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Original Article

Tight junction protein claudin 4 in gastric carcinoma and its relation to lymphangiogenic activity

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ABSTRACT

Background and study aims: Gastric cancer is the second most common cause of cancer-related death worldwide. Claudins are a family of tight junction proteins that are biologically relevant in many cancer progression steps. This study aimed to investigate the expression of the intestinal claudin (claudin 4) in gastric carcinoma and to evaluate its relation to the different clinicopathologic prognostic parameters, especially lymphangiogenesis (production of new lymphatic vessels, measured by lymphovascular density (LVD)) and lymphovascular invasion (LVI).

Patients and methods: Fifty-five gastric carcinoma specimens were immunohistochemically stained for claudin 4 and D2-40 (for detection of lymphatic vessel endothelium).

Results: High expression of claudin 4 was detected in 26 of 55 (47.3%) cases. Low expression of claudin 4 was related to poorly differentiated type ($p = 0.001$), non-intestinal (diffuse) type ($p = 0.001$), deeper tumour invasion ($p < 0.001$), lymph node metastasis ($p = 0.001$), and higher stage ($p = 0.001$). In addition, higher LVD was related to poorly differentiated types ($p = 0.001$), non-intestinal type ($p = 0.001$), lymph node metastasis ($p = 0.015$), and higher tumour, node, metastasis (TNM) stage ($p = 0.001$). LVI was related to lymph node metastasis ($p = 0.025$), higher TNM stage ($p = 0.001$), and LVD ($p = 0.001$). Claudin 4 significantly correlated with both LVD ($p = 0.009$) and LVI ($p = 0.009$).

Conclusions: High expression of claudin 4 was associated with the more differentiated intestinal-type gastric carcinoma and lost in poorly differentiated diffuse type. So, claudin 4 may be used as one of the differentiating markers between the two major types of gastric carcinoma (intestinal vs. diffuse). LVD and LVI were related to higher incidence of lymph node metastasis and therefore could be used as predictive markers for lymph node metastasis in limited specimens during early gastric carcinoma to determine the need for more invasive surgery. Low expression of claudin 4 was related to lymphangiogenesis. This may shed light on the relation of tight junction protein expression and lymphangiogenesis.

Introduction

Gastric cancer (GC) has symbolised a challenging health problem around the world and has remained the second most frequently diagnosed malignancy worldwide. Each year, GC has claimed 12% of all cancer-related deaths [1]. Although there is a decline in the incidence of GC, GC remains the second most common cause of cancer-related deaths. It is ranked eighth among cancers of Egyptians, representing 2.12% of all cancers (according to the statistics of the National Cancer Institute of Egypt (Cairo) 2003–2004) [2].

Gastric carcinoma can be histologically classified into two types: Intestinal gastric carcinoma (IGC) and diffuse gastric carcinoma (DGC) or classified into differentiated and undifferentiated types, respectively [3]. Intestinal-type carcinoma almost equates to differentiated-type carcinoma and diffuse-type carcinoma almost equates to undifferentiated-type carcinoma [4].

Tight junction (TJ) is one of the components of intercellular junctional complexes and plays an important role in tissue integrity by maintaining barrier function and cellular polarity. The loss of TJ proteins participates in carcinogenesis and tumour progression through epithelial mesenchymal transition with augmented cellular mobility and invasion [5]. Claudin family, the major TJ constituent, has at least 24 different tetraspan transmembrane proteins with a tissue-specific pattern of expression. To maintain the structure and function of the TJ, claudins interact with each other and with other TJ constituents along with cytoplasmic

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scaffolding proteins. Claudin subtypes 1–4 were found to be widely expressed in human tissue [6]. Claudin 4 was found to be overexpressed in a wide variety of cancers including breast, ovarian, pancreas, and prostate cancers. Claudin 4 was identified as one of the markers of gastric adenocarcinoma precursor lesions [7,8] and found to be the most prominently overexpressed among claudins in gastric adenocarcinoma [9]. However, it has remained unclear whether or not claudin 4 has a relation to the outcome in gastric carcinoma [8,10].

Lymph node metastasis is a major prognostic factor in gastric carcinoma [11]. Lymphovascular invasion (LVI) by tumour cells is a prerequisite for dissemination via the lymphatic vessels to lymph nodes. In addition, LVI was claimed to be an independent prognostic factor in gastric carcinoma by some authors [12]. Lymphatic metastasis was previously believed to occur through preexisting lymphatics. However, recent studies have suggested that lymphangiogenesis, the formation of new lymphatic vessels induced by tumours, is directly correlated to the extent of lymph node metastasis of solid tumours [11]. So, lymphangiogenesis, quantified by the lymphovascular density (LVD), can be used as an indicator for lymph node metastasis and therefore as a potentially useful prognostic factor, especially in small biopsies [13]. The D2-40 antibody, a marker for lymphatic endothelium, was identified as the specific antibody against human podoplanin (a glomerular antigen). Many studies have indicated that the immunostaining of D2-40 is specific for the evaluation of LVD and LVI in human cancers, as well as GC [13].

