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ORIGINAL ARTICLE – CANCER RESEARCH

Protein expression of close homologue of L1 (CHL1) is a marker for overall survival in non-small cell lung cancer (NSCLC)

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Abstract

Background The cell adhesion molecule close homologue of *L1* (CHL1) is a potential tumour suppressor and was recently detected in non-small cell lung cancer (NSCLC) specimens. The expression pattern, prognostic, and functional role of CHL1 in NSCLCs is unknown.

Methods We evaluated the protein expression of CHL1 by immunohistochemistry in 2161 NSCLC patients based on a tissue microarray. The results were correlated with clinical, histopathological, and patient survival data (Chi square test, *t* test, and log-rank test, respectively). A multivariate analysis (Cox regression) was performed to validate its impact on patients' survival.

Results CHL1 was expressed in NSCLC patients and was significantly overexpressed in lung adenocarcinomas and squamous cell carcinomas compared to neuroendocrine and large cell carcinomas of the lung ($p < 0.001$). CHL1 expression was associated with the T stage in adenocarcinomas ($p = 0.011$) and with metastatic lymph node status and UICC stage in squamous cell carcinomas ($p = 0.034$ and $p = 0.035$, respectively). Increased CHL1 expression was associated with improved survival in univariate ($p = 0.031$) and multivariate analyses (odds ratio 0.797, 95% confidence interval 0.677–0.939, $p = 0.007$).

Conclusion The prognostic significance of CHL1 makes it a potential prognostic and therapeutic target and underlines its role as a tumour suppressor. Further validation studies and functional analyses are needed to investigate its potential role in tumourigenesis and dissemination.

Keywords Lung cancer · NSCLC · CHL1 · L1 · Prognostic maker · Tumour marker

Introduction

Lung cancer is one of the most common cancers and causes of the world's most cancer-related deaths. Overall, it is estimated to be responsible for more deaths than colon, breast, and prostate cancers (Browse the SEER Cancer Statistics Review 1975). Almost 85% of all lung cancers are non-small cell lung cancer (NSCLC) with a dismal prognosis due to a high metastatic potential and a high rate of tumour recurrence resulting in a 5-year survival rate of only 11–15% (Shen and Ren 2017). The two major histologic subtypes of NSCLCs are adenocarcinomas (ADC) and squamous cell carcinomas (SQCC). In recent years, small but significant progress was made in the treatment of NSCLCs. Some genetic alterations, proven as key oncogenic factors, were validated as reliable targets for molecular-based therapy. Two of them, the epidermal growth factor receptor (EGFR) and the echinoderm microtubule-associated protein-like

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4-anaplastic lymphoma kinase (EML4-ALK) protein, are established in clinical routine. However, there is no diagnostic or prognostic serum marker of clinical relevance (Reck et al. 2014; Lo Russo et al. 2017). Known biomarkers such as the squamous cell carcinoma antigen (SCCA), Cytokeratin-19 fragments (CYFRA21-1), and the CarcinoEmbryonic Antigen (CEA) often fail to provide sufficient sensitivity, have no value for screening, and are cost-intensive (Zamay et al. 2017).

Tumourigenesis is a multi-determinant process in which cell adhesion molecules participate in the growth, invasive behaviour, and dissemination of a tumour. Members of the L1 adhesion molecule family, which were initially detected in neural cell interactions and are, therefore, summarised in the neural cell adhesion molecule group, are also involved in oncologic processes (Raveh et al. 2009). Particularly, the L1 Cell Adhesion Molecule (L1CAM) and the Neuronal Cell Adhesion Molecule (NRCAM) were detected in various tumour entities, such as colorectal carcinoma, malignant melanoma, papillary thyroid carcinoma, and endometrial adenocarcinoma. For these cancers, functional roles in increasing cell motility, enhancing growth rate, promoting cell transformation, and tumourigenicity were identified (Siesser and Maness 2009; Conacci-Sorrell et al. 2002; Górka et al. 2007; Klat et al. 2019). L1CAM and NRCAM are also crucial to embryonic development of the brain and peripheral nervous system in terms of cell proliferation and motility (Zecchini and Cavallaro 2010).

