α2,3 sialic acid processing enzymes expression in gastric cancer tissues reveals that ST3Gal3 but not Neu3 are associated with Lauren’s classification, angiolymphatic invasion and histological grade

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Gastric cancer (GC) is one of the leading causes of cancer-related deaths worldwide. Despite progress in the last decades, there are still no reliable biomarkers for the diagnosis of and prognosis for GC. Aberrant sialylation is a widespread critical event in the development of GC. Neuraminidases (Neu) and sialyltransferases (STs) regulate the ablation and addition of sialic acid during glycoconjugates biosynthesis, and they are a considerable source of biomarkers in various cancers. This study retrospectively characterized Neu3 and ST3Gal3 expression by immunohistochemistry in 71 paraffin-embedded GC tissue specimens and analyzed the relationship between their expression and the clinicopathological parameters. Neu3 expression was markedly increased in GC tissues compared with non-tumoral tissues (p<0.0001). Intratumoral ST3Gal3 staining was significantly associated with intestinal subtype (p=0.0042) and was negatively associated with angiolymphatic invasion (p=0.0002) and higher histological grade G3 (p=0.0066). Multivariate analysis revealed that ST3Gal3 positivity is able to predict Lauren's classification. No associations were found between Neu3 staining and clinical parameters. The in silico analysis of mRNA expression in GC validation cohorts corroborates the significant ST3Gal3 association with higher histological grade observed in our study. These findings suggest that ST3Gal3 expression may be an indicator for aggressiveness of primary GC.

Key words: Neuraminidase 3; primary gastric cancer; ST3 beta-galactoside alpha-2,3-sialyltransferase 3.

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Introduction

Gastric cancer (GC) is the sixth most common cancer worldwide. The average five-year survival rate for advanced-stage patients is only 20%, and it is accompanied by tumor metastasis and drug resistance. Despite progress in the last decades, factors such as late diagnosis, high molecular heterogeneity, and the absence of reliable biomarkers used in clinical practice for the prediction of patient outcome make GC the third leading cause of cancer-related deaths worldwide.

Recent data to evidence promising new biomarkers in GC with basis on their clinical implications, diagnostic methods, and the efficacy of targeted agents. Among these, circRNAs and lncRNAs are suggested as minimally invasive biomarkers capable of acting in the diagnosis and prediction for disease-free and overall survival for GC patients. Regarding the treatment effectiveness and usefulness prognostic, BMF, HAS2, SHB, AREG, EREG and HBEGF genes are suggested as predictive markers for response to anti-HER therapies, while ITGAL, HLA-E and GLP2R expression are considered as poor prognostic biomarker for GC patients.

Glycosylation modifications are usually associated with a poor prognosis in many cancer types. In addition, the abnormal expression and activity of glycosyltransferases and glycosidase enzymes has been consistently linked to dismal prognosis in cancer patients. In GC, the overexpression of sialylated glycans has been associated with tyrosine kinases hyperactivation receptors, resulting in pro-invasive phenotypes, chemoresistance, more aggressive tumors, and poor patient prognoses. In the context of GC, the remarkable match between the transcriptomic profile of cancer-relevant glycosyltransferase-coding genes and the expression of their respective glycan products makes the analysis of the expression of glycosyltransferases and glycosidases an important path towards the discovery of new biomarkers.

Sialyltransferases, known to catalyze the transfer of sialic acid residues to the oligosaccharide side chain of the glycoconjugates, have specific expression patterns in different cells and tissues, as well as differences in substrate specificities and types of linkages formed. Among the 20 sialyltransferases described to date, ST3Gal3, 4, and 6 have been connected to sialyl-Lewis antigen (sLe) formation during malignant transformation. In GC, there is a remarkable match between the transcriptomic profile of ST3Gal3 and ST3Gal4 genes and the expression of sialylated versions of the Lewis antigens, a fact that is related to the malignant phenotype of GC cells.

Unlike sialyltransferases, the neuraminidases (Neu) - also known as sialidases - cleave sialic acid residues from glycol-conjugates and are also associated with cancer progression. Among the four different mammalian sialidases (Neu1-Neu4) to date, Neu 1, 2, and 4 are down-regulated, while Neu3 is significantly up-regulated in many human cancers such as colon, renal, prostate, and ovarian tumors. Particularly by modifying the cellular ganglioside composition, Neu3 regulates different physiological phenomena such as proliferation, apoptosis, and tumor transformation. Ganglioside expression is higher in GC than in non-cancerous corresponding tissues, and its staining has been associated with an augmented tumor infiltration, presence of distant metastasis, and reduction in the patient’s overall survival rate after the tumor resection.

Taking into account that the irregular expression of alpha2,3-sialylation-related enzymes is a pathway implicated in GC development and chemoresistance, analyzing these changes may be a significant means of recognizing more specific phenotype markers and therapeutic targets in order to better enhance the diagnosis and treatment of the disease. In this work, we characterized for the first time the immunostaining of Neu3 and ST3Gal3 in gastric adenocarcinoma biopsies, and we also evaluated the association between the immunostaining pattern and clinicopathological features of patients.

Materials and Methods

Patients and samples

Biopsies previously fixed in buffered formalin and embedded in paraffin were obtained from 71 patients diagnosed with gastric adenocarcinoma who underwent surgical resection, from 2013-2016 years at the Pernambuco Cancer Hospital (HCP). Clinicopathological data, such as age, sex, lymph node involvement, histological grade, Lauren’s classification, nodal status, H. pylori infection, surgical staging, radiotherapy, as well as relapse and outcome parameters (overall survival and disease-free survival) were collected in medical charts (Supplementary Table 1). The flow of participants through the study is described in STARD diagram (Supplementary Figure 1).

