

ORIGINAL ARTICLE

Expression patterns of claudins 1, 4, and 7 and their prognostic significance in nasopharyngeal carcinoma

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Summary

Purpose: Tight junction (TJ) proteins in the cells organize paracellular permeability, and they have a critical role in apical cell-to-cell adhesion and epithelial polarity. In our study, the expression patterns of claudins 1, 4, and 7 and their relationship with prognosis were determined in patients with nasopharyngeal carcinoma.

Methods: Claudins 1, 4, and 7 were stained immunohistochemically in 18 biopsy samples of nasopharyngeal carcinomas that included non-neoplastic surface epithelium and dysplastic epithelium in addition to the tumor tissue. The files of these patients were scanned and the stage of disease and treatment received were obtained along with demographic data such as age and gender.

Results: Overexpression of claudins 1, 4, and 7 in non-neoplastic surface epithelium was found in 14 (77.7%), 16 (88.8%), and 10 (55.5%) cases respectively; in dysplastic surface epithelium overexpression was found in 8 (44.4%),

13 (72.2%), and 4 (22.2%) cases, respectively; and in invasive tumor areas overexpression was found in 13 (72.2%), 9 (50%), and 10 (55.6%) cases respectively. Increased claudin 4 expression was related to advanced stage ($p=0.014$). There was a significant relationship determined between claudin 4 and 7 expression and decreased survival ($p=0.018$, $p=0.024$, respectively).

Conclusion: The fact that a statistically significant relationship was found between claudin 4 expression and advanced stage, and similarly a statistically significant relationship was found between claudin 4 & 7 expression and decreased survival gives rise to thoughts that especially claudin 4 and 7 could have different tumorigenic effects on nasopharyngeal carcinoma besides their known adhesion characteristics.

Key words: Claudin 1, Claudin 4, Claudin 7, nasopharyngeal cancer, prognosis, tight junctions

Introduction

TJs are important for the polarity of epithelial and endothelial cells and perpetuation of the barrier function. As a result of the intense investigation performed on the molecular architecture of TJs, the claudin protein family is known to be an important component of tight junctions. The claudin family has 24 known members which have specific dispersion patterns [1,2]. Claudins 1, 4, 5, 7, 8, 11, 14, and 19 are impermeability claudins and increments in their expression strengthen the TJs between the epithelial cells [3-6]. Claudins 2,

10, and 16 are pore-forming claudins and an increment in their expression weakens the TJs among epithelial cells [7]. Other claudins (claudins 3, 6, 9, 12, 13, 15, 17, and 18) are able to form paracellular anion and cation pores and water channels [1,5].

Cell-to-cell adhesion loss is important in transformation and gain of metastatic potential [8]. It has recently been shown that the claudin protein family plays a role in a series of pathological events in carcinoma development [9].

Claudins 1, 4, and 7 are important members of the claudin protein family. It has been demon-

strated that their expression changes in many malignancies [9].

Even though epithelial, lymphoid, mesenchymal, and neurogenic tumors can be seen in the nasopharynx, the most common nasopharyngeal tumors are nasopharyngeal carcinomas (NFC). NFC accounts for 0.6% of all cancers. Even though there is an epidemiologically different racial and geographic distribution of NFC, Epstein-Barr virus (EBV), smoking, nitrosamines in foods, exposure to formaldehydes, some chemical powders, and exposure to radiation are important factors in the etiology of NFC [10]. Despite the knowledge of these factors in NFC, the molecular mechanisms that cause the formation and development of the disease are not currently understood. In our study, the expression patterns of claudin 1, 4, and 7 and their relationship with prognosis were determined.

Methods

Selection of patients

Cases of NFC that contained non-neoplastic surface epithelium and dysplastic surface epithelium in addition to tumor tissue were chosen from biopsy samples that were selected from the cases followed up at the Antalya Education and Research Hospital between January 2009 and October 2013 and histopathologically diagnosed with NFC according to endoscopic biopsy materials. Patients whose imaging and clinical staging were completed were staged histopathologically again according to the 7th edition of the American Joint Committee on Cancer (AJCC). Eighteen representative samples were chosen. Expression of claudins 1, 4, and 7 was analyzed via immunohistochemistry. The files of these patients were scanned and demographic data such as age and gender, stage of disease and treatment information were registered.

