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Immunohistochemical localization of basic fibroblast growth factor (bFGF) in oral squamous cell carcinoma (OSCC)

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Abstract
Objective: Basic fibroblastic growth factor (bFGF) is a potent angiogenic factor implicated in tumor growth and metastasis. To determine if bFGF is upregulated in Oral Squamous cell carcinoma (OSCC), we measured the level and intensity of expression of bFGF in various grades of oral squamous cell carcinoma specimens as compared to control specimens.
Methods: Oral squamous cell carcinoma of different grades and control tissue specimens were analyzed qualitatively (32 patients, 5 controls). Control specimen consisted of buccal mucosa tissue.
Results: Immunohistochemical analysis revealed that bFGF was strongly associated with OSCC specimens as compared to the control. The control specimens showed mild staining localized to the basal layers of epithelium. The intensity of expression and the number of cells showing bFGF expression increased significantly with increasing degree of morphological differentiation.
Conclusions: Oral squamous cell carcinoma cells express high levels of bFGF and suggest that these growth factors contribute significantly to cancer cell growth.

Keywords: Basic fibroblast growth factor, bFGF, oral squamous cell carcinoma, angiogenic factors

Introduction

Squamous cell carcinoma is the most common malignant tumor of the oral cavity, is one of the ten most common cancers in the world and accounts for about 10-20% of all the cancers detected in India. The prognosis for many of these patients is poor and is associated with high degree of morbidity and mortality even in cases that have undergone successful surgery. Hence, understanding of the disease process at the molecular level is important for the early diagnosis and successful management of oral malignancies.

Currently, the treatment decisions in oral and oropharyngeal tumors are guided mainly by clinico-pathologic factors such as age, sex, race, tumor-node metastasis stage and histologic grade. Although these factors are useful, it fails to provide definitive information regarding the overall aggressiveness of a tumor and its potential to recur.

The study of immunohistochemical changes in order to determine the tumor-associated antigenic constituents, commonly referred to as "tumor markers", has received considerable attention. Recently, angiogenic growth factors have been implicated for the growth of solid tumors.

Angiogenesis is a critical event in tumor growth and metastasis, mediated by several growth factors released by the tumor cells in the local environment. Experimental and clinical evidence suggest that after a relatively dormant prevascular phase, solid tumor enter a vascular or angiogenic phase resulting in ample supply of nutrients for rapid expansion of malignant population and allowing the tumor cells to metastasize [4]. Basic fibroblastic growth factor (bFGF) is a well-described angiogenic growth factor and its key role in tumor angiogenesis is well established. However, little is known about the role of bFGF in OSCCs. Expression of bFGF in various histological grades of oral squamous cell carcinoma in comparison to normal mucosa were examined immunohistochemically to find a correlation between them.

Materials and Method

32 oral carcinoma cases were compared to 5 controls (normal buccal mucosa) immunohistochemically. The carcinoma cases included 9 of well differentiated cases, 15 of moderately differentiated and 8 poorly differentiated cases. Diagnosis was made according to criteria set by Anneroth *et al.* [1] All tissues were formalin-fixed and paraffin embedded. All subsequent procedures were performed at room temperature. The sections were dewaxed, rehydrated, and endogenous peroxidase was blocked with 1% H_2O_2 in methanol for 30 minutes. Sections used for bFGF antibodies required antigen retrieval and were boiled in citrate buffer, were washed in PBS, followed by preincubation with 10% normal goat serum for 30 minutes. Sections were then incubated with primary antibodies (Monoclonal, mouse antihuman) (Sigma Aldrich chemicals) at 1: 500 dilution. After being washed in PBS, sections were incubated with biotinylated goat antimouse immunoglobin for 30 minutes then washed in PBS, followed by 30 minutes incubation with Streptavidin - Peroxidase Conjugate. Staining was visualized by immersing the sections in Diaminobenzidine tetrahydrochloride (DAB). The sections were counterstained with Mayer's hematoxylin and mounted by employing resinous media (DPX). Assessments of antigen-expressing cells were performed by using light microscope at 25X and 40X magnifications.

