ORIGINAL PAPER

Endoglin (CD105) and microvessel density in oral squamous cell carcinoma

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Abstract

Recent studies revealed that CD105 is intensively expressed in tumor vasculature, and may be an important prognostic indicator for the outcome in a number of malignancies. The aim of our study was to evaluate the CD105 expression and microvessel density in oral squamous cell carcinoma (OSCC). Nineteen surgical specimens with OSCC were immunohistochemical analyzed with CD105 (Endoglin). We determined the microvessel density (MVD) by "hot spot method". Endoglin was intensively expressed in vessels from the inner and invading front of all investigated OSCC. The highest value of MVD, about 30.89 ± 22.4, were record in peritumoral area of OSCC, since intratumoral MVD average was about 10.18 ± 4.7. We did not observe any significant association of MVD with age, sex, primary tumor's location, clinical stage or differentiation grade. In conclusion, CD105 expression is up-regulated in OSCC, and has a significant role in the development of such malignancies.

Keywords: angiogenesis, CD105, microvessel density, oral squamous cell carcinoma.

Introduction

Oral cancers is one of the major worldwide health problem due to high incidence, reduce survival rate and also to functional and cosmetic deficiency that accompany the disease and her treatment. Oral squamous cell carcinoma (OSCC) is the sixth commonest cancer in the world [1]. Although improvements have been achieved in surgical techniques, radiation therapy protocols, and chemo-therapeutic regimes, the overall 5-year survival rate for this disease remains at 50% and has not significantly improved in the past 30 years [2].

Angiogenesis is one of the major factors in the progression of OSCC [3–5]. Quantification of tissue angiogenesis can be made by counting microvasculars in a given area by immunohistochemical staining (microvascular density – MVD). During time were use a range of antibodies for the staining of vessel walls, most of which are aimed at, but not exclusively selective for, epitopes on endothelial cells. Endoglin (CD105), as a marker for neoangiogenesis, has certain advantages over the other commonly used panendothelial markers (CD31, CD34, and Factor VIII).

The purpose of our study was to study angiogenesis from OSCC by using endoglin (CD105) and also to assess the correlation of CD105-determined MVD with the most important clinicopathological characteristics of such tumors.

Material and methods

Tissues and clinical parameters

Nineteen formalin-fixed, paraffin-embedded archival tissue blocks of OSCC specimens were included in the current study. All these samples originated from complete resection material. By reviewed all Hematoxylin slides we selected the best section from each block showing central and peripheral areas of the tumor, avoiding areas with necrosis. The clinicopathological characteristics of these cases were reviewed from patients' records. Staging was established by IUCC system, and grading was performed according to WHO.

Immunohistochemistry

We performed immunostaining on formalin-fixed, paraffin embedded tissue sections using the Universal Immuno-enzyme Polymer (UIP) method (NICHIREI). Five-micrometer-thick serial sections were cut from each paraffin-embedded block. The sections were deparaffinized in xylene and rehydrated through graded concentrations of alcohol. As antigen retrieval, we used heat-induced epitope retrieval (HIER) technique by boiling tissue sections in 10 mM citrate buffer, pH 6.0 for 10–20 minutes, followed by cooling at RT for 20 minutes. Three-percent hydrogen peroxide in PBS for 15 minutes was then applied to block endogenous peroxidase activity. The sections were incubated overnight at 4^0C with anti-human CD105 rabbit

polyclonal antibody (diluted 1:50; Lab Vision Corporation, USA). Histofine Simple Stain MAX PO (MULTI) from NICHIREI was used for visualization of the expression of the antibody. Diaminobenzidine-tetrahydrochloride (DAB) was used as a chromogen. All sections were then counterstained with Hematoxylin. Negative-control staining was done by omitting the primary antibodies and as external positive control were used tonsil specimens.

