

Simultaneous Expression of Furin and Vascular Endothelial Growth Factor in Human Oral Tongue Squamous Cell Carcinoma Progression

Ricardo López de Cicco,¹ James C. Watson,²
Daniel E. Bassi,¹ Samuel Litwin,³ and
Andrés J. Klein-Szanto¹

Departments of ¹Pathology, ²Surgical Oncology, and ³Biomathematics and Biostatistics, Fox Chase Cancer Center, Philadelphia, Pennsylvania

ABSTRACT

Purpose: Squamous cell carcinoma (SCC) of the tongue is a common malignancy of the oral cavity. Furin convertase activates several precursor matrix metalloproteinases involved in the degradation of the extracellular matrix. The pattern of expression of furin and vascular endothelial growth factor-C (VEGF-C), two key molecules in neoplasm development, was examined during the progression from normal epithelium to invasive SCC.

Experimental Design: We evaluated furin and VEGF-C expression and microvessel density (MVD) by immunohistochemistry in human tongue sections harboring normal epithelium, dysplastic epithelium, and/or SCC. Sections from 46 glossectomy specimens were assessed for furin expression. A selected group of 15 cases, each containing normal epithelium, precursor lesions, and invasive SCC, were further studied for furin and VEGF-C expression and MVD quantification. We also evaluated the pattern of furin expression and VEGF-C processing by Western blot analysis in three SCC cell lines with different degrees of aggressiveness.

Results: Furin and VEGF-C expression was notably higher in most precursor lesions and SCCs than in normal epithelia. Approximately 60% ($n = 26$) and 100% ($n = 15$) of the normal epithelia showed low-intensity staining for furin and VEGF-C, respectively. Intense staining for furin and VEGF-C was detected in ~80% ($n = 34$) and 100% ($n = 15$) of the SCCs, respectively. A significant correlation was seen between the expression of these two markers (Spearman's test, $P < 0.00002$). We found a statistically significant increase in MVD when either dysplasia ($432 \pm$

19.06 ; $P < 0.05$) or SCC (546 ± 17.24) was compared with normal epithelium (315 ± 17.27 ; $P < 0.0001$). SCC71, the most aggressive cell line analyzed, was the one with the highest furin expression. This cell line totally processed the VEGF-C proform, whereas the less aggressive line SCC9, exhibiting the least furin expression, did not. SCC15, of intermediate aggressiveness and furin expression, showed intermediate pro-VEGF-C processing.

Conclusions: These findings suggest that furin is a useful marker of tumor progression and is responsible for VEGF-C processing. This in turn would enhance angiogenesis, leading to increased MVD associated with preinvasive and invasive neoplasia.

INTRODUCTION

Squamous cell carcinoma (SCC) accounts for >90% of the head and neck tumors and is one of the six most frequent cancers worldwide. It constitutes ~4% of all cancers in the United States and 5% in the United Kingdom. SCC of the tongue is a common malignancy of the oral cavity, with an annual rate of 1.51 among males and 0.99 among females per 100,000 persons in the United States (1).

The sequential changes that take place in malignant neoplasms, gradually giving rise to more aggressive malignant phenotypes, is usually known as tumor progression (2). In the case of SCC, tumor progression is frequently associated with partial or total loss of squamous differentiation and the eventual appearance of a more anaplastic or spindle cell morphology accompanied by a more invasive and metastatic behavior (3–5). The most common molecular changes associated with SCC progression are mutation of the p53 tumor-suppressor gene, inactivation of the cyclin-dependent kinase inhibitor p16, and overexpression of epidermal growth factor receptor. Some of these molecular changes appear in precursor lesions (6–8).

Proprotein convertases (PCs) of the subtilisin family are Ca^{2+} -dependent serine endoproteases that recognize and cleave precursor inactive proteins at the COOH terminus of basic paired amino acids within the consensus motif RXR/KR (9). The proteolytic activation of protein precursors renders mature bioactive molecules with many functions, such as matrix metalloproteinases, growth factors, growth factor receptors, neuropeptides, enzymes, and adhesion molecules (10–15). Many of these molecules have been implicated in the acquisition of the tumorigenic phenotype, invasion, and metastases.

To date, eight members have been identified in mammals, including furin, PC1/PC3, PC2, PC4, PACE4, PC5/PC6, PC7/LPC/PC8, and SKI-1/SIP (16–19). Furin, one of the best known members of this kexin-like PC family, has a transmembrane domain, cycles between the trans-Golgi network and the cell surface, and is involved in the processing of proteins secreted via the constitutive pathway (20–22).

Received 12/2/03; revised 3/2/04; accepted 3/19/04.

Grant support: NIH Grants CA75028 and CA06927 and an appropriation of the Commonwealth of Pennsylvania.

The costs of publication of this article were defrayed in part by the payment of page charges. This article must therefore be hereby marked *advertisement* in accordance with 18 U.S.C. Section 1734 solely to indicate this fact.

Requests for reprints: Andrés J. Klein-Szanto Department of Pathology, Fox Chase Cancer Center, Philadelphia, PA 19111. Fax: (215) 728-2899; E-mail: AJ_Klein-Szanto@fccc.edu.

Table 1 Microvessel density (MVD) quantification and results of immunostaining for furin and vascular endothelial growth factor-C (VEGF-C). Relevant statistical analyses are described in detail in the text.

Diagnosis	Percentage of samples (<i>n</i>) expressing furin (immunostaining intensity)			Average MVD ^a Mean (±SE)	Percentage of samples (<i>n</i>) expressing VEGF-C (immunostaining intensity)		
	Negative (0)	I	II		Negative (0)	I	II
Normal tongue epithelium (lateral)	41% (19)	56.8% (26), except for the basal cells	2.2% (1)	315 ± 17.27	0% (0)	100% (15)	0% (0)
Dysplastic lesions	19% (4)	57% (12)	24% (5)	432 ± 19.06	0% (0)	60% (9)	40% (6)
SCC ^b	0% (0)	23.81% (10)	76.19% (34)	546 ± 17.24	0% (0)	0% (0)	100% (15)

^a Vessels/mm², quantitated at ×200 magnification.

^b SCC, squamous cell carcinoma.

PCs play a key role in the genesis and progression of cancer. Elevated expression of PCs has been reported in many tumors and cell lines (23–28). Furin up-regulation occurs in advanced lung tumors, breast, and head and neck cancers (24–26). Recently we reported that levels of furin expression correlate with the aggressiveness of tumor cell lines derived from head and neck cancers. Furthermore, furin expression in metastatic head and neck primary SCCs was twice as much as the amount detected in nonmetastatic primary tumors (26) from the same sites.

