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ORIGINAL ARTICLE

The expression of Claudin-4 in gastric cancer tissue: A single center experience

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Summary

Purpose: *Gastric cancer (GC) is still one of the most common malignancies with the majority of the tumors diagnosed at advanced stage. The need for identification of prognostic and early detection biomarkers is thus compulsory. Claudins are biomarkers that are currently evaluated in the literature in the frame of epithelial-mesenchymal transition. The purpose of this investigation was to study the expression of claudin-4 in the various histological subtypes of GC and to evaluate its prognostic value.*

Methods: *This investigation was performed on gastric tumors obtained from 66 (46 men and 20 women) patients with documented gastric adenocarcinoma who underwent total or partial gastrectomy and regional lymphadenectomy from 2003 till 2011. Features such as tumor size, depth of invasion, grade and histological subtype, lymphovascular space invasion and regional lymph nodes involvement were also evaluated. Immunohistochemistry (IHC) was used for assessing the expression of claudin-4 with a semi-quantitative model.*

Results: *66.7% of our cases showed abnormal claudin-4 expression in IHC. Claudin-4 was significantly correlated with tumor T stage and with intestinal type classification. The correlation of claudin-4 tissue expression with patient overall survival survival (OS) or disease-free survival (DFS) was not statistically significant, as well as with age, gender, tumor N stage, grade, TNM stage, positive lymph node ratio or lymphovascular invasion.*

Conclusions: *Literature stands equivocal about the exact role and prognostic value of claudin-4 and histopathology and tumor invasiveness in patients with GC. Our results further strengthen the need of larger studies to fully elucidate the predictive role of claudin-4 in the natural history of GC.*

Key words: *gastric adenocarcinoma, claudin-4, claudins, epithelial–mesenchymal transition, immunohistochemistry, survival*

Introduction

Despite the emerging data that indicate a declining incidence and mortality, GC is still one of the most common malignancies in the world causing around 738,000 deaths worldwide [1]. Unfortunately the majority of these cancers at the time of diagnosis are at advanced stage and the treatment options are limited. Invasion and metastasis, the major causes of GC-related relapse and death, greatly impede the treatment efficiency [2]. A better understanding of the mechanism contributing to GC initiation and progression is warranting with the hope to improve early diag-

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nosis and treatment efficacies. Thus, the need for identification of prognostic and early detection biomarkers, possessing predictive value for survival of GC patients, is compulsory [3].

Claudin proteins play an essential role in the function of tight junction (TJ) and the maintenance of the polarity of epithelial cells, and 24 subtypes of the claudin have been identified [4-6]. For tumor progression and metastasis, the epithelial-to-mesenchymal transition (EMT), which includes loss or redistribution of tight junction proteins such as claudins or E-cadherin, is considered to be an important pathway and trigger mechanism for the ability to progress through the basement membrane [7,8]. Among the many tight junction proteins, claudins are key functional proteins and expression in humans varies in different cells and tissue. Furthermore, claudins have a significant influence on the biological behavior of tumor progression [9,10]. For the claudin protein family, claudin-1, claudin-2, claudin-3, and claudin-4 are frequently expressed in human tissue. Claudin-3 and claudin-4, previously classified as intestinal claudin phenotypes, have an important role in the metastatic pathway [11]. However, previous studies have demonstrated the heterogeneities of claudin immunoactivities in various cancer tissues and the biological function of the claudins has not been clarified.

In this study, we aimed to evaluate the expression pattern of claudin-4 protein in GC tissue.

Methods

Participants

This historical cohort survey was performed on gastric tumors obtained from 66 (46 men and 20 women) patients with documented gastric adenocarcinoma, aged from 27 to 96 years, who underwent radical resection at the Third Department of General Surgery, "Attikon" General Hospital, National and Kapodistrian University of Athens, Athens, Greece in 2003-2011. The study was approved by the Ethics Committee of "Attikon" General Hospital, National and Kapodistrian University of Athens, Athens, Greece, Ref. No 3/5-3-2012. No patient received any neoadjuvant therapy (neither chemotherapy nor radiotherapy). Information about age and gender were obtained from the patient records. Institutional review board approval was obtained through the same permission.