Antibody expression and MVD quantification

Two observers (PD and SA) without knowledge of the clinical data performed evaluation of the staining. Quantitative analysis of the intratumoral microvessel density was performed according to Weidner N *et al.* [6]. For the determination of MVD, the three most vascular areas (hot spots) within a section were selected at ×40 magnification and counted under a light microscope (Nikon Eclipse 55i) with a 200-fold magnification. Microvessel density was defined as the number of CD105-positive vessels per optical field (an optical field corresponds to an examination area of 0.7386 mm^2). Each three area was counted twice and the arithmetical mean in each area was used to calculate the mean for each tumor section. This procedure was repeated for each tumor sample in three different areas: in the inner tumor area, at the invading tumor edges and in the resection tumor edges (at least three optical fields far away from invading tumor edge).

Statistical analysis

For means comparison we have used nonparametric Kruskal Wallis performed using EPI–INFO 2000 package. The variances were significantly different across the groups and this was the reason we did not used a parametric test. The variances were tested using Barttlet test. Both Barttlet and Kruskal Wallis tests were considered to give a significant difference if *p*-value was less than 0.005.

Results

Clinicopathologic data

The major clinicopathologic characteristics of the patients included in the study are diagrammatic presented in Table 1. The median age of investigated patients was 66 (range 40–84). Related to sex, the majority of patients were male 14 (74%). Topographically, the most common location was at the lips with 12 cases (68%), followed by tongue with 5 cases (32%).

The investigation of risk factors revealed that 95% of patients had records of excessive alcohol consumption, 63% of patients were heavy smokers and 58% of patients had a double risk exposure to alcohol and tobacco. According to clinical stage, 14 of the patients (74%) had advanced (III and IV) clinical stage (Table 1).

There were 11 patients (58%) who had histological well-differentiated tumors (Figure 1a), four (21%) who had tumors with moderate differentiation (Figure 1b) and with four (21%) of the tumors being poorly differentiated (Figure 1c).

Table 1 – ***Correlation of clinicopathological characteristics with microvessel density***

		No. of patients (%)	Microvessel density			*p*-value
			Invading edge	*Inner area*	*Resection edge*	
		19	30.89 ± 22.4	22.5 ± 11.9	10.18 ± 4.7	0.000583
Sex	Male	14 (74)	34.1 ± 25.0	25.0 ± 12.4	10.7 ± 4.3	0.0023
	Female	5 (26)	21.8 ± 9.2	15.3 ± 7.3	8.6 ± 5.8	0.060
Age	<65 year	9 (47)	40.5 ± 28.7	29.0 ± 12.8	11.9 ± 2.9	0.010
	>65 year	10 (53)	22.2 ± 9.6	16.6 ± 7.6	8.6 ± 5.5	0.0038
Topography	Lips	13 (68)	31.7 ± 27.0	22.2 ± 14.1	10.1 ± 5.2	0.0143
	Tongue	5 (32)	27.4 ± 5.5	21.2 ± 4.6	10.1 ± 4.3	0.0044
Risk factors	Smokers (Sm)	12 (63)	35.0 ± 26.9	25.0 ± 13.5	9.8 ± 3.9	0.0052
	Drinkers (Drink)	18 (95)	30.8 ± 23.0	22.6 ± 12.3	10.3 ± 4.7	0.0011
	Sm + Drink	11 (58)	35.3 ± 28.2	25.4 ± 14.1	10.1 ± 4.0	0.011
TNM stage	II	5 (26)	26.2 ± 4.9	18.7 ± 6.3	9.3 ± 5.7	0.0103
	III	11 (58)	33.9 ± 29.4	24.6 ± 14.8	10.3 ± 4.7	0.0238
	IV	3 (16)	27.6 ± 4.8	21.1 ± 7.5	11.0 ± 4.1	0.0509
Histological grade	poorly	4 (21)	24.6 ± 10.1	18.1 ± 7.5	9.1 ± 4.7	0.043
	moderate	4 (21)	18.8 ± 12.1	14.7 ± 9.4	9.2 ± 6.6	0.3932
	well	11 (58)	36.8 ± 27.2	26.2 ± 13.5	10.4 ± 4.6	0.0060

CD105 expression

CD105-positive vascular endothelial cells were clearly identified by their brown staining. In normal oral mucosa and in the resection tumor edges microvessels rarely expressed CD105 and staining was faint and weak. These vessels were mainly located immediately underneath the epithelium, and were regularly distributed and had regular courses and cross-sectional shapes (Figure 1d). Neoplastic, inflammatory, and mesechymal cells were negative for CD105. We also notice a moderate CD105 staining in skeletal muscle cells on specimens with muscle invasion and a positive reaction in some keratin pearls.

