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ORIGINAL ARTICLE

Claudin-1 Protein Expression Is a Good Prognostic Factor in Non-Small Cell Lung Cancer, but only in Squamous Cell Carcinoma Cases

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Abstract The aim of the study was to investigate the correlation between claudin (CLDN) protein expression and clinicopathological parameters as well as survival in histological subtypes of non-small cell lung cancer. Archived surgical resection specimens of 137 pathologic stage I primary bronchial cancers including 49 adenocarcinomas of non-lepidic variants (ADC), 46 adenocarcinomas of lepidic variants (L-ADC), and 42 squamous cell carcinomas (SCC) were examined. Immunohistochemistry (IHC) using antibodies against CLDN1,-2,-3,-4,-7 proteins as well as semiquantitative estimation (IHC scores 0–5) were performed. Claudin IHC scores of L-ADC differed significantly from ADC (CLDN1: $p = 0.009$, CLDN2: $p = 0.005$, CLDN3: $p = 0.004$, CLDN4: $p = 0.001$, CLDN7: $p < 0.001$, respectively) and SCC (CLDN1: $p < 0.001$, CLDN3: $p < 0.001$, CLDN7: $p < 0.001$, respectively). Highly significant CLDN3-CLDN4 parallel expression could be demonstrated in ADC and L-ADC ($p < 0.001$ in both), which was not observed in SCC ($p = 0.131$). ADC and SCC showed no correlation with smoking, whereas in case of L-ADC heavier smoking correlated with higher CLDN3 expression ($p = 0.020$). Regarding claudin expression and survival, in SCC significant correlation could be demonstrated between CLDN1 IHC positivity and better survival ($p = 0.038$). In NSCLC as a whole, high CLDN2 expression proved to be a better prognostic factor when compared with cases where CLDN2 IHC score was 0–1 vs. 2–5 ($p = 0.009$), however, when analyzed separately, none of the histological subgroups showed correlation between CLDN2 expression and overall survival. The claudin expression pattern was significantly different not only between the SCC–ADC and SCC–L-ADC but also between the L-ADC and ADC histological subgroups, which strongly underlines that L-ADC represents a distinct entity within the ADC group. CLDN1 overexpression is a good prognostic factor in NSCLC, but only in the SCC subgroup.

Keywords Lung cancer · Claudin · Immunohistochemistry · Survival

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Abbreviations

NSCLC	Non-small cell lung cancer
ADC	Adenocarcinoma
L-ADC	Lepidic form of adenocarcinoma
SCC	Squamous cell carcinoma
IHC	Immunohistochemistry
CLDN1 – CLDN7	Claudin-1 – claudin-7

Introduction

Non-small cell lung cancer (NSCLC) accounts for about 80 % of lung cancers and consists of mainly adenocarcinomas (ADC), squamous cell carcinomas (SCC) and large cell carcinomas. Beside the presently used TNM classification, many efforts are made to reclassify or subclassify lung cancer by determining histological categories with prognostic differences that may be helpful in identifying candidates for adjunctive therapy [1, 2].

Claudins (CLDNs) are 22–27 kDa sized adhesion molecules that constitute tight junctions. They serve as a barrier regulating the passage of ions, water and different macromolecules to maintain homeostasis [3]. Certain claudins have prognostic significance in several human cancers. Although the prognostic relevance of the expression of different claudins has been demonstrated in many types of cancer, it is still under intensive investigation in case of lung cancer, yielding rather conflicting results so far. Chao et al. found that immunohistochemically confirmed low mRNA and protein expression levels of CLDN1 in case of lung ADC revealed shorter overall survival [4]. In the study of Merikallio et al. CLDN1 positivity predicted better survival in ADC and SCC [5]. Similarly, overexpression of CLDN1 indicated favorable prognosis in some patients with lung ADC [6]. On the contrary, in another study, Zhang et al. demonstrated that in stage N2 non-small cell lung cancer expression of CLDN1 was associated with poor prognosis [7]. These differing results may be explained by the differences in patient populations regarding histological subtype, tumor stage and smoking history. CLDN4 positivity predicted better survival in lung ADC, but not in SCC [5]. In SCC, CLDN7 positivity was associated with better survival [5, 8].