Angiogenesis, the growth of new capillaries from preexisting blood vessels (29), is essential for cancer to grow beyond minimal size and metastasize (30). This complex process involves extravasation of plasma proteins, degradation of extracellular matrix, endothelial cell migration and proliferation, and capillary tube formation. Vascular endothelial growth factor (VEGF) is regarded as the major angiogenesis factor during epithelial carcinogenesis in many malignant human cancers and in tumor metastases (31–38). Most human cancers have been characterized as containing a mixture of both VEGF-overexpressing tumor cells and VEGF receptor-overexpressing tumor-associated blood vessels (39). Microvessel density (MVD), as quantified in histological sections of tumors, has proven to be an independent prognostic indicator in various types of solid tumors, including SCC of the head and neck (40–42). Sauter *et al.* (43) determined that MVD correlated with the expression of VEGF during the progression from normal epithelium to invasive SCC.

Among the several vascular cell growth factors, VEGF-C has been identified as having a significant role in the lymphatic propagation of head and neck SCCs (44, 45). VEGF-C is mainly a ligand for the lymphatic endothelial receptor VEGF receptor-3 (Flt4), but it also binds to VEGF receptor-2, which is the major mitogenic signal transducer for VEGF in blood vessel endothelial cells (46, 47). Human VEGF-C cDNA encodes a protein of 419 residues with a predicted molecular mass of 59 kDa (46–49). The fact that VEGF-C is processed by furin at the RXXR↓ consensus motif (50) prompted us to examine the patterns of expression of furin and VEGF-C during the progression from normal epithelium to invasive SCC, and to determine whether the expression of these two tumor molecular markers, as well as the MVD, change simultaneously during the process of squamous cell carcinogenesis and progression. To minimize site-related variations, we selected SCCs of the oral tongue for this analysis.

MATERIALS AND METHODS

Cell Lines. Three SCC cell lines, SCC9, SCC15 and SCC71, were used in these experiments. The cell lines were obtained from the laboratory of Dr. J. G. Rheinwald (Harvard Skin Disease Center, Boston, MA). All cells were grown in Eagle's MEM, spinner modification (S-MEM), with the addition of 2 mM L-glutamine, 100 units/ml penicillin, 100 µg/ml streptomycin, and 10% fetal bovine serum. SCC9 and SCC15 are derived from well-differentiated SCC of the tongue, and SCC71 is derived from a moderately differentiated SCC of the soft palate. *In vivo* invasion assays and s.c. tumorigenesis assays showed that SCC9 and SCC15 have a low to moderate *in vivo* growth pattern, whereas SCC71 has a markedly invasive *in vivo* growth pattern (51).

Human Tongue Specimens. Oral tongue specimens from 46 subjects who underwent surgical excision at the Fox Chase Cancer Center (Philadelphia, PA) were evaluated. Cases were selected based on containing both the pathological lesion—either dysplasia (*n* = 21) or invasive carcinoma (*n* = 44)—and normal adjacent lateral tongue epithelium (*n* = 46). Of the 21 dysplasias, 13 were mild dysplasias, 7 were moderate dysplasias, and 1 was a severe dysplasia. All tissues were accrued by the Fox Chase Cancer Center Tumor Bank Facility after completing the process of informed consent and under the supervision of the Institutional Review Board.

Protein Analysis. We seeded 1×10^6 cells for each cell line in 100-mm plates and incubated them overnight in serum-free S-MEM for VEGF-C analysis. Conditioned medium was collected and concentrated 120 times down to 25 µl by filtration (Centriprep Microcon YM-10; Millipore). The whole concentrated conditioned medium harvested from each cell line was fractionated by 10% SDS-PAGE under reductive conditions and transferred to a nitrocellulose membrane. The membrane was immunoblotted with rabbit antihuman VEGF-C IgG (Santa Cruz Biotechnology, Santa Cruz, CA) and, after washing, with horseradish peroxidase-labeled antirabbit IgG.

After harvesting of the conditioned medium, cells were washed three times with cold PBS buffer. We added 0.4 ml of RIPA lysis buffer (1× PBS, 0.1% SDS, 0.5% sodium deoxycholate, 1% NP40) with the protease inhibitors aprotinin (1 mM), phenylmethylsulfonyl fluoride (100 mM), and Na₃VO₃ (100 mM) to the cultures and incubated them at 4°C for 15 min. Cells were scraped, passed through a 21-gauge needle, and incubated at 4°C for 30 min. Cell lysates were centrifuged at 10,000 × g

