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A high HIF-1α expression genotype is associated with poor prognosis of upper aerodigestive tract carcinoma patients

Carlos Alberto de Carvalho Fraga [a], Marcos Vinícius Macedo de Oliveira [a], Érica Silva de Oliveira [a], Lucas Oliveira Barros [a], Francis Balduino Guimarães Santos [b], Ricardo Santiago Gomez [c], Alfredo Maurício Batista De-Paula [a], André Luiz Sena Guimarães [a,*]

[a] Department of Dentistry, Universidade Estadual de Montes Claros, Montes Claros, Brazil
[b] Department of Medicine, Universidade Estadual de Montes Claros, Montes Claros, Brazil
[c] Department of Clinical, Surgery and Oral Pathology, School of Dentistry, Universidade Federal de Minas Gerais, Belo Horizonte, Brazil

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SUMMARY

The aim of the present study was to evaluate the role of HIF-1α genetic polymorphisms and protein expression in the development of metastasis in upper aerodigestive tract cancer (UADTC) patients. The expression of pro-angiogenic markers was also evaluated. Protein expression was analysed using immunohistochemistry, and RFLP analysis was used to investigate HIF-1α C1779T and G1790A polymorphisms in 52 patients with UADTC. Primary lesions were divided into 2 groups according to the absence or presence of metastasis. Lymph node samples were divided into 3 groups: metastatic lymph nodes, non-metastatic lymph nodes (both derived from patients with metastatic disease), and control lymph nodes, which were obtained from patients without any metastasis. The allele T was more frequently found in patients with metastatic disease. HIF-1α protein expression in the lymph nodes was increased in the presence of the T allele. Metastatic lymph nodes showed lower levels of HIF-1α, VEGFR1, and MMP-9 proteins compared to lymph nodes without metastasis, while VEGFR2 protein levels were increased. In agreement, HIF-1α expression was correlated with MMP-9. Cox regression analysis demonstrated that higher HIF-1α and MMP-9 protein expression levels and GA and GG genotypes were associated with poor survival. Our findings show that the C1772T and G1790A polymorphisms of the HIF-1α gene are associated with increased expression of the HIF-1α protein in UADTC. The present data indicate that non-metastatic tissues express higher levels of HIF-1α, VEGFR1, and MMP-9, while in metastatic lymph nodes, VEGFR2 protein expression is elevated. The present study also shows that the HIF-1α G1790A polymorphism and its protein expression have an impact on the prognosis of UADTC patients.

Introduction

Upper aerodigestive tract cancer (UADTC) is one of the most common cancers worldwide.[1] The aetiology of UADTC is complex owing to the multigenic nature of the disease and the number of potential environmental agents to which affected individuals may have been exposed. It is clear that the major aetiological agents are tobacco and alcohol exposure.[2] However, other factors such as genetic predisposition have an important role in disease genesis and progression.[3,4] The role of genetic polymorphisms in altering protein levels/function and predisposing patients to a variety of diseases has been demonstrated extensively.[5–10]

Rapid tumour expansion leads to an oxygen demand that cannot be supported by the existing tumour vasculature. Poorly oxygenated regions within a tumour are the main characteristics of the hypoxic phenomenon.[11] Hypoxia has not only been associated with resistance to treatment but also metastasis[12] and epithelial–mesenchymal transition.[13] The hypoxic response is regulated by hypoxia-inducible factor-1 (HIF-1), which is a basic helix-loop-helix transcription factor composed of 2 subunits, HIF-1α and HIF-1β.[14] HIF-1α is the main gene involved in the hypoxic phenomenon, and its polymorphisms have been associated with metastasis in several solid tumours.[15,16] In this context, non-neoplastic cells can modify specific sites for future metastatic adhesion; there is evidence that this cell population can portend a future metastatic site. This non-neoplastic cell population includes fibroblasts, haematopoietic progenitors and other cells that express factors that promote the establishment of metastatic cells. The identification of this cell population in human tissues prior to

* Corresponding author. Address: Universidade Estadual de Montes Claros, Hospital Universitário Clemente de Faria, Laboratório de Pesquisa em Saúde, Avenida Cula Mangabeira, 562, Montes Claros, Minas Gerais, cep 39401-001, Brazil.
E-mail address: andreluizguimaraes@gmail.com (A.L.S. Guimarães).