One representative tissue block from each case was used for immunohistochemical evaluation. Following the pathology report, histopathological features of the tumor comprising depth of tumor invasion, grade, histological subtype according to Lauren classification, lymphovascular space invasion and regional lymph node involvement were noted. TNM staging was performed according to the seventh edition of the American Joint Committee on Cancer Staging Manual [12]. Patients were followed-up to June 1st 2015. The clinical endpoints of the study were overall survival (OS) and DFS.

Immunohistochemistry

Staining was performed using the standard streptavidin–biotin–peroxidase complex method with an automated staining system (Autostainer Plus; Dako, Glostrup, Denmark). The tissue sections on the slides were de-paraffinized and rehydrated. For antigen retrieval, the slides were heated in a microwave oven for 15 min in 10 mM citrate buffer (pH 6.0). Endogenous peroxidase was blocked with 3% hydrogen peroxide for 20 min. The slides were then incubated with diluted primary antibody for 30 min at room temperature. After washing with Tris-buffered saline, tissue sections were incubated with biotinylated secondary antibody and then with diaminobenzidine substrate provided in a Dako Envision kit (Dako). Slides were counterstained with Harris hematoxylin. Appropriate positive controls, and negative controls by omitting the primary antibody, were included with each slide run.

Evaluation of samples

Protein expression was assessed using the widely accepted HSCORE system [13]. The evaluation of IHC was performed in a blinded fashion by a single expert observer. The proportion of neoplastic cells featuring a membranous staining throughout the tumor section was assessed using a low-power magnification (x40). The HSCORE was calculated using the following equation: HSCORE = $\sum Pi(I)$, where I represents the staining intensity score (i.e. 0 = no staining, 1 = weak staining, 2 = moderate staining, and 3 = strong staining) and Pi represents the percentage of stained cells (from 0 to 100%). The final HSCORE ranged from 0 to 300 and claudin-4 expression levels were classified as negative and positive using a cut-off value of 30 [13].

Statistics

Statistical analysis was carried out using R language and environment for statistical computing (http://www.R-project.org). In order to describe data, we used the mean ± standard deviation, median (range), or frequency, whereas x^2 test, Fisher's exact test, Student's or Welch's t-test, and Wilcoxon rank-sum test were used to compare results between groups when appropriate. The Kaplan–Meier method was used to estimate the OS and DFS rate, and survival differences were analyzed using log-rank tests. P values <0.05 were considered statistically significant.

Table 1. Clinicopathological data of patients and correlation with overall survival and disease-free survival

Characteristics	*Age, years (mean ±SD)*	*Patients n (%)*	*Overall survival Log-rank p*	*Disease-free survival Log-rank, p*
Gender			0.66	0.22
Male	70.5 (11.7)	46 (69.7)		
Female	70.2 (18.1)	20 (30.3)		
Overall	71.5 (13.8)	66 (100)		
Age, years			0.62	0.70
>70		36 (54.5)		
≤70		30 (45.5)		
Histological tumor location			0.01	0.98
Gastroesophageal junction		8 (12.1)		
Body		32 (48.5)		
Antrum		26 (39.4)		
pT *			<0.01	0.25
T1		11 (16.9)		
T2		8 (12.3)		
T3		19 (29.2)		
T4		27 (46.5)		
pN *			0.01	<0.01
N0		19 (29.2)		
N1		8 (12.3)		
N2		16 (24.6)		
N3		22 (33.8)		
Positive lymph node ratio*			<0.01	<0.01
Level of lymph node dissection*			0.02	0.20
D1		39 (60.0)		
D1+		18 (27.7)		
D2		8 (12.3)		
M*			0.01	0.24
M0		58 (87.9)		
M1		7 (12.1)		
Grade**			<0.01	0.47
1		2 (3.7)		
2		18 (32.7)		
3		35 (63.6)		
Stage*			<0.01	0.01
I		14 (21.5)		
II		13 (20.0)		
III		31 (47.7)		
IV		7 (10.8)		
Lauren classification***			0.07	0.81
Instestinal		27 (50.0)		
Diffuse		18 (33.3)		
Mixed		9 (16.7)		
Resection*			<0.01	0.88
R0		56 (86.2)		
R1		4 (6.2)		
R2		5 (7.7)		

* excluding 1 missing , ** excluding 11 missing, *** excluding 12 missing

Results

In the group of 66 patients with GC, men outnumbered women with a male/female ratio of 2.3:1. The mean age was 71.5±13.8 years (range 27-96).