Immunohistochemistry

To evaluate of ST3Gal3 and Neu3 expression we followed the methods described for de Souza et al. Briefly, biopsy slices were deparaffinized with xylol and rehydrated in graded ethanol. Antigen retrieval was done using citrate buffer in microwave for 15 min. Endogenous peroxidase blocker was performed with 1% phosphate-buffere saline for 30 min at room temperature. The sections were then incubated with rabbit polyclonal antibodies against human ST3Gal3 and Neu3 (CUSABIO, dilution 1: 100) at 4°C overnight. Next, sections were incubated with the amplification system (Easylink On, ImmPRESS ™, and DAKO EnVision ™) at 25°C for 1h and the reaction was visualized with diaminobenzidine for 30 min at room temperature. The sections were then stained in different degrees of intensity. Semi-quantitative analysis of the stained cells was done using immunoreactive score (IRS) classification 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. Samples with neoplastic cells staining less than 10% were denoted as negative.

Image analysis

Histomorphological analysis considered the enzyme staining site (cytoplasmic, membrane, perinuclear and nuclear). We analyzed the entire representative extension of the histological slide, considered positive when more than 10% of tumor cells were stained in different degrees of intensity. Semi-quantitative analysis of the stained cells was done using immunoreactive score (IRS) classification 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. Samples with neoplastic cells staining less than 10% were denoted as negative.

In silico analysis of validation cohorts

The validation cohort analyzed in this study was extracted from the cbioPortal PC genomic (www.cbioportal.org) Data from mRNA expression in Stomach Adenocarcinoma was obtained...
from TCGA Provisional, TCGA Nature and TCGA PanCancer cohorts, comprising for 415, 265 and 412 patients, respectively. Briefly, value normalized of ST3GAL3 and NEU3 mRNA expression was compared with clinical-pathological data (age, sex, lymph node involvement, histological grade, Lauren’s classification, nodal status, H. Pylori infection, surgical staging, radiotherapy and relapse) and with outcome parameters (overall survival and disease-free survival). Statistical association was performed using the Fisher’s Exact Test and Kaplan-Meier curves with long-rank test using GraphPad Prism version 7.0.

Statistical analysis

Fisher’s exact test was performed in GraphPad Prism version 7.0. A p-value ≤0.05 was considered statistically significant. Analysis of outcome was evaluated through Kaplan-Meyer curves with a long-rank test. Multivariate logistic regression analysis it was performed using STATA9.1, with stepwise forward selection.

Results

Neu3 expression is associated with malignant transformation in GC

In order to characterize the expression in GC, we evaluated Neu3 expression through immunohistochemistry in tumors and normal adjacent gastric tissues from 71 patients. As shown in Figure 1, Neu3 immunoreactivity was observed in 66 gastric adenocarcinoma samples, with staining predominantly located in the cytoplasmic region in 34 samples (51.51%) (Figure 1A) and different profile combinations such as membranous, cytoplasmic, perinuclear, and nuclear staining in 32 samples (48.48%) (Figure 1B). Sporadic positive staining on the stromal cells was also observed (Figure 1C). Furthermore, 15 areas of metaplasia were found in the specimens evaluated; in all of them the neoplastic tissue was also positive for Neu3 (Figure 1D), and 35 samples showed areas corresponding to normal tissue, in which only 12 were Neu3 positive. All normal Neu3-positive areas were in samples with positive neoplastic counterparts; the opposite did not happen. Compared with higher staining in gastric cells, Neu3 expression significantly decreased in metaplasics and normal mucosa tissues (p<0.0001) (Table 1). Collectively, these observations showed that Neu3 expression is increased in GC tissues compared with non-tumoral tissues. Association analyses revealed no significance differences between Neu3 expression and the clinicopathological parameters evaluated (Table 2).

No associations were found between Neu3 staining and overall survival. Additionally, in agreement with our immunohistochemistry results, there was no significant association between NEU3 mRNA expression and clinicopathological parameters, as shown in the validation cohort analysis (Table 3).

ST3Gal3 expression is associated with angiolymphatic invasion, histological grade and Lauren’s classification in GC

ST3Gal3 immunostaining was observed in cytoplasmic (17 samples, 94.44%) and membrane (1 sample, 5.56%) regions on GC cells (Figure 2A). In metaplasia areas, ST3Gal3 staining was positive in 20 samples (28.16%), including 7 samples (9.86%) with cytoplasmic staining (Figure 2B). Also, ST3Gal3 positivity was observed in 8 samples (22.87%) with areas corresponding to normal tissue; in two of them, the neoplasm was positive. Ducts and producer (faveolar) cells were positive in normal counterpart.

Figure 1. Immunohistochemistry of Neu3 on gastric adenocarcinomas. A) Neu3 staining predominantly located in the cytoplasmic region (blue arrows). B) Neu3 expression in membranous, cytoplasmic, perinuclear and nuclear localizations (blue arrows). C) Positive staining on the stromal cells (blue arrows). D) Metaplasia area positive for Neu3.
There was no significant difference in ST3Gal3 positivity among tumor tissues, metaplasia, and normal mucosa (Supplementary Table 2).

Association analysis between expression and clinicopathological parameters of GC patients revealed a positive association between ST3Gal3 expression and intestinal subtype Lauren’s classification (p=0.0042). As shown in Table 4, ST3Gal3 was negatively associated with angiolymphatic invasion (p=0.0002) and with higher histological grade G3 (p=0.0066). Multivariate analysis confirmed that the positivity to ST3Gal3 was able to predict Lauren’s classification (Table 5). Despite the ST3Gal3 expression indicating greater survival, there was no significant association (Supplementary Figure 3).

