Immunohistochemical Expression of Polo-like Kinase 1 in Oral Squamous Cell Carcinoma and Oral Submucous Fibrosis

Abstract
Context: Polo-like kinase 1 (PLK1) is a critical molecule in the proliferation of several human cancers. Overexpression of PLK1 has been correlated with cancer cell proliferation and lower overall survival rates. Although PLK1 has been studied in various tumors, information regarding its expression in oral cancer and precancer is limited. Aims: This study is aimed at evaluating the expression of PLK1 in a potentially malignant and malignant disorder of the oral cavity, namely, oral submucous fibrosis (OSMF) and oral squamous cell carcinoma (OSCC), respectively, using the immunohistochemistry technique. It also intended to evaluate the association of the various histological grades of OSCC with the intensity of PLK1 expression.

Subjects and Methods: Thirty OSMF, thirty OSCC tissues, and thirty control tissues were obtained, and the expression of PLK1 was detected by immunohistochemistry using rabbit anti-human PLK1 polyclonal antibodies (Abcam Ab47867). The association between staining intensity and histological grade of OSCC was evaluated. Statistical Analysis Used: Using SPSS 20 version, a test for proportions, nonparametric Chi-square/correlation analysis was used to compare differences in proportions of categorical variables of interest between groups. Results: PLK1 was positively expressed in 27 (90%) OSCC tissues. OSMF showed no detectable staining in 27 (90%) tissues and positive staining in 3 (10%) tissues. OSCC showed no staining (0%) in normal tissues. Statistically significant associations were not found between staining intensity and histological grade of OSCC. Conclusions: PLK1 could be a promising progression marker for OSCC. Therapeutically, targeting PLK1 may be a new approach to fight oral cancer.

Keywords: Biomarkers, cell signaling, gene expression, histochemistry

Introduction
Oral cancer is the sixth most common cancer worldwide.[1] Ninety percent of oral cancers are oral squamous cell carcinomas (OSCC).[2] Oral submucous fibrosis (OSMF) is a chronic, insidious oral mucosal condition with malignant transformation rate reported to be around 7.6% over a 10-year period.[3] The expanding field of molecular biology has revealed newer and more specific markers that would help determine the degree of cell alteration and malignant transformation. The pathogenesis of OSCC is a multistage process involving molecular and histological changes.[4,5] Novel oncogenes such as polo-like kinase 1 (PLK1) have oncogenic properties that are believed to be due to its role in dividing cell cycle progressions.[4] The overexpression of PLKs is seen in human tumors but not in healthy, nondividing cells.[2] High levels of PLK transcripts are expressed in about 80% of human tumors of various origins. Overexpression of PLK results in poor prognosis in several tumor types and lower overall survival rate.[2] Furthermore, PLK1 inhibitors have recently been developed and are being tested as potential anticancer agents.[6]

PLK1 expression in oral cancer though previously studied, information regarding the same is limited. Oral precancerous conditions such as OSMF are largely prevalent in the Indian subcontinent, due to betel quid chewing. Hence, we proposed a study to assess the expression of PLK1 in OSCC and OSMF by immunohistochemistry and also the association between the histological grade of OSCC and staining intensity.

Subjects and Methods
Patient and tissue samples
Thirty OSCC tissue samples, thirty OSMF samples, and thirty matched adjacent...
normal tissue samples from clinically diagnosed patients were obtained by incisional biopsy and histopathologically confirmed. The study was independently reviewed and approved by the Ethics Committee for Students Proposals (REF: CSP/13/JUN/29/129). Oral cancer patients suffering from infectious contagious disease, patients unable to undergo minor surgical procedures due to systemic health conditions, and other precancerous lesions and conditions were excluded from the study. The biopsy specimen was collected depending on the location of the lesion in precancer and cancer. The tissue samples were grouped as C1, C2, and C3.

Group C1 represents the control group. Patients reporting for minor oral surgical procedures such as preprosthetic surgery were taken as control, and the tissue samples were histologically confirmed.

Group C2 represents the OSMF group. In OSMF, sites with tough, leathery texture of the mucosa, and blanching of mucosa were selected.

Group C3 represents the OSCC group. In OSCC, sites with red or white, painless, nonhealing, and indurated ulcers were selected.

All the samples were formalin-fixed, paraffin-embedded, labeled appropriately, and submitted for immunohistochemical analysis, to assess the overexpression of PLK1.

Following the WHO classification (1997), the OSCC histopathological sections were analyzed by a pathologist and graded as 15 well-differentiated, 14 moderately differentiated, and 1 poorly differentiated.

