in epithelial turnover rate, saliva, and closeness to external environment. Such microenvironment has potential to modulate the pathogenesis of many oral pathologies including OSCC. Hence, it would be interesting to consider the effect of this unique microenvironment on disease progression of OSCC. Since microenvironment has potential to shape genetic expressions and malignant behavior,³ we have

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deliberated some of the aspects that are rather unique for OSCC in terms of heterogeneity and might impact the future development of targeted therapy.

Hypothesis

The microenvironment is a known cause for nonheritable heterogeneity in tumor cells. Oral cavity harbors unique microenvironment, which is attributed to site-specific diversified tissue composition, oral microflora, and direct communication to external environment. Moreover, OSCC is usually preceded by a myriad of oral potentially malignant disorders (OPMDs) with each one having different pathogeneses. These factors along with site-specific variation in epithelial turnover rate and diversified and repeated carcinogenic attacks on tumor cells contribute to the inter- and/or intra-tumoral heterogeneity in OSCC (Figure 1). Thus, it is hypothesized that OSCC has high degree of tumor heterogeneity than any other carcinomas of the body, which will impact the development of targeted therapy.

The discussions on the unique factors that can influence heterogeneity in OSCC are as follows:

**Tissue compositions**

Interactions of tumor cells with the microenvironment shape malignant behavior and promote tumor progression. The microenvironment within a tumor is not completely homogeneous and different regions of a tumor can have different densities of blood and lymphatic vasculature, different numbers and types of infiltrating normal cells, and different compositions of extracellular matrix. Therefore, tumor cells within a given tumor mass are expected to experience a range of microenvironmental signals, which would in turn translate into a range of phenotypic manifestations. Recent reports have highlighted the significance of the contribution of stromal gene expression and morphological structure as powerful prognostic determinants for a number of tumor types, emphasizing the importance of the tumor microenvironment in disease-related outcomes. Natrajan et al. measured microenvironmental heterogeneity with genomic alterations to predict breast cancer clinical outcome. They proposed a clinically relevant role of microenvironmental heterogeneity for advanced breast tumors and highlighted that ecological statistics can be translated into medical advances for identifying a new type of biomarker and, furthermore, for understanding the synergistic interplay of microenvironmental heterogeneity with genomic alterations in the cancer cells.

The oral cavity includes lips, gingivae, retromolar trigone, hard palate, soft palate, buccal mucosa, dorsal surface of tongue, ventral surface of tongue, and floor of the mouth. Each site is characterized by unique set of mucosal and submucosal tissue composition. For example, gingiva and hard palate are characterized by dense collagenous stroma attached to the periosteum of bone; soft palate and posterior part of the hard palate are characterized by the presence of minor salivary glands surrounded by dense stroma; buccal mucosa comprises adipose tissue, muscles, and minor salivary glands; tongue contains primarily muscle tissues; and so on. Hence, existence of site-specific inter-tumoral heterogeneity is quite conceivable in
OSCC. In this regard, Sathyan et al.\textsuperscript{11} studied buccal mucosal and tongue OSCC for possible heterogeneity in the cell cycle regulatory mechanism by comparing major cell cycle regulatory proteins. On comparison, tongue carcinoma showed significant down expression of p16 and p21. In combined analysis, simultaneous downregulation of p16 and p21 was seen in 47% of tongue cancer cases as against 28% in buccal carcinoma ($p = 0.004$). It was concluded that tongue and buccal mucosa cancers represent divergent biological subentities with prognostic and therapeutic significance. Similarly, Kannan et al.\textsuperscript{12} compared telomerase expression in OSCC of tongue and buccal mucosa. The majority (13 of 16 i.e. 81%) of tongue cancers exhibited weak or negative telomerase activity, irrespective of stage. Conversely, in OSCC of buccal mucosa, the majority (27 of 31) of tumors expressed various levels of telomerase activity ($\chi^2 = 4.24; p < 0.05$).

Local spread of tumor often leads to invasion into the surrounding areas carrying different sets of tissue composition, for example, gingival OSCC involving vestibule and buccal mucosa, alveolar OSCC invading into the floor of mouth or mucobuccal fold, and tongue OSCC spreading into the floor of mouth, and so on. Such scenario is particularly true in T3 and T4 stage of OSCC (personal observations). This variation in tissue composition (microenvironment), which is quite exclusive for the oral cavity, could be contributing to the heterogeneity in tumor cells.

