ORIGINAL PAPER

Angiogenesis in oral squamous cell carcinoma

Angiogenesi nel carcinoma squamocellulare della cavità orale

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Key words
Oral cavity • Carcinoma • Prognosis • Angiogenesis

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Summary

Many retrospective studies have recently shown that microvessel density could represent a valid independent prognostic factor for overall survival and disease-free survival for primary tumours. The fact that oral tumours with a higher microvessel density showed a tendency to present distant metastasis and a bad prognosis, suggested that angiogenetic activity would play a pivotal role also in oral carcinomas, exerting a negative effect on the clinical course and representing an independent negative prognostic factor also for this type of tumour. Based on these results, microvessel density was evaluated, in the present study, in 64 cases of squamous cell carcinoma of the oral cavity, using immunohistochemical analysis with anti-CD34 monoclonal antibody. Possible correlations between microvessel density and clinico-pathological parameters were analysed, such as: age, sex, tumour localization and size, TNM stage and histological grading. Statistical analysis has shown that microvessel density differs in the 3 histological groups (G1, G2, G3) ($p = 0.0331$), and between node-positive and node-negative patients ($p < 0.0001$). No statistical correlation was observed between microvessel density and other clinical parameters such as age, sex, tumour site and size.

Riassunto

Nell'ultimo decennio alcuni studi retrospettivi hanno mostrato che la densità di microvascolarizzazione (DMV) può costituire un buon marker prognostico indipendente in alcune neoplasie umane. L'osservazione di una maggiore densità microvascolare nelle neoplasie della cavità orale che presentavano una prognosi infausta e una tendenza alla metastatizzazione, ha suggerito che la attività angiogenetica possa svolgere un ruolo di primo piano anche nei carcinomi orali, influenzandone negativamente il decorso clinico e rappresentando un fattore prognostico negativo indipendente per questo tipo di neoplasie. Alla luce di quanto emerso dalla letteratura, nel presente studio abbiamo esaminato la densità della microvascolarizzazione in 64 casi di carcinoma squamocellulare della mucosa orale, valutando le eventuali correlazioni con altri parametri clinico-patologici come l'età, il sesso, la sede e la dimensione del tumore, lo stadio TNM ed il grading istologico. La valutazione della vascolarizzazione è stata fatta mediante colorazione immunoistochimica per il CD34. L'analisi statistica effettuata ha evidenziato una differenza significativa, in termini di densità di microvascolarizzazione, correlando i tre gruppi istologici (G1, G2 e G3) ($p = 0,0331$) e confrontando il gruppo di pazienti con linfonodi positivi, con il gruppo di pazienti che presentavano linfonodi negativi ($p < 0,0001$) al momento della diagnosi. Non sono state osservate differenze statisticamente significative correlando la DMV con i vari parametri clinici (età, sesso, sede del tumore) e dimensioni tumorali (T).

Introduction

Squamous cell carcinoma (SCC) of the oral cavity represents one of the ten most frequent tumours, and shows a difference in geographic incidence: 3-6% in Western countries and 30% in Eastern countries. Despite recent diagnostic and therapeutic improvements, prognosis of patients presenting this type of tumour still remains very poor, probably on account of the different biological behaviour of these tumours, which show a variable aggressiveness independently of clinico-pathological parameters of certain prognostic importance such as T and N stage and histological grading [1]. For this reason, the importance of new biological markers (oncogenes, growth factor, cell cycle and angiogenesis-related molecules) able to predict tumour aggressiveness and response to treatment is increasing. Growing tumours require increased blood supply in order to obtain sufficient oxygen and nutrients and to discard waste products. It has been demonstrated, in many experimental models, that carcinogenesis is associated with new vessel formation