Tissue preparation and immunohistochemical staining

Biopsy materials obtained just after nasopharynx surgery were immediately fixed in 10% formaldehyde and embedded in paraffin. Then, 4- μ m thick sections were obtained from paraffin blocks and were stained with hematoxylin-eosin for initial assessment. Immunohistochemical staining was applied on cross sections containing nominal tumor samples that were evaluated with hematoxylin-eosin staining. Cross sections 4- μ m thick prepared for immunohistochemical staining were deparaffinized in oven at 60°C for 2 hrs. Afterwards, they were kept in xylene for 30 min and 100% alcohol for 30 min, and washed with tap water. Sections were kept in a buffered solution with 10% citrate in the microwave at maximum power (800 W) for 15 min. Then, the power was decreased by half and samples were kept in the

microwave for an additional 20 min. Sections were brought out of the microwave and kept at room temperature for 20 min. Endogenous peroxidase activity was neutralized by incubation in 3% hydrogen peroxide for 10 min. Sections were washed with phosphate-buffered saline (PBS) and were kept in protein blocking solution for 10 min after washing with PBS 5 ml \times 3. Then, sections were incubated with primary antibodies against claudin 1 (rabbit polyclonal, clone ab15098, dilution 1:200, Abcam, Cambridge, MA, USA), claudin 4 (rabbit polyclonal, clone ab15104, dilution 1:200, Abcam) and claudin 7 (rabbit polyclonal, clone ab27487, dilution 1:200, Abcam) for 60 min, after which they were washed in PBS for 5 min. Afterwards, they were treated with biotinylated secondary antibody (Vector Laboratories, Burlingame, CA) for 20 min and washed in PBS for 5 min. Sections were then incubated with peroxidase-conjugated antibody for 20 min, and then washed in PBS for 5 min and were kept in chromogene (DAB) for 5 min. Sections were washed under tap water and then counterstained with hematoxylin, dehydrated, dried and covered with Entellan.

Microscopic examination of hematoxylin/eosin-stained sections

In all cases, tumor type and stage were determined according to 7th edition of AJCC. Also, lymphovascular invasion (LVI), perineural invasion (PNI), peritumoral lymphocytic response (PT-LR), intratumoral lymphocytic response (IT-LR), and necrosis were determined.

Evaluation of immunohistochemically stained sections

Expression of claudins 1, 4, and 7 were determined in NFC cases via immunohistochemistry. For each of the claudins, staining was membranous and if the staining was strong, concomitant cytoplasmic staining was observed. Only membranous staining was classified as positive. The evaluation was performed according to the method of Hsueh et al. [11]. Based on this, the expression of claudins 1, 4, and 7 was assessed by semi-quantitative scoring of the extent and intensity of the staining. The staining extent was represented by the percentage of positively stained tumor cells and graded as <10% (1+), 10–50% (2+), or >50% (3+). The staining intensity was recorded as absent (0), weak (1+), moderate (2+), or strong (3+) (Figure 1). The two scores were multiplied together to give a final score of 0-9. The staining scores were grouped as low (final score 0–2) and high (final score 3–9). The entire sections were examined by an expert pathologist who was blinded to all clinicopathological information.

Statistics

Statistical analyses were made using SPSS software for Windows 15.0. Suitability of variables to normal dispersion was observed by using visual (histograms and probability graphics) and analytical methods

