Lung Cancer: Targets and Therapy

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ORIGINAL RESEARCH

Reduced Claudin-3 Expression Is Linked to Unfavorable Tumor Features and Poor Prognosis in Non-Small Cell Lung Cancer

Nina Schraps[1], Martin Reck[2], Birgit Hantzsch-Kuhn[2], Till Olchers[2], David Benjamin Ellebrecht[3], Katharina Möller[4], Christoph Fraune[4,5], Maximilian Lennartz[4], Florian Lutz[4], Martina Kluth[4], Georgia Makrypidi-Fraune[4], Ronald Simon[4], Guido Sauter[4], Stefan Steurer[4], Sönke Von Weihe[3]

[1]General, Visceral and Thoracic Surgery Department and Clinic, University Medical Center Hamburg-Eppendorf, Hamburg, Germany; [2]Department of Thoracic Oncology, Airway Research Center North, German Center for Lung Research, LungenClinic, Grosshansdorf, Germany; [3]Department of Thoracic Surgery, Airway Research Center North, German Center for Lung Research, LungenClinic, Grosshansdorf, Germany; [4]Institute of Pathology, University Medical Center Hamburg-Eppendorf, Hamburg, Germany; [5]Institute of Pathology, Clinical Center Osnabrueck, Osnabrueck, Germany

Correspondence: Ronald Simon, Institute of Pathology, University Medical Center Hamburg-Eppendorf, Martinistr. 52, Hamburg, 20246, Germany, Tel +4940741057214, FAX +4940741055997, Email R.Simon@uke.de

Background: Claudin 3 (CLDN3) is a transmembrane protein which forms tight junctions (TJs) together with other claudins, occludin, and junctional adhesion molecules. Because of its membranous localization CLDN3 is a potential therapeutic target. Altered CLDN3 has been proposed as a prognostic feature in lung cancer and other tumors.

Material and Methods: To better understand the prevalence of expression and its impact on the prognosis of different lung cancer subtypes, we investigated CLDN3 expression by immunohistochemistry in a set of tissue microarrays containing 858 resected lung cancers.

Results: CLDN3 positivity was markedly more frequent and more intense in pulmonary adenocarcinoma (AC, 95.9% positive) than in squamous cell carcinoma (SCC, 53.3%; p<0.0001). Among 444 ACs, CLDN3 staining was strong in 64.2%, moderate in 25.0%, weak in 6.8% and negative in 4.1%. In 214 SCCs, CLDN3 staining was strong in 6.1%, moderate in 11.7%, weak in 35.5% and negative in 46.7%. Reduced CLDN3 staining was significantly linked to advanced pT stage in both AC and SCC (p<0.0001 each). The fraction of strongly positive cases decreased markedly from pT1 (AC: 67.6%; SCC: 11.1%) to pT4 (47.2%/1.8%). Low CLDN3 expression was also linked to nodal metastasis (p=0.0339) and R+ status (p = 0.0068) in SCCs. Absent or reduced CLDN3 staining was significantly associated with shortened overall survival in pulmonary ACs (p=0.0235) and SCCs (p=0.0330).

Conclusion: It is concluded that CLDN3 expression is common in lung cancer, that its level of expression is higher in AC than in SCC, and that a reduced expression level is associated with unfavorable outcome in both SCC and AC. Once anti-CLDN3 targeted drugs should prove to be safe and efficient, NSCLC may represent a major application for such treatments.

Keywords: CLDN3, NSCLC, TMA, IHC

Introduction

Despite a recent progress in the diversity of treatment options, lung cancer remains the leading cause of cancer-related deaths worldwide, with an estimated annual number of around 125,000 cancer deaths in the US alone.[1] Small cell (SCLC) and non-small cell lung carcinomas (NSCLC) are considered the two major clinical entities, with adenocarcinomas (AC) and squamous cell carcinomas (SCC) being the most common histological NSCLC subtypes.[2] Considering the poor prognosis of these tumors – only about 23% of patients survive for more than 5 years[3] – new systemic treatment options are urgently needed. Immunotherapies to improve the body's own cancer defenses and targeted therapies of oncogenic-driven NSCLC have led to substantial changes in the treatment of NSCLC.[4,5] Prognostic and predictive biomarkers are useful to identify patients who might benefit most from specific treatment options.[6]

