Role of cell proliferation and vascularity in malignant transformation of potentially malignant disorders

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Abstract

Background: Significant increase in cell proliferation and vascularity occurs during the transition from normal oral mucosa through differing degrees of dysplasia to oral squamous cell carcinoma (OSCC).

Aims: To evaluate the cell proliferation and vascularity in potentially malignant disorders and OSCC.

Settings and Design: Proliferating cell nuclear antigen (PCNA), vascular endothelial growth factor (VEGF) and CD34 were quantified immunohistochemically (IHC) using anti-PCNA, anti-VEGF and anti-CD34 antibody.

Materials and Methods: A total of 60 archival specimens included 10 oral lichen planus, 10 oral leukoplakia, 10 oral submucous fibrosis and 30 OSCC (well differentiated, moderately differentiated and poorly differentiated), and also, 10 normal oral mucosa as control group were taken. PCNA, VEGF and CD34 expression was assessed in relation to the localization and area of IHC-stained cells.

Statistical Analysis: One-way analysis of variance test and post hoc least significant difference test were assessed for statistical significance.

Results: Cell proliferation and vascularity appeared to increase gradually with disease progression.

Conclusion: Upregulation of cell proliferation and vascularity indicates their possible role in malignant transformation of potentially malignant disorders.

Keywords: Cell proliferation, microvessel density, vascularity

INTRODUCTION

The information obtained in the clinical and histopathological examinations is not always satisfactory for the diagnosis and prognosis of potentially malignant disorders. Therefore, more specific methods are used to allow the measurement of the cellular alterations by means of cellular and tissue markers. Several markers have been used to provide additional information about malignant transformation in potentially malignant disorders, including angiogenesis and cell proliferation markers, which have long been used in the study of cancer and are the focused of this study.[1] At present, angiogenesis is considered an essential process in tumor development. Angiogenesis, the formation of new blood vessels, is crucial to the growth, invasion and metastasis of a tumor.[2] Tumor angiogenesis, like the physiological one, is the process of creating new blood vessels starting from the already existing ones, either by

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recruiting precursor endothelial cells by multiplying the endothelial cells of the already existing capillaries.[9]

Cell proliferation is a biological process vitally important to all living organisms due to its role in the growth and maintenance of tissue homeostasis. The control of this important process is completely dysregulated in cancer, and the assessment of cell proliferation activity in tumors has become a common tool used by histopathologists to provide useful information for diagnosis, clinical behavior and therapy.[1]

The present study has been proposed to assess some aspects of the angiogenesis and cell proliferation processes based on vascular endothelial growth factor (VEGF), CD34 and proliferating cell nuclear antigen (PCNA) expression in oral lichen planus (OLP), oral leukoplakia (OL), oral submucous fibrosis (OSF) and oral squamous carcinoma (OSCC).

MATERIALS AND METHODS

Tissues

Sixty formalin-fixed paraffin-embedded archival biopsies of 10 OLP (Group I), 10 OL (Group II), 10 OSF (Group III) and 30 OSCC (10 well-differentiated SCC[WSCC], 10 moderately differentiated SCC [MSCC] and 10 poorly differentiated SCC [PSCC] [Group IV]) were obtained from the Department of Oral Pathology and Microbiology, Kamineni Institute of Dental Sciences, Narketpally. Ten cases of normal control group (Group V) were also included.

Histopathological and immunohistochemical analysis

All tissue biopsies were sectioned at 3μm thickness and taken onto a poly-L-lysine-coated glass slide, and further, immunohistochemistry (IHC) procedure was performed to detect VEGF, CD34 and PCNA expression. Sections were deparaffinized followed by rehydration and antigen retrieval was carried out. Thereby, sections were incubated with peroxidase block to block the endogenous peroxidase activity which was followed by protein block, primary antibody, post primary antibody, polymer and substrate chromogen application and finally counterstained with Mayer’s hematoxylin and mounted.

