

ppGalNAc-T15 in gastric adenocarcinoma: Characterization according to tissue architecture and cellular location

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Abstract

Gastric cancer (GC) is the second most common cause of cancer-related deaths in the world. This study aims to investigate the differential tissue expression of ppGalNAc-T15 and to evaluate its possible association with clinical-pathological parameters and outcome of gastric adenocarcinoma patients. For these 70 patients were evaluated the expression by immunohistochemistry to ppGalNAc-T15. Our results showed that 33 (47.1%) patients were ppGalNAc-T15 positive and 37 (52.9%) negative. Positive staining for ppGalNAc-T15 was significantly present in patients older than 60 years ($P=0.0306$) and submitted to total gastrectomy ($P=0.0087$). Also, some results remained at the limit of significance as surgical standing ($P=0.0562$) and histological grade ($P=0.0549$). Therefore, the ppGalNAc-T15 immunoreactivity can be useful to understand the prognosis of patients with gastric cancer.

Introduction

Gastric cancer (GC) is a heterogeneous disease and the endpoint of a long multistep process largely influenced by *Helicobacter pylori* infection, genetic susceptibility, and environmental factors. Despite the decrease in incidence, avoidance of gastric cancer remains a priority.^{1,2} Gastric carcinoma demonstrates marked heterogeneity at both

According to the World Health Organization guidelines, GC can be classified in four major histologic patterns of gastric cancers: tubular, papillary, mucinous, poorly cohesive and others uncommon histologic variants.⁵

Many secretory and cell surface proteins are modified through the addition of carbohydrate portion formed by mucin-type O-linked oligosaccharide structures, present in organs that have secretory characteristics like the stomach.⁶ The biosynthesis of mucin-type O-linked oligosaccharides is catalyzed by UDP-GalNAc:polypeptide N-acetylgalactosaminyltransferases (ppGalNAc-Ts), an enzyme family responsible for transferring GalNAc from UDP-GalNAc to a serine or threonine residue on the polypeptide acceptor.⁷ A total of 20 human GalNAc-T gene entries are available, in which 17 have been characterized.⁸ Several isoforms are expressed in various tissues and catalyze a broad spectrum of substrates (ppGalNAc-T1, T2), whereas the other isoforms are more restricted in expression and/or in substrate preference (ppGalNAc-T3, T4, T7, T9, T11 and T13).⁹

pp-GalNAc-T15 is mainly detected in Golgi apparatus where the enzyme catalyzes its reaction but also can be founded on endoplasmatic reticulum. Its transcript is broadly expressed in various tissues, mainly in small intestine, nervous and female reproductive systems.¹⁰ This enzyme has a homologous sequence to ppGalNAc-T2, however it exhibits different substrate specificities and diverges in the number of GalNAcs they incorporate into the acceptor peptide.⁹ In addition, there is little information about ppGalNAc-T15 importance on maintenance and development of normal and neoplastic cells. The clinical relevance of immunoreactivity of pp-GalNAc-T15 in gastric cancer and normal tissues was evaluated in this study.

Materials and Methods

Samples

Seventy patients sample of primary gastric adenocarcinoma, diagnosed between 2013 and 2016, were selected from the Service Registry of the Pernambuco Cancer Hospital. All samples obtained from this service were approved by the Certificate of Presentation for Ethical Assessment (CAAE: 39976214.90000.5205). Following variables were collected in medical charts: age, sex, extension of the surgery performed, therapeutic modality, surgical staging, lymph node involvement, histological grade, submission to chemotherapy and radiation therapy, and recurrence.

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Immunohistochemistry

Biopsy slices (4 μ m) were deparaffinized with xylol and rehydrated in graded ethanol (100%, 95%, 80% and 70%). Antigen retrieval was done using 100 mM citrate buffer, pH 6.0. Endogenous peroxidase blocker was performed with hydrogen peroxide followed by blocking the non-specific binding (phosphate-buffered saline-1% bovine serum albumin PBS-BSA). Incubation of samples was performed with polyclonal primary antibody anti-ppGalNAc-T15 (CUSABIO) 1:100 with 1% PBS-BSA for 18 h at 4°C or 2 h at 37°C. The amplification system (Easylink On, ImmPRESS™ and DAKO EnVision™) was applied. Reaction was visualized with diaminobenzidine (DAB-H₂O₂) and tissues were counterstained with hematoxylin. Positive control was used following the antibody manufacturer's designation and the negative controls were established by replacing the primary antibody with anti-human IgG (Dako) antibody (Supplementary Figure 1).