This study investigates the immunohistochemical expression of claudin 4 in gastric carcinoma, in a trial to find its relation with the relevant pathological prognostic factors, especially lymphangiogenesis and LVI (both assessed by D2-40 immunohistochemistry). It also evaluates lymphangiogenesis and LVI using D2-40 in gastric carcinoma, and their relation to other relevant pathological prognostic factors, especially lymph node metastasis.

Patients and methods

After the approval of research ethics committee (approval code 793-10-11), this study was conducted on 55 patients primarily diagnosed with gastric carcinoma, collected from the archives of the Pathology Department, Faculty of Medicine, Tanta University and private laboratories during the period from April 2011 to September 2013. The patients for this study were recruited based on the quality of the blocks and the presence of complete clinical records. Complete clinical data, such as age, gender, and clinical picture were obtained from clinical records of the patients. The cases were classified according to Lauren classification for gastric carcinoma [3] and the 2010 World Health Organization (WHO) histological classification of tumours of the stomach [14]. Cases were staged according to the 7th edition of the American Joint Committee on Cancer AJCC staging manual (tumour, node, metastasis (TNM)) [15].

Tumour paraffin blocks with the least amount of necrotic tissue were chosen for immunohistochemical (IHC) staining. Using streptavidin–biotin complex method, 4-μm-thick sections of formalin-fixed, paraffin-embedded tissue were immunostained for claudin 4 and D2-40 antibody. Immunohistochemical staining was performed using the following primary antibodies: claudin 4 (Clone 4E3) (dilution 1:50, rabbit-concentrated polyclonal immunoglobulin G2a (IgG2a) antibody (Lab vision, Thermo Scientific Inc., CA, USA)), and D2-40 (Clone D2-40) (dilution 1:100, mouse-concentrated monoclonal IgG1 antibody (Biocare Medical, Concord, CA, USA)).

The primary antibody was replaced with phosphate-buffered saline (PBS) for negative controls. Sections from tonsils and colonic adenocarcinoma were used as positive controls for claudin 4 and D2-40, respectively.

Assessment of claudin 4 immunostaining

In claudin 4 immunostaining, only membranous stain was considered positive [16]. Assessment was done as follows: Score 0, <10% of the tumour cells stained; score 1, 10–50% of the tumour cells stained; score 2, 50–90% of the tumour cells stained; and score 3, >90% of the tumour cells stained [16]. Cases were grouped into Group (1): low expression, fewer than 50% of tumour cells stained (score 0 or 1), and Group (2): high expression, >50% of tumour cells stained (score 2 or 3).

Assessment of LVD

Immunoreactivity of D2-40 was regarded as positive when brownish staining was localised in the cytoplasm of the endothelial cells in the lymphatic vessels. The luminal structures were the only ones counted as lymphatics. Separate stained cells were ignored as D2-40 cross-reacts with fibroblasts and smooth muscle cells [17]. Sections were scanned at low-power magnification (×100) to identify hot spots of lymphangiogenesis. Using light microscopy, five hot spots per tumour section were counted at ×400 magnifications. The mean value for the five fields was calculated as the LVD for each tumour. The impact of LVD was evaluated on various clinicopathological data; the cases were divided into two groups (low-expression and high-expression groups) according to a cutoff point represented by the mean LVD level (the mean LVD was 10.9) [11].

Assessment of LVI

Sections stained with D2-40 were screened, and LVI was established when at least one neoplastic cell was clearly visible inside a D2-40-positive lymphatic vessel either intratumourally or at the margin of the tumour (peritumoural lymphatics) [18].

Statistical analysis

The collected data were organised, tabulated, and statistically analysed using the statistical package for social studies (SPSS) version 19. The number and percentage distribution of variables were calculated. The relationship between variables was tested using chi-squared (χ^2) test. In the presence of small observations with expected values <5, Monte Carlo exact test (MCET) was used as chi-squared test was considered inappropriate for statistical analysis. The level of significance was adopted at $p < 0.05$.

Results

This study was conducted on 55 cases primarily diagnosed with gastric carcinoma (38 male and 17 female patients with a ratio of 2.2:1), with mean age of 53.25 (±13.26) years (range: 20–80 years). Table 1 summarises the demographic data and gross tumour characteristics (in relation to claudin 4 expression). Table 2 summarises the prognostic pathological features of tumours (in relation to claudin 4 expression).

Results of claudin 4 immunohistochemistry

The normal gastric mucosa was negative for claudin 4. The claudin 4 low-expression group included 29 of 55 (52.7%) cases and the high-expression group was represented by 26 of 55 (47.3%) cases. Tables 1 and 2 summarise the expression of claudin 4 in relation to different tumour parameters.

Claudin 4 expression was significantly higher in well-differentiated carcinoma (Fig. 1) ($p = 0.001$), intestinal-type