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Prognostic Significance of Claudin 4 in Completely Resected Adenocarcinoma of the Lung

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Background: The development of diagnostic techniques and an awareness of health examinations can bring about an early diagnosis of lung cancer. However, appropriate postoperative management and adjuvant chemotherapy remain under debate in postoperative therapeutic strategy. The present study was conducted to assess the clinicopathologic factors that influence recurrence and prognosis after complete resection of lung cancer. ***Methods:*** The present study analyzed 62 patients with lung cancer who underwent complete resection of diagnosed adenocarcinoma between 1994 and 2007. In addition to conventional factors, which include staging factor and histological evaluation, the present study also performed univariate and multivariate analyses to consider claudin, a cell adhesion molecule, as a prognostic factor by immunohistochemical staining. ***Results:*** There was no correlation between conventional factors, including lymphatic and vascular invasion, and recurrence. However, there was a significant correlation between high expression of claudin 4 and cancer recurrence. In particular, there was a correlation between high expressions of claudin 1, 4, and 5 and a reduction of disease-free survival. ***Conclusion:*** Increased expressions of claudin 4 were negative prognostic factors in adenocarcinoma of the lung and thus could be used to identify high-risk patients for adjuvant chemotherapy, even if they had early-stage lung cancer. The present findings collectively suggest that consideration of claudin as a prognostic factor in the active postoperative treatment in patients at high risk will lead to better therapeutic outcomes with fewer side effects.

Key words: 1. Lung neoplasms
2. Adenocarcinoma
3. Discard
4. Lung pathology
5. Claudin

INTRODUCTION

Lung cancer is the leading cause of cancer death. There have been histological, molecular, and genetic mutation studies aimed at increasing the survival rate and reducing the recurrence rate after lung cancer treatment [1-4]. More recently, studies on cell adhesion molecules (CAMs) have been in progress.

CAMs are proteins at the junction between cells. These proteins participate in the adhesion and transport of molecules between cells, which contribute to maintaining cell homeostasis when exposed to various conditions. In addition, CAMs

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participate in cell growth as well as cell cycle control, and loss of function in these junctions due to abnormalities in CAMs can cause pathological states. Studies based on the expression level of these proteins are being conducted for various cancer prognosis factors [5]. One of them is claudin, which consists of 24 transmembrane proteins, which exists at the tight junction of epithelial and endothelial cells [6,7]. Functions of claudin are structural maintenance of the junction, paracellular permeability control, maintenance of cell polarity, and other basic defense systems. Claudin is over- or underexpressed in pathological status although it is normally observed in normal tissues such as lung epithelial cells [8]. In addition, they affect tumerogenesis, recurrence, and metastasis by participating in cell cycle control and intracellular signaling through interaction between factors in the cytoplasm or the nucleus [9].

Since not all cancer patients have recurrence after complete resection, selective adjuvant treatment after surgery is necessary. Through careful evaluation of risk factors suggesting poor prognosis after surgery, selective adjuvant treatment among the high-risk group will be possible, which will lead to reduction of complications and enhanced curative effect. The authors in this study focus on factors that affect recurrence and prognosis in patients after complete resection.

METHODS

1) Patient selection

Between October 1994 and December 2007, 62 patients who were diagnosed with adenocarcinoma, received complete resection without neoadjuvant chemotherapy or radiotherapy at Dongsan Medical Center, Keimyeong University. The study was on patient's gender, operation method, size of tumor, pathological differentiation of tumor, visceral pleural invasion, lymph node metastasis, and the relationship between the recurrence rate and the degree of immunohistochemical staining, and the expression of claudin 1, 3, 4, 5, 7, and 10. Complete resection was defined as anatomical resection with systematic mediastinal lymph node dissection. The tumor-node-metastasis (TNM) staging system followed the 7th edition of the American Joint Committee on Cancer system. Recurrence included locoregional and distant recurrence diagnosed through computed tomography, positron emission tomography, bone scan, bronchoscopy, and if required, histological biopsy at anatomically contiguous sites of primary cancer, regional lymph nodes, or other organs. All clinical results' record of above mentioned examinations.