Staining was performed as per the IHC staining protocol. The presence of brown-colored end product at the site of target antigen was indicative of positive immunoreactivity.

PCNA expression was evaluated on the basis of number of positively stained cell; expression of PCNA was designated as positive (>5% of cells were stained) and negative (<5% of cells stained). Three high-power fields (×40) were selected from the stained sections to determine the stained cells per 100 counted cells in the basal and parabasal layers as positive and negative.

VEGF expression was quantified according to the area of staining in the connective tissue under low-power view (×10). The area of staining was scored as 0, no stained cells in any microscopic field, 1, <25% of tumor cells stained positively, 2, 25-50% of tumor cells stained positively, 3, 50-75% of tumor cells stained positively and 4, >75% of tumor cells stained.

CD34 expression was assessed as microvessel density, and the assessment was carried out at the level of endothelial cells lining the blood vessels by their brown cytoplasmic staining in the connective tissue. Microvessel density in areas showing the highest density of staining determined by low-power view (×10) was selected, and then, under three high-power view (×40), the number of CD34-positive endothelial lined blood vessels was counted.

Statistical analysis

SPSS (Statistical package for social sciences) is a software package used for statistical analysis. IBM SPSS Statistics for Windows, Version 22.0. (Armonk, NY: IBM Corp). The significance of the results obtained from the control and study group was statistically analyzed by Chi-squared test and one-way analysis of variance (ANOVA) test, and multiple comparisons between the groups were assessed for statistical significance using post hoc least significance difference (LSD) test.

RESULTS

The cell proliferation determined by PCNA expression was based on nuclear staining per 100 counted cells in

![Table 1: Descriptive analysis between the groups stained with proliferating cell nuclear antigen antibody](image-url)
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the basal and parabasal layer when viewed under three high-power views (×40) [Figures 1-7]. Based on the Chi-squared test among the study groups, normal group showed only 50% expression of PCNA, whereas OLP and OL groups showed 60% and 70% expression, respectively, while OSF showed 80% and OSCC showed majority (96.7%) expression. This difference in the expression was statistically significant (P = 0.035) [Tables 1 and 2, Graph 1]. The positivity for expression of PCNA in WSCC, MSCC and PSCC was 90%, 100% and 100%, respectively. Within the OSCC group, PCNA expression determined using Chi-squared test showed no statistical significance (P = 0.355) [Tables 3 and 4, Graph 2].

VEGF expression was confirmed by the presence of brown-stained cytoplasm in the connective tissue when viewed under low-power view (×10) [Figures 8-15]. The data assessed for significance between the groups using one-way ANOVA showed statistical significance (P = 0.000). Multiparametric post hoc LSD test was done between the study groups, and there was a statistical significance between the groups, but the results were not statistically significant (P = 0.068) between OL and normal oral mucosa group [Tables 5-7]. The data assessed for significance within the OSCC using one-way ANOVA and multiparametric post hoc LSD test showed statistical significance [Tables 8-10, Graphs 3 and 4].

Table 2: Chi-square test table between the groups stained with proliferating cell nuclear antigen antibody

| Group    | PCNA       | Significance |
|----------|------------|--------------|
|          | 0, n (%)   | 1, n (%)     | 0.035        |
| OLP      | 4 (40.0)   | 6 (60.0)     |              |
| OSMF     | 2 (20.0)   | 8 (80.0)     |              |
| OL       | 3 (30.0)   | 7 (70.0)     |              |
| OSCC     | 1 (3.3)    | 29 (96.7)    |              |
| Normal   | 5 (50.0)   | 5 (50.0)     |              |

PCNA: Proliferating cell nuclear antigen, OLP: Oral lichen planus, OL: Oral leukoplakia, OSCC: Oral squamous cell carcinoma, OSMF: Oral submucous fibrosis
Microvessel density was based on the CD34-positive endothelial cells lining the blood vessel in the connective tissue; at first, three microscopic fields of highest neovascularization under low-power view (×10) were selected and then counted under high-power view (×40) [Figures 16-21]. One-way ANOVA was performed for significance between the groups and \( P = 0.000 \) was considered statistically significant. Multiparametric post hoc LSD test done between the study groups also was statistically significant [Tables 11-13]. The data assessed for significance within the OSCC using one-way ANOVA and multiparametric post hoc LSD test showed statistical significance [Tables 14-16, Graphs 5 and 6].

