Protein profiling of angiogenesis-related growth factors in laryngeal carcinoma: Pattern of protein expression in relation to tumour progression

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Abstract. The expression of angiogenesis-related proteins was determined in laryngeal tumour tissue, associated tumour involved lymph nodes, apparently normal mucosa and control tissue and were related to tumour stage. Both laryngeal tumour tissue and associated metastatic nodes were obtained from seven patients undergoing surgical resection; in four cases apparently normal mucosa was also dissected from the tumour specimen margins. Control uvula mucosa was obtained from five healthy volunteers undergoing uvulopalatopharyngoplasty. The relative expression of 55 angiogenesis-related proteins was determined in tissue lysates using a Proteome Profiler human angiogenesis array kit. The level of 32/55 angiogenesis-related proteins was higher in tumour tissue compared with controls. Furthermore, in these tumour biopsies higher levels of proteins were associated with increasing tumour stage. A similar trend was seen for 29/32 of these proteins in the nodal tissue. In T4 stage tumour tissue samples, 29/55 angiogenensis-related proteins were more highly expressed compared with the adjacent normal mucosa from the same patient, and this decreased to 8 proteins in tumour tissue from the T1 stage patients. In contrast, the expression of 23 angiogenesis-related proteins in metastatic lymph node tissue from T4 stage patients was lower compared with that found in the normal mucosa adjacent to the tumour. In conclusion, this study has identified a number of factors involved in angiogenesis that are likely to contribute to the growth and metastasis of laryngeal tumours. Furthermore, a number of factors were also substantially altered in metastatic deposits compared with the primary tumour mass or adjacent normal tissue. This study requires confirmatory analysis of the selected key factors in a larger cohort of patients.

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Introduction

Head and neck squamous cell carcinoma (HNSCC), a term comprising a heterogeneous group of aggressive, mainly epithelial malignancies, represents the sixth most common solid tumour worldwide (1). HNSCC arises in distinct anatomical sites displaying varied histologies and clinical courses with tobacco and alcohol being the most common aetiological risk factors. Treatment usually involves a combination of surgery, chemo- and radiotherapy. Unfortunately both the anatomical location of the tumour and the subsequent treatment can severely affect a patient's quality of life, i.e., swallowing, breathing and speaking may all become difficult (2).

Although recent reports indicate a better prognosis for patients with human papilloma virus (HPV) positive oropharyngeal tumours (3), 5-year survival rates for HNSCC patients with HPV negative tumours remain less than 50%. This is despite advances in treatment, and is mainly due to late presentation, early loco-regional recurrence of the tumour and the development of second primaries (4). Advanced tumour stage and lymph node spread are widely accepted as the two strongest poor prognostic factors for HNSCC, although the relevance of a growing number of molecular markers are being investigated (5).

The concept of field cancerization was first introduced by Slaughter *et al* (6) who postulated that normal mucosa has the potential to undergo malignant transformation at multiple sites due to assault from various aetiological agents. The 'field of cancerization' is currently viewed as an area of cells in which a single stem cell, that has acquired initiating mutations caused by carcinogens, has given mutation to the daughter cells that grow out to form a patch and then a wider 'field' of premalignant cells (7).

In order for a tumour to grow and metastasise it needs to develop its own blood supply by a process known as angiogenesis. Factors secreted by both tumour and stroma stimulate the surrounding endothelial cells to form new blood vessels. Angiogenesis does not result from the action of a single growth factor, but is dependent on the interaction of multiple proteins with angiogenic properties. In HNSCC, like almost all other solid tumours, factors such as vascular endothelial growth factor (VEGF), fibroblast growth factor (FGF), hepatocyte growth factor (HGF) and platelet derived growth factor