Image analysis

Histomorphological analysis was performed with an integrated image system (BIOPTICA B20) microscope coupled to a CMOS camera (2584 x 1936 pixels resolution) with ISCapture image capture software and objectives 20x and 40x for image acquisition. Semi-quantitative analysis of

the stained cells was done using immunoreactive score (IRS) classification¹¹ by analyzing 5 random fields in each slide. The score evaluation was done by two independent evaluators through the analysis of images at 200x magnification, and the results expressed as negative, weak, intermediate and strong staining (supplementary Table 1).

Statistical analysis

Statistical analysis we carried out considering the results positive for ppGalNAc-T15 when the staining was weak, intermediate or strong. Fisher's exact test was performed in GraphPad Prism version 6.0. $P < 0.05$ was considered significant. Analysis of outcome was evaluated through log-rank method and Kaplan-Meier survival curves.

Results

Gastric cancer patients included in this study had a mean age of 59.4 ± 12.9 (range = 30-89) years and 47 (66.1%) were male and 24 (33.9%) were female. Evaluation of ppGalNAc-T15 using immunohistochemistry in paraffin sections of a series of 70 patients showed expression of this enzyme in 33 (47.14%) cases and in 37 (52.86%) its absence. Among these samples 12 had weak staining, 16 intermediate and 5 strong.

The four histological lesions of gastric cancer were found in this study (Figure 1 A-D). These lesion types were present in individual samples and simultaneously in the same sample. Immunohistochemical analysis showed that (23/70) 32.86% of the samples had a cytoplasmic staining pattern while (10/70) 14.29% presented a combination of cytoplasmic, nuclear and perinuclear staining (Figure 2). Thirty-four patients (48.6%) exhibit samples with normal gastric glands areas. This non-cancerous gastric tissue showed expression in 24 cases with cytoplasmic staining restricted to basal portion (Figure 3 C,D). When comparing enzyme staining between tumor and its adjacent normal area the patterns are well heterogeneous (Supplementary Table 2).

Statistics analysis revealed significant association between the ppGalNAc-T15 immunoreactivity and the parameters age ($P = 0.0306$) and extension of the surgery performed ($P < 0.05$). Association with surgical staging ($P = 0.0562$) and histological grade ($P = 0.0549$) was close to significance. Of analyzed samples 32 (31.42%) were positive for ppGalNAc-T15 and belonged to patients older than 60 years, while 23 (32.85%) were ppGalNAc-T15-negative and younger than 60 years. In relation to the extension of surgery performed, 25

(35.71%) samples that expressed ppGalNAc-T15 was obtained from a total gastrectomy, while 21 (30%) samples ppGalNAc-T15 negative came from partial gastrectomy (Table 1). In the paired analysis of total positive cases, tumor staining with adjacent normal tissue showed marking concordance in 17 cases (51.51%), and the remaining 16 (48.48%) cases were discordant. Associations with overall patient survival 496 days for the negative group and 414 days for the positive group ($P = 0.6672$) and relapse-free time 11 months for the negative group and 12 months for the positive group ($P = 0.6195$) do not show

statistics significance (supplementary Figure 2).

Discussion

Changes in the glycosylation patterns occur on cell surface and secreted glycoproteins during cancer tumorigenesis and progression. Modifications in glycosyltransferase and/or glycosidase expression, activity, and structure play a key role in the onset and progression of cancer, epithelial-mesenchymal transition (EMT), and metastasis.¹²

Table 1. Association analysis of ppGalNAc-T15 expression with clinicopathological features of gastric cancer patients.