**Oral microbial flora**

Increased growth of microbial flora of the oral cavity is considered as a known risk factor of OSCC development.\textsuperscript{13} Many bacterial species have been found to interfere directly with cellular signaling which acts as tumor promoters. Formation of carcinogenic N-nitroso compounds by bacteria in situ is one possible mechanism that may play an etiological role in various cancers of the body.\textsuperscript{14–16} A number of studies have now unequivocally revealed that bacterially mediated formation of nitrosamines can occur in vitro and in vivo, both in animal models and in human cancer cases.\textsuperscript{17,18} Recently, oral cavity has been viewed as extragastric reservoir of Helicobacter pylori, a microaerophilic gram-negative spiral organism associated with various malignancies including OSCC.\textsuperscript{19,20} Tateda et al.\textsuperscript{21} found Streptococcus anginosus in the gingival samples and reported its strong association with carcinogenesis of OSCC. The role of the viruses (especially human papilloma virus 16 and 18) in carcinogenesis has also been well documented. Candida species found in the oral cavity are known to secret nitrosamines which act as carcinogens. Interestingly, oral cavity shows a vast inter-individual diversity in terms of microbial colonization.\textsuperscript{22} This microenvironmental variability, attributed to microbial flora, has potential to modulate tumor cell genotype as well as phenotype leading to heterogeneity in tumor cells, a feature that might be quite exceptional for OSCC and not hold true for other cancers of the body.

Majority of the cases of OSCC are associated with secondary infection of the tumor site derived from microorganisms of the oral microbial flora (personal observation), which can evoke “inflammation-associated” mutational changes in already mutated cells, thus fueling already existing heterogeneity and cancer drug resistance.

**Diversified carcinogenic attack**

One of the unique features of OSCC is its association with varied nature of carcinogenic attack derived from smokeless tobacco, smoked tobacco, betel quid, alcohol, chronic trauma, and so on.\textsuperscript{23} All the factors alone and in various permutations and combinations produce wide-ranging carcinogenic attacks leading to molecular alteration, which could add to the already existing inter-tumoral heterogeneity.

**Continued carcinogenic attack**

Majority of the patients are diagnosed at late stages due to diagnostic delay on patient’s part.\textsuperscript{24} Persistent habit with OSCC in the oral cavity often causes repeated carcinogenic attacks on already transformed tumor cells leading to further mutations in the already mutated cells. This could be one of the sources for intra-tumoral heterogeneity, which is quite unique for oral cancer.

**Association with potentially malignant disorders**

OSCC is one of the tumors that may be preceded by a vast number of OPMDs. This vastness can be very well appreciated in our recently proposed pathogenesis-based classification of OPMDs.\textsuperscript{4} Each OPMD carries different sets of microenvironment which is carried forward during malignant transformation. For example, oral submucous fibrosis is characterized by dense fibrosis of the connective tissue, which is attributed to the presence of myofibroblasts.\textsuperscript{25} OSCC associated with oral submucous fibrosis is one of the most common malignancies in South and Southeast Asian countries.\textsuperscript{26,27} In this regard, Chourasia et al.\textsuperscript{28} reported 25.77% of OSCC cases associated with oral submucous fibrosis. Chaturvedi et al.\textsuperscript{29} in their study recognized that most of these patients are younger males with better prognostic factors such as better grade of tumor differentiation, lesser incidence of nodal metastasis and extra-capillary spread and better prognosis. Thus, it makes oral submucous fibrosis associated OSCC a unique type of oral cancer. Similarly, each OPMD can create its own unique microenvironment, which will have potential to modulate the tumor phenotype as well as clinicopathological behavior. And thus OSCC in this regard can be considered unique as compared to other carcinomas of the body.

**Pro-tumorigenic cytokines**

Tumor necrosis factor-alpha and interleukin-6 are known for their tumor-promoting and mutagenic nature. Factors such as unique microenvironment, poor oral hygiene, presence of microflora, OPMDs, tumor-associated inflammation, and advanced age, which are unique for OSCC, are known for
elevating tumor-promoting cytokines. The additive effect of all these factors in elevating pro-tumoral cytokines could make OSCC unique in terms of microenvironment diversity and hence the heterogeneity.