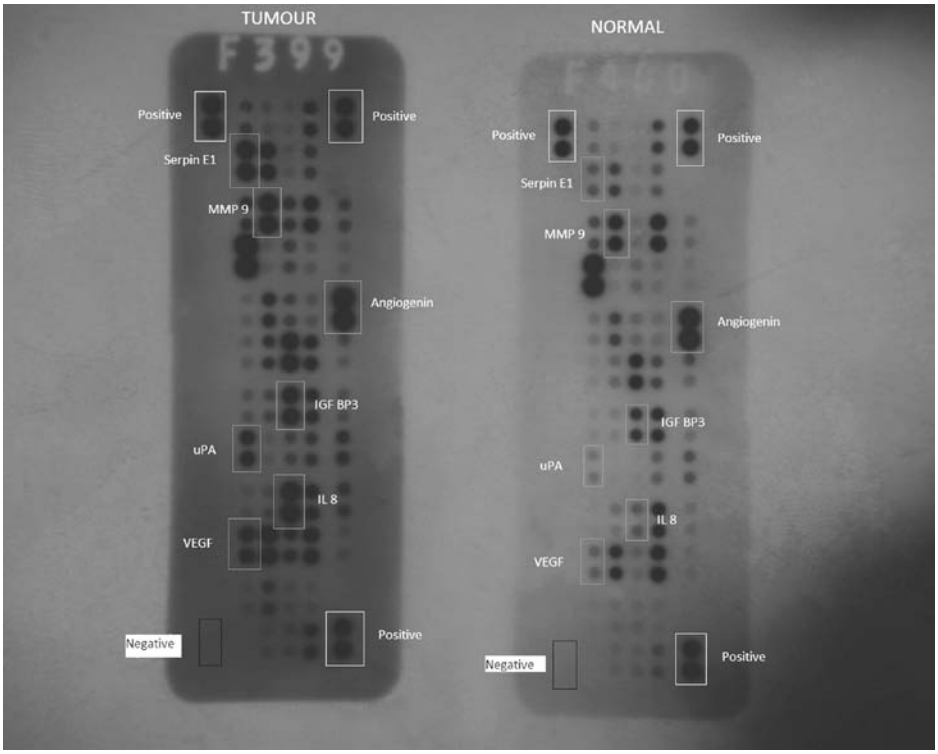

Figure 1. X-ray film of angiogenesis antibody arrays processed to detect proteins in tumour and normal mucosal tissue taken following 10 min of exposure. Grey boxes show examples of differential expression of proteins between tumour and normal mucosal tissue, white boxes are positive control spots and black boxes are negative control spots.

(PDGF) have been shown to induce angiogenesis and are negative prognostic factors for patient survival (8).

VEGF is the most widely studied angiogenic factor and is considered a key regulator of tumour-induced angiogenensis. The VEGF family consists of several factors known to act directly on vascular endothelial cells stimulating formation of new blood vessels (9). However, more recently it has been shown that VEGF proteins are also involved in tumour progression, immune-suppression and immune-tolerance (10). Similarly, FGF plays a role in angiogenesis by inducing endothelial proliferation and by regulating proteases like uPA and PAI-1 (11,12). Similarly, PDGF stimulates angiogenesis, plays a crucial role in tumour growth and raised levels have has been correlated with VEGF in HNSCC (13,14).

A recent phase I/II clinical trial by Cohen and colleagues (15), investigated the efficacy of anti-angiogenic therapies and demonstrated that a combination of erlotinib (epidermal growth factor receptor inhibitor) and bevacizumab (anti-VEGF antibody) is well tolerated in recurrent metastatic HNSCC (15). A better understanding of the network of angiogenic proteins present in distinct subsets of HNSCC is necessary, however, to underpin further anti-angiogenic therapies.

The aim of the current study was to investigate the expression of a panel of angiogenesis-related proteins in laryngeal tumour tissue, associated metastatic nodes and clinically normal mucosa from the same patient, in comparison with their expression in control uvula mucosa. The results were also correlated with tumour stage with a view to identifying potential markers of HNSCC cancer progression and metastatic spread.