ST3Gal3 mRNA expression analysis in validation cohorts composed of gastric adenocarcinoma patients revealed a significant association of its expression in patients older than 60 years

Table 1. Paired comparison of Neu3 staining in non-tumoral, neoplastic cells and metaplasia adjacent gastric tissue.

| Clinicopathological parameters | Non-tumoral | Neoplastic | Metaplasia | p-value |
|--------------------------------|-------------|------------|------------|---------|
| Age (years)                    |             |            |            |         |
| ≥60                            | 10          | 66         | 20         | <0.0001 |
| <60                            | 20          | 5          | 0          | <0.0001 |
| Sex                            |             |            |            |         |
| Female                         | 21 (29.58)  | 3 (4.23)   | 2 (2.82)   | 0.3275  |
| Male                           | 45 (63.38)  | 3 (4.23)   | 2 (2.82)   |         |
| Surgery                        |             |            |            |         |
| Total gastrectomy              | 30 (42.52)  | 2 (2.82)   |            | >0.9999 |
| Partial gastrectomy            | 36 (50.70)  | 3 (4.23)   |            |         |
| Neoadjuvant treatment          |             |            |            |         |
| I                              | 5 (7.04)    | 0 (0.00)   |            | >0.9999 |
| III                            | 61 (85.92)  | 5 (7.04)   |            |         |
| Surgical staging (TNM)         |             |            |            |         |
| (I and II)                     | 49 (69.01)  | 3 (4.23)   |            |         |
| (III and IV)                   | 17 (23.94)  | 2 (2.82)   |            | 0.6049  |
| Lymph node involvement         |             |            |            |         |
| Yes                            | 44 (61.97)  | 2 (2.82)   | 3 (4.23)   |         |
| No                             | 22 (30.99)  | 3 (4.23)   |            |         |
| Histological grade             |             |            |            |         |
| GI + GII                       | 2 (2.82)    | 2 (2.82)   |            | 0.3369  |
| GIII                           | 35 (49.30)  | 0 (0.00)   | 5 (7.04)   | 0.0539  |
| Chemotherapy                   |             |            |            |         |
| Yes                            | 37 (52.11)  | 2 (2.82)   |            | 0.6518  |
| No                             | 29 (40.85)  | 3 (4.23)   |            |         |
| Radiotherapy                   |             |            |            |         |
| Yes                            | 21 (29.58)  | 1 (1.41)   |            | >0.9999 |
| No                             | 45 (63.38)  | 4 (5.63)   |            |         |
| Recurrence                     |             |            |            |         |
| Yes                            | 16 (22.54)  | 0 (0.00)   | 5 (7.04)   | 0.5809  |
| No                             | 50 (70.42)  |            |            |         |
| Lauren’s classification        |             |            |            |         |
| Intestinal                     | 32 (47.06%) | 2 (2.94%)  | 3 (4.11%)  | >0.9999 |
| Diffuse                        | 31 (45.59%) | 3 (4.11%)  |            |         |
| Angiolymphatic invasion        |             |            |            |         |
| Detected                       | 29 (40.00%) | 0 (0.00%)  | 5 (7.46%)  | 0.0666  |
| Not detected                   | 34 (42.65%) |            |            |         |
| H. pylori infection            |             |            |            |         |
| Yes                            | 9 (13.94%)  | 0 (0.00%)  |            | >0.9999 |
| No                             | 52 (78.79%) | 5 (7.35%)  |            |         |

Lauren’s classification, N=68; Angiolymphatic invasion, N=67; H. pylori infection N=66.

Table 2. Association analysis of Neu3 expression with clinicopathological parameters of gastric cancer patients.

| Clinicopathological parameters | NEU3 (+)  n (%) | NEU3 (-)  n (%) | p Neu3 |
|--------------------------------|----------------|----------------|--------|
| Age (years)                    |                |                |        |
| ≥60                            | 33 (46.48)     | 3 (4.72)       | >0.9999|
| <60                            | 33 (46.48)     | 2 (2.82)       |        |
| Sex                            |                |                |        |
| Female                         | 21 (29.58)     | 3 (4.72)       | 0.3275 |
| Male                           | 45 (63.38)     | 2 (2.82)       |        |
| Surgery                        |                |                |        |
| Total gastrectomy              | 30 (42.52)     | 2 (2.82)       |        |
| Partial gastrectomy            | 36 (50.70)     | 3 (4.72)       |        |
| Neoadjuvant treatment          |                |                |        |
| I                              | 61 (85.92)     | 5 (7.04)       |        |
| III                            | 5 (7.04)       | 0 (0.00)       |        |
| Surgical staging (TNM)         |                |                |        |
| (I and II)                     | 49 (69.01)     | 3 (4.72)       |        |
| (III and IV)                   | 17 (23.94)     | 2 (2.82)       | 0.6049 |
| Lymph node involvement         |                |                |        |
| Yes                            | 44 (61.97)     | 2 (2.82)       | 0.3369 |
| No                             | 22 (30.99)     | 3 (4.72)       |        |
| Histological grade             |                |                |        |
| GI + GII                       | 35 (49.30)     | 0 (0.00)       | 0.0539 |
| GIII                           | 31 (43.66)     | 5 (7.04)       |        |
| Chemotherapy                   |                |                |        |
| Yes                            | 37 (52.11)     | 2 (2.82)       | 0.6518 |
| No                             | 29 (40.85)     | 3 (4.72)       |        |
| Radiotherapy                   |                |                |        |
| Yes                            | 21 (29.58)     | 1 (1.41)       | >0.9999|
| No                             | 45 (63.38)     | 4 (5.63)       |        |
| Recurrence                     |                |                |        |
| Yes                            | 16 (22.54)     | 0 (0.00)       | 0.5809 |
| No                             | 50 (70.42)     | 5 (7.04)       |        |
| Lauren’s classification        |                |                |        |
| Intestinal                     | 32 (47.06%)    | 2 (2.94%)      | >0.9999|
| Diffuse                        | 31 (45.59%)    | 3 (4.11%)      |        |
| Angiolymphatic invasion        |                |                |        |
| Detected                       | 29 (40.00%)    | 0 (0.00%)      | 0.0666 |
| Not detected                   | 34 (42.65%)    | 5 (7.46%)      |        |
| H. pylori infection            |                |                |        |
| Yes                            | 9 (13.94%)     | 0 (0.00%)      | >0.9999|
| No                             | 52 (78.79%)    | 5 (7.35%)      |        |
increased in samples with higher grade G3 (p<0.0001), and with histological grade where ST3Gal3 expression was significantly associated with angiolymphatic invasion, histological grade, and Lauren’s classification in GC.