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Claudin 3 (CLDN3) is of interest both as a therapeutic target and a prognostic molecular parameter in lung cancer. Claudin 3 is one of 27 members of the claudin family.[7] Claudins are transmembrane proteins which, together with occludin and junctional adhesion molecules form the tight junctions (TJs) which are part of the apical membranes in epithelial and endothelial sheets.[8,9] TJs regulate intercellular permeability and maintain tissue homeostasis.[9] Most claudins are either required to form the paracellular barrier or have a channel function within the TJs.[7] Their expression pattern is highly tissue type specific.[10] CLDN3 is a barrier protein that is found in most epithelial tissues.[11,12] As one of the most abundant claudins in the alveolar epithelium,[13] CLDN3 plays a role in the acinar development and alveolar epithelial cell differentiation.[14] Aberrant claudin expression has been found in several cancer types and both elevated[15–17] or reduced[18–20] expression can be associated with poor patient prognosis. Only few studies have investigated CLDN3 expression in pulmonary cancers.[20–23] These described varying expression levels in the different subtypes of lung cancer[21] and proposed a link between low expression and poor prognosis in a cohort of 103 SCC.[20]

Given these promising data, our study intended to collect further information on the prevalence and potential prognostic role of CLDN3 expression in different NSCLC subtypes. We therefore used immunohistochemistry (IHC) to examine the relationship between CLDN3 expression and clinicopathological parameters of tumor aggressiveness as well as patient outcome in more than 850 lung cancers in a tissue microarray (TMA) format.

Materials and Methods

Tissue Microarrays (TMAs)

Our TMA set was composed of one sample each from 858 lung cancers from patients that were operated at LungenClinic Grosshansdorf. All cancers had been diagnosed and classified at the Institute of Pathology, University Medical Center Hamburg, Germany. The distribution of histopathological parameters such as tumor stage (pT), histologic grade, lymph node status (pN) and resection margin (R) are shown in Table 1. Clinical follow up data (overall survival; OS) were available from 431 patients with AC (range: 1 to 45 months; median: 13 months) and 207

Table 1 Patient Cohort

	Study Cohort (n=858)		
Histological subtype	Adenocarcinoma	470 (54.8%)	
	Squamous cell carcinoma	235 (27.4%)	
	Mesothelioma	50 (5.8%)	
	Carcinoid	52 (6.1%)	
	Large cell neuroendocrine carcinoma	16 (1.9%)	
	Large cell carcinoma	7 (0.8%)	
	Carcinosarcoma	2 (0.2%)	
	Pleomorphic carcinoma	14 (1.6%)	
	Adenosquamous carcinoma	8 (0.9%)	
	Low-grade mucoepidermoid carcinoma	1 (0.1%)	
	Lymphoepithelial carcinoma	1 (0.1%)	
	SMARCA4-deficient undifferentiated tumor	2 (0.2%)	
Adenocarcinoma	Follow up	Months (mean)	15.2
		Censored (alive)	383 (88.9%)
		Failed (dead)	48 (11.1%)
	Gender	Female	258 (55.8%)
		Male	204 (44.2%)
	Stage	pT1	157 (36.0%)
		pT2	165 (37.8%)

(*Continued*)

Table 1 (Continued).

	Study Cohort (n=858)		
		pT3	58 (13.3%)
		pT4	56 (12.8%)
	Nodal stage	pN0	280 (68.6%)
		pN+	128 (31.4%)
	Grade	1-2	69 (37.9%)
		3	113 (62.1%)
	Resection margin	R0	420 (93.7%)
		R+	28 (62.2%)
Squamous cell carcinoma	Follow up	Months (mean)	14.9
		Censored (alive)	172 (83.1%)
		Failed (dead)	35 (16.9%)
	Gender	Female	70 (30.3%)
		Male	161 (69.7%)
	Stage	pT1	58 (25.5%)
		pT2	66 (29.1%)
		pT3	43 (18.9%)
		pT4	60 (26.4%)
	Nodal stage	pN0	134 (59.8%)
		pN+	90 (40.2%)
	Grade	1-2	35 (38.0%)
		3	57 (61.9%)
	Resection margin	R0	204 (90.7%)
		R+	21 (9.3%)

Notes: Percent in the column "study cohort on TMA" refers to the fraction of samples across each category. Because of cases with missing data, the numbers in the different categories do not always add up to the total number of cases.

patients with SCC (range: 1 to 63 months; median: 13 months). Tissues were fixed in 4% buffered formalin and then embedded in paraffin. The TMA manufacturing process was described earlier in detail.[24,25] In brief, one tissue spot (diameter: 0.6 mm) per patient was taken from a cancer containing tumor block by using a semi-automated homemade tissue arrayer. The use of archived remnants of diagnostic tissues for TMA manufacturing, their analysis for research purposes, and the use of patient data were according to local laws (HmbKHG, §12) and analysis had been approved by the local ethics committee (Ethics commission Hamburg, WF-049/09). All work has been carried out in compliance with the Helsinki Declaration.

