

**Keywords:** polypeptide *N*-acetylgalactosaminyl transferase-5 (GalNAc-T5); prognostic; glycosylation; gastric cancer; overall survival

# Clinical significance of polypeptide *N*-acetylgalactosaminyl transferase-5 (GalNAc-T5) expression in patients with gastric cancer

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**Background:** The family of polypeptide *N*-acetylgalactosaminyl transferases is responsible for the altered *O*-linked glycosylation occurring during the development of various cancers and their progression via altering *O*-glycan biosynthesis. Our studies were designed to investigate the expression and prognostic values of GalNAc-T5 and improve the risk stratification in patients with gastric cancer.

**Methods:** Tissue samples from a training set and a validation set of patients with gastric adenocarcinoma from China were used for analyses. GalNAc-T5 expression was retrospectively analysed by immunohistochemistry. Results were assessed for association with clinicopathological parameters and overall survival by using Kaplan–Meier analysis. Prognostic values of GalNAc-T5 expression and clinical outcomes were evaluated by Cox regression analysis. A molecular prognostic stratification scheme incorporating GalNAc-T5 expression was determined by using receiver operating characteristic analysis.

**Results:** GalNAc-T5 expression was markedly reduced in gastric cancer tissues compared with non-malignant gastric mucosa. Low intratumoral GalNAc-T5 density, which was associated with tumour cell differentiation, T classification, N classification, and TNM stage in the two independent sets, was an independent prognosticator for poor prognosis of gastric cancer patients. Applying the prognostic value of intratumoral GalNAc-T5 density to the conventional clinicopathologic TNM stage system showed a better prognostic value in patients with gastric cancer.

**Conclusions:** Intratumoral GalNAc-T5 expression was recognised as an independent prognostic marker for the overall survival of gastric cancer patients. Detection of GalNAc-T5 expression in gastric cancer tissues might add some prognostic information for patients with this disease and lead to a more accurate classification under the TNM stage system.

Despite a decreasing overall incidence, gastric cancer remains the fourth most common neoplasm and the second leading cause of cancer-related mortality worldwide with a frequency that varies

widely across geographic locations (Hartgrink *et al*, 2009; Jemal *et al*, 2011). In China, gastric cancer is also a major public health issue, some 400 000 new cases are diagnosed every year and 95% of

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Received 5 September 2013; revised 30 November 2013; accepted 23 January 2014; published online 11 March 2014

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the total number of gastric malignancies is adenocarcinoma (Yang, 2006). The tumour–node–metastasis (TNM) classification of the International Union Against Cancer based on the post-operative clinicopathological status of patients with gastric cancer establishes a predictive model for accurately predicting patient survival and guiding therapy decisions (Washington, 2010). Nonetheless, some gastric cancer patients may experience recurrence and ultimately die from the disease, even with a favourable prognostic background (Lim *et al*, 2005). This considerable heterogeneity of gastric cancer presents at the molecular level and has a genetic predisposition to it (Stock and Otto, 2005). Therefore, molecular approaches for stratifying patients with gastric cancer, through incorporation of molecular information, including post-translational modification, into the conventional clinicopathologic TNM stage system will improve current prognostic stratification and provide important clinically relevant insights into predicting which patients are prone to develop recurrence and mortality after surgery.

Glycosylation is the most common post-translational modification of proteins (Dennis *et al*, 1999). Two major types of protein glycosylation, *N*-linked and *O*-linked, exist in mammalian cells (Dube and Bertozzi, 2005). Glycosylation alterations, including both under- and overexpression of naturally occurring glycans, as well as neoexpression of glycans normally restricted to embryonic tissues, is a well-described hallmark of many human cancers, prominently including gastric cancer, and has been shown to have multiple effects on many cellular properties, including cell differentiation, proliferation, apoptosis, transformation, migration, and invasion (Fuster and Esko, 2005; Raman *et al*, 2005). *O*-glycosylation may affect structural and functional properties of secreted and membrane-bound proteins and peptides, and have a major role in the tumorigenesis of gastric cancer (Kobayashi *et al*, 2009). A critical aspect of *O*-glycosylation, the serine and threonine residues at which target proteins are glycosylated with *N*-acetylgalactosamine (GalNAc), is catalysed by a large family of polypeptide GalNAc-transferases (GalNAc-Ts) that are normally located in the Golgi complex (Ten Hagen *et al*, 2003). Thus, through regulating the initial step of *O*-glycosylated protein biosynthesis and determining sites of *O*-glycosylation on proteins, GalNAc-Ts are important for understanding normal and carcinoma-associated *O*-glycosylation (Brockhausen, 2006). Prior studies have revealed that strong GalNAc-T3 expression in gastric cancer tissues was significantly correlated with good prognosis of gastric cancer patients (Onitsuka *et al*, 2003). Besides, according to the previous literatures, it had been demonstrated that GalNAc-T10 was a useful indicator of tumour differentiation in gastric cancer (Gao *et al*, 2013). The expression of GalNAc-T6 was reported to be an immunohistochemical marker associated with venous invasion in gastric carcinoma (Gomes *et al*, 2009). However, to date, no study has assessed GalNAc-T5 expression in gastric cancer, and the correlation between GalNAc-T5 expression and the prognosis of patients with gastric cancer remains largely unclear and needs to be further investigated.