The median follow-up period was 59.1 months with a total of 65198 person-days included in the study. At the end of follow-up, 4 patients were lost (6.1%), 15 (22.7%) were alive and 47 dead, indicating a disease-specific mortality of 71.2% and an overall median survival of 32.6 months. The median censorship interval was 29.6 months.

As far as the location of the tumors is concerned, 32 lesions were found at the body of the stomach, 26 at the antrum and 8 at the gastroesophageal junction (Siewert III). Consequently, 2 patients underwent central gastrectomy, 43 underwent subtotal gastrectomy, and 21 underwent total gastrectomy.

Histologically, all GC were adenocarcinomas. TNM stage, grade, resection status, histological subtype according to Lauren classification and extent of lymph node dissection are shown in Table 1.

OS rates were negatively correlated with T stage at diagnosis (p<0.01), N stage (p=0.01), positive lymph node ratio (p<0.01), M (p<0.01), TNM stage (p<0.01), grade (p<0.01) and the absence of R0 resection (p<0.01). DFS rates were affected by N stage at diagnosis (p<0.01) and positive lymph node ratio (p<0.01).

Immunohistochemistry data analysis

This study analyzed 63 surgical specimens (3 cases lacked staining). Of those, 42 cases (66.7%) showed abnormal claudin-4 expression in IHC. The correlation of claudin-4 tissue expression with patient OS or DFS was not statistically significant, as well as with age, gender, tumor N stage, grade, TNM stage, positive lymph node ratio or lymphovascular invasion. Of interest though was the finding that claudin-4 expression was significantly correlated with T stage and Lauren classification (p=0.04 for both comparisons) (Table 2).

Table 2. Claudin-4 positivity (>30) and correlation with clinicopathological data, overall survival and disease-free survival (n=63)

Characteristics	*OR / HR (95% CI)*	*Log rank, p*
Gender (female)	1.11 (0.86 – 1.43)	0.63
Age > 70	1.02 (0.81 – 1.30)	1.00
pT		0.04
T1	(reference)	
T2	1.45 (1.04 – 2.25)	
T3	1.27 (1.08 – 1.82)	
T4	1.15 (1.01 – 1.62)	
pN		0.57
N0	(reference)	
N1	0.80 (0.54 – 1.19)	
N2	0.91 (0.66 – 1.25)	
N3	1.03 (0.76 – 1.39)	
Grade		0.16
G1	(reference)	
G2	0.85 (0.33 – 2.15)	
G3	0.66 (0.26 – 1.66)	
Stage		0.27
I	(reference)	
II	1.26 (0.88 – 1.80)	
III	1.08 (0.79 – 1.46)	
IV	0.83 (0.54 – 1.28)	
Lauren classification		0.04
Diffuse	(reference)	
Intestinal	1.68 (1.07 – 1.69)	
Mixed	1.24 (0.86 – 1.78)	
Positive lymph node ratio	1.23 (0.87 – 1.74)	0.34
Lymphovascular invasion	1.02 (0.81 – 1.30)	1.00
Overall survival*	1.01 (0.54 – 1.90)	0.97
Disease-free survival*	1.03 (0.37 – 2.81)	0.96

* hazard ratios calculated using Cox proportional hazards model (multivariate)

Discussion

The epithelial-to-mesenchymal transition (EMT) has been considered an important mechanism for cancer progression and metastasis. For the EMT pathway, tight junction proteins such as claudins, E-cadherin, CD44 and vimentin are important proteins that are required to preserve the integrity of the cell layer and to control cell proliferation [14,15]. The functions of tight junction proteins in tumor progression are complex in action and are associated with multiple interactions with other proteins. However, the loss or downregulation of tight junction proteins in cancer cells has been reported in previous studies [16,17].