Another member of the L1 molecule family is the Close Homologue of L1 (CHL1, GenBank Accession No. NM_006614.2). CHL1 plays a significant role in different neurological diseases, in general cognitive activities, and was also found in various tumour tissues (Ross et al. 2000; Wei et al. 1998; Frints et al. 2003; Sakurai et al. 2002). Compared to L1CAM and NRCAM, little is known of its function in oncological processes. Its expression has been reported to promote cancer cell growth, migration, and invasion in different cancer types. In 2013, He and colleagues described a down-regulation of CHL1 in breast cancer and an association with lower tumour grading. Overexpression, on the other hand, seemed to suppress the invasion and proliferation of tumour cells (He et al. 2013). Furthermore, Martín-Sánchez et al. were able to show that down-regulation of CHL1 by hypermethylation in breast cancer correlates with a significantly shorter progression-free time (Martín-Sánchez et al. 2017). Ognibene et al. confirmed the latter findings in recent work by demonstrating the tumour suppressive role of CHL1 in neuroblastomas (Ognibene et al. 2018). Additionally, down-regulation of CHL1 via overexpression of miR-21-5p also promotes the propagation and invasion of tumour cells in colon adenocarcinomas (Yu et al. 2018). Very recently, Tang et al. identified a significant correlation between CHL1 expression and better overall survival for oesophageal cancer patients and also described a tumour suppressive role of CHL1 in the Akt Pathway (Tang et al. 2019).

Senchenko et al. published the most extensive study on CHL1 gene expression in different tumours; the CHL1-encoding mRNA was screened in 19 tumour entities and corresponding metastases using cancer-profiling array and real-time qPCR. Their study demonstrated that CHL1 could act as a suppressor during initial tumour growth and re-expression could promote invasive growth as well as metastasis in ovarian, colon, and breast cancer. Additionally, their work showed a CHL1 expression in 64% of lung cancer samples (Senchenko et al. 2011).

Hence, the aim of the current study was to investigate the expression of CHL1 in a large number of NSCLC specimens on a tissue microarray (TMA) and analyse its correlation with the clinical and pathological parameters to evaluate the prognostic impact on patients' survival.

Methods

Patients and clinical data

We evaluated tissue samples of 2279 patients with lung cancer who underwent curative oncologic surgery (lobectomy or pneumectomy with lymphadenectomy). Information about neoadjuvant radio- or radiochemotherapy are available for 246 patients only, and out of this, 7 received neoadjuvant treatment (2.8%). Retrieval of tissue and clinical data was performed according to the regulations of the local medical association's ethics committee and data safety laws. Patient data were anonymous. Tumour staging of the resected specimens including histological findings, depth of tumour invasion (T), lymph node metastases (N), distant metastases (M), residual tumour (R), tumour grading (G), lymphatic (L) and vascular (V) infiltration, as well as tumour type were classified according to the classification of the sixth edition of the International Union Against Cancer (UICC). Overall survival (OS) was defined as the time between the date of surgical resection and death or last follow-up. These data were obtained by reviewing the clinical and pathological record, by reviewing outpatient clinic medical records and direct communication with patients and their attending physicians. Due to cases with lack of tissue samples and the missing of unequivocal cancer tissue in the TMA section, a total of 118 patients had to be excluded. Hence, we investigated a total of 2161 patients. The presented numbers of variables within the analyses do not necessarily add up to the total sum of investigated patients because of missing clinicopathological data.

Tissue microarrays (TMA)

For immunohistochemical analyses, two sets of previously described TMAs were used (Tachezy et al. 2011; Went et al. 2006). Tissue samples were fixed in 4% buffered formalin, embedded in paraffin, and used for tissue microarray construction as described previously (Dancau et al. 2016). Hematoxylin and eosin stained sections were generated from selected primary tumour blocks (donor blocks) with representative tumour regions. Tissue cylinders (diameter of 600 µm) from a peripheral or central area with a high number of vital tumour cells and little necrosis were then punched from that region of the donor block using a semi-automated tissue array. Control samples included lung, normal oesophagus mucosa, endometrium, skin, skeletal muscle, heart muscle, colon mucosa, lymph node, prostate, and kidney tissue. Adjacent sections of the complete TMA with a thickness of 3 µm were made using the Paraffin Sectioning Aid System (Instrumentics, Hackensack, NJ).