2) Production of tissue microarray block

Formalin-fixed, paraffin-embedded tissue samples were for tissue microarray (TMA). Representative areas of each tumor were marked on each hematoxylin and eosin-stained slide, and the corresponding area of the tissue blocks was sampled. The designated area of each donor block was collected using a tissue cylinder punch (diameter, 3 mm), and the samples were transferred to a recipient block.

3) Immunohistochemical staining

Sections (thickness, 4 μm) from TMAs were cut from 10% formalin buffer, embedded in paraffin, mounted onto Superfrost Plus glass slides (VWR Scientific, West Chester, PA, USA) and incubated at 60°C for 15 minutes. The slides were deparaffinized in xylene, rehydrated in graded alcohol solutions, and washed in tap water. Endogenous peroxidase activity was blocked by the addition of 3% H_2O_2. Slides were placed in a steam cooker filled with 10-mM sodium citrate buffer, pH 6.0, for antigen retrieval after treatment with a blocking agent (DAKO, Carpinteria, CA, USA) for 10 minutes to block nonspecific protein binding. Immunohistochemistry for each antigen (claudin 1, 3, 4, 5, 7, and 10) was performed using an autostainer (LV360-2D; LabVision Co., Fremont, CA, USA). Reagents and the secondary antibody from the LP Kit (TL-125-HD, LabVision) were used as provided by the manufacturer. For the primary antibody, rabbit polyclonal antibodies against claudin 1 (1:500, ab15098; Abcam, Cambridge, MA, USA), claudin 3 (1:100, ab15102; Abcam), claudin 4 (1:200, ab15104; Abcam), claudin 7 (1:200, ab27487; Abcam), and claudin 10 (1:100, ab24792; Abcam), and a mouse polyclonal antibody against claudin 5 (1:200, 18-7364; Invitrogen, Carlsbad, CA, USA) were applied. Claudin immunopositivity was evaluated by staining sensitivity (0, negative; 1, weak; 2, moderate; 3, strong) and the proportions of positive cells (0, 0% positive; 1, ≤10%; 2, >10% and ≤50%; and 3, >50%). Samples were consid-

Table 1. Patient characteristics

Characteristic	Value
Age (yr)	64.2±8.3
Gender	
Male	38 (61.2)
Female	24 (38.8)
Operation	
Lobectomy	55 (88.7)
Bilobectomy, pneumonectomy	4 (6.4)
Segmentectomy	3 (4.9)
T status	
T1a	12 (19.3)
T1b	22 (35.5)
T2a	17 (27.4)
T2b	5 (8.0)
T3	6 (9.8)
N status	
N0	53 (85.5)
N1	6 (9.7)
N2	3 (4.8)
Pathologic stage	
IA	31 (50.0)
IB	14 (22.6)
IIA	10 (16.2)
IIB	3 (4.8)
IIIA	3 (4.8)
IIIB	1 (1.6)
Microscopic findings	
Differentiation	
Well	16 (25.8)
Moderate	36 (58.0)
Poor	10 (16.1)
Necrosis	30 (48.4)
Bronchial invasion	0
Pleural invasion	26 (42)
Lymphatic invasion	25 (40.3)
Vascular invasion	18 (29)
Perineural invasion	4 (6.45)

Values are presented as mean±standard deviation or number (%).

ered positive for claudin staining intensity of >1 (moderate to strong), and >50% of the cells were positive.

4) Statistical analysis

The chi-squared test and Fisher's exact test were used to analyze the association between the degree of claudin expression and recurrence. The disease-free survival rate was calculated using the Kaplan-Meier method. A univariate analysis for each risk factor was carried out using the log-rank test; and a multivariate analysis, by the Cox proportional hazards regression model to identify the prognostic factor. The statistical significance level was set at 0.05, and all statistical analyses utilized PASW SPSS ver. 18.0 (SPSS Inc., Chicago, IL, USA).