Clinicopathological features	ppGalNAc-T15 ⁽⁺⁾ n (%)	ppGalNAc-T15 ⁽⁻⁾ n (%)	P value
Age (years)			
≥60	22 (31.42)	13 (18.57)	0.0306*
<60	12 (17.14)	23 (32.85)	
Sex			
Female	10 (14.28)	14 (20)	0.4570*
Male	24 (34.28)	22 (31.42)	
Surgery			
Total gastrectomy	25 (35.71)	15 (21.42)	0.0087*
Partial gastrectomy	9 (12.85)	21 (30)	
Neoadjuvant treatment			
I	32 (45.71)	33 (47.14)	1.0000*
III	2 (2.85)	3 (4.28)	
Surgical staging (TNM)			
(I and II)	5 (7.14)	13 (18.57)	0.0562*
(III and IV)	29 (41.42)	23 (32.85)	
Lymph node involvement			
Yes	24 (34.28)	21 (30)	0.3260*
No	10 (14.28)	15 (21.42)	
Positive/retrieved (Node ratio)	4.8/18.45 (0.28)	4.9/18.64 (0.34)	0.5238
Lymphadenectomy			
D1	16 (22.86)	15 (21.43)	0.0667
D2	18 (25.71)	19 (27.14)	
D3	0	2 (2.85)	
Lauren classification			
Intestinal	18 (26.47)	16 (23.53)	0.4664
Diffuse	14 (20.59)	20 (29.41)	
Histological grade			
GI + GII	21 (30)	13 (18.57)	0.0549*
GIII	13 (18.57)	23 (32.85)	
Chemotherapy			
Yes	19 (27.14)	20 (28.57)	1.0000*
No	15 (21.42)	16 (22.85)	
Radiotherapy			
Yes	12 (17.14)	10 (14.28)	0.6086*
No	22 (31.42)	26 (37.14)	
Recurrence			
Yes	7 (10)	9 (12.85)	0.7785*
No	27 (38.57)	27 (38.57)	

*Fisher's exact test.

GalNAc transferases (GALNTs) are crucial *O*-glycosyltransferases that initiate the formation of mucin-type *O*-glycan are differentially expressed in various tissues. This enzyme transfers GalNAc from UDP-GalNAc to a serine or threonine residue on the polypeptide acceptor mucin-type *O*-gly-

cosylation processing, forming the Tn antigen.⁹ This glycoconjugate is an immature structure that is modified or elongated to produce *O*-glycans like mucin. In some tumor cells, *O*-glycosylation is dramatically altered, resulting in expression of incomplete *O*-glycans, as represented by the Tn

and STn antigens.¹³ These structures are markers for poorly differentiated adenocarcinomas and mucinous carcinomas, whose increased occurrence is associated with advanced cancer, invasive and highly proliferative tumors, metastasis and a poor clinical outcome.¹⁴ Here, we show that the

