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ORIGINAL ARTICLE

Glycine decarboxylase and HIF-1α expression are negative prognostic factors in primary resected early-stage non-small cell lung cancer

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Abstract Glycine decarboxylase (GLDC) was recently described as a critical enzyme of tumor-initiating cells and, thus, a driver of tumorigenesis in lung non-small cell cancer (NSCC). It is important in metabolism under hypoxic conditions. Hypoxia-inducible factor 1-alpha (HIF-1α) is the unique subunit that determines HIF system activity, thereby regulating the adverse effects of hypoxia on cancer outcome. We examined the expression and prognostic significance of GLDC and HIF-1α in primary resected stage I/II NSCC. Immunohistochemistry for GLDC and HIF-1α was validated on two lung NSCC cell lines (A549, NCI-H460) and evaluated on a tissue microarray with 428 lung NSCC: 184 adenocarcinomas, 211 squamous cell carcinomas, and 33 large cell carcinomas (LCC). The results were correlated with clinicopathological parameters. High levels of GLDC expression were detected in 33/428 cases (7.7%). HIF-1α was expressed in 71 (16.6%) cases and more frequently in squamous cell carcinoma ($p < 0.001$). Significantly longer survival was seen in younger patients ($p = 0.007$), patients with non-LCC histology ($p = 0.006$), lower primary tumor category ($p = 0.002$), and Union for International Cancer Control (UICC) stage ($p = 0.001$). Both GLDC and HIF-1α were significantly associated with worse tumor-related survival ($p = 0.013$, $p = 0.021$, respectively), although not independent from each other in multivariate models. The combination of low-GLDC/negative HIF-1α expression was significantly prognostic for longer survival ($p = 0.002$) and emerged as an independent prognostic factor in multivariate analysis ($p = 0.007$, HR 2.052), next to UICC stage and age. We show that the combination of GLDC and HIF-1α expression is an independent prognostic factor in early-stage NSCC. Our results will assist future development of therapeutic approaches targeting GLDC or exploiting tumor hypoxia.

Keywords NSCC · Glycine decarboxylase · Hypoxia · HIF-1α · Immunohistochemistry

Introduction

Lung cancer is a highly aggressive neoplasm and accounts for the most cancer-associated deaths worldwide. Most cases are non-small cell cancer (NSCC), which can be further subdivided histologically into the major groups of adenocarcinoma (AC), squamous cell carcinoma (SqCC), and large cell carcinoma (LCC) [1]. The 5-year survival rate in the low-stage situation (Union for International Cancer Control (UICC) 2009, 7th edition stage I/II tumors) ranges from 83 to 49% for stage I and stage II tumors, respectively [2, 3].

Tumor-initiating cells (TICs) are responsible for the increased tumorigenicity, therapy resistance, and recurrent disease (reviewed in [4]). TICs have a stem cell phenotype, and their characterization and prospective isolation are based on the expression of various cell surface markers (reviewed in [4]). Expression of those markers is plastic (e.g., non-TIC cells can acquire a TIC phenotype and vice versa [5, 6]). Hence, conflicting results were published concerning the prognostic significance of cell surface TIC markers in lung cancer [7, 8].

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The enzymatic activity of glycine decarboxylase (GLDC) conveys stem cell characteristics, drives NSCC TICs, and thus presents an alternative to identifying TICs based on cell surface marker expressions [7]. In short, mutational inactivation of the catalytic activity of GLDC abolished tumor formation in a mouse model, and knockdown of GLDC abolished soft-agar colony formation and tumor formation from human lung cancer spheres [7]. Similarly, in non-lung malignancies, inhibiting GLDC abolished formation of tumorigenic glioblastoma neurospheres with increased GLDC expression, but the survival of non-tumorigenic glioblastoma cells, characterized by low levels of GLDC expression, was not affected [9].

GLDC encodes the central component of the glycine-cleavage complex, which catalyzes the degradation of glycine into ammonia, carbon dioxide, and a methylene group that enters the folate pool. Increased GLDC expression was described in a variety of tumor tissues, including lung cancer [7]. However, to the best of our knowledge, the significance of increased GLDC protein expression has not been evaluated in homogeneous large cohorts of early-stage NSCC to date.