Immunohistochemical staining for CHL1 and evaluation of its expression

The CHL1 staining protocol for the paraffin tissue was optimised in an extensive multistep procedure on various benign and malignant tissues, modifying the staining protocol until selective staining with the lowest background signal were established (Simon et al. 2010). The immediately processed TMA sections, as described above, were deparaffinised and exposed to heat-induced antigen retrieval for 5 min by the aid of an autoclave (121 °C, pH 7.8 Tris–EDTA citrate buffer). Subsequently, specific CHL1 primary antibody (goat, polyclonal antibody, R&D Systems, USA, cat# AF2126, dilution 1:450) was adjoined for 60 min (37 °C, pH 9.1). To visualise the bound antibody, we applied the EnVision Kit (Dako, Glostrup, Denmark) according to the manufacturer's directions. A quantitative (0–100%) and qualitative (grade 1–3) analysis of each spot on the TMA was performed and different scores were calculated. However, only quantitative scoring demonstrated a significant correlation with clinicopathological data. A cutoff with > 10% immunopositivity for CHL1 within the tumour cells demonstrated an optimal separation for the investigated parameters. Hence, specimens were dichotomized into positive or negative for CHL1 expression, accordingly.

Statistical analysis

All statistical analyses were carried out using IBM SPSS Statistics for Mac (Version 20, IBM Corporation, Armonk, New York, USA). Chi square test and Fisher's exact test were used to depict the calculated interdependence between immunostaining and clinical data in cross tables. Kaplan–Meier plots and log-rank tests were used to examine the association of CHL1 expression with overall survival. A multivariate Cox regression model was applied to evaluate the independent contribution of each separate parameter to patient outcome. Therefore, odds ratios (OR) and 95% confidence intervals (95% CI) were used. Patients that died within the first 30 days after surgery were excluded from the survival analyses. All tests were two-sided, and the statistical significance was set to $p < 0.05$.

Results

Patients' characteristics and CHL1 protein expression in NSCLC

We analysed a total of 2161 primary NSCLC samples in a TMA analysis. Positive CHL1 protein expression was found in 854 (39.5%) samples whereas 1307 (60.5%) samples were not considered immunopositive (Fig. 1). We were able to detect a significantly stronger expression in adenocarcinomas and squamous cell carcinomas compared to the other NSCLC subtypes ($p < 0.001$).

The median age at the time of diagnosis was 62.0 years with 76.7% male and 23.3% female patients (Table 1). The median follow-up time was 23.0 months (range 0.0–200.2 months) and the median calculated overall survival was 47.0 months (95% CI 39.9–54.0 months). During the observation period, 47.9% deceased, and within the first 30 days after surgery, 5.1% of the patients died.

Association of CHL1 protein expression and clinical and pathological parameters

CHL1 expression showed no significant association with the analysed clinical and histopathological parameters such as sex, age, tumour size, grading, lymph nodes, and metastasis status in all tumour cell types. Vascular invasion was not significantly associated with CHL1 expression by a small margin ($p = 0.054$). We then analysed CHL1 expression of adenocarcinomas ($n = 563$) and squamous cell carcinomas ($n = 958$) separately. Significant associations were found between sex ($p = 0.034$) as well as T stage ($p = 0.011$) for adenocarcinomas while in squamous cell carcinomas a positive correlation with lymph node status ($p = 0.034$) and UICC stage ($p = 0.035$) was observed (Supplemental Tables 1 and 2).