In the current study, we evaluated the expression of GalNAc-T5 in gastric cancer specimens and their correlation with the clinicopathologic features of the patients. The results indicated that low intratumoral GalNAc-T5 expression was a significant negative prognostic predictor for patients with gastric cancer. Moreover, integration of intratumoral GalNAc-T5 expression into the conventional clinicopathologic TNM stage system could refine prognostic stratification of patients with gastric cancer.

## MATERIALS AND METHODS

**Clinical specimens.** We prospectively recruited consecutive patients with gastric cancer, collected the clinicopathologic data

and the specimens, and retrospectively analysed the samples in detail for markers correlating with survival and their role in refining gastric cancer prognostic stratification (Wang *et al*, 2013). Two independent sets comprising of 223 patients, who had undergone total or partial gastrectomy for gastric adenocarcinoma at the Zhongshan Hospital of Fudan University (Shanghai, China), were enrolled in the study. Patients were excluded if they had previously been exposed to any targeted therapy, chemotherapy, radiotherapy, or intervention therapy for gastric cancer. Specimens of the training set ( $n = 97$ ) were obtained between January 2000 and December 2005, and specimens of the validation set ( $n = 126$ ) were obtained between January 2006 and December 2008. Detailed clinical characteristics of the two independent sets are summarised in Supplementary Table S1. There were more patients with late-stage gastric cancer (TNM III and IV, 61.11% vs 49.48%,  $P = 0.016$ ) and poorer overall survival ( $P = 0.026$ ) in the validation set compared with the training set. Besides, there were more patients with old age ( $P = 0.002$ ) and deep tumour invasion (T3 and T4,  $P = 0.001$ ) in the validation set. Such heterogeneity may help to ensure that the predictor has real-world applicability. Tissue samples of human gastric cancer, which had been formalin-fixed, paraffin-embedded, and clinically and histopathologically diagnosed, were collected at the time of surgical resection. Non-tumoral gastric tissues were obtained at least 5 cm from the tumour at the same time. After operation, routine chemotherapy had been given to the patients with advanced-stage disease, but no radiation treatment was done in any of patients included in our study. All specimens were pathologically reassessed independently by two gastroenterology pathologists blinded to the clinical data. Institutional review board approval from Zhongshan Hospital of Fudan University and written informed consent from all patients were obtained for this study.

**Tissue microarray and immunohistochemistry.** Tissue microarrays were constructed as previously described (Zhu *et al*, 2008). Primary anti-GalNAc-T5 antibody (Sigma-Aldrich, St Louis, MO, USA) was used for immunohistochemistry staining. Before immunohistochemistry staining, the expression of GalNAc-T5 was detected by RT-PCR, quantitative real-time PCR, and western blot analysis in four human gastric cancer cell lines (NCI-N87, SGC7901, AGS, and BGC823). The protein expression level of GalNAc-T5 detected by the antibody parallels its mRNA expression level in human gastric cancer cells, and the antibody is specific for it (Supplementary Figure S1). The intensity of immunohistochemistry staining of GalNAc-T5 was scored independently by two gastroenterology pathologists using the semi-quantitative immunoreactivity scoring system as previously described (Weichert *et al*, 2008; Wang *et al*, 2013). Negative controls were treated identically but with the primary antibody omitted.

**Statistical analysis.** Statistical analysis was performed with MedCalc Software (version 11.4.2.0; MedCalc, Mariakerke, Belgium). Numerical data were analysed using Student's *t*-test, whereas categorical data were studied using  $\chi^2$  or Fisher's exact test. Cumulative survival time was calculated using the Kaplan–Meier method and analysed using the log-rank test. Numbers at risk were calculated for the beginning of each time period. The Cox proportional hazards regression model was used to perform univariate and multivariate analyses. Receiver operating characteristic analysis was used to compare the sensitivity and specificity for the prediction of overall survival by the parameters. All *P*-values were two sided, and differences were considered significant at values of  $P < 0.05$ . Results are reported according to REMARK (Reporting Recommendations for Tumor Marker Prognostic Studies) guidelines (McShane *et al*, 2005).

Other materials and methods are detailed in the Supplementary Materials and Methods.