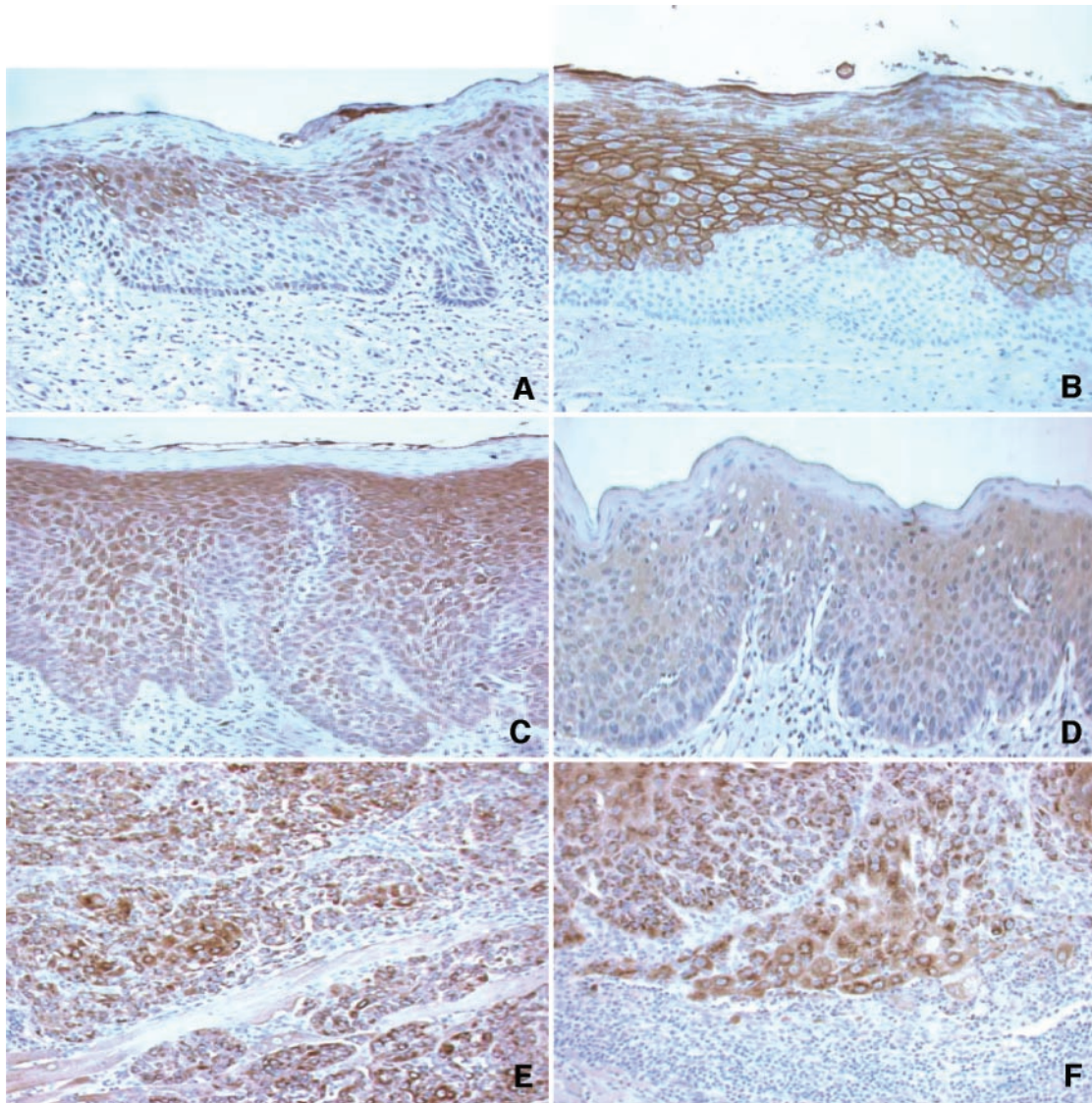


Fig. 1 Immunohistochemical detection of furin. *A*, when present (56.8% of the cases), the normal tongue epithelium showed mild furin expression. Note the cytoplasmic staining in the upper spinous layer. *B*, 40% of normal epithelia showed membrane-bound furin-associated immunostaining localized within the spinous layer. Note the absence of stain in the basal and parabasal cells. This micrograph corresponds to the only sample of normal oral mucosa that exhibits intense furin immunostaining (grade II). *C*, moderate dysplastic lesion shows intense immunostaining in the spinous layer with milder expression in the basal and parabasal keratinocytes. *D*, moderate to severe dysplasia shows mild immunostaining in all layers. *E*, furin is expressed intensely in all squamous cell carcinoma (SCC) cells. No SCC was negative when stained for furin. *F*, furin staining is present in the invading front of a SCC. Furin immunohistochemistry with hematoxylin counterstain (magnification, $\times 100$).

for 5 min, and the supernatants were separated. Fifty μl of the cell lysates were fractionated by 8% SDS-PAGE. The proteins were electroblotted onto a nitrocellulose membrane and probed with the antifurin monoclonal antibody MON-152 (Alexis, San Diego, CA) and, after washing, with horseradish peroxidase-conjugated antimouse IgG.

An enhanced chemiluminescence detection system (Amersham Biosciences, Piscataway, NJ) was used for band detection.

Immunohistochemistry. Furin and VEGF immunohistochemistry was performed on histological sections from paraffin-embedded cases. After rehydration, sections were subjected to antigen retrieval by microwave boiling at a low setting in

distilled water for 10 min. Mon-152 was used as primary antibody at a 1:50 dilution to reveal furin convertase in 46 glossectomy specimens. In addition, 15 selected samples that contained normal epithelium, dysplastic lesions, and SCC were chosen to assess VEGF-C staining and determine MVD. A rabbit polyclonal antibody directed to human VEGF-C (Santa Cruz Biotechnology) at 1:100 dilution was used. To determine MVD, slides were incubated with a mouse monoclonal antibody to factor VIII-related antigen (Dako Corp., Carpinteria, CA). In the latter, enzyme predigestion was performed by incubating deparaffinized sections for 15 min in Pronase. In all of the cases, an avidin-biotin-peroxidase kit (Bio Genex, San Ramon, CA) was

then used, followed by the chromogen 3',3'-diaminobenzidine to develop the immunostain. Negative controls, not incubated with primary antibody, were used. As positive controls, sections from known specimens containing salivary glands (positive for furin) and SCC of the head and neck (positive for VEGF-C and factor VIII) were used (26, 43). All sections were counterstained with hematoxylin, mounted, and analyzed.

MVD Determination. The subepithelial vascular plexus localized within 100 μm of the epithelial basement membrane of normal epithelium and preinvasive lesions were evaluated. Subepithelial vascular plexus within 100 μm of the peripheral edge of invasive lesions were counted. An image analysis system (Fairfield Imaging, United Kingdom) was used to assist in the quantitation (0.104 mm^2/field) of any brown-staining endothelial cells or endothelial cell clusters that were differentiable from adjacent vasculature, tumor cells, and other connective-tissue elements, and they were considered a single, countable microvessel based on the criteria of Weidner *et al.* (41). Vessel lumens were not necessary for a structure to be defined as a microvessel, and red cells were not used to define a vessel lumen. Three noncontiguous $\times 200$ fields associated with the area of interest (normal epithelium, dysplastic epithelium, or tumor) were randomly selected without bias toward areas of "highest neovascularization." Recognizing that tumors are often heterogeneous in their MVD, we determined average MVD after the lowest and highest manual counts were discarded; this allowed the counts to be more representative of the histology as a whole. MVD was expressed as number of microvessels/ mm^2 .

Quantification of Furin and VEGF Immunostaining.

Grading of furin and VEGF-C immunostaining was based on semiquantitative evaluation of stain intensity from 0 to II. No or marginal staining of $<5\%$ of cells was called negative (0), mild to moderate stain of 5–50% of cells was graded as I, and moderate to intense staining comprising $>50\%$ of the cells was classified as II. Section grading was based on stain intensity per field (for tumors) or epithelial layer (for epithelia). Staining evaluation of the slides was performed in a blind fashion.

RESULTS

Furin Expression Patterns Associated with Different Lesions.

Furin immunohistochemistry was performed on 46 specimens to assess its expression during the process of tumor development. As shown in Table 1, 56.8% of the normal epithelia showed mild furin immunostaining (grade I; Fig. 1A). Only one case showed moderate immunostaining (grade II; Fig. 1B). Except for the cells in the basal layer, which did not stain at all, all other layers of the normal epithelia showed a mild expression of this PC. The pattern of furin staining in the normal epithelium was not uniform in all cases. Sixty percent of the specimens had cytoplasmic immunostaining (Fig. 1A), whereas another 40% showed membrane-bound furin-associated immunostaining (Fig. 1B).

