CLINICAL SCIENCE

Differential expression of hypoxia-inducible factor 1α in non-small cell lung cancer and small cell lung cancer

Eleni Karetsi,[I] Maria G. Ioannou,[II] Theodora Kerenidi,[I] Markos Minas (*in memoriam*), Paschalis A. Molyvdas,[III] Konstantinos I. Gourgoulianis,[I] Efrosyni Paraskeva[III]

[I] University of Thessaly, Medical School, Department of Respiratory Medicine, Biopolis, Larissa, Greece. [II] University of Thessaly, Medical School, Department of Pathology, Biopolis, Larissa, Greece. [III] University of Thessaly, Medical School, Department of Physiology, Biopolis, Larissa, Greece.

OBJECTIVES: The aim of this study was to compare the expression of hypoxia-inducible factor 1α and vascular endothelial growth factor in small cell lung cancer and subtypes of non-small cell lung cancer and examine their relationships with clinicopathologic factors, response to treatment and survival.

METHODS: We examined samples obtained by bronchial endoscopic biopsy from 55 patients with inoperable lung cancer (16 with adenocarcinoma, 17 with squamous cell carcinoma, and 22 with small cell lung cancer). Hypoxia-inducible factor 1α and vascular endothelial growth factor were detected using immunohistochemistry. The diagnosis, treatment, and follow-up of patients were conducted according to the standard practice.

RESULTS: A significant difference ($p = 0.022$) in hypoxia-inducible factor 1α expression was observed between non-small cell lung cancer (75.8% positive) and small cell lung cancer (45.5% positive). The frequency of hypoxia-inducible factor 1α nuclear expression was 88.2% in squamous cell carcinoma, 62.5% in adenocarcinoma, and 45.5% in small cell lung cancer. A significant correlation was observed between hypoxia-inducible factor 1α and vascular endothelial growth factor expression (Fisher's exact test, $p = 0.001$) when all types of lung cancer were examined, either collectively or separately.

CONCLUSIONS: The expression of hypoxia-inducible factor-1α differs significantly between subtypes of lung cancer. These findings could help elucidate the biology of the different types of non-operable lung carcinomas and have implications for the design of new therapeutic approaches for lung cancer.

KEYWORDS: Hypoxia-Inducible Factor 1α; Lung Cancer; Vascular Endothelial Growth Factor; Small Cell Lung Cancer; Non-small Cell Lung Cancer.

INTRODUCTION

Lung cancer is the primary type of cancer responsible for patient death worldwide. Lung cancer is divided into two major subtypes, non-small cell lung cancer (NSCLC) and small cell lung cancer (SCLC). NSCLC is further divided into four histological subtypes - adenocarcinoma, squamous cell carcinoma, large cell carcinoma, and NSCLC NOS (not otherwise specified) - of which adenocarcinoma and squamous cell carcinoma are the most common and represent approximately 80% of all cases. The different types of lung cancer have distinct histological features and biological behavior, which influence the treatment and prognosis of lung cancer patients (1). It is therefore important to understand the molecular profiles of the different types of lung cancer to improve the repertoire of therapies used for their treatment.

The transcriptional activator hypoxia-inducible factor 1 (HIF-1) is a key mediator of the cellular response to hypoxia. HIF-1 consists of a regulatory alpha subunit and a constitutive beta subunit and controls the expression of many genes involved in angiogenesis, erythropoiesis, glucose uptake, cell metabolism, apoptosis, invasion and metastasis (2-4). The importance of HIF-1α and its target gene vascular endothelial growth factor (VEGF) as biomarkers and therapeutic targets in cancer therapy is currently emerging (3,5,6).

Increased expression of HIF-1α has been observed in several human cancers, including NSCLC (7-9), and is, in some cases, associated with a poor prognosis (10,11). In

addition, the expression of HIF-1α in SCLC has been recently reported (12). These findings highlight the possible use of HIF-1α as a prognostic factor for the outcome of lung cancer patients. Moreover, biological agents against VEGF have been approved for clinical use as a first-line treatment in several cancer types, including advanced NSCLC (13,14). Consequently, HIF-1α could be considered an additional target for the treatment of lung cancer in cases in which its expression is elevated.

In this study, we evaluated the expression of HIF-1α and VEGF in bronchial biopsies from patients with inoperable NSCLC and SCLC and examined the correlation between these markers, the different cancer types, clinicopathologic factors, response to treatment, and survival. This comparative analysis could assist with the clarification of the unique HIF-1α and VEGF expression profiles in SCLC and subtypes of NSCLC and assist with their evaluation as prognostic factors and therapeutic targets.