Materials and methods

Patients and samples. Newly-presenting patients with moderately differentiated squamous cell carcinoma of the larynx (T1N2, n=2; T3N1, n=1; T3N2, n=1; T4N2, n=3) were recruited into the study. Following written informed consent, and prior to chemotherapy and radiotherapy, fresh tissue specimens were obtained intraoperatively from the tumour (n=7) and secondary lymph node (n=7). Where possible, normal-looking mucosa was also dissected away from the resected tumour specimen (n=4). Patients were all male with a mean age of 66.4 (range 46-85) years. A sample of control uvula mucosa (n=5) was removed from healthy volunteers undergoing surgery for uvulopalatopharyngoplasty. Approval for the study was gained from both the South Humber Research Ethics committee and Hull and East Yorkshire Hospitals R&D (06/Q1105/63 and 07/H1305/70).

Lysate preparation. Tissue was transported to the laboratory in Dulbecco's Modified Eagle's Medium (DMEM; PAA, Somerset, UK), supplemented with 10% (v/v) fetal bovine serum (FBS; Biosera, East Sussex, UK), penicillin/streptomycin (final concentrations: 0.1 U/ml, 0.1 mg/ml, respectively PAA). Tissue lysates were prepared by finely mincing the tissue, on ice, using scalpels in ProteoJET mammalian cell lysis reagent (500 μl/100 mg tissue; Fermentas Life Sciences, York, UK), containing protease inhibitor cocktail (1 ml/20 g tissue; Calbiochem/Merck, Nottingham, UK). The lysate was then subjected to sonication for 15 min on ice before centrifugation at 400 x g for 15 min to pellet cell debris. The resulting

Table I. Percent expression of angiogenic factors from stage T1 to stage T4 in tumour and nodal tissue.

Angiogenic factor	Tumour			Node		
	T1	T3	T4	T1	T3	T4
Actin A	ND	37	84	ND	23	25
ADAMTS[a]	ND	ND	ND	ND	ND	ND
Angiogenin	125	103	53	97	83	93
Angiopoietin-1[a]	ND	ND	ND	ND	ND	ND
Angiopoietin-2	ND	ND	38	ND	ND	35
Amphiregulin	ND	47	131	ND	ND	26
Antemin	ND	ND	124	ND	ND	31
Coagulation factor-3	34	66	182	28	47	77
Cxcl16	ND	43	129	ND	24	44
Dpp-iv	68	94	182	65	74	81
EGF[a]	ND	ND	ND	ND	ND	ND
EG-VEGF[a]	ND	ND	ND	ND	ND	ND
Endoglin[c]	22	94	192	40	61	102
Endostatin	33	68	223	25	66	82
Endothelin	ND	46	233	ND	ND	99
FGF acidic	44	64	250	ND	36	84
FGF basic	29	58	303	ND	29	51
FGF-4[a]	ND	ND	ND	ND	ND	ND
FGF-7	ND	31	70	ND	ND	23
GDNF[a]	ND	ND	ND	ND	ND	ND
GM-CSF[a]	ND	ND	ND	ND	ND	ND
HB-CSF	ND	55	77	ND	ND	44
HGF	ND	38	23	ND	22	37
IGFBP-1[d]	28	40	56	ND	ND	ND
IGFBP-2	46	90	156	40	69	98
IGFBP-3	ND	109	170	ND	69	89
IL-1β	ND	45	53	ND	ND	21
IL-8	ND	103	238	ND	42	71
LAP	ND	44	129	ND	25	29
Leptin	ND	ND	30	ND	ND	ND
MCP-1	ND	22	81	ND	ND	ND
MIP-1α[a]	ND	ND	ND	ND	ND	ND
MMP-8[b]	42	ND	94	29	60	64
MMP-9[d]	104	120	158	100	74	110
NRG1-B1[a]	ND	ND	ND	ND	ND	ND
Pentraxin3[b]	ND	73	31	ND	41	32
PD-ECGF[b]	ND	56	38	ND	34	51
PDGF-AA	ND	24	36	ND	ND	ND
PDGF-BB[a]	ND	ND	ND	ND	ND	ND
Persephin[a]	ND	ND	ND	ND	ND	ND
Plasminogen	ND	52	99	ND	20	67
Platelet factor 4[c,d]	40	95	204	79	76	109
PIGF	ND	30	145	ND	ND	26
Prolactin	ND	ND	73	ND	ND	ND
Serpin B5	ND	37	130	ND	ND	63
Serpin E1[c]	46	124	159	62	62	110
Serpin F1	21	56	33	ND	22	45
TIMP1	117	87	66	125	69	125
TIMP4[a]	ND	ND	ND	ND	ND	ND
Thrombospondin-1[e]	ND	ND	36	ND	39	58
Thrombospondin-2[a]	ND	ND	ND	ND	ND	ND
uPA[b]	ND	111	104	ND	52	119
Vasohibin[a]	ND	ND	ND	ND	ND	ND
VEGF	ND	58	193	ND	ND	68
VEGF-C[a]	ND	ND	ND	ND	ND	ND

