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FUCA2 and TSTA3 expression in gastric cancer: candidate biomarkers of malignant transformation

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Abstract

Introduction. Aberrant fucosylation is closely related to malignant transformation, cancer detection, and evaluation of treatment efficacy. The fucosylation process requires GDP-L-fucose, fucosyltransferases, and fucosidases. In gastric cancer (GC), fucosylation alterations were associated with tumor formation, metastasis inhibition, and multi-drug resistance. It is not clear whether tissue-specific transplantation antigen P35B (TSTA3) and alpha-L-fucosidase 2 (FUCA2) have any effect on the development of GC.

Materials and methods. We used immunohistochemistry to assess the expression of TSTA3 and FUCA2 in 71 gastric adenocarcinoma samples and their relationship with clinicopathological parameters.

Results. TSTA3 expression was associated with lower histological grade I and II ($P = 0.0120$) and intestinal type Lauren classification ($P = 0.0120$). TSTA3 immunopositivity could predict Lauren's classification. Analysis of mRNA expression in GC validation cohorts corroborates the significant TSTA3 association with histological grade observed in our study. However, no associations were found between TSTA3 staining and overall survival. FUCA2 expression was markedly increased in GC tissues compared with non-tumoral tissues ($P < 0.0001$) and was associated with surgical staging III and IV ($P = 0.0417$) and advanced histological grade tumor states ($P = 0.0125$).

Conclusions. Alterations of FUCA2 and TSAT3 immunoexpression could lay the basis for future studies using cell glycosylation as a biomarker for the planning of therapeutic strategy in primary gastric cancer. *(Folia Histochemica et Cytobiologica 2022, Vol. 60, No. 4, 335–343)*

Keywords: gastric cancer; glycomics; fucosidase 2; TSTA3; IHC

Introduction

Gastric cancer, the fifth in incidence and third cancer in mortality worldwide [1, 2], is characterized by non-specific symptoms, late diagnosis, and metastasis, which are correlated to poor prognosis, high recurrence, and a five-year overall survival rate of less than 30% [3]. In this cancer type, α1,3/4 fucosylation is upregulated compared with normal tissue counterparts [4, 5], and increased core fucosylation is correlated to multi-drug resistance [6].

Abnormal fucosylation is closely related to malignant transformation and has been associated with tumor development and metastatic capability in multiple types of cancer [7–9]. It has also been used as a tumor marker for cancer detection and evaluation of treatment efficacy [10, 11]. The fucosylation process requires GDP-L-fucose, which is the substrate of most

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fucosyltransferases (FUTs), and many studies showed that their altered expression plays an important role in tumorigenesis, invasion, and metastasis of various cancers [10, 12].

GDP-L-fucose synthase, also named tissue-specific transplantation antigen P35B (TSTA3), participates in *de novo* pathways to synthesize GDP-L-fucose and plays a key role in the course of glycosylation [13, 14]. TSTA3 plays a critical role in tumor progression due to the significant correlation with FUTs expression [15], induction of the elevation of core-fucosylated and fucosylated glycoproteins including Sialyl Lewis X (a fucosylated glycoprotein that mediates cell-to-cell recognition processes [16–19], clinical-stage lymph node metastasis, and poor prognosis for patients with esophageal squamous cell carcinoma [20].

TSTA3 is a member of the protein family, alpha-L-fucosidases (FUCA). FUCA2 increases the expression of Lewis x antigens important for *H. pylori* bacterial adhesion and allows bacterial camouflage as an immune system defense which catalyzes the removal of terminal L-fucose residues linked to oligosaccharides on cellular surfaces [21–23]. Additionally, alterations in FUCA expression have been involved in early diagnosis, good prognosis, and survival of patients with hepatocellular carcinoma, colorectal carcinoma, intrahepatic cholangiocarcinoma, and breast cancer [24–29].