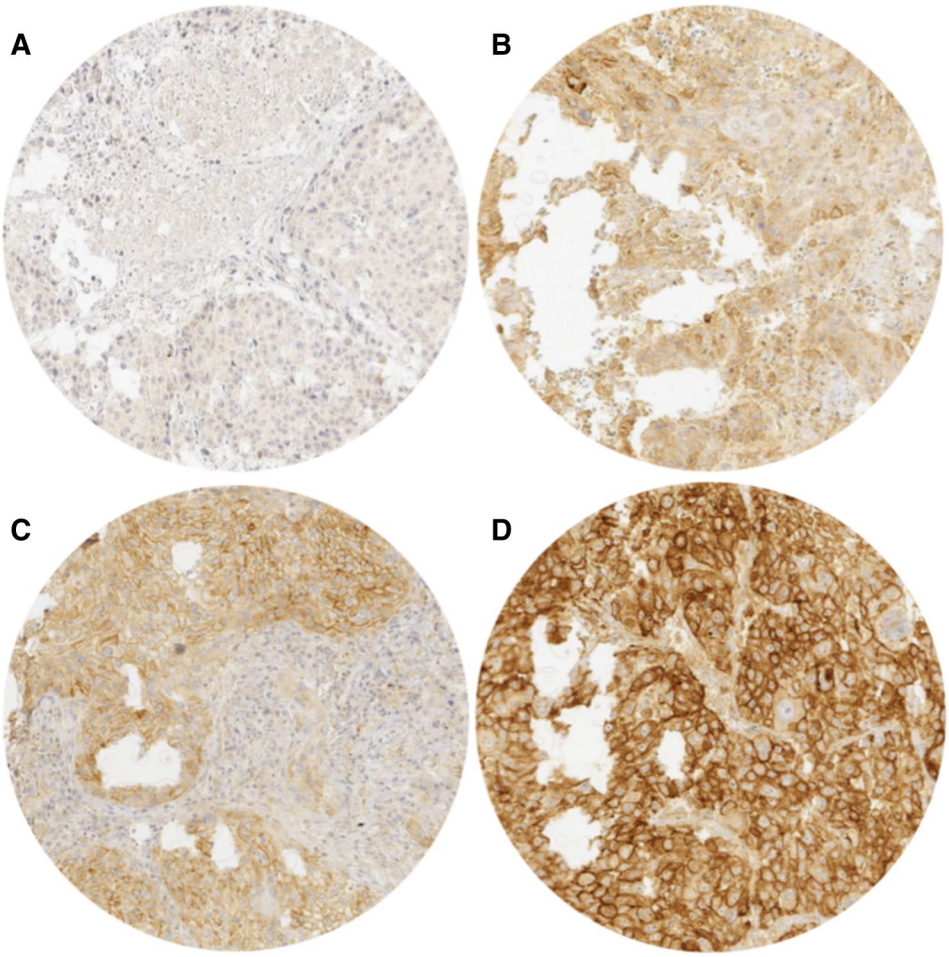

Fig. 1 Representative immunohistochemical CHL1 stainings. **a** No expression (< 10% immunopositivity), **b** low expression (immunopositivity grade 1 in 80% of tumour cells), **c** intermediate expression (immunopositivity grade 2 in 90% of tumour cells) and **d** high expression (immunopositivity grade 3 in 100% of tumour cells)

CHL1 protein expression and survival in NSCLC patients

The log-rank analysis revealed a significant difference for overall survival between patients with CHL1-positive and CHL1-negative tumours ($p = 0.031$, Fig. 2a). CHL1-positive tumours demonstrated improved survival of 60.3 months (95% CI 47.6–72.3 months) as compared to survival of 43.2 months (95% CI 35.1–50.9 months) in CHL1-negative tumours. This difference remained significant when only adenocarcinomas of the NSCLCs were taken into account ($p = 0.048$; Fig. 2b) demonstrating a survival of 60.2 months (95% CI 42.1–78.3 months) for CHL1 positive and a survival of 43.1 months (95% CI 30.3–56.4 months) for CHL1-negative tumours. However, subgroup analysis for overall survival in squamous cell carcinomas failed to reach significance by a small margin ($p = 0.052$; Fig. 2c).