The aim of this study was to evaluate the correlation between expression of CLDN1,-2,-3,-4,-7 proteins and the prognosis in homogeneous groups of lung adenocarcinoma and squamous cell carcinoma patients operated on for pathologic stage I disease. Considering that adenocarcinoma can be divided into further histological subclasses, we compared adenocarcinomas without lepidic growth pattern (ADC) with those characterized by pure or predominant lepidic growth (L-ADC). The possible correlations between claudin expression and gender as well as claudin expression and smoking history were also analyzed.

Patients and Methods

Patients

A total of 137 pathologic stage I primary bronchial cancers including 49 non-lepidic predominant adenocarcinomas (ADC), 46 lepidic predominant adenocarcinomas (L-ADC), which constituted minimally invasive adenocarcinomas of pathologic stage IA (40 cases) and adenocarcinomas of lepidic predominant forms of stage IB (6 cases), as well as 42 squamous cell carcinomas (SCC) were studied. Within the L-ADC subgroup there were 22 mucinous and 24 non-mucinous cases. All tumors were surgical resection specimens obtained between 1991 and 2000 and all were formalin-fixed and paraffin-embedded tissue samples. Tumors were reclassified according to the IASLC/ATS/ERS classification (International Association for the Study of Lung Cancer/American Thoracic Society/European Respiratory Society) [9]. Mixed or anaplastic tumors were excluded from the study. None of the patients received neoadjuvant and/or adjuvant chemo- or radiotherapy. Smoking history was available in 85 cases. Patient survival was measured in months from the day of surgery until death or the final visit. Follow up period varied from 6 months to 192 months. The clinical and histopathological data for all cases are summarized in Table 1.

Permission to use the archived tissue blocks was obtained from the Regional Ethical Committee (N^{o} 510/2013). All slides contained normal lung tissue as well, which served as a endogenous positive control.

Claudin Immunohistochemistry and Scoring

Tissue sections of 4 μm thickness were deparaffinized in xylene and rehydrated. Immunohistochemical examination was performed using standard avidin-biotin-peroxidase complex method. Endogenous peroxidase was inhibited in 1 % H_2O_2-methanol for 30 min at 37 °C. Antigen retrieval for CLDN1 and −3 was achieved by using two different solutions: 1 % Vector [H3300] antigen retrieval solution (Vector Laboratories, Burlingame, CA) and microwave heating [800 W] for 20 min, as well as Dako (Dako, Glostrup, Denmark) antigen retrieval solution and microwave heating [800 W] for 40 min. For CLDN2,4 and −7 antigen retrieval was performed with Vector antigen retrieval solution and microwave heating [800 W] for 20 min. The sections were then incubated for 1 h at room temperature with the primary antibodies (Zymed, San Francisco, CA): CLDN1,-2,-3,-4 and −7 diluted 1:100. CLDN2 and −4 antibodies were monoclonal antibodies, while CLDN-1,3 and −7 were rabbit polyclonal antibodies. For each claudin a negative control with omission of the primary antibody was included. The antigen-antibody complexes were visualized using Streptavidin-HRP detection system (DAKO LSAB2, K0675), then 3,3′–diaminobenzidine

Table 1 Clinicopathological characteristics of patients

Clinicopathological characteristics	ADC	L-ADC	SCC
Number of cases studied	49	46	42
Mean age (years) (range)	56.96 (37–73)	58.98 (56–71)	56.67 (33–72)
Sex (male / female)	25/24	19/27	32/10
Stage IA/IB	23/26	40/6	1/41
Smoking history	non-smoker: 8	non-smoker: 14	non-smoker: 1
(available in 85 cases)	ex-smoker: 6	ex-smoker: 8	ex-smoker: 3
	current smoker: 27	current smoker: 10	current smoker: 8
Mean follow up time (months)	58	103	48

tetrahydrochloride (DAB) as chromogen. All sections were counterstained with Mayer's hemalaun, dehydrated, and coversliped. On all slides normal bronchial epithelial cells served as positive internal controls and stained appropriately. All samples were examined by two pathologists.