Alterations in the expression of fucosylated proteins are currently considered a promising source of new biomarkers of cancer initiation, progression, and response to treatment [30–35]. Since, to the best of our knowledge, there are no reports on the expression or activity of TSTA3 and FUCA2 in gastric adenocarcinomas, this study evaluated TSTA3 and FUCA2 immunoexpression in gastric adenocarcinoma and its relationship with patients' clinicopathological parameters.

Materials and methods

Patients and samples. Paraffin-embedded biopsies were obtained from 71 patients diagnosed with gastric adenocarcinoma who underwent surgical resection at the Pernambuco Cancer Hospital (HCP), Recife-PE, Brazil. Ethical approval was obtained from the human ethics committee of HCP (CAAE: 39976214.90000.5205).

Immunohistochemistry. To evaluate TSTA3 and FUCA2 expression we followed the methods described by De Souza *et al.* [36]. Briefly, biopsy slices were deparaffinized with xylol and rehydrated in graded ethanol. Antigen retrieval was done using citrate buffer pH 6.0 in the microwave at 95ºC for 15 min. Inactivation of endogenous peroxidase was performed with 3% hydrogen peroxide for 30 min at room temperature, followed by blocking the nonspecific binding with 1% phosphate-buffered saline for 30 min at room temperature. Next, tissue sections were then incubated with rabbit polyclonal antibodies against human TSTA3 and FUCA2 (CUSABIO, dilution 1: 100) at 8°C overnight. Next, sections it was incubated with the amplification system (Easylink On, ImmPRESS™, and DAKO EnVision™) at 25°C for 1 h and the reaction was visualized with diaminobenzidine (DAB, SigmaAldrich). Nuclei were counterstained with Mayer's hematoxylin and specimens were dehydrated in graded alcohol and mounted. The positive control used was breast and colon cancer tissues according to the antibody manufacturer's designation (CUSABIO).

Image analysis. Histomorphological analysis was performed with Pannoramic MIDI II automatic digital slide scanner (3DHISTECH, Ltd., Budapest, Hungary). If more than 10% of tumor cells were stained in different degrees of intensity, expression was considered positive. According to De Souza *et al.* [36], staining below 10% was denoted as negative. Cancer samples that presented a counterpart of normal tissue were studied and mucosa was described. The cellular localization (cytoplasmic, membrane, perinuclear, and nuclear) of TSTA3 and FUCA2 were also analyzed. The relationship between the clinicopathological parameters and the different patterns of enzyme staining was verified.

***In silico* analysis.** We used the data obtained from the cBioPortal PC genomic (http://www.cbioportal.org) [37]. The information from mRNA expression in Stomach Adenocarcinoma TCGA Provisional (https://www.cbioportal.org/study/summary?id=stad_tcga_pub) and TCGA Nature (https://www.cbioportal.org/study/summary?id=stad_tcga), comprising 415 and 265 patients, respectively, were used. In summary, the TSTA3 and FUCA2 mRNA expression values were compared with clinicopathological data (age, sex, lymph node involvement, histological grade, Lauren classification, nodal status, *H. pylori* infection, surgical staging, radiotherapy, and relapse) and with outcome parameters (overall survival, OS, and disease-free survival, DFS).

Statistical analysis. Fisher's exact test and analysis of the outcome were evaluated through Kaplan-Meyer curves with a long-rank test performed in GraphPad Prism version 7.0. A P-value < 0.05 was considered statistically significant. Multivariate logistic regression analysis was performed using Stata 9.1 software with stepwise forward selection.

Results

Clinicopathological data such as age, sex, lymph node involvement, histological grade, Lauren classification, nodal status, *H. pylori* infection, surgical staging, radiotherapy, as well as relapse and outcome parameters (OS and DFS) were collected from medical charts and presented in Table 1.