MATERIALS AND METHODS

Patients and Tissue Samples

This study was performed on 216 consecutive patients who underwent bronchoscopy for diagnostic purposes in the Respiratory Medicine Department of the Medical School of the University of Thessaly in Larissa between May and December 2008. Of these patients, 134 were excluded from the study because they did not have endobronchial findings. Of the rest that underwent bronchial endoscopic biopsy, eight patients were excluded because they had operable lung cancer, 14 patients were excluded because their bronchial endoscopic biopsy showed histological types other than SCLC or NSCLC, and five patients were excluded because data were missing in their medical records. The remaining 55 patients (52 men and three women), who were diagnosed with SCLC or NSCLC (16 with adenocarcinoma, 17 with squamous cell carcinoma, and 22 with SCLC) and had not received any treatment, were evaluated. The study protocol was approved by the local ethics committee, and all subjects gave their written informed consent. Subject characteristics are presented in Table 1. Tumor staging was based on the staging system of the American Joint Committee on Cancer (AJCC), Revised Definition of TNM classification. Only patients with inoperable lung cancer were included (24 patients with stage III and 31 patients with stage IV). The mean age of patients was 62.8 ± 9.8 years. The treatment and follow-up of patients were conducted according to the standard practice. The surveillance included follow-up visits every three months during the first and second years and every six months during third to fifth years. Patients with incomplete medical records or inadequate follow-up were excluded from study. Overall survival was calculated from the day of first-line treatment initiation to the day of the last follow-up (death or follow-up visit). All histological samples were obtained using bronchoscopy techniques for diagnostic purposes and were fixed in 10% buffered formalin, processed, and embedded in paraffin. All biopsies were retrieved from the Pathology Department of the University of Thessaly.

HIF-1α and VEGF immunohistochemistry and evaluation of immunostaining

HIF-1α was detected using the monoclonal antibody 54/HIF-1α (BD Transduction Laboratories, San Diego, dilution 1:20), and VEGF was detected using the primary antibody

Table 1 - Characteristics and response to treatment of patients with SCLC and NSCLC.

	SCLC	NSCLC	p-value
	N = 22	N = 33	
Gender (male)	21 (95.5%)	31 (93.9%)	0.808
Age (years)	60.7 ± 10.0	64.2 ± 9.5	0.198
≤65 years	16 (72.7%)	17 (51.5%)	
>65 years	6 (27.3%)	16 (48.5%)	
Smoking status			0.354
S	16 (72.7)	20 (60.6%)	
NS/ES	6 (27.3)	13 (39.4%)	
Weight loss (≥5%)	9 (40.9%)	12 (36.4%)	0.734
Performance status			0.825
• 0	12 (54.5%)	17 (51.5%)	
• 1	10 (45.5%)	16 (48.5%)	
Stage			0.375
• III	8 (36.4%)	16 (48.5%)	
• IV	14 (63.6%)	17 (51.5%)	
T			0.907
T1-T2	7 (31.8%)	11 (33.3%)	
T3-T4	15 (68.2%)9	22 (66.7%)	
N			0.216
N0-N1	4 (18.2%)	11 (33.3%)	
N2-N3	18 (81.8%)	22 (66.7%)	
M			0.375
M0	8 (36.4%)	16 (48.5%)	
M1	14 (63.6%)	17 (51.5%)	
Response to treatment			**0.034**
• PD, SD	7	21	
• PR, CR	14	12	

Data were obtained at the time of diagnosis, prior to the initiation of any treatment. Data are presented as the mean $\pm$ S.E.M with the p-value. N = number of patients. Abbreviations: NSCLC, non-small cell lung cancer; SCLC, small cell lung cancer; S, smokers; NS, non-smokers; ES, ex-smokers; PD, progressive disease; SD, stable disease; PR, partial response; CR, complete response. Bold letters indicate statistically significant differences.

clone JH121 (Neomarkers, UK, dilution 1:50) as previously described (15).

Briefly, the assessment of HIF-1α immunoreactivity scores was based on the percentage of cells showing positive nuclear immunostaining in the whole tumor area of the section. The percentage of tumor cells with cytoplasmic VEGF staining was recorded to assess VEGF reactivity. HIF-1α and VEGF expression was considered positive when immunostaining was observed in at least 10% of the tumor cells.

