Immunoexpression of programmed cell death 4 protein in normal oral mucosa, oral epithelial dysplasia and oral squamous cell carcinoma

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Abstract

**Background:** Oral squamous cell carcinoma (OSCC) is the frequently reported cancer of the head and neck. Recent studies are being conducted to evaluate the role of potential markers for diagnosing the stages of development of OSCC from normal cells.

**Aim:** The aim of this study is to evaluate and compare the immunoexpression of programmed cell death 4 (PDCD4) protein in normal oral mucosa, oral epithelial dysplasia (OED) and OSCC.

**Materials and Methods:** Histologically diagnosed, formalin-fixed paraffin-embedded archived cases ($n = 100$) of normal mucosa ($n = 10$), OED ($n = 60$) and OSCC ($n = 30$) were analyzed immunohistochemically in the present retrospective study using monoclonal rabbit antihuman PDCD4. OED and squamous cell carcinoma were graded according to the World Health Organization and Broder’s histological grading criteria, respectively. Clinical parameters and immunohistochemical results were analyzed by Fisher exact test using SPSS software. $P < 0.05$ was indicative of significant differences.

**Results:** PDCD4 expression was observed in the normal oral mucosa, OED and OSCC. The maximum expression was observed in the normal oral mucosa, which reduced significantly in OED and OSCC ($P = 0.017$). With the increase in the transformation from normal cells to cancer cells, a shift from nuclear to cytoplasmic staining was observed indicating predominant cytoplasmic localization of stain as a feature of altered cells.

**Conclusion:** The present study delineates the molecular difference between the normal, dysplastic and carcinomatous cells; and points toward the role of PDCD4 localization in the proliferation of cells. This study thus highlights the need for further research with inclusion of long follow-up period and other pathological criteria such as inflammation and microenvironment, immune status of patient and tumor stage, which could aid in the development of prospective diagnostic options.

**Keywords:** Oral epithelial dysplasia, oral squamous cell carcinoma, programmed cell death 4

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INTRODUCTION

Oral squamous cell carcinoma (OSCC) is among the multiple health disorders caused due to tobacco consumption. Its incidence increases with every passing year and is a frequently reported form of malignant cancer in the head and neck region worldwide. OSCC constitutes 3% of overall cancer types and approximately 40% of the total cancer count in India. As a result, management of OSCC, especially with the aid of surgery, has become the most preferred method. However, its timely detection is the most researched area of study due to the problem of recurrence after surgery.

Potentially malignant lesions such as leukoplakia and erythroplakia have often been observed to precede OSCC and reported to advance into oral carcinoma even though approximately 50% of cases of oral carcinomas are reported to arise from normal mucosa. In routine examination, dysplastic features are among the main factors of malignant potential. Several studies have stated that advancement in the degree of dysplasia increases the risk of malignancy in the patients. Due to great disparity among observers for grading the stages of dysplasia, there is a need of detecting unique biological markers to confirm the malignancy and predict the malignant potential.

Proteins such as programmed cell death 4 (PDCD4), a well-known suppressor of tumor genesis, has also been established as regulator of OSCC invasion. PDCD4 protein is known to localize in the nucleus of proliferating cells and may have an important role in transcription, translation and signal transduction pathways of the cell cycle such as apoptosis. However, the reduction in the expression of PDCD4 has been strongly associated with the progress of a tumor and metastasis of multiple types of human cancers, including the lung, colon, breast, esophagus, stomach and ovary. PDCD4 has also been studied in association with OSCC. Reduced expression of PDCD4 protein has often been correlated with poor survival of OSCC patients. This suggests that PDCD4 may be a clinically significant prognosticator and the loss of PDCD4 may be one of the important steps required for invasion and metastasis of OSCC along with initiation of tumor.

Very little literature is available on the role of PDCD4 and therefore, it was hypothesized that PDCD4 immunostaining is important in understanding the transformation of the oral dysplastic lesions to carcinoma and its expression with respect to change from the normal to premalignant and malignant conditions. Thus, using molecular technique, such as immunohistochemistry, an attempt was made to evaluate and compare the immunoenexpression of PDCD4 protein in the normal oral mucosa, oral epithelial dysplasia (OED) and OSCC.