**DISCUSSION**

The results from the present study indicate a significant upregulation of VEGF, CD34 and PCNA expression during the transition from normal oral mucosa through OLP, dysplasia, OSF and OSCC. An overall increase in mean scores from normal to OLP, OL, OSF and different grades of OSCC was similar to other studies.[2-20]
Previous studies\textsuperscript{[2‑20]} included most but not all the parameters as in the present study, providing evidence of variation in anti-VEGF, anti-CD34 and anti-PCNA antibody staining. However, few studies\textsuperscript{[2‑7,12,15,18‑20]} have not shown statistically significant results among the parameters considered.

A probable explanation could be that changes in the proliferative capacity may be an early consequence of carcinogen exposure and simultaneous field cancerization, a phenomenon that could occur before the appearance of morphologically apparent hyperplasia or dysplasia. It is generally accepted that increased proliferation is associated with more advanced lesions and that the distribution of proliferating cells in tissue may tell us more about the regulatory mechanism that becomes dysfunctional during the multi-step process of carcinogenesis.\textsuperscript{[21]} Along with cell proliferation, at present, angiogenesis is considered an essential process in oral cancer development. Significance of angiogenesis because the exact quantification of tumor vessels is useful for assessing the lesion prognosis and metastasization ability.\textsuperscript{[3]}

In OLP, an increase in proliferation might be related to the release of cytokines and inflammatory mediators from injured keratinocytes or inflammatory cells following immunological reactions. This increase may result in

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Table 4: Chi-squared test table within the oral squamous cell carcinoma groups stained with proliferating cell nuclear antigen antibody

| Group    | PCNA 0, n (%) | PCNA 1, n (%) | Significance |
|----------|---------------|---------------|--------------|
| WSCC     | 1 (10.0)      | 9 (90.0)      | 0.355        |
| MSCC     | 0             | 10 (100.0)    |              |
| PSCC     | 0             | 10 (100.0)    |              |

PCNA: Proliferating cell nuclear antigen, WSCC: Well-differentiated squamous cell carcinoma, MSCC: Moderately differentiated squamous cell carcinoma, PSCC: Poorly differentiated squamous cell carcinoma
chronic progression, OLP satisfies all the prerequisites of hypoxia which is essential for angiogenesis. If angiogenesis is increased, it leads to more recruitment and retention of lymphocytes or inflammatory infiltrate or progression of disease or recurrence of lesions. Inflammatory infiltrates in turn can progress to carcinogenesis. \[9\]

In OL, accumulation of mutations in growth regulatory genes may result in an increased proliferative activity. \[23\]
Table 10: Multiple comparisons post hoc least significant difference test table within the oral squamous cell carcinoma group stained with vascular endothelial growth factor antibody

| Group (I) | Group (J) | Mean difference (I−J) | SE     | Significant | 95% CI          |
|-----------|-----------|------------------------|--------|-------------|----------------|
| WSCC      | MSCC      | −0.50000               | 0.15635| 0.004       | −0.8208 −0.1792|
| PSCC      | WSCC      | −1.1000*               | 0.15635| 0.000       | −1.4208 −0.7792|
| MSCC      | PSCC      | −0.60000               | 0.15635| 0.001       | −0.9208 −0.2792|
| PSCC      | WSCC      | 1.00000                | 0.15635| 0.000       | 0.7792 1.4208 |

CI: Confidence interval, SE: Standard error, WSCC: Well-differentiated squamous cell carcinoma, MSCC: Moderately differentiated squamous cell carcinoma, PSCC: Poorly differentiated squamous cell carcinoma