Increased GLDC protein expression is exploited by malignant tumors to adapt their metabolism under hypoxic conditions and is, thereby, associated with aggressiveness [9]. In solid cancers, hypoxic regions arise from limited oxygen diffusion (reviewed in [10]). Hypoxia affects various aspects of tumor biology including energy metabolism, cell survival, chemo/radiosensitivity, differentiation, invasion, and metastasis. Hypoxia induces the expression of hundreds of genes, including many that are regulated by hypoxia-inducible factors (HIFs). Under normoxic conditions, the alpha subunit of HIF-1 (HIF-1α) is hydroxylated by the O_2-dependent prolylhydroxylase and subsequently degraded. Under hypoxic conditions, the HIF-1α subunit dimerizes with the HIF-1β subunit, which allows the HIF-1 heterodimer to bind to hypoxia response elements to activate the transcription of hypoxia-inducible genes. Although the prognostic significance of HIF-1α protein expression has been evaluated in several solid tumors, conflicting results have been reported in lung cancer (reviewed in [11]).

In the present study, we evaluated the expression and prognostic significance of the TIC marker GLDC and hypoxia, visualized by HIF-1α expression, in a homogeneous cohort of NSCC (i.e., stages I/II).

Materials and methods

Patient collective

We evaluated a cohort of patients with primary resected node-negative early-stage NSCC (UICC 7th edition 2009 stage IA-IIB) [3], treated with curative surgery and diagnosed at the Institute of Pathology, University of Bern, Switzerland, and the Institute of Pathology, University Hospital Basel, Switzerland, between January 1988 and August 2008. The retrospective study was approved by both Institutions' local ethics committees.

The detailed staging work-up for this cohort of 544 patients was already reported [12]. We excluded cases with lymph node metastases, neoadjuvant treatment, rare tumor types other than AC, SqCC, and LCC, and insufficient tumor tissue for further analysis. Our final samples included tissue from 428 patients. The median age of the patient cohort was 67 years (range 39–83 years), and the male/female ratio was 317/111. The histologic subtype was AC in 184 cases (43%), SqCC in 211 cases (49.3%), and LCC in 33 cases (7.7%). The primary tumor (pT) category [3] was as follows: pT1a, 20 cases (4.7%); pT1b, 26 cases (6.1%); pT2a, 285 cases (66.6%); pT2b, 59 cases (13.8%); and pT3, 38 cases (8.9%), with corresponding UICC stage IA for 46 cases (10.7%), IB for 285 cases (66.6%), IIA for 59 cases (13.8%), and IIB for 38 cases (8.9%). Survival data were available for 355 patients. The median tumor-related overall survival, which was calculated from the day of surgical resection until last contact or death, was 186 months (95% CI 140–231 months).

Tissue microarray

A tissue microarray (TMA) was constructed as reported [12]. In short, formalin-fixed and paraffin-embedded (FFPE) tissue blocks were retrieved from the archives of the Institutes of Pathology. One punch (diameter, 0.6 mm) from the tumor center of the best-preserved block was transferred to the receptor TMA block. From the Bern sub-cohort (i.e., that part of the cohort with tissue samples from the Institute of Pathology, University of Bern), 37 patients were selected for evaluation of staining heterogeneity. A TMA was constructed as reported elsewhere, using eight randomly selected cores (diameter, 0.6 mm) from two FFPE blocks per tumor [13, 14].

Cell lines, reagents, and treatment

The NSCC cell lines A549 (CCL-185) and H460 (HTB-177) were purchased from American Type Culture Collection (ATCC, Manassas, VA) and cultured as described [15]. In brief, cells were cultured in Dulbecco's modified Eagle's medium nutrient mixture F-12 Ham (cat. #D6421, Sigma-Aldrich, St. Louis, MO), supplemented with 10% fetal bovine serum (cat. #10270-106; Life Technologies, Grand Island, NY), 1% penicillin/streptomycin solution (cat. #P0781, Sigma-Aldrich), and 1% L-glutamine (cat. #25030-024, Sigma-Aldrich) at 37 °C in a humidified 5% CO_2 incubator. To exclude cross-contamination, cell lines were DNA fingerprinted (Microsynth, Bern, Switzerland). The medium was changed every 3 days.