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the spread of neoplastic cells supports the targeting of proteins associated with angiogenesis such as vascular endothelial growth factor A (VEGF-A) and its receptors VEGFR1, VEGFR2[17], or extracellular matrix degradation proteins such as metallopeptidase-9 (MMP-9).[18] Based on these data, the present study aimed to evaluate the role of HIF-1α genetic polymorphisms and protein expression in the development of nodal metastases in UADTC patients. The findings of previous studies[15,19,16,12,20–22] led to the hypothesis that the HIF-1α C1779T and G1790A polymorphisms could enhance the HIF-1α pathway by up-regulating HIF-1α protein expression and facilitate the spread of tumour cells and their attachment to lymph nodes. Thus, we also evaluated angiogenesis by assessing the protein expression of VEGF-A, VEGFR1, VEGFR2, MMP-9, and CD105 at the primary tumour and metastatic and non-metastatic nodes.

Patients and methods

Patients and ethical aspects

In this retrospective study, we analysed 52 patients who were diagnosed at a stomatological clinic and referred to the Head and Neck Service of the Universidade Estadual de Montes Claros (Montes Claros, Minas Gerais State, Brazil) between 1998 and 2008.[23,5] Only cases with available primary tumour and lymph node tissues for molecular and immunohistochemistry analysis were included in the study. The health records of these patients were retrieved, and socio-demographic, clinical, and outcome data were obtained (Supplementary Table 1). All UADTC patients were classified on the basis of the primary site according to the International Union Against Cancer (UICC)-TNM classification of malignant tumours, as described in the International Classification of Diseases for Oncology.[24] One sample from the primary malignant tumour and at least 1 cervical lymph node were obtained from each UADTC patient and included in the analysis. Ethical approval for this study was obtained from the local ethics committees (Unimontes, CEP 1852/2010). All patients underwent surgical resection and were treated with postoperative radiotherapy.

Evaluation and classification of locoregional lymph nodes

All samples were fixed in formalin, embedded in paraffin, and serially sectioned (5-μm thickness). The sections were stained with haematoxylin and eosin (H&E) and evaluated under a conventional light microscope. Cervical lymph nodes were analysed using morphological and immunohistochemical methods to identify a metastatic focus. A high-molecular weight cytokeratin primary antibody was used to identify micrometastases. Immunohistochemistry staining, DNA isolation, and HIF-1α genotyping were performed as described in the Supplementary Materials.

Primary cancer lesion classifications

Primary lesions were divided into 2 groups according to the presence ($n = 26$) or absence of metastasis ($n = 26$). Patients in the 2 groups were matched by tumour size, WHO grade, anatomical site, and age. Other clinical characteristics were also similar (Supplementary Table 1).

Lymph node classifications

Histological, immunohistochemical, and clinical examination revealed lymph node metastases in 26 patients. In this group, the metastatic and non-metastatic lymph nodes were analysed. In addition, the lymph nodes from 26 patients without metastasis were analysed (control group).

Statistical analysis

Chi-square and Fisher's exact statistical tests were used to evaluate the association between HIF-1α polymorphic variants and metastasis. In addition, a multivariate analysis using binary logistic regression was performed to build a model of variables to evaluate the risk of locoregional metastasis.

Immunolocalization analysis of HIF-1α, VEGFA, VEGFR1, VEGFR2, and MMP-9 proteins assumed non-parametrical distribution, and comparisons between groups were performed using the Mann–Whitney test. The analysis of CD105 protein expression in tissues assumed parametrical distribution, and comparisons between groups were performed using the Student's *t*-test. The Spearman correlation test was used to evaluate the correlation between the expression levels of different proteins. For survival analyses, the Kaplan–Meier test was performed, and the variables were compared using the log-rank test. Variables with $p \leqslant 0.25$ were included in the Cox proportional hazards regression to estimate predictive factors of crude survival. All statistical analyses were performed with the statistical software package SPSS®, version 13.0 for Windows®. *P* values <0.05 were considered significant.