(angiogenesis) [2] and that solid tumours need a rich vascular network in order to reach a clinically evident size and also to acquire the ability to metastasize. For this reason, tumours with a higher vessel density seem to show a growth advant-age and to progress earlier than tumours with a poor vascular background [3]. In the last decade, many retrospective studies have shown that microvessel density (MVD) could represent a valid independent prognostic factor for overall survival and disease-free survival in primary tumours showing a significant correlation between high intratumoural micro-vascularization, the presence of metastasis and poor prognosis, not only in breast cancer but also in other types of solid tumours [4-7]. On the other hand, few studies have suggested a correlation between a high degree of vascularization and better prognosis in solid tumours [8 9]. These contrasting results could be due to the lack of a standardized method for evaluating tumoural angiogenesis. The greatest difficulty in the study of angiogenesis, in human tumours, is the lack of direct methods to evaluate angiogenetic activity in neoplastic tissues. One of the most used indirect methods was introduced by Weidner et al. [6] and consists in counting routine immunohistochemically stained vessel wall profiles in tissue sections of tumours, thus obtaining the tumoural microvessel density (MVD): this finding would represent the angiogenetic potential of the neoplasia [10]. As is well known, the first step consists in the identification, at low magnification, of a domain of high staining intensity, the so-called neovascular hot-spot; the exact MVD is subsequently determined at high power magnification. Using this method, a higher MVD has been demonstrated in oral tumours with poor prognosis and high metastatic potential. This observation has suggested that angiogenetic activity could exert a key role also in oral carcinomas, with a negative effect upon the clinical course, thus representing an independent negative prognostic factor also in this type of cancer [11]. Albeit, the results of recent studies focusing on this aspect are controversial [12-16]. Aim of the present study was to evaluate angiogenetic activity in ACC of the oral cavity, by assessing MVD and to demonstrate possible correlations with the clinico-pathological parameters, such as age, sex, site and size of the tumour, TNM staging and histological grading.

Material and methods

For this retrospective study, 64 patients with primitive epidermoidal carcinoma of the oral cavity were studied. All had been submitted to surgical treatment at the Maxillofacial Surgical Unit of the Azienda Ospedaliera-Ospedali Riuniti "Umberto I – Lancisi-Salesi" of Torrette (Ancona, Italy) between 2000 and 2002. All patients underwent surgical removal of the primitive site of the tumour, with excision of lymph nodes. At the time of diagnosis, surgical specimens, from each patient, had been fixed in formalin and sent to the Anatomic Pathology Institute of the Polytechnic University of Marche where they were paraffin-embedded for histological evaluation. For the purposes of the present investigation, all histological slides of the patients under study were retrieved from the Archives of the Anatomic Pathology Institute. Haematoxylin-Eosin (H&E) stained sections were re-examined in order to identify the most representative areas of the lesion and to select the most appropriate blocks for the immunohistochemical analysis. Briefly, 4 µm sections of representative blocks were deparaffinized with xylene and rehydrated with a graded alcohol series. Endogenous peroxidase was blocked with incubation for 5 min in 3% hydrogen peroxide. Microwave pre-treatment for 20 minutes at 750W, with citrate buffer pH 6 was used as antigen retrievement. The sections were then cooled for 20 minutes at room temperature and incubated with anti-CD34 monoclonal antibody (BI-3C5; 1:100, DAKO) for 12 hours. Immunolabelling of CD34 was detected using an LSAB positive peroxidase kit (DAKO) applied for 20 minutes. 3-3 diaminobenzidine was used as chromogen and the sections counterstained with Mayer Haematoxylin. The immunohistochemical reaction was considered positive in cases showing brownish dots in the cytoplasm of the endothelial cells. MVD was quantitatively assessed by one of us (C.R). Ibas-At imaging analysis method was used to evaluate vessel number. The microvessel count was performed analysing the peripheral areas of tumour infiltration, previously identified at low magnification. MVD was expressed as the mean number of microvessels in 1 mm^2 at high power field (400x). The following clinico-pathological parameters were recorded in each patient: age, sex, site and size of tumour, TNM staging and histological grading and possible correlations with MVD. Statistical analysis was performed with non parametric Mann-Whitney U, Kruskal-Wallis and Chi-square tests. A p value of $p<0.05$ was considered significant.