Statistical analysis

Statistical analysis was performed using the SPSS software package, version 16.0 (SPSS Inc., Chicago, IL, USA). Spearman's rank correlation test was used to assess the relationship between ordinal variables. A linear regression analysis was used to study the association between HIF-1α and VEGF expression. The association between HIF-1α and VEGF was assessed using HIF-1α as a continuous variable. Pair-wise comparisons were performed with the non-parametric Mann-Whitney U test. For all tests, $p \leq 0.05$ was considered statistically significant.

RESULTS

Detection of HIF-1α in NSCLC and SCLC

After immunohistological examination, 75.8% (n = 33) of NSCLC cases (i.e., 88.2% (n = 17) of squamous cell carcinoma cases and 62.5% (n = 16) of adenocarcinoma cases) and

45.5% (n = 22) of SCLC cases were scored as HIF-1α positive (Figure 1). The percentage of HIF-1α-positive cases was significantly higher in NSCLC compared to SCLC ($p = 0.022$, Figure 1B).

Detection of VEGF and correlation with HIF-1α in NSCLC and SCLC

Cytoplasmic VEGF immunostaining was detected in 76.4% of all cases. The VEGF-positive biopsies were observed in 26 cases (78.8%) of NSCLC (i.e., 14 cases (82%) of squamous cell carcinoma and 12 cases (75%) of adenocarcinoma) and 16 cases (72.7%) of SCLC (Figures 2A and 2B). VEGF immunoreactivity showed no association with the histological types of lung cancer. A significant correlation was observed between HIF-1α with VEGF expression when all types of lung cancer were included in the analysis ($R^2 = 0.52$, $p < 0.001$, Fig. 2C). In addition, further analysis of the lung cancer subtypes revealed a significant correlation between HIF-1α and VEGF expression in squamous cell carcinoma ($R^2 = 0.80$, $p < 0.001$), adenocarcinoma ($R^2 = 0.45$, $p < 0.001$), and SCLC ($R^2 = 0.59$, $p < 0.001$).
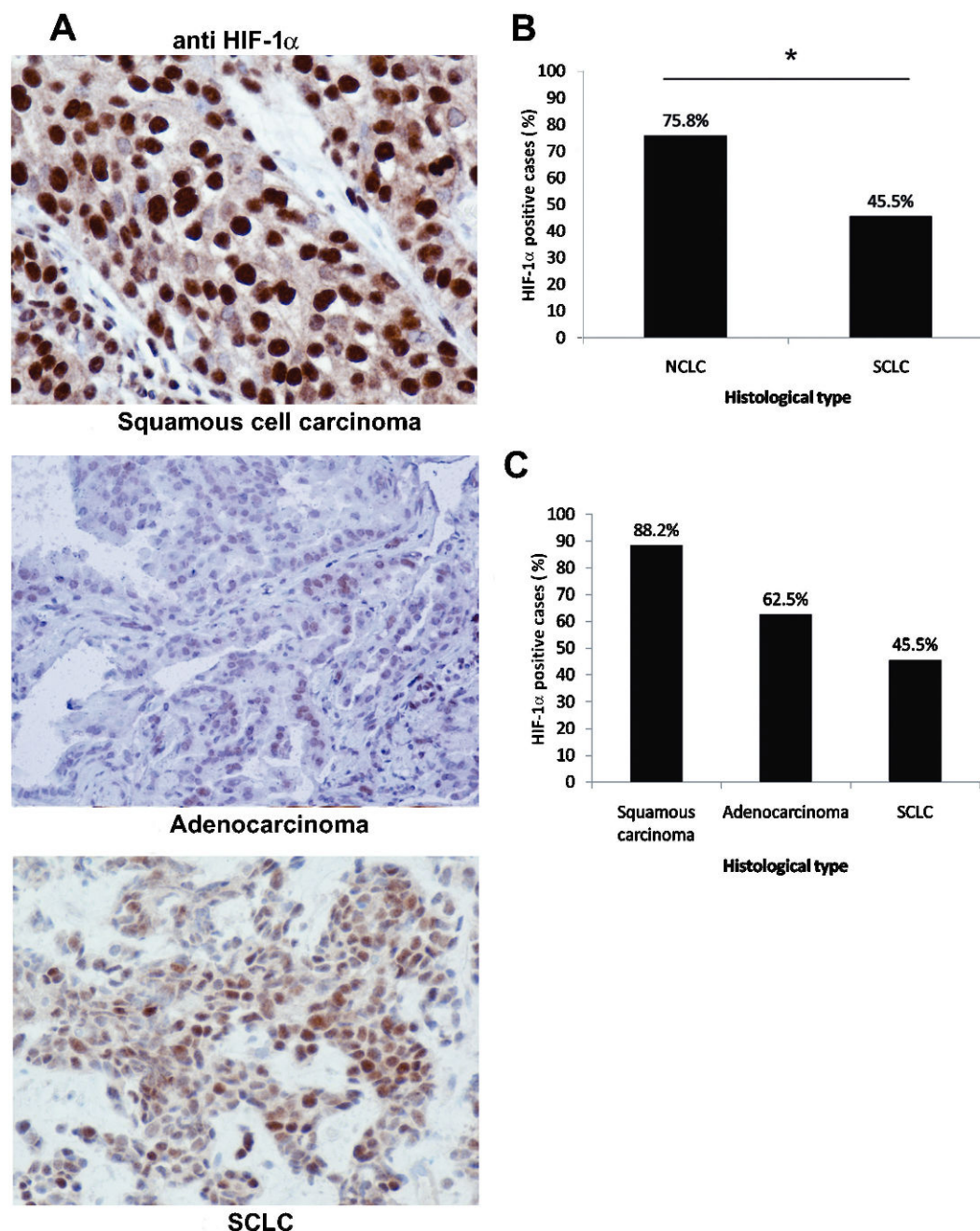