TSTA3 staining characterization

TSTA3 staining was observed in 58 (81.69%) samples in different cell compartments. The cytoplasmic region was stained in 35 samples (60.35%); another 23 samples (39.65%) showed nuclear and cytoplasmic staining at the same time (Fig. 1A), plasma membrane, and perinuclear (Fig. 1B). In addition, 37 samples showed areas of normal tissue adjacent to the neoplastic tissue (Table 2). Among them, 13 were negative and 24 positive (41.37%) for TSTA3 (Fig. 1C). Of the 24 that were stained, in 12 the neoplastic counterpart was positive (Fig. 1D). Areas of metaplasia were detected in 17 samples: 14 (82.35%) were positive, and 3 (17.64%) were negative. TSTA3 staining in cancer samples was significantly associated with histological grades G1 and G2 (P = 0.0120) and intestinal type Lauren classification (P = 0.0120) (Table 3). No associations were found between TSTA3 staining and overall survival (Fig. 2A, B).

Analysis in a validation cohort (TCGA) composed of 415 patients with gastric adenocarcinomas extracted from cBio Cancer Genomics Portal, revealed that TSTA3 mRNA expression was associated with histological grade (P = 0.0186) in well and moderately differentiated cancer. Other analyses involving clinicopathological parameters (age, sex, staging, radiotherapy, nodal invasion) and overall survival and disease-free survival did not show statistical significance (P > 0.05) (Table 4).

It was also found that TSTA3 was able to predict Lauren's classification. This effect was maintained in the multivariate analysis model, which considered the age and sex of the patients (Table 5).

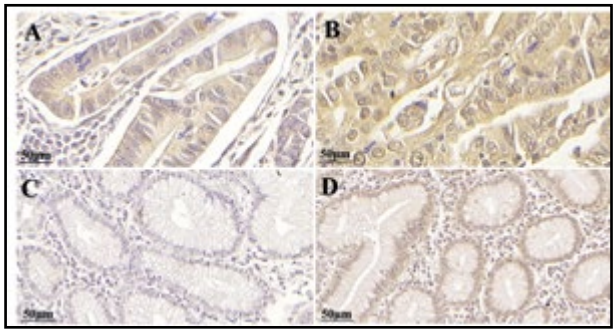

Figure 1. Immunohistochemistry of TSTA3 in gastric adenocarcinoma. **A.** Cytoplasmic immunoreactivity of TSTA3 (arrows). **B.** TSTA3 staining was also detected in the plasma membrane, nucleus and in the perinuclear region (arrows). **C.** Negative reactivity in adjacent normal gastric tissue. **D.** Positive immunoreactivity in adjacent normal gastric tissue. Microphotographs were captured at 19.7×, 30.5×, 31.1×, and 34.8× magnification, respectively.

FUCA2 staining characterization

Immunoreactivity of FUCA2 was observed in 56 tumor samples (78.87%). There was cytoplasmic staining in 37 samples, and membranous immunoreactivity in two. In 18 samples there was a combination

Table 1. Clinicopathological characteristics of patients with gastric adenocarcinoma

Clinical and pathological parameters	Nº of patients (total n = 71)
AGE	
≥ 60 years	36 (50.70)
< 60 years	35 (50.69)
Gender	
Male	47 (66.19)
Feminine	24 (33.80)
Surgery type	
Total gastrectomy	32 (54.79)
Partial gastrectomy	39 (49.29)
Initial Treatment	
I	66 (92.95)
III	5 (7.04)
Surgical Staging (TNM)	
(I and II)	19 (26.76)
(III and IV)	52 (73.23)
Lymph node involvement	46 (64.78)
Yes	25 (35.21)
No	
Histological grade	
GI + GII	35 (49,29)
GIII	36 (50.71)
Chemotherapy	
Yes	39 (54.92)
No	32 (45.07)
Radiotherapy	
Yes	22 (30.98)
No	49 (69.01)
Recurrence	
Yes	16 (22.53)
No	55 (77.46)
Lauren's classification	
Diffuse	33 (48.52)
Intestinal	35 (51.47)
Angiolymphatic invasion	
Detected	29 (43.28)
Not detected	38 (56.71)
***H. pylori* infection**	
Positive	9 (13.63)
Negative	57 (86.36)

Classification Angiolymphatic *H. Pylori* Infection N-66 of Lauren N-68 Invasion N-67