CD105 stained intensively intratumoral and peritumoral (at the tumor invasion front) microvessels. The tumor vessels are mostly of aberrant morphology, tortuous, without clear lumen and with

large gaps between endothelial cells (Figure 1, e and f). In addition, they varied greatly in size, predominating microvessels of small-caliber (smaller than 15 µm diameter) (Figure 2a). The highest density is near to the carcinomatous proliferations and in the inflammatory tumor stromal areas (Figure 2b).

In two cases, the tumoral microvessel architecture was more complex as a vascular network with multiple vascular loops and increased vessel tortuosity (Figure 2c). In many tumoral areas, we identify individual CD105 positive cells (Figure 2d). Typically, at the invasion front of OSCC we observed the greatest CD105 positive vessels with irregular courses and elongated cross-sectional shapes (Figure 2, e and f). These aspects are more obvious in areas with inflammatory reaction.

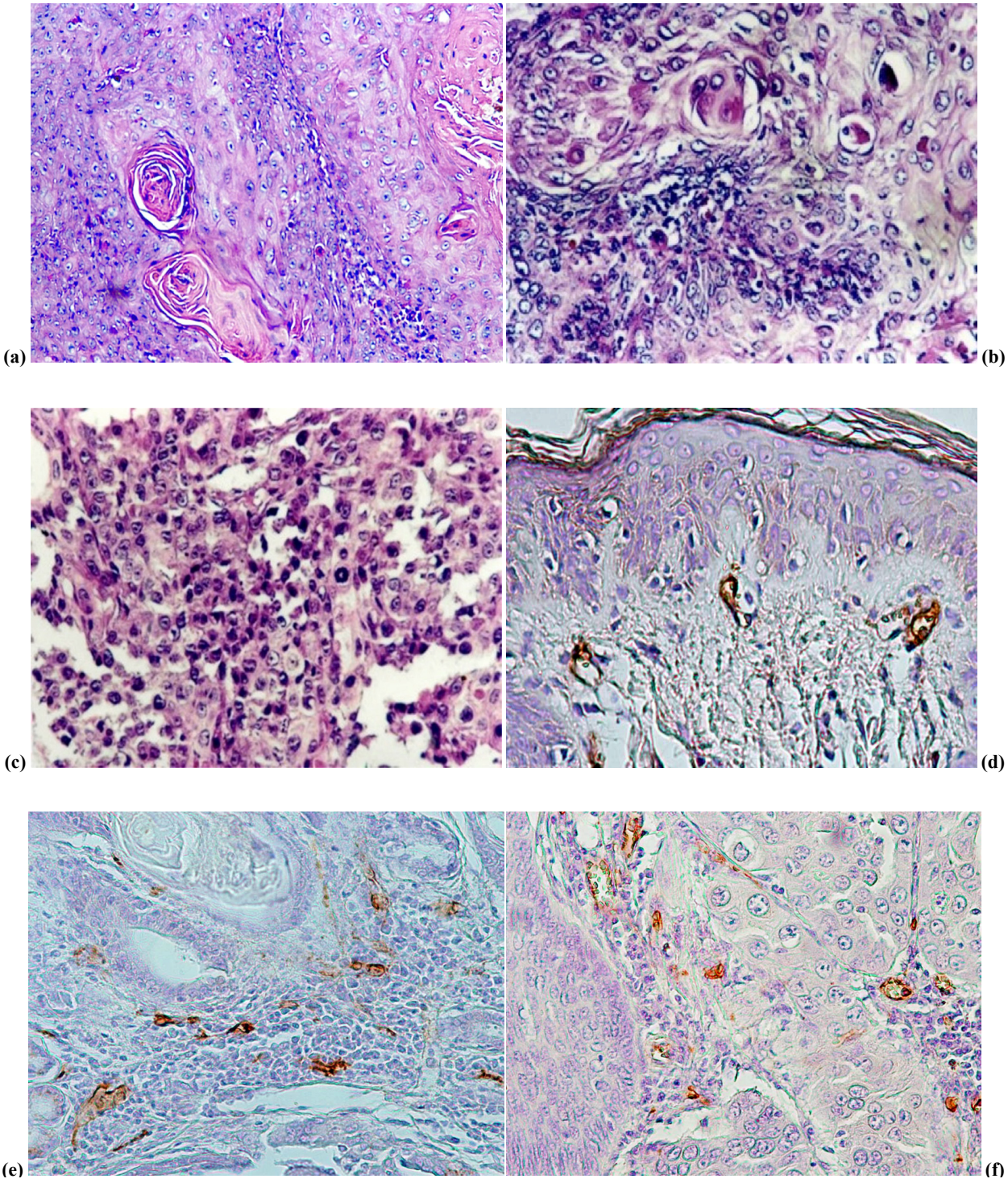

Figure 1 – ***(a) Typical well-differentiated squamous cell carcinoma of the tongue (HE staining, ×100); (b) Moderate differentiated squamous cell carcinoma of the lower lip (HE staining, ×200); (c) Poor differentiated squamous cell carcinoma of the tongue (HE staining, ×200); (d) CD105 positive vessels located immediately underneath the normal oral epithelium, ×200; (e, f) CD105 positive vessels adjacent and between tumoral proliferations with aberrant morphology, ×200***