Results and Observation

Expressions of bFGF were observed in 100% of the OSCC specimens tested. Immunohistochemical staining of tumor cells for bFGF was granular with the entire cytoplasm/ nucleus/ both staining positively. Among the various grades of OSCC, poorly differentiated cases showed a significantly more intense staining as compared to moderately differentiated and well differentiated cases (Bar chart 2). The number of cells expressing bFGF also increased significantly with increasing grades that is, more number of cells in poorly differentiated cases showed bFGF expression as compared to moderately and poorly differentiated cases (Bar chart 1). It was also observed that in well differentiated cases, bFGF expression was mainly cytoplasmic as compared to moderately and poorly differentiated cases where it was more in nucleus (Bar chart 3). The control specimens showed mild staining localized to the basal cell layers.

Discussion

The pathogenesis of oral squamous cell carcinomas (OSCCs) is multifactorial, little is known about the risk factors and the multistep process of tumorigenesis. Researchers have identified several molecular factors that are involved in malignant transformation, including alterations in expression of tumor suppressor genes and oncogenes. Recently, amplifications and overexpression of growth factors have been demonstrated in human Squamous cell carcinomas and are thought to play a biological role in tumor progression.

Growth factors are polypeptides that stimulate cell proliferation through binding to specific high affinity cell membrane receptors; these are present in a wide variety of tissues, both adult and embryonic and are thought to be released by many, if not all cells in culture [14].

Among the members of the family of growth factors, fibroblast growth factor is now known to influence growth of wide variety of tissues including epithelia and plays a potential role in tumorigenesis.

In the present immunohistochemical study, localization of tumor-associated bFGF was studied in different grades of OSCCs, both qualitatively and quantitatively. Among the 32 cases of OSCCs, most of the cases showed nuclear and cytosolic synthesis of bFGF protein, similar to the observation made by Myoken Y *et al*. 1994 [9] in their study on SCC cell lines, which expressed bFGF, contributing to the proliferation of cells. Of particular importance is the observation made by Schweigerer *et al*. 1987. [13] In the *in-vitro* studies, that bFGF stimulate endothelial proliferation, a potent inducer of angiogenesis. Thus the result of the present study perhaps suggest that neoplastic cell initially exhibit an increased cell proliferation and angiogenesis, necessary for tumor growth by synthesis of bFGF. Interestingly, tumor associated bFGF localization was significantly higher in poorly differentiated squamous cell carcinoma when compared to moderately and well differentiated squamous cell carcinoma. This may be explained by the fact that initially tumor cells are synthesizing large amount of tumor-associated growth factors to promote growth. This in turn may stimulate the release of pro-tumorigenic factors such as proliferative factor and angiogenic factor to enhance metastasis, supporting the views of Zoya *et al*. 1997 [15]. Furthermore, the localization of endogenous bFGF was observed in greater number in poorly differentiated OSCC than in moderately and well-differentiated OSCCs and the protein were expressed more in nucleus than in cytoplasm of the cells. This is consistent to the observation made by Renko *et al*. 1990 [11] that higher molecular weight forms of bFGF are found preferentially in ribosomal and nuclear fraction, added to this, in few cases only cytoplasmic staining was evident with lack of nuclear staining. This may be explained by the fact that a relatively low level of nuclear bFGF may be seen in proliferating cells which is probably below the limits of immunocytochemical detection in formalin fixed paraffin embedded tissues.

However, in the present study the bFGF expression was patchy and heterogenous in distribution with increased staining in poorly differentiated OSCCs, mainly in tumor cells with basaloid morphology. This was in agreement with other studies wherein they suggested the bFGF positive cells might be involved in mitosis; this observation also supports the view of Ornitz *et al*. 1996 [10] and Miller *et al*. 2000 [6] that FGF regulates cell proliferation, differentiation and function, in a number of processes including normal development, carcinogenesis and metastasis. Thus bFGF may influence increased mitotic activity by increased expression in SCC than in normal epithelium.

Likewise, several authors identified bFGF in basal layer of normal epithelium and an intense staining in the superficial layers with little or lack of staining in basal layer[8]. However, in the present study in all the 5 cases of normal oral epithelium included as control showed a staining of bFGF similar to the observation made by earlier studies, an intense staining in basal and parabasal layers [4] in contrast to the observation made by other workers of the positive staining in superficial layers [8]. The presence of bFGF protein in basal and parabasal layers suggest that this endogenous protein is perhaps involved in proliferation but not in differentiation of keratinocytes. Interestingly in few cases of SCC, the paraffin section showed severely dysplastic epithelium. Huges *et al*. 1994 [6] in their study on epithelial dysplasia observed a progressive increase in bFGF-mRNA with increasing severity of dysplasia; similar staining reaction was observed in basal, parabasal and superficial layers with greater intensity in these layers of dysplastic epithelium.

