Claudin-1 correlates with poor prognosis in lung adenocarcinoma

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Keywords
CLDN1; EGFR; lung adenocarcinoma; Ras.

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Received: 22 April 2016;
Accepted: 1 May 2016.
doi: 10.1111/1759-7714.12368

Thoracic Cancer 7 (2016) 556–563

Introduction

Adenocarcinoma represents the most common histological subtype of lung cancer. Dysfunction of the airway epithelial barrier has been reported to be involved in the development and progression of cancer, including lung cancer. The epithelial barrier is composed of two essential elements, an intact epithelial monolayer and the intercellular tight junctions that connect epithelial cells to their neighboring cells. Tight junctions are composed of several different components, including transmembrane, peripheral, and cytoskeletal proteins, which act in concert to control paracellular permeability. The transmembrane proteins that mediate cell-to-cell contacts include claudins, occludin, tricellulin, and junctional adhesion molecules. Claudins (23 kDa) have four transmembrane domains and two extracellular loops and now have a family of 27 isoforms. They are expressed differentially among various tissues, and their expression pattern impacts on epithelial barrier function. It has been reported that claudin-1 (CLDN1), CLDN 4, CLDN 5, CLDN 14, and CLDN 18 are tightening junctional proteins with sealing function, whereas CLDN 2 and CLDN 8 are loosening junctional molecules with leaky function.

Recent studies have suggested that CLDN1 is downregulated in lung adenocarcinoma and that low CLDN1 messenger ribonucleic acid (mRNA) expression leads to shorter overall survival (OS). The aim of this study was to evaluate the clinical significance of CLDN1 expression in patients with lung adenocarcinoma; however, our results differed from those of other studies.

Methods

Patients and tissue samples

The study analyzed 258 paraffin-embedded lung adenocarcinoma tissues in a tissue microarray, collected from the...
Tianjin Cancer Institute & Hospital, Tianjin Medical University, Tianjin, China between 2005 and 2010. Patient medical records were retrospectively reviewed to assess clinical characteristics and survival. Patients who had smoked < 100 cigarettes in their lifetime were defined as never smokers. The routine preoperative workup included pulmonary function tests, contrast chest computed tomography (CT), flexible bronchoscopy, and brain magnetic resonance imaging; 59 patients underwent positron emission tomography (PET)-CT scans. Surgical procedures included: lobectomy or bilobectomy in 243 patients; pneumonectomy in 15; and subsequent mediastinoscopy or endobronchial ultrasound/esophageal endoscopic ultrasound-transbronchial aspiration (EBUS/EUS-TBNA) in 38 patients. In our hospital, if a contrast CT or PET-CT shows no mediastinal lymph node enlargement, a cervical mediastinoscopy or EBUS/EUS-TBNA is not routinely performed. No neoadjuvant therapy was administered to any of the 258 patients. All patients underwent systematic lymph node dissection or sampling. Patient consent and approval from the Research Ethics Committee of Tianjin Cancer Institute & Hospital of Tianjin Medical University was obtained. Histological classification was defined according to World Health Organization histologic classification. Clinicopathological variables are summarized in Table 1.

Lung cancer cell lines were either originally purchased from the American Type Culture Collection (ATCC, Rockville, MD, USA; H358), or obtained from Dr. Zhenyi Ma (Tianjin Medical University, Tianjin, China; A549).

**Immunohistochemical staining and assessment**

Paraffin sections (4 μm) from samples were deparaffinized in 100% xylene and rehydrated in descending ethanol dilutions according to standard protocols. Heat-induced antigen retrieval was performed in ethylene-diamine-tetraacetic acid buffer (pH 9.0) for three minutes at 100°C. Endogenous peroxidase activity and non-specific antigens were blocked with peroxidase blocking reagent containing 3% hydrogen peroxide and serum, followed by incubation with each antibody: rabbit anti-CLDN1 (Abcam, Cambridge, UK) at a dilution of 1:200; mouse anti-epidermal growth factor receptor (EGFR; Santa Cruz Biotechnology, Santa Cruz, CA, USA) at a dilution of 1:200; and rabbit anti-Ras (Bioss, Beijing, China) at a dilution of 1:100. Sections were counterstained using DAB counterstained with hematoxylin, mounted in neutral gum, and analyzed using a bright field microscope.