**Diversity in epithelial turnover rate**

Time required to replace all the cells in the epithelium is known as turnover time. It is obtained by estimating the time taken by a cell to divide and pass through the entire epithelium. Intriguingly, oral mucosa shows site-specific variation in epithelial turnover rate. The turnover time has been estimated as 41–57 days in the gingiva, 24 days in hard palate, 20 days in floor of mouth, 14 days in buccal mucosa, and 5–6 days in junctional epithelium. A variation in the expression of epidermal growth factor, keratinocyte growth factor, interleukin-1, and transforming growth factors \( \alpha \) and \( \beta \) is attributed to the variation in the site-specific epithelial turnover rate. Interestingly, these factors are overexpressed during malignant transformation. This site-specific inherent genetic makeup related to epithelial turnover rate could be carried forward during malignant transformation and thus could be a possible cause for intra and inter-tumoral heterogeneity.

**Tumoral heterogeneity measurement techniques: Recent advances**

While the problem of identifying and characterizing tumor heterogeneity is still under active research, some effective strategies have been proposed, including both experimental and computational solutions. The most widely used method in the literature for tumor heterogeneity is multiregional sequencing. However, recently, single-cell genomics is emerging as the most reliable technology for measurement of tumor heterogeneity and has an advantage of direct sequencing of each clone. Realizing the high cost for this technology, researchers have developed computational methods wherein bulk-tumor sequencing data are used to aggregate metadata of each clone’s genomic information. Usually, daughter cell carries exactly the same parental genomic information. However, there are always DNA replication system malfunctions, mutational signatures, copy number alterations, and loss of heterozygosity, which remain from generation to generation. This enables the back tracking of genomic signature and thus investigating subclones for tumor heterogeneity from genomic landscape of bulk tumor sequencing is a widely used strategy.

Computational methods, such as PyClone and EXPANDS, are current highly sophisticated tools that use mutational information to infer subclonal populations.

**Evaluation of hypothesis**

As different locations of the oral cavity show variations in tissue composition, tumor cells of OSCC are expected to experience site-specific variations in microenvironment and thus heterogeneity in population. Hence, while conducting any molecular studies in oral cancer in future, it is important to consider individual site as a separate entity. Future genomic and proteomic studies on molecules of proliferation, invasion, migration, angiogenesis, cell adhesion, and so on, in OSCC of different sites of oral cavity will help in better understanding the inter-tumoral heterogeneity.

Using polymerase chain reaction and cloning strategies that target 16S rRNA genes, it is feasible to define the bacterial composition and diversity of any given environment. Isolated DNA is amplified by polymerase chain reaction using universally conserved primers for 16S rRNA genes. The 16S rRNA amplicons are cloned into *Escherichia coli*, and the cloned 16S rRNA inserts are sequenced to establish the species identity or closest relative. It would be interesting to compare oral microbial flora composition (both qualitative and quantitative) with the molecular characteristics of OSCC. This will help us in unveiling the correlation between particular microbial combinations with molecular alteration patterns in the tumor cells.

Looking at the role of pro-tumoral cytokines in the development and progression of OSCC, we recommend correlative studies on cytokine profiling and molecular characterization of tumor cells. This will help us in elucidating the role of cytokines in heterogeneity in tumor cells, which has not yet investigated in any malignancy.

**Implications**

All the parameters discussed in this article will contribute to the heterogeneity in OSCC. Enhanced heterogeneity could signify poor prognosis, as all tumor cells might not respond to the chemotherapy or targeted therapy. Heterogeneous tumors may exhibit different sensitivities to cytotoxic drugs among different clonal populations. This is attributed to the clonal interactions that may inhibit or alter therapeutic efficacy, posing a challenge to successful therapies in heterogeneous tumors (and their heterogenic metastases). The viewpoints discussed in the article will help researchers in exploring and unrevealing heterogenetic uniqueness of OSCC, which would impact the future targeted drug development.

Due to the genetic differences within and between the tumors, biomarkers that may predict treatment response or prognosis may not be widely applicable. However, it has been suggested that the level of heterogeneity can itself be used as a biomarker since heterogeneous tumors may be more likely to contain treatment-resistant subclones. In this regard, the hypothesis presented in this article, if investigated, will enable us to know the attributes of heterogeneity and their magnitude in OSCC. This will help in designing predictable biomarkers and effective therapeutic strategies.

**Declaration of Conflicting Interests**

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**Translational Value**

The viewpoints discussed in the article will help researcher in exploring and unraveling intra- and inter-tumoral heterogenetic uniqueness of OSCC, which would impact the future targeted drug development. If investigated, it will enable to know the attributes of heterogeneity and their magnitude in OSCC that will help in designing predictable biomarkers.