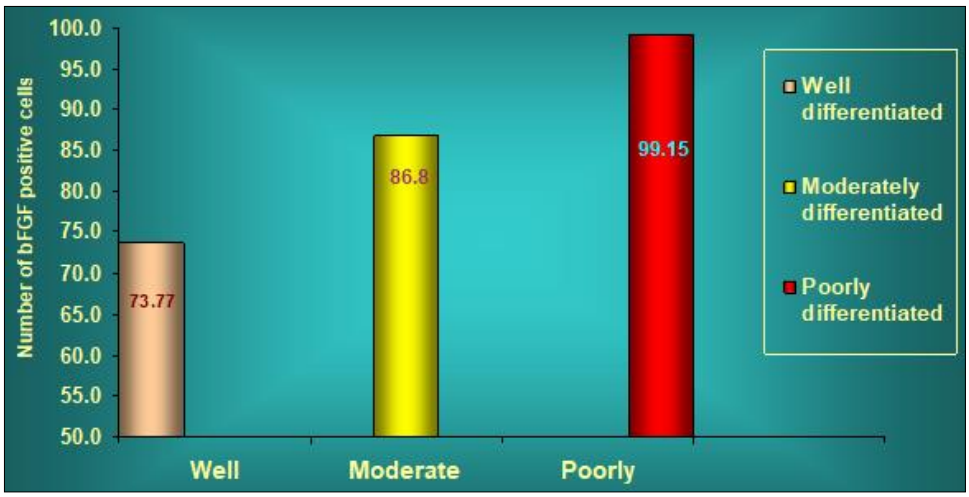
Neovascularization is a necessary phenomenon for both normal and neoplastic growth. Tumor growth is dependent

upon the ability of the tumor to recruit vasculature. Earlier studies have suggested that active form of bFGF stimulates endothelial cell mitoses, migration and remodeling of basement membrane, the 3 major steps in angiogenesis [2] of particular importance is the observation made by Davidson *et al*. [3] 1985 in bFGF, that it stimulates the endothelial cell proliferation *in vitro* and also a potent inducer of blood vessel growth *in vivo*. In the present study, bFGF was consistently demonstrated in the matrix around the proliferating blood vessels as well as in the matrix outside the tumor nests. Close proximity to the vasculature suggests the role of fibroblast growth factor in angiogenic process. Conversely, a reverse process might have taken place that tumor cells might release unknown mediators that can stimulate the production of angiogenic factors, bFGF from endothelial cell, a self-stimulatory growth factor inducing neovascularization.

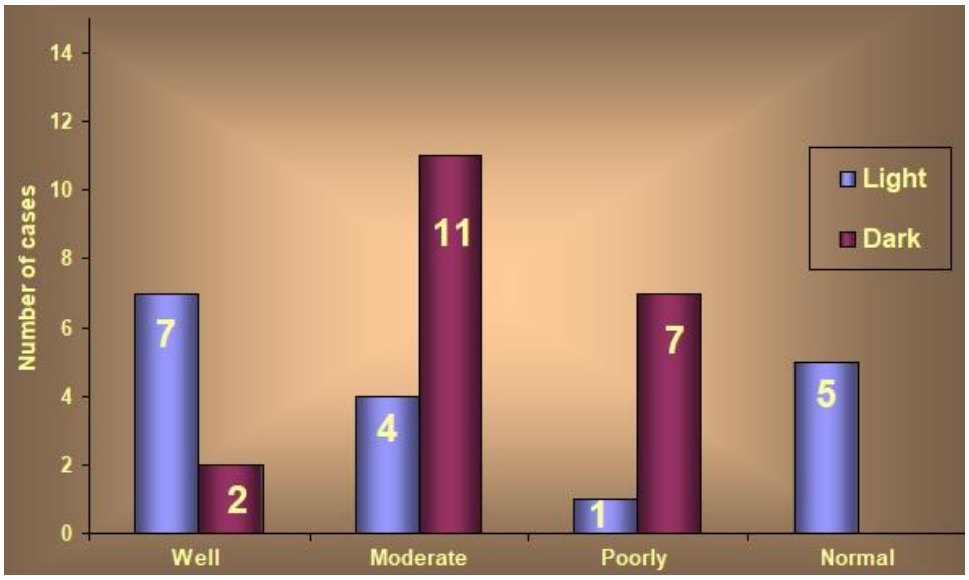
However, in the present study staining reaction of bFGF was also noted to be intensified outside the tumor nests and in invasive front of the tumor, both in moderately differentiated SCC and poorly differentiated squamous cell carcinoma. This observation supports the view of Saksela *et al*. [12] 1988 wherein bFGF stimulates the synthesis and release of proteases by endothelial cells, proteases like collagenases and plasminogen activator which may provide the proteolytic activity in the matrix which is necessary for locomotion and penetration of tumor cells into the surrounding tissues.