Two independent investigators performed the analysis of immunohistochemical staining. Extensiveness and intensity of staining in tumor cells were assessed for each sample using a semiquantitative scale. The extent of tumor

| Variables                  | n  | 5-year OS (%) | P     | 5-year DFS (%) | P     |
|----------------------------|----|---------------|-------|----------------|-------|
| Pathology                  |    |               |       |                |       |
| AIS                        | 25 | 65.2          | 0.020 | 65.2           | 0.023 |
| MIA + LPA                  | 123| 45.0          | 0.000 | 61.3           | 0.000 |
| ADE                        | 110| 36.7          | 0.000 | 33.8           | 0.000 |
| Ras                        |    |               |       |                |       |
| −                          | 106| 54.9          | 0.000 | 54.8           | 0.000 |
| +                          | 152| 34.9          | 0.000 | 31.3           | 0.000 |
| Claudin-1                  |    |               |       |                |       |
| −                          | 106| 54.8          | 0.000 | 53.4           | 0.000 |
| +                          | 152| 34.6          | 0.000 | 32.5           | 0.000 |
| EGFR                       |    |               |       |                |       |
| −                          | 149| 46.2          | 0.000 | 45.5           | 0.000 |
| +                          | 109| 38.4          | 0.000 | 34.1           | 0.000 |
| Gender                     |    |               |       |                |       |
| Male                       | 123| 37.4          | 0.074 | 36.1           | 0.075 |
| Female                     | 135| 48.6          | 0.074 | 46.1           | 0.075 |
| Age                        |    |               |       |                |       |
| < 60                       | 142| 43.0          | 0.941 | 42.0           | 0.920 |
| > 60                       | 116| 43.0          | 0.941 | 40.5           | 0.920 |
| Smoking                    |    |               |       |                |       |
| No                         | 138| 47.6          | 0.036 | 45.6           | 0.039 |
| Yes                        | 120| 38.0          | 0.036 | 36.8           | 0.036 |
| T stage                    |    |               |       |                |       |
| T1                         | 111| 60.9          | 0.000 | 56.9           | 0.000 |
| T2                         | 88 | 39.4          | 0.000 | 39.8           | 0.000 |
| T3, 4                      | 59 | 16.8          | 0.000 | 16.0           | 0.000 |
| N stage                    |    |               |       |                |       |
| N0                         | 140| 58.1          | 0.000 | 55.7           | 0.000 |
| N1                         | 31 | 37.1          | 0.000 | 34.8           | 0.000 |
| N2                         | 87 | 21.0          | 0.000 | 20.8           | 0.000 |
| Adjuvant chemotherapy      |    |               |       |                |       |
| No                         | 98 | 52.6          | 0.062 | 51.7           | 0.013 |
| Yes                        | 160| 39.0          | 0.062 | 35.0           | 0.013 |
| Laterality                 |    |               |       |                |       |
| Left                       | 111| 41.3          | 0.889 | 40.4           | 0.957 |
| Right                      | 147| 44.8          | 0.889 | 41.9           | 0.957 |
| Location                   |    |               |       |                |       |
| Peripheral                 | 219| 44.5          | 0.587 | 42.3           | 0.427 |
| Central                    | 39 | 36.0          | 0.587 | 35.0           | 0.427 |
| Operation                  |    |               |       |                |       |
| Lobectomy                  | 243| 44.9          | 0.019 | 42.7           | 0.016 |
| Pneumonectomy              | 15 | 17.8          | 0.019 | 20.0           | 0.016 |

ADE, invasive adenocarcinoma not including LPA; AIS, adenocarcinoma in situ; DFS, disease-free survival; LPA, lepidic predominant adenocarcinoma; MIA, minimally invasive adenocarcinoma; OS, overall survival.
staining was scored as follows: 0 (< 5% immunoreactive), 1+ (< 33% immunoreactive), 2+ (33–66% immunoreactive), and 3+ (> 66% immunoreactive). The staining intensity was graded using the following scale: 0 (negative), 1+ (weak), 2+ (moderate), and 3+ (strong). For each tumor, a combined score was calculated by multiplying the scores for extensiveness and intensity, using the following scale: 0+ = score 0, 1+ = 1–3, 2+ = 4–6, 3+ = 7–9. Scores 0+ and 1+ were defined as negative, while scores 2+ and 3+ were considered positive expression.

**Quantitative real-time reverse transcription-polymerase chain reaction**

Quantitative real-time reverse transcription-polymerase chain reaction (RT-PCR) was used to test the levels of CLDN1 expression in lung cancer cells. Real-time RT-PCR was performed on CLDN1 and the control gene, glyceraldehyde 3-phosphate dehydrogenase (GAPDH), with the use of specific TaqMan probes and primer sets. Gene expression was quantified in relation to GAPDH expression using sequence detector software with the relative quantification method (Applied Biosystems, Foster City, CA, USA). Briefly, RNA (2 μg/reaction) was used to generate cDNA and then the appropriate individual pairs of oligonucleotides (40 pmol/reaction) for the test genes were used to amplify DNA. Semiquantitative PCR was performed using 100 μL reaction volumes and taking 33 μL aliquots at 25, 30, and 35 cycles. GAPDH mRNA expression was determined for RNA samples to control for variations in RNA quantity.7

**Statistical analysis**

The χ² test was used to evaluate differences between the groups. Survival curves were obtained by the Kaplan–Meier method. Survival values were compared using the log-rank test. The Cox proportional hazards ratio model was used to investigate the simultaneous effect of multiple predictors on survival. All statistical tests were two-sided, and a P value less than 0.05 was considered statistically significant. All statistical analyses were performed using SPSS version 17.0 (SPSS Inc., Chicago, IL, USA).

