Impact of p16 status on pro- and anti-angiogenesis factors in head and neck cancers

Running title: *Angiogenesis factors in head and neck cancer*

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Abstract

Background: Head and neck cancers (HNC) are aggressive tumours. Overexpression of p16 in HNC correlates with Human Papilloma Virus (HPV) associated HNC that carry a better prognosis than HPV-negative tumours. Angiogenesis is an important factor in tumour progression. Our aim was to dissect the impact of p16 expression on angiogenesis factors in HNC.

Methods: 18 newly diagnosed HNC patients and controls were analysed. Eleven pro- and anti-angiogenesis factors were quantified by multiplex ELISA in HNC patients and controls. Angiogenesis factors were analysed in tumour tissue by immunohistochemistry.

Results: Circulating levels of endostatin (anti-angiogenesis factor) were higher in the HNC group compared to healthy donors. Interestingly, the pro-angiogenesis factors angiopoietin-1 and vascular endothelial growth factor (VEGF) were significantly higher in patients with p16-negative compared to p16-positive HNC. Moreover, the major source of VEGF in p16-positive HNC tissue was tumour stromal cells. In contrast, both tumour cells and stromal cells expressed VEGF in p16-negative tissue.

Conclusions: We show that p16-negative tumours associate with increased circulating levels of pro-angiogenic VEGF and angiopoietin-1. Tissue expression of VEGF differs between p16-positive and p16-negative tumours. These findings may explain differences in the biological behaviour of p16-positive and p16-negative HNC. Better understanding of mechanisms by which p16 status influences tumour angiogenesis may guide development of targeted therapies.

Key words: angiogenesis factors; head and neck cancer; HPV; p16 expression

Abbreviations: head and neck cancer (HNC); human papilloma virus (HPV)

Introduction

Head and neck squamous cell carcinomas (HNC) constitute an important proportion of cancers worldwide. These are aggressive tumours and survival rates have remained poor in spite of advances in surgical techniques, radiotherapy and chemotherapy. Interestingly, certain subtypes of head and neck cancer are associated with a better prognosis. Sixty percent of oro-pharyngeal cancers associate with the oncogenic human papilloma virus (HPV) (subtypes 16 and 18)(Venuti et al., 2004). Of note, this cohort of patients has a better disease-free survival than the patients who do not express the viral protein (Ang et al., 2010). The reasons underlying this are not known and further understanding of the biology of these tumours is necessary in order to develop new therapeutic options.

It is well known that tumour growth depends on the establishment of a robust vascular supply to support the oxygen and nutrient requirements of cancer cells. This formation of blood vessel network or angiogenesis also acts as a conduit to allow spread of cancer cells to the rest of the body (metastases) and thereby promote aggressive tumour progression (Folkman et al., 1989, Hanahan and Folkman, 1996). Vessel turnover in healthy tissue is maintained in the steady state by interactions between molecules which promote angiogenesis (pro-angiogenesis factors) and those that inhibit it (anti-angiogenesis factors)(Hanahan and Weinberg, 2011). Tumours exploit this phenomenon and can produce a wide variety of pro-angiogenesis factors to promote their own growth, and the levels of these factors in the tumour microenvironment could determine the outcome of cancer therapy. Indeed anti-angiogenesis therapies in the form of antibodies (e.g. anti-Vascular Endothelial Growth Factor (VEGF) antibody) or recombinant proteins (e.g. Endostatin) are being actively used in the treatment of solid tumours (Cohen et al., 2009, Ye et al., 2014).

The aim of this work was to dissect the impact of prognostic factors like p16, tumour size and nodal status on the angiogenesis profile of HNC. To this purpose we studied the circulating levels of a panel of eleven angiogenesis factors in patients with HNC and correlated the levels with known prognostic factors including p16 status. In addition we explored the effect of p16 status on the cellular source of angiogenesis factors in the tumour tissue.

Materials and methods

Study population

Blood samples were obtained from 18 patients with newly diagnosed HNC cancers and 12 healthy controls. The demographic details of patients and controls are provided in **Supplemental Table 1**. Strict exclusion criteria were applied to reduce the impact of other inflammatory conditions on the serum levels of angiogenesis factors. Patients with co-existing inflammatory disorders such as autoimmune diseases, diabetes, renal failure and cardiac disease were excluded from the study. The study was approved by the local research ethics committee and informed consent was obtained from all study subjects.

Serum isolation and storage

Blood was allowed to coagulate for at least 30 minutes at room temperature, and then centrifuged at 1500g for 10 minutes and the serum was stored at a temperature below -20°C until analysis. In one HNC patient plasma was used instead of serum.

Multiplex analysis of cytokines

The multiplex Luminex Performance angiogenesis panel was purchased from R&D Systems. The quantification of cytokines was performed according to the manufacturer's instructions using a Luminex analyser. The following cytokines were

analysed: VEGF, VEGF-D, Angiopoietin-1, Angiogenin, Thrombospondin-2, Endostatin, Placental Growth Factor (PlGF), Platelet Derived Growth Factor-aa (PDGF-aa), Platelet Derived Growth Factor- bb (PDGF-bb), Fibroblast Growth Factor –acidic (FGF-a) and Fibroblast Growth Factor-basic (FGF-b). Angiopoietin-1 and VEGF could not be quantified in plasma and therefore one HNC patient was excluded from those analyses.