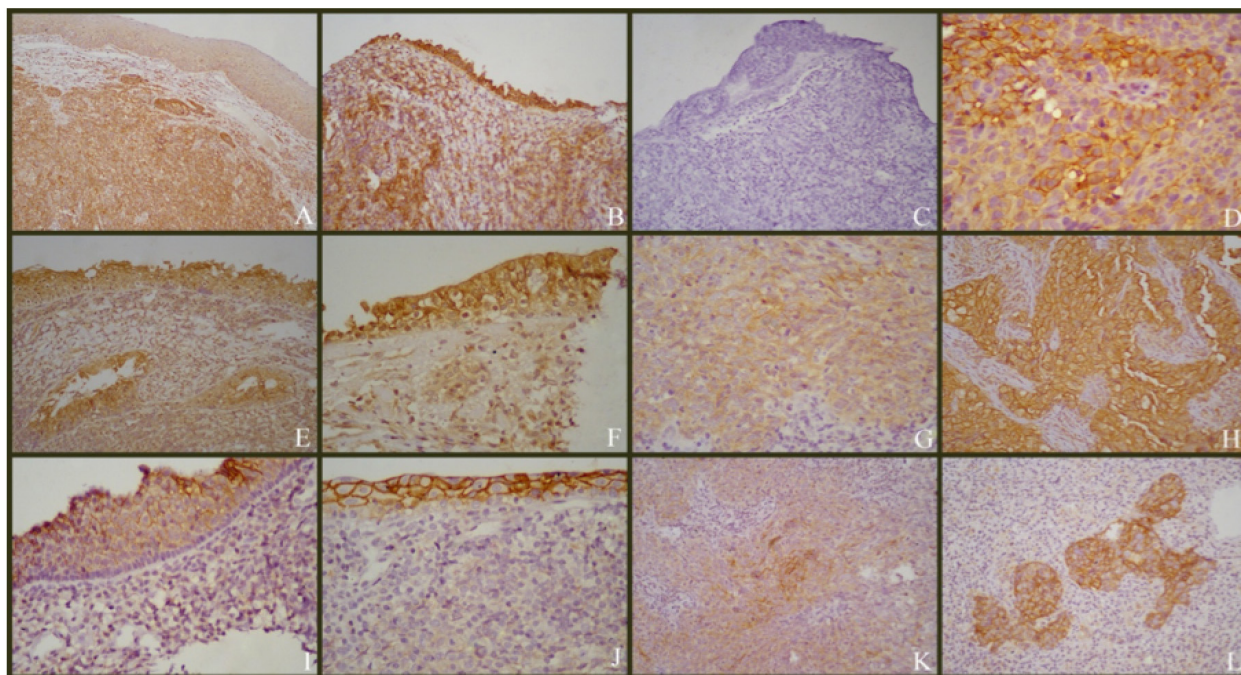


Figure 1. **A:** Strong Claudin 1 positivity in carcinoma, whereas there is no staining in non-neoplastic surface epithelium (Claudin 1 $\times 40$); **B:** Strong Claudin 1 positivity in dysplastic surface epithelium and in carcinoma (Claudin 1 $\times 100$); **C:** Complete loss of Claudin 1 in non-neoplastic surface epithelium and in carcinoma (Claudin 1 $\times 40$); **D:** Strong Claudin 1 expression in carcinoma (Claudin 1 $\times 400$); **E:** Strong Claudin 4 positivity in non-neoplastic epithelium (Claudin 4 $\times 200$); **F:** Strong Claudin 4 positivity in dysplastic surface epithelium (Claudin 4 $\times 200$); **G:** Weak Claudin 4 positivity in carcinoma (Claudin 4 $\times 400$); **H:** Strong Claudin 4 expression in carcinoma (Claudin 4 $\times 400$); **I:** Strong Claudin 7 positivity in non-neoplastic surface epithelium (Claudin 7 $\times 400$); **J:** Strong Claudin 7 positivity in dysplastic surface epithelium (Claudin 7 $\times 200$); **K:** Moderate staining with Claudin 7 in carcinoma (Claudin 7 $\times 200$); **L:** Strong Claudin 7 positivity in carcinoma (Claudin 7 $\times 200$).

(Kolmogorov-Smirnov/Shapiro-Wilk tests). In the Kolmogorov-Smirnov test, a p -value >0.05 was considered as normal dispersion. Differences between groups were assessed by using the Chi-square and Mann-Whitney tests. The relationship of each immunohistochemically positive and negative result with survival was determined by Kaplan-Meier survival analysis and statistical differences were assessed with log-rank test. A p value <0.05 was considered as statistically significant.

Results

Clinicopathological characteristics

A total of 18 patients, 5 of whom (27.8%) were female and 13 (72.2%) male were included in the study. The patient mean age was 54.1 ± 11.8 years (range 27–72). Keratinized squamous cell carcinoma was observed in 2 patients (11.1%) and non-keratinized squamous cell carcinoma in 16 (88.9%). When the specimens were evaluated in terms of histological grade, low grade tumor was observed in 9 patients (50%) and high grade in 9 (50%). LVI was found in 8 (44.4%) patients, PT-LR in 14 (77.8%), IT-LR in 8 (44.4%), and necrosis in 8 (44.4%) patients. Staging information was available in 13 patients. Stage 2a and 2b were deter-

mined in 1 patient each, stage 3 and stage 4a were determined in 2 patients each, stage 4b was in 1 patient and stage 4c was determined in 6 patients (Table 1).

