Original Paper

Immunoexpression of Claudin 4 in Gastric Adenocarcinomas

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ABSTRACT: Disturbance of the intercellular adhesion system represents a basic biomolecular mechanism in gastric carcinogenesis. Claudin 4 is member of a protein family involved in maintaining homeostasis and epithelial integrity. In this study, we analyzed the immunoexpression of Claudin 4 in 58 cases of gastric adenocarcinomas, in relation to the main histopathological parameters of aggressiveness, the reactions obtained being evaluated through the intensity of the reactions and the number of positive cells. Positive membranous reactions of Claudin 4 were observed in all cases, in tumor cells and some stromal elements, but in some high grade gastric adenocarcinomas also cytoplasmic immunostaining was present. Claudin 4 high scores were associated with tubular, tubulopapillary and hepatoid adenocarcinomas, of low grade and in early stages, aspects that suggest the usefulness of the marker in evaluating the aggressiveness of gastric epithelial tumors.

KEYWORDS: *Gastric adenocarcinomas, Claudin 4, histopathological parameters.*

Introduction

Gastric carcinomas represent one of the most frequent human malignant neoplasms in the world, being reported in the year 2020 more than 1 million new cases [1].

Also, these malignant gastric tumors represent a problem through the increased mortality rate, gastric carcinomas being the fourth most lethal type of cancer globally, responsible for approximately 769,000 deaths [2].

Although the incidence has decreased for the general population, in recent years it has been identified an increase in patients under 50 years [1].

Among the neoplasias identified at the gastric level, the most common are gastric adenocarcinomas, associated with a high incidence, progression, metastasis rate, as well as a poor prognosis [3], representing a problem in the health field and a major challenge for the efficiency of diagnostic and treatment methods and protocols [2].

Numerous studies have focused on understanding the biomolecular mechanisms involved in carcinogenesis, respectively in the disruption of intercellular adhesion in carcinogenesis in general, including at the gastric level, and adhesion molecules are complex proteins that play a central role in maintaining tissue integrity [3,4].

Claudins are adhesion proteins expressed on the surface of tumor cells and some stromal elements, important components in maintaining the structure and function of the epithelium, and the reduction of immunoexpression for these proteins is correlated with disruptions of intercellular connections, destabilizing the tissue and, thus, favoring tumor progression [3,5,6].

In recent years, numerous studies have demonstrated the alteration of claudin immunoexpression in various malignant epithelial tumors (mammary gland, pancreas, liver, esophagus, and ovary) [5-7], including Claudin 4, which is the most expressed protein in some malignant tumors [8].

Claudin 4 seems to be involved in biomolecular processes that control proliferation, cell migration and apoptosis in carcinogenesis [5-7].

However, the results and significance of Claudin 4 immunoexpression in gastric adenocarcinomas are controversial [3,9].

In this study we analyzed the immunoexpression of Claudin 4 in gastric adenocarcinomas in relation to the aggressiveness parameters.

Material and Methods

The study included 58 cases of gastric adenocarcinomas from patients admitted to the Clinical Emergency County Hospital Craiova,

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on the General Surgical Departments, over a period of 4 years (2017-2020).

The biological material was represented by specimens of total gastrectomy, fixed in 10% buffered formalin, processed by the classical histopathological technique and stained with hematoxylin and eosin. The criterion for inclusion in the study was represented by the diagnosis of primitive gastric adenocarcinoma, without other tumor history or oncological treatment.

The diagnosis was made in the Pathology Department within the Clinical Emergency County Hospital Craiova and the lesions were classified according to the latest classification for tumors of the digestive system, developed by the specialized working group within the WHO (World Health Organization) [10].

In this study, the immunoexpression of Claudin 4 was followed in relation to the aggressiveness parameters of gastric adenocarcinomas, represented by the histopathological type, the tumor grade and the tumor stage.