Values shown are the percent expression relative to positive control samples. [a]Angiogenic factors excluded from the analysis (level of expression was less than the standard deviation of the positive control spots in all samples tested). [b]Factors expressed more highly in nodal tissue from stage T1 compared to matched tumour. [c]Factors in nodal tissue showing variable expression throughout tumour progression. [d]Factors with variable expression in tumour and nodal tissue. [e]Thrombospondin-1 is expressed more highly in nodal tissue compared with tumour at stage T3 and T4 in nodal tissue compared to tumour. ND, not detected; expression was <20% of the positive control.

supernatant was aliquoted into 0.5 ml lysates and stored at -80˚C for antibody array processing.

Protein determination and antibody array. The protein concentration in each lysate was quantified immediately before use in the antibody array using the Coomassie Brilliant Blue plus assay kit (Thermo Scientific, Pierce, Rockford USA) following the manufacturer's instructions. The relative expression of 55 angiogenesis-related proteins was determined in each lysate using a Proteome Profiler™ human angiogenesis array kit (R&D Systems, Abingdon, UK) following the manufacturer's protocol. Briefly, following blocking of the nitrocellulose membrane spotted with antibodies against angiogenesis-related proteins, 200 μg of lysate was mixed with a cocktail of biotinylated detection antibodies (15 μl) and added to the membrane before incubating overnight at 4˚C. Streptavidin-horseradish peroxidase (HRP, 1.5 ml) was then added to the membrane, incubated for 30 min before chemiluminescence detection reagents were added in equal volumes for 1 min. Residual detection reagent was carefully blotted off the membrane before covering it in plastic wrap and exposing to X-ray film for 5 and 10 min (Fig. 1). The light produced at each spot is proportional to the amount of analyte bound and the average pixel density of the duplicate spots produced on the film was determined using a UVP Bio-Imaging system fitted with LabWorks 4.0 Image acquisition and analysis software.

In addition to the 55 angiogenesis-related proteins, the membrane contained three pairs of positive control and one pair of irrelevant negative control antibodies. Following subtraction of the average optical density of the negative control spots from

all values, the level of the angiogenesis-related proteins were expressed as a percentage relative to the mean of the positive controls, which was assigned a value of 100%. The mean optical density and the standard deviation (SD) for the six positive control spots were determined for each of the membranes (n=7) studied and the SD was found to be ~20% in all cases. Therefore factors, which had an expression level within 20% of the positive control in all the T stages, were deemed not to have altered protein levels (n=15, Table I).

Relative expression of all 55 angiogenesis-related proteins was compared between: i) tumour and lymph node from the same patient (n=7) and uvula control tissue (n=5); ii) clinically normal looking mucosa (n=4), and malignant tissue (tumour and node); and iii) clinically normal looking mucosa and uvula control mucosa (n=5).

Results

Comparison of angiogenesis-related protein expression between tumour tissue and corresponding lymph node. Fifteen of the 55 angiogenesis-related factors showed no changes in expression (within 20% of positive control) across all T stages and were not further examined (Table I). Forty of the 55 angiogenesis-related factors investigated demonstrated a level of expression which was >20% of the positive control in at least one tumour stage. Of these 40 angiogenesis-related proteins 32 showed an increase from stage T1 through T4 (Table I). In most samples expression was detectable in all samples at all stages, alternatively in some cases it only became detectable in stage T3 (and then increased in T4), or was only present in T4. The same overall trend was observed for 29/32 of these factors in the nodal tissue from the same patient (excluding platelet factor 4, MMP-9 and IGFBP-1 which showed a variable expression through the stages). An example of the increasing trend is illustrated in Fig. 2A.