Immunohistochemistry

The following primary antibodies were used: rabbit polyclonal VEGF (A-20, Santa Cruz Biotechnology); goat polyclonal Angiopoietin-1 (R&D Systems); and goat polyclonal Endostatin (R&D Systems). Paraffin sections of tumour tissue were cut at 4μm and heated for 45 minutes at 60°C prior to staining. Heat antigen retrieval was carried out using Epitope Retrieval Solution 1 (VEGF and Endostatin), pH 6 or Epitope Retrieval Solution 2 (Angiopoietin-1), pH 9 at 100°C for 20 or 30mins, according to the antibody. Antibodies were diluted 1:50 and incubated for 15 minutes. Negative controls used antibody diluent in place of primary antibody. Visualisation for rabbit polyclonal antibodies was by Bond Polymer Refine kit, an HRP-conjugated 3,3'-diaminobenzidine (DAB) detection system, supplied by Leica Microsystems. Visualisation for goat polyclonal antibodies was by Bond Intense R kit, a Biotin/streptavidin HRP-conjugated DAB detection system, supplied by Leica Microsystems and secondary antibody biotinylated rabbit anti-goat, supplied by Dako UK Ltd. All immunohistochemistry staining was carried out using Bond III Fully automated staining system and associated reagents, supplied by Leica Microsystems, Newcastle-Upon-Tyne, UK. Images were captured using an Olympus BX50 microscope with UPlan Appo lenses and equipped with an Olympus digital camera.

Statistical analysis

Data were compared using the Student *t* test. Probability values (p) of less than 0.05 were considered statistically significant. Statistical analysis was performed using the GraphPad Prism software version 5.02.

Results

Angiopoietin-1 is higher in the circulation of patients with p16-negative HNC

Angiopoietins are a well-described family of vascular growth factors that play a role in embryonic and tumour angiogenesis (Suri et al., 1996, Holash et al., 1999). We examined the levels of the pro-angiogenesis factor angiopoietin-1 in the serum of head and neck cancer (HNC, n=17) patients and healthy individuals (n=13) **(Figure 1)**. No significant differences were observed in the levels of angiopoietin-1 when the total patient cohort was compared to healthy individuals. However, further analysis revealed that angiopoietin-1 levels in the circulation of patients with p16-negative tumours were significantly higher compared to those present in healthy individuals **(Figure 1B)**. In contrast, serum angiopoietin-1 levels in patients with p16-positive tumours did not differ from healthy individuals (**Figure 1B).** We also examined if the tumour size or nodal status had any impact on the circulating angiopoietin-1 level, neither of which was found to have any influence (**Figure 1C,D).**

Vascular Endothelial Growth Factor (VEGF) is higher in the circulation of patients with p16-negative HNC

VEGF is up-regulated in several tumours and is a potent pro-angiogenesis factor (Ferrara and Davis-Smyth, 1997, McMahon, 2000). We next evaluated the levels of circulating VEGF in patients with HNC (n=17) and healthy individuals (n=13). No

differences in serum levels of VEGF were observed between the total patient cohort and the healthy controls **(Figure 2A)**. However, in line with our observation with angiopoietin-1, the levels of VEGF were significantly increased in patients with p16-negative tumours compared to healthy individuals (**Figure 2B**). Moreover, circulating VEGF levels in patients with p16-positive tumours were comparable to those present in healthy individuals **(Figure 2B)**. In contrast to the p16 status, tumour and nodal stage had no influence on circulating VEGF levels (data not shown). Furthermore, whilst circulating levels of VEGF (total) showed a difference between patients carrying p16-positive or negative tumours, no such difference was observed in VEGF-D levels (**Figure 2C, D**).

Endostatin levels are higher in patients with HNC compared to controls

We next examined the levels of circulating endostatin in patients with HNC (n=18) and healthy individuals (n=13). Endostatin is a fragment of collagen 18 and is a potent anti-angiogenesis agent (O'Reilly et al., 1997). Endostatin was found to be significantly higher in the serum of patients with HNC compared to healthy individuals (**Figure 3A**). Unlike VEGF and angiopoietin-1, a significant increase in endostatin was present in both p16-negative and p16-positive HNC patients **(Figure 3B)**. Additionally, tumour stage and nodal stage had no influence on endostatin levels (not shown).

Levels of circulating thrombospondin-2, FGF-b, PDGF-aa, PDGF-bb and PlGF in patients with p16-negative and p16-positive HNC

In addition to the previously described angiogenesis factors we examined the levels of a panel of other angiogenesis factors: thrombospondin-2, Placental Growth Factor

(PlGF), Platelet-derived Growth Factor (PDGF-aa, PDGF-bb), Fibroblast Growth Factor-basic (FGF-b) and angiogenin. Thrombospondin-2 is an anti-angiogenesis factor (Iruela-Arispe et al., 2004). While thrombospondin-2 levels in the whole patient cohort (n=18) did not differ from controls (n=13) **(Figure 4A)**, its levels were found to be lower in sera from patients with p16-positive tumours in comparison to the levels detected in healthy individuals or patients with p16-negative tumours **(Figure 4B**). This however did not reach statistical significance. Patients with T1/2 tumours had lower levels of thrombospondin-2 compared to patients with T3/4 tumours and healthy donors **(Figure 4C)** but again this did not reach statistical significance. Serum levels of thrombospondin-2 were comparable in patients with N0 or node-positive disease (**Figure 4D**). Levels of FGF-b, PDGF-aa, PDGF-bb and PlGF were similar in HNC patients compared to the control group (**Figure 5A,C,E,G**). In addition, the p16 status did not have any influence on the levels of these angiogenesis factors (**Figure 5B,D,F,H**). No differences were observed for angiogenin and FGF-a (data not shown). A summary of all angiogenic factors is included in **Supplemental Table 2**.