Results

Immunohistochemical and molecular data associated with metastasis

The distribution of HIF-1α genotypes according to the presence of metastasis is shown in Supplementary Table 2. The data reveal an association between the C1772T polymorphism and the presence of metastasis ($p = 0.023$). The frequency of the CT genotype was higher in patients that developed metastasis. The allele T was more frequently found in patients who had metastasis. However, the G1790A polymorphism was not associated with metastasis ($p = 0.172$).

To further define the factors that contribute to metastasis, we performed binary logistic regression for all lesions and lymph nodes. In primary lesions, there was no association between the polymorphism findings and protein expression levels. The binary logistic regression showed an association between the CT genotype and the presence of metastasis ($p = 0.009$). In addition, low expression of VEGFR1 was correlated with metastasis ($p = 0.023$) (Table 1).

Genotypes and protein expression in lymph nodes

We next investigated whether the C1772T and G1790A polymorphisms could independently induce a change in protein expression levels. HIF-1α protein expression was higher in the TT genotype than the CC genotype (79.575 ± 23.293 and 22.625 ± 41.101, respectively; $p = 0.027$) in all lymph nodes groups. In metastatic lymph nodes, HIF-1α protein expression in lymph nodes increased in the presence of the T allele. Similarly, HIF-1α protein expression was higher in the AA genotype than in the GG genotype (63.025 ± 47.199 vs. 44.139 ± 41.948; $p = 0.028$).

Expression of angiogenesis-associated proteins in the different lymph node groups

Increased expression levels of HIF-1α, VEGFR1, and MMP-9 were observed in non-metastatic lymph nodes ($p = 0.047$, $p = 0.003$, and $p = 0.022$, respectively). VEGFR2 expression was higher in tissues from metastatic lymph nodes than in non-metastatic lymph nodes (Fig. 1). There was no difference in the expression of any of the proteins studied in non-metastatic lymph nodes between patients with metastasis and those in the control group. No difference in neo-vessels (CD105 staining) was observed between the different lymph node groups.

Table 1
Lymph node parameters associated to risk of locoregional metastasis evaluated by binary logistic regression in the UADTC patients.

Variables	95 CI			
	OR	Lower	Upper	*p* Value
Polymorphism CT				
CC		Referent		
CT	5.481	1.532	19.606	0.009[a]
TT	NA	NA	NA	0.999
Polymorphism GA				
GG		Referent		
GA	0.679	0.165	2.791	0.592
AA	5.594	0.390	80.268	0.205
Immunohistochemistry				
HIF-1 α	0.993	0.980	1.005	0.255
VEGFA	0.989	0.965	1.014	0.394
VEGFR1	0.962	0.930	0.995	0.023[a]
VEGFR2	1.009	0.985	1.035	0.457
MMP9	0.998	0.970	1.026	0.884
CD105	1.034	0.972	1.100	0.292

OR: odds ratio; CI: confidence interval; *n*: total number; NA: not applicable. The model was fitted to the best-fit model.
[a] Results statistically significant.

Expression of angiogenesis-associated proteins in the primary cancer lesion

The expression pattern of HIF-1α, VEGF-A, VEGFR1, VEGFR2, MMP-9, and CD105 in primary lesions analysed by immunohistochemistry is shown in Fig. 2. CD105 was used to identify neo-vessels. The immunohistochemical expression of CD105 in primary tumours with metastasis was significantly higher than in the group without metastasis (Fig. 2).

Protein expression correlations

To understand the effect of HIF-1α on the levels of the other proteins, we next analysed the correlation between the expression levels of the different proteins studied in all groups. A positive correlation was found between HIF-1α and MMP-9 in both the primary tumour and metastatic samples ($r = +0.374$, $p = 0.004$ and $r = +0.640$, $p = 0.001$, respectively). In the neoplastic cells, the expression of VEGFA was correlated with VEGFR2 in the metastatic lymph nodes ($r = +0.418$, $p = 0.034$). The expression of VEGFR1 was correlated with the expression of MMP-9 in lymph nodes of patients without metastasis ($r = +0.304$, $p = 0.029$). In all lymph node groups analysed, the expression of HIF-1α was correlated with the expression of the VEGFR1 protein ($r = +0.232$, $p = 0.042$). In addition, VEGFR1 was correlated with MMP-9 when all lymph node groups were analysed ($r = +0.343$, $p = 0.002$).