Tumour tissues from stage T3 and stage T4 patients had a higher relative expression of 31 (excluding thrombospondin-1) of the 32 angiogenesis-related proteins compared with the corresponding nodal tissues. Tumour tissues from stage T1 patients also showed a higher relative expression of 29 (excluding endoglin, platelet factor 4 and serpin E1) of the 32 proteins compared with the corresponding nodal tissue, although the level of difference was less apparent than at the advanced stages (Table I).

Of the remaining 8 proteins, which had detectable levels, angiogenin and TIMP1, showed a consistent decrease in expression in tumour tissue from stage T1 to stage T4 (Fig. 2B). These proteins in the corresponding nodal tissue showed variable expression; with higher expression in stage T1 and stage T4 tumours (Table I).

Comparison of angiogenesis-related protein expression between tumour patients (HNSCC primary and associated lymph nodes) and controls. Of the 40 angiogenesis-related proteins analysed the number of factors showing higher relative expression in tumour tissue compared with healthy controls increased from 2 in T1 stage, to 22 in T3 stage and 33 in T4 stage. Similarly, the number of angiogenic factors with a higher relative expression in the nodal tissue compared with healthy controls increased from 2 in stage T1 to 16 in stage T4.

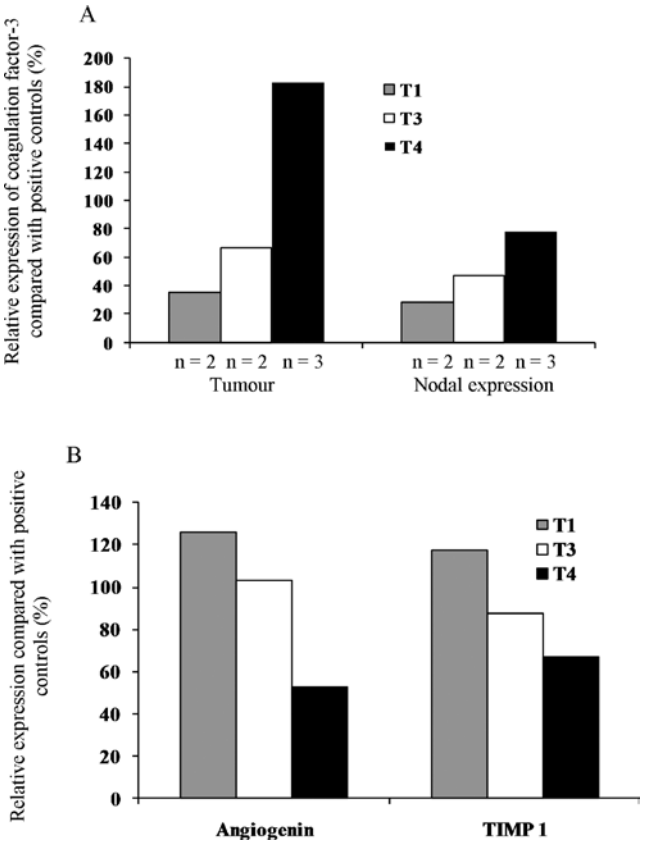

Figure 2. (A) An example of increasing protein expression with disease stage in both tumour and node. Relative expression of coagulation factor-3 relative to the mean of the positive controls is shown. (B) Relative expression of angiogenin and TIMP1 in relation to the positive control in HNSCC tumour tissue from different T stages.

Table II. Angiogenesis-related proteins which demonstrate increasing expression in normal mucosa with advancing tumour stage of the patient (matched samples).