RESULTS

1) General characteristics of the patient group

The median follow-up time for all patients was 1,518.8± 1,094.4 days. Of the 62 patients, 38 were male (61.2%) and 24 were female (38.8%), and the average age was 64.2±8.3 years. In the surgical excision range, 55 patients (88.7%) underwent lobectomy; 4 patients (6.4%), bilobectomy and pneumonectomy; and 3 patients (4.9%), segmentectomy. On the basis of the TNM pathological stage, 12 patients were T1a (19.3%), 22 patients were T1b (35.5%), 17 patients were T2a (27.4%), 5 patients were T2b (8%), and 6 patients were T3 (9.8%). Further, 53 patients were N0 (85.5%), 6 patients were N1 (9.7%), and 3 patients were N2 (4.8%). According to the comprehensive pathological stage categorization, 31 patients belonged to IA (50.0%), 14 patients to IB (22.6%), 10 patients to IIA (16.2%), 3 patients to IIB (4.8%), 3 patients to IIIA (4.8%), and 1 patient to IIIB (1.6%). Based on the degree of differentiation, 36 patients showed moderate differentiation (58.0%), 16 patients showed good differentiation (25.8%), and 10 patients showed poor differentiation (16.1%) (Table 1).

2) Relapse patient group analysis

Among 62 patients, 31 had locoregional recurrence or distant recurrence (50%). The disease-free interval was determined as the interval from the date of surgery to the date of first recurrence, and the median disease-free interval was 1,152.5±914.7 days. The chi-squared test and Fisher's exact test showed that claudin 4 was associated with relapse (p=0.016) (Table 2). A univariate analysis of the relapse and gender, tumor and lymph node status, grade of tumor differentiation, tumor necrosis, lymphatics and blood vessel invasion, visceral pleura invasion, and claudin expression levels was carried out to verify the recurrence risk factor related to

Table 2. Analysis of claudin and pathologic stage related with recurrence

Variable	Recurrence (%)	p-value
Claudin 1		0.084
Low	17 (41.5)	
High	13 (65.0)	
Claudin 3		0.445
Low	15 (45.5)	
High	16 (55.2)	
Claudin 4		0.016
Low	16 (39.0)	
High	15 (71.4)	
Claudin 5		0.141
Low	14 (43.8)	
High	17 (63.0)	
Claudin 7		0.277
Low	19 (45.2)	
High	12 (60.0)	
Claudin 10		0.492
Low	31 (51.7)	
High	0	
Pathologic stage		0.010
I	18 (40)	
II, III	13 (50)	

Table 3. Univariate analysis of prognostic factors in a total of 62 patients by log-rank test

Factor	Variable	p-value
Gender	Male/female	0.182
T status	T1/T2, T3	0.176
N status	N0/N1, N2	0.002
Pathologic stage	I/II, III	0.000
Differentiation	Well, moderate/poor	0.270
Necrosis	-/+	0.394
Bronchial invasion	-/+	0.452
Pleural invasion	-/+	0.383
Lymphatic invasion	-/+	0.423
Vascular invasion	-/+	0.762
Perineural invasion	-/+	0.438
Claudin 1	Low/high	0.035
Claudin 3	Low/high	0.404
Claudin 4	Low/high	0.026
Claudin 5	Low/high	0.024
Claudin 7	Low/high	0.210
Claudin 10	Low/high	0.134

Table 4. Multivariate analysis of prognostic factors in a total of 62 patients by Cox proportional hazards regression model

Factor	Hazard ratio	95% confidence interval	p-value
Claudin 1 (high)	1.014	0.388–2.650	0.874
Claudin 4 (high)	4.609	1.209–17.574	0.030
Claudin 5 (high)	1.128	0.317–4.019	0.680
T status (T2, T3)	1.298	0.651–2.588	0.247
N status (N1, N2)	0.550	0.201–1.504	0.486
Pathologic stage (II, III)	4.922	1.329–18.231	0.117
Necrosis (+)	0.395	0.170–0.916	0.255
Lymphatic invasion (+)	2.065	1.094–3.896	0.101