Immunohistochemical study findings

A similar staining profile was found in non-neoplastic squamous or ciliated columnar surface epithelium. In non-neoplastic surface epithelium, high expression of claudins 1, 4, and 7 was found in 14 (77.7%), 16 (88.8%), and 10 cases (55.5%), respectively. In dysplastic surface epithelium, claudin 1 and 4 staining was generally variable; however, even though claudin 7 staining demonstrated similar staining to the tumor cells, it decreased in both tumor and dysplastic epithelium. In dysplastic surface epithelium, high expression of claudins 1, 4, and 7 was found in 8 (44.4%), 13 (72.2%), and 4 (22.2%) cases, respectively. As for tumor cells, there was predominant membranous expression for all three claudins. Cytoplasmic expression was sometimes observed when the staining intensity was strong. The distribution of the staining was usually heterogeneous, but claudin 1 was more often located at the periphery of the

Table 1. Patient characteristics

Characteristics	Patients N	%
Gender		
Female	5	27.8
Male	13	72.2
Age		
Median	54.1	
Range	27-72	
Tumor grade		
Low	9	50
High	9	50
Stage		
2a	1	5.6
2b	1	5.6
3	2	11.1
4a	2	11.1
4b	1	5.6
4c	6	33.3
Missing	5	27.7

Table 2. Immunohistochemical expression patterns of claudins

Expression	N	%
Claudin 1		
Low	5	27.8
High	13	72.2
Claudin 4		
Low	9	50.0
High	9	50.0
Claudin 7		
Low	8	44.4
High	10	55.6

tumor cell islands compared to claudins 4 and 7. Generally, expression of claudins 1 and 4 was similar in tumor cells compared to non-neoplastic epithelium or was more severe in tumor cells, whereas claudin 7 staining was absent or less when compared to non-neoplastic epithelium. Claudin 1 expression was evaluated as negative in 1 (5.6%) patient, 1+ in 4 (22.2%), 2+ in 2 (37.1%), and 3+ in 11 (61.1%) patients. The final claudin 1 score was determined as low in 5 (27.8%) patients and high in 13 (72.2%). Staining intensity was 1+ in 7 (38.9%) patients, 2+ in 3 (16.7%), and 3+ in 8 (44.4%) patients. The final claudin 4 score was low

in 9 (50%) patients and high in 9 (50%). Claudin 7 expression was negative in 3 (16.7%) patients, 1+ in 3 (16.7%), 2+ in 7 (38.9%), and 3+ in 5 (27.8%). The final claudin 7 score was low in 8 (44.4%) patients and high in 10 (55.6%;Table 2). In Figure 1, immunohistochemical expression patterns in non-neoplastic surface epithelium, dysplastic surface epithelium, and tumors are shown for each of the three claudins.

Correlation between clinical parameters and expression of claudins

There was no significant relationship determined between claudin 1 expression and gender, histological type, grade, LVI, PT-LR, IT-LR, necrosis, or stage ($p=0.058$, $p=0.352$, $p=0.599$, $p=0.196$, $p=0.131$, $p=0.889$, $p=0.648$, $p=0.410$, respectively). There was no significant relationship between claudin 4 expression with the same parameters ($p=0.146$, $p=0.599$, $p=0.134$, $p=0.637$, $p=0.343$, $p=0.287$, $p=0.599$, $p=1.0$, respectively), except advanced disease stage ($p=0.014$). Similarly, no significant relationship was found between increased claudin 4 expression and advanced stage ($p=0.014$). Also, no significant relationship was found between claudin 7 expression and gender, histological type, grade, LVI, PT-LR, IT-LR, necrosis, or stage ($p=0.819$, $p=0.094$, $p=1.0$, $p=0.671$, $p=0.375$, $p=0.196$, $p=0.596$, $p=0.135$, respectively). The mean patient follow-up was 16 ± 15.2 months (median 10.9, range 1.31-52.8). The mean overall survival time was 28.8 ± 5.7 months (95% CI: 17.4-40.1). Survival was not related to claudin 1 expression ($p=0.7$). However, a significant negative relationship was observed between increments in claudin 4 and 7 expression and survival ($p=0.018$, $p=0.024$, respectively;Table 3).

Discussion

In our study, there was no correlation between claudin 1 expression and clinicopathological factors, whereas there was a statistically significant relationship determined between claudin 4 overexpression and advanced stage, as well as between claudin 4 and 7 overexpression and decreased survival.