To establish a positive control for the HIF-1α immunohistochemical (IHC) staining, A549 cells were grown for 3 days as described above, and the culture medium was subsequently spiked with 1 mM dimethyloxalylglycine (DMOG, Sigma-Aldrich). Untreated cells were used as a negative control. After 3.5 h, cells were non-enzymatically detached (TrypLE™ Select Enzyme, Thermo Fisher Scientific, South San Francisco, CA), and 5×10^6 cells were pelleted by centrifugation at 500*g* for 5 min and fixed in 4% formalin/PBS for 2 h at room temperature (RT). Subsequently, cell pellets were embedded in paraffin and stained by IHC as described below (Fig. 1).

Immunohistochemical staining

IHC staining was carried out in the automated system BOND RX® (Leica Biosystems, Newcastle, UK). All sections were deparaffinized and rehydrated using Dewax solution (Leica Biosystems) at 72 °C for 30 s. Endogenous peroxidase activity was blocked with 3% H_2O_2 solution (Leica Biosystems) for 5 min. Samples were incubated with the following primary antibodies at RT for 30 min: anti-GLDC rabbit polyclonal antibody (Sigma Aldrich, #HPA002318) at a 1:200 dilution and anti-HIF-1α mouse monoclonal antibody, clone GT10211 (Genetex, Irvine, CA, #GTX628480), at a 1:300 dilution. The antigen retrieval was performed using Tris-EDTA buffer (pH 9) and citrate buffer (pH 6) at 95 °C for 30 min, respectively.

The slides were incubated with the secondary antibody with the Bond Polymer Refine Kit (Leica Biosystems) for 15 min. Subsequently, samples were incubated with the chromogen DAB (3-3′-diaminobenzidine) for 10 min, counterstained with hematoxylin for 5 min, and finally mounted with Aquatex® (Merck Millipore).

The stainings were read by a pathologist specialized in lung pathology (SB). For GLDC, the intensity of the staining was very homogeneous in the cores, and stains were scored semi-quantitatively as scores 0–3 according to intensity in at least 10% of tumor cells, as exemplified in Fig. 2. Scores 0–1 were considered low expression, and scores 2–3 were combined as high expression pattern. Nuclear expression of any intensity in at least 10% of cells was considered positive for HIF-1α.