![Image](87x576 to 583x707)

**Figure 2.** Immunohistochemistry of ST3Gal3 on gastric adenocarcinomas. A) ST3Gal3 staining located in the membrane and cytoplasmic regions (blue arrows). B) ST3Gal3 staining negative (blue arrows).

**Table 3.** Association analysis of NEU3 expression with clinicopathological features of gastric cancer patients in silico study.

| Clinical data                           | TCGA Nature n=293 | TCGA PanCancer n=412 | TCGA Provisional n=415 |
|-----------------------------------------|-------------------|----------------------|-------------------------|
|                                         | NEU3(+) n (%)     | NEU3(-) n (%)        | p                       |
| Age (years)                             |                   |                      |                         |
| <60                                     | 36 (13.74)        | 43 (16.41)           | 0.0045                  |
| ≥60                                     | 88 (33.59)        | 95 (36.26)           |                         |
| Sex                                     |                   |                      |                         |
| Female                                  | 47 (17.74)        | 55 (20.75)           | 0.7049                  |
| Male                                    | 80 (30.19)        | 83 (31.32)           |                         |
| Surgical staging (TNM)                  |                   |                      |                         |
| (I and II)                              | -                 | -                    | 79 (20.05)              |
| (III and IV)                            | -                 | -                    | 101 (25.6)              |
| Nodal invasion                          | -                 | -                    | 102 (25.89)             |
| 0                                       | 176 (46.67)       | 193 (48.9)           | 0.3037                  |
| >1                                      | -                 | -                    | 121 (33.99)             |
| Angiolymphatic invasion                 |                   |                      |                         |
| Not detected                            | -                 | -                    | 69 (17.00)              |
| Detected                                | -                 | -                    | 124 (30.5)              |
| Histological grade                     |                   |                      |                         |
| GI + GII                               | -                 | -                    | 69 (17.00)              |
| GII                                     | -                 | -                    | 124 (30.5)              |
| Lauren’s classification                 |                   |                      |                         |
| Intestinal                              | 88 (34.64)        | 85 (32.46)           | 0.2230                  |
| Diffuse                                 | 25 (9.84)         | 40 (15.74)           |                         |
| Mixed                                   | 7 (2.75)          | 9 (3.54)             |                         |
| Radiotherapy                            |                   |                      |                         |
| Yes                                     | -                 | -                    | 32 (8.58)               |
| No                                      | -                 | -                    | 138 (37.00)             |
| Relapse                                 | -                 | -                    | 164 (43.9)              |
| Yes                                     | -                 | -                    | >0.9999                 |
| No                                      | -                 | -                    | 61 (15.27)              |
| H. pylori infection                     | -                 | -                    | 29 (14.15)              |
| Yes                                     | -                 | -                    | >0.9999                 |
| No                                      | -                 | -                    | 86 (41.52)              |

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Discussion

Aberrant sialylation in glycoconjugates is a characteristic feature of malignancy.\textsuperscript{27} Human sialidases have been implicated in cancer progression.\textsuperscript{34,44} In this scenario, Neu3 expression is markedly upregulated in different human tumors when compared to non-tumor tissues, a fact that evidences its potential utility as a diagnosis biomarker.\textsuperscript{39,45-47} Additionally, this increased Neu3 expression is associated with migration, invasion, tumor progression, and therapeutic resistance in many cancer types.\textsuperscript{44,48,49}

In the present study, no associations were observed between Neu3 expression and clinical-pathological parameters, disease-free progression, or overall survival. So far, there are no studies linking significant Neu3 expression with survival outcomes. The immunohistochemistry analyses of Neu3 expression in 71 patients with clear cell adenocarcinoma of the ovary showed no significant difference in survival outcomes.\textsuperscript{50} Other studies revealed that increased NEU3 mRNA expression in ovarian, prostate, colorectal, and GC samples was not significantly correlated with clinicopathological parameters.\textsuperscript{5} Even so, tissue availability and clinical-related information, as well as the state of conservation, were some of the factors limiting the sample size during the work period. Considering this context, we performed an in silico analysis to corroborate our findings. As expected, there was no significant association between NEU3 mRNA expression and clinicopathological data of validation cohorts in this evaluation.