Table 2. Paired comparison of tissue-specific transplantation antigen P35B (TSTA3) staining in non-tumoral, neoplastic cells and metaplasia adjacent gastric tissue

	Non-cancerous	Neoplastic	Metaplasia	P-value
TSTA3 (+)	24	58	14	
TSTA3 (–)	13	13	3	0.1221

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Table 3. Association analysis of TSTA3 expression with clinicopathological features of gastric cancer patients

Clinicopathological features	TSTA3 (+) n (%)	TSTA3 (–) n (%)	P-value
Age (years) ≥ 60 < 60	 28 (39.44%) 30 (42.25%)	 8 (11.27%) 5 (7.04%)	0.5414
Sex Female Male	 17 (23.94%) 41 (57.75%)	 8 (11.27%) 5 (7.04%)	0.0507
Surgery Total gastrectomy Partial gastrectomy	 33 (46.48%) 25 (35.21%)	 6 (8.45%) 7 (9.86%)	0.5469
Neoadjuvant treatment I III	 54 (76.06%) 4 (5.63%)	 12 (16.90%) 1 (1.41%)	> 0.9999
Surgical staging (TNM) (I e II) (III e IV)	 15 (21.13%) 10 (14.08%)	 3 (4.23%) 43 (60.56%)	> 0.9999
Lymph node involvement YES NO	 38 (55.07%) 18 (26.09%)	 8 (11.59%) 5 (7.25%)	0.7476
Histological grade GI + GII GIII	 33 (46.48%) 25 (35.21%)	 2 (2.82%) 11 (15.49%)	0.0120
Chemotherapy YES NO	 31 (43.66%) 27 (38.03%)	 8 (11.27%) 5 (7.04%)	0.7601
Radiotherapy YES NO	 17 (23.94%) 41 (57.75%)	 5 (7.04%) 8 (11.27%)	0.5236
Recurrence YES NO	 14 (19.72%) 44 (61.97%)	 2 (2.82%) 11 (15.49%)	0.7181
Classification of Lauren Intestinal Difuse	 31 (45.59%) 24 (35.29%)	 2 (2.94%) 11 (16.18%)	0.0120
Angiolymphatic invasion Detected Not detected	 26 (38.24%) 29 (42.65%)	 4 (5.88%) 9 (13.24%)	0.3598
H. Pylori infection YES NO	9 (13.64%) 0 (0.00%)	44 (66.67%) 13 (19.70%)	0.1865
Fisher's exact test			

of cytoplasmic/membrane (Fig. 3A), perinuclear, and nuclear staining (Fig. 3B). Furthermore, we found 32 samples (57.14%) with areas of normal tissue adjacent to tumor tissue; among them, 17 were positive (53.12%) and 15 negative (46.87%) for FUCA2. Areas of metaplasia were detected in 14 samples; all presented FUCA2 staining. It was possible to observe that the number of positive cases for FUCA2 was significantly different in metaplasia and neoplasia when compared to normal tissue (Table 6). FUCA2 staining was significantly associated with surgical staging III and IV (P = 0.0417); it was accentuated in advanced and, similarly, in early and advanced histological grade tumor states (P = 0.0125) (Table 7).

We analyzed a validation cohort composed of 262 patients with gastric adenocarcinomas extracted from cBio Cancer Genomics Portal. FUCA2 mRNA expression was associated with intestinal type Lauren classification (P = 0.0034). Other analyses involving clinical and pathological parameters (age, sex) and OS and DFS were not statistically significant (P > 0.05) (Table 8). No association was found between FUCA2 staining and overall disease-free survival (Fig. 2C, D). There was no significant difference in multivariate analyses for FUCA2 expression (data not shown).

