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VEGF and ki 67 expression in squamous cell carcinoma of the tongue: An immunohistochemical and computerized image analysis study

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SUMMARY

Over-expression of ki 67 and vascular endothelial growth factor (VEGF) is a frequent finding in squamous cell carcinoma (SCC) of the oral mucosa. The expression of VEGF and ki 67 proteins was studied in a cohort of 87 patients with primary, previously untreated SCC of the tongue, using computerized image analysis (CIA) in order to determine the potential prognostic significance of these factors. Immunohistochemical analysis was performed with monoclonal anti-ki 67 (MIB 1) and anti-VEGF antibodies. A digital image analysis assay was applied for the evaluation of the results. Using CIA, VEGF over-expression was observed in 24/87 (27.5%) of the examined cases and this finding correlated to the stage of the disease ($p = 0.05$). ki 67 was over-expressed in 49/87 (56.3%) of the cases and correlated to the size of the tumors ($p = 0.05$). Cox regression analysis showed that there was no prognostic significance associating VEGF protein expression to survival status of the examined patients ($p = 0.77$), whereas ki 67 over-expression was strongly correlated to poor prognosis ($p = 0.017$). The size of the primary tumors was also strongly correlated to survival status of the patients ($p = 0.024$), whereas stage of disease showed a borderline statistical significance ($p = 0.091$).

Introduction

Head and neck squamous cell carcinoma (HNSCC) is the sixth most common cancer worldwide with an estimated 700,000 cases reported annually. The role of anti-tumor immunity in relation to HNSCC has been intensely studied during the past years. It is now becoming clear that the complex interaction between HNSCC and immune system plays an important role in determining tumor growth and disease progression influencing locoregional and distant disease control and survival of the patient.[1,2] The neoplastic transformation of squamous epithelial cells is mediated by the deregulation of crucial molecular pathways. During carcinogenesis, normal epithelia accumulate a variety of genetic alterations due to viral infections or exposure to carcinogens such as tobacco and alcohol.[3,4] Hyperplasia, dysplasia, carcinoma in situ, and finally, invasive carcinoma are the representative stages in the progression of SCC.[5]

VEGF has been recognized as the key mediator of angiogenesis in different type of cancers.[6] VEGF gene is located on chromosome 6 (6p12). Its protein product – a member of the PDGF/VEGF growth factor family – is a glycosylated mitogen acting as an endothelial cell growth factor, promoter of cell migration, and inhibitor of apoptosis.[7] Although this cytokine normally induces endothelial proliferation and increases vascular permeability, it is also involved, by its over-expression, in tumor-associated angiogenesis.[8] Under hypoxic conditions, hypoxia-inducible factor-1 alpha (HIF 1α), a transcription factor responsible for the regulation of oxygen homeostasis, is activated through PI3 kinase–AKT and MAPK–ERK pathways, binding with its complementary factor HIF 1β to the promoters of genes that mediate glycolysis and angiogenesis.[9] Aberrant secretion of VEGF due to hypoxia, activation of oncogenes, and epidermal growth factor receptor (EGFR) or an abnormal hormonal activity, leads to an uncontrolled binding to specific receptors such as VEGFR-1 or VEGFR-2.[10] This process triggers a sequence of reactions including phosphorylation of intracellular tyrosine-kinase chains, and finally, leads to tumor angiogenesis.[11] In SCC of the tongue (TSCC), it was shown that over-expression of VEGF is associated with an aggressive phenotype (advanced stage, poor survival rates and minimal response to chemotherapy), but there are controversial results regarding the prognostic significance of its protein expression in contrast to other markers of angiogenesis, such as furin convertase or endoglin (CD 105).[12,13] It is also well known that excessive proliferation is

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correlated to carcinogenesis and affects the biological behavior in a variety of cancers.[14] ki 67 gene located on chromosome 10 (10q25) encodes a protein which is expressed in the nucleolus in all cell cycle phases except Go (arrest phase).[15] In fact, ki 67 expression increases as a cell progresses through the cell cycle, with highest expression being seen in G2/M phase cells.[16] In TSCC, ki 67 is frequently over-expressed but its prognostic significance is under investigation.[17–19]

In the current study, we co-evaluated the VEGF and ki 67 protein expression in TSCC in order to identify potential correlations to clinicopathological parameters.