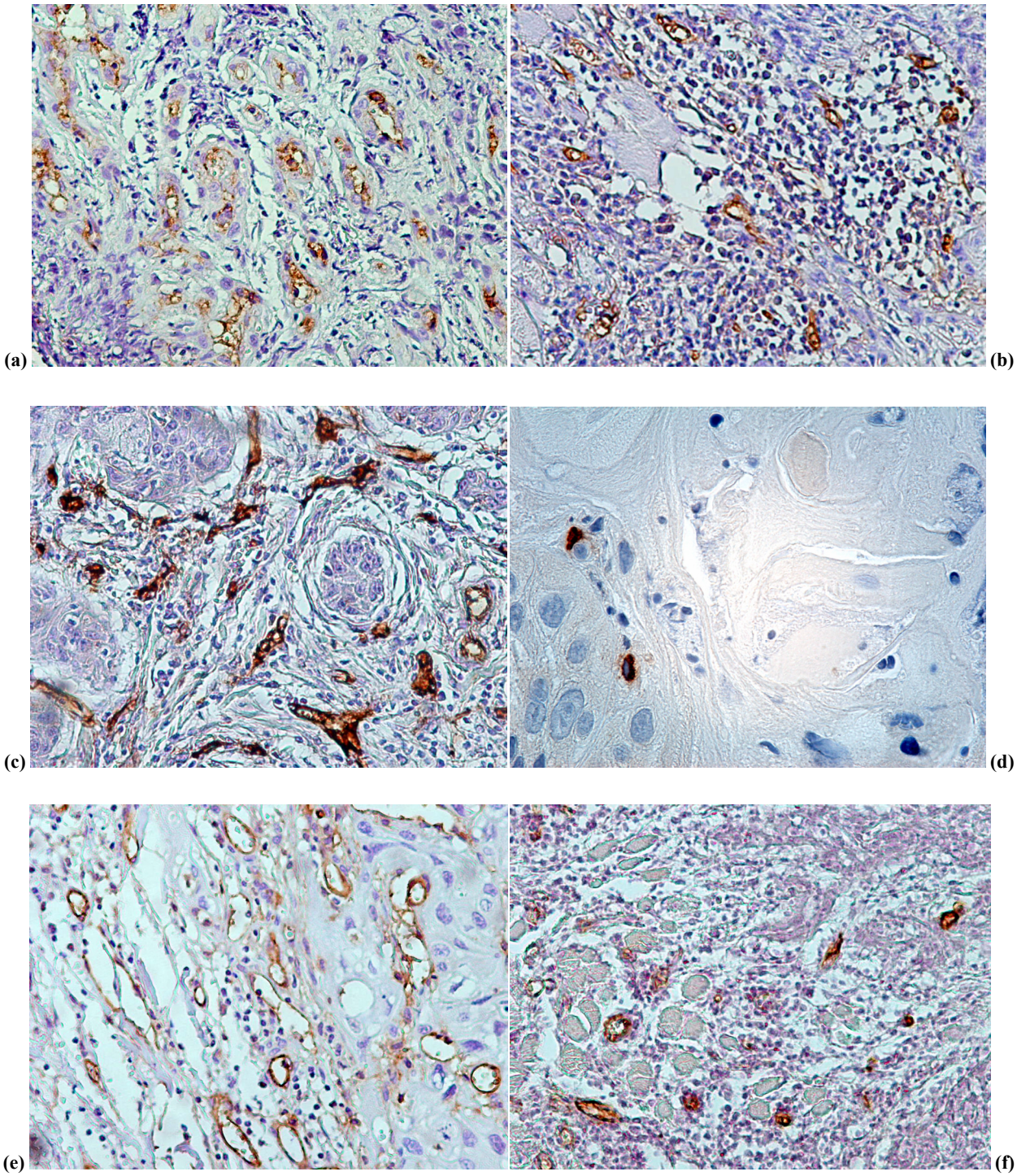

Figure 2 – ***(a) CD105 positive tumoral vessels of small-caliber, ×200; (b) CD105 positive vessels in the inflammatory tumor stromal areas, ×200; (c) CD105 positive tumoral vessels with complex architecture, ×200; (d) intratumoral individual CD105 positive cells, ×600; (e, f) CD105 positive vessels at the invasion front of OSCC with irregular courses and elongated cross-sectional shapes, ×200***

Evaluation of MVD and statistical data

Microvessel density varied among tissue samples from 10 to 170 (median 49). In accordance with the location of neoangiogenesis we notice a variability of MVD. Thus, we found that highest value of MVD, about 30.89 ± 22.4, were record in peritumoral area of OSCC, since intratumoral MVD average was about 10.18 ± 4.7. The correlation of the microvessel density with clinicopathologic parameters is summarized in Table 1. We did not observe any significant association of MVD with age, sex, primary tumor's location, clinical stage or differentiation grade.

Discussion

One of the crucial events in tumorigenesis, as was experimental proved [7] is achievement of nutrition sanguine network basis on new blood vessels formation from the pre-existent one angiogenesis.

In many experiments becoming classically, Folkman J [8] proved that solid tumors do not grow up more than 2–3 mm in diameter without any self-induction of blood supply.