The expression rate and clinicopathologic correlation of E-cadherin expression has been frequently reported for gastric cancer and other malignancies [16]. However, expression of claudin family proteins has been rarely reported and

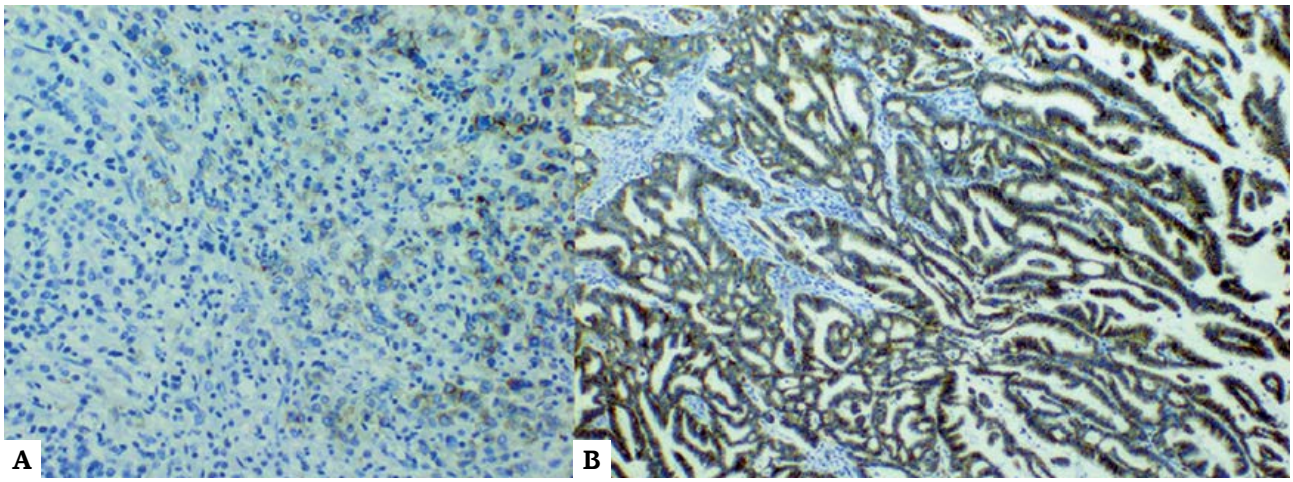

Figure 1. A: Immunohistochemical staining indicative of low claudin-4 expression in diffuse type (x200). **B:** Immunohistochemical staining indicating high claudin-4 expression in well differentiated intestinal type (x200).

outcomes have been limited to evaluation of one or two claudin proteins. Furthermore, the expression of claudin has been reported for numerous types of malignancies such as breast, pancreas, liver, and esophagus in previous studies [16-20]. Studies over the function of claudin-4 have not provided consistent results. It was reported that claudin-4 expression was significantly correlated with improved rates of patient survival in GC [21]. However, Resnick et al. [18] suggested that moderate to strong staining for claudin-4 in GC was associated with decreased survival rates.

Claudins, however, could be clinically considered as feasible molecular markers for targeting progression or metastasis in GC. Matsuda et al. have proposed a novel three-phenotype classification according to the type of claudin expression in GC [7,11]. In another study, immunohistochemical staining was performed for four subtypes of the claudin family (claudin-1, claudin-2, claudin-3, and claudin-4); expression patterns were evaluated and the probability of interaction was predicted [21]. Though the sample size was small compared with other single claudin studies, variable expression of claudin family members was demonstrated in GC specimens. The lowest frequency of expression was seen for claudin-4 (44.4%) and the most prominent expression was seen for claudin 2 (73.6%) in the GC specimens [21]. Satake et al. [22] have reported that claudin-3 and claudin-4 have been pathologically classified as intestinal claudins, with expression not only in the intestinal metaplasia of gastric mucosa but also in GC, with regulation of the proteins by Cdx2. Similarly, another study demonstrated that claudin-4 expression was present in only 15.9% normal gastric samples, but expression of claudin-4 in the intestinal metaplasia lesions and dysplasia lesions was 90.5 and 95.2%, respectively [23]. In another study, the expression rates of claudin-3 (51.4%) and claudin-4 (44.4%) were lower compared with the two other claudins [21]. However, expression of claudin-3 and claudin-4 was significantly weaker for cases with positive lymphatic invasion [21]. It is suggested that claudin-3 and claudin-4 may be involved as important proteins during the lymphatic invasion process in GC.