Approximately 24% of dysplastic lesions expressed moderate to high levels of furin immunostaining (grade II; Fig. 1C), whereas 19% were negative for furin immunostaining. The remaining lesions had mild immunostaining (grade I; Fig. 1D). Approximately 50% of furin-positive dysplastic lesions exhib-

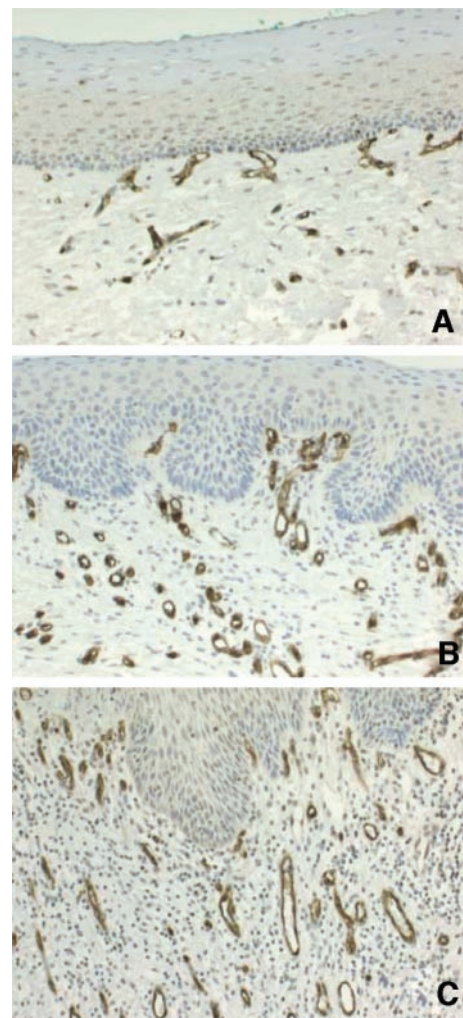


Fig. 2 Microvessel density staining pattern in subepithelial plexus of normal epithelium (A), subepithelial plexus of dysplastic epithelium (B), and peritumoral vasculature of squamous cell carcinoma (C). Note that the number of vessels increases from A to C with progressive stages of squamous carcinogenesis of the human tongue. Factor VIII immunohistochemistry with hematoxylin counterstain (magnification, $\times 60$).

ited a cytoplasmic staining pattern. Similarly, the basal layer stained positively for furin in nearly 50% of the lesions.

Previous investigators have proposed that furin expression is up-regulated in more aggressive tumors and cell lines (26). In the present study, 100% of the tumors tested were positive for furin staining (Fig. 1E). It is important to note that the intensity of furin staining was higher in the tumors than in the normal epithelium. Most invasive SCCs analyzed (76.19%) showed strong immunostaining (grade II). The outermost or basaloid cells of the tumors facing the connective tissue were positive when stained for furin (Fig. 1F).

MVD Associated with Different Lesions. MVD was measured in normal and abnormal human tongue tissues by immunohistochemical staining for factor VIII-related antigen (Fig. 2). Immunohistochemistry was performed on 15 specimens, each containing normal, dysplastic, and neoplastic epi-

Table 2 Microvessel density data and results of immunostaining for furin and vascular endothelial growth factor-C (VEGF-C) in 15 of the 46 cases depicted in Table 1, each containing normal epithelium, precursor lesions, and invasive squamous cell carcinoma (SCC)

Tissue type		Patient						
		1	2	3	4	5	6	7
Normal	Furin expression (grade)	I	0	I	I	I	I	0
	VEGF-C expression (grade)	I	I	I	I	I	I	I
	MVD ^a	357.16 ± 27.96	335.47 ± 88.6	444.57 ± 104.5	392.30 ± 62.81	369.80 ± 25.82	313.03 ± 96.57	355.13 ± 63.92
Dysplastic	Furin expression (grade)	II	I	I	I	I	I	II
	VEGF-C expression (grade)	II	I	I	I	I	II	II
	MVD	406.13 ± 21.21	421.63 ± 28.77	393.33 ± 79.79	374.43 ± 24.28	392.40 ± 25.36	688.30 ± 75.34	430.77 ± 53.94
SCC	Furin expression (grade)	II	II	II	II	I	II	II
	VEGF-C expression (grade)	II	II	II	II	II	II	II
	MVD	659.07 ± 31.63	565.80 ± 29.4	621.70 ± 77.95	424.07 ± 72.66	669.17 ± 28.62	538.83 ± 24.76	614.57 ± 4.3

^a MVD, microvessel density (vessels/mm²) quantitated at ×200 magnification. Mean ± SE of three values.

thelial tissues. Normal tongue epithelium contained few capillaries distributed within the lamina propria contiguous to the epithelium basal cell layer (Fig. 2A). The number and distribution of vessels in the dermis was altered in the dysplastic lesions (Fig. 2B) and carcinomas (Fig. 2C). The vessels were more densely packed and closer to the basal layer of the lesions than in the normal epithelium. Quantification of vascularization in tongue sections at each stage of neoplastic progression revealed a statistically significant increase in MVD when normal epithelium (315 ± 17.27) was compared with dysplasia (432 ± 19.06 ; $P < 0.05$) or carcinoma (546 ± 17.24 ; $P < 0.0001$; Table 1). Table 2 shows the MVD data and grading of immunostaining for furin and VEGF-C in 15 of the 46 cases depicted in Table 1,

each containing normal epithelium, precursor lesions, and invasive SCC.

VEGF-C Expression Patterns Associated with Different Lesions. Immunohistochemical staining for VEGF-C was performed on 15 specimens, each containing three distinct tissues, *i.e.*, normal epithelium, dysplastic epithelium, and invasive SCC (Fig. 3). The results are summarized in Tables 1 and 2. Low or mild expression (grade I) of VEGF-C was found in all normal epithelia (Fig. 3A). Strong (grade II) staining was detected in 40% of dysplastic epithelia (Fig. 3C). The remaining 60% of specimens showed a low or mild staining intensity for VEGF-C (grade I; Fig. 3B). All of the SCC showed strong staining (grade II; Fig. 3D). No tumors showed low expression of VEGF-C.