Figure 1 - HIF-1α immunostaining in lung carcinoma specimens. A) Strong nuclear and weak cytoplasmic HIF-1α staining in squamous cell carcinoma, HIF-1α nuclear staining in adenocarcinoma cells and HIF-1α nuclear staining in SCLC (immunoperoxidase staining, original magnification ×400). B) Percentage of HIF-1α-positive samples in NSCLC compared to SCLC samples ($p = 0.022$). C) Percentage of HIF-1α-positive samples in squamous cell carcinoma, adenocarcinoma and SCLC.
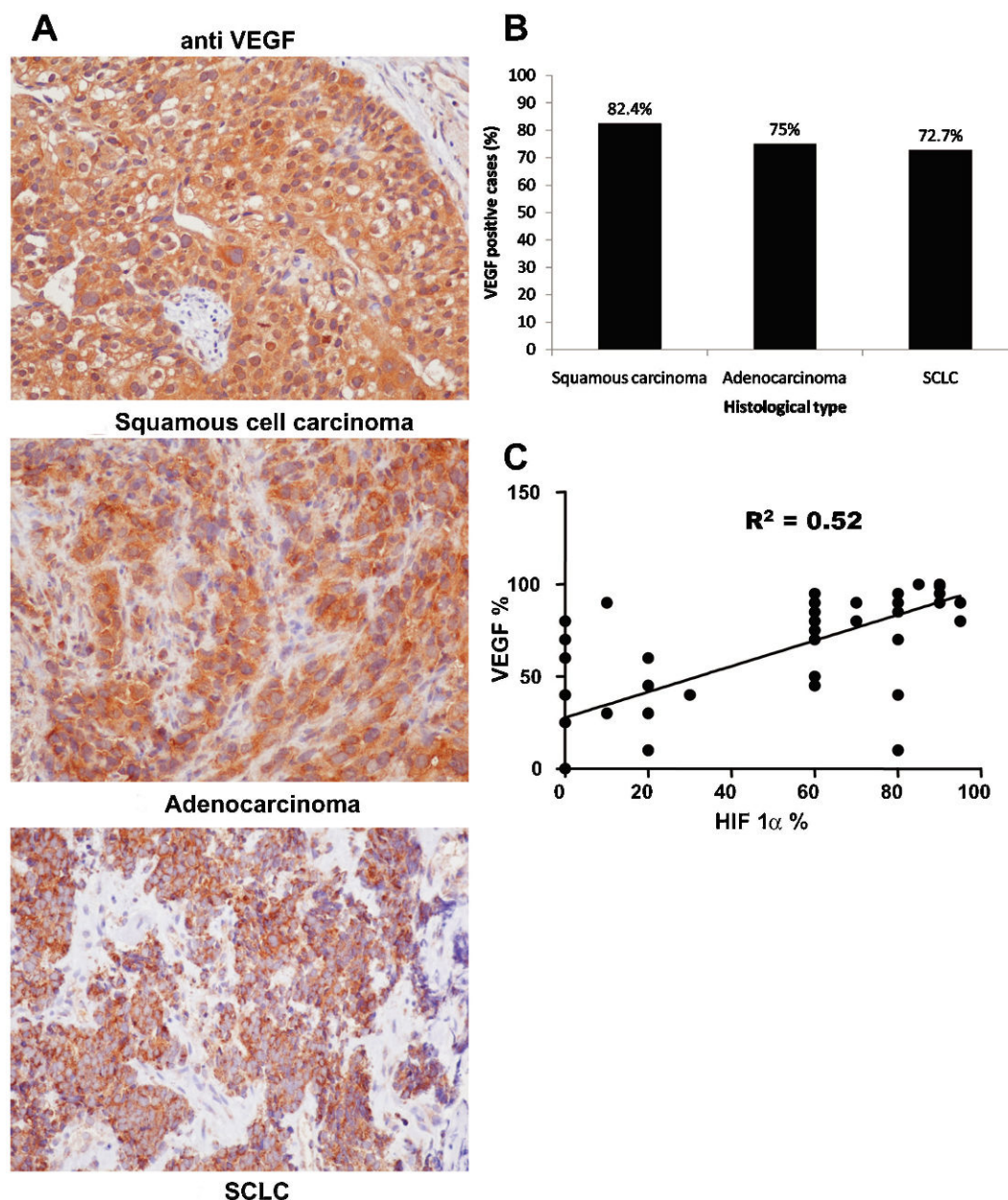
Figure 2 - VEGF immunoreactivity in lung carcinoma specimens. **A)** Diffuse cytoplasmic staining in many malignant cells in squamous cell carcinoma, adenocarcinoma and SCLC (immunoperoxidase staining, original magnification ×200). **B)** Percentage of VEGF-positive samples in squamous cell carcinoma, adenocarcinoma and SCLC. **C)** Linear regression analysis of the percentage of HIF-1α-positive cells with VEGF-positive cells (r²: correlation coefficient).