Risk of death and molecular findings

The mean survival for all patients was 1106.2 days after diagnosis. High MMP-9 expression in primary lesions was associated with poor survival. GG and GA genotypes and elevated HIF-1α protein expression in lymph nodes had a negative impact on survival (Table 2).

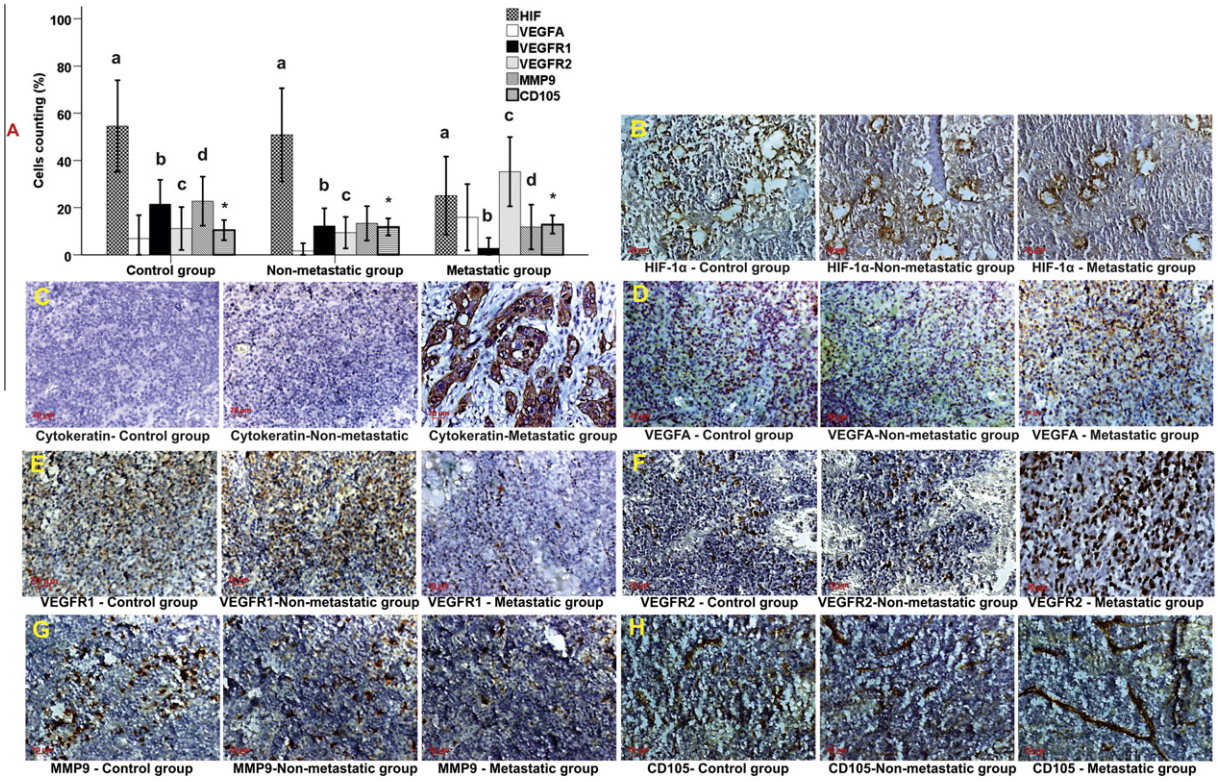

Figure 1 Expression of HIF-1α, cytokeratin, VEGF-A, VEGFR1, VEGFR2, MMP-9, and CD105 proteins in lymph node samples. (A) Analyses of HIF-1α, VEGF-A, VEGFR1, VEGFR2, MMP-9, and CD105 protein expression levels in leukocyte cells of lymph node samples. Analysis by Mann–Whitney test showed statistically significant differences between metastatic and non-metastatic groups in the protein expression levels of HIF-1α (a) ($p = 0.047$), VEGFR1 (b) ($p = 0.003$), and VEGFR2 (c) ($p = 0.003$). Similarly, there were statistically significant differences between metastatic and control groups in the protein expression levels of HIF-1α (a) ($p = 0.029$), VEGFR1 (b) ($p < 0.001$), VEGFR2 (c) ($p = 0.006$), and MMP-9 (d) ($p = 0.022$). There were no statistically significant differences in protein expression levels between non-metastatic and control groups. Expression of HIF-1α (B), cytokeratin (C), VEGF-A (D), VEGFR1 (E), VEGFR2 (F), MMP-9 (G), and CD105 (H) proteins in the control group (left panel), non-metastatic group (middle panel) and metastatic group (right panel). *P* values of HIF-1α, VEGF-A, VEGFR1, VEGFR2, and MMP-9 protein expressions were calculated using the Mann–Whitney test. The *P* value of CD105 protein expression was calculated by Student's *t* test. ∗Counting performed by microvessel density. Scale bar, 20 μm.