Tumors were scored from 0 to 5 according to the percentage of the tumor cells with positive membranous immunostaining, except for CLDN2, which gave a cytoplasmic reaction: 0: < 5 %, 1: 5–20 %, 2: 21–40 %, 3: 41–60 %, 4: 61–80 %, 5: 81–100 %.

Statistical Analysis

Statistical analysis was performed using GraphPad Prism 5 software (GraphPad Software Inc., San Diego, US) for Mann-Whitney U test and Spearman non-parametric correlation. The Mann-Whitney U test was used to compare immunohistological expression of individual claudins and other protein markers in the different groups. Correlation between claudin expression and the degree of smoking was analyzed with Spearman's correlation. Kaplan-Meier survival analysis was used to estimate the effect of different claudin expressions on survival. The value of $p < 0.05$ was considered statistically significant.

Results

The cellular localization of claudin immunostaining as well as claudin expression profiles in ADCs and SCCs was found to be similar to our previous results [10]. In general, all positive tumors stained diffusely and in fairly uniform manner (Fig. 1a-i). The intensity of immunostaining was usually similar to – or even stronger than – the adjacent normal bronchial epithelium (Fig. 1a). Heterogeneity of expression in different areas within the same tumor was observed only in very few cases. Considering that the intensity of claudin immunostaining in IHC positive tumors did not vary significantly, IHC intensity scores were not determined.

Results of Semiquantitative Evaluation of Claudin Scoring

When comparing claudin IHC scores in the examined three NSCLC subgroups, highly significant differences could be observed. In case of CLDN1, ADC differed from L-ADC ($p = 0.009$) and SCC also differed from L-ADC ($p < 0.001$). ADC differed from L-ADC ($p = 0.005$) in case of CLDN2 as well. In case of CLDN3, significant differences were observable regarding all subgroups: ADC – L-ADC ($p = 0.004$), L-ADC – SCC ($p < 0.001$) and ADC – SCC ($p < 0.001$). Regarding CLDN4, the ADC – L-ADC difference ($p = 0.001$), while in case of CLDN7 the ADC – L-ADC ($p < 0.001$) and L-ADC – SCC differences ($p < 0.001$) were found to be statistically significant. The claudin expression differences in the histologic subtypes of NSCLC are demonstrated in Fig. 2.

Regarding ADC, highly significant positive correlation could be observed between CLDN1 and CLDN3 as well as CLDN3 and CLDN4 expressions ($p < 0.001$).

Concerning L-ADC, significant positive correlation was observed between CLDN1 and CLDN4 expression ($p = 0.029$) as well as CLDN4 and CLDN7 expressions ($p = 0.004$). The correlation was even more pronounced in case of CLDN3 and CLDN4 expressions ($p < 0.001$).

As regards SCC, significant negative correlation was observed between CLDN2 and CLDN3 expressions ($p = 0.027$) as well as between CLDN2 and CLDN7 expressions ($p = 0.008$). In contrast to ADC and L-ADC, no correlation between CLDN3 and CLDN4 expressions was observable in case of SCC ($p = 0.131$).

Overall Survival

With regard to the NSCLC subgroups and overall survival, significant differences could be demonstrated between ADC – L-ADC ($p = 0.065$), L-ADC – SCC ($p = 0.001$) and ADC – SCC ($p = 0.013$). The longest survival was found in case of L-ADC followed by ADC and SCC. In L-ADC, the mucinous subtype was found to be a bad prognostic factor, as significant correlation between mucinous/non-mucinous status and overall survival could be demonstrated ($p = 0.035$).