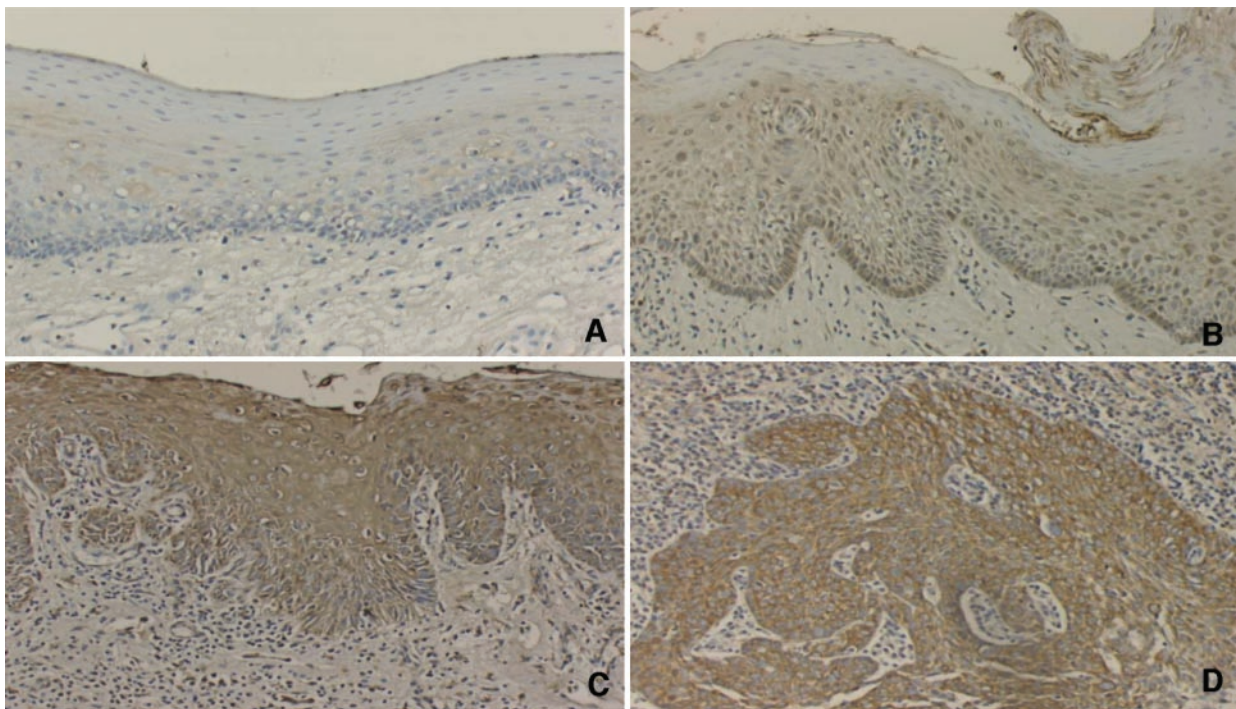


Fig. 3 Assessment of vascular endothelial growth factor-C (VEGF-C) immunostaining. A, normal epithelium showing mild VEGF-C staining. B, mild dysplasia showing moderate VEGF-C staining. C, moderate dysplasia exhibiting intense immunostaining. D, intense VEGF immunostaining in an invasive squamous cell carcinoma. VEGF-C immunohistochemistry with hematoxylin counterstain (magnification, ×100).

Table 2 Continued

Patient							
8	9	10	11	12	13	14	15
0	I	I	I	0	I	I	I
I	I	I	I	I	I	I	I
151.50 ± 30.92	229.60 ± 56.67	269.0 ± 18.5	257.57 ± 44.75	290.17 ± 34.8	203.00 ± 30.74	320.74 ± 45.49	435.43 ± 43.13
I	I	I	I	0	II	II	I
I	I	I	I	II	II	II	I
418.13 ± 37.56	417.44 ± 75.29	294.6 ± 25.11	351.80 ± 29.74	273.37 ± 32.17	541.64 ± 53.73	588.84 ± 62.33	438.50 ± 59.48
I	II	II	II	II	II	II	II
II	II	II	II	II	II	II	II
501.87 ± 113.84	433.57 ± 76	405.23 ± 27.76	563.60 ± 66.41	527.13 ± 39.59	611.37 ± 77.94	597.87 ± 77.55	472.17 ± 31.88

Correlation between Furin and VEGF-C Expression.

We compared furin expression with VEGF-C expression in normal and dysplastic epithelia and SCCs; the results are summarized in Table 1. VEGF-C showed increased expression in association with a simultaneous increase in furin immunostaining. A strong relationship was seen between the expression of these two markers (Spearman's test, $P < 0.00002$). The normal epithelium showed mild or absent furin and VEGF expression. On the other hand, almost 80% of the invasive carcinomas showed strong staining for both markers.

VEGF-C Processing in SCC Cell Lines. To confirm that furin-expressing SCC-derived cells are able to process VEGF-C, we analyzed whole conditioned medium from three SCC cell lines by Western blotting. We also assessed furin expression in these cell lines. As shown in Fig. 4A, SCC9, SCC15, and SCC71 cells, expressing low, intermediate, and high amounts of furin (Ref. 26; Fig. 4B), respectively, were capable of processing the VEGF-C precursor. The band corresponding to the mature form of the growth factor (~30 kDa) was markedly more prominent in SCC71. SCC9, the least aggressive of the three cell lines and the one that expressed the least amount of furin (Fig. 4B; Ref.26), showed a predominance of the band corresponding to the proform (~59 kDa), and no processing of the precursor could be detected. On the other hand, SCC15 showed intermediate processing of the growth factor, in accordance to its intermediate aggressiveness and furin expression level (Fig. 4B) compared with the other two cell lines.

DISCUSSION

Tumor progression is driven by complex mechanisms that produce an enhanced malignant phenotype. Among the processes involved in the acquisition of the invasive/metastatic phenotype, angiogenesis plays a major role by increasing tumor cell oxygenation by providing a conduit for delivery of nutrition and by providing increased blood and lymphatic vessel surface areas for tumor cells to penetrate into the newly formed vessels, reach the general circulation, and metastasize (32, 33, 52). In previous studies we have shown that furin is able to enhance tumor progression by processing several metalloproteinases and growth factors (53–55). In addition, Siegfried *et al.* (50) have recently demonstrated that furin is able to process VEGF-C in a colon cancer cell line. To date, of the five members of the VEGF

family, VEGF-C is the only demonstrated furin substrate. VEGF-C plays a significant role by enhancing the neoformation of lymphatic and blood vessels in several human malignancies, including cancer of the head and neck (45, 56, 57). Regional lymph node metastasis is a significant factor in the therapy and prognosis of oral cavity SCC (58). Among the VEGF family, VEGF-C has a role in inducing both angiogenesis and lymphangiogenesis via VEGF receptor-2 and, more importantly, via VEGF receptor-3 (47, 59). Expression of VEGF receptor-3 is highly restricted to the lymphatic endothelial cells and is stimulated mainly by VEGF-C and to some extent by VEGF-D (60). Kishimoto *et al.* (44) reported that VEGF-C expression in oral SCC biopsies strongly correlated with lymph node metastasis. Two recent studies showed that an up-regulation of VEGF-C