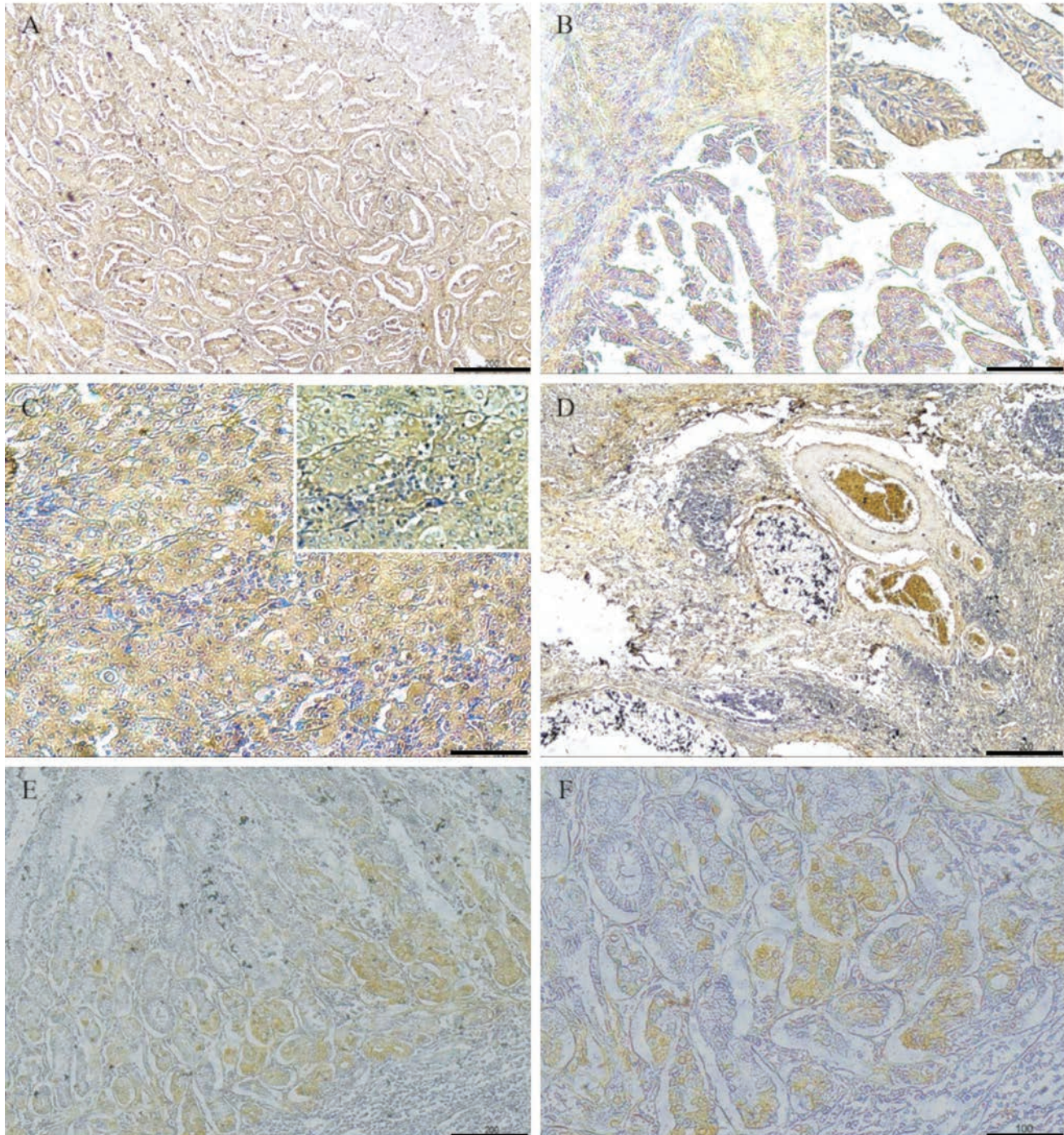


Figure 1. Histological classification of gastric cancer evidenced by PpGalNAc-T15 immunohistochemistry. Representative areas of tubular region (A), papillary region (B), poorly differentiated region (C) and delimited mucine region (D). E,F) Normal gastric glands. Scale bars: A-E) 200 μ m; F) 100 μ m.

ppGalNAc-T15 expression was present in approximately half of the GC samples, presenting a general cytoplasmic pattern, and in smaller percentages, nuclear, perinuclear and membrane staining. Observation of non-cancerous gastric tissue revealed the same cytoplasmic profile but restricted to basal and productive portion of gastric glands. Besides, ppGalNAc-T15 immunoreactivity was associated with the following clinical parameters: age, extension of the surgery performed, surgical staging and histological grade. Age was a factor analyzed in our study. Gastric cancer patients with ppGalNAc-T15 expression were relatively older than those without ppGalNAc-T15 expression. Some protein biomarkers are important in regulating metabolism, stress resistance and aging. It is likely that ppGalNAc-T15 may be an important element in the aging process through regulation of metabolism by reducing stress-related cell damage. This hypothesis, however, needs further experiments to elucidate the detailed mechanism.

ppGalNAc-T15 staining profile in gastric cancer samples was mostly cytoplasmic. Similarly, a study in 2016 reported the same pattern for ppGalNAc-T2.⁷ Normal gastric tissue had a higher ppGalNAc-T2 expression than the gastric cancer samples and was present to basal portion of gastric

glands. This is likely due to the homology between the two enzymes, which has been described by a previous study.⁹

It was observed that the expression of several ppGalNAcTs is increased in colon and other carcinomas.¹⁵ Shibao *et al.*¹⁶ reported that GalNAc-T3 expression was not associated with age, gender, tumor size, tumor location, or disease stage but was related to histologic differentiation and depth of invasion. Moreover, they showed that enzyme expression was related to enhance the likelihood of survival and as an independent prognostic factor. According to Brockhausen,¹⁵ the arrangement of biosynthetic enzymes in the cis-Golgi is an important factor controlling O-glycan biosynthesis and can vary between cell types. However, in cancer cells ppGalNAcTs could be present in medial Golgi and trans-Golgi compartments. Therefore, the altered Golgi localization of enzymes in cancer cells contribute to a disturbance in the assembly line and to the synthesis of truncated or aberrant glycans. However, the nuclear and membrane localization remains unknown.