Thus the observation of the present study suggests bFGF may influence increased mitotic activity by increased expression by tumor cells i.e. increased immunohistochemical staining for bFGF in poorly differentiated SCC as compared to moderately and well-differentiated SCC. Further bFGF may have a pleotropic influence on tumor invasion by the release of proteolytic enzyme by endothelial cells, which is essential for tumor progression and metastasis.

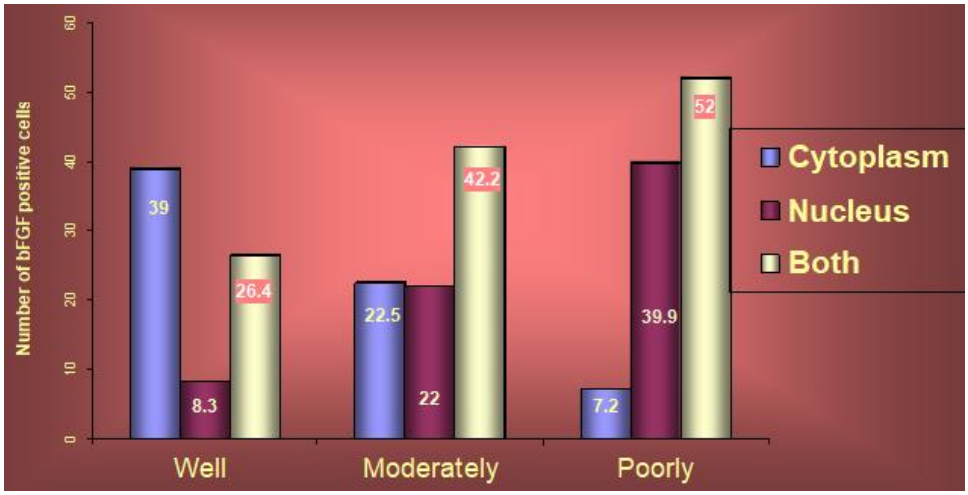
However further studies by increasing the sample with appropriate clinical correlation may be useful in detecting the relationship between bFGF and prognosis.



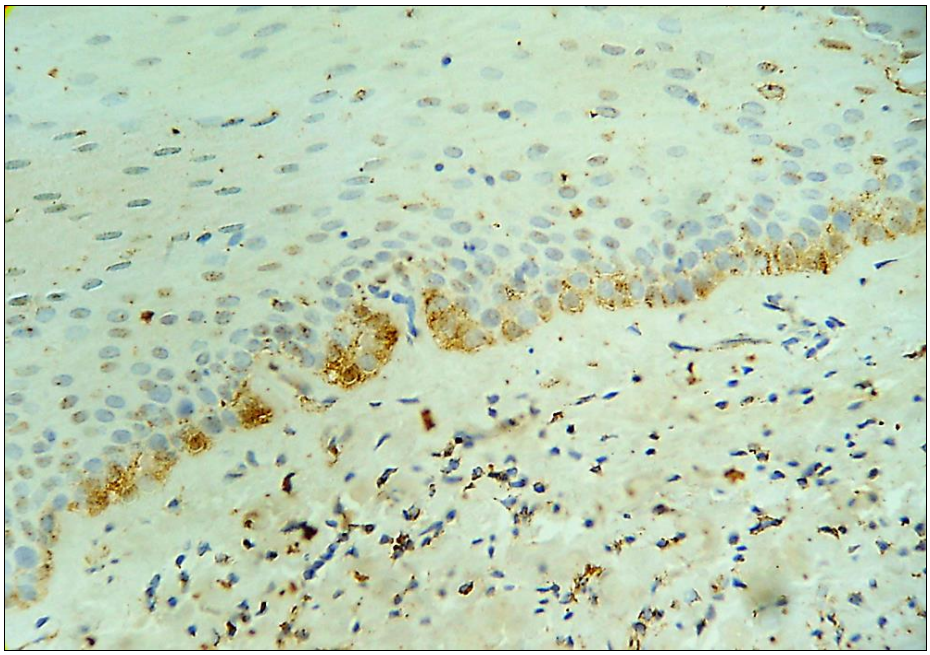
Bar Chart 1: Shows mean number of bFGF immunopositive cells in different grades of OSCCs.