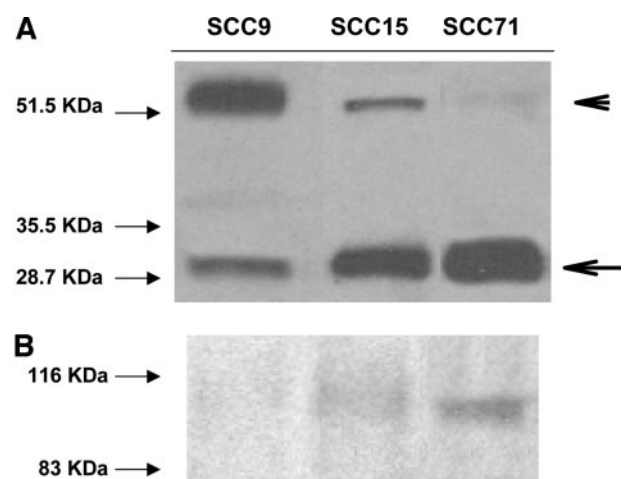


Fig. 4 A, Western blot analysis of vascular endothelial growth factor-C (VEGF-C). Concentrated total conditioned medium from SCC9, SCC15, and SCC71 human head and neck cell lines was fractionated by 10% SDS-PAGE. Note the higher proportion of processed form in SCC71 (arrow) and the higher proportion of the proform in SCC9 (arrowhead). SCC15 showed intermediate processing of the VEGF-C proform. B, Western blot analysis of furin expression. Fifty μ l of cell lysates were submitted to 8% SDS-PAGE. Immunoblotting was performed with the antifurin monoclonal antibody MON-152. Note the higher furin expression in the most invasive cell line (SCC71) than in the less aggressive cell line (SCC15). The least invasive cell line (SCC9) showed the lowest furin expression.

(56) and a high intratumoral lymphatic MVD (61) positively correlated with lymph node metastasis in oral cancers. Sedivy *et al.* (62) demonstrated a significant correlation of VEGF-C expression with an elevated lymphatic MVD and with lymph node metastasis in patients with oral SCC. Mandriota *et al.* (60) reported that VEGF-C-induced lymphangiogenesis mediates tumor cell dissemination and the formation of lymph node metastases in studies *in vivo*. Thus, in oral SCC progression, VEGF-C is a very significant factor that influences regional lymph node metastasis and survival.

The fact that furin-expressing head and neck cell lines and primary tumors showed increased aggressiveness *in vivo* (26, 54) may be explained in part by enhancement of VEGF processing, resulting in increased tumor-associated angiogenesis. This hypothesis is supported by results obtained by Siegfried *et al.* (50), who found that CHO cells stably expressing VEGF-C, when s.c. injected into nude mice, enhanced angiogenesis and lymphangiogenesis compared with nontransfected cells.

In the present report we show that expression of furin and VEGF-C increases simultaneously along with MVD in a series of preinvasive lesions and SCCs of the tongue. Although angiogenesis in head and neck SCC is a complex phenomenon driven by many stimulatory factors (63), it is reasonable to hypothesize that increased furin expression promotes the processing of several members of the PDGF/VEGF family, including VEGF-C, that are known substrates of this convertase (50, 64). This increase in the available mature form could thus induce the increase in neovascularization seen in dysplastic epithelia and SCCs. This is further supported by the finding that three head and neck SCC cell lines known to express furin process VEGF-C.

Sauter *et al.* (43) found, in a limited series of cases from several head and neck sites, that VEGF expression correlated with MVD of preinvasive and invasive lesions of the head and neck. Similarly, in a small series of head and neck lesions from the oral cavity, larynx, and pharynx, Ninck *et al.* (63) described that the expression of the furin substrates VEGF and platelet-derived growth factor-AB correlated with poor survival in SCC patients. In addition, they observed that the tumors expressed angiogenic growth factors, whereas the normal adjacent epithelium did not.

To avoid site heterogeneity in vascularization within the head and neck, we focused on lesions of the oral tongue. For the purpose of this study, we evaluated glossectomy specimens that showed stages of tumorigenesis from normal epithelium to invasive SCC, including dysplasia. Our data confirmed that angiogenesis, as measured by MVD, occurs in association with preneoplastic changes in human tongue epithelium before invasion. No significant differences in furin or VEGF-C expression and MVD were seen among the lesions of different degrees of dysplasia. The increment in MVD was associated with an increase of VEGF-C.

In this study we found that there is a positive correlation between furin and VEGF-C up-regulation during tumor progression. We also analyzed the staining intensity ratio between the basaloid (or peripheral) cells of the tumors and the basal cells of normal epithelium. The results obtained showed that in 81% of the specimens studied, the ratio of intensity (ratio of intensity of basaloid cells of the tumor to intensity of basal cells of normal

epithelium) was >1 . This result suggests that furin expression is increased at the invasive front of the tumors, where processing of tumor progression-related substrates, such as metalloproteinases and VEGF-C, takes place. We confirmed with *in vitro* studies that the ability to process VEGF-C (Fig. 4A) is in direct relationship with the degree of malignancy and furin expression (Fig. 4B) of the cell lines analyzed. SCC9, the least malignant cell line, did not process all of the available proform of the growth factor, whereas SCC71 did. SCC15 showed an intermediate processing of the growth factor, in accordance with its intermediate aggressiveness and furin expression. It is important to note that furin expression was highest in SCC71 and lowest in SCC9 (Fig. 4B).

The hypothesis that neoplastic cells stimulate angiogenesis early during their progression to an invasive cancer has been suggested by recent studies combining *in vitro* angiogenesis assays and specimens taken from transgenic mice. These studies suggest that angiogenesis begins before the emergence of invasive cancer (65–67). Furthermore, increased MVD has been reported in other models of preinvasive progression, such as human colon and precursor lesions of the mouse skin (68, 69).

The present study shows that there is a close association between progressive squamous carcinogenesis in the human tongue, epithelial furin expression, VEGF-C expression, and neovascularization. This suggests that furin may promote tumor-associated angiogenesis through enhanced VEGF-C processing.

ACKNOWLEDGMENTS

We thank C. F. Renner and FangPing Chen for excellent technical assistance.

REFERENCES

- Canto MT, Devesa SS. Oral cavity and pharynx cancer incidence rates in the United States, 1975–1998. *Oral Oncol* 2002;38:610–7.
- Foulds L. Neoplastic development. New York: Academic Press, 1969.
- Portella G, Liddell J, Crombie R, et al. Molecular mechanisms of invasion and metastasis during mouse skin tumour progression. *Invasion Metastasis* 1994;14:7–16.
- Portella G, Cumming SA, Liddell J, et al. Transforming growth factor beta is essential for spindle cell conversion of mouse skin carcinoma *in vivo*: implications for tumor invasion. *Cell Growth Differ* 1998;9:393–404.
- Klein-Szanto AJ, Larcher F, Bonfil RD, Conti CJ. Multistage chemical carcinogenesis protocols produce spindle cell carcinomas of the mouse skin. *Carcinogenesis (Lond)* 1989;10:2169–72.
- Gleich LL, Salamone FN. Molecular genetics of head and neck cancer. *Cancer Control* 2002;9:369–78.
- Helliwell TR. Molecular markers of metastasis in squamous carcinomas. *J Pathol* 2001;194:289–93.
- Forastiere A, Koch W, Trotti A, Sidransky D. Head and neck cancer. *N Engl J Med* 2001;345:1890–900.
- Hosaka M, Nagahama M, Kim WS, et al. Arg-X-Lys/Arg-Arg motif as a signal for precursor cleavage catalyzed by furin within the constitutive secretory pathway. *J Biol Chem* 1991;266:12127–30.
- Khatib AM, Siegfried G, Chretien M, Metrakos P, Seidah NG. Proprotein convertases in tumor progression and malignancy: novel targets in cancer therapy. *Am J Pathol* 2002;160:1921–35.
- Pei D, Weiss SJ. Furin-dependent intracellular activation of the human stromelysin-3 zymogen. *Nature (Lond)* 1995;375:244–7.