Results

The study population comprised 49 males and 15 females, mean age 66.6 years (range: 43-87; SD = 10.8). Tumours were most frequently located on the tongue (42.2%), followed by gums (17.2%) and cheek mucosa (15.6%) (Fig. 1). Node metastases were present in 27/64 patients (42.2%). The histological grade of differentiation was G1 in 19 cases (29.7%), G2 in 22 (34.4%) and G3 in 23 (35.9%). Thirteen cases (20.3%) were at pathological stage T1, 20 (31.2%) at T2, 14 (21.9%) at T3 and 17 (26.6%) at T4. Mean MVD was

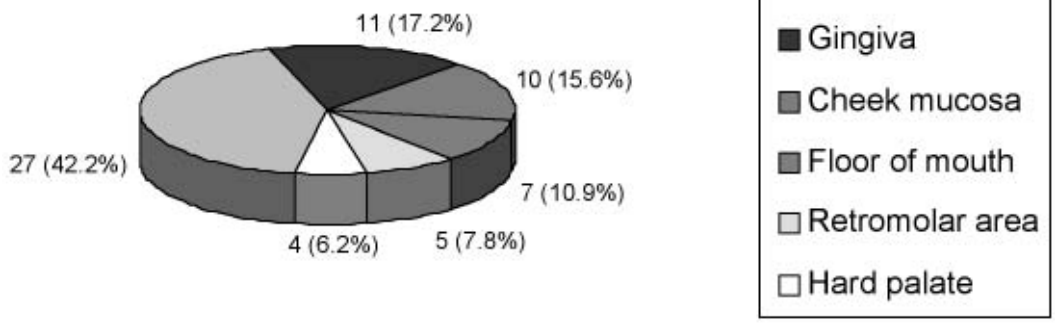

Fig. 1. Site distribution of oral squamous cell carcinoma.

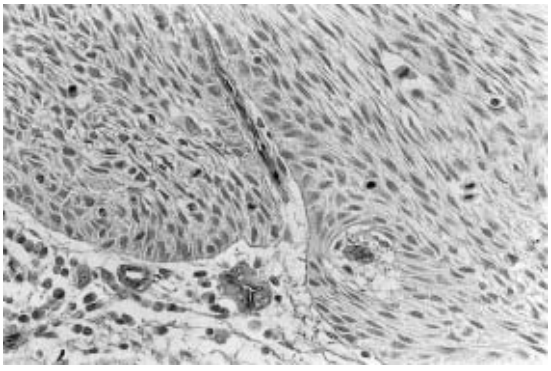

Fig. 2. Immunohistochemical vascular staining in peripheral area of the tumour in a node-negative patient.

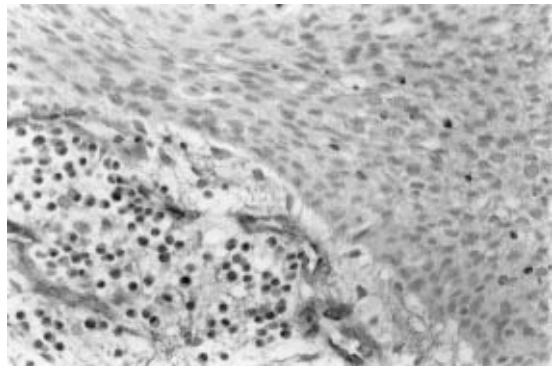

Fig. 3. Immunohistochemical vascular staining in peripheral area of the tumour in a node-positive patient.

26.66 (range: 10.5-39.5, SD = 6.8). MVD appeared to increase with the increase in histological grading, on account of the higher MVD detected in tumours showing a lower degree of differentiation. G1, G2 and G3 cases showed a statistically different MVD with a mean value of 20.16, 21.06 and 26.14, respectively ($p = 0.0331$). In patients without node metastasis (Fig. 2) mean MVD appeared to be statistically lower than in cases with node metastasis (Fig. 3) (19.22 *vs.* 27.73, $p<0.0001$). Mean MVD values and relationship with histological grading and nodal status are shown in Table I. No statistical relationship was found between MVD and age, sex and tumour location.