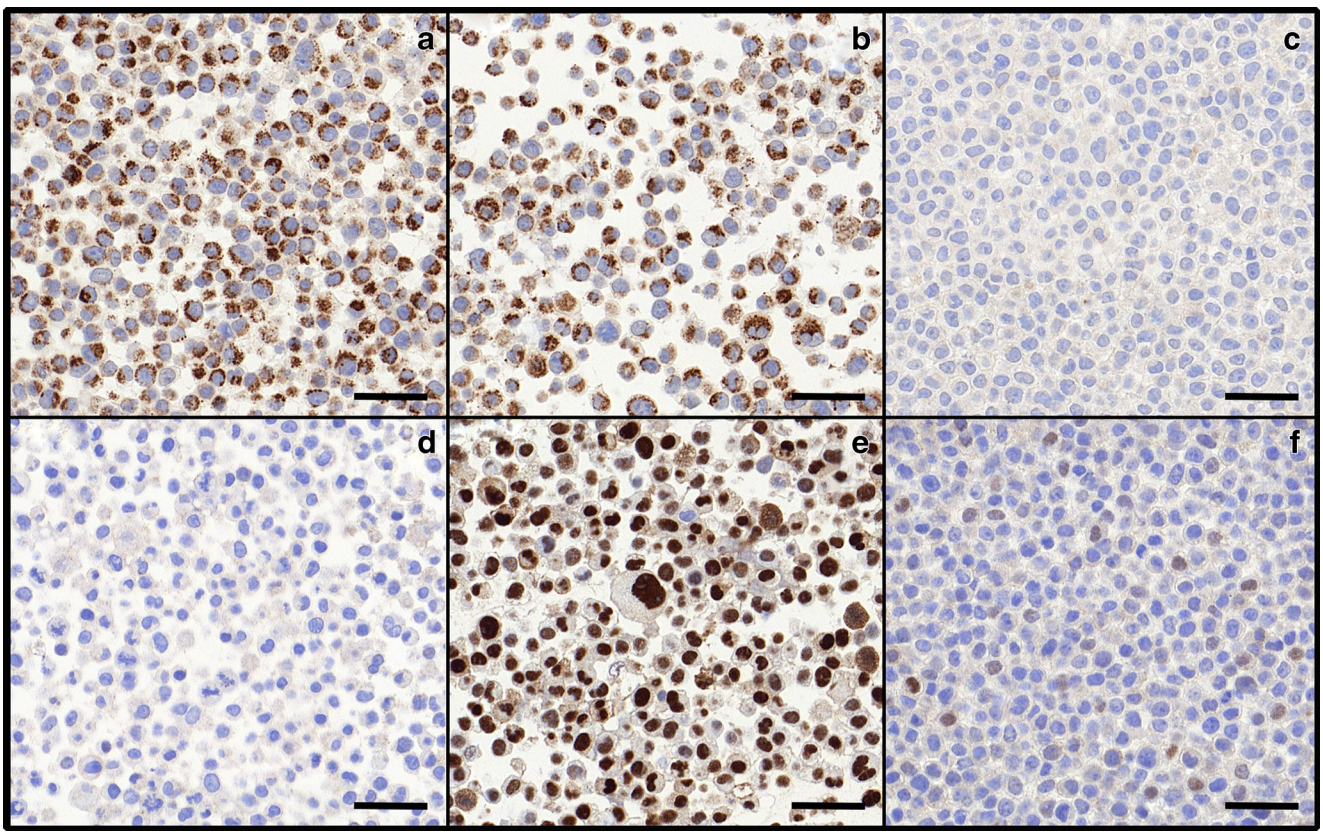

Fig. 1 Immunohistochemical staining for GLDC (*upper panel*) and HIF-1α (*lower panel*) validated in A549 and H460 cells. For GLDC, **a**, **b** cytoplasmic granular staining was confirmed in FFPE pellets of A549 cells and **c** FFPE pellets of H460 cells serving as negative control. Anti-HIF-1α antibody specificity was confirmed with **e** strong nuclear positivity after DMOG treatment, and **d** no staining of untreated A549 cells. **f** A faint nuclear positive signal was visible in a small percentage of H460 cells. All photomicrographs were taken at an objective magnification of ×40 (*scale bar* 50 μm)