For immunohistochemical reactions, there were obtained from the paraffin blocks 3µm sections and mounted on slides with poly-l-lysine. After deparaffinization in xylene, sections were rehydrated and subjected to endogenous enzyme blocking with hydrogen peroxide, nonspecific site blocking with bovine serum albumin, and antigen retrieval by microwave boiling for 20 min, according to the protocols indicated by the manufacturers.

The working system used for polymer amplification was represented by the EnVision™ FLEX+System (code K8002, Dako).

To visualize the reactions, the chromogen DAB (3, 3'-diaminobenzidine tetrahydrochloride) was used. Claudin 4 polyclonal antibody was used in a dilution of 1:150 and the IHC reactions were validated by positive external controls (kidney) and the sections were counterstained with Hematoxylin.

For semiquantitative assessment of immunohistochemical reactions, we used a final staining score (FSS) obtained by multiplying the percentage of labeled cells in a 40x microscopic field (MF) with the intensity of the reaction. There were analyzed 10 MF for each case.

For the number of labeled cells, the score assigned was 1 (5-25% cells), 2 (26-50% cells), 3 (>50% cells), while the score for the intensity of reactions was 1 (weak reaction), 2 (moderate reaction) and 3 (strong reaction).

The FSS had values between 1-9, of which the interval 1-4 establishes low scores, and the interval 6-9 high scores.

The threshold value for positivity was given by the presence of at least 5% immunostained tumor cells, below this value, the reactions being considered negative. The images were obtained with Motic Panthera DL microscope equipped with Motic Images Plus 3.0 ML software.

For the statistical analysis were used comparison tests represented by chi square ($\chi 2$) within the SPSS 10 software (Statistical Package for the Social Sciences), the results being considered significant for values of $p<0.05$. To establish the mean values and standard deviations, there were used numerical values of the markers obtained for all cases, including the negative ones.

In the scientific research, the ethical aspects were respected, based on the informed consent of the patients, the study being approved by the Local Ethics Commission (no. 151/24.09.2021).

Results

The study included 58 patients diagnosed with gastric adenocarcinoma, most of them being males (65.5%), with age between 40-86 years and an average of 68.3±10.4 years.

Most cases were represented by tubular adenocarcinomas (39.7%), high grade (62.1%) and in stage III tumors (51.7%) (Table 1).

Claudin 4 immunoexpression was identified in all investigated cases. The marked cells were observed both at the membrane and cytoplasmic level, as well as in some stromal elements (lymphocytes, macrophages, fibroblasts). Cytoplasmic labelled cells were present in high grade gastric adenocarcinomas, respectively hepatoid, poorly cohesive non-signet-ring cell carcinomas (PCC-NOS), mucinous and tubular types. For the entire analyzed group, the average number of marked tumor cells was 28.53±16.49, the reactions revealed variable intensity and the average final staining score (FSS) value was 3.1.

Table 1. The relation between the histopathological parameters and the average scores of Claudin 4.

Parameters / No. cases/ p value		FSS Claudin 4
Histopathological type	Tubular (23)	3.6
	Tubulopapillary (3)	3.6
	PCC-NOS (6)	1.3
	PCC-SRC (9)	3.3
	Mixed (6)	3
	Mucinous (7)	1.5
	Micropapillary (2)	2.5
	Hepatoid (2)	6.5
	p value	0.291

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Tumor grade	Low (22)	4.2
	High (36)	2.4
	p value	0.053
Tumor stage	I (4)	4.2
	II (18)	3.2
	III (30)	3.1
	IV (6)	2.1
	p value	0.183

In relation to the histopathological type, tubular and tubulopapillary adenocarcinomas had a number of marked cells of 34.7±15.6 and 35±15, variable intensity, and for both lesions the average FSS was 3.6 (Table 1) (Figure 1A, B).

In the case of poorly cohesive carcinomas with signet-ring cell type (PCC-SRC), the number of immunopositive cells was 31.6±11.7, variable intensity and average final score of 3.3 (Table 1) (Figure 1C).