Immunohistochemistry (IHC)

Freshly cut TMA sections were immunostained on one day and in one experiment. Slides were deparaffinized with xylol, rehydrated through a graded alcohol series and exposed to heat-induced antigen retrieval for 5 min in an autoclave at 121°C in pH 7.8 Tris-EDTA-Citrat (TEC) puffer. Endogenous peroxidase activity was blocked with Dako REAL Peroxidase-Blocking Solution (Agilent Technologies, Santa Clara, CA, USA; #S2023) for 10 min. Primary antibody specific against CLDN3 protein (rabbit recombinant monoclonal, HMV-309, ardoci GmbH, Hamburg, Germany) was applied at 37°C for 60 min at a dilution of 1:150. Bound antibody was then visualized using the Dako REAL EnVision Detection System Peroxidase/DAB+, Rabbit/Mouse kit (Agilent Technologies, Santa Clara, CA, USA; #K5007)

according to the manufacturer's directions. The sections were counterstained with hemalaun. For each tumor, the percentage of positive neoplastic cells was estimated, and the staining intensity was semi-quantitatively recorded (0, 1 +, 2+, 3+). For statistical analyses, the staining results were categorized into four groups. Tumors without any staining were considered negative. Tumors with 1+ staining intensity in ≤70% of tumor cells and 2+ intensity in ≤30% of tumor cells were considered weakly positive. Tumors with 1+ staining intensity in >70% of tumor cells, 2+ intensity in 31–70%, or 3+ intensity in ≤30% of tumor cells were considered moderately positive. Tumors with 2+ intensity in >70% or 3+ intensity in >30% of tumor cells were considered strongly positive.

Statistics

Statistical calculations were performed with JMP17® software (SAS®, Cary, NC, USA). Contingency tables and the chi^2-test were performed to search for associations between CLDN3 immunostaining and tumor phenotype as well as PD-L1 immunostaining. In addition a bonferoni corrections was performed. Survival curves were calculated according to Kaplan-Meier. The Log Rank test was applied to detect significant differences between groups. Cox proportional hazards analysis as well as hazard ration analysis were performed to test the potential of CLDN3 immunostaining as an independent prognostic factor.

Results

CLDN3 Immunohistochemistry

CLDN3 immunostaining was strong in normal respiratory epithelium and moderate to strong in alveolar cells of the lung. CLDN3 staining varied both in intensity and in its pattern between tumor samples. Most CLDN3 positive tumors showed a purely membranous staining pattern but some tumors showed an additional cytoplasmic positivity. A CLDN3 staining was seen in 630 (79.4%) of the 793 interpretable cancers. Of these cancers, 368 (58.4%) showed purely membranous staining and 262 (41.6%) showed membranous staining with an additional weaker cytoplasmic staining. Overall, 115 (14.5%) tumors showed a weak, 154 (19.4%) a moderate, and 361 (45.5%) a strong positivity (Table 2). Representative images are shown in Figure 1. CLDN3 positivity was markedly more frequent and more intense in AC (95.9% positive)

Table 2 CLDN3 Immunostaining in Lung Cancer

		CLDN3 Immunostaining Results					
	on TMA (n)	Analyzable (n)	Negative (%)	Weak (%)	Moderate (%)	Strong (%)	P
Adenocarcinoma	470	444	4.1	6.8	25	64.2	<0.0001
Squamous cell carcinoma	235	214	46.7	35.5	11.7	6.1	
Mesothelioma	50	34	94.1	2.9	0	2.9	
Carcinoid	52	52	0	0	25	75	
Large cell neuroendocrine carcinoma	16	16	6.3	0	6.3	87.5	
Large cell carcinoma	7	7	42.9	0	14.3	42.9	
Carcinosarcoma	2	2	50	0	0	50	
Pleomorphic carcinoma	14	13	38.5	38.5	15.4	7.7	
Adenosquamous carcinoma	8	7	42.9	28.6	0	28.6	
Low-grade mucoepidermoid carcinoma	1	1	0	0	100	0	
Lymphoepithelial carcinoma	1	1	0	0	0	100	
SMARCA4-deficient undifferentiated tumor	2	2	50	50	0	0	