Tissue expression of VEGF differs between p16-positive and p16-negative tumours

We next examined the expression of angiopoietin-1, VEGF and endostatin in the HNC tumour tissue using immunohistochemistry. Angiopoietin-1 was localised in tumour tissue to both tumour cells and stromal cells (**Figure 6A**). This pattern of expression of angiopoietin-1 was similar in both p16-positive and p16-negative tumours. Strong VEGF expression was noted in tumour cells and stromal cells in tissue specimens from p16-negative tumours (**Figure 6B left panel**). In contrast, expression of VEGF in p16-positive tissue was primarily localised to stromal cells

(**Figure 6B right panel**). Tumour cells in p16-positive HNC tissue did not express VEGF in four out of five tissue samples examined. We also examined endostatin in the tumour tissue. Endostatin was expressed in stromal cells resembling macrophages, while tumour cells in HNC did not express endostatin. No differences were observed in the tissue expression of endostatin in p16-positive or p16-negative tumour tissue.

Discussion

This study explored the impact of p16 status on the profile of angiogenesis factors in HNC, both in terms of circulating levels of these factors and their expression in the tumour tissue. We show an interesting effect of p16 status on angiogenesis factors in HNC. First the pro-angiogenesis factors VEGF and angiopoietin-1 are elevated in the serum of patients with p16-negative tumours compared to healthy individuals, whilst this increase was not observed in patients with p16-positive HNC. Second we show via immunohistochemistry that VEGF production in p16-positive tumours is mainly derived from stromal cells while in p16-negative tumours VEGF is produced both by tumour cells and stromal cells.

p16 is considered a good surrogate marker to identify HPV-positive tumours and is widely used in clinical practice. It has been reported that p16 status correlates with a better prognosis in oropharyngeal cancers (Weinberger et al., 2004). Additionally, a good correlation between HPV positivity and p16 status has been demonstrated in oropharyngeal cancers (Nichols et al., 2009), which form the majority of our patient cohort (15 of 18 patients). However, detection of p16 by immunohistochemistry carries a false positive rate of 3-4% as some HPV-negative tumours could continue to express p16 (Schlecht et al., 2011, Seiwert, 2013, Thavaraj et al., 2011). Although detection of HPV by PCR is the most accurate test for

identifying HPV infection, p16 expression by immunohistochemistry continues to be a widely accepted method for HPV detection due to low costs and availability of technical infrastructure in most head and neck units. Of note, ongoing clinical trials such as De-ESCALaTE HPV stratify patients for inclusion based on HPV status as determined by p16 immunohistochemistry (Masterson et al., 2014). Interestingly, while p16 status does not correlate with HPV positivity in HNC outside the oropharynx such as the oral tongue, it still associates with a better prognosis irrespective of HPV status (Harris et al., 2011).

It is well established that patients with HPV-positive tumours fare better than patients with HPV-negative tumours (Ang et al., 2010). This has resulted in discussion of altering therapy (de-escalation) of HPV-positive tumours by reducing the dose of radiation or trials of cetuximab rather than cisplatin in an effort to reduce the side effects of chemo-radiation therapy (Masterson et al., 2014, Tornesello et al., 2014). However, the precise reasons for the different biological behaviour of HPV-positive versus HPV-negative tumours are not certain. Our findings that VEGF and angiopoietin-1 levels are raised in the serum of patients with p16-negative tumours but not those with p16-positive tumours, indicates that differences in angiogenic pathways may account, at least in part, for the distinct biological behaviour of p16-positive HNC.

The tumour stroma has emerged as an important contributor to the angiogenesis and progression of cancer (Semenza, 2013). Our immunohistochemistry analysis confirms the important role of the stroma in the production of angiogenesis factors in the HNC microenvironment. Stromal cells such as macrophages are known to produce angiogenesis factors such as VEGF (Lin and Pollard, 2007). We demonstrate that in p16-positive tumour tissue, the stromal cells are the predominant

source of VEGF, with no expression of VEGF in tumour cells. In contrast, in p16-negative tumours both tumour and stromal cells produce VEGF. This suggests that future work into the mechanisms of VEGF induction in stromal cells vis-a-vis tumour cells may reveal novel strategies to specifically target stromal-derived VEGF. Though our results need to be validated in a larger patient cohort, our findings may have therapeutic potential. Indeed, anti-VEGF antibodies have been used as adjuvants to chemotherapy in the treatment of HNC (Cohen et al., 2009) but are unfortunately associated with potentially fatal complications such as haemorrhage and high blood pressure (Peng et al., 2014, Qi et al., 2013). Inhibitors specific for tumour stromal cell-derived VEGF could allow for inhibition of angiogenesis within p16-positive HNC while leaving normal vasculature intact and avoiding catastrophic side-effects.

