BCL-X Expression in Oral Cancer: Comparison between Oral Squamous Cell Carcinoma and Verrucous Carcinoma

Abstract
Background: Verrucous carcinoma (VC) should be considered a distinct clinicopathologic entity different from the more common oral squamous cell carcinoma (OSCC) because of its unique biological behavior. Best way to understand the behavior of these carcinomas is to study them by means of molecular methods, especially in tumor progression tests and Bcl-X is an important antiapoptotic member of the Bcl-2 family and is one of the newest and most useful markers to determine the aggressiveness of many carcinomas. The relationship between this Bcl-X protein and carcinomatous behavior toward it is not studied extensively, which we attempted to evaluate using immunohistochemical analysis in selected carcinomas of the head and neck region. Method: We studied Bcl-X protein expression in sections of thirty OSCC and ten VC samples and correlated this with tumor differentiation. Results: There was a significant difference in cytoplasmic staining of Bcl-X expression with statistical analysis (P < 0.005) for VC and OSCC when compared as a group. No significance was seen among the different histological grades of OSCC and when compared with VC individually. Conclusion: The significant result between OSCC and VC suggests that their biologic course is comparable and can be helpful in differentiating them with each other for establishment of a better treatment protocol.

Keywords: Bcl-2, Bcl-X, oral squamous cell carcinoma, verrucous carcinoma

Introduction
Verrucous carcinoma (VC) was first defined by Ackerman in 1948, as a diagnostically problematic squamous cell neoplasia involving lip, oropharyngeal, and laryngeal mucosa. As a result, this neoplasm was named as “Ackerman’s tumor.” It is considered a slowly growing neoplasm that can reach a significant size before being brought to medical attention.[1,2]

The clinico-histopathological diagnosis of VC is often exclusionary and extremely difficult in doubtful cases. Distinction from classical squamous cell carcinoma (SCC) is a frequent problem also for the clinicians because of the extensive nature of the lesion mimicking invasive cancer.[3] It shows distinct clinical and histological features and whose molecular alterations have not yet been extensively studied. An important help could be offered by molecular approaches such as immunohistochemical studies determining the factors responsible for this epithelial proliferation that can help in investigating the differences between biological behaviors of these carcinomas.[4-6]

Current studies of tumor biology suggest several basic mechanisms that may be used by neoplastic cells to provide a growth advantage over normal tissue. Defects in the control of apoptosis causing either the survival of unwanted cells or inappropriate killing of vital cells underlie a multitude of disorders and carcinomas.[7] Although such programmed cell deaths were described many decades ago, the significance of apoptosis had been largely overlooked, particularly its relevance to development and growth of a disease process. Certainly, defects in apoptosis are now considered to be a hallmark of most, if not all, carcinomas. It is believed that neoplastic cells show a normal level of cell division and an increased expression of antiapoptotic proteins which is responsible for their extensive growth and development.[8]

An imbalance among the Bcl-2 family members could induce dysregulation of apoptosis, which would contribute to oncogenesis and one such specific
marker to identify the antiapoptosis activity and tumor aggressiveness by immunohistochemistry (IHC) is Bcl-X, a 20 kDa protein, newly discovered antiapoptotic member of Bcl-2 family whose elevated expression is associated with the neoplastic process.[9]

Very few studies exist in the literature correlating the role of antiapoptosis in oral SCCs (OSCCs) and VC, and this is the first study that demonstrates the correlation of the newly discovered antiapoptotic protein Bcl-X, in OSCC, and VC.

The aim of the present study is to assess the expression of Bcl-X in OSCC and VC, to compare its expression in both of these carcinomas, and to investigate the possible correlation of Bcl-X expression in different histological grades of OSCC.

Materials and Methods
The samples for this study involved the use of formalin-fixed, paraffin-embedded tissues of histopathologically diagnosed thirty cases of OSCC and ten cases of VC retrieved from the Department of Oral Pathology and Microbiology, Yenepoya Dental College, Mangalore, India. These ten cases of VC and thirty cases of OSCC which included the different grades based on Broader’s classification, i.e., well-differentiated (10 cases), moderately differentiated (10 cases), and poorly differentiated carcinoma (10 cases), were confirmed and taken for IHC evaluation.

Immunohistochemistry
For IHC detection of Bcl-X, two to three serial sections of 4 μm thickness were cut and mounted on poly-L-lysine-coated slides and were dried for 24 h at 37°C. Then, the sections were deparaffinized and rehydrated in xylene and descending grades of alcohol, respectively. Antigen heat retrieval was done by keeping the slides in a pressure cooker filled with boiling trisodium citrate buffer (pH 6.0) for 20 min. Peroxidase block is applied for 10 min. It is then washed with Tris buffer twice for 5 min each. Monoclonal anti-Bcl-X antibody (Ready-to-use vial, BioGenex) was used. The Super Sensitive™ Polymer HRP IHC detection system (BioGenex Life Sciences Pvt. Ltd.,) was used for application of the biotinylated link antibody and peroxidase-labeled streptavidin, according to the manufacturer’s instructions for the procedure. Visualization was performed using freshly prepared 3,3’-diaminobenzidine tetrahydrochloride chromogen for 10 min. The slides were then counterstained with Mayer’s hematoxylin stain. For each batch of staining, a negative control, where the primary antibody was replaced by Tris buffer saline, and a positive control of normal tonsil tissue was used. Cytoplasmic staining was considered positive for Bcl-X staining. The positive cases were further analyzed for the intensity of staining, which were graded on a scale of 0–3, where 0 indicated negative staining, 1 indicated mild staining, 2 indicated moderate staining, and 3 indicated intense staining. Two other observers carried out all observations to eliminate the interobserver bias.