**Results**

**Patient survival**

Claudin-1 and EGFR showed membrane expression (Fig 1a,b, respectively), while Ras was expressed in the cytoplasm (Fig 1c). Univariate analysis revealed that pathologic subtype, CLDN1 expression, Ras, smoking, T stage, and lymph node involvement were prognostic factors (P = 0.020, P = 0.001, P = 0.000, P = 0.036, P = 0.000, P = 0.000 for 5-year OS; P = 0.023, P = 0.001/ P = 0.000, P = 0.039, P = 0.000, P = 0.000 for 5-year disease-free survival [DFS], respectively; Fig 2). Patients with positive expressions of CLDN1/Ras had significantly poorer survival than those with negative expression (Table 1).

Table 2 summarizes the multivariate analysis of the prognostic value of the prognostic factors, which were determined by univariate analysis (P < 0.05) of OS in the 258 patients. In this study, a significant OS value was observed for pathologic subtype (P = 0.026), CLDN1 expression (P = 0.008), T stage (P = 0.014), and lymph node involvement (P = 0.003).

**Correlation between claudin-1 (CLDN1) and clinical-pathological characteristics**

Because our results indicated that CLDN1 was a poor prognostic factor, we investigated the relationship between CLDN1 and other factors that may affect prognostic outcome. Our results demonstrated that CLDN1 expression

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**Figure 1** Typical photomicrographs show Claudin-1 (CLDN1), epidermal growth factor receptor (EGFR), and Ras immunohistochemistry expression in tissue microarray. Diaminobenzidine solution is the stain used in all images. (a) CLDN-1+ (original magnification ×100); (b) EGFR+ (original magnification ×100); (c) Ras+ (original magnification ×100).
was correlated with Ras \((P = 0.000)\) and EGFR expression \((P = 0.000)\). There was no association between CLDN1 and other clinical prognostic factors, such as T or N stage (Table 3).

### Combination of CLDN1 and Ras/epidermal growth factor receptor

Because CLDN1 is associated with poor prognosis and has an association with Ras/EGFR, we hypothesized that Ras and EGFR signal transduction pathways may regulate the role of CLDN1 in terms of prognosis. We conducted survival analysis with a combination of these factors. Our results indicated that both CLDN1(+) and Ras(+) prognoses were worse than CLDN1(+) Ras(−), CLDN1(−) Ras (+), and CLDN1(−) Ras (−); and both CLDN1(+) and EGFR(+) prognoses were worse than CLDN1(+) EGFR(−), CLDN1(−) EGFR(+), and CLDN1(−) EGFR(−) (Tables 4–5, Figs 3–4).

### Messenger ribonucleic acid expression was lower in the H358 cell line compared with A549

The International Association for the Study of Lung Cancer, the American Thoracic Society, and the European Respiratory Society sponsored a new international multi-disciplinary classification, in which non-mucinous bronchioloalveolar carcinoma (BAC, ≤ 3 cm) is classified as adenocarcinoma in situ (AIS, formerly pure BAC). Some authors have suggested that atypical adenomatous hyperplasia (AAH), AIS, minimally invasive adenocarcinoma (MIA, formerly BAC with focal invasion), and lepidic predominant adenocarcinoma (LPA, formerly adenocarcinoma with BAC features) represent the developmental sequence of bronchioloalveolar stem cells, rather than a classification system.[]

We hypothesized that CLDN1 expression was higher in adenocarcinoma than in AIS and MIA. mRNA expression in CLDN1 was lower in the H358 cell line compared with the lung adenocarcinoma cell line, A549 (Fig 5, Table 6).
Discussion

Disruption of the cell-cell junction and detachment of tumor cells from the primary site is the first step of cancer cell invasion and metastasis. Therefore, CLDN1, a key component of tight junctions, which is abnormally regulated in lung adenocarcinoma, plays an important role in cancer progression and prognosis in lung adenocarcinoma.