Our results have also uncovered interesting facets of endostatin, which is an anti-angiogenesis factor that has been shown to be elevated in a variety of cancers (Kantola et al., 2014, Koc et al., 2006). We also found significantly elevated levels of endostatin in our HNC patient cohort, albeit there was no difference between p16-positive and p16-negative tumours. We found that endostatin is expressed in tumour stroma in cells resembling macrophages. To our knowledge, this is the first demonstration of endostatin production within the cancer stromal tissue, which warrants further investigation to define the role of endostatin in the tumour microenvironment. Recombinant endostatin therapy has shown good response in mouse models in early studies (Blezinger et al., 1999) but human tumour response was not encouraging (Thomas et al., 2003), possibly due to a failure to achieve adequate local concentrations of endostatin required to inhibit tumour growth. Endostatin delivery to tumour tissue using viral vectors is currently in trials (Ye et al., 2014).

p16 status was also not found to impact on the production of other angiogenesis factors such as FGF-b, PDGF and PlGF. However, thrombospondin-2 levels showed a trend to be lower in the serum of patients with p16-positive tumours compared to patients with p16-negative tumours and even healthy donors (not statistically significant). High levels of thrombospondin-2 in lung cancer have been correlated to poorer prognosis (Naumnik et al., 2015), while down-regulation of thrombospondin-2 in gastric cancer correlates with poorer prognosis (Sun et al., 2014). The relationship between thrombospondin-2 and p16 status is not clear currently, but future work could shed light on this.

In summary, alterations in angiogenesis pathways in p16-positive versus p16-negative HNC could be one of the factors underlying the different biological behaviour of these two types of HNC. Future studies in larger patient cohorts and direct demonstration of HPV by PCR techniques within tumour tissue will provide further confirmation of the role of HPV in this process. Additionally, the identification of stromal cells as important producers of angiogenesis factors, in particular VEGF and endostatin, opens new avenues for research on targets for selective inhibition or induction of angiogenesis factors from stromal cells in HNC.

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Titles and legends to figures

Figure 1. Angiopoietin-1 levels in patients with head and neck cancer and controls. Circulating levels of angiopoietin-1 were quantified by multiplex ELISA in sera from healthy individuals (Ctrl, n=13) and patients with head and neck cancer (HNC, n=17). **A.** The scatter plots show Angiopoietin-1 concentration in the two study groups (horizontal bars show the mean value for each group). **B.** Angiopoietin-1 levels were compared between p16-positive (HNC p16+) or p16-negative (HNC p16-) HNC patients and controls. *$p<0.05$ **C.** The plots display Angiopoietin-1 in patients with early tumour stage (T1/2), advanced tumour stage (T3/4) and controls. **D.** The scatter plots display Angiopoietin-1 in patients without nodal disease (N0), nodal disease (N+) and controls.

Figure 2. VEGF levels in patients with head and neck cancer and controls. Circulating levels of VEGF were quantified by multiplex ELISA in sera from healthy individuals (Ctrl, n=13) and patients with head and neck cancer (HNC, n=17). **A.** The scatter plots show VEGF concentration in the two study groups (horizontal bars show the mean value for each group). **B.** VEGF levels were compared between p16-positive (HNC p16+) or p16-negative (HNC p16-) HNC patients and controls. *$p<0.05$ **C.** The plots display VEGF-D in the two study groups. **D.** The scatter plots display VEGF-D in p16-positive (HNC p16+) and negative HNC (p16-) patients and controls. VEGF=vascular endothelial growth factor

Figure 3. Endostatin levels in patients with head and neck cancer and controls. Circulating levels of endostatin were quantified by multiplex ELISA in sera from healthy individuals (Ctrl, n=13) and patients with head and neck cancer (HNC, n=18).

A. The scatter plots show endostatin concentration in the two study groups (horizontal bars show the mean value for each group). **B.** Endostatin levels were compared between p16-positive (HNC p16+) or p16-negative (HNC p16-) HNC patients and controls. ***p<0.001, **p<0.01, *p<0.05

Figure 4. Thrombospondin levels in patients with head and neck cancer and controls. Circulating levels of thrombospondin-2 were quantified by multiplex ELISA in sera from healthy individuals (Ctrl, n=13) and patients with head and neck cancer (HNC, n=18). **A.** The scatter plots show thrombospondin-2 concentration in the two study groups (horizontal bars show the mean value for each group). **B.** Thrombospondin-2 levels were compared between p16-positive (HNC p16+) or p16-negative (HNC p16-) HNC patients and controls. **C.** The plots display thrombospondin-2 in patients with early tumour stage (T1/2), advanced tumour stage (T3/4) and controls. **D.** The scatter plots display thrombospondin-2 in patients without nodal disease (N0), nodal disease (N+) and controls.

Figure 5. Other angiogenic factors levels in patients with head and neck cancer and controls. Circulating levels of angiogenic factors were quantified by multiplex ELISA in sera from healthy individuals (Ctrl, n=13) and patients with head and neck cancer (HNC, n=18). **A.** The scatter plots show FGF-b concentration in controls and HNC patients. Horizontal bars show the mean value for each group. **B.** FGF-b levels in p16-positive (HNC p16+) or p16-negative (HNC p16-) HNC patients and controls. **C.** The scatter plots show PDGFaa concentration in controls and HNC patients. **D.** PDGFaa levels in p16-positive or p16-negative HNC patients and controls. **E.** The scatter plots show PDGFbb concentration of controls and HNC patients. **F.** PDGFbb

levels in p16-positive or p16-negative HNC patients and controls. **G.** The scatter plots show PlGF concentration controls and HNC patients. **H.** PlGF levels in p16-positive or p16-negative HNC patients and controls. FGF-b=fibroblast growth factor-basic; PDGF=platelet-derived growth factor; PlGF=placental growth factor

Figure 6. Expression of Angiopoietin-1, VEGF and Endostatin in p16-negative and p16-positive head and neck cancer tissue. Expression of angiogenic factors (angiopoietin-1, VEGF and endostatin) was assessed in tissue from five patients with p16-negative and five patients with p16-positive head and neck cancer (HNC) using immunohistochemistry (see Methods for details). **A**. Angiopoietin-1 expression in tumour cells (arrow heads) and tumour stroma (arrows) **B**. VEGF expression in tumour tissue (arrow heads) and stromal tissue (arrows) **C**. Expression of endostatin in the tumour stroma (arrows). **D.** p16 staining in the tumour tissue used for analysis of angiogenesis factors expression.