Table 11: Descriptive analysis between the groups stained with CD34 antibody

| Group  | n  | Mean     | SEM    | Median  | Mode  | SD    | Range  | Minimum | Maximum |
|--------|----|----------|--------|---------|-------|-------|--------|---------|---------|
| OLP    | 10 | 17.2000  | 0.69602| 16.5000 | 15.00 | 2.20101| 6.00   | 15.00   | 21.00   |
| OSM    | 10 | 32.8000  | 1.15277| 32.5000 | 30.00 | 3.64539| 11.00  | 28.00   | 39.00   |
| OL     | 10 | 23.8000  | 0.66332| 24.0000 | 22.00 | 2.09762| 7.00   | 20.00   | 27.00   |
| OSCC   | 30 | 51.766   | 1.49842| 51.0000 | 50.00 | 8.20716| 35.00  | 35.00   | 70.00   |
| Normal | 10 | 7.4000   | 0.37118| 7.5000  | 6.00  | 1.73799| 3.00   | 6.00    | 9.00    |

SEM: Standard error of the mean, SD: Standard deviation, OLP: Oral lichen planus, OL: Oral leukoplakia, OSCC: Oral squamous cell carcinoma, OSM: Oral submucous fibrosis

Figure 13: Anti-vascular endothelial growth factor antibody staining in moderately differentiated squamous cell carcinoma

Figure 14: Anti-vascular endothelial growth factor antibody staining in poorly differentiated squamous cell carcinoma

Figure 15: Anti-vascular endothelial growth factor antibody staining in normal oral mucosa

Figure 16: Anti-CD34 antibody staining in oral lichen planus
As cells transform from normal to dysplastic, the balance between proangiogenic and antiangiogenic factors is altered and the dysplastic epithelial cells themselves acquire transient angiogenic properties. Thereby, shifting to angiogenic phenotype occurs as early as mild dysplasia.\cite{12} As appearance of OSCC is gradually preceded by epithelial dysplasia,\cite{13} a gradual increase of VEGF in OL is considered to satisfy the criteria of a potentially malignant disorder progressing into a malignancy.

In OSF, the increased cell proliferation could be induced by direct stimulation from the mitogen-like compounds contained in areca quid or by there generative proliferation after cell death. Secondly, as PCNA is associated with DNA excision repair, PCNA expression may also increase after DNA damage is induced by areca quid components.\cite{15} As the stroma becomes more and more hyalinized due to progressive deposition and cross-linkage of mature collagen bundles, the tissue suffers resultant ischemia/hypoxia due to physical and biochemical effects of the process. Pursuing further the pathological mechanism, the tissue tries to cope up with hypoxia by actively promoting neovascularization as

Figure 17: Anti-CD34 antibody staining in normal oral mucosa

Figure 18: Anti-CD34 antibody staining in well-differentiated squamous cell carcinoma

Figure 19: Anti-CD34 antibody staining in moderately differentiated squamous cell carcinoma

Figure 20: Anti-CD34 antibody staining in poorly differentiated squamous cell carcinoma

Figure 21: Anti-CD34 antibody staining in normal oral mucosa
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Table 12: One-way analysis of variance table between the groups stained with CD34 antibody

| Sum of squares | df | Mean square | F      | Significant |
|---------------|----|-------------|--------|-------------|
| Between groups | 20419.219 | 4 | 5104.805 | 153.010 | 0.000 |
| Within groups  | 2168.567  | 65 | 33.363   |         |     |
| Total          | 22587.786 | 69 |         |         |     |

Graph 1: Comparison of cell proliferation between study groups with proliferating cell nuclear antigen. OLP: Oral lichen planus, OL: Oral leukoplakia, OSM: Oral submucous fibrosis, OSCC: Oral squamous cell carcinoma