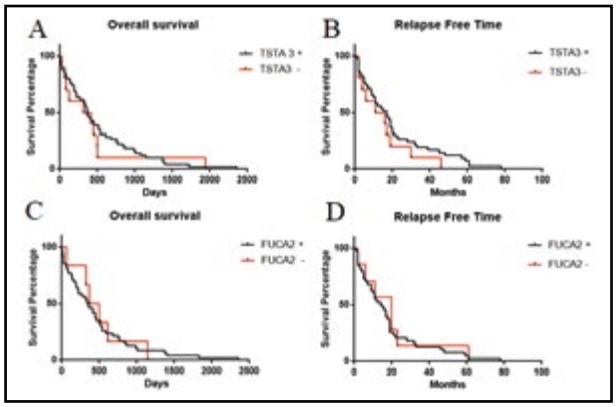

Figure 2. Associations between the immunoreactivity of tissue-specific transplantation antigen P35B (TSTA3) and alpha-L-fucosidase 2 (FUCA2) and the outcome parameters. Overall survival TSTA3 (P = 0.5208) (**A**) and disease-free survival TSTA3 (P = 0.4298) (**B**). Overall survival FUCA2 (P = 0.5341) (**C**) and disease-free survival FUCA2 (P = 0.6121) (**D**). Kaplan-Meier curves were prepared as described in Materials and methods.

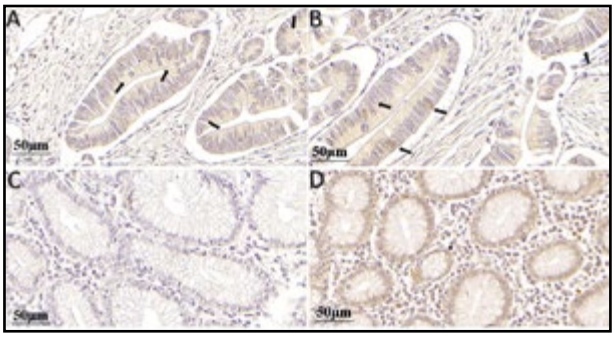

Figure 3. Immunohistochemistry of FUCA2 in gastric adenocarcinoma. **A.** FUCA2 staining located in region cytoplasmic and plasma membrane. **B.** FUCA2 expression was also identified in perinuclear and nuclear region (arrows). **C.** Negative reactivity in adjacent normal gastric tissue. **D.** Positive immunoreactivity in adjacent normal gastric tissue. Microphotographs were captured at 33.2×, 42.7×, 36.6×, and 31.5× magnification, respectively.

Table 4. Association analysis of TSTA3 mRNA with clinicopathological features of gastric cancer patients — *in silico* study based on the Cancer Genome Atlas) — TCGA PROVISIONAL

	Number of patients = 415		
Clinical data	**TSTA3 (+)** **n (%)**	**TSTA3 (–)** **n (%)**	**P-value**
Age (years) < 60 ≥ 60	 63 (15.37) 171 (41.71)	 59 (14.39) 117 (28.54)	 0.1571
Sex Female Male	 87 (20.96) 151 (36.39)	 60 (14.46) 117 (28.19)	 0.6048
Surgical Staging (TNM) (I and II) (III and IV)	 117 (29.55) 69 (17.42)	 114 (28.79) 96 (24.24)	 0.1022
Nodal invasion 0 > 1	 67 (18.31) 142 (38.80)	 39 (10.66) 118 (32.24)	 0.1622
Histological **Grade** GI + GII GIII	 103 (25.37) 129 (31.77)	 57 (14.04) 117 (28.82)	 0.0186
Radiotherapy YES NO	 31 (1520) 88 (43.14)	 17 (8.33) 68 (33.33)	 0.4028
H. Pylori infection YES NO	 13 (7.34) 94 (53.11)	 7 (3.95) 63 (35.59)	 0.8094

Lack of data in TCGA on angiolymphatic invasion, Lauren classification, and relapse.