Many studies used pan-endothelial markers (CD34, CD31, and von Willebrand factor) with low sensitivity and specificity in evaluation of intratumoral vessels density; therefore, the results were conflicting and contradictory [9–13]. The CD105 (endoglin) seems to reacts specifically with angiogenic endothelial cells and consequently an increasing amount of studies proved a significant association of high MVD with poor prognosis in various neoplasms [14–19].

Our study proves that endoglin stained intensively intratumoral and invading tumoral front vessels with high sensitivity, whereas vessels in non-neoplastic tissue were not or were weakly expressing CD105. This confirms previous observations that endoglin reacts specifically with angiogenic endothelial cells from the tumor [20, 21].

Quantification of microvessel density with CD105 in our study reveals that the highest density was present at the invading tumoral front, followed by intratumoral location. This confirms previous observations that CD105 expression declines as we move away from the invasive front of the tumor [21].

The statistical analysis in our study did not show any significant association of MVD with age, sex, primary tumor's location, clinical stage or differentiation grade. This fact is owned mainly to small number of investigated cases. There have been a few numbers of studies evaluating the prognostic significance of microvessel density with CD105 in head and neck squamous cell carcinoma [20–23]. In all of these studies, a significant association of high MVD with poor prognosis was observed, and investigators agree that assessment of microvessel counts with endoglin seems to be better than the use of any other pan-endothelial marker.