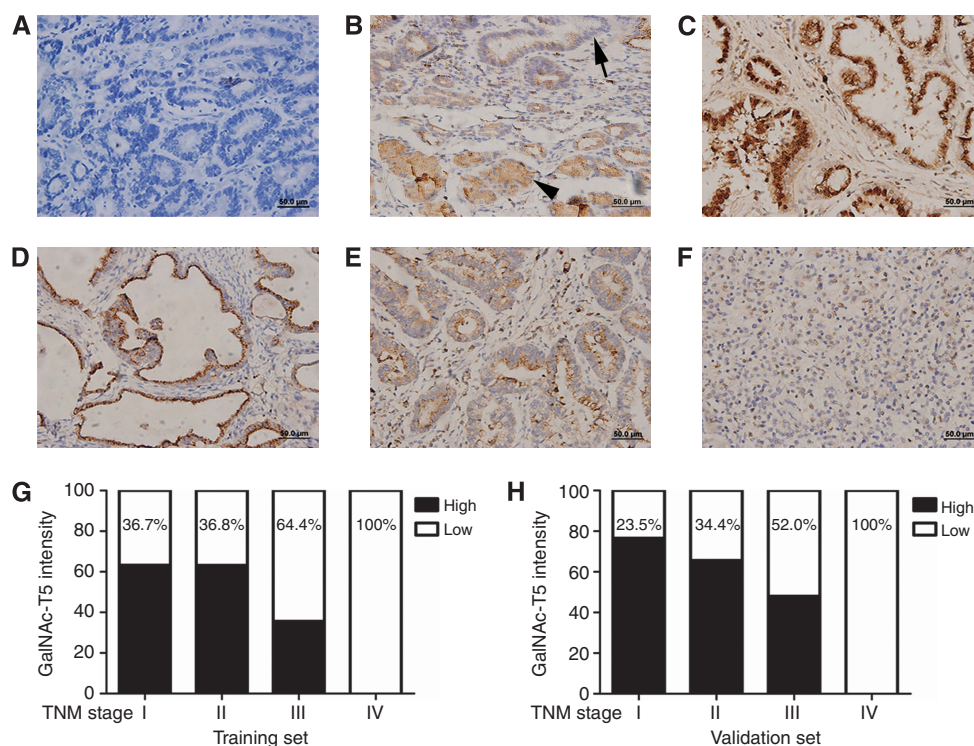
## RESULTS

**Immunohistochemical findings.** In order to ascertain whether the expression of GalNAc-T5 protein is linked to clinical progression of gastric cancer, we first evaluated GalNAc-T5 expression by immunohistochemical analyses in tumour and non-tumoral specimens from 223 patients with gastric cancer in the training and validation sets. As shown in Figure 1, GalNAc-T5 immunoreactivity was predominantly located in the cytoplasm of gastric epithelia (Figure 1B) and cancer cells (Figure 1C–F), and the intensity of the immunohistochemical staining was variable. Sporadic positive staining on the stroma cells was also observed (Figure 1B–F). Compared with higher non-tumoral GalNAc-T5 density in gastric epithelial cells (Figure 1B), intratumoral GalNAc-T5 expression in gastric cancer cells decreased gradually accompanied with disease progression from well-differentiated adenocarcinoma (Figure 1C), moderately differentiated adenocarcinoma (Figure 1D), poorly differentiated adenocarcinoma (Figure 1E) to undifferentiated adenocarcinoma (Figure 1F). Collectively, these observations suggest that GalNAc-T5 expression is decreased in gastric cancer tissues compared with the non-tumoral tissues, and decreased GalNAc-T5 expression in tumour cells might be associated with histologic progression of gastric cancer.

**Associations between GalNAc-T5 expression and clinicopathologic parameters of patients with gastric cancer.** According to the immunoreactivity scoring criterion, ~51.55% (training set, 50 of 97) and 44.44% (validation set, 56 of 126) tumours were scored as low GalNAc-T5 expression. Immunohistochemical staining of GalNAc-T5 levels was statistically analysed to determine their

relationship with various clinicopathologic features of patients with gastric cancer. As shown in Table 1, intratumoral GalNAc-T5 expression was significantly associated with tumour cell differentiation ( $P=0.041$  and  $P=0.020$ , respectively), T classification ( $P=0.033$  and  $P=0.032$ , respectively), N classification ( $P=0.013$  and  $P=0.022$ , respectively), and TNM stage ( $P=0.018$  and  $P=0.036$ , respectively) in the two independent sets. Besides, in the validation set, intratumoral GalNAc-T5 expression was also significantly related with the Lauren classification ( $P=0.028$ ). In addition, the per cent of patients with high intratumoral GalNAc-T5 expression decreased gradually accompanied with disease progression from TNM stage I to IV, and there were no patients with high intratumoral GalNAc-T5 expression in the TNM stage IV in the two independent sets (Figure 1G and H). Besides, quantitative real-time PCR also confirmed that the mRNA expression level of GalNAc-T5 decreased gradually accompanied with disease progression (Supplementary Figure S2), which is consistent with the results of immunohistochemistry staining. However, non-tumoral GalNAc-T5 expression was not associated with any clinicopathologic parameters of gastric cancer patients in the training set (Supplementary Table S2).