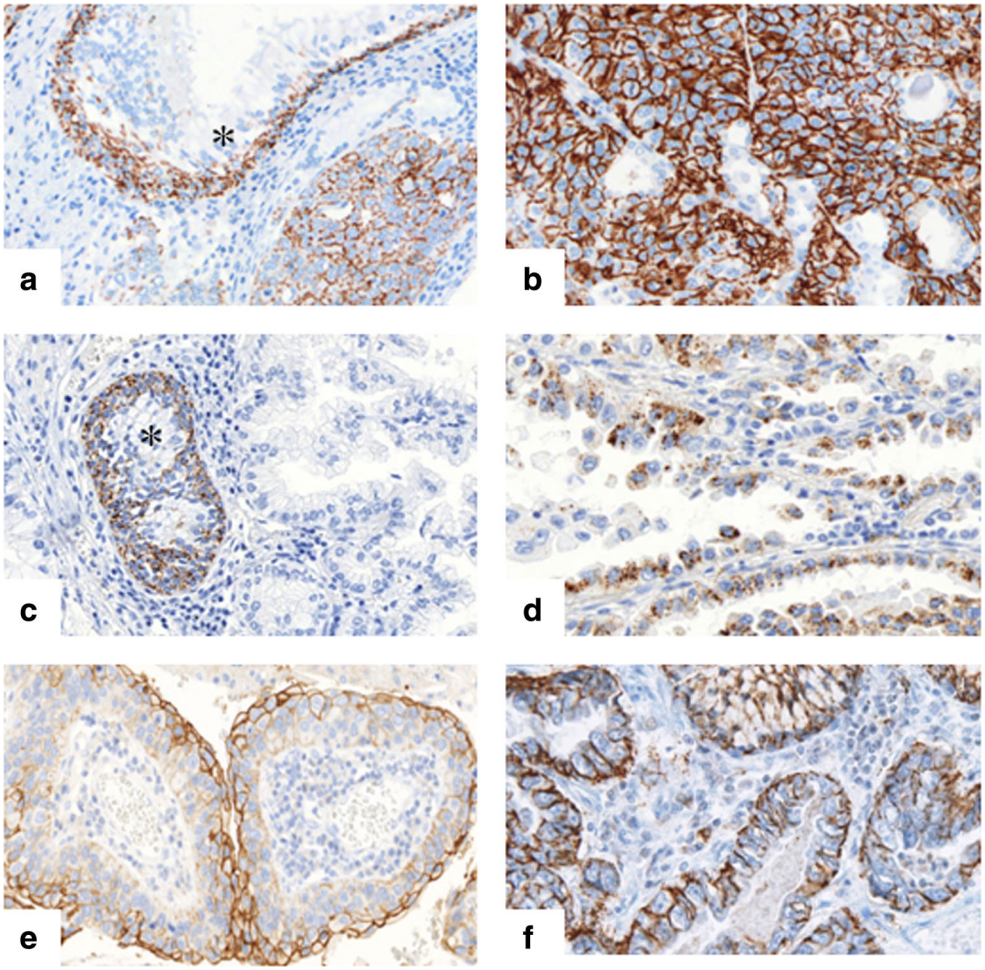

Fig. 1 Claudin expression in histologic subtypes of NSCLC. **a** SCC CLDN1 ×20, IHC score 4. * represents normal bronchus with intensive CLDN1 staining. **b** SCC CLDN7 ×20, IHC score 5. **c** L-ADC CLDN1 ×30, IHC score 0. * represents normal bronchus with intensive CLDN1 staining. **d** L-ADC CLDN2 ×30, IHC score 4. **e** ADC CLDN4 ×40, IHC score 4. Note the more intensive immunostaining in the apical cell layer. **f** ADC CLDN7 ×40, IHC score 5

Upon examining claudin expression and overall survival in NSCLC as a whole, high CLDN2 expression proved to be a better prognostic factor when compared with the CLDN2 IHC score of 0–1 vs. 2–5 ($p = 0.009$). When analyzed separately, however, none of the histologic subgroups showed correlation between CLDN2 expression and overall survival.

In case of ADC, no correlation was found between CLDN1,-2,-3,-4,-7 and overall survival.

Regarding L-ADC, a trend of high CLDN4 expression and better overall survival was observed when compared with cases showing CLDN4 IHC scores of 0–1 vs. 2–5 ($p = 0.087$).