We then confirmed the latter results with multivariate analysis showing that CHL1 protein expression in NSCLC is an independent prognostic factor (OR 0.797, 95% CI 0.677–0.939, $p = 0.007$) for survival. As expected, other established histopathological variables like the TNM status and grading were strongly associated with survival (Table 2).

Discussion

In the current study, we were able to detect significant protein expression of CHL1 in 39.3% of NSCLC specimens and also found significantly higher expression in the adeno- and squamous cell carcinoma subtypes (44.3% and 42.9%, respectively). This is in line with a study of Tian et al. demonstrating a significant link between the CHL1 gene and lung cancer susceptibility (Tian et al. 2018). Another study by Senchenko et al. also found an upregulation of the CHL1 gene in lung cancer patients. When they separately investigated the mRNA expression in squamous cell carcinomas, and adenocarcinomas of the lung, a significant mRNA decrease in SQCC compared to ADC was found, though, no association to tumour progression was reported (Senchenko et al. 2011). In our study, however,

Table 1 Association of clinicopathological characteristics of NSCLC patients and CHL1 expression in all tumour cell types

	Total ($n=2161$)	All tumour cell types				p value
		CHL1 negative		CHL1 positive		
		($n=1307$; 60.5%)		($n=854$; 39.5%)		
Age in years (SD)		62.0	(9.3)	62.3	(9.1)	0.682
Sex	1617					
Male	1241	709		532		
Female	376	233		143		0.107
Grade	1011	633	62.6%	378	37.4%	
1	137	90	65.7%	47	34.3%	
2	646	392	60.7%	254	39.3%	
3	228	151	66.2%	77	33.8%	0.240
T	1993	1189	59.7%	804	40.3%	
1	423	231	54.6%	192	45.4%	
2	1126	693	61.5%	433	38.5%	
3	292	170	58.2%	122	41.8%	
4	152	95	62.5%	57	37.5%	0.075
N	1947	1161	59.6%	786	40.7%	
0	984	584	59.3%	400	40.7%	
1	480	291	60.6%	189	39.4%	
2	425	252	59.3%	173	40.7%	
3	58	34	58.6%	24	41.4%	0.965
M	2002	1169	59.7%	751	40.3%	
0	1880	1129	60.1%	55	39.9%	
1	122	67	54.9%	806	45.1%	0.295
L	307	183	59.6%	124	40.4%	
0	15	8	53.3%	7	46.7%	
1	292	175	59.9%	117	40.1%	0.603
V	36	22	61.1%	14	38.9%	
0	26	13	50.0%	13	50.0%	
1	10	9	90.0%	1	10.0%	0.054
R	198	114	57.6%	84	42.4%	
R0	162	96	59.3%	66	40.7%	
R1 and R2	36	18	50.0%	18	50.0%	0.353
UICC classification	1986					
Stage Ia	287	161	56.1%	126	43.9%	
Stage Ib/IIa	546	332	60.8%	214	39.2%	
Stage IIb	458	278	60.7%	180	39.3%	
Stage IIIa	403	247	61.3%	156	38.7%	
Stage IIIb	156	88	56.4%	68	43.6%	
Stage IIIc	20	13	65.0%	7	35.0%	
Stage IV	116	65	56.0%	51	44.0%	0.675
Tumour cell type	2025					
Adenocarcinoma	563	314	55.7%	249	44.3%	
Bronchioalveolar	83	42	50.6%	41	49.4%	
LCLC	359	266	74.1%	93	25.9%	
NET	62	46	74.2%	16	25.8%	
SQCC	958	547	57.1%	411	42.9%	0.001

Numbers of variables do not add up to total sample number due to missing clinical or histopathological data. Age is presented as mean value

CHL1 close homologue of L1, *SD* standard deviation, *LCLC* large cell lung cancer, *NET* neuroendocrine tumor, *SQCC* squamous cell carcinoma