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Figure 1

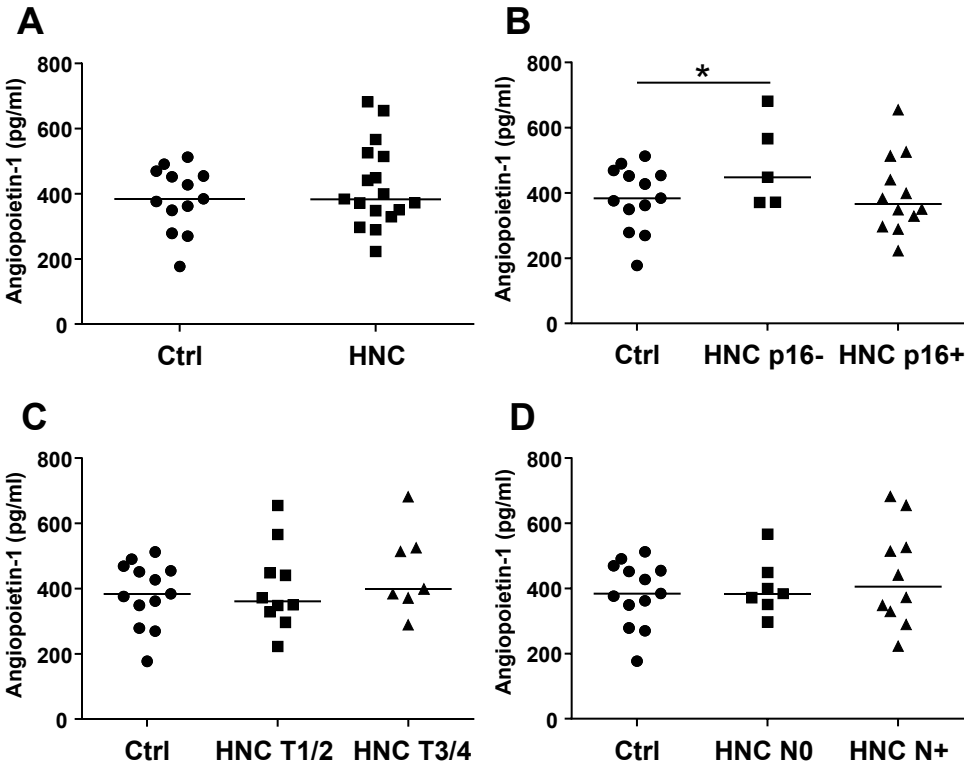

A
B
C
D
Angiopoietin-1 (pg/ml)
800
600
400
200
0
Ctrl
HNC
HNC p16-
HNC p16+
HNC T1/2
HNC T3/4
HNC N0
HNC N+
*