Immunohistochemistry

Immunohistochemistry was performed using labeled 3-µm thick sections of formalin-fixed and paraffin-blocked samples that were mounted on glass slides, deparaffinized, and immersed in methanol with 3% hydrogen peroxidase for 5 min to eliminate endogenous peroxidase activity. For antigen retrieval, sections used for PLK1 protein immunostaining were microwaved in ethylenediaminetetraacetic acid (pH 8.0) and 96°C (heat 2 cycles of 12 min = 24 min) microwave method (BioGenex EZ Retriever system). Next, the sections were incubated for 8 h (overnight) with rabbit antihuman PLK1 polyclonal antibody (Abcam Ab47867), diluted 1:50-fold in Tris-buffered saline (pH 7.6) with 1% bovine serum albumin at room temperature. All slides were incubated in sequence with secondary antibody for 30 min (BioGenex automated supersensitive antibodies in the processor). The reaction products were visualized by immersing the sections for 3–10 min in 0.03% diaminobenzidine solutions containing 2 mM hydrogen peroxide. Finally, the sections were counterstained with Mayer’s hematoxylin and examined under a light microscope.

Evaluation of immunohistochemistry

Staining intensity was interpreted independently by two pathologists. For interpretation, guidelines were followed and placenta control tissue was standardized for the PLK1 expression of Ab47867 antibodies. The field chosen was the area of tissue that showed the maximum intensity of staining. The cytoplasmic and nuclear positively stained cells in the epithelium of the OSCC samples were identified. The OSCC samples were graded as negative (−ve), weak (1+) [Figure 1], intermediate (2+) [Figure 2], and strong (3+) [Figure 3]. The PLK1 cells were interpreted using a microscope at 10× and 20× magnification. The OSMF samples that expressed PLK1 was graded as PLK1 positive [Figure 4] and the ones that did not express PLK1 as negative [Figure 5].

Statistical analysis

Using Statistical Package for the social sciences version 20 (SPSS version 20, New York USA, used for statistical analysis in research) a test for proportions, nonparametric Chi-square/correlation analysis was used to compare differences in proportions of categorical variables of interest between groups. Statistical values of P < 0.05 were considered as significant.

Results

Expression of polo-like kinase 1 in oral cancer, oral submucous fibrosis, and adjacent normal tissues

In OSMF tissues, PLK1 stained negatively in almost all cases except three tissues that showed positive PLK1 staining. All the normal oral tissues stained negative for PLK1. Positive staining of PLK1 in OSCC tissues was significantly higher than either OSMF or normal oral tissues, i.e. 90% (27/30) in cancer tissues versus 10% (3/30) in OSMF and 0% (0/10) in normal oral tissues. In oral cancer tissues, among the 27 PLK1 positively stained OSCC tissues, 29.7% stained

Figure 1: Polo-like kinase 1 weak expression 1+ in oral squamous cell carcinoma tissue
cytoplasmically and 70.3% cytoplasmic with nuclear staining.

**Association between polo-like kinase 1 and histopathological grade of oral squamous cell carcinoma**

Statistically significant associations were not observed between PLK1 expression and histopathological grade of OSCC ($P = 0.443$).

**Discussion**

Prognosis of head and neck cancer patients is based mainly on clinicopathological and imaging parameters. In recent years, growing spectrums of investigations have focused attention on the impact of markers for cellular proliferation. The pathogenesis of OSCC involves enhanced function of several oncogenes and/or the deactivation of tumor suppressor genes, resulting in the loss of cell cycle checkpoints.$^9$ Mutations that convert proto-oncogenes to oncogenes typically increase the expression of the normal gene or increase the activity of the encoded protein.

In vitro and in vivo data on PLK1 expression reveal that PLK1 overexpression is involved in carcinogenesis.$^{10}$ PLK1, the best characterized member of serine/threonine protein kinases family, is a pivotal regulator of the cell cycle and involved in centrosome maturation, regulation of anaphase-promoting complex, and bipolar spindle formation.$^4$ PLK1 is detected at S phase, continues to increase at G2 phase, and reaches a peak during mitosis.$^{11}$

Because of the close correlation between the high level of PLK1 and cancer cell proliferation, PLK1 expression has prognostic value for predicting outcomes in patients with squamous cell carcinoma of head and neck cancer. PLK inhibitors have been observed to interfere with different stages of mitosis, such as centrosome maturation, spindle formation, chromosome separation, and cytokinesis. Therefore, they induce mitotic chaos and severely perturb cell cycle progression, eventually leading to cancer cell death.$^{12}$ Apoptotic pathway may be regulated by PLK1, and cell proliferation would be inhibited by PLK1 silencing.$^{13}$