Table 5. Univariate and multivariate regression analysis of Lauren classification in gastric cancer patients

	Univariate				**Multivariate**			
Variable	**OR**	**95% CI**		**P-value**	**OR**	**95%**	**CI**	**P-value**
TSTA3	7.10	1.43	35.1	0.016	7.18	1.37	37.5	0.020
Age	1.25	0.49	3.21	0.633	1.77	0.63	4.98	0.277
Gender	0.46	0.16	1.26	0.134	0.67	0.22	2.02	0.483

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Discussion

TSTA3 activity significantly influences tumor incidence and affects the phenotype and invasion ability of tumor cells [20, 38]. Studies demonstrated that the abnormal expression of TSTA3 can make it a new tumor marker and therapeutic target in many cancer types such as esophageal squamous cell carcinoma (ESCC), lung, non-small-cell lung, and breast cancers [39, 40]. To the best of our knowledge, this is the first study to evaluate TSTA3 expression in gastric adenocarcinoma and to analyze its association with clinicopathological parameters of patients. TSTA3 expression was observed in cell membrane, cytoplasm, nuclear, and perinuclear regions, among which the perinuclear region is the cellular location of the Golgi apparatus, where fucosylation reactions occur. This staining pattern is similar to that observed in colorectal cancer [41] and

Table 6. Paired comparison of alpha-L-fucosidase 2 (FUCA2) immunostaining in non-tumoral, neoplastic cells and metaplasia adjacent gastric tissue

	Non-cancerous	Neoplastic	Metaplasia	P-value
$FUCA2^{(+)}$ $FUCA2^{(-)}$	17 15	56 7	14 0	< 0.0001

Table 7. Association analysis of FUCA2 immunoexpression with clinicopathological features of gastric cancer patients

Clinicopathological features	FUCA2(+) n (%)	FUCA2(–) n (%)	P-value
Age (years) ≥ 60 < 60	 29 (46.03) 27 (42.86)	 4 (6.35) 3 (4.76)	> 0.9999
Sex Female Male	 19 (30.16) 37 (58.73)	 3 (4.76) 4 (6.35)	0.6871
Surgery Total gastrectomy Partial gastrectomy	 32 (50.79) 23 (36.51)	 2 (3.17) 6 (9.52)	0.1286
Neoadjuvant treatment I III	 54 (85.71) 2 (3.17)	 6 (9.52) 1 (1.59)	0.3020
Surgical staging (TNM) (I e II) (III e IV)	 14 (22.22) 42 (65.08)	 4 (7.94) 3 (4.76)	0.0470
Lymph node involvement YES NO	 36 (57.14) 20 (31.75)	 4 (6.35) 3 (4.76)	0.6991
Histological grade GI + GII GIII	 29 (46.03) 27 (42.86)	 0 (0.00) 7 (11.11)	0.0125
Chemotherapy YES NO	 28 (44.44) 28 (44.44)	 6 (9.52) 1 (1.59)	0.1122
Radiotherapy YES NO	 17 (26.98) 39 (61.90)	 2 (3.17) 5 (7.94)	> 0.9999
Recurrence YES NO	 14 (22.22) 42 (66.67)	 2 (3.17) 5 (7.94)	0.5364
Lauren classification Intestinal Difuse	 29 (47.54%) 25 (40.98 %)	 2 (3.28%) 5 (8.20%)	0.2554
Angiolymphatic invasion Detected Not detected	 2 5(40.98%) 29 (47.54%)	 2 (3.28%) 5 (8.20%)	0.4483
H. Pylori Infection YES NO	9 (15.25%) 0 (0.00%)	43 (72.88%) 7 (11.86%)	0.5808
Fisher's exact test.			

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Table 8. Association of FUCA2 mRNA with clinicopathological features of gastric cancer patients (*in silico* study based on The Cancer Genome Atlas) — TCGA NATURE

Nº of patients = 262			
Clinical data	**FUCA2 (+) n (%)**	**FUCA2 (–) n (%)**	**P-value**
Age (years)			
< 60 ≥ 60	35 (13.36) 94 (35.88)	44 (17.79) 89 (33.97)	0.3462
Sex			
Female Male	51 (19.25) 79 (29.81)	51 (19.25) 84 (31.70)	0.8996
Lauren Classification INTESTINAL DIFFUSE MIXED	 93 (37.58) 20 (7.87) 10 (3.93)	 80 (31.49) 45 (17.71) 6 (2.36)	0.0034

Lack of data in TCGA on surgical staging (TNM), nodal invasion, angiolymphatic invasion, histological grade, radiotherapy, relapse.

it presents the first evidence of TSTA3 expression in GC cell fucosylation.