**Correlation of GalNAc-T5 expression with prognosis of patients with gastric cancer.** To further investigate the prognostic value of GalNAc-T5 expression in gastric cancer patients, we compared overall survival according to intratumoral GalNAc-T5 expression, and Kaplan–Meier survival analysis was performed. Clearly, gastric cancer patients with low intratumoral GalNAc-T5 expression have a poor prognosis than those with high intratumoral GalNAc-T5 expression in the two independent sets ( $P<0.001$  and  $P<0.001$ , respectively; Figure 2A and B; Supplementary Table S3), which



**Figure 1.** GalNAc-T5 expression in sections of gastric tissue. (A) Negative control. (B) Gastric cancer and adjacent peritumoral tissues. Arrowhead shows gastric peritumoral epithelial cells with strong expression of GalNAc-T5, and arrow shows gastric cancer tissue with moderate GalNAc-T5 expression. (C) Well-differentiated adenocarcinoma showing strong expression of GalNAc-T5. (D) Moderately differentiated adenocarcinoma showing moderate expression of GalNAc-T5. (E) Poorly differentiated adenocarcinoma showing moderate or weak expression of GalNAc-T5. (F) Undifferentiated adenocarcinoma showing weak expression of GalNAc-T5. (G, H) The per cent of patients with high intratumoral GalNAc-T5 expression decreased gradually accompanied with disease progression from TNM stage I to IV in (G) training set ( $n=97$ ) and (H) validation set ( $n=126$ ). Scale bar:  $50.0\ \mu\text{m}$ .

**Table 1.** Relation between intratumoral GalNAc-T5 expression and clinical characteristics in the two independent sets

Factors	Training set			Validation set		
	GalNAc-T5 expression High	GalNAc-T5 expression Low	P-value	GalNAc-T5 expression High	GalNAc-T5 expression Low	P-value
All patients	47	50		70	56	
Age (years) <sup>a</sup>			0.443			0.808
≤63	29	27		29	22	
>63	18	23		41	34	
Gender			0.685			0.314
Female	16	19		52	37	
Male	31	31		18	19	
Localisation			0.617			0.966
Proximal	5	5		16	12	
Middle	24	21		26	22	
Distal	18	24		28	22	
Differentiation			0.041			0.020
Well	3	2		12	5	
Moderately	23	13		24	10	
Poorly	21	35		34	41	
Lauren classification		0.784			0.028	
Intestinal type	35	36		54	33	
Diffuse type	12	14		16	23	
T classification			0.033			0.032
T1	18	8		8	2	
T2	5	5		10	2	
T3	3	1		5	9	
T4	21	36		47	43	
N classification			0.013			0.022
N0	24	14		26	16	
N1	11	8		12	2	
N2	6	8		12	10	
N3	6	20		20	28	
Distant metastasis		0.088			0.111	
No	47	47		70	54	
Yes	0	3		0	2	
TNM stage			0.018			0.036
I	19	11		13	4	
II	12	7		21	11	
III	16	29		36	39	
IV	0	3		0	2	
Tumour size (cm) <sup>a</sup>			0.380			0.523
<3.5	25	31		31	28	
≥3.5	22	19		39	28	

Abbreviations: GalNAc-T5 = polypeptide N-acetylgalactosaminyl transferase 5; TNM = tumour–node–metastasis.  
<sup>a</sup>Split at median.

indicates a crucial impact of intratumoral GalNAc-T5 expression on clinical outcome in patients with gastric cancer. However, Kaplan–Meier survival analysis showed that non-tumoral GalNAc-T5 expression was not associated with overall survival of gastric cancer patients in the training set (data not shown). In addition, in order to determine whether intratumoral GalNAc-T5 expression could stratify patients with TNM stage stratum, we evaluated the