Recently, many scientific papers have demonstrated the involvement of Neu3 in oncogenic transformation mediated by EGFR.\textsuperscript{40,47,50-52} EGFR is overexpressed in 27-64% of gastric tumors; it is well known that EGRF signaling is directly associated with chemoresistance in this cancer type.\textsuperscript{53-56} However, as enzyme activity is highly context-dependent, which severely limits the extrapolation of relevant findings from one pathological setting to another,\textsuperscript{18} the detailed mechanism and consequences of Neu3 expression in malignant transformation of GC requires further elu-

| Clinical and pathological parameters | ST3Gal3\textsuperscript{1,2} n (%) | ST3Gal3\textsuperscript{3,4} n (%) | p |
|--------------------------------------|----------------------------------|----------------------------------|---|
| Age (years)                         |                                  |                                  |   |
| ≥60                                 | 11 (15.49)                       | 25 (35.21)                       | 0.4148 |
| <60                                 | 7 (9.86)                         | 28 (39.44)                       |   |
| Sex                                 |                                  |                                  |   |
| Female                              | 4 (5.63)                         | 20 (19.72)                       | 0.3647 |
| Male                                | 14 (28.17)                       | 33 (46.48)                       |   |
| Surgery                             |                                  |                                  |   |
| Total gastrectomy                   | 8 (11.27)                        | 24 (33.80)                       | >0.9999 |
| Partial gastrectomy                 | 10 (14.08)                       | 29 (40.85)                       |   |
| Initial treatment                   |                                  |                                  |   |
| I                                   | 17 (23.94)                       | 49 (69.01)                       | >0.9999 |
| II                                  | 1 (1.41)                         | 5 (6.53)                         |   |
| Surgical staging (TNM)              |                                  |                                  |   |
| I and II                            | 2 (2.82)                         | 16 (22.54)                       | 0.1297 |
| III and IV                          | 16 (22.54)                       | 57 (22.11)                       |   |
| Lymph node involvement              |                                  |                                  |   |
| Yes                                 | 13 (18.31)                       | 33 (46.48)                       | 0.5719 |
| No                                  | 5 (7.04)                         | 20 (28.17)                       |   |
| Histological grade                  |                                  |                                  |   |
| GI + GII                            | 14 (19.72)                       | 21 (29.58)                       | 0.0066 |
| GIII                                | 4 (5.63)                         | 32 (45.07)                       |   |
| Chemotherapy                        |                                  |                                  |   |
| Yes                                 | 10 (14.08)                       | 28 (39.44)                       | >0.9999 |
| No                                  | 8 (11.27)                        | 25 (35.21)                       |   |
| Radiotherapy                        |                                  |                                  |   |
| Yes                                 | 8 (11.27)                        | 13 (18.31)                       | 0.1389 |
| No                                  | 10 (14.08)                       | 40 (56.34)                       |   |
| Recurrence                          |                                  |                                  |   |
| Yes                                 | 3 (4.23)                         | 15 (21.13)                       | 0.7450 |
| No                                  | 13 (14.23)                       | 40 (56.34)                       |   |
| Lauren's classification             |                                  |                                  |   |
| Intestinal                          | 14 (20.59%)                      | 20 (28.41%)                      | 0.0042 |
| Diffuse                             | 3 (4.41%)                        | 31 (45.59%)                      |   |
| Angiolymphatic invasion             |                                  |                                  |   |
| Detected                            | 9 (13.64%)                       | 20 (28.45)                       | 0.0002 |
| Not detected                        | 8 (12.12%)                       | 30 (44.78%)                      |   |
| *H pylori* infection                |                                  |                                  |   |
| Yes                                 | 1 (1.56%)                        | 7 (10.49%)                       | 0.6673 |
| No                                  | 15 (23.44%)                      | 41 (64.06%)                      |   |
An increase in sialic acid moieties on the cell surface is a shared characteristic of many tumors.\textsuperscript{5,8} In GC, this increased cell sialylation is considered to be a potential mechanism for invasive phenotypes and differential efficacy of targeted therapy.\textsuperscript{23-24,61} Previous work has disclosed that an increase of α2,3-sialylation by high expression of ST3Gal4 leads to SL\textsuperscript{c} expression and induces c-Met activation, invasive phenotypes, and higher therapeutic resistance in GC.\textsuperscript{23-24,61} Additionally, the overexpression of ST3Gal4 is associated with MET and RON signaling activation, which are frequently altered in GC, leading to a pro-inflammatory phenotype.\textsuperscript{5,23-24,61}

In our study, we found an association between the absence of ST3Gal3 and no angiolymphatic invasion, and also with a higher histological grade. In GC patients, stages II-IV are associated with high recurrence rates, ranging from 25\% to 40\%, and with metastatic cases not amenable to re-resection.\textsuperscript{62-64} Our in-silico analysis of ST3GAL3 mRNA expression corroborated the negative association with higher histological grade of gastric adenocarcinoma.

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

| Variable          | Univariate | Multivariate |
|-------------------|------------|--------------|
|                   | OR         | 95\% CI      | p   | OR         | 95\% CI      | p   |
| ST3Gal3           | 7.23       | 1.84 - 28.4  | 0.005 | 0.14       | 0.03 - 0.57  | 0.006 |
| Surgical stage    | 1.00       | 0.34 - 2.92  | 1.000 | 1.08       | 0.61 - 1.91  | 0.777 |
| Age               | 1.25       | 0.49 - 3.21  | 0.633 | 1.14       | 0.40 - 3.23  | 0.804 |
| Gender            | 0.46       | 0.16 - 1.26  | 0.134 | 0.57       | 0.19 - 1.73  | 0.328 |

### Table 6. Association analysis of ST3GAL3 expression with clinicopathological parameters of gastric cancer patients in-silico study.