Results
Bcl-X expression was detected in all the four groups and intensity of the staining varied from weak to strong in the studied sections. IHC results of the qualitative analysis for Bcl-X expression in studied groups are summarized in Table 1, and microscopic findings of them have been shown in Figure 1a-d.

Bcl-X positivity was seen in 36 cases (90%) of total 40 cases studied. Seven cases of ten cases of VC were immunopositive for Bcl-X (70%) and 29 cases of 30 cases of OSCC were immunopositive for Bcl-X (96%). Among the various grades of OSCC, well-differentiated carcinoma (10 cases; Figure 1), moderately differentiated carcinoma (10 cases; Figure 2), and poorly differentiated carcinoma (9 cases) were positive and OVC (7 cases) was positive. The intensity of staining was graded as seen in Table 1.

Statistical analysis
In this study, statistical analysis was performed by Chi-square test using SPSS software version 10.05 (SPSS Inc. Chicago, Illinois, USA). P < 0.05 was considered statistically significant. The results of our study showed statistical significance at P < 0.05 for Bcl-X expression when compared between OSCC and VC as a group, with a positive correlation having correlation coefficient r = 0.632 [Figure 2], but there were no significant differences seen in VC when compared with different histological grades

Table 1: Statistical analysis in various groups of carcinomas

| Group       | Total cases | Mild | Moderate | Intense | Negative | P      |
|-------------|-------------|------|----------|---------|----------|--------|
| OSCC        | 30          | 7    | 6        | 16      | 1        | 0.048  |
| VC          | 10          | 3    | 0        | 4       | 3        | 0.198  |
| WDSCC       | 10          | 2    | 2        | 6       | 0        | 0.398  |
| MDSCC       | 10          | 5    | 2        | 3       | 0        | 0.055  |
| PDSCC       | 10          | 0    | 2        | 7       | 1        | 0.133  |
| VC          | 10          | 3    | 0        | 4       | 3        | 0.130  |
| WDSCC       | 10          | 2    | 2        | 6       | 0        | 0.055  |
| MDSCC       | 10          | 5    | 2        | 3       | 0        | 0.078  |
| PDSCC       | 10          | 0    | 2        | 7       | 1        | 0.133  |

OSCC=Oral squamous cell carcinoma, VC=Verrucous carcinoma, WDSCC=Well-differentiated squamous cell carcinoma, MDSCC=Moderately differentiated squamous cell carcinoma, PDSCC=Poorly differentiated squamous cell carcinoma, NS=Not significant, S=Significant
of OSCC. The different grades of OSCC were also compared with each other but did not show any significant results except for comparison of Bcl-X staining intensity between moderately differentiated and poorly differentiated OSCC which almost showed statistical significance of $P = 0.055$ [Table 1].

**Discussion**

VC should be considered a distinct entity from OSCC because of its innocuous appearance and unique biologic behavior. It is considered to be a low-grade well-differentiated variant of SCC characterized by silent clinical and pathological features that sometimes renders diagnostic dilemmas for the clinicians as well as for the pathologists in doubtful cases. Adequately optimized and validated immunohistochemical examination is highly specific and considered as a reliable diagnostic tool to differentiate between similar lesions. The correct diagnosis of these carcinomas is necessary for significant influence on the prognosis prediction and selection of the best prevalent treatment protocol.

The basis of the carcinoma formation lies in the fact that there is activation of growth promoting oncogenes, inactivation of tumor suppressor genes, and alterations in the genes that regulate apoptosis, leading to unregulated cell proliferation and decreased apoptosis. Mutations of any of the genes encoding antiapoptotic proteins or any changes in the levels of their expression can lead to increased cell survival, and perhaps the best studied regulatory proteins are the Bcl-2 family of molecules which includes the newly discovered Bcl-X protein.

Dysregulated expression of Bcl-X oncoprotein in oral preneoplastic and neoplastic lesions results in a resistant mechanism against apoptotic stimuli induced by chemotherapeutic agents characterizing such ones as highly aggressive ones, generally exhibiting a poor prognosis, and suggesting its possible role in the progression of oral cancer.

In addition, initial studies revealed that cells expressing Bcl-X demonstrated evidence for genomic instability and abnormal expression of cell cycle and apoptosis-associated genes can result in gene products that promote clonal expansion leading to neoplasias, facilitate invasion and spread, maintain tumor microenvironment, and promote cancer growth.