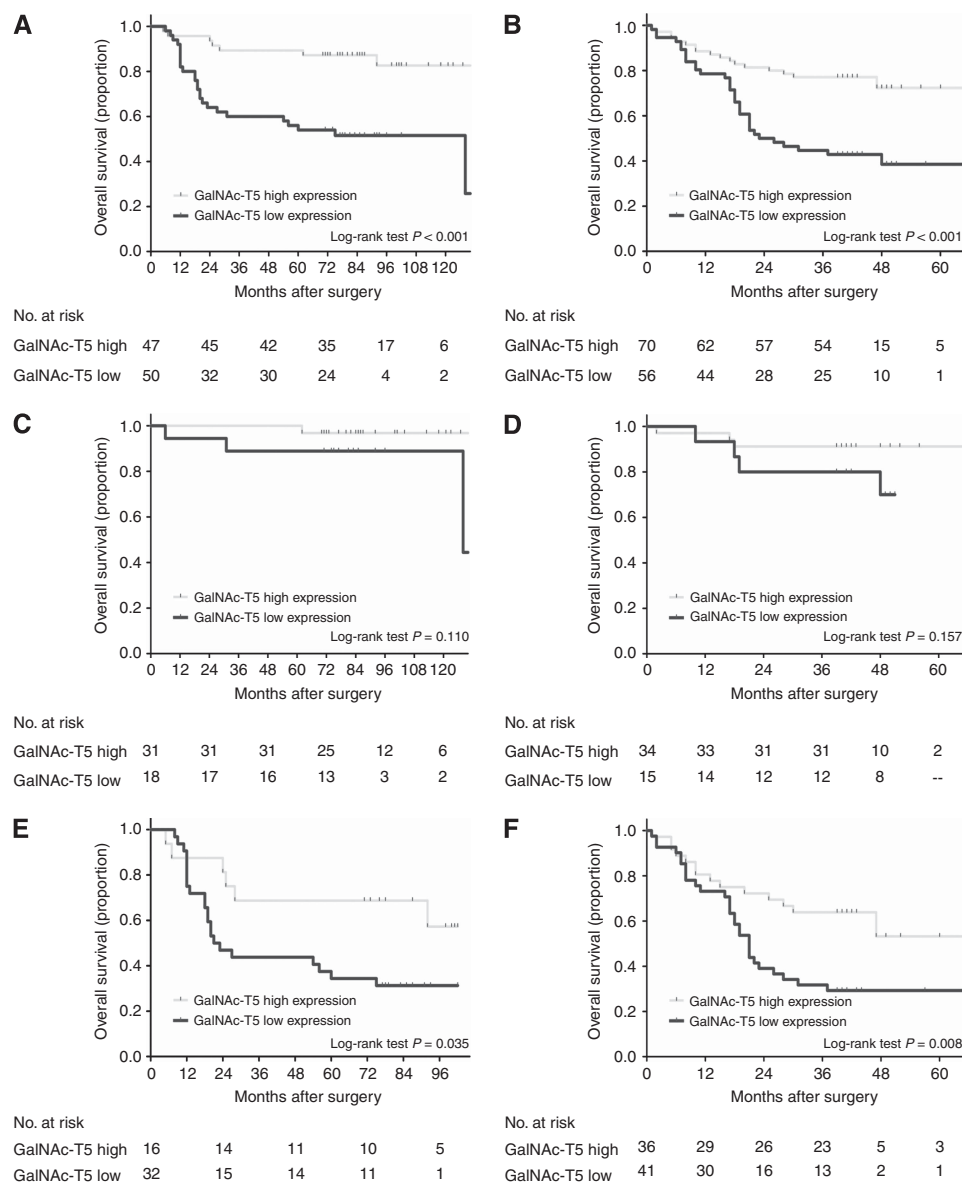
prognostic value of intratumoral GalNAc-T5 expression and performed stratified analyses of gastric cancer patients with TNM stage I + II and TNM stage III + IV, respectively. As shown in Supplementary Table S3, only the patients with TNM stage III + IV could be significantly stratified by intratumoral GalNAc-T5 expression; the prognosis of TNM stage III + IV patients with low intratumoral GalNAc-T5 expression was significantly poorer than those with high intratumoral GalNAc-T5 expression in the two independent sets ( $P=0.035$  and  $P=0.008$ , respectively; Figure 2C–F; Supplementary Table S3).

**Low intratumoral GalNAc-T5 expression is an independent predictor of poor prognosis in patients with gastric cancer.** In order to estimate the clinical significance of intratumoral GalNAc-T5 expression that might influence survival in the study population, univariate analyses was performed for overall survival in the two independent sets of gastric cancer patients. As shown in Table 2, low intratumoral GalNAc-T5 expression is a significant negative prognostic predictor for patients with gastric cancer in the training set (hazard ratio (HR), 2.92; 95% CI, 1.63–5.26;  $P<0.001$ ) and the validation set (HR, 4.41; 95% CI, 1.90–10.26;  $P<0.001$ ). In addition, T classification ( $P=0.002$  and  $P=0.009$ , respectively), N classification ( $P=0.001$  and  $P=0.002$ , respectively), distant metastasis ( $P<0.001$  and  $P<0.001$ , respectively), and TNM stage ( $P<0.001$  and  $P<0.001$ , respectively) were also statistically significant factors affecting overall survival of patients with gastric cancer in the two independent sets. To evaluate the robustness of the prognostic value of intratumoral GalNAc-T5 expression, Cox multivariate regression analyses were performed to derive risk estimates related to overall survival with the same clinicopathological parameters of the training set that show significance in univariate analyses to control for confounders. As shown in Table 3, intratumoral GalNAc-T5 expression ( $P=0.022$  and  $P=0.003$ , respectively) and TNM stage ( $P<0.001$  and  $P<0.001$ , respectively) were both recognised as independent prognostic factors for overall survival of gastric cancer patients in the two independent sets. Taken together, our findings indicate that intratumoral GalNAc-T5 expression may be a useful marker to predict the overall survival of patients with gastric cancer.

**Extension of the TNM stage prognostic model with intratumoral GalNAc-T5 expression.** To develop a more sensitive predictive tool, we constructed a prognostic model combining the two independent prognostic factors, intratumoral GalNAc-T5 expression and TNM stage, and compared its prognostic validity with the intratumoral GalNAc-T5 expression alone and TNM stage alone models by means of receiver operating characteristic analyses. Combination of intratumoral GalNAc-T5 expression and the TNM stage (AUC (95% CI), 0.840 (0.752–0.907) and 0.780 (0.698–0.849), respectively) showed a better prognostic value than did intratumoral GalNAc-T5 expression (AUC (95% CI), 0.698 (0.597–0.787) and 0.679 (0.590–0.759), respectively) or TNM stage (AUC (95% CI), 0.784 (0.688–0.861) and 0.706 (0.619–0.784), respectively) alone in the two independent sets (Figure 3).

