

ORIGINAL PAPER

CLAUDIN 6 IS ASSOCIATED WITH A SHORT SURVIVAL AND A SHORT RECURRENCE-FREE INTERVAL IN NON-SMALL CELL LUNG CARCINOMAYLERMI SOINI¹, RISTO PIRINEN², KUMI TAKASAWA³, MAKOTO OSANAI³, AKIRA TAKASAWA³¹Department of Pathology and Forensic Medicine, Institute of Clinical Medicine, Pathology and Forensic Medicine, School of Medicine, University of Eastern Finland, Cancer Center of Eastern Finland, Kuopio, Finland²Department of Pathology, North Karelia Central Hospital, Joensuu, Finland³Department of Pathology, Sapporo Medical University School of Medicine, Sapporo, Japan

We investigated the expression of claudin 6 in non-small-cell lung carcinomas (NSCLC) by immunohistochemistry.

Samples of 164 patients with NSCLC were studied by immunohistochemistry.

Claudin 6 was expressed in 42 % of cases. Its expression was significantly more frequent in adeno- than in squamous cell carcinoma ($p = 0.002$). There was no association between the TNM status and claudin 6 expression. Claudin 6 associated with a poor prognosis of the patients and with a short recurrence-free interval ($p = 0.002$, $p < 0.001$). The association with survival had independent prognostic value ($p = 0.011$).

The results show that claudin 6 can be regarded as a marker of poor prognosis in lung cancer. This is different to some other cancers, such as breast and cervical carcinoma suggesting that claudin 6 probably induces other cellular pathways in neoplastic lung cells than in those tumors. In lung cancer, adenocarcinomas were most abundantly positive indicating a higher linkage of claudin 6 to glandular differentiation.

Key words: claudin, lung, carcinoma, survival, immunohistochemistry.

Introduction

Claudins are tight junction proteins serving two main functions in epithelial cells: the barrier and the fence function [1]. They regulate the paracellular permeability of solutes across epithelial layers which is important for the function of the kidneys or the gut [2]. They also take part in immune defense by creating secluded areas for the immune system through formation of the blood brain barrier or the blood retinal barrier [3]. By creating a tight junctional zone on the apicolateral border of the cell claudins separate the apical cellular membrane from other parts thus

preventing membrane bound proteins from entering a wrong membrane compartment [1].

There are 27 known claudins [1, 2, 3]. In addition to epithelial cells they are also found in endothelial and mesothelial cells [2, 4]. They are proteins with a molecular weight of 20-34 kDa [1, 3]. Claudins are able to selectively heterodimerize and homodimerize, whereby they selectively link to the claudin of the neighbouring cell and to the claudin adjacent to them [3]. Claudins consist of four transmembrane domains which create two extracellular loops, and one intracellular loop [1, 2, 3]. The extracellular loops are associated with many functional properties, such as paracel-

lular charge selectivity, and possess receptors for *Clostridium perfringens* toxin or hepatitis C [1, 3]. The inner carboxyterminal tail of claudins contains the PDZ recognition part which attaches to scaffolding proteins ZO 1-3 [1, 3]. The carboxyterminal tail also contains serine, threonine, and tyrosine which through modification can trigger signals to the cell by inducing various cellular pathways such as PI3K and MAPK [1]. Thus signal transduction appears to be yet another feature of claudin function [1]. In cancer, the function of claudins is disturbed. Loss of barrier function enables membrane bound receptors to move to other compartments of the cell with complex consequences on cell signalling [1].

The most studied claudins present in human tissues are claudins 1-5 and 7. Claudins 1-4 and 7 are found in epithelia of different kind while claudin 5 is mostly present in endothelia [5]. Of other claudins, claudin 11 is found in oligodendroglia and in testis tissues [3]. Claudin 6 is mostly not or only weakly present in mature human tissues. Tumors, however, show variable claudin 6 expression. In serous ovarian carcinomas claudin 6 expression was detected in 69% and it showed no association with prognostic factors [6]. Curiously, in one study the expression of claudin 6 in gastric cancer was lower than in adjacent nonneoplastic tissues with 55% and 79% frequency, respectively, and did not show any association with prognostic factors [7]. Claudin 6 has also been reported in nonepithelial tumors such as rhabdoid tumors, germinomas, Wilms tumors and even meningiomas [8]. In breast cancer methylation of claudin 6 induces cancer invasion [9]. Claudin 6 also induces apoptosis through ASK1 in breast carcinoma [10] and appears to be associated with a better prognosis [11]. In lung cancer the reports concerning the prognostic value of claudin 6 are conflicting [12, 13]. Because of this we analysed a large set of non-small cell lung carcinomas and compared the results with the clinical data of the tumors.