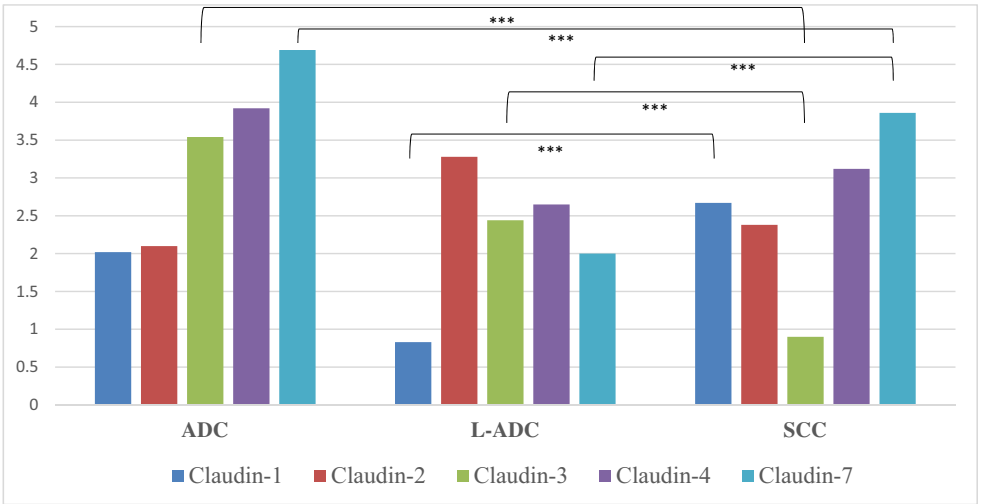

Fig. 2 Claudin expression differences in histologic subtypes of NSCLC (***: $p < 0.001$; only highly significant results were presented)

In case of SCC, significant correlation could be demonstrated between CLDN1 IHC positivity and better survival (p = 0.038) (Fig. 3). As regards CLDN3 expression, there was a trend between high CLDN3 expression and worse overall survival (p = 0.070) that became statistically significant when comparing cases with CLDN3 IHC scores of 0–1 vs. 2–5 (p = 0.029).

Claudin Expression – Clinicopathological Parameters

Comparing claudin expression and the degree of smoking in NSCLC cases, we found that lower CLDN2 IHC scores correlated with heavier smoking (r = 0.227, p = 0.036). On the contrary, higher CLDN3 IHC scores showed correlation with heavier smoking (r = 0.251, 0.026).

In case of ADC, there was no correlation between CLDN1,-2,-3,-4,-7 and gender, age or smoking status.

Regarding L-ADC, no correlation was observable between CLDN1,-2,-3,-4,-7 and gender, age, or mucinous/non-mucinous status. With relation to claudin expression and smoking, significant difference could be demonstrated between ever and never smokers (p = 0.020). The degree of smoking showed positive correlation with the expression of CLDN3. Similarly, near-significant correlation was found between higher CLDN4 expression and heavier smoking (p = 0.054).

In case of SCC, no correlation was found between CLDN1,-2,-3,-4,-7 and gender, age or smoking.

Discussion

The prognostic significance of claudin expression differs in the different types of cancer. In breast cancer for example, the loss of CLDN7 was found to correlate with histologic grade in both ductal carcinoma in situ and invasive ductal carcinoma [11]. In gastric cancer CLDN1 expression was independently associated with poor post-operative prognosis [12]. Szasz et al. demonstrated in breast cancer patients that decrease or loss of CLDN1 and expression of CLDN4 in lymph node metastases correlated with reduced disease-free survival [13]. On the contrary, in triple-negative breast carcinomas, CLDN4 positivity was recently found to be a probable biomarker of favorable prognosis [14]. In our study, decreased CLDN1 expression was found to be a bad prognostic factor in case of NSCLC but only in the SCC subgroup. When examining CLDN4 expression, significant differences could be observed in the ADC subgroups, which may represent certain similarities with breast cancer cases.