	Normal mucosa expressed factors subdivided by T stage		
	T1 (n=1)	T3 (n=2)	T4 (n=1)
Angiopoietin-1	ND	ND	94
Angiopoietin-2	ND	ND	105
Amphiregulin	ND	ND	125
Antemin	ND	ND	86
Dpp-iv	63	76	235
FGF acidic	45	65	269
MMP-9	80	96	224
Plasminogen	ND	ND	83
Platelet factor 4	63	77	232
Serpin F1	ND	24	128
TIMP1	87	94	167

ND, not detected, expression was <20%.

Comparison of angiogenesis-related protein expression between normal looking mucosa, primary tumour and lymph nodes from the same patient. Macroscopically normal

Table III. Angiogenesis-related protein expression in normal mucosa compared with matched samples of tumour and nodal tissue.

	Tumour (n=4)			Node (n=4)			Normal mucosa (n=4)		
Angiogenic factor	T1	T3	T4	T1	T3	T4	T1	T3	T4
Actin A[a-c,f]	21	37	170	ND	23	40	ND	ND	79
Angiogenin[b,d,e]	92	103	ND	74	83	81	110	99	ND
Angiopoietin-2[f]	ND	ND	83	ND	ND	28	ND	ND	105
Amphiregulin[b,c,f]	ND	47	282	ND	ND	38	ND	ND	125
Antemin[c,f]	ND	ND	330	ND	ND	37	ND	ND	86
Coagulation factor-3[a-d,f]	68	66	390	53	47	84	63	34	272
Cxcl16[a,b,f]	22	43	68	ND	24	69	ND	ND	100
Dpp-iv[a-c,e,f]	86	94	286	97	74	81	63	76	235
Endoglin[b,c,f]	30	94	302	64	61	93	54	38	102
Endostatin[b-d,f]	66	68	222	48	66	92	69	57	142
Endothelin[b-d,f]	22	46	173	ND	ND	86	28	ND	126
FGF acidic[a,c-f]	85	64	282	ND	36	106	45	65	269
FGF basic[c-f]	58	58	474	ND	29	92	78	59	242
FGF-7[b,c,f]	ND	31	141	ND	ND	ND	21	ND	33
HB-CSF[c,d]	ND	55	175	ND	ND	75	ND	ND	ND
HGF[b]	ND	38	ND	25	22	50	23	ND	ND
IGFBP-1[a,b,f]	54	40	ND	ND	ND	ND	ND	ND	71
IGFBP-2[b-f]	76	90	270	43	69	146	108	89	226
IGFBP-3[b-d]	ND	109	296	ND	69	119	76	41	116
IL-1B[b,d]	ND	45	ND	ND	ND	ND	46	ND	ND
IL-8[b-d]	ND	103	232	ND	42	84	101	ND	ND
LAP[b,c]	ND	44	201	ND	25	47	ND	ND	ND
Leptin[c]	ND	ND	65	ND	ND	ND	ND	ND	ND
MCP-1[b,c,f]	ND	22	133	ND	ND	ND	ND	ND	22
MMP-8[b-d,f]	30	101	116	41	60	50	56	27	94
MMP-9[b,c,e,f]	80	120	261	100	74	106	80	96	224
Pentraxin3[b,d]	ND	73	ND	ND	41	21	34	27	ND
PD-ECGF[b]	ND	56	31	ND	ND	54	ND	ND	50
PDGF-AA[b]	ND	24	ND	ND	ND	27	ND	ND	ND
Plasminogen[b,c,f]	ND	52	178	ND	ND	77	ND	ND	83
Platelet factor 4[a-c,e,f]	79	95	407	89	76	110	65	77	232
PIGF[b-d]	ND	30	310	ND	ND	37	23	ND	26
Prolactin[c]	ND	ND	122	ND	ND	ND	ND	ND	ND
Serpin B5[b-f]	ND	37	318	ND	ND	93	41	23	227
Serpin E1[b-d,f]	40	124	295	46	62	109	81	21	142
Serpin F1[a,b,e,f]	37	56	59	ND	22	48	ND	24	128
TIMP1[e,f]	82	87	71	102	69	116	87	94	167
Thrombospondin-1[b,c]	ND	ND	ND	ND	39	47	ND	ND	ND
uPA[b-d]	ND	111	200	ND	52	133	63	ND	ND
VEGF[b-d]	ND	58	474	ND	ND	123	25	ND	42