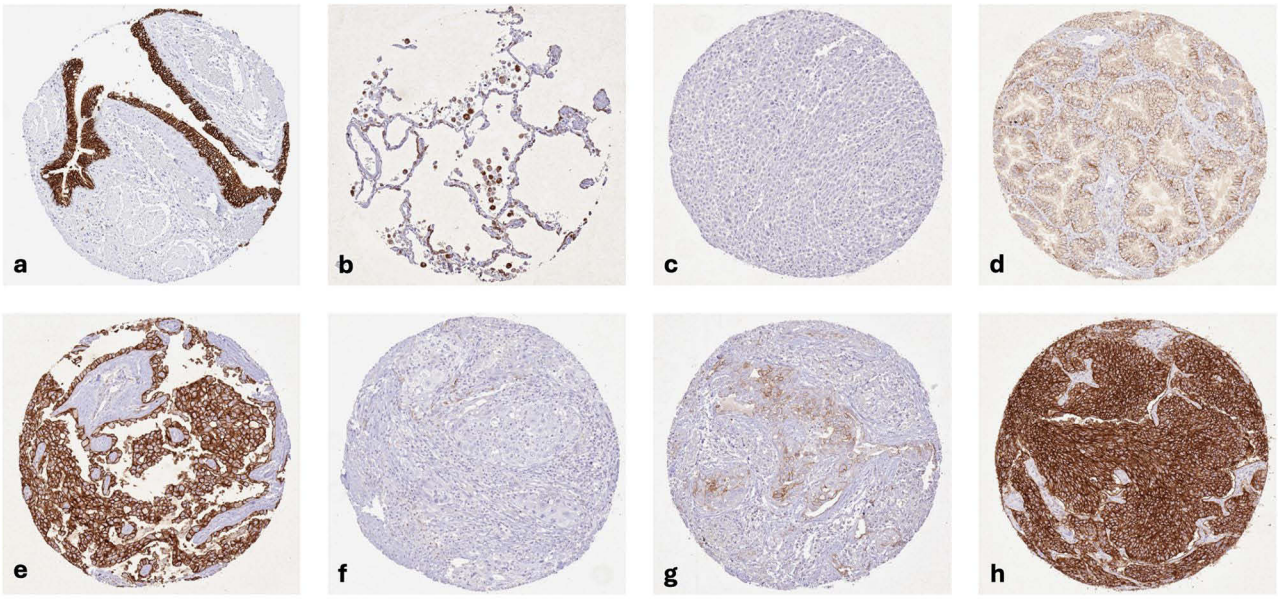

Figure 1 CLDN3 immunostaining in normal and neoplastic lung tissue. The panels show a strong CLDN3 staining in the respiratory epithelium of the bronchus (**a**) and a weak to moderate staining in alveolar cells of lung (**b**), representative images of a lung adenocarcinoma with negative (**c**), weak to moderate (**d**) and strong staining (**e**), and lung squamous cell carcinomas with negative (**f**), weak to moderate (**g**) and strong staining (**h**).

than in SCC (53.3%; p<0.0001). The fraction of positive cases was also lower in large cell carcinomas (57.1%), and in malignant mesotheliomas (5.9%) than in AC. Associations between CLDN3 staining and tumor phenotype are given in Table 3 for SCC and AC. Reduced CLDN3 positivity was significantly linked to advanced pT stage in both AC and SCC (p<0.0001 each). The fraction of strongly positive cases decreased markedly from pT1 (67.6% of AC, 11.1% of SCC) to pT4 (47.2%/1.8%). Low CLDN3 expression was also linked to nodal metastasis (p=0.0339) in SCCs. Reduced CLDN3

Table 3 CLDN3 and Cancer Phenotype

		on TMA (n)	CLDN3 Immunostaining Results						Bonferoni correction
			Analyzable (n)	Negative (%)	Weak (%)	Moderate (%)	Strong (%)	p	
Adenocarcinomas	pT1	157	145	0.7	3.4	28.3	67.6	<0.0001	<0.0001
	pT2	165	160	1.3	6.3	18.1	74.4		
	pT3	58	54	9.3	13	29.6	48.1		
	pT4	56	53	15.1	9.4	28.3	47.2		
	pN0	280	263	4.2	6.5	21.7	67.7	0.1788	0.7152
	pN+	128	122	4.1	8.2	31.1	56.6		
	grade 1-2	69	65	0	1.5	18.5	80	0.4757	1.9028
	grade 3	113	110	0.9	3.6	23.6	71.8		
	R0	420	396	3.3	6.6	24.5	65.7	0.1842	0.7368
	R+	28	27	11.1	7.4	33.3	48.1		