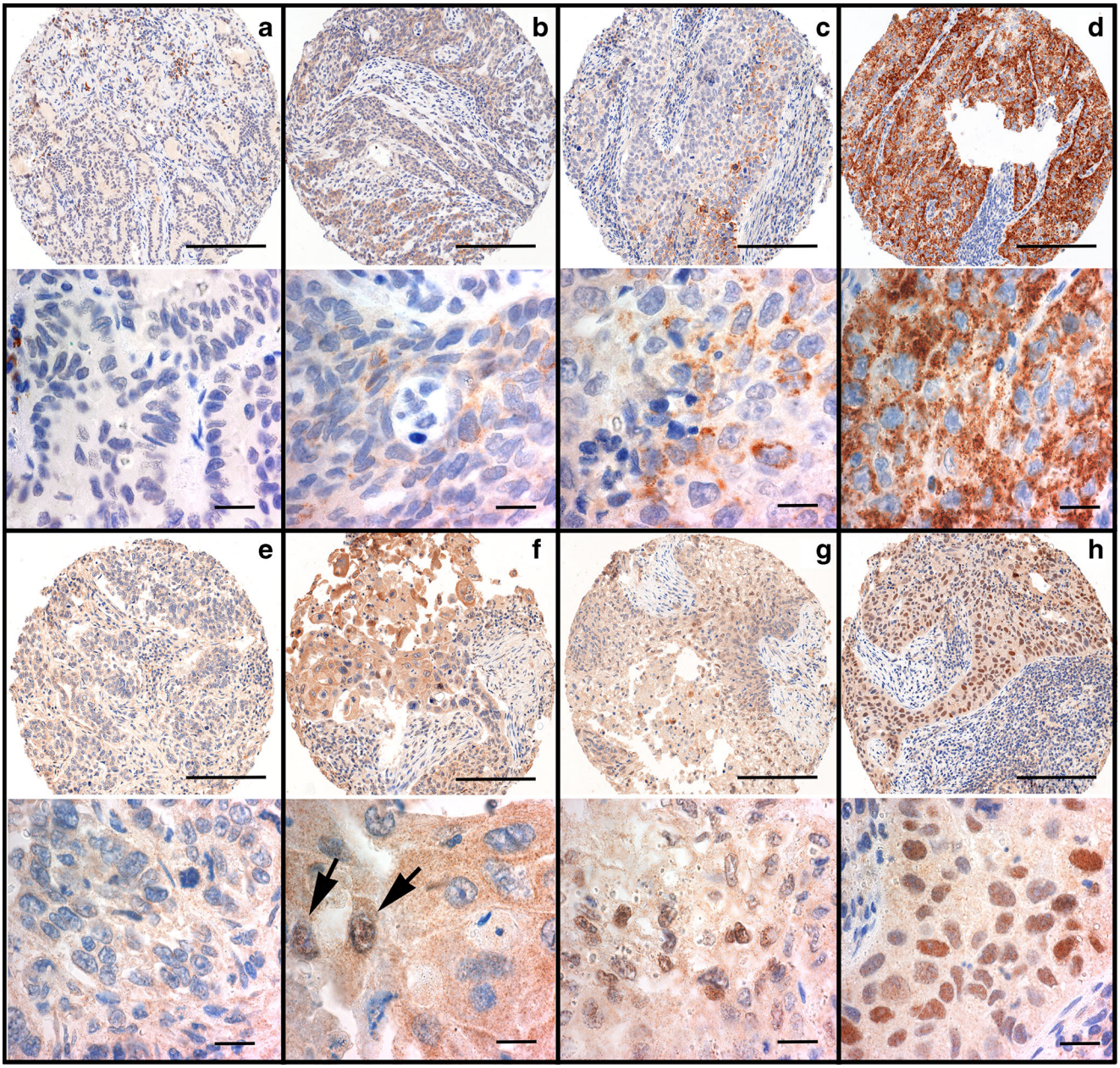

Fig. 2 Immunohistochemical staining scores of GLDC (*upper panel*) and HIF-1α (*lower panel*). GLDC low-grade staining comprised **a** completely negative or **b** weak (score 1) expression. GLDC high-grade staining cases showed **c** medium (score 2) and **d** strong (score 3) staining patterns. HIF-1α was evaluated as negative in case of **e** no nuclear staining or **f** faint nuclear staining in less than 10% of nuclei. **g**, **h** HIF-1α-positive cases showed a spectrum of intensity. All photomicrographs were taken at an objective magnification of ×20 (*upper panel*, *scale bar* 200 μm) and ×100 (*lower panel*, *scale bars* 20 μm)

Statistical analysis

The SPSS 23 software (SPSS, Chicago, IL) was used for descriptive and comparative and statistical analyses. Associations between staining patterns and pathologic parameters were evaluated using simple cross tabs (chi^2 test or Fisher's exact test). Log-rank test and Cox regression analysis were applied for survival analysis. The significance level was set at 0.05.

Results

Validation of GLDC and HIF-1α immunohistochemical staining

We used two lung NSCC cell lines to validate IHC stainings. A549 cells showed strong GLDC staining in the vast majority of cells (Fig. 1a), as reported [16]. The staining pattern was cytoplasmic and granular, as expected for the mitochondrial

protein GLDC [7]. GLDC staining was significantly less intense in NCI-H460 cells (Fig. 1c), in agreement with the reported fivefold lower GLDC protein levels in NCI-H460 cells than in A549 cells, based on mass spectrometry analysis [17].

HIF-1α protein was undetectable in A549 cells under normoxic conditions (Fig. 1d). Since hypoxia-induced stabilization of HIF-1α is reversible under normoxic conditions within minutes, we treated A549 cells with the prolylhydroxylase inhibitor DMOG, which chemically mimics hypoxia and stabilizes HIF-1α under normoxic conditions [18, 19]. After treatment with DMOG, A549 presented strong nuclear HIF-1α staining in most cells (Fig. 1e). DMOG treatment did not significantly affect GLDC staining (Fig. 1b). Only rare NCI-H460 cells stained faintly positive for HIF-1α in the absence of DMOG (Fig. 1f).

Staining patterns in patient cohort

GLDC and HIF-1α expression was evaluated in 428 patients (Table 1). For GLDC, 374 cases (87.4%) were negative, 21 cases (4.9%) were scored 1, 24 cases (5.6%) scored 2, and nine cases (2.1%) scored 3 (Fig. 2, upper panel). High cytoplasmic and granular expression of GLDC (scores 2 and 3) was detected in 33 cases (7.7%). For HIF-1α, 357 cases (83.4%) were negative, and 71 cases (16.6%) were positive. In the positive group, 41 cases (9.6%) showed only very weak and focal staining, and 30 cases (7%) had a stronger nuclear staining pattern (Fig. 2, lower panel). There was no significant correlation between GLDC and HIF-1α expression ($p = 0.326$).