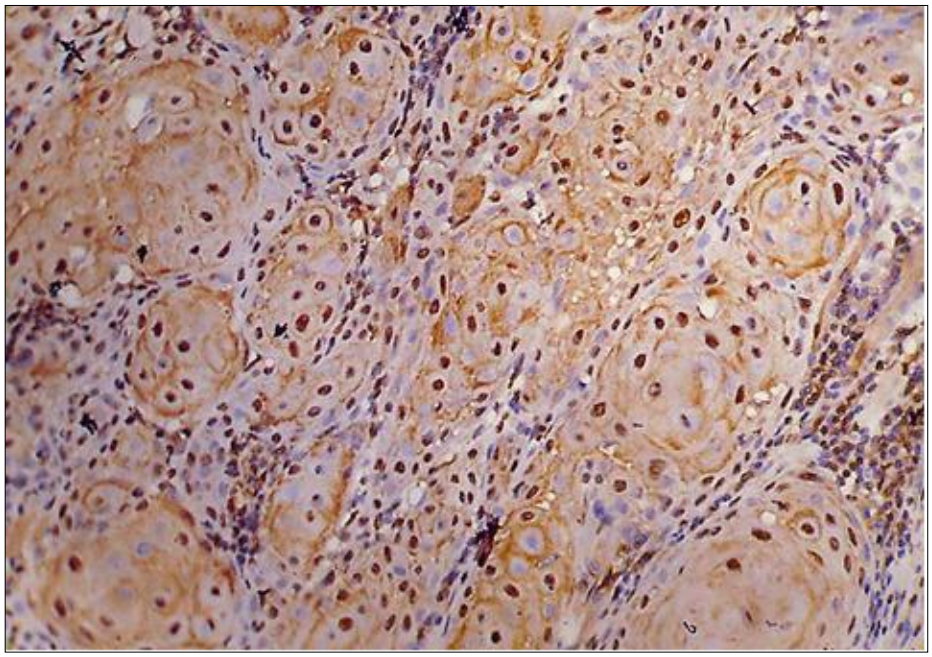
Bar Chart 2: shows intensity of expression of bFGF positive cells in different grades of OSCCs.



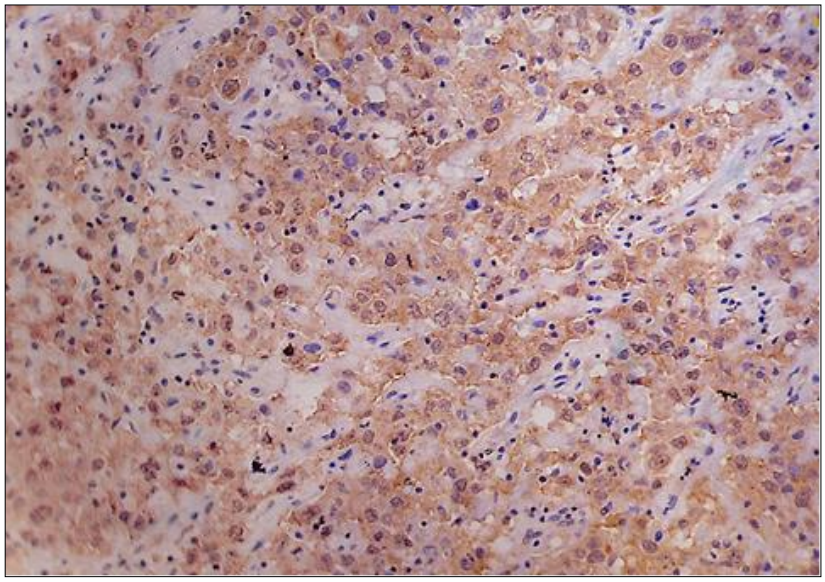
Bar Diagram 3: Shows mean number of tumor cells expressing bFGF in cytoplasm/ nucleus/ both in different grades of OSCCs.