The strongest reactions were observed in gastric hepatoid adenocarcinomas, with a number of 60±14.1 positive cells, moderate and strong intensity and an average FSS score of 6.5 (Figure 1D).

For mixed and micropapillary tumors, the values were 26.6±5.1 and 30±14.1 tumor cells, with weak/moderate intensity and mean FSS of 3, respectively 2.5 (Table 1).

Mucinous and PCC-NOS adenocarcinomas presented a number of marked cells of 10.7±5.3 and 8.3±4, with weak/moderate intensity and average FSS score of 1.5 and 1.3 (Table 1).

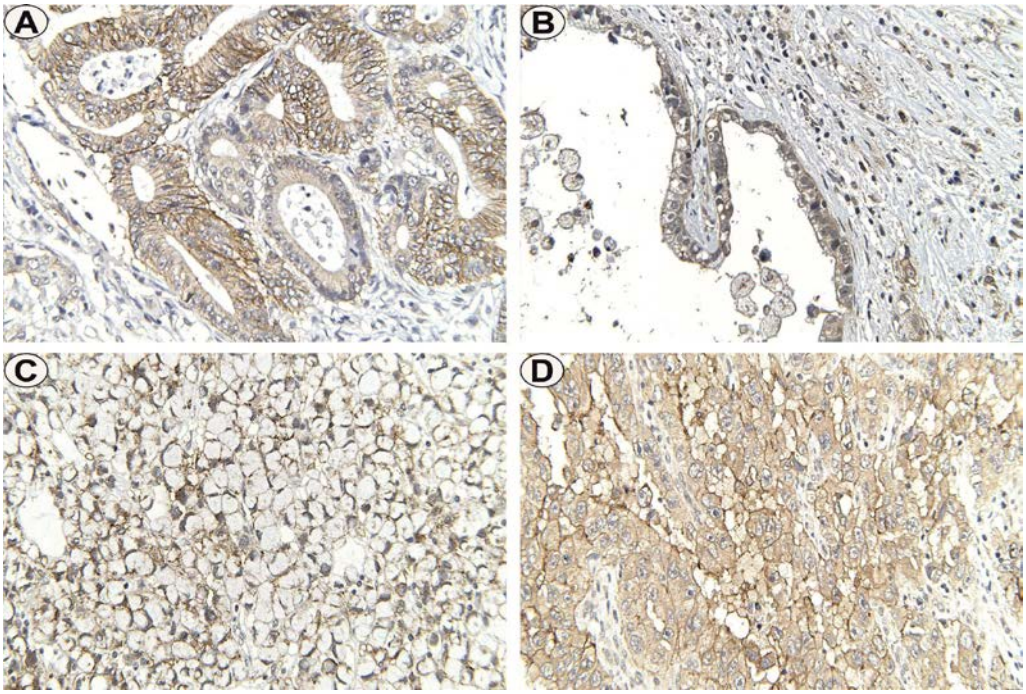

Figure 1. Gastric adenocarcinoma, Claudin 4 immunostaining, x40.
A. Tubular adenocarcinoma. B. Tubulopapillary adenocarcinoma. C. PCC-SRC. D. Hepatoid adenocarcinoma.

Referring to the tumor grade, low grade lesions presented higher values of Claudina 4 immunoexpression, respectively a number of 39±13.2, with variable intensity and average FSS of 4.2 (Figure 2A).

Comparatively, the high grade tumors indicated a number of 22±14.9 marked cells, variable intensity and an average FSS score of 2.4 (Table 1) (Figure 2B).