Staining heterogeneity was assessed in 37 cases, with eight randomly selected tumor punches each, taken from two different tissue blocks. GLDC was scored as high versus low and HIF-1α as positive or negative as described for the whole case collective. The stainings were concordant among at least 75% of the cores evaluated per case in all cases (100%) evaluated for GLDC and in 33/37 (89%) of cases scored for HIF-1α.

Correlation with clinico-pathological parameters

High GLCD staining did not correlate with age, sex, histological subtype, pT category, or UICC stage. HIF-1α expression was significantly more frequent in SqCC (50/211 cases, 23.7%) than in AC (16/168 cases, 9.5%) or LCC (5/33, 15.2%; $p < 0.001$), but did not correlate with any other parameters mentioned above.

Survival analysis

We found longer survival for younger patients ($p = 0.007$), patients with AC and SqCC histology versus LCC ($p = 0.006$), lower pT category ($p = 0.002$), and lower UICC stage ($p = 0.001$). There was a trend for longer survival for females ($p = 0.061$). Patients with high tumoral GLDC expression, as well as HIF-1α expression, had significantly shorter tumor-related survival (GLDC $p = 0.013$, HIF-1α $p = 0.021$, Fig. 3a, b).

In a multivariate model encompassing the additional parameters age, histology, gender, and UICC stage, GLDC and HIF-1α were independent prognostic parameters if separately included, but were not independent from each other (Table 2 A–C).

Moreover, separating the cases into four groups, according to combined high or low GLDC expression and absent or present HIF-1α staining, rendered not only significantly prognostic groups in univariate analysis ($p = 0.013$, Fig. 3c) but was also independently prognostic in a multivariate model ($p = 0.005$, HR 1.545). This result was even more pronounced after dichotomizing the staining combinations, with significantly longer survival for GLDC low/HIF1-α-negative tumors versus all other combinations (univariate $p = 0.002$, Fig. 3d; multivariate $p = 0.007$, HR 2.052, Table 2).

Discussion

In this paper, we show that the combination of GLDC and HIF-1α expression is an independent prognostic factor in early-stage NSCC. Both GLDC and HIF-1α were associated with worse tumor-related survival, but they were not independent of each other in multivariate models. The combination of low-GLDC/negative HIF-1α expression was significantly prognostic for longer survival and emerged as an independent prognostic factor, next to UICC stage and age.

As a first step, we thoroughly validated the IHC staining, with adequate positive and negative controls. In particular,

Table 1 GLDC and HIF-1α expression in all cases of low-stage NSCC

Parameter		GLDC score				Total, *N* (%)
		0	1	2	3	
HIF-1α score	Negative	316	17	18	6	357 (83.4)
	Positive	58	4	6	3	71 (16.6)
Total, *N* (%)		374 (87.4)	21 (4.9)	24 (5.6)	9 (2.1)	428 (100)