Graph 2: Comparison of cell proliferation within oral squamous cell carcinoma study group with proliferating cell nuclear antigen. WSCC: Well-differentiated squamous cell carcinoma, MSCC: Moderately differentiated squamous cell carcinoma, PSCC: Poorly differentiated squamous cell carcinoma

Graph 3: Comparison of vascularity between study groups with vascular endothelial growth factor. OLP: Oral lichen planus, OL: Oral leukoplakia, OSM: Oral submucous fibrosis, OSCC: Oral squamous cell carcinoma

Graph 4: Comparison of vascularity within oral squamous cell carcinoma study group with vascular endothelial growth factor. WSCC: Well-differentiated squamous cell carcinoma, MSCC: Moderately differentiated squamous cell carcinoma, PSCC: Poorly differentiated squamous cell carcinoma

Graph 5: Comparison of microvessel density between study groups with CD34. OLP: Oral lichen planus, OL: Oral leukoplakia, OSM: Oral submucous fibrosis, OSCC: Oral squamous cell carcinoma

Graph 6: Comparison of microvessel density within oral squamous cell carcinoma study group with CD34. WSCC: Well-differentiated squamous cell carcinoma, MSCC: Moderately differentiated squamous cell carcinoma, PSCC: Poorly differentiated squamous cell carcinoma

In OSCC, the correlation between PCNA and cell proliferation is probably because of the PCNA involvement in DNA repair which is active and ongoing function so that it might be upregulated in nonproliferating cells. The increase in VEGF expression within the OSCC group supports the idea that VEGF is involved in increasing vascularity with an adaptive response on the part of the mucosa in survival of the atrophic epithelium. [24]
This could be supported by the fact that VEGF secreted by tumor cells does not stimulate growth directly but leads to increased growth and permeability of endothelial cells, and as vascular permeability increases, microvessels in tumor environment may become leaky, thereby making them more penetrable by tumor cells.\cite{12}

**CONCLUSION**

Cell proliferation and angiogenesis can be considered a paramount for the assessment of the behavior of potentially malignant disorder. In fact, the malignant transformation of a potentially malignant disorder

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**Table 13: Multiple comparisons post hoc least significant difference test table between the groups stained with CD34 antibody**

| Group (I) | Group (J) | Mean difference (I−J) | SE  | Significant | Lower bound | Upper bound |
|----------|-----------|-----------------------|-----|-------------|-------------|-------------|
| OLP      | OL        | −6.60000*             | 2.5831 | 0.013       | −11.7588    | −1.4412     |
| OLP      | OSF       | −15.60000*            | 2.5831 | 0.002       | −20.7588    | −10.4412    |
| OLT      | OSF       | −34.56667*            | 2.10911 | 0.000       | −38.7788    | −30.3545    |
| OLT      | Normal    | 9.80000*              | 2.5831 | 0.013       | 4.6412      | 14.9588     |
| OL       | OLP       | 6.60000*              | 2.5831 | 0.013       | 1.4412      | 11.7588     |
| OL       | OSF       | −9.00000*             | 2.5831 | 0.001       | −14.1588    | −3.8412     |
| OSCC     | −27.96667* | 2.10911  | 0.000 | 0.01        | −32.1788    | −23.7545    |
| OSCC     | Normal    | 16.40000*             | 2.5831 | 0.04        | 11.2412     | 21.5588     |
| OSF      | OLP       | 15.60000*             | 2.5831 | 0.000       | 10.4412     | 20.7588     |
| OSF      | OL        | 9.00000*              | 2.5831 | 0.001       | 3.8412      | 14.1588     |
| OSF      | OSCC      | −18.96667*            | 2.10911 | 0.01        | −23.1788    | −14.7545    |
| OSF      | Normal    | 25.40000*             | 2.5831 | 0.012       | 20.2412     | 30.5588     |
| OSCC     | OLP       | 34.56667*             | 2.10911 | 0.000       | 30.3545     | 38.7788     |
| OSCC     | OL        | 27.96667*             | 2.10911 | 0.012       | 23.7545     | 32.1788     |
| OSCC     | OSF       | −18.96667*            | 2.10911 | 0.001       | −23.1788    | −14.7545    |
| OSCC     | Normal    | 44.36667*             | 2.10911 | 0.012       | 40.1545     | 48.5788     |
| Normal   | OLP       | 16.40000*             | 2.5831 | 0.001       | 10.4412     | 20.7588     |
| Normal   | OSF       | −16.90000*            | 2.5831 | 0.001       | −20.9443    | −10.4557    |
| Normal   | OSCC      | −25.40000*            | 2.5831 | 0.000       | −30.5588    | −20.2412    |
| Normal   | OLT       | −44.36667*            | 2.10911 | 0.001       | −48.5788    | −40.1545    |