## DISCUSSION

In general, it is often asymptomatic or causes only non-specific symptoms in its early stages of gastric cancer. By the time specific symptoms occur, the disease has often reached an advanced stage (Shou *et al*, 2012). Advanced gastric cancer prognosis tends to be dismal, despite aggressive therapy (Hartgrink *et al*, 2009). Various clinicopathologic features, including biological markers, have been proposed as prognostic indicators, although the results remain inconsistent and inconclusive to date (He *et al*, 2012, 2013). Defining molecular subgroups may identify patients who could benefit from aggressive and targeted therapies, and might be used



**Figure 2.** Analyses of overall survival according to the expression of intratumoral GalNAc-T5 in gastric cancer patients. Kaplan–Meier analyses of overall survival according to intratumoral GalNAc-T5 expression in patients with gastric cancer in **(A)** training set, all patients ( $n = 97$ ), **(B)** validation set, all patients ( $n = 126$ ), **(C)** training set, TNM stage I + II ( $n = 49$ ), **(D)** validation set, TNM stage I + II ( $n = 49$ ), **(E)** training set, TNM stage III + IV ( $n = 48$ ), and **(F)** validation set, TNM stage III + IV ( $n = 77$ ).  $P$ -value was calculated using the log-rank test.

to select specific treatment approaches for patients with gastric cancer (Kasaian and Jones, 2011). For this discrimination at the molecular level, in the present study, we investigated the expression and prognostic values of GalNAc-T5 in patients with gastric cancer. To our knowledge, this is the first study to identify low intratumoral GalNAc-T5 expression as an independent poor prognostic factor for overall survival of gastric cancer patients following gastrectomy, and only the patients with TNM stage III + IV could be significantly stratified by intratumoral GalNAc-T5 expression. Moreover, in this study, incorporation of intratumoral GalNAc-T5 density into the current clinicopathologic TNM stage system improved the prognostic value for overall survival. These data suggest that the intratumoral GalNAc-T5 density might have good discriminatory power as a supplementary risk factor in patients with late-stage gastric cancer and lead to a more accurate classification under the TNM stage system. Gastric cancer patients with low intratumoral GalNAc-T5 expression should have aggressive therapies and a closer follow-up. However, the results

of integration of intratumoral GalNAc-T5 expression into the current prognostic model and the potential clinical practice changing should be validated in an independent and larger data set. The profound molecular roles of GalNAc-T5 in gastric cancer progression remain far from being fully elucidated and await further investigation.

Until now, clinicopathological parameters including invasion depth, lymphnode and distant metastases, and TNM stage have been considered to be the prognostic factors for gastric cancer. Tumour invasion, lymphnode metastases, and distant metastases are also primary causes for death or treatment failure among gastric cancer patients (Morabito *et al*, 2009). They are enormously complex and multistep processes involving regulation at the post-transcriptional level of adhesive molecules, proteolytic enzymes, and cell growth and angiogenesis factors. Altered glycoforms of these proteins with changed biological activity may have a pivotal role in these processes and contribute to the invasion and metastases of gastric cancer (Hakomori, 2002). Glycosylation

Table 2. Univariate Cox regression analyses for overall survival in the two independent sets

Factors	Overall survival			
	Training set		Validation set	
	Hazard ratio (95% CI)	P-value	Hazard ratio (95% CI)	P-value
Age (years) <sup>a</sup>		0.142		0.332
≤ 63	1.00 (reference)		1.00 (reference)	
> 63	1.71 (0.84–3.51)		1.34 (0.74–2.40)	
Gender		0.948		0.222
Female	1.00 (reference)		1.00 (reference)	
Male	0.98 (0.46–2.05)		0.70 (0.39–1.24)	
Localisation		0.251		0.240
Proximal + middle	1.00 (reference)		1.00 (reference)	
Distal	1.52 (0.74–3.12)		0.70 (0.39–1.27)	
Differentiation		0.206		0.076
Well + moderately	1.00 (reference)		1.00 (reference)	
Poorly	1.63 (0.76–3.50)		1.71 (0.95–3.11)	
Lauren classification		0.025		0.723
Intestinal type	1.00 (reference)		1.00 (reference)	
Diffuse type	2.31 (1.11–4.80)		1.11 (0.61–2.02)	
T classification		0.002		0.009
T1 + T2	1.00 (reference)		1.00 (reference)	
T3 + T4	23.36 (3.18–171.71)		6.63 (1.61–27.35)	
N classification		0.001		0.002
N0	1.00 (reference)		1.00 (reference)	
N1 + N2 + N3	25.79 (3.51–189.58)		3.35 (1.57–7.15)	
Distant metastasis		<0.001		<0.001
No	1.00 (reference)		1.00 (reference)	
Yes	14.54 (3.84–55.06)		16.34 (3.67–72.81)	
TNM stage		<0.001		<0.001
I + II	1.00 (reference)		1.00 (reference)	
III + IV	13.12 (3.97–43.41)		5.41 (2.42–12.09)	
Tumour size (cm) <sup>a</sup>		0.736		0.095
< 3.5	1.00 (reference)		1.00 (reference)	
≥ 3.5	1.13 (0.55–2.33)		1.64 (0.92–2.92)	
GalNAc-T5 expression		<0.001		<0.001
High	1.00 (reference)		1.00 (reference)	
Low	2.92 (1.63–5.26)		4.41 (1.90–10.26)	

Abbreviations: CI = confidence interval; GalNAc-T5 = polypeptide *N*-acetylgalactosaminyl transferase 5; TNM = tumour–node–metastasis.