Studies have suggested that the role of CLDN1 in the behavior of cancer cells differs in different types of cancers, and even in the same cancer. Several studies have reported a robust increase in CLDN1 expression in colon cancer.\(^\text{10--12}\) The causal association of high CLDN1 expression with cancer progression, invasion, and metastasis has been demonstrated in colon and colorectal cancer lesions.\(^\text{12,13}\) Other studies have reported that low CLDN1 expression is associated with lymphatic involvement, pathological differentiation, the extent of the poorly differentiated component, and reduced DFS and OS in cancers.\(^\text{14--16}\)

Our study demonstrated different results than those found in previous studies; however, the reason for this is unclear.\(^\text{4,5}\) Warrier \textit{et al.} reported that CLDN1 was expressed both in the cytoplasm and at the plasma membrane in breast carcinoma cells.\(^\text{17}\) Cytoplasmic predominant expression of CLDN1 is associated with a favorable prognosis. In contrast, membranous predominant expression is associated with larger tumors, histological grade 3, and a poor outcome; however, we did not find a correlation between this kind of expression location and clinical outcome. We hypothesized that other molecular pathways associated with lung cancer development may affect the role of CLDN1. We examined Ras and EGFR protein expression by immunohistochemistry in the tissue microarray of 258 patients. We found that CLDN1 expression was correlated with Ras and EGFR, and the combination of CLDN1 and Ras/EGFR had more powerful clinical significance. Therefore, we suggest that Ras and EGFR signal pathways may regulate the role of CLDN1 in lung adenocarcinoma. If CLDN1 does not activate Ras and EGFR signal pathways, it may not negatively affect the clinical outcome in lung adenocarcinoma.

Other studies have also suggested that CLDN1 plays a role in cancer via different signal transduction pathways.
Warrier et al. suggested that CLDN1 is a direct target of Hedgehog pathway activation in breast cancer. The transcription factor, RUNX3, is a gastric tumor suppressor; CLDN1 has gastric tumor suppressive activity and is a direct transcriptional target of RUNX3. CLDN1 is downregulated during the epithelial-mesenchymal transition.

**Figure 3** Prognosis of patients with both Claudin-1 (CLDN1)(+) and Ras(+) were worse than those with CLDN1(+) Ras(−), CLDN1(−) Ras(+), and CLDN1(−) Ras(−) (a) Overall survival (OS), (b) disease-free survival (DFS). a: CLDN1+/Ras−, b: CLDN1+/Ras− and CLDN1→Ras+, and c: CLDN1+/Ras−.

**Figure 4** Prognosis of patients with both Claudin-1 (CLDN1)(+) and epidermal growth factor receptor (EGFR)(+) were worse than those with CLDN1 (+) EGFR(−) CLDN1(−) EGFR(+) and CLDN1(−) EGFR(−). (a) Overall survival, (b) disease-free survival (DFS). a: CLDN1−/EGFR−, b: CLDN1+/EGFR− and CLDN1−/EGFR+ and c: CLDN1+/EGFR.

**Table 6** Claudin-1 primers for RT-PCR

| Gene  | Primer (5′–3′)                  | Genbank         | Product size (bp) |
|-------|---------------------------------|-----------------|-------------------|
| CLDN1 | Forward GATAGCAATCTTTGTGGCCACCT | NC_000003.12    | 205               |
|       | Reverse TTCGTACCTGGCATTGACTGGG  |                 |                   |

CLDN1, claudin-1; RT-PCR, reverse transcriptase-polymerase chain reaction.
In lung cancer cells, CLDN1 small interfering RNA significantly reduced tumor necrosis factor-enhanced cell migration and fibroblast-like morphology. Furthermore, CLDN1 overexpression enhanced cell migration in human lung cancer cells. AAH, AIS, MIA, and LPA might represent the developmental sequence of bronchioloalveolar stem cells, rather than a classification system. During the malignant transformation of normal colonic epithelium to colon adenocarcinoma, CLDN1 levels increased. We hypothesized that CLDN1 expression was higher in adenocarcinoma and LPA; however, our immunohistochemistry results showed no difference in protein expression. mRNA expression was lower in the H358 compared with the lung adenocarcinoma cell line (A549), which may be attributed to the relatively low number of BAC cases (25) compared with the high number of lung adenocarcinoma cases (233) in this study. No subgroup analysis of BAC, such as pathologic subtype (mucinous or non-mucinous) or tumor size was conducted, because of the small number of cases.

Our results indicate that CLDN1 is associated with poor prognosis and has an association with Ras/EGFR. The combination of CLDN1 and Ras/EGFR had more powerful clinical significance. CLDN1 may be involved in the progression of lung adenocarcinoma and Ras and EGFR signal pathways may regulate its role.

Acknowledgments

We thank Dr. Zhenyi Ma for his contribution of the lung adenocarcinoma cell line (A549). This research was supported by the National Natural Science Foundation of China (NO. 81201649, NO. 81470137) and the Key Program for Anti-cancer Research of Tianjin Municipal Science and Technology Commission (12ZCDZSY15400).

Disclosure

No authors report any conflict of interest.

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