Relationships between HIF-1α and VEGF expression and clinicopathologic factors, response to treatment and survival

We observed no significant association between HIF-1α or VEGF expression and various clinical factors, including age, smoking habit, performance status, weight loss, stage, and N (lymph nodes) or M (metastases) stage or response to treatment in adenocarcinoma, squamous cell carcinoma or SCLC (Table 2). However, the analysis revealed a significant positive association between T stage and HIF-1α expression ($p = 0.003$) and VEGF expression ($p = 0.008$) in adenocarcinoma but not squamous cell carcinoma or SCLC (Table 2). We observed no significant associations between HIF or

VEGF expression and overall survival for any of the histologic subtypes (data not shown).

DISCUSSION

Individual lung cancer patients respond differently to chemotherapy and have different survival rates. This variability is related to the lung cancer histological subtype and its individual biological characteristics (Table 1). Therefore, it is important to improve our knowledge of the pathophysiology and molecular profiles of the different histological types of lung cancer, thus allowing personalization of the available therapies and the design of new, efficient ones. In this respect, understanding the contribution of

Table 2 - The relationships between HIF-1α and VEGF expression and clinicopathologic factors in three types of lung cancer.

	Squamous carcinoma				Adenocarcinoma				SCLC			
	HIF-1α⁺		VEGF⁺		HIF-1α⁺		VEGF⁺		HIF-1α⁺		VEGF⁺	
	N	p-value	N	p-value	N	p-value	N	p-value	N	p	N	p-value
Age		0.11		0.45		0.51		0.77		0.48		0.69
≤65 years	6		6		5		7		8		12	
>65 years	9		8		5		5		2		4	
Smoking status		0.78		0.76		0.18		0.55		0.48		0.69
S	9		8		5		7		8		12	
NS/ES	6		6		5		5		2		4	
Weight loss ≥5%		0.26		0.93		0.79		0.55		0.34		0.59
	6		5		4		4		3		9	
Perform.status		0.92		0.45		0.30		0.25		0.69		0.48
0	8		8		4		7		5		8	
1	7		6		6		5		5		8	
Stage		0.63		0.21		0.33		0.35		0.57		0.07
III	10		10		4		3		3		4	
IV	5		4		6		9		7		12	
T		0.21		0.76		**0.003**		**0.008**		0.095		0.92
T1-T2	7		6		0		1		5		5	
T3-T4	8		8		10		11		5		11	
N		0.33		0.87		0.42		0.074		0.19		0.91
N0-N1	5		5		3		6		3		3	
N2-N3	10		10		7		6		7		13	
M		0.64		0.21		0.33		0.35		0.57		0.07
M0	10		10		4		3		3		4	
M1	5		4		6		9		7		12	
Response to treatment		0.92		0.60		0.79		0.24		0.14		0.24
PD, SD	8		7		7		8		2		7	
PR, CR	7		7		2		3		8		9	