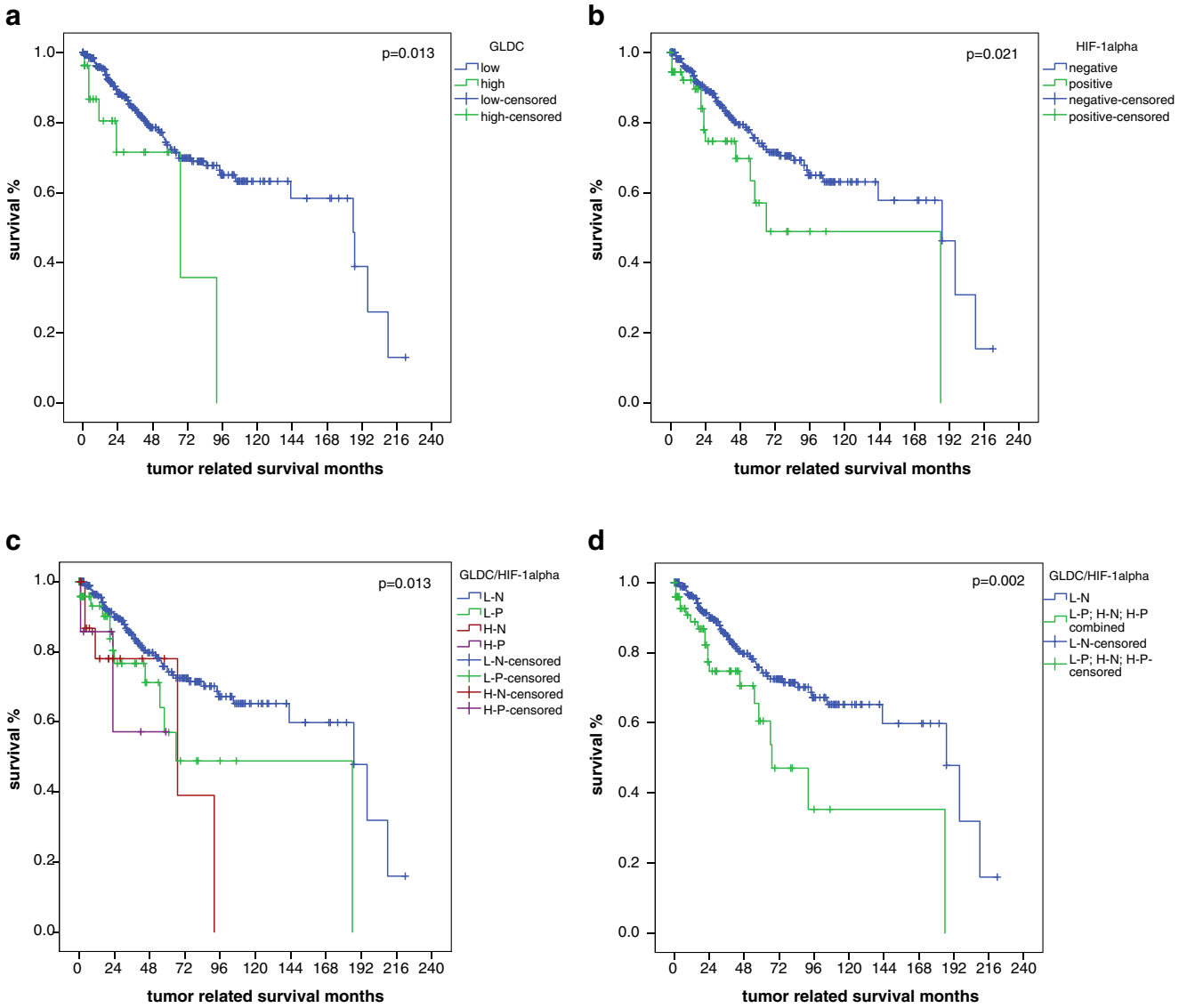

Fig. 3 Survival analysis. Kaplan-Meier curves for tumor-related overall survival assessed for **a** GLDC low versus high expression ($p = 0.013$) and **b** HIF-1α absent versus present expression ($p = 0.021$). The combination of GLDC and HIF-1α staining showed longer survival for GLDC low tumors with absent HIF-1α as assessed by **c** different combinations of GLDC and HIF-1α staining patterns (low-negative, *blue*; low-positive, *green*; high-negative, *red*; high-positive, *purple*; $p = 0.013$) and **d** GLDC low/absent HIF-1α versus all other staining combinations ($p = 0.002$)

after chemical stabilization of HIF-1α protein by DMOG treatment, A549 cells served as an HIF-1α-positive control. Basal HIF-1α levels were slightly higher in H460 cells, consistent with low constitutive activation in a subset of tumors [11].

Only few normal tissues significantly express GLDC, including postmitotic liver cells, kidney cells, placenta cells, and olfactory bulb neurons [16]. Conversely, GLDC mRNA and protein expression is higher in a variety of tumor tissues, including lung cancer [7, 20]. In our study, 7.7% of all 428 tumors showed high GLDC expression, not associated to any of the investigated clinico-pathological characteristics, including histological type. Clearly though, high GLDC staining was associated with shorter tumor-related survival in our cohort. This is in line with previous results, linking high expression of the metabolic oncogene GLDC with a threefold higher risk of lung cancer mortality in a cohort of 143 NSCC patients (stages I–III) [7].