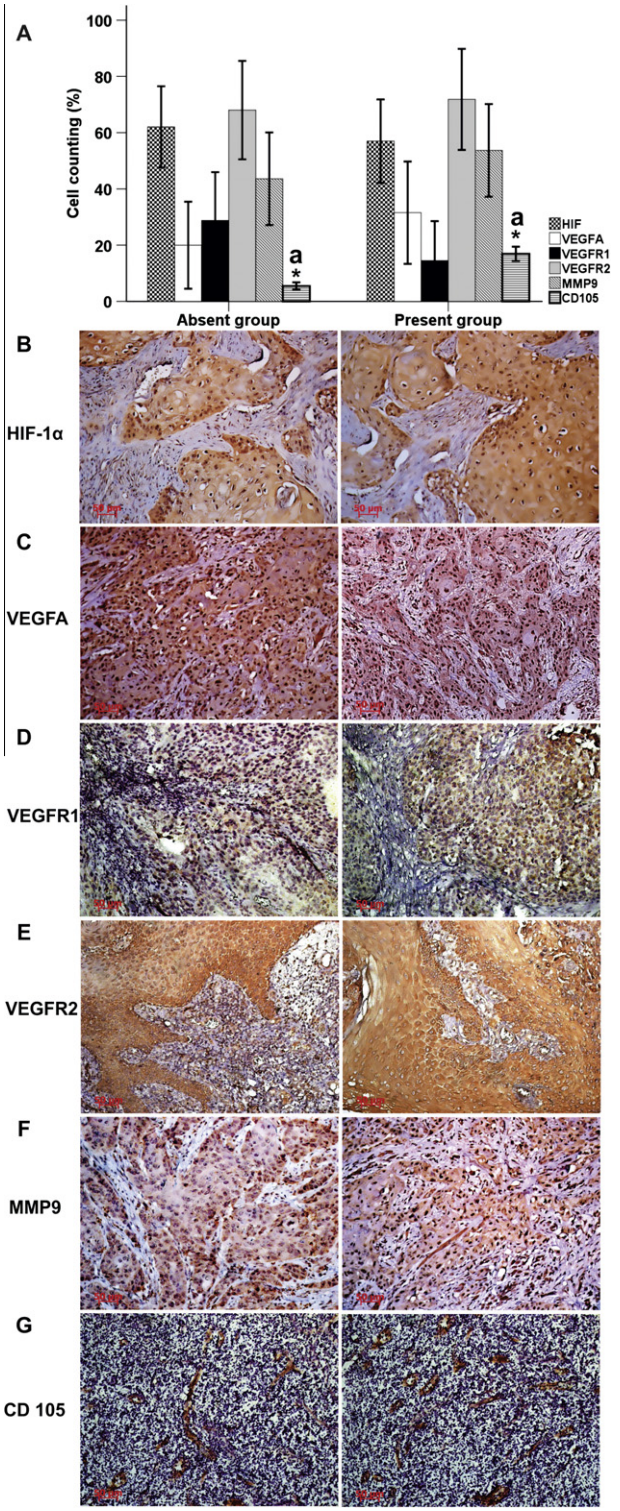

Figure 2 Expression of HIF-1α, VEGF-A, VEGFR1, VEGFR2, MMP-9, and CD105 proteins in primary lesions. (A) Counting was carried out in neoplastic cells of primary lesions. Note that there was a statistically significant increase in the expression of the CD105 protein in primary lesions that present metastasis (a) ($p < 0.001$). The right panel shows the expression levels of HIF-1α (B), VEGF-A (C), VEGFR1 (D), VEGFR2 (E), MMP-9 (F), and CD105 (G) proteins in primary lesions of patients with metastasis. The left panel shows the expression of HIF-1α (B), VEGF-A (C), VEGFR1 (D), VEGFR2 (E), MMP-9 (F), and CD105 (G) proteins in primary lesions of patients without any metastasis. *P* values of HIF-1α, VEGF-A, VEGFR1, VEGFR2, and MMP-9 protein expression levels were calculated using the Mann–Whitney test. The *p* value for CD105 protein expression was calculated by Student's *t* test. ∗Counting performed by microvessel density. Scale bar, 50 μm.

Discussion

Metastasis is one of the hallmarks of malignant disease and the cause of death for the majority of cancer patients. The molecular mechanisms underlying the early onset of metastasis are complex, involving both genetic and epigenetic alterations in malignant cells and the tumour environment. Tumour cells have many mechanisms to escape the host defence mechanisms, and metastasis development requires tumour cells to lose adhesion to surrounding cells, cross the basement membrane, migrate through lymphatic channels, and subsequently, extravasate into foreign nodal tissue.[25,21] Hypoxia has been shown to promote regional metastasis in several solid tumours.