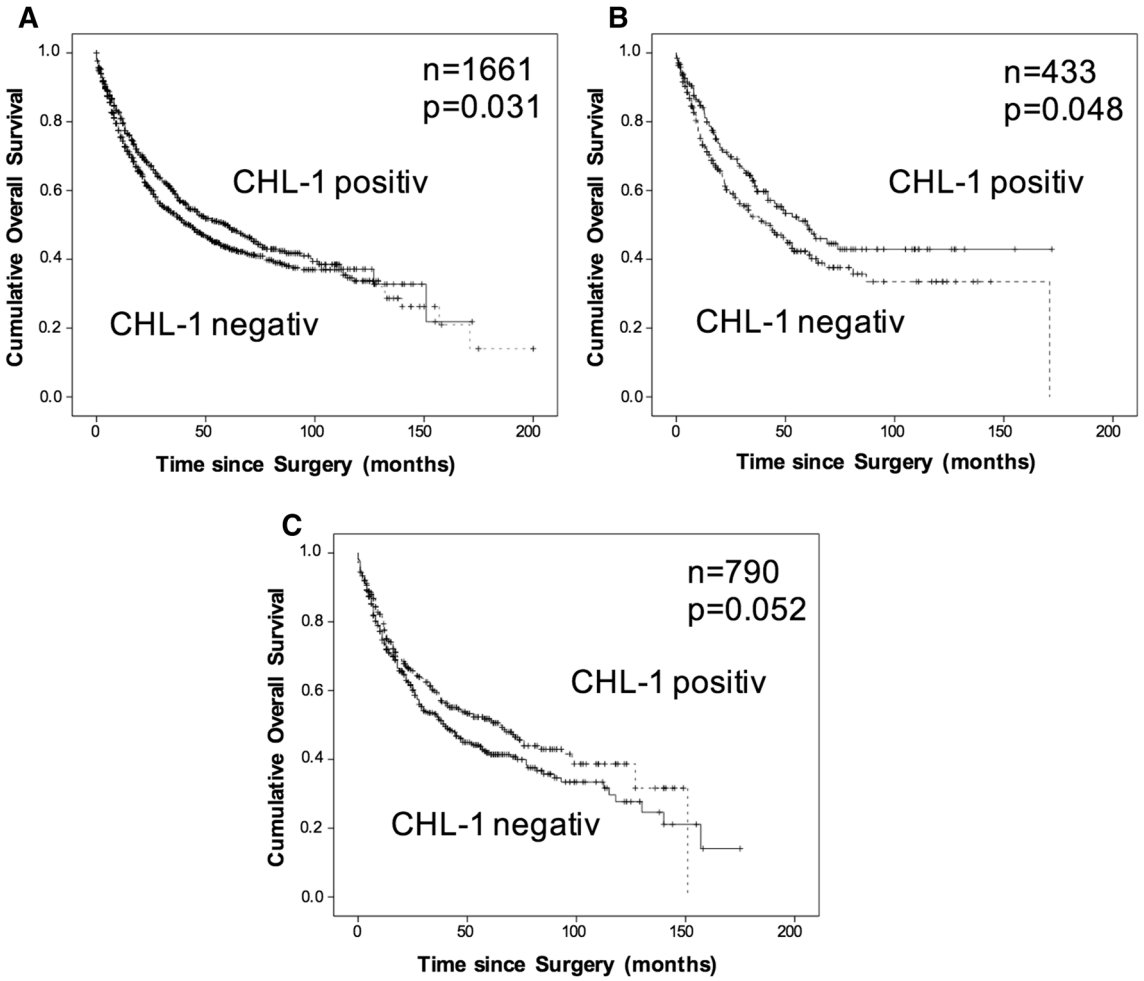

Fig. 2 Kaplan–Meier survival analyses of CHL1 expression. **a** All tumour types, **b** adenocarcinomas, **c** squamous cell carcinomas. *CHL1* close homologue of L1

Table 2 Prognostic value of CHL1 expression for overall survival in NSCLCs

	Univariate			Multivariate		
	OR	95% CI	*p* value	OR	95% CI	*p* value
Sex (male vs. female)	0.853	0.695/1.047	0.128	0.889	0.692/1.132	0.326
Age in years	1.273	1.110/1.460	0.001	1.234	1.052/1.448	0.100
CHL1 expression	0.859	0.742/0.987	0.032	0.797	0.677/0.939	0.007
Grading	1.578	1.234/2.016	0.001	2.069	1.492/2.869	0.001
T	1.863	1.596/2.175	0.001	2.066	1.732/2.465	0.001
N	2.162	1.861/2.511	0.001	2.239	1.879/2.669	0.001
M	2.523	2.016/3.159	0.001	2.748	2.125/3.554	0.001