In case of kidney cancer, CLDN1 expression was found to be associated with markers of unfavorable tumor biology in clear cell renal cell carcinoma, whereas the opposite was valid for papillary renal cell carcinoma [15]. This is in line with our findings that different cancers in the same organs demonstrate different patterns in the prognostic value of claudin expression.

In stage II colonic cancer low expression levels of CLDN1 were associated with higher tumor grade as well as with poor survival [16]. In another study, the expressions of CLDN3 and −4 were significantly lower in cases with positive lymphatic invasion, and CLDN4 expression was related to good overall survival rate [17]. Interestingly, in our work low CLDN1 expression was observed in L-ADC, which had the longest overall survival among the three NSCLC subtypes, nonetheless CLDN1 expression was not found to be a prognostic factor within the L-ADC subgroup. Similarly, lower CLDN3 and −4 expression levels were found in the L-ADC subgroup when compared with ADC without prognostic significance.

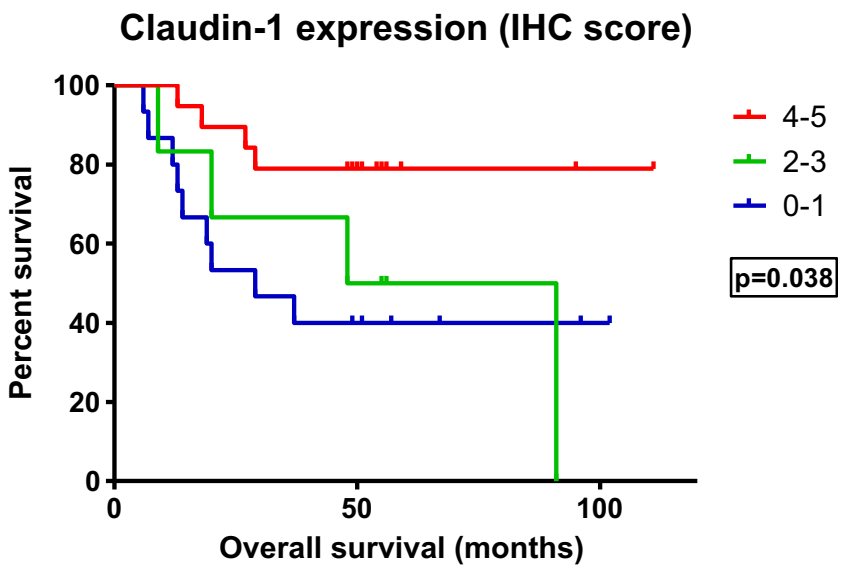

Fig. 3 High CLDN1 score correlates with better overall survival in lung SCC

In nasopharyngeal carcinoma low CLDN4 and high CLDN7 expressions were associated with distant metastases. Elevated CLDN7 expression also correlated with high tumor stage. Furthermore, decreased CLDN4 and increased CLDN7 expressions independently predicted shorter distant metastases [18]. In the present work we studied a very homogeneous group of lung cancer patients, i.e. only pathologic stage I tumors in order to minimize the effect of different metastatic steps and histologic subtypes.

The prognostic significance of CLDN7 expression is divergent. Recently, in oral and oropharyngeal squamous cell carcinoma, lack of CLDN7 expression in the tumor center was found to identify patients at high risk for regional recurrence [19]. On the contrary, high CLDN7 expression in epithelial ovarium carcinoma correlated with shorter progression-free survival as demonstrated by Kim et al. [20]. In our study we could not demonstrate any prognostic significance of CLDN7 expression, however, it is of note that within the ADC subgroups highly significant expression difference could be observed.

In case of prostate cancer, CLDN1 and −4 were found to be useful in distinguishing between patients who had and those who did not have metastases [21]. Our results might be of similar value in the lung cancer management of patients with SCC and low CLDN1 expression, since in such cases this immunohistochemical result might necessitate closer patient follow-up.

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Compliance with Ethical Standards

Conflict of Interest Statement Authors declare no conflict of interest in relation to the content of this manuscript.

Contributorship Statement All authors included in the paper fulfill the criteria of authorship. All authors have reviewed the final version of the manuscript and approve it for publication.

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