MATERIALS AND METHODS

The study was performed after obtaining the Institutional Ethical Clearance and the waiver of informed consent. Formalin-fixed tissue block samples (embedded in paraffin) of 90 patients, histologically diagnosed with OED and OSCC, were retrieved from departmental archives of Oral pathology and Microbiology, KLE VK Institute of Dental Sciences. The normal mucosal tissue collection from the oral region of the patients was done during periodontal crown lengthening and impaction procedures. All cases of OED and OSCC were reevaluated to confirm the grades based on the WHO 2005 grading criteria and Broder's histological grading criteria, respectively.

Demographic data (age, sex, site of cancer and habit history) of the cases were collected from departmental records and tabulated. The samples were categorized into three groups: group I– normal oral mucosa (control group; n = 10), Group II– OED (n = 60) and Group III– OSCC (n = 30). The cases of OED were further classified as mild, moderate and severe dysplasia with 20 samples each. Similarly, the OSCC samples were grouped as well-differentiated, moderately differentiated and poorly differentiated carcinoma with 10 cases in each category.

Tissue sections (4 µm) from the paraffin blocks were obtained on aminopropyl triethoxy silane-coated slides (Sigma Aldrich). Immunohistochemical (IHC) slides were stained using avidin-biotin method and incubated with monoclonal rabbit antihuman PDCD4 protein (Clone EPR3432; catalog no AN524-5M; BioGenex Lab) against PDCD4. The primary protein was detected using polymer HRP kit (super sensitive polymer-HRP IHC detection system, BioGenex) by avidin-biotin complex method as described by Mäkitie et al. Use of Harris’s hematoxylin for counterstaining was the sole modification from the method followed. The color was developed due to the presence of the chromogen, diaminobenzidine.

Intensity and percentage of protein expression in the nucleus and cytoplasm were evaluated for comparing the amount of PDCD4 expressed in all the three cases. Intensity of expression, defined as the amount of protein expressed, was determined intensity of color and percentage of expression defined as the number of cells expressing the protein. Total score was analyzed by taking into account
of both the intensity and percentage scores. Slides were analyzed for intensity and percentage of expression in the cells in different fields by three independent observers under ×10 and ×40 magnification followed by the tabulation of average score. The epithelium of oral mucosa (control slide) and OED were assessed along with the tumor islands and cells in OSCC cases according to Reis and Tomenson criteria. The percentage of expression (both nuclear and cytoplasmic) of PDCD4 in stained cells was considered minimal within the range of 1–4 and overexpressed in the range of 5–7. Total scores were evaluated individually for both the nuclear, as well as cytoplasmic staining pattern.

The association between the clinical parameters and IHC results was analyzed with Fisher exact test. Statistical analysis was done using SPSS software, and significant differences were indicated by \( P < 0.05 \).

**RESULTS**

Out of 60 patients with OED, 42 were men (mean age 53.9 ± 14.68 years) and 18 were women (mean age 51.4 ± 12.92 years). Out of 30 patients with OSCC, 23 were men (mean age 55 ± 11.51 years) and 7 were women (mean age 54.1 ± 6.38 years).

Maximum cases of OED were observed in the buccal mucosa region followed by the tongue and labial mucosa region. However, the buccal and alveolar mucosa and the tongue region were the most common sites in OSCC cases. The distribution of OED was also observed in the alveolar mucosa, gingivobuccal sulcus and palate region. Cases of OSCC were also reported in the gingivobuccal sulcus, labial mucosa and the floor of the mouth.

The nuclear and cytoplasmic intensity and percentage scores comparison among the control, OED and OSCC groups have been represented in Table 1. Nuclear intensity and percentage along with cytoplasmic percentage scores among all the groups were significant.

The nuclear and cytoplasmic intensity and percentage scores among the subdivisions of OED and OSCC are represented in Tables 2 and 3. Among the OED cases, nuclear percentage scores were highly significant. On the other hand, the nuclear percentage and intensity scores among the subgroups of OSCC were significant along with the cytoplasmic intensity scores.

The total nuclear and cytoplasmic scores among all the groups and the subgroups have been represented in Table 4. The total nuclear score among all the groups was highly significant (\( P < 0.001 \)).