Discussion and conclusions

Clinical and experimental evidence has demonstrated that new vessel formation is an essential condition for tumour growth and progression [3 17 18] since neoplastic lesions with a more abundant microvascular background show a growth advantage compared to tumours with a less developed microvascular network. Intratumoural neoformed vessels give the tumour the possibility to metastasize, representing a key factor in defining the overall aggressiveness and the prognosis of the tumour itself [4 19]. Following the studies of Folkman and Weidner [3 4 6], microvessel density, an indirect marker of tumour angiogenetic activity, appears to be of prognostic value in breast carcinoma [6 20], non-small cell lung carcinoma [5], prostatic carcinoma [21] and melanoma [22]. As far as concerns SCC of the oral cavity, studies focused on the possible prognostic value of MVD in this type of neoplasia, have led to conflicting results [13-16]. Pazouki et al. [16] showed an increase in vascularization during transformation from normal oral mucosa, through dysplasia, to in situ and infiltrating carcino-

Table I. Mean MVD related to histological grading and nodal status.

		Cases (%)	Mean MVD (range)	
Grading	G1	19 (29.7)	20.16 ± 6.19 (10.5-32.3)	
	G2	22 (34.4)	21.06 ± 5.68 (12.3-32.3)	p = 0.0331
	G3	23 (35.9)	26.14 ± 7.06 (13.2-39.5)	
Nodal status	Negative	37 (57.8)	19.22 ± 5.13 (10.5-29.1)	
	Positive	27 (42.2)	27.73 ± 5.73 (17.7-39.5)	p < 0.0001

ma, supporting the pivotal role of angiogenesis in malignancy progression in oral carcinoma. Many studies have shown a correlation between intratumoural MVD and prognosis in oral carcinoma, demonstrating a relationship between high MVD and tumour size, relapse rate [23-25] and presence of node metastases [15 26 27]. In agreement with these results, our study showed that the neoangiogenetic process is strictly related to the histological grade of differentiation and to the presence of loco-regional metastases in oral carcinoma. Recently, Hannen et al. [11] evaluated not only the number of new intratumoural vessels, but also their diameter, observing that vessels with a lower cross-sectional area were found primarily in non-metastasizing lesions while those with a higher cross-sectional area appeared to be mostly localized in cases with nodal metastases. From these observations, it might be suggested that MVD is a useful marker to identify those patients with a more aggressive tumour, for whom a more adequate therapeutic approach should be taken into consideration. On the other hand, not all Authors have shown a statistical relation between MVD and oral carcinoma prognosis, thus denying the usefulness of this parameter as a prognostic marker in this type of neoplasia [13 14 28]. These conflicting results are probably due to absence of a standardized protocol for the evaluation of tumoural angiogenesis and to the high vascularization of the oral mucosa, which could create difficulties in distinguishing between pre-existing and newly formed intratumoural vessels [16 29]. With regard to the former problem, the major difficulty concerns the low reproducibility of the methods used for evaluating the angiogenetic activity of neoplastic tissue. The method, currently most used, has been introduced by Weidner et al. [6] and consists in counting the microvessels on histological sections of the tumour, thus obtaining the tumoural MVD and considering this number as the angiogenetic potential of the neoplasia [10]. A wide panel of monoclonal antibodies are available for endothelial cell staining. In the past, monoclonal antibody anti-CD31 was widely used, but its cross reaction with plasma cells led to the preference of other endothelial markers, in order to avoid false positive results due to the large percentage of plasma cells usually present in the intra-tumoural inflammatory infiltrate of oral neoplasia. At present, for this type of tumour, anti-CD34 monoclonal antibody is that most commonly used, since it stains both the lymphatic and the blood vessels. Although the results of many studies, including ours, appear to be of great value for the statistically significant results, the immuno-histochemical methods now available, do not allow us to define an absolute number of intra-tumoural microvessels, as an exact cut-off, over which the risk of metastasis significantly increases. Thus the standardization of a valid and reproducible immuno-histochemical or flow cytometry method [30] is worthwhile, in order to correctly evaluate MVD. MVD would represent an independent prognostic factor [15 26] to determine which node-negative patients would present a greater risk of metastasis during follow-up and for whom adjuvant therapy would be of benefit.

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