Finally, some insight into the controversy regarding the role and the effect of radiation therapy on VC is crucial. It is suggested that surgical resection remains the choice of treatment for this neoplasm. Neck dissection is not indicated for any pure VC and given the absence of nodal metastases; cervical adenopathy may be associated with VC, representing reactive changes and not metastatic disease. The literature supports the concept that radiotherapy is contraindicated in the treatment of VC for the occurrence of radiation-induced anaplastic transformation, manifesting 2–8 months following the therapeutic cycle. Therefore, there is a need of the hour that VC should be correctly diagnosed and differentiated from the classical OSCC as it influences the treatment protocol to a greater extent.

The present study utilized the Bcl-X monoclonal antibody on OSCC and VC to illuminate the role of this antiapoptotic protein in both of these carcinomas, and the expression was correlated between the two entities and in different histologic grades of OSCC.

In our study, 29 cases (96%) of 30 cases of OSCC were positive for Bcl-X protein which was in high accordance with Pena et al., who studied OSCC cases, demonstrated enhanced expression of Bcl-2 and Bcl-X compared to surrounding normal epithelium suggesting disruption of apoptotic control pathways as an important event in the evolution of SCC.

In this study, the intensity of Bcl-X positive cells was more in the poorly differentiated SCC [Figure 1d] than in well-differentiated SCC [Figure 1b]. An increase of Bcl-2
expression in poorly differentiated SCC was also seen by Yu Chen et al. and Muzio et al. A proportional increase in the intensity with increase in the cytological grade was observed. This observation of an increased expression of Bcl-2 positive cells with a decrease in the differentiation is similar for Bcl-X, as it belongs to the same protein family and is expected to behave in the similar manner. It probably reflects that Bcl-2 and Bcl-X are expressed more in keratinocytes that have an increased capacity for survival.\[13\]

In a previous study, de Vicente et al.\[16\] showed immunoreactivity of Bcl-2 in 35 OSCC cases mainly in peripheral cells of epithelial tumor islands with decreasing expression toward the center of the neoplastic nests and associated with aggressive disease, neck lymph node metastasis, and poor prognosis which was in high agreement with the present study as the expression of Bcl-X was strongly seen in the peripheral areas of the tumor islands of 29 cases of OSCC whereas there was a decrease in the intensity of staining toward the center of the tumor islands [Figure 1b-d].

VC includes one of the diagnostic challenges to pathologists. The lack of objective criteria and nonspecific histopathologic features for using in prepared tissue sections from biopsies, particularly very small biopsy specimens or poorly orientated specimens, can cause difficulty in correct histopathologic diagnosis of noninvasive VC from invasive well-differentiated OSCC which has a very important influence in the treatment planning.\[17,18\]

A study of twenty cases of VC and 23 cases of SCC by Saghafi et al. showed immunoreactivity for Bcl-2 in the cytoplasm of cells with a significant difference in the mean of Bcl-2 expression between the lesions.\[19\] Our study included thirty cases of OSCC with 96% of positivity and ten cases of VC, of which seven cases (70%) were positive for Bcl-X expression with a statistical significance of \(P < 0.05\). VC had expression significantly lower than OSCC and can be helpful in differentiating VC and OSCC which was also reflected from the present study.

According to a study by Angadi and Krishnapillai, the pattern of expression of cyclin D1 in VC was predominantly observable in the pushing margins of VC, probably reflecting the maximum proliferative activity of this tumor in these areas. The staining tends to diminish in the superficial areas, which could represent the more differentiated part of the tumor where the cells have stopped proliferation.\[20\] To our surprise, this unique and similar pattern of expression was detected for Bcl-X protein in the present study with positive expression in the whole thickness of the epithelium of OSCC, whereas it was more seen in the pushing margins in case of VC [Figure 1a] and individual cell cytoplasm staining for Bcl-X protein was more for OSCC than VC.

The immunohistochemical results of Bcl-X protein suggest the biologic course of VC is comparable to SCC in the oral cavity and can be considered in differentiating them. The different levels and patterns of gene expression and cell turnover between OSCC and VC undoubtedly correlate with the different biology and prognosis of these lesions.

No study exists on VC and OSCC where the staining intensity of Bcl-X has been compared and considered as a parameter for the differentiation of these carcinomas with each other at the molecular level and we hypothesize from our findings that Bcl-X can be proved to be an important antiapoptotic immunohistochemical marker which can be helpful in differentiating between VC and OSCC, so our results are not comparable with any other studies.

Conclusion

The overexpression of Bcl-X protein correlated with OSCC and VC can be useful in differentiating classical SCCs from the more silent and extensive VCs of the oral cavity and assessing their unique biologic behavior. It can be beneficial in identifying the common pitfalls during the routine assessment of surgical or biopsied tissue with the pathologically underdiagnosed cases or the clinically and surgically overtreated cases of VC treated as a conventional invasive OSCC, with redundant surgical resection and needless lymphadenectomy.

However, further comparative studies on larger samples and molecular mechanisms with other panel of antiapoptotic markers are needed to confirm our study results and suggested reasons.

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Conflicts of interest

There are no conflicts of interest.

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