(*Continued*)

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Table 3 (Continued).

		on TMA (n)	CLDN3 Immunostaining Results						Bonferoni correction
			Analyzable (n)	Negative (%)	Weak (%)	Moderate (%)	Strong (%)	p	
Squamous cell carcinomas	pT1	58	54	40.7	33.3	14.8	11.1	<0.0001	<0.0001
	pT2	66	63	33.3	36.5	25.4	4.8		
	pT3	43	34	41.2	50	2.9	5.9		
	pT4	60	55	67.3	30.9	0	1.8		
	pN0	134	118	40.7	38.1	16.9	4.2	0.0339	0.1356
	pN+	90	85	52.9	32.9	5.9	8.2		
	grade 1-2	35	32	34.4	46.9	15.6	3.1	0.3583	1.4332
	grade 3	57	52	46.2	28.8	17.3	7.7		
	R0	204	185	41.6	39.5	12.4	6.5	0.0068	0.0272
	R+	21	19	78.9	10.5	10.5	0		

Abbreviations: pT, pathological tumor stage; G, Grade; pN, pathological lymph node status; R, resection margin status.

staining was significantly associated with shortened overall survival in both ACs (p=0.0235) and in SCCs (p=0.0330; Figure 2a–c) in a univariate analysis independent from pT stage groups (Supplementary figure 1). The differences in patient outcome became even more evident if negative cancers were compared to tumors with any (weak, moderate, strong) positivity (Figure 2d–f). No differences were found in survival and tumor phenotype between cases with purely membranous and additional cytoplasmic staining (data not shown). In the multivariate analysis – including pT state, lymph node status, grade, and resection margin status, CLDN3 immunostaining was not independently related to overall survival (Table 4 and Supplementary table 1). CLDN3 immunostaining were unrelated to PD-L1 immunostaining in both ADCs and SCCs (Supplementary Figure 2).

Discussion

The successful analysis of more than 850 pulmonary tumors revealed that CLDN3 expression is common in lung cancer and that the rate of positivity and strong positivity was markedly higher in AC than in SCC. The higher prevalence of CLDN3 expression in AC (our result: 95.9%) than in SCC (53.3%) is in line with earlier studies describing CLDN3 positivity in 41.4–97.0% of AC[22,23] but in only 50.0–65.0% of SCC.[20,22] Discrepancies in the rate of positive cases between our results and some data from earlier studies are likely due to differences in antibodies used, IHC protocols, thresholds for defining positivity, or the composition of patient cohorts. For example, Jung et al[22] only distinguished between a low and a strong expression group, whereby tumors with 0–25% of positive tumor cells were considered low level expression. It is of note that our CLDN3 IHC assay has earlier been extensively validated for sensitivity and specificity by a comparison with RNA expression and with immunostaining results obtained by an independent second antibody on more than 600 samples from 76 different normal tissue types.[26] Such an extensive validation of IHC assays has been proposed by the International Working Group for Antibody Validation (IWGAV).[27] A comparative analysis of a very broad range of normal tissues exposes antibodies to most proteins (and their posttranslational modifications) expressed in adult human tissues and enables the identification of most antibody cross-reactivities.

The significant association between reduced CLDN3 expression and aggressive tumor phenotype and unfavorable prognosis in both AC and SCC represents the key result of our study although we were unable to correlate these findings treatment information. For SCC, our findings are in line with earlier data by Che et al[20] describing significant