<sup>a</sup>Split at median.

alterations most often arise from changes in the expression levels of glycosyltransferases of cancerous cells (Dube and Bertozzi, 2005). One of the most common changes is an increase in the size and branching of *N*-glycans, which is often attributed to the increased activity of  $\beta$ 1,6 *N*-acetylglucosaminyltransferase V (MGAT5). Our previous study has demonstrated that intratumoral MGAT5 expression predicted independently post-operative overall survival of patients with gastric cancer (Wang *et al*, 2013). In addition to abnormal *N*-glycosylation, alterations in the *O*-glycosylation patterns of some proteins have been associated with cancerous cellular transformation, and may have a critical role in determining the malignant behaviour of tumour cells through regulating biochemical and functional properties of cell-surface proteins (Slawson and Hart, 2011). The initiating step of *O*-glycosylation is catalysed by a large family of up to 20 distinct polypeptide GalNAc-Ts (Gill *et al*, 2011). GalNAc-Ts could lead to the

formation of less-complex structures and an increase in the simple short determinants through catalysing the initiation of *O*-glycosylation at normally unoccupied potential glycosylation sites. The GalNAc-Ts are usually expressed only in the Golgi complex and it is dependent on post-translational modifications, including glycosylation. But recently a new mode of regulation has emerged where activation of Src kinase selectively redistributes Golgi-localised GalNAc-Ts to the endoplasmic reticulum (Gill *et al*, 2010). These results imply that the localisation of GalNAc-Ts may differ in different conditions and different cells, and may express in other organelles besides the Golgi complex. However, the exact molecular mechanisms of ectopic expression of GalNAc-Ts remain to be defined.

It has been shown that several GalNAc-Ts are useful markers for the development and progression of various tumours, such as renal cell carcinoma (Kitada *et al*, 2013), neuroblastoma (Berois *et al*, 2013),

pancreatic cancer (Li *et al*, 2011), and gastric cancer. Prior studies had revealed that patients with strong GalNAc-T3 expression in gastric tumour tissues were significantly correlated with good prognosis (Onitsuka *et al*, 2003). Besides, GalNAc-T6 was reported to be an immunohistochemical marker associated with venous invasion in gastric carcinoma (Gomes *et al*, 2009). GalNAc-T10 had

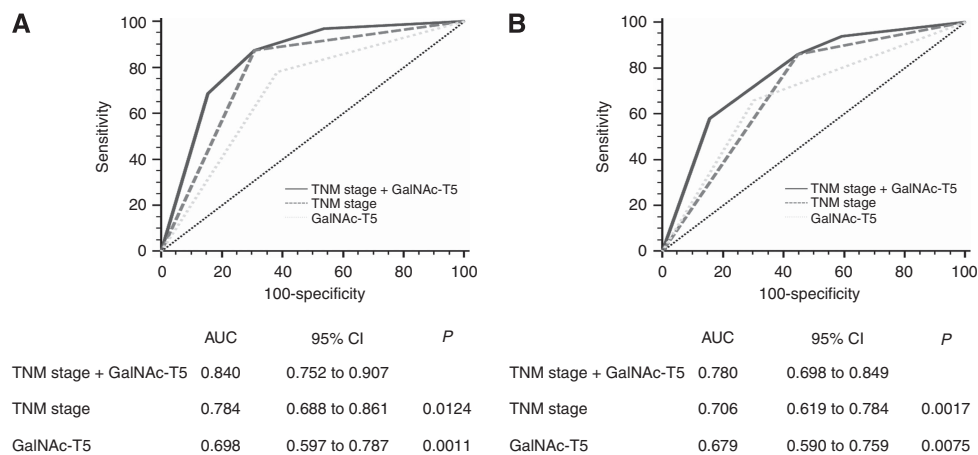
been demonstrated as a useful indicator of tumour differentiation in gastric cancer (Gao *et al*, 2013). Nonetheless, to date, no study has assessed GalNAc-T5 expression in gastric cancer. GalNAc-T5 accumulates in a very specific subset of cells, expresses in a highly tissue-specific manner, and glycosylates a restricted subset of peptides (Ten Hagen *et al*, 1998, 2003). It has been demonstrated that GalNAc-T5 could extend its catalytic domain into the lumen of the Golgi complex and/or mediate interaction with other proteins within the Golgi complex (Breton *et al*, 2001; Ten Hagen *et al*, 2003). In the present study, we first identified that GalNAc-T5 immunoreactivity was predominantly located in the cytoplasm of gastric epithelia (Figure 1B) and cancer cells (Figure 1C–F), and the intensity of the immunohistochemical staining decreased accompanied with dedifferentiation process in gastric cancer cells. Non-tumoral and well-differentiated gastric cancer tissues show strong expression of GalNAc-T5 (Figure 1B and C), whereas the less differentiated show moderate or poor expression (Figure 1D–F). These results imply that the expression status of GalNAc-T5 might be associated with the structure of gastric mucosa and the differentiated type of gastric cancer. When the normal structure was destroyed, such as carcinogenesis, the expression of GalNAc-T5 decreased. Thus, we assume that GalNAc-T5 may be an important factor that could reflect the normal form and function of gastric tissues. However, the molecular mechanisms underlying the altered expression of GalNAc-T5 remain poorly determined and await further characterisation.