Previous studies have shown that TSTA3 expression varies according to cancer type and can be associated with prognosis depending on the cancer type being studied. Yang *et al.* [40] demonstrated that high TSTA3 expression was related to more advanced stages of ESCC. Other studies have found that increased TSTA3 expression alters fucosylation and is related to poor prognosis, metastasis, and low survival rates in hepatocellular carcinoma, non-small-cell lung cancer, and breast cancer [39, 42, 43]. In this study, the increased expression of TSTA3 was significantly associated with lower histological grade, which reinforces that the elevated activity of this enzyme is highly context-dependent and corroborates the fact that relevant findings cannot be extrapolated from one pathological setting to another [44].

Increased or altered fucosylation on cell surfaces is correlated with oncogenic transformation [45, 46]. In this scenario, previous studies demonstrated that the balance between alpha-L-fucose and alpha-L-fucosidase (FUCA) may affect the prognosis of malignancies. FUCA expression has been shown to be associated with the diagnosis, prognosis, and survival of patients with hepatocellular carcinoma, colorectal carcinoma, intrahepatic cholangiocarcinoma, and breast cancer [24–27, 47–49].

To date, there are no reports regarding the association of FUCA2 immunoexpression with gastric carcinogenesis nor correlation with clinicopathological parameters of patients. However, FUCA2 secretion by gastric mucosa cells in response to *H. pylori* infection is significantly associated with bacterial adhesion, growth, and pathogenicity through the expression of the Le^x antigen [53]. These findings are associated with the well-established involvement of H. pylori infection as the primary cause of gastric cancer. Together with the results of our study, they support the idea that FUCA2 can be a potential target for clinical diagnosis and therapeutic intervention in gastric cancer.

This study is the first to demonstrate FUCA2 staining in the cytoplasmic region of gastric cancer cells and to report the significant association between its expression and histological grade early and advanced stages of gastric adenocarcinomas. Similar to TSTA3 expression, previous studies showed that the correlation between FUCA expression and clinicopathological parameters depends on the cancer type being studied. [24–26, 51, 52].

Recently, low alpha-l-fucosidase (FUCA) serum levels in esophageal squamous cell carcinoma patients were significantly associated with a pathological early stage and longer overall survival compared to patients with high FUCA levels [54]. This, considered in association with significant FUCA2 expression in metaplastic and gastric neoplastic cells compared to non-tumor cells observed in our study, leads us to suggest that FUCA2 can be involved in the malignant transformation of gastric cancer. However, this hypothesis should be investigated in future studies.

Conclusions

In summary, this is the first study describing significant FUCA2 expression in primary gastric cancer compared to non-tumoral tissues. Our results suggest an interesting association between TSTA3 staining and intestinal type according to Lauren classification, and they lay the basis for future studies using cell glycosylation as a biomarker that can be used for planning therapeutic strategy in cases of primary gastric cancer.

Data availability

The data, in free formats, used to support the findings of this study are available from the corresponding author upon request.

Conflicts of interest

The authors declare no conflict of interest.

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Authors contributions

Michael Williames Leal Quirino, Amanda Pinheiro de Barros Albuquerque and Moacyr Jesus Barreto de Melo Rêgo were responsible for the conception of the study. Michael Williames Leal Quirino, Amanda Pinheiro de Barros Albuquerque, Maria de Fátima Deodato de Souza, Antônio Felix da Silva Filho, Mário Rino Martins, Maira Galdino da Rocha Pitta and Michelly Cristiny Pereira designed the experiments and analyzed the data. Michael Williames Leal Quirino performed the experiments. Michael Williames Leal Quirino and Amanda Pinheiro de Barros Albuquerque were involved in the preparation of the manuscript. Moacyr Jesus Barreto de Melo Rêgo and Maira Galdino da Rocha Pitta were involved in critical revision of the manuscript.

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