[a]Factors expressed more highly in tumour tissue in stage T1 compared to normal mucosa. [b]Factors expressed more highly in stage T3 tumour tissue compared to normal mucosa. [c]Factors expressed more highly in tumour tissue in stage T4 compared to normal mucosa. [d]Factors expressed more highly in normal mucosal tissue in stage T1 compared to nodal tissue. [e]Factors expressed more highly in stage T3 normal mucosal tissue compared to nodal tissue. [f]Factors expressed more highly in normal mucosal tissue in stage T4 compared to nodal tissue. ND, not detected, expression was <20% of the positive control.

mucosa, tumour and metastatic nodal tissue from the same patient were obtained in four cases (T1, n=1; T3, n=2; T4, n=1). Eleven of the 40 angiogenesis-related proteins analysed increased in relative expression in normal looking mucosa with increasing tumour stage of the patient from which the normal tissue was obtained (Table II).

Of the 40 angiogenesis-related factors analysed there were only 8 which had a higher relative expression in tumour tissue from T1 patients compared to normal mucosa from the same patient, however the number of factors with a higher expression in the tumour tissue compared to the normal mucosa increased in the later stage tumours (T3, n=32; and T4, n=29).

Table IV. Percent expression relative to positive controls of factors expressed more highly in normal mucosa from tumour patients compared with uvula tissue from healthy donors.

Factor	Normal (n=4)	Control tissue (n=5)
Amphiregulin	33	27
Coagulation factor-3	103	97
Dpp-iv	114	91
FGF acidic	114	84
FGF basic	109	83
IGFBP-1	36	32
IGFBP-2	123	102
Platelet factor 4	112	108
TIMP1	101	83

In contrast expression of some angiogenesis-related proteins in tumour adjacent normal tissue was higher than in the nodal tissue from the same patient: out of the 40 angiogenesis-related factors analysed, 24 showed a higher expression in normal mucosa from T4 patients compared with the corresponding node, whereas adjacent normal mucosa from stage T3 and stage T1 biopsies had 10 and 18 factors, respectively which showed a higher expression than the corresponding nodal tissue (Table III).

Comparison of angiogenesis-related protein expression between normal looking mucosa and uvula control tissue. Nine of the 40 angiogenesis-related proteins analysed had a higher relative expression in normal mucosa obtained from the tumour patient compared with control uvula tissue (Table IV). Of these nine factors, FGF acidic, FGF basic, IGFBP-2 and serpin B5 were also higher in normal mucosa compared with the nodal deposits (Table III).

Discussion

A number of proteins are known to play an important role in angiogenesis, which is vital for the growth and advancement of solid tumours. Many studies have demonstrated the production of angiogenic proteins by HNSCC; however, the majority of these have only explored the expression of a single angiogenic factor (13,16,17). To date there have been only four studies which have looked at multiple angiogenesis-related factors in lysates of HNSCC (8,18-20).

The current pilot study has investigated the expression of a panel of angiogenesis-related proteins in lysates from tumour tissue, associated lymph nodes, adjacent normal mucosa and uvula mucosa from normal controls. It was found that a large number of these proteins increase in expression in both tumour and metastatic nodes with increasing disease stage and therefore may be involved in promoting tumour progression. In addition we have shown that there is increased expression of some of these factors in the normal mucosa adjacent to the tumour compared with both the metastatic nodal tissue and uvula control tissue, suggesting that the processes may be occurring in a wider area than just the main tumour mass, potentially predisposing the patient to recurrence or the development of a second primary tumour supporting the 'field of cancerization' hypothesis. The angiogenic growth factors VEGF, IL-8, bFGF, HGF are commonly found to be over-expressed in HNSCC in lysates (8) and systemically (21,22). In the current study 32 of the 40 angiogenesis-related proteins analysed had an increasing expression in the tumour tissue with advancement of the tumour stage. This is in agreement with previous studies in HNSCC, which showed increased expression of VEGF (18,23), MMP-9 (24) HGF and FGF (8) in tissue lysates with tumour progression. However, the current study has identified many more.