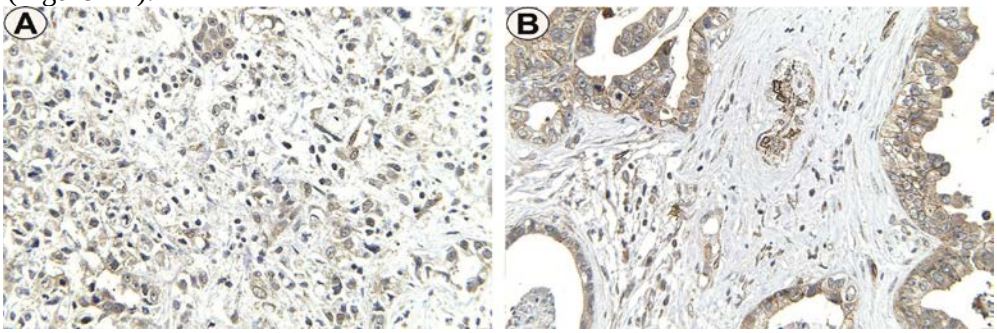

Figure 2. Gastric adenocarcinoma, Claudin 4 immunostaining, x40.
A. Low grade adenocarcinoma. B. High grade adenocarcinoma.

In relation to the tumor stage, gastric adenocarcinomas in stage I had a higher number of positive tumor cells than the other stages, respectively 42.5±25, the intensity of the reactions being variable with an average FSS score of 4.2.

Stages II and III had a number of positive tumor cells of 30±24.4, respectively 27±16.4, variable intensity of reactions, and the average value of the final scores was 3.2 and 3.1 (Table 1).

Gastric adenocarcinomas in stage IV was identified a number of 22.5±14.7 marked cells the intensity of reactions was weak/moderate and the average final score was 2.1 (Table 1).

The statistical analysis of Claudin 4 immunoexpression indicated values at the limit of significance in relation to the tumor grade (p=0.053, $\chi 2$ test), without associations with the histopathological type (p=0.291, $\chi 2$ test), and tumor stage (p=0.183, $\chi 2$ test).

Thus, high scores of Claudin 4 were identified in gastric adenocarcinomas of tubular, tubulopapillary and hepatoid type, of low grade and in the incipient stage (Figure 3A-C).

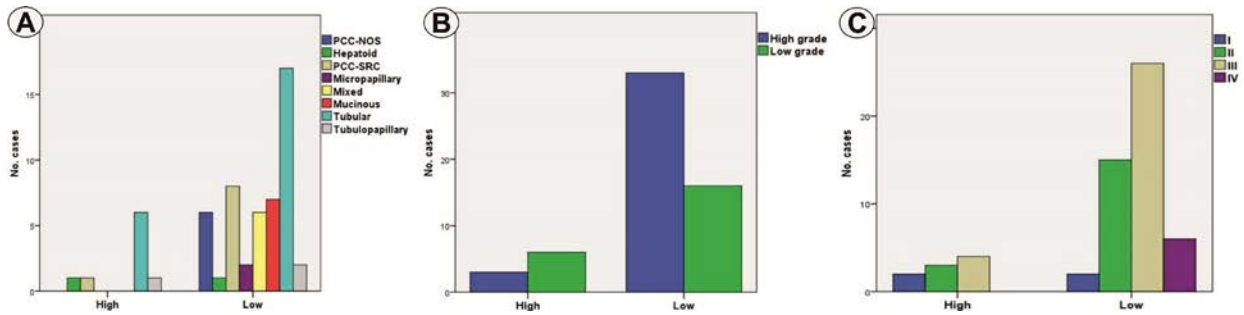

Figure 3. Distribution of cases in relation to Claudin 4 immunoexpression and tumor type (A), tumor grade (B) and tumor stage (C).

Discussions

Gastric adenocarcinomas are responsible for numerous deaths every year, both due to the heterogeneity of the lesions and the late advanced stages diagnosis [3].

Claudins represent one of the most important classes of proteins involved in cell adhesion, and disruption of the expression of these adhesion molecules is correlated with tumor progression [11].