Age is presented as mean value

CI confidence interval, *CHL1* close homologue of L1, *CHL1* univariate analysis: non-expression vs. expression, *Grading univariate analysis* stage 1 and 2 vs. stage 3, *N stage univariate analysis* stage 0 and 1 vs. stage 2 and 3, *OR* odds ratio, *T stage univariate analysis* stage 1 and 2 vs. stage 3 and 4,

we did not find any significant differences in expression patterns between both entities at the protein level.

For the first time, we were able to demonstrate a significantly better overall survival rate for elevated CHL1 protein expression in NSCLCs independent of age, sex, TNM status, and grading. Interestingly, an effect of CHL1 on the biologic function of cancer cells has been reported in a recent study demonstrating that the inhibition of CHL1 expression by microRNA-21 leads to increased invasiveness of tumour cells in colon adenocarcinomas (Yu W, Zhu K, Wang Y, Yu H, Guo J. Overexpression of miR-21-5p promotes proliferation and invasion of colon adenocarcinoma cells through targeting CHL1. Mol Med [Internet] 2018). Similar results have been shown for breast cancer cells in which CHL1 deficiency led to tumour formation, and a knockdown of CHL1 expression led to increased proliferation and invasion (He et al. 2013). This invasiveness might be caused by an alteration of cell adhesion due to the loss of CHL1 or L1 leading to increased local proliferation and higher metastatic potential as recently described for oesophageal cancers (Tang et al. 2019). The latter study not only found an association of decreased CHL1 expression with distant metastases but also a correlation to lymph node metastasis and reduced overall survival. In our study, we also found a significant association between CHL1 expression and histopathological parameters like tumour size in lung adenocarcinomas and lymph node metastases and UICC stage in lung squamous cell carcinomas. Hence, our data imply a similar mechanism in NSCLCs and underline the general tumour suppressive role of CHL1.

In this study, we focused on tissue protein expression only. However, similar to L1 cell adhesion molecule (L1CAM), the ectodomain of CHL1 is shed via the disintegrin and metalloproteinase domain-containing protein 8 (ADAM8) and can be detected in the blood (Naus et al. 2004). Interestingly, in a recent study by Kotani et al., a significant association between elevated CHL1's blood-secretion and tumour size in a lung cancer xenograft mouse model was described (Kotani et al. 2018). Hence, not only local levels of CHL1 but also systemic levels of CHL1 might serve as potential markers in NSCLCs and should be investigated in the future.

In conclusion, increased protein expression of CHL1 in primary NSCLCs is associated with prolonged survival. Hence, CHL1 might be a potential biomarker especially in combination with other clinical staging markers to improve patients' prognosis and help tailor the best individual treatment. Further studies are needed to investigate its potential function in the development and progression of NSCLCs in greater detail. However, considering CHL1 as a potential candidate for targeted therapies, the physiologic expression of CHL1 in normal and developing tissues must be taken into account.

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Compliance with ethical standards

Conflict of interest Jenny Hötzel declares that she has no conflict of interest. Nathaniel Melling declares that he has no conflict of interest. Julia Müller declares that she has no conflict of interest. Adam Polonski declares that he has no conflict of interest. Gerrit Wolters-Eisfeld declares that he has no conflict of interest. Jakob R. Izbicki declares that he has no conflict of interest. Karl-F. Karstens declares that he has no conflict of interest. Michael Tachezy declares that he has no conflict of interest.

Ethical approval Retrieval of tissue and clinical data was performed according to the regulations of the local medical association's ethics committee and with the 1964 Helsinki Declaration and its later amendments or comparable ethical standards as well as data safety laws.

Informed consent Informed consent was obtained from all patients enrolled in the study.

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