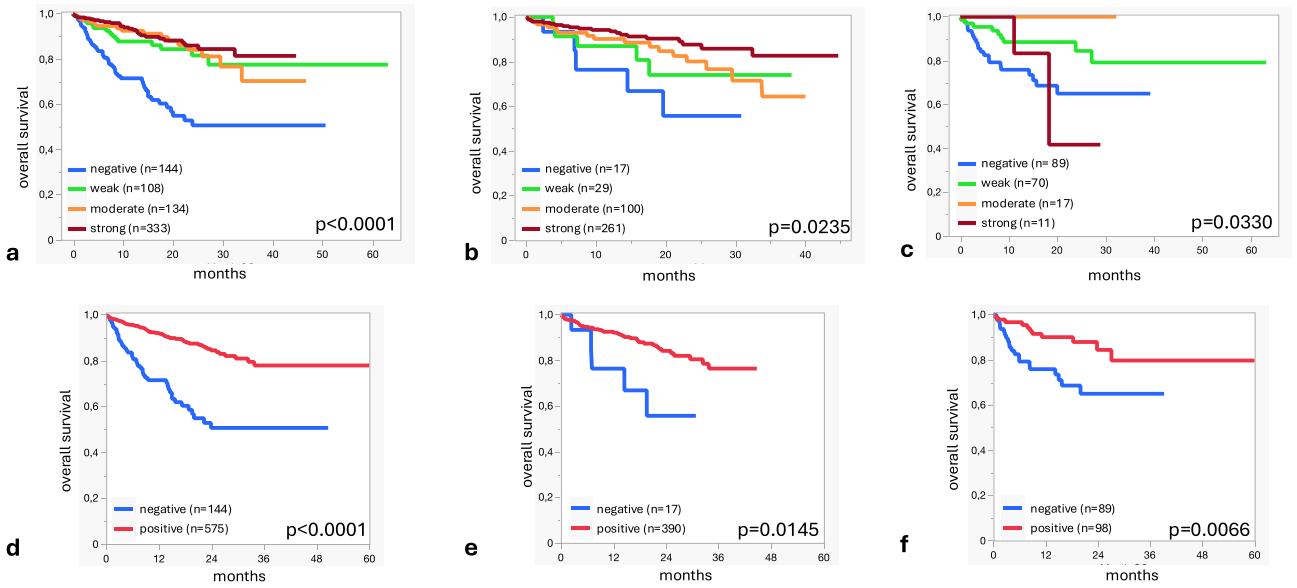

Figure 2 CLDN3 expression and patient survival. The panels show the impact of CLDN3 staining levels on overall survival in all lung cancers (**a**), AC (**b**), and SCC (**c**). The panels (**d**–**f**) describe the same patient cohorts after combining tumors with weak, moderate, and strong positivity in one category (positive) for all cancers (**d**), AC (**e**), and SCC (**f**).

associations between low CLDN3 expression and poor patient outcome as well as advanced stage, nodal metastasis and disease recurrence in a series of 103 SCCs. However, Jung et al[22] could not confirm a significant association with patient prognosis and unfavorable tumor features in a cohort of 171 pulmonary SCC. A previous study investigating CLDN3 expression in pulmonary AC found opposite results compared to our data. Zhang et al[28] found a significant link between high CLDN3 expression levels and distant metastasis, as well as reduced overall survival in 261 patients with pulmonary AC. In this study, the antibody, controls, and staining conditions for IHC were not disclosed but functional data also supported a tumor promoting effect of CLDN3.[28] In contrast, Jung et al[22] could not find significant associations between either high or low CLDN3 expression and aggressive tumor features in AC.

Previous studies evaluating the prognostic role of CLDN3 expression in other tumor entities have described associations between reduced CLDN3 expression and aggressive tumor features or poor prognosis in gastric,[29] prostatic,[18] and hepatic cancer[19] while CLDN3 upregulation correlated with features of tumor progression in studies on urothelial carcinomas,[30] breast,[17] and ovarian cancer.[31] A tumor type dependent relationship between CLDN3 expression levels and cancer aggressiveness is also supported by a study from our laboratory analyzing 14,966 tumors from 133 different tumor entities. Under fully standardized conditions for IHC staining and interpretation these data linked low CLDN3 to aggressive phenotype in clear cell and papillary renal cell carcinoma while high CLDN3 was associated with aggressive disease in urothelial carcinomas.[26]

Table 4 Cox Proportional Analysis

	Analyzable (n)	p-value				
		pT stage	lymph node status	grade	resection Margin status	CLDN3 immunostaining
All subtypes	258	0.2597	0.0288	0.3184	0.5368	0.4491
Adenocarcinoma of the lung	143	0.0325	0.1624	0.6778	0.5765	0.4648
Squamous cell carcinoma of the lung	64	0.7309	0.2017	0.9802	0.7645	0.3148