[26,27] To maintain cell survival in a microenvironment that is low in oxygen, cells can alter their protein expression.[28] Under normal oxygen tension, HIF-1α is degraded by ubiquitin dependent proteolysis, whereas HIF-1β is stable.[29] Angiogenesis, glycolytic metabolism, and cell survival and invasion are regulated through HIF-1α signalling.[22] During early tumourigenesis, HIF-1α expression is required for the induction of angiogenesis although other factors can facilitate tumour angiogenesis. Liao et al.[20] reported that HIF-1α expression acts as an accelerating factor in tumour progression and metastasis.[20]

Polymorphisms in the HIF-1α gene have been associated with an increased risk of developing certain solid tumours.[30,15,16] However, the role of single-nucleotide polymorphisms in the oxygen-dependent degradation domain of the HIF-1α gene in carcinogenesis appears complex and is not currently known. To date, no conclusive results have established a link between HIF-1α polymorphism and carcinogenesis.[19,18,31–33] Genetic polymorphisms at C1772T and G1790A of HIF-1α have been associated with increased transcriptional activity[16,32] and high expression levels of HIF-1α in oral cancer.[32] Munoz-Guerra et al. demonstrated that the frequencies of the heterozygous GA and homozygous AA genotypes were high in patients with oral cancer.[34] Our study showed that the CT heterozygous genotype is associated with lymph node metastasis. Moreover, based on lymph node analyses, the homozygous TT genotype is associated with a higher number of HIF-1α$^+$ leukocyte cells than the homozygous CC genotype, and HIF-1α$^+$ leukocyte cell numbers are higher in homozygous AA genotypes than in homozygous GG genotypes.