From prophase to metaphase, PLK1
localizes to centrosomes and kinetochores and regulates different aspects of spindle assembly including bipolar spindle formation. Interfering with PLK1 function in human cells leads to a prominent prometaphase/metaphase-like arrest, which is dependent on the activation of the spindle assembly checkpoint. Overexpressed in various types of tumors, including oropharyngeal carcinoma, melanoma, squamous cell carcinomas of the head and neck, non small lung cancer, and ovarian and endometrial carcinomas, PLK1 has been proposed as novel marker for metastatic disease. Zhao et al. identified that the expression of PLK1 in esophageal squamous cell carcinoma tissue was high compared to normal adjacent tissue in 56 patients by performing western blotting and immunohistochemistry. Shi et al. observed PLK1 overexpression in human nasopharyngeal cancer, using immunohistochemistry, which in turn was associated with a higher likelihood of recurrence. PLK expression was found to be significantly higher in metastatic head and neck squamous cell carcinomas (HNSSCs) than nonmetastatic HNSSC on the basis of an observation period of 5-year post therapy. In the same study, it was demonstrated that determination of the PLK mRNA levels is of prognostic value for the patient population. Kim et al. demonstrated that the level of PLK1 was significantly higher in human OSCC compared with that of normal tissue by performing an immunohistochemistry assay for PLK1 in five OSCC tissues. PLK1 expression in the cytoplasmic and nuclear fractions were assessed and found to be higher in the nucleus compared to the cytoplasm, suggesting that PLK1 may play a major role in the former. PLK1 might also be a chemo- or radio-therapeutic target for cancer. The targeted inactivation of essential kinases is commonly by ATP-competitive small-molecule inhibitors that block their enzymatic activity. This indicates that PLK1 is an attractive kinase target for cancer drug development. Since it is overexpressed in many cancers, it can serve as a biomarker to monitor treatment efficacy of PLK1 inhibitors and is, therefore, an intriguing molecule both as a biomarker and a relevant target for highly specific cancer therapy.

The role and significance of PLK1 in cancer have been established as seen in the studies mentioned above. In the present study, the evaluation of PLK1 expression in OSMF and in the various histological grades of OSCC was carried out against control tissue samples, using immunohistochemistry. The results show that PLK1 is overexpressed in 90% (27/30) cases of OSCC. This finding is similar to the study results of Kim et al. and Knecht et al. However, in the present study, PLK1 was not positively expressed in any (0/30) of the normal tissues, hence the overexpression of PLK1 is seldom appreciated in normal healthy oral mucosa.

PLK1 was found to be overexpressed in 10% (3/30) OSMF tissue samples, in the cytoplasm of the epithelial cells. DNA damage occurs when atrophy, which is a classical histological feature of OSMF, progresses to dysplasia. This progression suggests a possible reason for the positive expression of PLK1 in the atrophic epithelium of OSMF as genetic and molecular changes precede dysplasia histopathologically.

Expression of PLK1 intensity in the different histological grades of OSCC and the association between the two was not found, suggesting that the overexpression of PLK1 does not seem to correlate with the histological grades of OSCC tissues. These results are harmonious with previous studies done, in which the levels of PLK1 expression did not correlate with tumor stage.

Kim et al. demonstrated that PLK1 expression was higher in the nucleus compared to the cytoplasm. However, in the present study of the expression of PLK1 in OSCC tissues, the expression was predominantly cytoplasmic.

Therefore, in the current study, the overexpression of PLK1 is strongly appreciated in OSCC. The lack of correlation between expression of PLK1 and various histological grades of OSCC suggests that PLK1 might be a crucial marker for cancer progression. However, its expression in OSMF needs to be studied further before any conclusive remarks are made.

**Conclusion**

As demonstrated, PLK1 is seen to be overexpressed in the OSCC tissues as compared to the OSMF and normal oral tissues. It could be a promising biomarker for oral cancer and a therapeutic target. The overexpression of this marker appears to be independent of the histologic grade of OSCC. This has to be further evaluated and will go a long way in in the use of this important biomarker in the follow-up and management of this dreaded malignancy. Its expression in OSMF needs to be studied further before establishing the role of PLK1 in precancerous conditions. PLK1 is definite to play a significant role in the future of oral oncology. It can be used as a routine immunohistochemical marker for OSCC.

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**Conflicts of interest**

There are no conflicts of interest.
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