Material and methods

Material

The representative samples of primary lung tumors were collected from 164 patients, diagnosed and treated for non-small cell lung carcinoma (NSCLC) during 1978-1996 at the University Hospital of Kuopio. The samples had been fixed in buffered formalin and embedded in paraffin. For analysis, cases were re-evaluated in haematoxylin-eosin section and punctures for tissue microarray (TMA) were obtained from four representative tumor areas. All clinical data from the patients' files were re-evaluated and the stage of the disease was recorded according

to TNM classification. An experienced histopathologist re-examined and classified the sections of the primary tumors according to the WHO classification (YS), being unaware of the clinical data. There were 101 squamous cell carcinomas, 41 adenocarcinomas and 22 large cell carcinomas in the study. The mean age of the patients was 62.5 years. There were 150 males and 14 females in the study. The ethical consent to use the material was obtained from the Ethical Committee of the Northern Savo Hospital District (110/2010).

Treatment and follow-up

The patients were treated mainly with radical lobectomy or radical pneumectomy. Palliative operation or only explorative thoracotomy was performed in less than 5% of patients. None of the patients received preoperative radio- or chemotherapy. Postoperative radiotherapy and chemotherapy were given to 42 and 6 patients, respectively. The patients were followed-up by a senior physician according to a routine protocol. Time between the radical operation and any documented sign (radiological or clinical) of recurrence was considered as disease-free survival (DFS). For overall survival (OS), corrected survival rates were used, i.e. only deaths due to NSCLC were considered as outcome events and all other deaths as censored events.

Immunohistochemistry

The TMA slides were pretreated in microwave oven (800 W, pH 9, 10 mmol EDTA). The primary rabbit anti-human antibody was used in a dilution of 1:500 (18865, Immuno-Biological Laboratories, Gunma, Japan). For detection the MACH1 Universal HRP polymer detection kit was used (Biocare Medical, Concord, CA). Both membranous and cytoplasmic staining was assessed by evaluating the percentage of positively stained cells. Three punctures of separate tumor areas were assessed. The percentage of positivity was graded into four groups: 0 ≤ 1%, 1 = 1-10%, 2 = 11-50%, 3 = 51-80%, 4 = 81-100%. A limit for cellular positivity was when 1% or more of tumor cells expressed positivity. The staining was evaluated by two experienced pathologists (AT, YS).

Statistical analysis

Statistical analyses were done with SPSS Statistics (IBM SPSS Statistics 21.0). Associations between immunostainings and histopathological parameters were analysed with χ^2 and Fisher's exact test. Survival analyses were carried out with Kaplan-Meier log rank test and Cox regression model. P-values < 0.05 were considered statistically significant.

Table I. Claudin 6 expression in different types of NSCLC

CLAUDIN 6 EXPRESSION	PAD			TOTAL
	SQ	AC	LC	
0	66 (40%)	15 (9%)	14 (9%)	95 (58%)
1	35 (21%)	26 (16%)	8 (5%)	69 (42%)
Total	101 (61%)	41 (25%)	22 (14%)	164 (100%)

0 – negative; 1 – positive; SQ – squamous cell carcinoma; AC – adenocarcinoma; LC – large cell carcinoma; PAD – pathologic anatomical diagnosis; NSCLC – non-small cell lung carcinoma

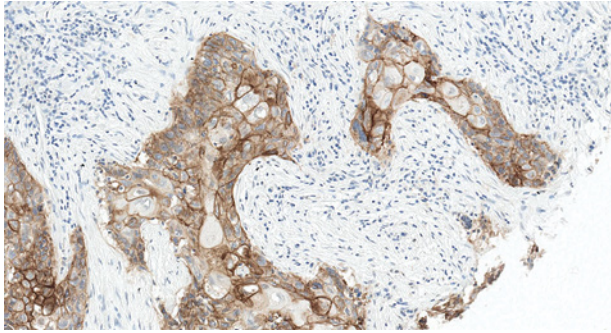


Fig. 1. Claudin 6 in squamous cell carcinoma. There is strong membranous staining present (Immunohistochemical stain $\times 220$)

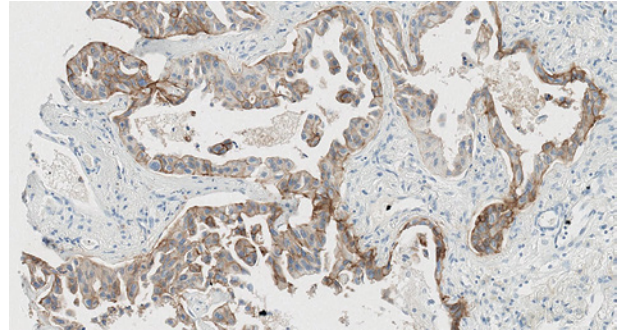


Fig. 2. Claudin 6 in adenocarcinoma. In this case there is also strong membranous staining which, however, is discontinuous and partly lacking (Immunohistochemical stain $\times 220$)