Figure 2

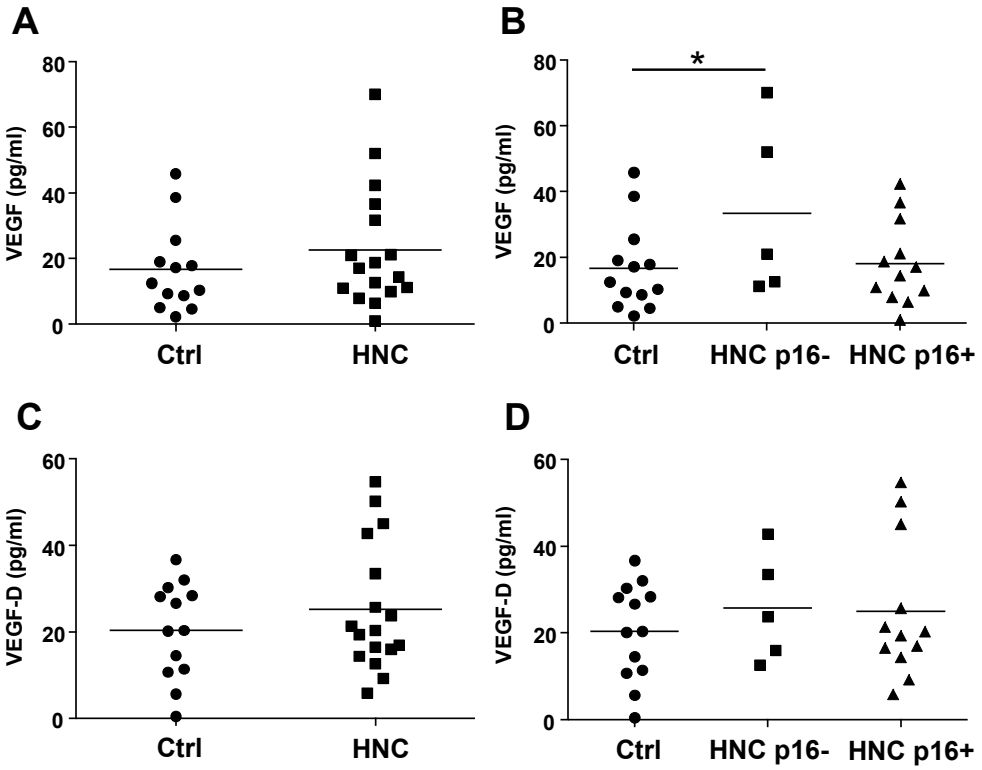


Figure 3

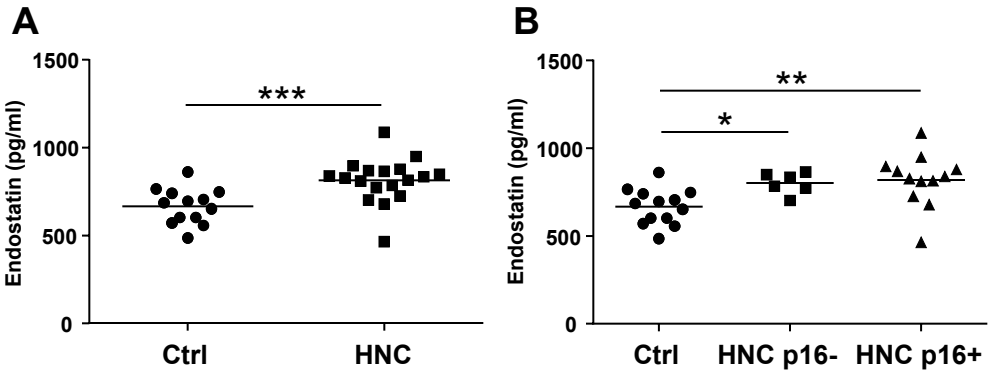

A
1500
1000
500
0
Endostatin (pg/ml)
***
Ctrl
HNC
B
1500
1000
500
0
Endostatin (pg/ml)
**
*
Ctrl
HNC p16-
HNC p16+

Figure 4

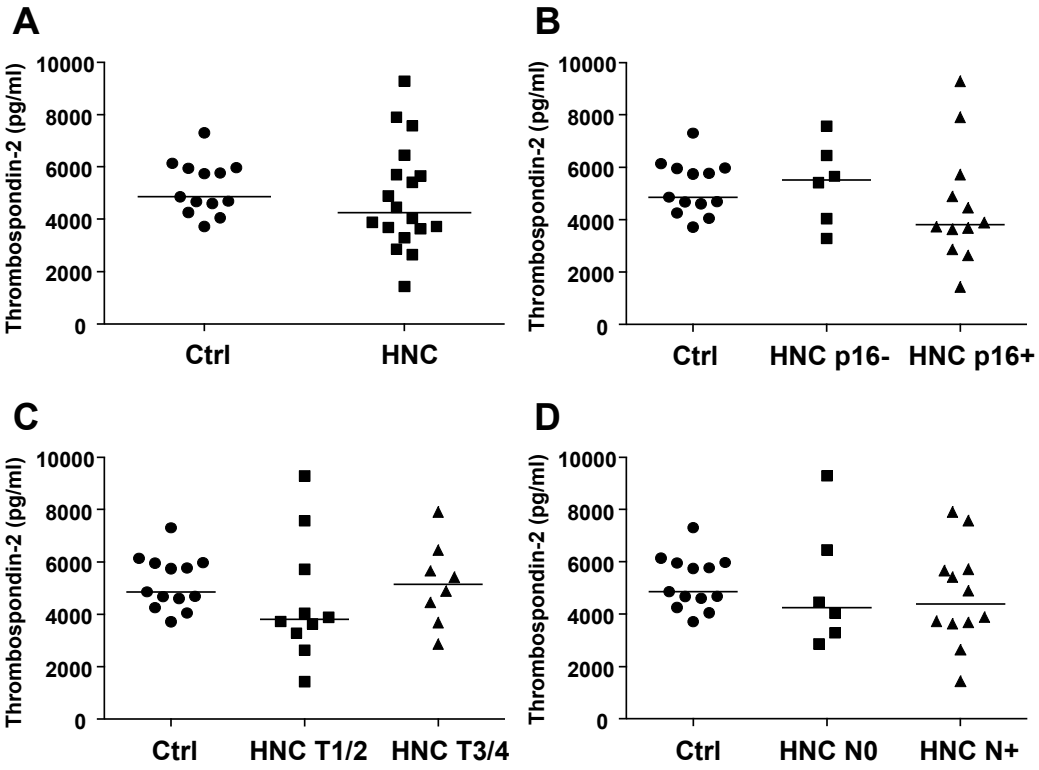

A
B
C
D
Thrombospondin-2 (pg/ml)
10000
8000
6000
4000
2000
0
Ctrl
HNC
HNC p16-
HNC p16+
HNC T1/2
HNC T3/4
HNC N0
HNC N+