12. Yana I, Weiss SJ. Regulation of membrane type-1 matrix metalloproteinase activation by proprotein convertases. *Mol Biol Cell* 2000;11:2387–401.
13. Dubois CM, Blanchette F, Laprise MH, Leduc R, Grondin F, Seidah NG. Evidence that furin is an authentic transforming growth factor-beta1-converting enzyme. *Am J Pathol* 2001;158:305–16.
14. Duguay SJ, Jin Y, Stein J, Duguay AN, Gardner P, Steiner DF. Post-translational processing of the insulin-like growth factor-2 precursor. Analysis of O-glycosylation and endoproteolysis. *J Biol Chem* 1998;273:18443–51.
15. Lehmann M, Andre F, Bellan C, et al. Deficient processing and activity of type I insulin-like growth factor receptor in the furin-deficient LoVo-C5 cells. *Endocrinology* 1998;139:3763–71.
16. Seidah NG, Chretien M. Eukaryotic protein processing: endoproteolysis of precursor proteins. *Curr Opin Biotechnol* 1997;8:602–7.
17. Nakayama K. Furin: a mammalian subtilisin/Kex2p-like endoprotease involved in processing of a wide variety of precursor proteins. *Biochem J* 1997;327(Pt 3):625–35.
18. Zhou A, Webb G, Zhu X, Steiner DF. Proteolytic processing in the secretory pathway. *J Biol Chem* 1999;274:20745–8.
19. Seidah NG, Mowla SJ, Hamelin J, et al. Mammalian subtilisin/kexin isozyme SKI-1: a widely expressed proprotein convertase with a unique cleavage specificity and cellular localization. *Proc Natl Acad Sci USA* 1999;96:1321–6.
20. Molloy SS, Thomas L, VanSlyke JK, Stenberg PE, Thomas G. Intracellular trafficking and activation of the furin proprotein convertase: localization to the TGN and recycling from the cell surface. *EMBO J* 1994;13:18–33.
21. Schafer W, Stroth A, Berghofer S, et al. Two independent targeting signals in the cytoplasmic domain determine trans-Golgi network localization and endosomal trafficking of the proprotein convertase furin. *EMBO J* 1995;14:2424–35.
22. Shapiro J, Sciaky N, Lee J, Bosshart H, Angeletti RH, Bonifacio JS. Localization of endogenous furin in cultured cell lines. *J Histochem Cytochem* 1997;45:3–12.
23. Schalken JA, Roebroek AJ, Oomen PP, et al. fur gene expression as a discriminating marker for small cell and nonsmall cell lung carcinomas. *J Clin Invest* 1987;80:1545–9.
24. Mbikay M, Sirois F, Yao J, Seidah NG, Chretien M. Comparative analysis of expression of the proprotein convertases furin, PACE4, PC1 and PC2 in human lung tumours. *Br J Cancer* 1997;75:1509–14.
25. Cheng M, Watson PH, Paterson JA, Seidah N, Chretien M, Shiu RP. Pro-protein convertase gene expression in human breast cancer. *Int J Cancer* 1997;71:966–71.
26. Bassi DE, Mahloogi H, Al-Saleem L, Lopez De Cicco R, Ridge JA, Klein-Szanto AJ. Elevated furin expression in aggressive human head and neck tumors and tumor cell lines. *Mol Carcinog* 2001;31:224–32.
27. Jin L, Kulig E, Qian X, et al. Distribution and regulation of proconvertases PC1 and PC2 in human pituitary adenomas. *Pituitary* 1999;1:187–95.
28. Kajiwara H, Itoh Y, Itoh J, Yasuda M, Osamura RY. Immunohistochemical expressions of prohormone convertase (PC)1/3 and PC2 in carcinoids of various organs. *Tokai J Exp Clin Med* 1999;24:13–20.
29. Diaz-Flores L, Gutierrez R, Varela H. Angiogenesis: an update. *Histol Histopathol* 1994;9:807–43.
30. Folkman J. Angiogenesis—retrospect and outlook. *EXS* 1992;61:4–13.
31. Folkman J. Proceedings: tumor angiogenesis factor. *Cancer Res* 1974;34:2109–13.
32. Folkman J, Merler E, Abernathy C, Williams G. Isolation of a tumor factor responsible for angiogenesis. *J Exp Med* 1971;133:275–88.
33. Brown LF, Detmar M, Claffey K, et al. Vascular permeability factor/vascular endothelial growth factor: a multifunctional angiogenic cytokine. *EXS* 1997;79:233–69.
34. Ferrer FA, Miller LJ, Andrawis RI, et al. Vascular endothelial growth factor (VEGF) expression in human prostate cancer: in situ and in vitro expression of VEGF by human prostate cancer cells. *J Urol* 1997;157:2329–33.
35. Takahashi Y, Kitadai Y, Bucana CD, Cleary KR, Ellis LM. Expression of vascular endothelial growth factor and its receptor, KDR, correlates with vascularity, metastasis, and proliferation of human colon cancer. *Cancer Res* 1995;55:3964–8.
36. Bancher-Todesca D, Obermair A, Bilgi S, et al. Angiogenesis in vulvar intraepithelial neoplasia. *Gynecol Oncol* 1997;64:496–500.
37. Abu-Jawdeh GM, Faix JD, Niloff J, Tet al. Strong expression of vascular permeability factor (vascular endothelial growth factor) and its receptors in ovarian borderline and malignant neoplasms. *Lab Invest* 1996;74:1105–15.
38. Brown LF, Berse B, Jackman RW, et al. Expression of vascular permeability factor (vascular endothelial growth factor) and its receptors in breast cancer. *Hum Pathol* 1995;26:86–91.
39. Dvorak HF, Brown LF, Detmar M, Dvorak AM. Vascular permeability factor/vascular endothelial growth factor, microvascular hyperpermeability, and angiogenesis. *Am J Pathol* 1995;146:1029–39.
40. Shpitzer T, Chaimoff M, Gal R, Stern Y, Feinmesser R, Segal K. Tumor angiogenesis as a prognostic factor in early oral tongue cancer. *Arch Otolaryngol Head Neck Surg* 1996;122:865–8.
41. Weidner N, Semple JP, Welch WR, Folkman J. Tumor angiogenesis and metastasis—correlation in invasive breast carcinoma. *N Engl J Med* 1991;324:1–8.
42. Murray JD, Carlson GW, McLaughlin K, et al. Tumor angiogenesis as a prognostic factor in laryngeal cancer. *Am J Surg* 1997;174:523–6.
43. Sauter ER, Nesbit M, Watson JC, Klein-Szanto A, Litwin S, Herlyn M. Vascular endothelial growth factor is a marker of tumor invasion and metastasis in squamous cell carcinomas of the head and neck. *Clin Cancer Res* 1999;5:775–82.
44. Kishimoto K, Sasaki A, Yoshihama Y, Mese H, Tsukamoto G, Matsumura T. Expression of vascular endothelial growth factor-C predicts regional lymph node metastasis in early oral squamous cell carcinoma. *Oral Oncol* 2003;39:391–6.
45. Neuchrist C, Erovcic BM, Handisurya A, et al. Vascular endothelial growth factor C and vascular endothelial growth factor receptor 3 expression in squamous cell carcinomas of the head and neck. *Head Neck* 2003;25:464–74.
46. Huang HY, Ho CC, Huang PH, Hsu SM. Co-expression of VEGF-C and its receptors, VEGFR-2 and VEGFR-3, in endothelial cells of lymphangioma. Implication in autocrine or paracrine regulation of lymphangioma. *Lab Invest* 2001;81:1729–34.
47. Joukov V, Pajusola K, Kaipainen A, et al. A novel vascular endothelial growth factor, VEGF-C, is a ligand for Flt4 (VEGFR-3) and KDR (VEGFR-2) receptor tyrosine kinases. *EMBO J* 1996;15:290–8.
48. Karkkainen MJ, Makinen T, Alitalo K. Lymphatic endothelium: a new frontier of metastasis research. *Nat Cell Biol* 2002;4:E2–5.
49. Joukov V, Sorsa T, Kumar V, et al. Proteolytic processing regulates receptor specificity and activity of VEGF-C. *EMBO J* 1997;16:3898–911.
50. Siegfried G, Basak A, Cromlish JA, et al. The secretory proprotein convertases furin, PC5, and PC7 activate VEGF-C to induce tumorigenesis. *J Clin Invest* 2003;111:1723–32.
51. Caamano J, Zhang SY, Rosvold EA, Bauer B, Klein-Szanto AJ. p53 alterations in human squamous cell carcinomas and carcinoma cell lines. *Am J Pathol* 1993;142:1131–9.
52. Ishibashi H, Shiratuchi T, Nakagawa K, et al. Hypoxia-induced angiogenesis of cultured human salivary gland carcinoma cells enhances vascular endothelial growth factor production and basic fibroblast growth factor release. *Oral Oncol* 2001;37:77–83.
53. Bassi DE, Lopez De Cicco R, Mahloogi H, Zucker S, Thomas G, Klein-Szanto AJ. Furin inhibition results in absent or decreased invasiveness and tumorigenicity of human cancer cells. *Proc Natl Acad Sci USA* 2001;98:10326–31.
54. Bassi DE, Mahloogi H, Lopez De Cicco R, Klein-Szanto A. Increased furin activity enhances the malignant phenotype of human head and neck cancer cells. *Am J Pathol* 2003;162:439–47.