Results

Immunostaining with claudin 6

Claudin 6 was positive in 69/164 (42.1%) of cases (Table I). Adenocarcinomas had significantly more positive cases for claudin 6 than squamous cell carcinomas ($p = 0.002$; Table I, Figs. 1, 2). A similar tendency was observed when adenocarcinomas were compared with large cell carcinomas ($p = 0.062$). Claudin 6 did not associate with the grade of the tumors ($p = 0.45$). There was no significant association between claudin positivity and the size of the tumors ($p = 0.12$), the number of lymph node metastases ($p = 0.66$) or distant metastases ($p = 0.51$). There was no difference in the expression between smokers and nonsmokers ($p = 0.51$).

In the whole material those cases with claudin 6 positivity had a worse survival ($p = 0.002$ log rank, $p = 0.004$ Breslow, $p = 0.003$ Tarone-Ware) than the rest (Fig. 3). Cases with claudin 6 positivity had significantly more recurrences than the rest of the cases ($p < 0.001$, log rank, $p < 0.001$ Breslow, $p < 0.001$ Tarone-Ware; Fig. 4). When Cox regression analysis was used including TNM status, patient gender, diagnosis, grade, lung site, smoking status and lobe site as covariates claudin 6 had an independent prognostic value ($p = 0.011$) along with the presence of regional metastases ($p = 0.011$).

Qualitative claudin 6 expression also associated with a worse survival ($p = 0.009$ log rank).

Discussion

In this study we investigated the expression of claudin 6 in a set of NSCLCs. In our sample set 42% of cases were claudin 6 positive and 61% of adenocarcinomas were positive. According to the results claudin 6 was associated with a worse survival of the patients and a shorter recurrence-free interval.

In the study of Micke *et al.* distinct claudin 6 expression in adenocarcinoma was associated with a worse survival and it was associated with TTF1 positivity [13]. Since adenocarcinomas are mostly TTF1 positive their association with claudin 6 suggests that it preferentially directs tumor differentiation towards adenocarcinoma lineage. In the same study it was found that cases with claudin 6 positivity did not overlap with claudin 18 positive cases suggesting different pathways of carcinogenesis [13]. Interestingly, claudin 18 is associated with a better survival while claudin 6 showed a worse prognosis in adenocarcinomas [13].

Previously it has been shown that pluripotent stem cells express claudin 6 and that the expression diminishes when the cells differentiate [14]. Knockout of claudin 6 downregulates endodermal transcription factors such as Sox17, Foxa2, Gata4 and AFP while mesodermal genes were upregulat-

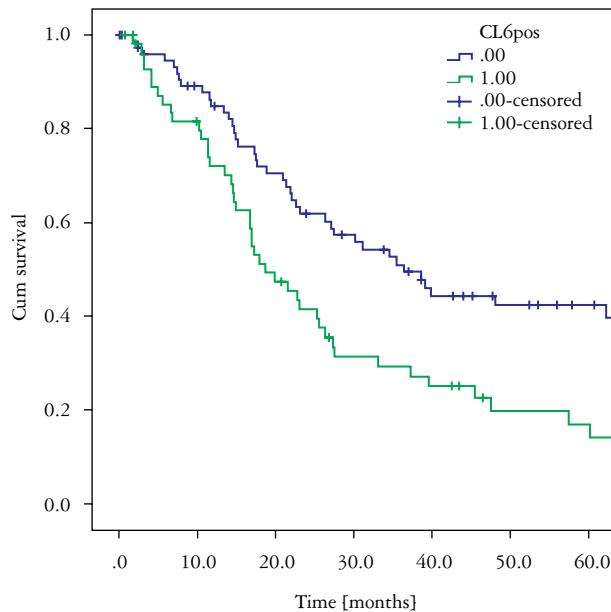


Fig. 3. Claudin 6 expression associates with a worse survival of the patients ($p = 0.002$ log rank, $p = 0.004$ Breslow, $p = 0.003$ Tarone-Ware). The blue line shows claudin 6 negative and green line claudin 6 positive cases. Small vertical lines indicate censored cases. Cumulative survival indicates the proportion of patients alive in relation to time