Claudin expression loss causes suppression of TJ functions and accordingly it plays a role in carcinogenesis by causing cell proliferation, motility, and invasiveness in cancer cells [9]. In a study in NFC cell culture, it was demonstrated that claudin 1 expression was increased in NFC cell lines under fluorouracil (5-FU) treatment and

Table 3. Statistical correlation between clinical parameters and claudins

Clinical parameters	Claudin 1	Claudin 4	Claudin 7
	<i>p</i> value	<i>p</i> value	<i>p</i> value
Gender	0.058	0.146	0.819
Histological type	0.352	0.599	0.094
Grade	0.599	0.134	1.0
LVI	0.196	0.637	0.671
PT-LR	0.131	0.343	0.375
IT-LR	0.889	0.287	0.196
Necrosis	0.648	0.599	0.596
Stage	0.410	1.0	0.135
Survival	0.70	0.018	0.024

LVI: lymphovascular invasion, PT-LR: peritumoral lymphocytic response, IT-LR: intratumoral lymphocytic response

decreased apoptosis [12]. Claudin 1 interacts with ZO 1 (Zona Occludens 1) and affects other signaling pathways, thus causing neoplastic transformation [13]. Again, it was demonstrated that increased claudin 1 expression prevented NFC cell line apoptosis [12].

One of the possible mechanisms is that claudins affect the neoplastic transformation through matrix metalloproteinases (MMPs). Upregulation of claudin 1 in oral squamous cell carcinoma enhances invasion via the activation of MMP-2 and MMP-1, and overexpression of claudins 3 and 4 in ovarian surface epithelial cells promotes invasion by increasing MMP-2 activity [14,15].

Another hypothesis is through the alteration of the mixing ratios of claudin proteins because the barrier function of TJs is controlled by a specific combination of claudins. This is supported by the observation that upregulated claudin 2 decreases the tightness of TJ strands with resultant leakage in Madin-Darby canine kidney I cells [16].

There are studies demonstrating that claudin expression loss, rather than overexpression, also plays a role in carcinogenesis. In invasive ductal carcinomas, head and neck cancers, and metastatic breast cancers, claudin 7 expression was found to be decreased [17-19].

Claudin overexpression or loss of expression varies in different cancer types. In hepatocellular carcinoma and renal cell carcinomas, claudin 4 and 5 expression is lost, whereas claudin 3 and 4 overexpression increases in various cancer types such as pancreatic ductal adenocarcinoma and bladder, uterine, ovarian, and breast cancers [20-22]. A low release of claudin 2 was shown in breast and bladder carcinomas, whereas claudin 1 and 7 expression, which is not possible to detect in nor-

mal cervical squamous epithelium, increased in cervical neoplasia [23].

Claudin expression in NFC has been examined in a few studies only. In a study where claudin expression was assessed in 18 patients with EBV-related non-keratinized NFC, claudin 1 and 4 expression was positive in all patients [24]. In our study, claudin 1 negativity was determined in only one patient, whereas claudin 4 was determined positive in all patients.

Hsueh et al. [11] studied claudins 1, 4, and 7 in NFC cases, similar to our study. They demonstrated claudin 1, 4, and 7 overexpression in non-neoplastic surface epithelium in 29.6, 57.9, and 69.6% of the cases, respectively. In dysplastic epithelium, they found claudin 1, 4, and 7 overexpression in 42.9, 71.4, and 7.1% of the cases, respectively. We determined claudin 7 expression at a higher ratio compared to this study both in non-neoplastic surface epithelium and dysplastic epithelium. In the tumor, high claudin 1, 4, and 7 expression was determined in 72.2, 88.1, and 17.6% of the cases respectively. According to our study, claudin 7 expression was found to be at a lower ratio when compared to that study. The fact that the patients involved in the Hsueh et al. study had a higher ratio of non-keratinized-type NFC and that the antibodies used were different might be the reason for this difference. Claudin 1 expression was not found to be related to any of the clinical factors, similar to our study [11]. Claudin 4 and 7 were found to be related to stage and distant metastasis in the Hsueh et al. study, whereas only claudin 4 was related with stage and metastasis in our study.

Conclusion

The main function of claudin family members is cell-to-cell adhesion. Because of this, a decrease in the cellular expression of claudins is thought to cause increase in motility in tumor cells and therefore facilitates their invasion capacity. However, in some cancer types, expression of the claudin family is increased. Some claudin family members may cause decrease in the invasion ability of tumor cells, and increased expression can cause decreased apoptosis in some cancer types and also increased cell survival in some cancer types. This indicates that claudins have other important functions besides their known adhesion functions. In our study, the fact that increased claudin 4 expression had a statistically significant relationship with advanced stage and that clau-

din 4 and 7 expression had a statistically significant relationship with decreased survival may indicate that, especially claudins 4 and 7, have different tumorigenic effects besides their known

adhesion attributes. Owing to this, we believe that these specific members of the claudin family are promising prognostic molecular indicators in nasopharyngeal cancer cases.

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