The photomicrographs of control group and different grades of OED and OSCC have been depicted in Figures 1-7. The basal cell layers of mild OED showed predominant nuclear staining with weak-to-moderate cytoplasmic staining similar to that of normal mucosa. In moderate OED, a slight increase in the cytoplasmic staining pattern of basal cells was noted. While one case of severe OED exhibited absence of staining in the basal cell layer along with reduced nuclear staining pattern. Moreover, the increased keratinization of the epithelium of the OED cases did not present with any PDCD4 immunoexpression. Likewise, analysis of the cases of OSCC displayed a predominant nuclear expression with mild-to-moderate cytoplasmic expression in well-differentiated carcinoma cases (WDSCC) with a reduction in the nuclear staining pattern and shift towards a more cytoplasmic staining pattern. Keratin pearls in the OSCC cases did not show any reactivity toward PDCD4 protein.

| Subgroup | Score 0 (%) | Score 1 (%) | Score 2 (%) | Score 3 (%) | Score 4 (%) | \( P \) |
|----------|------------|------------|------------|------------|------------|------|
| Nuclear intensity scores comparison among all the groups |  |  |  |  |  |  |
| Control group | 0 | 0 | 0 | 10 (100) | - | 0.017 |
| Oral epithelial dysplasia | 0 | 6 (10) | 29 (48.3) | 25 (41.7) | - |  |
| Oral squamous cell carcinoma | 0 | 3 (10) | 14 (46.7) | 13 (43.3) | - |  |
| Nuclear percentage scores comparison among all the groups |  |  |  |  |  |  |
| Control group | - | 0 | 0 | 7 (70) | 3 (30) | <0.001 |
| Oral epithelial dysplasia | - | 25 (41.7) | 29 (48.3) | 5 (8.3) | 1 (1.7) |  |
| Oral squamous cell carcinoma | - | 4 (13.3) | 8 (26.7) | 11 (36.7) | 7 (23.3) |  |
| Cytoplasmic intensity scores comparison among all the groups |  |  |  |  |  |  |
| Control group | 1 (10) | 7 (70) | 2 (20) | 0 | - | 0.182 |
| Oral epithelial dysplasia | 2 (3.3) | 32 (53.3) | 25 (41.7) | 1 (1.7) | - |  |
| Oral squamous cell carcinoma | 0 | 23 (76.7) | 7 (23.3) | 0 | - |  |
| Cytoplasmic percentage scores comparison among all the groups |  |  |  |  |  |  |
| Control group | 1 (10) | 7 (70) | 2 (20) | 0 | - | <0.001 |
| Oral epithelial dysplasia | 2 (3.3) | 54 (90) | 3 (5) | 1 (1.7) | - |  |
| Oral squamous cell carcinoma | 0 | 15 (50) | 12 (40) | 3 (10) | - |  |
DISCUSSION

This retrospective study was conducted to evaluate and compare the expression of PDCD4 protein in OED and OSCC with respect to that in normal oral mucosa. Study based on this comparison was hypothesized to provide a link between the grades of OED and OSCC leading to early diagnosis of patients with suspected malignancy.

On studying the sex distribution of dysplasia and carcinoma cases, the present study revealed higher preponderance of OED and OSCC in men than women. Similar results were observed in research studies based on cancer. A possible reason may be the higher incidence of tobacco consumption in various forms by men than women. Annual statistics in India has revealed that the risks of developing malignant disorders are more in men than in women.

Upon evaluation of age in cases of both men and women, it was observed that the mean age in patients with OED and OSCC was more than 50 years. The previous research studies on epidemiological patterns of oral cancer have made some similar observations.

Site distribution analysis in the study revealed that the buccal, labial and alveolar mucosa, tongue, palate region and gingivobuccal sulcus were the predominant sites at risk of OED and OSCC. However, some cases OSCC were also observed in the floor of the mouth. Literature survey also indicated that these sites were among the most commonly affected regions observed in patients with OED and OSCC patients.
The present study demonstrated the presence of both nuclear and cytoplasmic PDCD4 expression along with nonepithelial cells such as the endothelial cells and inflammatory cells in the stroma. This was similar to the expression noted in the esophageal and colon normal mucosa and carcinomas as reported by Fassan et al.\textsuperscript{23,24} A predominant nuclear expression along with weak-to-moderate cytoplasmic expression was a feature of normal epithelium, and as the grade progressed to carcinoma, an increase in the cytoplasmic expression was noted.