N = patient number. Abbreviations: SCLC, small cell lung cancer; S, smokers; NS, non-smokers; ES, ex-smokers; PD, progressive disease; SD, stable disease; PR, partial response; CR, complete response. Bold letters indicate statistically significant differences.

VEGF, the major angiogenic factor, and its key transcriptional regulator, HIF-1, is of great importance. Unfortunately, most of the studies performed to date on the expression of HIF-1α and VEGF in lung cancer concern operable NSCLC, and mainly, the adenocarcinoma subtype (16,17). In contrast, few studies have evaluated the expression of these factors in subtypes of operable NSCLC (8) or bronchial biopsies of SCLC (12).

Because the majority of lung cancer patients are diagnosed at an advanced stage of the disease and carry inoperable tumors, it is very important to diagnose their tumor molecular profile using specimens obtained from less invasive methods, such as bronchoscopy. To the best of our knowledge, the present study is the first to compare the expression of the angiogenic and pro-metastatic factors HIF-1α and VEGF in bronchial biopsies obtained from inoperable adenocarcinoma, squamous cell carcinoma and SCLC (Figure 1A). We detected convincing HIF-1α immunoreactivity in slightly less than half of the SCLC samples. A significantly higher incidence of HIF-1α expression was observed in NSCLC (squamous cell carcinoma and adenocarcinoma) compared to SCLC (Figure 1B). We also observed a higher frequency of HIF-1α-positive squamous cell carcinoma cases than adenocarcinoma cases (Figure 1C). However, VEGF expression was equally high in subtypes of NSCLC and SCLC (Figure 2B), and there was a correlation between HIF-1α and VEGF expression in all types of lung cancer collectively (Figure 2C) as well as in each type of lung cancer separately.

Interestingly, we found biopsies that were positive for VEGF staining and negative for HIF-1α expression. In HIF-1α-negative, VEGF-positive cases, it is possible that HIF-1α-independent pathways are responsible for the induction of VEGF. VEGF transcription in these samples might be controlled by HIF-2, which contains the HIF-2α isoform (3,6). The relative importance of the HIF 1α and 2α subunits in different tissues and cell types is still under intense study (18). The expression of HIF-2α has been previously shown in early operable NSCLC specimens (7), but there are no data concerning SCLC or subtypes of NSCLC. In HIF-1α-negative, VEGF-positive adenocarcinoma or SCLC cases, HIF-2α or other molecules and pathways may be responsible for VEGF induction.

In the present study, we found that the expression of both HIF-1α and VEGF was associated with T stage in adenocarcinoma. A previous report also indicated that HIF-1α expression in operable non-small cell lung cancer was marginally associated with T stage (7). We found no association between HIF-1α or VEGF expression and response to treatment or survival for the different types of lung cancer. This could be in part due to the relatively small number of biopsies examined. Previous studies have also reported a diverse degree of association between HIF-1α expression and the survival of lung cancer patients (7,8,12), while HIF-1α-positive, VEGF-positive SCLC biopsies were associated with poor survival (12).

In conclusion, this study demonstrated that HIF-1α was differentially expressed across lung cancer subtypes and that HIF-1α and VEGF had varying associations with

clinicopathological markers in different lung cancer subtypes. It also highlights the importance of using small bronchial biopsy specimens to evaluate the expression of important molecular markers, which can contribute to optimal therapy design for patients with advanced inoperable lung cancer.

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AUTHOR CONTRIBUTIONS

Karetsi E conceived the study, performed the experiments, analyzed the data and wrote the manuscript. Ioannou MG performed the experiments and analyzed the data. Kerenidi T analyzed the data. Minas M analyzed the data. Molyvdas PA and Gourgoulianis KI conceived the study and wrote the manuscript. Paraskeva E conceived and designed the study and wrote the manuscript.

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