Materials and methods

Study group

Eighty seven ($n = 87$) paraffin-embedded tissue samples of histologically confirmed primary TSCC deriving from equal number of patients, treated surgically in our department between 1998 and 2006 were included in the study. Fifty-eight ($n = 58$) patients were male with a mean age of 58.5 years and 29 ($n = 29$) female with a mean age of 62.5 years. Follow up ranged between 16 and 127 months (mean 77). Additionally, 10 sites ($n = 10$) of healthy looking oral epithelium, adjacent to the cancerous lesion and 10 sites ($n = 10$) of normal squamous epithelium were used as a control group. None of the patients had a familial history of cancer or other inherited cancer syndromes, so they were characterized as sporadic cases. The Hospital Ethical Committee consented to the use of these tissues for research purposes, according to Helsinki statement on medical protocols and ethics. The tissue samples were fixed in 10% neutral-buffered formalin. hematoxylin and eosin (H&E)-stained slides of the corresponding samples were reviewed for confirmation of histopathological diagnoses. All lesions were graded and staged according to the histological classification criteria of World Health Organization (WHO, 2002) and the TNM system for head and neck cancers. Clinicopathological data of the examined cases are presented in Table 1.

Table 1
Clinicopathological data of the examined TSCC

Characteristic		$n = 87$	%
Gender	Male	58	66.6
	Female	29	33.3
Tumor site	Posterior 2/3	60	68.9
	Anterior 1/3	27	31.1
Grade	1	38	43.6
	2	34	39.1
	3	15	17.2
Stage	I	16	18.4
	II	33	37.9
	III	24	27.6
	IV	14	16.1
Smoking	Yes	55	63.2
	No	32	36.8
Alcohol	Yes	33	37.9
	No	54	62.1
Tumor size (max diam)	T1 (0–2 cm)	20	22.9
	T2 (>2–4 cm)	47	54
	T3 (>4–6 cm)	10	11.5
	T4 (>6 cm)	10	11.5
Survival status	Alive no disease	40	45.9
	Dead from all causes	47	54
	Dead from disease	34	39.1

Antibodies and immunohistochemistry (IHC)

Monoclonal antibodies including anti-ki 67 (clone MIB 1-DAKO, Denmark) at dilution of 1:50, and anti-VEGF (clone VG1-Diagnostic Biosystems, CA, USA) at dilution of 1:40 were used. IHC protocol for those antigens was carried out on 3-μm-thick paraffin sections of the corresponding blocks. Two tissue sections initially deparaffinized in xylene and rehydrated via graded ethanol, were immunostained for each of the applied markers according to the EN Vision® (DAKO, Denmark) assay using an automated staining system (I 6000 – Biogenex, CA, USA) and according to corresponding manufacturer's instructions. This specific assay is based on a soluble, dextran-polymer system preventing endogenous biotin reaction and increasing the quality of stained slides. The sections, after peroxidase blocking, were incubated with primary antibody for 30–60 min, depending on the corresponding antibody, at room temperature and then incubated with horseradish peroxidase labeled polymer – HRP LP for 30 min. The antigen–antibody reaction was visualized using 3-3′diaminobenzidine tetrahydrochloride (DAB) as a chromogen substrate. Finally, tissue sections were slightly counterstained with hematoxylin for 30 s, dehydrated and mounted. In the control group slides, the primary antibodies were omitted. Diffuse membranous, cytoplasmic and nuclear staining pattern was considered to be acceptable for VEGF and ki 67 proteins, respectively (Fig. 1).

Computerized image analysis (CIA)

In order to evaluate the IHC results, we performed CIA by using a semi-automated system with the following hardware features: Intel Pentium IV, MATROX II Card Frame Grabber, Digital Camera

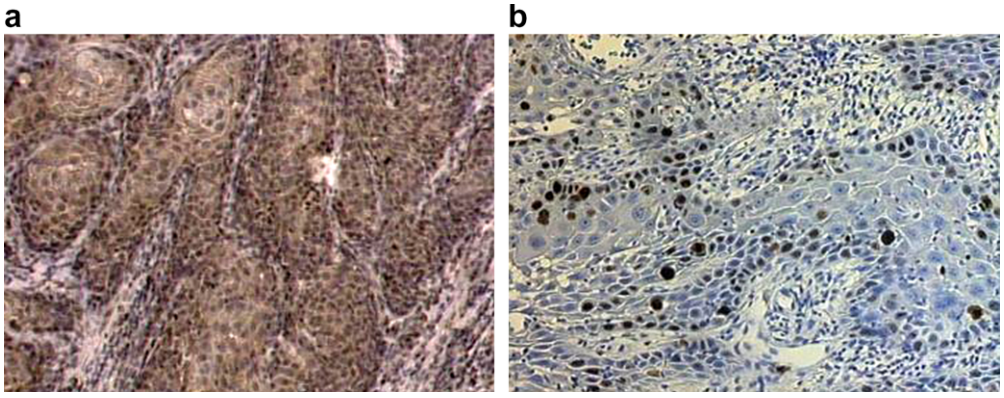

Figure 1 Over-expression of the examined markers. VEGF (a) and ki 67-MIB1 (b) demonstrate diffuse cytoplasmic/membrane and also nuclear immunostaining pattern, respectively (original magnification 200×).