Figure 5

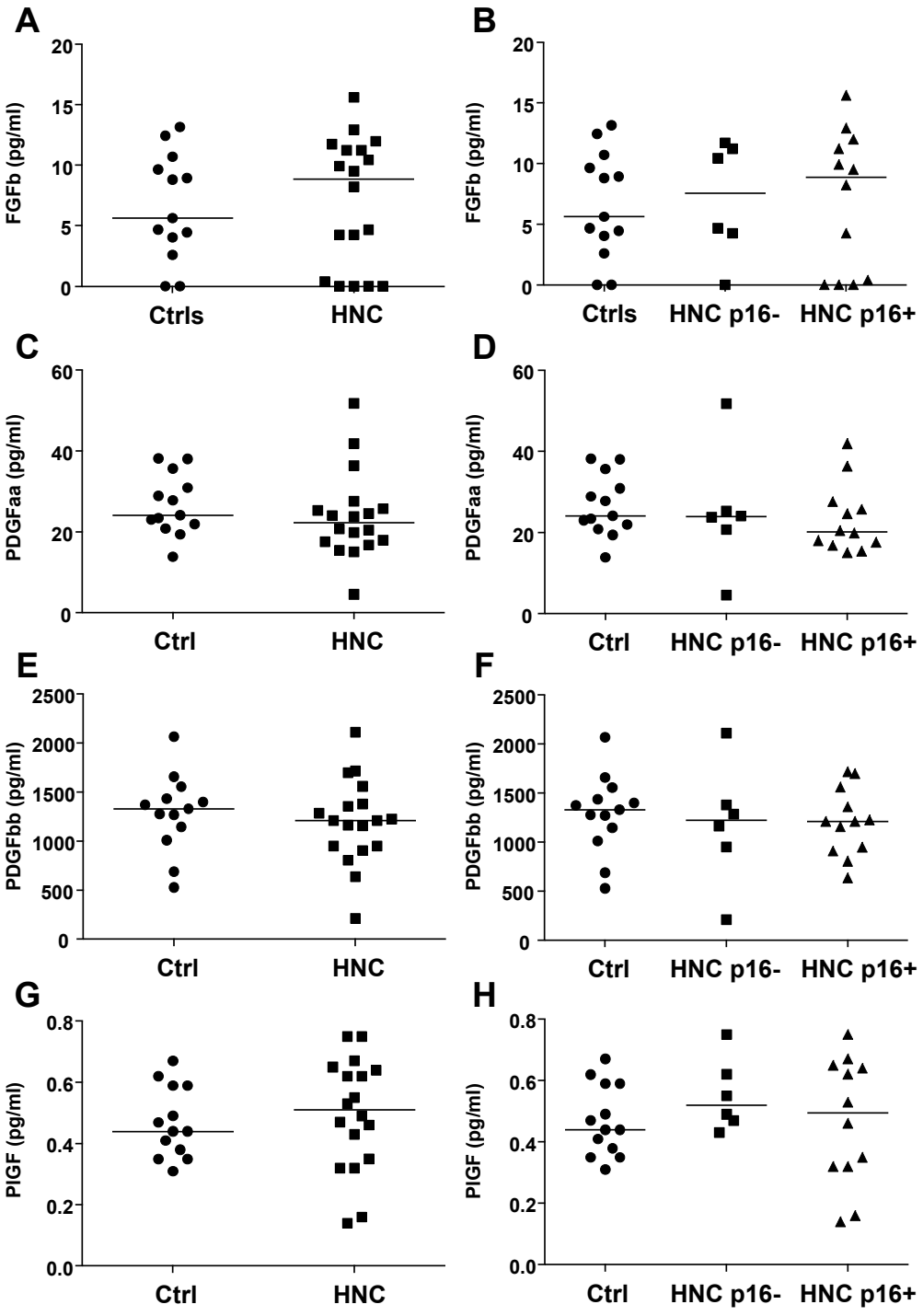
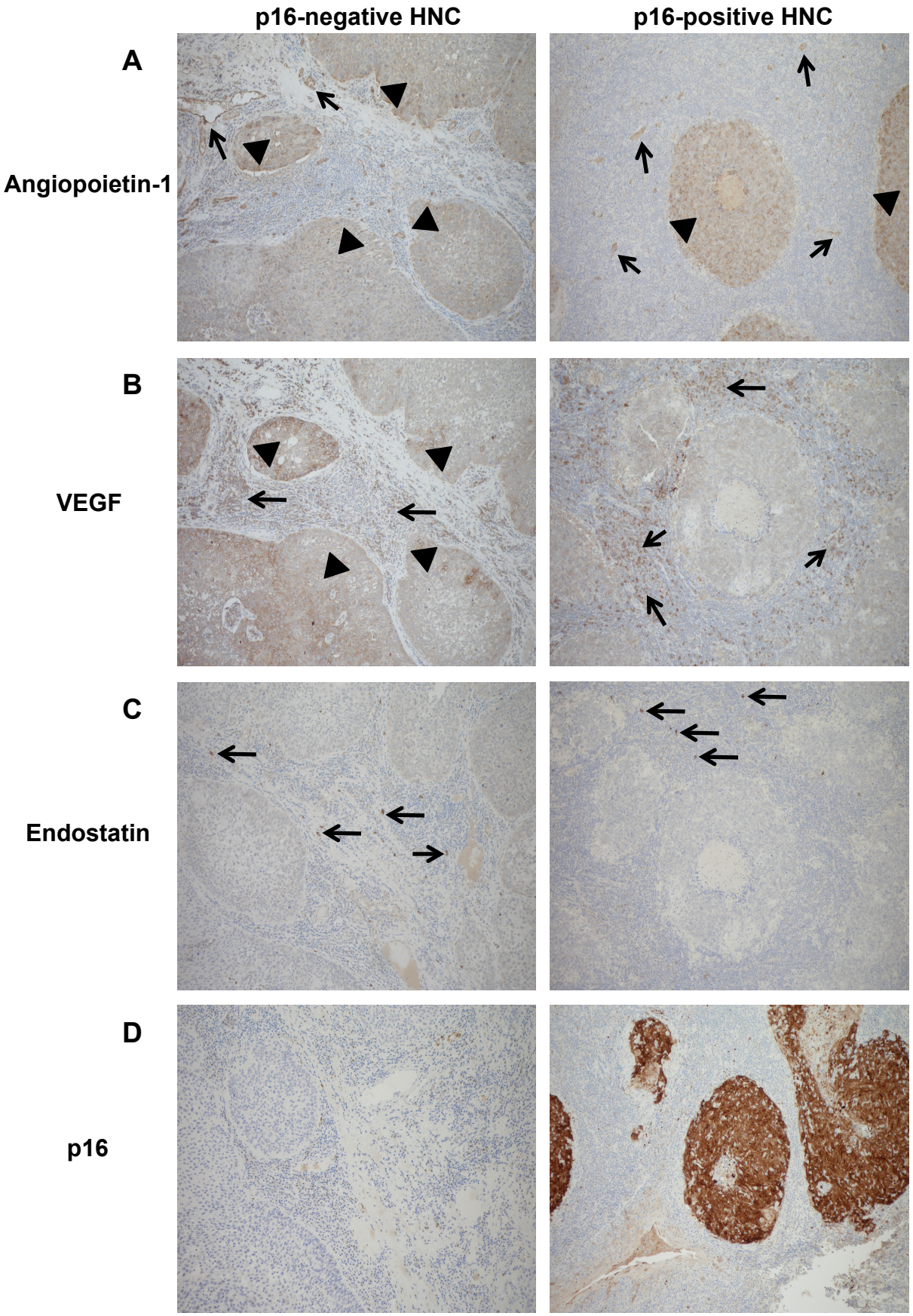