Mucins are secreted or cell-surface-bound heavily *O*-glycosylated glycoproteins are produced by various epithelial cell types (Kufe, 2009). The changes in the biochemical characteristics of mucins are accompanied by malignant transformation of glandular epithelial cells. These profound changes include an alteration in *O*-glycans of mucin core peptides and an altered expression of mucin genes (Tarp and Clausen, 2008). The incomplete elongation of *O*-glycan saccharide chains in mucins could be induced by the downregulation of GalNAc-Ts expression, that can lead to the expression of shorter carbohydrate structures, such as Tn and sialyl-Tn-antigens (Wu *et al*, 2010). Tn antigen, usually masked by additional sugar residues in normal tissues, has a relatively simple structure composed of *N*-acetyl-D-galactosamine with a glycosidic  $\alpha$  linkage to serine/threonine residues in glycoproteins, and it was detected in ~90% of human carcinomas (Springer *et al*, 1995). A direct correlation has been shown between carcinoma aggressiveness and the density of Tn expression in the tumour (Springer, 1997). Thus, expression of the Tn determinant could be one of the

**Table 3.** Multivariate Cox regression analyses for overall survival in the two independent sets

Factors	Overall survival			
	Patients		Multivariate	
	No.	%	Hazard ratio (95% CI)	P-value
Training set	97	100		
Lauren classification				0.007
Intestinal type	71	73.20	1.00 (reference)	
Diffuse type	26	26.80	2.72 (1.32–5.61)	
TNM stage				<0.001
I + II	49	50.52	1.00 (reference)	
III + IV	48	49.48	11.08 (3.29–37.35)	
GalNAc-T5 expression				0.022
High	47	48.45	1.00 (reference)	
Low	50	51.55	2.75 (1.16–6.57)	
Validation set	126	100		
Lauren classification				0.530
Intestinal type	87	69.05	1.00 (reference)	
Diffuse type	39	30.95	0.82 (0.45–1.51)	
TNM stage				<0.001
I + II	49	38.89	1.00 (reference)	
III + IV	77	61.11	4.78 (2.11–10.78)	
GalNAc-T5 expression				0.003
High	70	55.56	1.00 (reference)	
Low	56	44.44	2.47 (1.35–4.50)	

Abbreviations: CI = confidence interval; GalNAc-T5 = polypeptide *N*-acetylgalactosaminyl transferase 5; TNM = tumour–node–metastasis.



**Figure 3.** ROC analyses for the prediction of overall survival in patients with gastric cancer. ROC analyses of the sensitivity and specificity for the prediction of overall survival by the combined TNM stage and GalNAc-T5 expression model, the TNM stage model, and the GalNAc-T5 expression model in (A) training set ( $n = 97$ ) and (B) validation set ( $n = 126$ ). *P*-values show the area under the ROC curves (AUC) of the combined TNM stage and GalNAc-T5 expression model vs AUCs of the TNM stage model or the GalNAc-T5 expression model.

results of GalNAc-Ts deregulation via changes in enzyme activity and/or in substrate specificity (Freire *et al*, 2005). For instance, in hepatocellular carcinoma cells, GalNAc-T2 can suppress EGF-induced malignant phenotypes through decorating the EGFR with short O-glycans, preferentially sialyl-Tn (Wu *et al*, 2011). In addition, recent studies focusing on the modulation of GalNAc-Ts indicated that GalNAc-Ts deregulation could affect leukocyte adhesion through modulating E-selection and P-selection counter receptors (Tenno *et al*, 2007). However, the exact mechanism to explain the tight relationship between GalNAc-T5 status and the progression of gastric cancer remains to be elucidated. And a better understanding of the molecular mechanism that regulates the GalNAc-T5 gene expression or enzyme activity is important for developing novel-targeted treatments for gastric cancer.

In conclusion, our results indicate that decreased intratumoral GalNAc-T5 expression predicts independently poor post-operative overall survival of patients with gastric cancer. Integration of intratumoral GalNAc-T5 density into the current clinicopathologic TNM stage system might add some prognostic information for patients with gastric cancer and could help to identify those patients in need of more aggressive treatment and closer follow-up.

## ACKNOWLEDGEMENTS

This work was supported by grants from the National Natural Science Fund (31100629, 31270863, 31300671), the Program for New Century Excellent Talents in University (NCET-13-0146), the Shanghai Rising-Star Program (13QA1400300), the Key Project of Science and Technology Commission of Shanghai Municipality (09DZ1950101, 11411951000), and the Shanghai Municipal Health Bureau Fund (20124290).

## CONFLICT OF INTEREST

The authors declare no conflict of interest.

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