| Clinical data                  | TCGA Nature n=293 | TCGA PanCancer n=412 | TCGA Provisional n=415 |
|--------------------------------|--------------------|----------------------|------------------------|
|                                | n (%)              | n (%)                | n (%)                  |
| Age (years)                    |                    |                      |                        |
| <60                            | 53 (20.23)         | 78 (19.12)           | 78 (18.89)             |
| ≥60                            | 38 (14.50)         | 56 (13.73)           | 56 (13.56)             |
| Sex                            |                    |                      |                        |
| Female                         | 31 (11.70)         | 44 (10.68)           | 44 (10.60)             |
| Male                           | 61 (23.02)         | 91 (22.09)           | 91 (21.93)             |
| Surgical Staging (TNM)         |                    |                      |                        |
| (I and II)                     | -                  | 56 (14.21)           | 56 (14.36)             |
| (III and IV)                   | -                  | 124 (31.4)           | 124 (31.7)             |
| Nodal invasion                 |                    |                      |                        |
| 0                              | 13 (3.30)          | 12 (3.05)            | 31 (8.54)              |
| >1                             | 108 (38.95)        | 251 (63.7)           | 83 (22.87)             |
| Angiolymphatic invasion        |                    |                      |                        |
| No detected                    | -                  | -                    | -                      |
| Detected                       | -                  | -                    | -                      |
| Histological grade             |                    |                      |                        |
| GI and GIIL                    | -                  | -                    | -                      |
| LAI                            | 31 (7.64)          | 31 (7.54)            | 31 (7.64)              |
| Lauren’s classification        |                    |                      |                        |
| Intestinal                     | 38 (14.96)         | 135 (53.14)          | 99 (24.38)             |
| Diffuse                        | 45 (17.71)         | 20 (7.97)            | 147 (36.2)             |
| Mixed                          | 6 (2.36)           | 10 (3.93)            |                        |
| Radiotherapy                   |                    |                      |                        |
| Yes                            | 20 (5.36)          | 51 (13.67)           | 34 (16.59)             |
| No                             | 107 (38.95)        | 195 (52.2)           | 123 (60.0)             |
| Relapse                        |                    |                      |                        |
| Yes                            | -                  | -                    | -                      |
| No                             | -                  | -                    | -                      |
| H. pylori infection            |                    |                      |                        |
| Yes                            | -                  | -                    | -                      |
| No                             | -                  | -                    | -                      |

[European Journal of Histochemistry 2022; 66:3330]
observed in immunohistochemistry findings and evidence that molecules negatively related to higher histological grades have potential utility as indicators of good tumor phenotype. However, further studies addressing this hypothesis are warranted.

GC is a heterogeneous disease, and the different molecular subtypes have been linked to distinct patterns of disease progression, prognosis, survival outcome, and recurrence patterns after surgery. Diffuse-subtype tumors with a molecular classification of mesenchymal-like type have the worst prognosis, a tendency to occur at an earlier age, and the highest recurrence frequency, while intestinal-subtype tumors with a molecular classification of microsatellite-unsatble type have the best overall prognosis and the lowest frequency of recurrence (22%).

Also, in GC, aberrant sialylation has been considered a source for biomarkers with potential consequences for patient stratification, survival outcomes, and chemoresistance.

To the best of our knowledge, this is the first study in which ST3Ga13 expression is significantly related and can predict Lauren’s classification of intestinal subtypes in GC. However, further investigations are needed to determine whether the relationship between ST3Ga13 expression and GC subtypes is significant, particularly in identifying new prognostic and diagnostic markers as well as therapeutic targets.

Given that immunostaining in non-tumoral tissues is lower than in metaplastic and tumoral tissues, we hypothesize that the increase in Neu3 expression may be an indicator of malignant consequences for patient stratification, survival outcomes, and chemoresistance.

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References

1. Sung H, Ferlay J, Siegel RL, Laversanne M, Soerjomataram I, Jemal A, et al. Global Cancer Statistics 2020: GLOBOCAN estimates of incidence and mortality worldwide for 36 cancers in 185 countries. CA Cancer J Clin 2021;71:209-49.

2. Shah M, Telang S, Raval G, Shah P, Patel PS. Serum fucosylation changes in oral cancer and oral precancerous conditions: a-L-fucosidase as a marker. Cancer 2008;113:336-46.

3. Zhou B, Zhou Z, Chen Y, Deng H, Cai Y, Rao X, et al. Plasma proteomics-based identification of novel biomarkers in early gastric cancer. Clin Biochem 2020;76:5-10.

4. Waddell T, Verheij M, Allum W, Cunningham D, Cervantes A, Arnold D. Gastric cancer: ESMO-ESSO-ESTRO clinical practice guidelines for diagnosis, treatment and follow-up. Eur J Surg Oncol 2014;40:584-91.

5. Britain CM, Holdbrooks AT, Anderson JC, Willey CD, Bellis SL. Sialylation of EGFR by the ST6Gal-I sialyltransferase promotes EGFR activation and resistance to gefitinib-mediated cell death. J Ovarian Res 2018;11:1-11.

6. Fattahi S, Nikbaksh N, Taheri H, Ranaee M, Akhavan-Niaki H. RNA sequencing of early-stage gastric adenocarcinoma reveals multiple activated pathways and novel long non-coding RNAs in patient tissue samples. Reports Biochem Mol Biol 2021;9:478-89.

7. Hossain MT, Li S, Reza MS, Feng S, Zhang X, Jin Z, et al. Identification of circRNA biomarker for gastric cancer through integrated analysis. Front Mol Biosci 2022;9:857320.