However, for a reliable and reproducible assessment of MVD with CD105 in OSCC, we must keep in mind that there are many difficulties, such as inter-observer variability for the identification and selection of the "hot spots", differences between immunohistochemical protocols, selection of paraffin block, section within the block, and counting procedure. Moreover measuring microvessel density by examining small sections of archival tissue at a single point in time does not necessarily represent the angiogenic status of the tumor.

Therefore, more studies with validation procedures and quality control protocols are needed to confirm the importance of MVD (determined with CD105) as a prognostic factor.

Conclusions

The results suggest that CD 105 (endoglin) is strongly expressed in blood vessels of OSCC compared with normal healthy oral mucosa. Endoglin seems to be a more sensitive and specific marker for microvessel highlights than the usually pan-endothelial markers and have a significant role in the development of OSCC. For these reasons, CD105 may represent a novel target for prognostic purposes and for novel therapeutic approaches.

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References

[1] PARKIN D. M., BRAY F., FERLAY J., PISANI P., *Estimating the world cancer burden: Globocan 2000*, Int J Cancer, 2001, 94(2):153–156.

[2] COOPER J. S., PAJAK T. F., FORASTIERE A. A., JACOBS J., CAMPBELL B. H., SAXMAN S. B., KISH J. A., KIM H. E., CMELAK A. J., ROTMAN M., MACHTAY M., ENSLEY J. F., CHAO K. S., SCHULTZ C. J., LEE N., FU K. K.; RADIATION THERAPY ONCOLOGY GROUP 9501/ INTERGROUP, *Postoperative concurrent radiotherapy and chemotherapy for high-risk squamous-cell carcinoma of the head and neck*, N Engl J Med, 2004, 350(19):1937–1944.

[3] KUNIYASU H., TAHARA E., Molecular pathology of esophageal cancer. In: KELSEN D. P., DALY J., KERN S. E., LEVIN B., TEPPER J. E. (eds), *Gastrointestinal Oncology: Principles and Practice*, Lippincott Williams & Wilkins, Philadelphia, 2001.

[4] LÓPEZ-GRANIEL C. M., TAMEZ DE LEÓN D., MENESES-GARCÍA A., GÓMEZ-RUIZ C., FRIAS-MENDIVIL M., GRANADOS-GARCÍA M., BARRERA-FRANCO J. L., *Tumor angiogenesis as a prognostic factor in oral cavity carcinomas*, J Exp Clin Cancer Res, 2001, 20(4):463–468.

[5] SCHLIEPHAKE H., *Prognostic relevance of molecular markers of oral cancer – a review*, Int J Oral Maxillofac Surg, 2003, 32(3):233–245.

[6] WEIDNER N., CARROLL P. R., FLAX J., BLUMENFELD W., FOLKMAN J., *Tumor angiogenesis correlates with metastasis in invasive prostate carcinoma*, Am J Pathol, 1993, 143(2):401–409.

[7] HAHN W. C., COUNTER C. M., LUNDBERG A. S., BEIJERSBERGEN R. L., BROOKS M. W., WEINBERG R. A., *Creation of human tumor cells with defined genetic elements*, Nature, 1999, 400(6743):464–468.

[8] FOLKMAN J., Tumor angiogenesis. In: MENDELSOHN J., HOWLEY P. M., ISRAEL M. A., LIOTTA L. A. (eds), *The molecular basis of cancer*, W. B. Saunders, Philadelphia, 1995, 206–232.

[9] AEBERSOLD D. M., BEER K. T., LAISSUE J., HUG S., KOLLAR A, GREINER R. H., DJONOV V., *Intratumoral microvessel density predicts local treatment failure of radically irradiated squamous cell cancer of the oropharynx*, Int J Radiat Oncol Biol Phys, 2000, 48(1):17–25.