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Cancer driving mechanisms associated with a reduced expression of TJ proteins such as CLDN3 include impaired epithelial barrier function[32] and reduced cohesion,[5] an important step for tumor cell invasion and metastasis, as well as cellular dedifferentiation.[32,33] Data by Che et al[20] suggested that reduced CLDN3 expression may contribute to pulmonary SCC development and progression through epithelial-mesenchymal transition (EMT) signaling. They also found that CLDN3 knockdown promoted Wnt/ß-catenin signaling, thereby enhancing EMT and suppressing the expression of the cell adhesion molecule E-cadherin.[34] In ADC cell lines, downregulation of CLDN3 prevented the EGF-induced increase of cell proliferation, whereas overexpression of CLDN3 activated the EGF-activated MEK/ERK and PI3K-Akt pathways.[28] The loss of CLDN3 and the associated downregulation of E-cadherin and activation of the ß-catenin signaling pathway have also been demonstrated in ovarian carcinomas.[35] In ovarian cancer CLDN3 knockdown was associated with impaired TJ function, reduced cell adhesion and thus increased migration and invasion.[35] In hepatocellular carcinoma mouse models, downregulation of CLDN3 significantly suppressed metastasis by inactivation the Wnt/ß-Catenin/EMT axis.[19] Ahmad et al[32] showed that the loss of CLDN3 in colonic epithelium promoted colorectal cancer by inducing Wnt/β-catenin signaling and Stat-3-activation.

Much of the topical interest in CLDN3 is derived from its potential role as a therapeutic target due to its membranous localization. Even though CLDN3 is expressed in a broad range of vital normal tissues,[9] the accessibility of CLDN3 and of other TJ proteins appears to be limited for anti-CLDN3 antibodies due to the orchestrated cell growth under physiological conditions and a blockage of several claudins by other components of TJs.[36–39] Due to extrajunctional mislocalization, claudins appear to become more accessible during malignant transformation.[40] Claudins have two extracellular domains which can serve as potential target sites.[33] The ECL2 motive of CLDN3 is known to serve as a receptor for the Clostridium perfringens enterotoxin (CPE).[41,42] Therefore CLDN3 has been tested as a potential therapeutic target in cancer through CPE mediated cytolysis and as a carrier to specifically deliver therapeutic drugs.[43] Several studies have shown anti-tumor effects of CPE in prostate,[42] breast,[44] and ovarian cancer cells.[45] The ECL1 and 2 domains of CLDN3 are also targeted by the human monoclonal antibodies KM3907,[46] IgGH6,[47] and h4G3,[36] developed for treating cancer.[36,46,48] In non-squamous NSCLC, CLDN3 targeted therapies using small molecules showed promising results as they suppressed cancer stemness and decreased chemoresistance.[49]

Conclusions

In summary, our results show that CLDN3 expression is common in lung cancer, that the level of expression is higher in AC than in SCC, and that a reduced expression level is associated with an unfavorable prognosis in both SCC and AC. Once anti-CLDN3 targeted drugs are proven to be safe and efficient NSCLC may represent a major application for such treatments.

Abbreviations

AD, Adenocarcinoma; CLDN3, Claudin 3; CPE, Clostridium perfringens enterotoxin; EMT, Epithelial-mesenchymal transition; G, Grade; IHC, Immunohistochemistry; NSCLC, Non-small cell lung cancer; pT, Pathological tumor stage; pN, Pathological lymph node status; R, Resection margin status; SCC, Squamous cell carcinoma; SCLC, Small cell lung cancer; TJ, Tight Junction; TMA, Tissue microarray.

Data Sharing Statement

All data generated or analyzed during this study are included in this published article.

Ethics Approval

The use of archived remnants of diagnostic tissues for manufacturing of TMAs and their analysis for research purposes as well as patient data analysis has been approved by local laws (HmbKHG, §12) and by the local ethics committee (Ethics commission Hamburg, WF-049/09). All work has been carried out in compliance with the Helsinki Declaration.

Patient Consent

Patient consent was waived due to local laws (HmbKHG, §12,1) that permit research with anonymized diagnostic left-over tissue samples.

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Author Contributions

All authors made a significant contribution to the work reported, whether that is in the conception, study design, execution, acquisition of data, analysis and interpretation, or in all these areas; took part in drafting, revising or critically reviewing the article; gave final approval of the version to be published; have agreed on the journal to which the article has been submitted; and agree to be accountable for all aspects of the work.

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Disclosure

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