8. Reis-das-Mercês L, Vinasco-Sandoval T, Pompeu R, Ramos AC, Anaissi AKM, Demachki S, et al. CircRNAs as potential blood biomarkers and key elements in regulatory networks in gastric cancer. Int J Mol Sci 2022;23:650.

9. Song Q, Lv X, Ru Y, Dong J, Chang R, Wu D, et al. Circulating exosomal gastric cancer-associated long noncoding RNA1 as a noninvasive biomarker for predicting chemotherapy response and prognosis of advanced gastric cancer: A multi-cohort, multi-phase study. eBioMedicine 2022;78:103971.

10. Ebert K, Haffiner I, Zwingenberger G, Keller S, Raimúndez E, Geffers R, et al. Combining gene expression analysis of gastric cancer cell lines and tumor specimens to identify biomarkers for anti-HER therapies - the role of HAS2, SHB and HBEGF. BMC Cancer 2022;22:1-17.

11. Fu M, Huang Y, Peng X, Li X, Luo N, Zhu W, et al. Development of tumor mutation burden-related prognostic model and novel biomarker identification in stomach adenocarcinoma. Front Cell Dev Biol 2022:10:1-17.

12. Morinaga T, Iwatsuki M, Yamashita K, Matsumoto C, Harada K, Kurashige J, et al. Evaluation of HLA-E expression combined with natural killer cell status as a prognostic factor for advanced gastric cancer. Ann Surg Oncol 2022;29:4951-60.

13. Zhang J, Wang H, Yuan C, Wu J, Xu J, Chen S, et al. ITGAL as a prognostic biomarker correlated with immune infiltrates in gastric cancer. Front Cell Dev Biol 2022;10:1-15.

14. Hakomori SI, Handa K. GM3 and cancer. Glycoconjug J 2015;32:1-8.

15. Kim YJ, Varki A. Perspectives on the significance of altered glycosylation of glycoproteins in cancer. Glycoconjug J 1997;14:569-76.

16. Taniguchi N, Hancock W, Lubman DM, Rudd PM. The second golden age of glycomics: From functional glycomics to clinical applications. J Proteome Res 2009;8:425-6.

17. Reis CA, Osorio H, Silva L, Gomes C, David L. Alterations in glycosylation as biomarkers for cancer detection. J Clin Pathol 2010;63:322-9.

18. Pinho SS, Reis CA. Glycosylation in cancer: Mechanisms and clinical implications. Nat Rev Cancer 2015;15:540-55.

19. Agrawal P, Fontanals-Cirera B, Sokolova E, Jacob S, Vaiana CA, Argibay D, et al. A systems biology approach identifies FUT8 as a driver of melanoma metastasis. Cancer Cell 2018;31:804-19.

20. Ferreira JA, Magalhães A, Gomes J, Peixoto A, Gaiteiro C, Fernandes E, et al. Protein glycosylation in gastric and colorectal cancers: Toward cancer detection and targeted therapeutics. Cancer Lett 2017;387:32-45.

21. Magalhães A, Duarte HO, Reis CA. aberrant glycosylation in cancer: A novel molecular mechanism controlling metastasis. Cancer Cell 2017;31:733-5.

22. Nakagoe T, Sawai T, Tsuji T, Jibiki M, Nanashima A, Yamaguchi H, et al. Pre-operative serum levels of sialyl Tn antigen predict liver metastasis and poor prognosis in patients with gastric cancer. Eur J Surg Oncol 2001;27:731-9.

23. Gomes C, Osório H, Pinto MT, Campos D, Oliveira MJ, Reis CA. Expression of ST3Gal4 leads to SLe expression and induces c-Met activation and an invasive phenotype in gastric carcinoma cells. PLoS One 2013;8:e1-13.