Although the precise molecular and cellular mechanisms that control tumour metastasis have not been defined, several studies have demonstrated that tumours have a predilection for metastasis to specific organs. Kaplan et al.[17] introduced the concept that tumour metastasis is initiated by a well-defined sequence of events that depend on the expression patterns of fibronectin and VEGFR1$^+$VLA4$^+$ clusters, which dictate organ-specific tumour spread. These clusters can modify the microenvironment by regulating the production of MMP-9, resulting in the attraction of tumour cells and their establishment in a niche.[17] HIF-1α might enhance this signalling by up-regulating MMP-9[18] and VEGFR1 expression in these sites. In the present study, increased expression of HIF-1α, VEGFR1, and MMP-9 was detected in non-metastatic lymph nodes compared to metastatic samples. In addition, VEGFR1 protein expression was correlated with HIF-1α and MMP-9 protein levels in non-metastatic lymph nodes. According to the pre-metastatic niche theory, non-metastatic lymph nodes prepare for tumour cell spreading by up-regulating some proteins. After the establishment of the metastases, a down-regulation of the proteins is observed.[17,35] Notably, a decrease in protein levels after metastasis does not occur in some types of tumours.[36] Therefore, distinct time-dependent events may or may not occur before, during, and after the establishment of a metastatic niche.

Despite contradictory results on the clinical significance of microvessel density in UADTC, this parameter has been regarded

Table 2
Cox regression analyses in the UADTC patients with a follow-up of 3554 days taken primary lesions and all lymph nodes from this study.

Variables	95 CI			
	OR	Lower	Upper	*p* Value
Polymorphism CT				
CC		Referent		
CT	1.708	0.586	4.978	0.327
TT	NA	NA	NA	0.982
Polymorphism GA				
AA		Referent		
GG	27.651	2.151	355.519	0.011[a]
GA	37.898	3.497	410.702	0.003[a]
Immunohistochemistry – Primary lesions				
HIF-1 α	0.985	0.970	1.000	0.053
VEGFA	0.988	0.976	1.000	0.052
VEGFR1	0.996	0.984	1.009	0.546
VEGFR2	1.001	0.988	1.014	0.857
MMP9	1.016	1.004	1.029	0.007[a]
CD105	1.011	0.935	1.094	0.781
Immunohistochemistry – Lymph nodes				
HIF-1 α	1.013	1.003	1.023	0.014[a]
VEGFA	0.994	0.965	1.023	0.673
VEGFR1	0.976	0.948	1.004	0.089
VEGFR2	1.004	0.990	1.017	0.585
MMP9	0.993	0.971	1.016	0.554
CD105	0.967	0.924	1.013	0.159

OR: odds ratio; CI: confidence interval; *n*: total number; NA: not applicable. The model was fitted to the best-fit model.
[a] Results statistically significant.

as an independent negative prognostic factor. These discrepancies may be due to differences in treatment protocols and the endothelial markers used for immunohistochemical staining. CD105 binds preferentially to activate endothelial cells undergoing angiogenesis, and it has been found to be a better marker for evaluating ongoing tumour angiogenesis than other pan-endothelial markers.[37] Our data show that higher expression levels of CD105 are significantly associated with lymph node metastases, which implies that increasing the number of vessels in a tumour provides an environment in which the tumour is susceptible to being disseminated. The molecular basis for CD105 up-regulation is not clear, but the CD105 gene promoter has been shown to be predominantly active in proliferating endothelial cells undergoing hypoxia via the HIF-1 complex, which binds a functional consensus hypoxia response element (HRE) in the CD105 promoter.[38] In our study, HIF-1α showed higher expression levels in non-metastatic lymph nodes than in metastatic lymph nodes. The distribution and expression of CD105 in UADTC samples was not consistent with that of HIF-1α in lymph node samples. These findings provide compelling evidence that CD105, but not HIF-1α, is required for angiogenesis in lymph nodes after metastatic spread. These results are supported by the correlation between VEGFR2, the main transducer of VEGFA-mediated angiogenic signals, VEGFA protein expression in metastatic tissues, and by the higher expression of VEGFR2 in metastatic compared with non-metastatic lymph nodes. Consistent with an increase in tumour cell dissemination, VEGFR2 stimulates neovascularization of the tumour after the arrival of $VEGFR1^+$ cells and metastasis spread. Furthermore, $VEGFR2^+$ cells are essential for the growth and progression of malignant cells in the new organ.[17]