Table 4: Comparison of total nuclear and cytoplasmic score of programmed cell death 4 expression among all the groups

| Group                        | Minimal expression | Over expression | \(P\) |
|------------------------------|--------------------|-----------------|-------|
| Total nuclear score among all the groups |                     |                 |   |
| Control group                | 0                  | 10 (100)        | <0.001 |
| Oral epithelial dysplasia    | 42 (70)            | 18 (30)         |      |
| Oral squamous cell carcinoma | 9 (30)             | 21 (70)         |      |
| Total cytoplasmic score among all the groups |                     |                 |   |
| Control group                | 10 (100)           | 0               | 0.158 |
| Oral epithelial dysplasia    | 60 (100)           | 0               |      |
| Oral squamous cell carcinoma | 28 (93.3)          | 2 (6.7)         |      |
| Total nuclear score among different grades of oral epithelial dysplasia |                     |                 |   |
| Mild                         | 12 (20)            | 8 (13.3)        | 0.386 |
| Moderate                     | 14 (23.3)          | 6 (10)          |      |
| Severe                       | 16 (26.7)          | 4 (6.7)         |      |
| Total cytoplasmic score among different grades of oral epithelial dysplasia |                     |                 |   |
| Mild                         | 20 (33.3)          | 0               | 1.000 |
| Moderate                     | 20 (33.3)          | 0               |      |
| Severe                       | 20 (33.3)          | 0               |      |
| Total nuclear score among different grades of oral squamous cell carcinoma |                     |                 |   |
| Well differentiated           | 1 (13.3)           | 9 (30)          | 0.077 |
| Moderately differentiated     | 2 (6.7)            | 8 (26.7)        |      |
| Poorly differentiated         | 6 (20)             | 4 (13.3)        |      |
| Total cytoplasmic score among different grades of oral squamous cell carcinoma |                     |                 |   |
| Well differentiated           | 10 (33.3)          | 0               | 0.585 |
| Moderately differentiated     | 9 (30)             | 1 (13.3)        |      |
| Poorly differentiated         | 9 (30)             | 1 (13.3)        |      |

The PDCD4 expression in the nucleus was present in all the cases of normal mucosa, thus depicting that its nuclear localization regulates the normal functioning of the cells while shuttling to the cytoplasm was indicative of cellular derangement or cell division. The maximum intensity of nuclear staining was observed in the normal mucosa and the maximum cytoplasmic intensity was observed in the cases of OED and OSCC.

The present study also indicated the localization of the nuclear stain in the normal mucosa, predominantly in the basal cell layers, extending up to the superficial layers of the mucosa. This was in accordance with the expression of PDCD4 in the normal esophageal mucosa and colon as put forth by Fassan et al.\textsuperscript{23,24} Contrary to these, Nagao et al. observed exclusive nuclear staining in the pancreatic normal tissues.\textsuperscript{11}
Desai and Kale: Expression of PDCD4 protein in oral mucosa, OED and OSCC

OED cases demonstrated a reduced nuclear expression when compared to normal mucosa. On comparison of results within the groups, a significant difference in the percentage of cells expressing nuclear PDCD4 expression was observed. Further, a reduction in the number of cases showing overall strong nuclear expression was detected within the groups with maximum expression in mild OED to least in severe OED group. The discrepancies in the expression pattern, as noted in OED cases, could be attributed to the presence of inflammation, which was not evaluated separately in all the groups and hence warrants further research for justification of the same.

Distinct linear and peripheral pattern of cytoplasmic staining was observed in cases of WDSCC and moderately differentiated carcinoma cases suggestive of the proliferative activity at the tumor islands. Mitotic figures demonstrated a characteristic cytoplasmic staining, which suggests the role of this shuttling phenomenon of PDCD4 in cell multiplication. This is similar to the immunostaining of epidermal growth factor receptor and transferrin receptor, which depicted a marked expression in the periphery of the tumor cell islands.\cite{25}

The cytoplasmic localization of PDCD4 in the periphery or invasive front indicates the proliferative activity (or the increased mitosis) shown by peripheral tumor cells. A parallel finding in some cases of dysplasia and carcinoma was the pattern of immunostaining in cells showing mitosis, wherein a distinct cytoplasmic staining with absence of nuclear expression was observed. This was in accordance with the results put forth by Yoshinaga \textit{et al.}\cite{26} and Hayashi \textit{et al.}\cite{27} which stated that cells undergoing multiplication show a predominant cytoplasmic shift of PDCD4.
An overall reduction and shuttling of PDCD4 from nucleus to cytoplasm was found from normal mucosa to OED to OSCC. These findings suggest the role of PDCD4 as a proliferative marker in OED and squamous cell carcinoma. Further research can include long follow-up period and other pathological criteria such as inflammation and microenvironment, immune status of patient and tumor stage could aid in the development of prospective diagnostic options.

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Conflicts of interest
There are no conflicts of interest.

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