the disease-free survival rate. Patients' gender and histological findings such as lymphatic invasion, blood vessel invasion, and visceral pleura invasion did not show a significant result. However, lymph node status, pathologic stage, and expression of claudin 1, 4, and 5 had a significant correlation with recurrence according to the log-rank test (Table 3). The multivariate analysis using Cox proportional hazards regression model was used for analyzing the relationship among relapse rate, expression of claudin, T status, N status, pathologic stage, necrosis, and lymphatic invasion. Overexpression of claudin 4 and pathologic stage had a significant correlation with recurrence (Table 4). A survival analysis between recurrence and claudin expression levels was done with the Kaplan-Meier method (Figs. 1–3).

DISCUSSION

The increasing occurrence of cancer is a worldwide phenomenon, and among them, lung cancer is a major cause of death due to cancer. There are many ongoing studies on the improvement of the prognosis and treatment result [10]. This study shows that the pathologic stage is related to recurrence. The TNM staging system is a typical index for comprehending the prognosis, and studies on this system are in progress. In particular, the 2012 National Comprehensive Cancer Network guideline suggests chemotherapy after surgery in case of non-small lung cell carcinoma in stage IB for the high-risk group, where the high-risk group is referred as that of patients who have poorly differentiated tumor, vessel invasion, visceral pleura invasion, and tumor size of more than 4 cm [11]. If studies on factors affiliated with the high-risk group are continued, better prognosis are expected by deciding the implementation of pre- or post-surgery chemotherapy

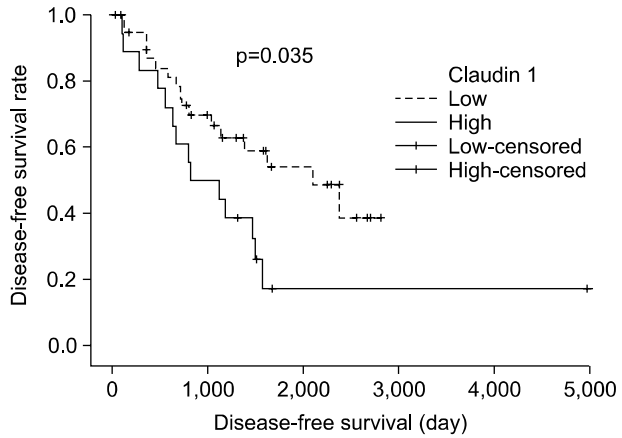

Fig. 1. Disease-free survival rate curve related to expression of claudin 1.

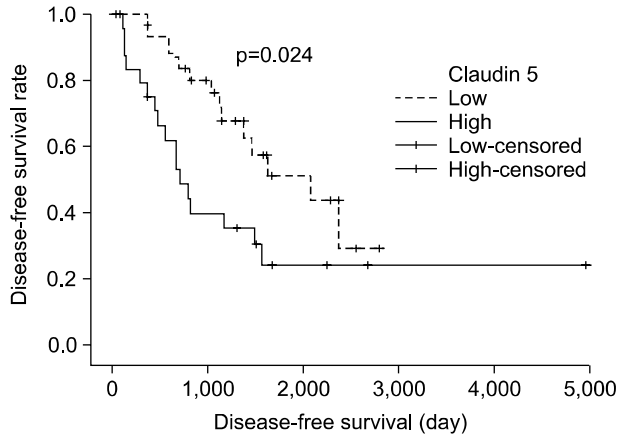

Fig. 2. Disease-free survival rate curve related to expression of claudin 4.

and radiation therapy through an accurate understanding of the high-risk group.