55. Mercapide J, Lopez De Cicco R, Bassi DE, Castresana JS, Thomas G, Klein-Szanto AJ. Inhibition of furin-mediated processing results in suppression of astrocytoma cell growth and invasiveness. *Clin Cancer Res* 2002;8:1740–6.
56. O-charoenrat P, Rhys-Evans P, Eccles SA. Expression of vascular endothelial growth factor family members in head and neck squamous cell carcinoma correlates with lymph node metastasis. *Cancer (Phila)* 2001;92:556–68.
57. Salven P, Lymboussaki A, Heikkila P, et al. Vascular endothelial growth factors VEGF-B and VEGF-C are expressed in human tumors. *Am J Pathol* 1998;153:103–8.
58. Kalnins IK, Leonard AG, Sako K, Razack MS, Shedd DP. Correlation between prognosis and degree of lymph node involvement in carcinoma of the oral cavity. *Am J Surg* 1977;134:450–4.
59. Ohta Y, Shridhar V, Bright RK, et al. VEGF and VEGF type C play an important role in angiogenesis and lymphangiogenesis in human malignant mesothelioma tumours. *Br J Cancer* 1999;81:54–61.
60. Mandriota SJ, Jussila L, Jeltsch M, et al. Vascular endothelial growth factor-C-mediated lymphangiogenesis promotes tumour metastasis. *EMBO J* 2001;20:672–82.
61. Beasley NJ, Prevo R, Banerji S, et al. Intratumoral lymphangiogenesis and lymph node metastasis in head and neck cancer. *Cancer Res* 2002;62:1315–20.
62. Sedivy R, Beck-Mannagetta J, Haverkamp C, Battistutti W, Honigschnabl S. Expression of vascular endothelial growth factor-C correlates with the lymphatic microvessel density and the nodal status in oral squamous cell cancer. *J Oral Pathol Med* 2003;32:455–60.
63. Ninck S, Reisser C, Dyckhoff G, Helmke B, Bauer H, Herold-Mende C. Expression profiles of angiogenic growth factors in squamous cell carcinomas of the head and neck. *Int J Cancer* 2003;106:34–44.
64. Siegfried G, Khatib AM, Benjannet S, Chretien M, Seidah NG. The proteolytic processing of pro-platelet-derived growth factor-A at RRKR(86) by members of the proprotein convertase family is functionally correlated to platelet-derived growth factor-A-induced functions and tumorigenicity. *Cancer Res* 2003;63:1458–63.
65. Arbeit JM. Transgenic models of epidermal neoplasia and multi-stage carcinogenesis. *Cancer Surv* 1996;26:7–34.
66. Folkman J, Watson K, Ingber D, Hanahan D. Induction of angiogenesis during the transition from hyperplasia to neoplasia. *Nature (Lond)* 1989;339:58–61.
67. Hanahan D, Folkman J. Patterns and emerging mechanisms of the angiogenic switch during tumorigenesis. *Cell* 1996;86:353–64.
68. Bolontrade MF, Stern MC, Binder RL, Zenklusen JC, Gimenez-Conti IB, Conti CJ. Angiogenesis is an early event in the development of chemically induced skin tumors. *Carcinogenesis (Lond)* 1998;19:2107–13.
69. Watson JC, Cooper H, Babb J, Sigurdson ER, Klein-Szanto AJP. Angiogenesis is an early event in the adenoma-carcinoma sequence in human colon cancer. *Surg Forum* 1998;50:440–2.