In the present study, we focused on metastatic and non-metastatic lymph nodes from UADTC patients. As head and neck cancers refer to a group of biologically similar cancers that may affect different regions, including the oral cavity, nasal cavity, paranasal sinus, pharynx, and larynx, we restricted our selection criteria to tumours located in the oral cavity, pharynx, and larynx. Although there are no reports of HIF-1α levels in metastatic and non-metastatic lymph nodes from UADTC patients, some studies have compared HIF-1α expression in primary lesions and metastatic lymph nodes. Shim et al. demonstrated that the expression of HIF-1α was higher in metastatic lymph nodes than in primary lesions. We did not find any differences in HIF-1α protein expression between primary lesions and metastatic sites (data not shown). However, patients with metastatic disease and a homozygous TT genotype showed higher HIF-1α protein expression. A previous study demonstrated that bone metastasis from human breast carcinoma can modify the microenvironmental signals controlled by COX-2 induction and HIF-1α activation. In agreement with these findings, $HIF\text{-}1\alpha^+$ bone marrow supportive cells were detected in metastasis-bearing animals.[39] We observed the highest HIF-1α protein expression in non-metastatic samples. These results, in conjunction with previous studies,[40] suggest that HIF-1α has an important role in the initial stages of metastasis or in a more hypoxic environment in non-metastatic lymph nodes. Taken together, our results suggest that HIF-1α polymorphisms can alter HIF-1α protein expression and simultaneously enhance VEGFR1 and MMP-9 protein signals in a non-metastatic niche, thereby altering the microenvironment and supporting metastasis at that site, with the consequent negative impact on survival. A proposed model of

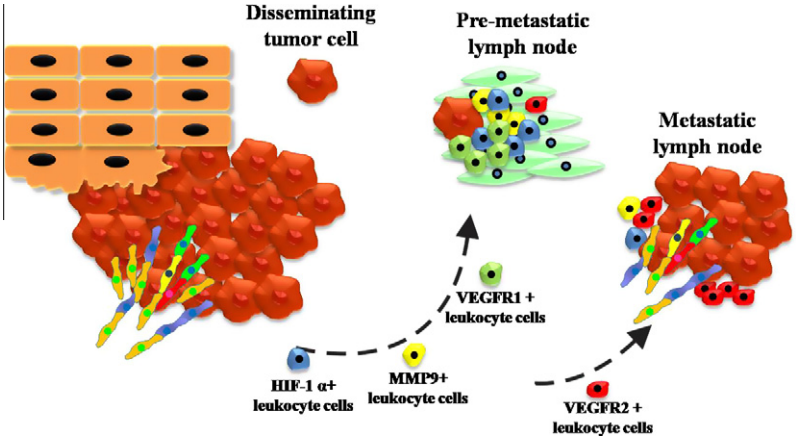

Figure 3 Proposed model of HIF-1α, MMP-9, VEGFR1, and VEGFR2 + leukocyte cells in UADTC metastasis. In response to factors secreted by the primary tumour, HIF-1α, MMP-9, and VEGFR1 proteins are up-regulated in pre-metastatic lymph nodes. These cells secrete a variety of pre-metastatic factors that alter the microenvironment and support the establishment of metastases. Recruitment of VEGFR2 + leukocyte cells to the early metastatic niche mediates the angiogenic switch and enables progression and colonization of macrometastases.

HIF-1α, MMP-9, VEGFR1, and VEGFR2 + leukocyte cells in UADTC metastasis is shown in Fig. 3.

Conclusion

In conclusion, our study demonstrated that the C1772T and G1790A polymorphisms of the HIF-1α gene are associated with increased expression of the HIF-1α protein in UADTC. Our data indicate that the expressions of HIF-1α, VEGFR1, and MMP-9 increase in non-metastatic tissues, while the VEGFR2 protein is expressed at higher levels in metastatic lymph nodes. Finally, the present study shows that the HIF-1α G1790A polymorphism and its protein expression have an impact on the prognosis of patients with UADTC. These findings suggest an additional role for HIF-1α in UADTC. Further studies are necessary to elucidate the HIF-1α pathway, which would facilitate the development of novel therapeutic strategies for the prevention and treatment of metastases in UADTC and other solid tumours.

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Appendix. . Supplementary data

Supplementary data associated with this article can be found, in the online version, at doi:10.1016/j.oraloncology.2011.08.023.

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