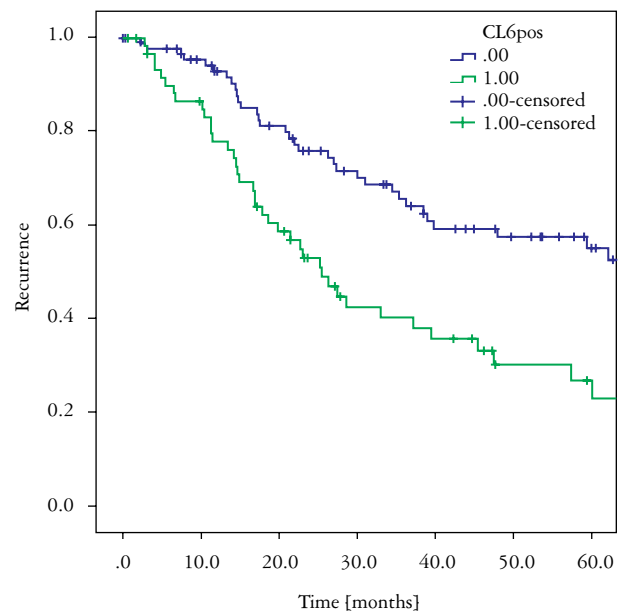


Fig. 4. Claudin 6 associates with a shorter recurrence-free interval of the patients ($p < 0.001$, log rank, $p < 0.001$ Breslow, $p < 0.001$ Tarone-Ware). The blue line shows claudin 6 negative and green line claudin 6 positive cases. Small vertical lines indicate censored cases

ed [14]. In the development of the lung, claudin 6 takes part in epithelial and bronchial morphogenesis by induction of transcriptional factors such as TTF1 but its expression is absent in mature lung [13, 15]. In non-committed cells claudin 6 induces epithelial differentiation by induction of other tight junction proteins such as claudin 7 or occludin [16].

Claudin 6 was associated with worse survival and a shorter recurrence-free interval in lung tumors. In our study there was, however, no association with tumor size, presence of metastases or the grade of the tumors. In endometrial carcinoma high claudin 6 expression was associated with aggressive parameters of the tumor and was an independent prognostic factor [17]. In a recent study on gastric cancer and cancer cell lines claudin 6 immunoreactivity and mRNA level were increased compared to normal tissues and the investigators found that claudin 6 induced translocation of YAP1 to the nucleus and promoted YAP1-snail1 interaction leading to increased invasion [18]. Yu *et al.* found in lung NSCLC cell lines that Yap1 regulates slug expression and in this way affects EMT in NSCLC [19]. Even though not studied so far, claudin 6 might thus promote EMT in NSCLC similar to gastric carcinoma.

In breast cancer tissues high claudin 6 expression was found in 23% of cases and it has been regarded as a tumor suppressor in both breast and cervical carcinoma [9, 22]. In breast cancer it was lower in metastatic tissue compared to primary tumor suggesting

that claudin 6 suppresses breast cancer progression [9]. The inhibitory effect of claudin 6 in breast cancer invasion is partly based on Er β which promotes tumor autophagy through claudin 6-beclin 1 axis [20]. In breast carcinoma cell lines MCF-7 and SKBR-3, Li *et al.* showed that claudin 6 downregulates MMP2 and MMP9 [21]. In cervical carcinoma claudin 6 associated with ASK1 thus putatively promoting apoptosis [22]. Also, in breast cancer ASK1 and claudin 6 are related [10]. The inverse effect of claudin 6 in these tumors compared to lung carcinoma suggests that it triggers different kinds of cellular pathways leading to more aggressive behaviour in lung tumors. In fact, claudins may show different prognosis in different types of tumors and tumor sites. Claudin 3 overexpression, for instance, predicts a worse prognosis in renal clear cell carcinoma [23] while a low claudin 3 expression is associated with poor prognosis in gastric cancer [24].

Even though most lung adenocarcinomas showed claudin 6 expression, squamous cell carcinomas had also a considerable expression. In dermal squamous tissue of claudin 6 overexpressing mice there is severe dysfunction of the epidermal barrier [25]. This is because claudin 6 replaces other claudins in the skin thus disturbing its barrier function [25]. Also structural proteins such as filaggrin, loricrin, transglutaminase 3 and involucrin were aberrantly expressed in claudin 6 overexpressing mice [20]. Even though such defects were found in squamous cells of the skin,

claudin 6 expression in squamous cell carcinoma probably hampers the contacts between the neoplastic cells thus adding to aberrant adhesion of the cells.

In conclusion, expression of claudin 6 in lung carcinomas indicated a poorer prognosis of the patients and might be used as a marker of more aggressive tumor type since the association had independent prognostic value. Adenocarcinomas had a significantly higher number of cases with claudin 6 expression suggesting that upregulation of claudin 6 in these tumors might be associated with its regulation by TTF1 [15].

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