Numerous studies have analyzed the immunoexpression of claudins in different malignant tumors [12,13].

However, there are relatively few studies regarding the immunoexpression of these proteins in gastric adenocarcinomas, and the results regarding the role and importance of these markers in tumor progression and prognosis are limited and inconsistent [11].

Some studies reported different aspects regarding the immunoexpression of Claudin 4 and the survival rate of patients with gastric adenocarcinoma.

Thus, increased values were associated with a low survival rate [5,14], but also with lymphatic metastasis [12,15].

On the other hand, there are studies that observed the association of Claudina 4 immunoexpression with a favorable prognosis [9,15,16].

Regarding the localization of Claudin 4 in the tumor cells, there is few information about this aspect.

The majority of studies indicate the membranous immunoexpression of Claudin 4, but with cytoplasmic immunoreaction of reduced intensity in some cases [17-19].

Although the mechanisms are not fully known, it has been suggested a possible role of claudins in the interaction between the cell and the matrix [18].

Kwon MJ et al. reported that the membranous immunostaining of Claudin 4 was associated with favorable prognosis, and the cytoplasmic immunoreaction had no significant association [19].

In this context, the cytoplasmic and membrane translocation of claudins suggests a possible role as a transcription cofactor involved in tumor development and proliferation [20-22].

In our study, membrane immunoreaction was observed in all cases of gastric adenocarcinomas, although, in the case of high grade gastric adenocarcinomas, hepatoid, PCC-NOS, mucinous and tubular type, there were also present cytoplasmic immunostaining of Claudin 4.

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Most of the studies conducted to analyze the immunoexpression of Claudin 4 in gastric adenocarcinomas, indicated an increased number of positive tumor cells in intestinal type, compared to the diffuse type [23-25].

Some studies carried out on gastric adenocarcinomas have suggested the utility of Claudin 4 as a histopathological differentiation marker between the intestinal and diffuse types, the percentage of positive tumor cells being inferior in diffuse type lesions [26,27].

Also, Kuo WL et al. and Soini Y et al. have suggested a possible role of Claudin 4 in determining the diffuse phenotype of gastric adenocarcinomas, in the sense that the decrese of immunoexpression is correlated with the alteration of cell cohesion, glandular structures and tumor differentiation [24,28,29].

Similar results were also obtained by Resnick MB et al., which reported strong Claudin 4 immunoexpression in intestinal gastric tumors compared to diffuse type, considering the marker useful both in establishing the diagnosis and as a potential therapeutic target [14].

In our study, high scores of Claudin 4 were identified in tubular, tubulopapillary and hepatoid adenocarcinomas.

In relation to the gastric adenocarcinomas grade and tumor stage, the studies are limited and contradictory.

Some authors have reported a significant association between Claudin 4 expression and tumor differentiation in gastric adenocarcinomas.

In poorly differentiated tumors, a low immunoexpression was observed [5,16].

By contrary, Wang H et al. indicated a low expression of Claudin 4 in well-differentiated lesions [30].

In our study, the lowest values of Claudin 4 were identified in high grade adenocarcinomas.

Some authors indicated a significant association of Claudina 4 immunoexpression in relation to the tumor stage [3,31].

Morris D et al. observed in gastric adenocarcinomas in advanced stage a low percentage of immuno-labelled cells.

On the other hand, some studies have reported in advanced stages an increased immunoexpression [9,31].

Finnaly, another study did not identify a significant association between the immunoexpression of Claudin 4 and the tumor stage of gastric adenocarcinomas [17], an aspect similar to the results of our study.

Conclusions

In this study, we observed high scores of Claudin 4 immunoexpression in low grade, incipient stages gastric adenocarcinomas, with tubular and hepatoid architecture.

The marker used can contribute to the evaluation of lesions aggressiveness, in order to improve the stratification criteria for targeted oncological therapy, as well as the prognosis of pacients.

Conflict of interests

None to declare.

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