Molecular biological approaches are also being pursued, and one of them is the study of a protein that exists in the intracellular junction. Claudin is a protein that exists in a tight junction that plays an important role in maintaining a cell's polarity and paracellular transport [6,7]. Claudin exists normally in normal cells, but in a distressed cell condition, it is often over- or underexpressed [8]. Claudin expression differs for various types of tumors, and even tumors that occur in the same organ show a difference in Claudin expression in different tissues. Therefore, claudin expression not only affects the accuracy of the diagnosis but is also an important

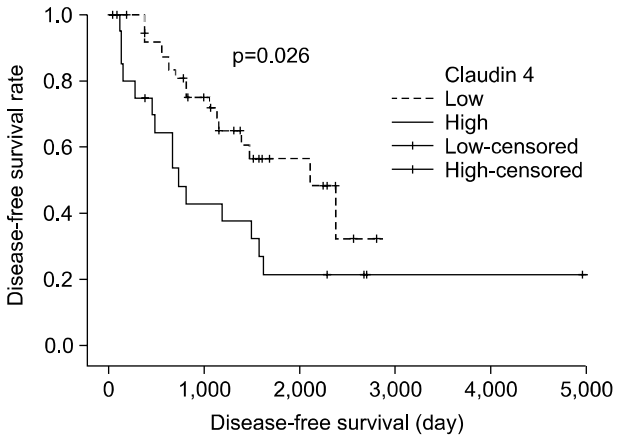

Fig. 3. Disease-free survival rate curve related to expression of claudin 5.

index for prognosis [12]. In lung cancer, claudin 1 is expressed the most in squamous cell carcinoma and claudin 4 and 5 are expressed the most in adenocarcinoma [13]. In addition, the overexpression of claudin 1 is observed in the cases of pancreatic cancer, hepatic cell carcinoma, prostatic cancer, and some head and neck cancers. In contrast, claudin 1 repressed in the cases of breast cancer and some ovarian cancers. Claudin 4 is overexpressed in the cases of breast cancer, biliary cancer, and gastric cancer, and underexpressed in the cases of hepatic cell carcinoma and malignant mesothelioma [14]. Claudin 5 is observed to be overexpressed in the cases of lung adenocarcinoma, gastric cancer, and ovarian cancer [15].

The results of this study show that overexpression of claudin 4 has significant correlation with the recurrence and overexpression of claudin 1, 4, and 5 and is relevant to recurrence after surgery in the survival analysis. It is not easy to find a reason that explains how the expression of claudin relates to the prognosis of lung cancer. Chao et al. [16] reported that the overexpression of claudin 1 inhibits cancer invasion and metastasis. Shiozaki et al. [17] stated that claudin 1 mediates TNF-α-induced cell morphological change and cell migration in a lung carcinoma cell. There are some reports that claudin interrupts intracellular fusion due to phosphorylation of membrane protein and weakens the defense function of membrane protein. Moreover, in association with the signal conduction system, it blocks the stopping of the cell cycle and thus influences apoptosis [8,18]. The relation-

ship between the overexpression of claudin 4 and the recurrence of tumor shown in this study can be explained not through the increase in invasiveness due to increased intercellular adhesion function, but through the loss of functionality and change in the signal conduction system related to tumor cell proliferation and cell migration. Claudin is a transmembrane protein that interacts with the intracellular and extracellular environments. It is possible that it engages with the signaling pathway through an intracellular loop and interacts with extracellular molecules that promote cell motility through an extracellular loop [19]. Claudin expression has also been associated with the activation of matrix metalloproteinase, which involved cell migration via degradation of the extracellular matrix [20]. These mechanisms may play a role in the recurrence and metastasis of a tumor. In order to clarify the relationship between claudin and recurrence, an additional molecular biological study is required.

The prognosis analysis of tumor using the degree of claudin expression is still not clear, particularly in the case of claudin 4, which is expressed in lung cancer. Thus, precise comprehension of the prognosis correlation and mechanism is needed, and further research and experiments are also necessary. Efforts to find various prognostic factors will lead to better studies that will enable a more selective and aggressive treatment before and after surgery by precisely categorizing the high-risk group by using a poor prognosis factor, and will improve tumor treatment on the whole.

In conclusion, this study suggests that the overexpression of claudin 4 is associated with poor disease-free survival and is identified as a high risk factor in lung adenocarcinoma. Further study on claudin is necessary to identify it as a prognostic factor in lung cancer.

CONFLICT OF INTEREST

No potential conflict of interest relevant to this article was reported.

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