HIF-1α protein is overexpressed in lung cancer versus benign tissue [11]. Increasing HIF-1α protein level as metabolic adaptation to O_2 deprivation is an important event in the progression of cancer. HIF-1α protein overexpression is associated with poor treatment response and worse outcome in a wide range of human tumors [10]. Although its clinical significance in lung cancer is controversial, Ren et al. found higher expression associated with higher stage and worse

Table 2 Multivariate analysis

	Parameter	HR	95% confidence interval		*p* value
			Min	Max	
A	Age (median)	1.612	1.015	2.561	*0.043*
	Gender	0.74	0.4	1.37	0.338
	Histology	1.346	0.929	1.948	0.116
	UICC stage	1.378	1.072	1.771	*0.012*
	GLDC	2.357	1.069	5.195	*0.033*
B	Age (median)	1.669	1.048	2.659	*0.031*
	Gender	0.728	0.394	1.347	0.312
	Histology	1.295	0.885	1.897	0.183
	UICC stage	1.37	1.067	1.758	*0.014*
	HIF-1α	1.81	1.013	3.234	*0.045*
C	Age (median)	1.686	1.057	2.69	*0.028*
	Gender	0.762	0.41	1.415	0.389
	Histology	1.291	0.881	1.891	0.190
	UICC stage	1.367	1.061	1.762	*0.016*
	HIF-1α	1.714	0.951	3.091	0.073
	GLDC	2.161	0.973	4.8	0.058
D	Age (median)	1.673	1.051	2.663	*0.030*
	Gender	0.763	0.411	1.416	0.391
	Histology	1.302	0.892	1.899	0.171
	UICC stage	1.369	1.062	1.764	*0.015*
	GLDC/HIF-1α combination	1.545	1.138	2.097	*0.005*
E	Age (median)	1.668	1.048	2.655	*0.031*
	Gender	0.757	0.408	1.404	0.377
	Histology	1.306	0.892	1.914	0.170
	UICC stage	1.364	1.06	1.755	*0.016*
	GLDC low/HIF-1α low	2.052	1.214	3.47	*0.007*

Parameters included histology (AC versus SqCC versus LCC), age, UICC stage 2007, 7th edition (IA versus IB versus IIA versus IIB), and GLDC/HIF-1α staining combinations (GLDC low/HIF-1α negative versus GLDC low/HIF-1α positive versus GLDC high/HIF-1α negative versus GLDC high/HIF-1α positive). Items set in italic show statistical significance at the 0.05 probability level

outcome in a meta-analysis summarizing 30 studies published until 2012 [11].

Our correlation with clinico-pathological parameters revealed that neither GLDC staining nor HIF-1α expression significantly correlated with stage, which might be due to the fact that our homogenous cohort consisted of stage I/II cases but no late-stage cases (i.e., stage III/IV). HIF-1α expression was significantly more frequent in SqCC than in AC in our cohort, in line with published data [11]. Expression of HIF-1α was also associated with shorter tumor-related survival, which proved an independent prognostic marker in multivariate analysis, regardless of tumor histology. Remarkably though, the prognostic value of HIF-1α was not independent from expression of the metabolic oncogene GLDC (Table 2 C). Finally, the combination of GLDC and HIF-1α staining yielded the best prognostic results. Combined low GLDC expression and HIF-1α negativity was associated with a significantly longer tumor-related survival and proved an independent prognostic factor in multivariate analysis (Table 2 D, E, Fig. 3c, d). In this context, modulation of GLDC expression is exploited by malignant tumors for adapting their metabolism under hypoxic conditions, thereby being associated with aggressiveness [7].

Therapeutic approaches might emerge that target GLDC enzymatic activity [21]. Various bio-reductive pro-drugs are in clinical development to exploit tumor hypoxia (reviewed in [10]). Our data provide extra rational to this therapeutic approach by adding to the body of evidence that high GLDC and HIF-1α expression is associated with higher tumor aggressiveness. Additional studies will be required to determine the predictive significance of GLDC and HIF-1α expression in the setting of the primary disease and also in recurrent NSCC and metastasis. Nevertheless, the development of GLDC-targeting strategies and bio-reductive pro-drugs might focus on GLDC and HIF-1α protein levels in the search after predictive biomarkers, warranting the prior assessment of

expression patterns and prognostic value reported in the present study.

In conclusion, we assessed GLDC and HIF-1α expression on a large collective of 428 primary resected early-stage node-negative lung NSCC. We show that expression of HIF-1α and GLDC are associated with shorter tumor-related survival, although not independent from each other. It is the combination of GLDC and HIF-1α expression that emerged as an independent prognostic factor in multivariate models.

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Compliance with ethical standards This study was approved by the local ethics committees of both institutions and has been performed in accordance with the ethical standards laid down in the 1964 Helsinki Declaration and its later amendments.

Conflict of interest The authors declare that they have no conflict of interest.

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