Our study added another brick in the wall of emerging knowledge on the field, since we confirmed the equivocity of the role of claudin-4 expression and the survival of patients with GC. In another study, tumors expressing claudin-4 were only related to good prognosis in 4-year OS results [21]. The expression of claudin-4 was significantly associated with histological differentiation ($p<0.001$) and tumor growth patterns ($p<0.001$) but not associated with patient survival [23]. However, intermediate type staining of claudin-4 exhibited a trend of correlation with patients' survival ($p=0.023$). The 5-year OS survival rate with low expression of claudin-4 in intermediate type (76.4%) was similar to the expanding type (64.5%), while the high expression group (46.6%) was closer to the infiltrative type (50.7%) [23]. In the same frame, claudin-4 expression was significantly decreased in tumors with undifferentiated type adenocarcinoma, advanced T stage, lymph node metastasis, and peritoneal metastasis. OS was significantly shorter in patients with low claudin-4 expression. Cox multivariate analysis revealed that low claudin-4 expression was independently associated with significantly decreased OS [24].

The present study demonstrated a relatively

high claudin-4 expression in patients with GC, especially in those with intestinal type. A recent study demonstrated a lower expression of claudin-4 (47.3% of GC patients). Low expression of claudin-4 was related to poorly differentiated type (p=0.001), non-intestinal (diffuse) type (p=0.001), deeper tumor invasion (p<0.001), lymph node metastasis (p=0.001), higher stage (p=0.001) and lymphovascular invasion (p=0.009) [25]. Similar to our results, Kuo et al. [26] found that the overexpression of claudin-4 was greater in the intestinal type rather than in the diffuse type GC. A trend was observed between the overexpression of claudin-4 and lymph node metastasis, however, this association was not statistically significant. The results showed that the expression of claudin-4 was lower in the diffuse type GC. Possibly it played a role in determining the diffuse phenotype and loose cohesion of cells in diffuse type GC in a similar manner as E-cadherin [14]. Contrasting our results, Tokuhara et al. demonstrated that the expression rate of claudin-4 in poorly differentiated GC was comparable to that of the well-to-moderately well differentiated gastric adenocarcinomas; therefore, claudin-4 was not significantly associated with any clinicopathological factors [27].

Hwang et al. [28,29] showed that the expression of claudin-4 in GC cells was found to be correlated with increased cell invasion and migration. Moreover, the claudin-4 expression was found to be related to increased matrix metalloproteinase (MMP)-2 and -9 expression, indicating that claudin-mediated increased invasion may be mediated through the activation of the MMP proteins [28,29]. Overall, the results suggest that claudin-4 overexpression may promote GC metastasis through the increased invasion of gastric cancer cells [28,29].

A recent meta-analysis included 9 studies with a total of 1265 GC patients. Overall, the pooled results showed that overexpression of claudin-4 was associated with poor survival in GC patients. Overexpression of claudin-4 was also associated with advanced stage and lymph node metastasis [30]. In the same tune, another meta-analysis included 14 studies containing 2,106 patients with GC. The overall analysis showed that claudin-4 expression was associated with increasing pT category, tumor size, and lymph node metastasis in patients with GC [31]. Additionally, claudin-4 expression was associated with histological differentiation as well as gender and age. This meta-analysis found no significant association between claudin-4 expression and prognosis for OS in patients with GC [31].

Conclusions

In conclusion, the patterns of claudin-4 expression in GC were diverse and controversial. The reasons for the upregulation or downregulation in gastric tumorigenesis were unclear. Literature stands equivocal about the exact role and prognostic value of claudin-4 and histopathology and tumor invasiveness in patients with GC. Our results further strengthen the need of larger studies to fully elucidate the predictive role of claudin-4 in the natural history of GC.

Author contributions

Schizas D, Alexandrou A and Liakakos T designed the study; Schizas D, Moris D, Michalinos A and Kanavidis P collected the patient's clinical data; Moris D, Schizas D, Dimitrokallis N, Misiakos E and Kanavidis P analyzed the data and wrote the paper, and Liakakos T supervised the manuscript. Moris D and Schizas D contributed equally to this work.

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Conflict of interests

The authors declare no confict of interests.

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