A
FGFb (pg/ml)
20
15
10
5
0
Ctrls
HNC
B
FGFb (pg/ml)
20
15
10
5
0
Ctrls
HNC p16-
HNC p16+
C
PDGFaa (pg/ml)
60
40
20
0
Ctrl
HNC
D
PDGFaa (pg/ml)
60
40
20
0
Ctrl
HNC p16-
HNC p16+
E
PDGFbb (pg/ml)
2500
2000
1500
1000
500
0
Ctrl
HNC
F
PDGFbb (pg/ml)
2500
2000
1500
1000
500
0
Ctrl
HNC p16-
HNC p16+
G
PIGF (pg/ml)
0.8
0.6
0.4
0.2
0.0
Ctrl
HNC
H
PIGF (pg/ml)
0.8
0.6
0.4
0.2
0.0
Ctrl
HNC p16-
HNC p16+

Figure 6


p16-negative HNC
p16-positive HNC
A
Angiopoietin-1
B
VEGF
C
Endostatin
D
p16

Supplemental material

Impact of p16 status on pro- and anti-angiogenesis factors in head and neck cancers

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Supplemental material inventory:

Supplemental Table 1. Demographic characteristics of head and neck cancer patients					
Patient	**Age**	**Gender**	**Tumour site**	**TNM**	**p16**
P1	50	F	BOT	T4N2cM0	Negative
P2	60	M	Tonsil	T4N2bM0	Negative
P3	50	M	PCA	T4N0M0	Positive
P4	83	M	PFS	T4N0M0	Negative
P5	72	M	Tonsil	T4N2bM0	Positive
P6	54	M	Tongue	T1N0M0	Negative
P7	49	M	Tonsil	T2N0M0	Positive
P8	56	M	Tonsil	T2N2aM0	Positive
P9	42	F	Nose	T4N2bM0	Positive
P10	49	F	Uvula	T1N0M0	Negative
P11	60	M	Tonsil	T3N0M0	Positive
P12	62	M	Tonsil	T1N2aM0	Positive
P13	71	M	Tonsil	T2N1M0	Positive
P14	63	F	Tonsil	T4N2cM0	Positive
P15	60	M	FOM	T1N2bM0	Negative
P16	61	F	Tonsil	T1N2bM0	Positive
P17	60	M	BOT	T2N2cM0	Positive
P18	66	M	BOT	T2N1M0	Positive
Abbreviations: BOT=base of tongue; FOM=floor of mouth; PCA=postcricoid area; PFS=pyriform sinus					

Supplemental Table 2. Levels of circulating angiogenic factors in head and neck cancer patients compared to healthy controls

Factor	HNC p16-	HNC p16+	HNC T1/2	HNC T3/4	HNC N0	HNC N+
Angiogenin	Equal[a]	Equal	Equal	Equal	Equal	Equal
Angiopoietin-1	**Higher**[b]	Equal	Equal	Equal	Equal	Equal
Endostatin	**Higher**	**Higher**	Equal	Equal	Equal	Equal
FGF-acidic	Equal	Equal	Equal	Equal	Equal	Equal
FGF-basic	Equal	Equal	Equal	Equal	Equal	Equal
PDGF-aa	Equal	Equal	Equal	Equal	Equal	Equal
PDGF-bb	Equal	Equal	Equal	Equal	Equal	Equal
PlGF	Equal	Equal	Equal	Equal	Equal	Equal
Thrombospondin-2	Equal	**Lower**[c]	**Lower**	Equal	Equal	Equal
VEGF	**Higher**	Equal	Equal	Equal	Equal	Equal
VEGF-D	Equal	Equal	Equal	Equal	Equal	Equal

Abbreviations: HNC=head and neck cancer; p16-=p16-negative; p16+=p16-positive; T1/2=early tumour stage; T3/4=advanced tumour stage; N0=without nodal disease; N+=with nodal disease

[a]Equal=similar levels than those detected in healthy controls

[b]Higher=significantly higher levels than those detected in healthy controls

[c]Lower=lower levels than those detected in healthy controls ($p>0.05$, not significant)