[10] GALLO O., MASINI E., BIANCHI B., BRUSCHINI L., PAGLIERANI M., FRANCHI A., *Prognostic significance of cyclooxygenase-2 pathway and angiogenesis in head and neck squamous cell carcinoma*, Hum Pathol, 2002, 33(7):708–714.

[11] KYZAS P. A., CUNHA I. W., IOANNIDIS J. P., *Prognostic significance of vascular endothelial growth factor immunohistochemical expression in head and neck squamous cell carcinoma: a meta-analysis*, Clin Cancer Res, 2005, 11(4):1434–1440.

[12] PAZOUKI S., CHISHOLM D. M., ADI M. M., CARMICHAEL G., FARQUHARSON M., OGDEN G. R., SCHOR S. L., SCHOR A. M., *The association between tumour progression and vascularity in the oral mucosa*, J Pathol, 1997, 183(1):39–43.

[13] SALVEN P., HEIKKILÄ P., ANTTONEN A., KAJANTI M., JOENSUU H., *Vascular endothelial growth factor in squamous cell head and neck carcinoma: expression and prognostic significance*, Mod Pathol, 1997, 10(11):1128–1133.

[14] BREWER C. A., SETTERDAHL J. J., LI M. J., JOHNSTON J. M., MANN J. L., MCASEY M. E., *Endoglin expression as a measure of microvessel density in cervical cancer*, Obstet Gynecol, 2000, 96(2):224–228.
[15] DALES J. P., GARCIA S., CARPENTIER S., ANDRAC L., RAMUZ O., LAVAUT M. N., ALLASIA C., BONNIER P., CHARPIN C., *Long-term prognostic significance of neoangiogenesis in breast carcinomas: comparison of Tie-2/Tek, CD105, and CD31 immunocytochemical expression*, Hum Pathol, 2004, 35(2):176–183.
[16] LI C., GARDY R., SEON B. K., DUFF S. E., ABDALLA S., RENEHAN A., O'DWYER S. T., HABOUBI N., KUMAR S., *Both high intratumoral microvessel density determined using CD105 antibody and elevated plasma levels of CD105 in colorectal cancer patients correlate with poor prognosis*, Br J Cancer, 2003, 88(9):1424–1431.
[17] MINEO T. C., AMBROGI V., BALDI A., RABITTI C., BOLLERO P., VINCENZI B., TONINI G., *Prognostic impact of VEGF, CD31, CD34, and CD105 expression and tumour vessel invasion after radical surgery for IB–IIA non-small cell lung cancer*, J Clin Pathol, 2004, 57(6):591–597.
[18] SAAD R. S., JASNOSZ K. M., TUNG M. Y., SILVERMAN J. F., *Endoglin (CD105) expression in endometrial carcinoma*, Int J Gynecol Pathol, 2003, 22(3):248–253.
[19] YAGASAKI H., KAWATA N., TAKIMOTO Y., NEMOTO N., *Histopathological analysis of angiogenic factors in renal cell carcinoma*, Int J Urol, 2003, 10(4):220–227.
[20] KYZAS P. A., AGNANTIS N. J., STEFANOU D., *Endoglin (CD105) as a prognostic factor in head and neck squamous cell carcinoma*, Virchows Arch, 2006, 448(6):768–775.
[21] SCHIMMING R., MARMÉ D., *Endoglin (CD105) expression in squamous cell carcinoma of the oral cavity*, Head Neck, 2002, 24(2):151–156.
[22] MARIONI G., MARINO F., GIACOMELLI L., STAFFIERI C., MARIUZZI M. L., VIOLINO E., DE FILIPPIS C., *Endoglin expression is associated with poor oncologic outcome in oral and oropharyngeal carcinoma*, Acta Otolaryngol, 2006, 126(6):633–639.
[23] MARTONE T., ROSSO P., ALBERA R., MIGLIARETTI G., FRAIRE F., PIGNATARO L., PRUNERI G., BELLONE G., CORTESINA G., *Prognostic relevance of CD105+ microvessel density in HNSCC patient outcome*, Oral Oncol, 2005, 41(2):147–155.

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