*: Significant at 0.05, OLP: Oral lichen planus, OL: Oral leukoplakia, OSF: Oral submucous fibrosis, OSCC: Oral squamous cell carcinoma, CI: Confidence interval, SE: Standard error

**Table 14: Descriptive analysis within the oral squamous cell carcinoma group stained with CD34 antibody**

| Group     | n  | Mean   | SEM    | Median | Mode  | SD       | Range  | Minimum | Maximum |
|-----------|----|--------|--------|--------|-------|----------|--------|---------|---------|
| WSCC      | 10 | 43.2000| 1.35647| 44.0000| 44.00 | 4.28952  | 14.00  | 35.00   | 49.00   |
| MSCC      | 10 | 52.0000| 0.68313| 51.5000| 50.00 | 2.16025  | 6.00   | 49.00   | 55.00   |
| PSCC      | 10 | 60.1000| 1.87646| 60.5000| 65.00 | 5.93390  | 20.00  | 50.00   | 70.00   |

SEM: Standard error of the mean, SD: Standard deviation, WSCC: Well-differentiated squamous cell carcinoma, MSCC: Moderately differentiated squamous cell carcinoma, PSCC: Poorly differentiated squamous cell carcinoma

**Table 15: One-way analysis of variance table within the oral squamous cell carcinoma group stained with CD34 antibody**

| Sum of squares | df | Mean square | F      | Significant |
|----------------|----|-------------|--------|-------------|
| Between groups | 1428.867 | 2 | 714.433 | 36.777 | 0.000 |
| Within groups  | 524.500 | 27 | 19.426 |        |        |
| Total          | 1953.367 | 29 |        |        |        |

**Table 16: Multiple comparisons post hoc least significant difference test table within the oral squamous cell carcinoma group stained with CD34 antibody**

| Group (I) | Group (J) | Mean difference (I−J) | SE     | Significant | Lower bound | Upper bound |
|-----------|-----------|-----------------------|--------|-------------|-------------|-------------|
| WSCC      | MSCC      | −8.80000*             | 1.97109| 0.003       | −12.8443    | −4.7557     |
| WSCC      | PSCC      | −16.90000*            | 1.97109| 0.000       | −20.9443    | −12.8557    |
| MSCC      | WSCC      | 8.80000*              | 1.97109| 0.002       | 4.7557      | 12.8443     |
| MSCC      | PSCC      | −8.10000*             | 1.97109| 0.001       | −12.1443    | −4.0557     |
| PSCC      | WSCC      | 16.90000*             | 1.97109| 0.000       | 12.8557     | 20.9443     |
| PSCC      | MSCC      | 8.10000*              | 1.97109| 0.004       | 4.0557      | 12.1443     |

*: Significant at 0.05, CI: Confidence interval, SE: Standard error, WSCC: Well-differentiated squamous cell carcinoma, MSCC: Moderately differentiated squamous cell carcinoma, PSCC: Poorly differentiated squamous cell carcinoma
can be predicted based on cell proliferation rate and degree of vascularity. In turn, therapies that focus on targeting various molecules and pathways involved in cell proliferation and vascularity may provide better control of the progression of potentially malignant disorders to malignancies.

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**Conflicts of interest**
There are no conflicts of interest.

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