In a study which looked into multiple angiogenesis-related growth factors, Ninck *et al* (20) reported an increased expression of a combination of factors in 80% of the HNSCC tumours and identified a distinct pattern of secretion which always included either VEGF or PDGF-AB, with G-CSF or GM-CSF. Montag *et al* (8) studied the expression of eight angiogenic factors in tissues and reported detectable expression of at least 4 factors in 90% of the tumours. In addition there was a significant association between factors VEGF-A and PDGF-BB two of the most common factors and with bFGF, HGF or G-CSF as well as with PDGF-AB. Chen *et al* (18) investigated the expression of angiogenic factors in tumour tissue, HNSCC cell lines and serum and established that the factors IL-8, VEGF, GM-CSF and a number of other pro-inflammatory cytokines (not included in the current angiogenesis array) produced by HNSCC are consistent with the pathology of the neoplasm. Petruzzelli *et al* (19) studied the expression of bFGF, VEGF, TGF and PGE2 in HNSCC cell lines and reported that HNSCC produce these factor(s) to stimulate endothelial cell proliferation and that VEGF may be involved in HNSCC-induced endothelial cell mitogenesis. In the current study it has been observed that a panel of proteins showed increased expression in tumour and associated nodes with the progression of tumour stage. However, further studies of large cohort of HNSCC patients should be considered to validate the significance of these factors.

In addition we demonstrated a higher level of expression in many of the angiogenic factors in both the tumour tissue and the associated nodal tissue, compared with tissue from the healthy control group, which is in accordance with previous studies where angiogenic factor levels of MMP-9 (24), HGF, VEGF (21,25) and IL-8 (22) were increased in patients with HNSCC compared with the control group (21). In contrast to the angiogenesis-related proteins, which increased with tumour progression, angiogenin and TIMP1 were found to decrease with disease progression. One study which supports this finding is that of Homer *et al* (25) who demonstrated a lower serum angiogenin expression associated with locoregional disease recurrence in HNSCC. However, Ruokolainen *et al* (26) reported that high levels of TIMP1 are associated with tumour progression. The investigations showed an increased expression of TIMP1 in serum and tumour tissue in patients with stage T3 and T4 tumours compared with stage T1.

Furthermore, in the current study a higher expression of angiogenin and TIMP1 in tumour and associated nodal tissue compared with healthy control tissue was demonstrated, which is in agreement with the study by Charoenrat *et al* (24) who investigated levels in tumours compared with normal

mucosa and also found a significant correlation between the levels of MMP-9 and TIMP1 with advanced T stage.

A number of angiogenic factors have also been shown to differ between tumour and normal tissue, and with disease stage including uPA and its inhibitor, cxcl16, endothelin, IGFBP-1 which all increase with tumour progression. The role of these factors have also been studied in other malignancies including colon (27,28), renal cell (29), ovarian (30) and gastric cancer (31). However, there is little evidence for their role in HNSCC, suggesting that the role requires further elucidation.

Roesch-Ely *et al* (32) have carried out proteomic analysis on both tumour and adjacent healthy mucosa and found that 72% of the healthy mucosa adjacent to the tumour had a significant association between aberrant profiles and tumour relapse events. In the current study the expression of angiogenesis-related proteins in the adjacent normal mucosa compared with that of the tumour tissue, metastatic node and healthy control tissue support the fact that although the adjacent mucosa may have a normal appearance, molecular events have occurred which are influencing protein production and could be important for subsequent tumour development.

In conclusion, many angiogenesis-related proteins may be involved in the growth, spread and progression of laryngeal tumours and tissues which may have a clinically normal appearance are likely to be part of the affected tumour zone. Further investigation into the level of expression of specific factors in a larger cohort of patients is required to validate the findings of this study.

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