It has been reported¹⁷ that Src protein, after stimulation with EGF (Epidermal Growth Factor) and PDGF (Platelet Derivate Growth Factor), regulates O-glycosylation through redistribution of the

GalNAc-Ts from the Golgi apparatus to the ER indicating that Src activates a COP-I-dependent trafficking event. This change increases the GalNAc addition on polypeptide acceptor and produces higher density of Tn antigen. Therefore, GalNAc-T relocation could favor shorter glycan chain lengths. This mechanism could affect mucin-type protein synthesis. Alteration of mucin expression is a hallmark of numerous epithelial cancers and has often been correlated to bad prognosis of the tumor. Muc2 expressed by goblet cells is the most abundant secreted gastrointestinal mucin, the protein component of the viscous-elastic mucus that protects this epithelium against mechanical and chemical aggressions. Yang *et al.*¹⁸ demonstrated that Muc2 deficiency results in the spontaneous development of tumors along the entire gastrointestinal tract through an inflammation related pathway. According to a previous study,¹⁹ chronic inflammatory status of the stomach play an important role in the initiation and progression of gastric cancer.

The expression of ppGalNAc-T15 in gastric lesions was identified an association between an enzyme staining with relatively elderly people and the type of surgical procedure. These conclusions may indicate that decreasing metabolism with age may alter ppGalNAc-T15 expression. The type of surgery to be performed on a gastric cancer patient is always a challenge to the surgeon and biomarkers that help in this decision are always welcome. This result indicates that ppGalNAc-T15 has relevant characteristics for these patients. However, further studies are needed to uncover the role of ppGalNAc-T15 in gastric cancer.

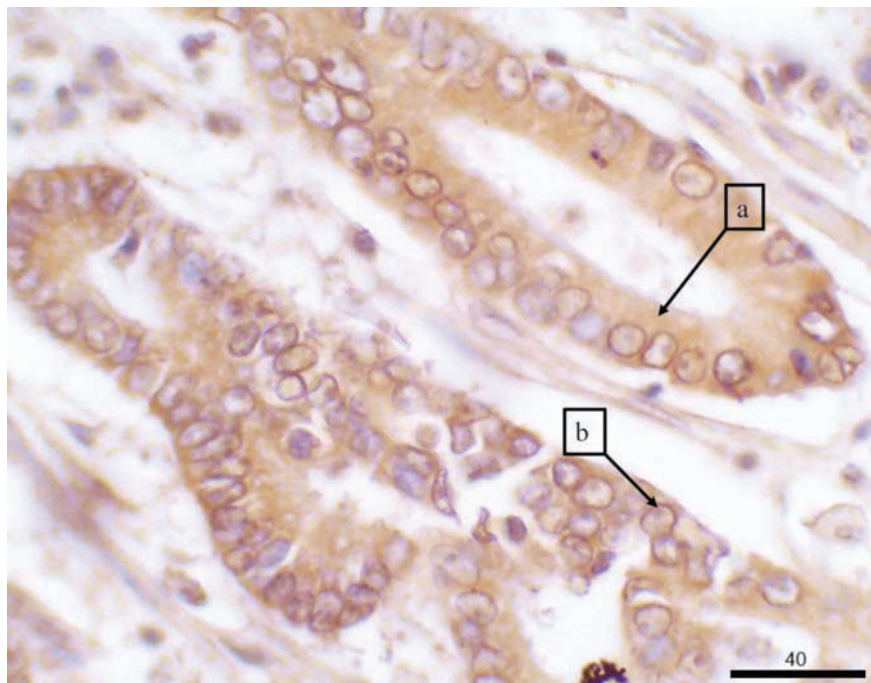


Figure 2. PpGalNAc-T15 subcellular staining in gastric cancer. a, cytoplasmic staining; b, perinuclear staining. Scale bar: 40 μ m.

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