24. Mereiter S, Magalhães A, Adamczyk B, Jin C, Almeida A, Drici L, et al. Glycomic and sialoproteomic data of gastric carcinoma cells overexpressing ST3Gal4. Data Brief 2016;7:814-33.
25. Munkley J. The role of sialyl-Tn in cancer. Int J Mol Sci 2016;17:275.
26. Balmaña M, Diniz F, Feijão T, Barrias CC, Mereiter S, Reis CA. Analysis of the effect of increased α2,3-sialylation on RTK activation in MKN45 gastric cancer spheroids treated with crizotinib. Int J Mol Sci 2020;21:722.
27. Duarte HO, Balmaña M, Mereiter S, Osorió H, Gomes J, Reis CA. Gastric cancer cell glycosylation as a modulator of the ErbB2 oncogenic receptor. Int J Mol Sci 2017;18:2262.
28. Harduin-Lepers A, Mollicone R, Delannoy P, Oriol R. The animal sialyltransferases and sialyltransferase-related genes: A phylogenetic approach. Glycobiology 2005;15:805-17.
29. Sperandio M, Fromhold D, Babushkina I, Ellis LG, Olson TS, Smith ML, et al. α2,3-sialyltransferase-IV is essential for L-selectin ligand function in inflammation. Eur J Immunol 2006;36:3207-15.
30. Carvalho AS, Harduin-Lepers A, Magalhães A, Machado E, Mendes N, Costa LT, et al. Differential expression of α-2,3-sialyltransferases and α-1,3/4-fucosyltransferases regulates the levels of sialyl Lewis a and sialyl Lewis x in gastrointestinal carcinoma cells. Int J Biochem Cell Biol 2010;42:80-9.
31. Colomb F, Krzewinski-recchi M, El F, Mensier E, Jaillard S, Steenackers A, et al. TFN regulates sialyl-Lewis x and 6-sulfosialyl-Lewis x expression in human lung through up-regulation of ST3GAL4 transcript isoform BX. Biochemie 2012;94:2045-53.
32. Li J, Wang Y, Xie Y, Xu Z, Yang J, Wang F, et al. Altered mRNA expressions of sialyltransferases in human gastric cancer tissues. Med Oncol 2012;29:84-90.
33. Miyagi T, Yamaguchi K. Mammalian sialidases: Physiological and pathological roles in cellular functions. Glycobiology 2012;22:880-96.
34. Li F, Ding J. Sialylation is involved in cell fate decision during development, reprogramming and cancer progression. Protein Cell 2019;10:550-65.
35. Monti E, Bonten E, D’Azzo A, Bresciani R, Venerando B, Borsani G, et al. Sialidases in vertebrates: a family of enzymes tailored for several cell functions. Adv Carbohydr Chem Biochem 2010;64:403-79.
36. Monti E, Miyagi T. Yamaguchi K. Biological and pathological roles of ganglioside sialidases. Top Curr Chem 2015;366:183-208.
37. Monti E, Arienti C, Pignatta S, Tesei A. Epidermal growth factor receptor family and its role in gastric cancer. Front Oncol 2017;7:226.
38. Kim MA, Lee HS, Lee HE, Jeon YK, Yang HK, Kim WH. EGFR in gastric carcinomas: Prognostic significance of protein overexpression and high gene copy number. Histopathology 2012;50:663-72.
39. Shiga K, Takahashi K, Sato I, Kato K, Saijo S, Moriya S, et al. Upregulation of sialidase NEU3 in head and neck squamous cell carcinoma associated with lymph node metastasis. Cancer Sci 2015;106:1544-53.
40. Yoshinaga A, Kajiyama N, Oishi K, Kamada Y, Ikeda A, Chigwechokha PK, et al. NEU3 inhibitory effect of naringin suppresses cancer cell growth by attenuation of EGFR signaling through GM3 ganglioside accumulation. Eur J Pharmacol 2016;782:21-9.
41. Kim MA, Lee HS, Lee HE, Jeon YK, Yang HK, Kim WH. EGFR in gastric carcinomas: Prognostic significance of protein overexpression and high gene copy number. Histopathology 2012;50:663-72.
42. Dulak AM, Schumacher SE, van Lieshout J, Imamura Y, Fox C, Shim B, et al. Gastrointestinal adenocarcinomas of the esophagus, stomach, and colon exhibit distinct patterns of genome instability and oncogenesis. Cancer Res 2012;72:4383-93.
43. Xu H, Miao ZF, Wang ZN, Zhao TT, Xu YY, Song YX, et al. HCRP1 downregulation confers poor prognosis and induces chemoresistance through regulation of EGFR-AKT pathway in human gastric cancer. Virchows Arch 2017;471:743-51.
44. Arienti C, Pignatta S, Tesei A. Epidermal growth factor receptor family and its role in gastric cancer. Front Oncol 2019;9:1308.
45. Marcos NT, Bennett EP, Gomes J, Magalhães A, Gomes C, David L, et al. ST6GalNAc-4 controls expression of sialyl-Tn antigen in gastrointestinal tissues. Front Biosci (Elite Ed) 2011;3:1443-55.
59. Pearce OMT, Läubli H. Sialic acids in cancer biology and immunity. Glycobiology 2015;26:111-28.
60. Liu N, Zhu M, Linhai Y, Song Y, Gui X, Tan G, et al. Increasing HER2 α2,6 sialylation facilitates gastric cancer progression and resistance via the akt and ERK pathways. Oncol Rep 2018;40:2997-3005.
61. Liu SY, Shan CT, Hung KY, Juan HF, Hsu CL, Huang MC, et al. Mucin glycosylating enzyme GALNT2 suppresses malignancy in gastric adenocarcinoma by reducing MET phosphorylation. Oncotarget 2016;7:11251-62.
62. Kim S, Lim DH, Lee J, Kang WK, MacDonald JS, Park CH, et al. An observational study suggesting clinical benefit for adjuvant postoperative chemoradiation in a population of over 500 cases after gastric resection with D2 nodal dissection for adenocarcinoma of the stomach. Int J Radiat Oncol Biol Phys 2005;63:1279-85.
63. Bang YJ, Kim YW, Yang HK, Chung HC, Park YK, Lee KH, et al. Adjuvant capecitabine and oxaliplatin for gastric cancer after D2 gastrectomy (CLASSIC): A phase 3 open-label, randomised controlled trial. Lancet 2012;379:315-21.
64. Lee J, Lim DH, Kim S, Park SH, Park JO, Park YS, et al. Phase III trial comparing capecitabine plus cisplatin versus capecitabine plus cisplatin with concurrent capecitabine radiotherapy in completely resected gastric cancer with D2 lymph node dissection: The ARTIST trial. J Clin Oncol 2012;30:268-73.
65. Cristescu R, Lee J, Nebozhyn M, Kim KM, Ting JC, Wong SS, et al. Molecular analysis of gastric cancer identifies subtypes associated with distinct clinical outcomes. Nat Med 2015;21:449-56.
66. Mereiter S, Polom K, Williams C, Polonia A, Guergova-Kuras M, Karlsson NG, et al. The Thomsen-Friedenreich antigen: A highly sensitive and specific predictor of microsatellite instability in gastric cancer. J Clin Med 2018;7:256.
67. Freitas D, Campos D, Gomes J, Pinto F, Macedo JA, Matos R, et al. O-glycans truncation modulates gastric cancer cell signaling and transcription leading to a more aggressive phenotype. EBioMedicine 2019;40:349-62.