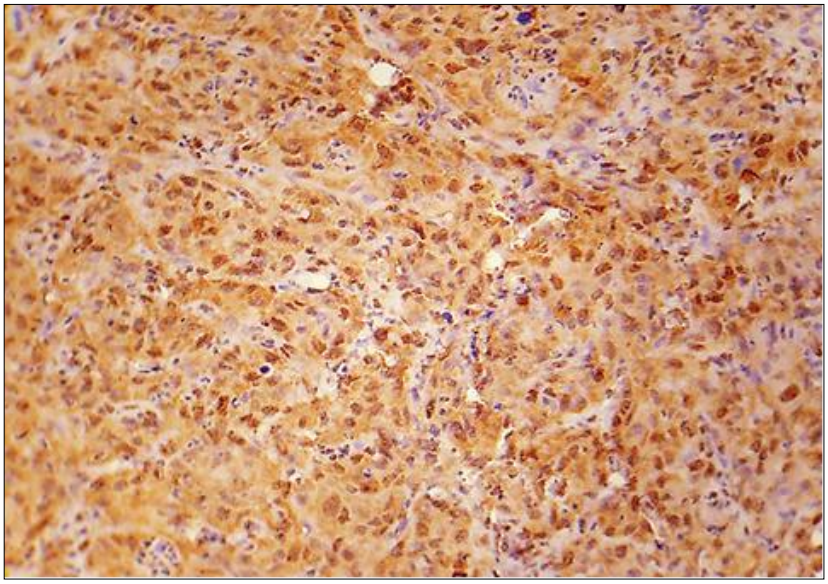
Photomicrograph 1: Normal buccal mucosa showing bFGF expression in the basal cell layer (40X).



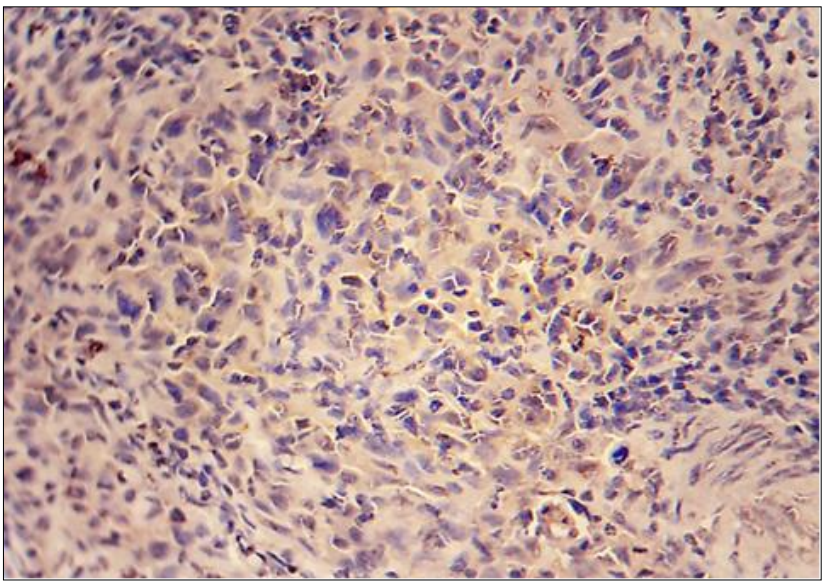
Photomicrograph 2: Well differentiated OSCC showing bFGF expression in the cytoplasm and few nucleus of tumor islands(25X).



Photomicrograph 3: Strands and sheets of tumor cells showing cytoplasmic and nuclear expression of bFGF in moderately differentiated OSCC (25X).



Photomicrograph 4: Showing sheets of tumor cells with intense bFGF immunopositivity of both cytoplasm and nucleus in poorly differentiated OSCC (25X).



Photomicrograph 5: Negative reagent control showing absence of bFGF immunopositivity in tumor cells in the absence of primary antibody (40X).

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