Altered expression of different GalNAc-transferases is associated with disease progression and poor prognosis in women with high-grade serous ovarian cancer

RAZAN SHETA[1,2], MAGDALENA BACHVAROVA[2], MARIE PLANTE[2,3], JEAN GREGOIRE[2,3], MARIE-CLAUDE RENAUD[2,3], ALEXANDRA SEBASTIANELLI[2,3], ION POPA[4,5] and DIMCHO BACHVAROV[1,2]

[1]Department of Molecular Medicine, Laval University, Quebec, Quebec G1V 0A6; [2]CHU de Québec Research Center, Hotel-Dieu de Québec, Quebec, QC G1R 3S3; Departments of [3]Obstetrics and Gynecology and [4]Molecular Biology, Medical Biochemistry, and Pathology, Laval University, QC G1V 0A6; [5]CHU de Québec Research Center, Hospital Saint-Sacrement, Quebec, QC G1S 4L8, Canada

Abstract. Protein glycosylation perturbations are implicated in a variety of diseases, including cancer. Aberrant glycosylation in cancer is frequently attributed to altered expression of polypeptide GalNAc transferases (GalNAc-Ts) - enzymes initiating mucin-type O-glycosylation. A previous study from our group demonstrated that one member of this family (GALNT3) is overexpressed in epithelial ovarian cancer (EOC), and GALNT3 expression correlated with shorter progression-free survival (PFS) in EOC patients with advanced disease. As considerable degree of redundancy between members of the GalNAc-Ts gene family has been frequently observed, we decided to investigate whether other members of this family are essential in EOC progression. *In silico* analysis based on publically available data was indicative for altered expression of five GalNAc-Ts (GALNT2, T4, T6, T9 and T14) in ovarian high-grade serous carcinoma (HGSC) samples compared to non-tumoral (control) ovarian tissue. We analyzed protein expression of these GalNAc-Ts in EOC cells and tumors by western blotting, followed by immunohistochemical (IHC) evaluation of their expression in EOC tumor and control samples using tissue microarrays (TMAs). Western blot analyses were indicative for low expression of GALNT2 and strong expression of GALNT6, T9 and T14 in both EOC cells and tumors. These observations were confirmed by IHC. GALNT2 displayed significantly lower expression, while GALNT6, GALNT9 and GALNT14 showed significantly higher expression in HGSC tumors compared to control tissue. Importantly, GALNT6 and GALNT14 expression correlated with poor prognosis of serous EOC patients. Moreover, our results suggest for overlapping functions of some GalNAc-Ts, more specifically GALNT3 and GALNT6, in directing EOC progression. Our results are indicative for a possible implication of different members of the GalNAc-T gene family in modulating EOC progression, and the potential use of GALNT6 and GALNT14 as novel prognostic EOC biomarkers. These data warrant future studies on the role of members of the GalNAc-Ts gene family in ovarian tumorigenesis.

Correspondence to: Dr Dimcho Bachvarov, CHU de Québec Research Center, Hotel-Dieu de Québec, 9 rue McMahon, Quebec, Quebec G1R 3S3, Canada
E-mail: dimtcho.batchvarov@crhdq.ulaval.ca

Abbreviations: EOC, epithelial ovarian cancer; GalNAc-Ts, GalNAc transferases; HGSC, high-grade serous carcinoma; HOSE, human ovarian surface epithelial; IHC, immunohistochemistry; KD, knockdown; LMP, low-malignant potential; MD, moderately differentiated; MCA, multiple correspondence analyses; PD, poorly differentiated; PTM, post-translational modifications; PFS, progression-free survival; SOC, serous ovarian carcinomas; SNO, superficial scrapings from normal ovaries; TMAs, tissue microarrays

Introduction

Epithelial ovarian cancer (EOC) accounts for 4% of all cancers in women and is the leading cause of death from gynecologic malignancies (1). Despite medical and surgical improvements, long-term survival rates for EOC patients with advanced disease remain disappointing, primarily due to its asymptomatic nature and the lack of reliable methods for early diagnosis (2,3). Indeed, most women are diagnosed with EOC when micro- and macro-metastases are already present and currently, the 5-year EOC survival rate is rather disappointing (<40%) (1). Thus, the identification of novel pro-metastatic EOC molecules and associated pathways could provide additional therapeutic targets for improved management of this deadly disease.

There are many documented studies investigating different post-translational modifications (PTMs) and their

association with cancer; among these, aberrant glycosylation has displayed rather important roles in cancer progression (4,5), including EOC dissemination (6,7). Mucin-type O-glycosylation of proteins represents the most diverse PTM form, and is considered to be a conserved type of glycosylation found in many species and organ types (8). This type of PTM is rather complex and involves the transfer of different monosaccharides to each of the six O-linked glycans (9). O-glycosylation is initiated by 20 GalNAc-transferases, a family of enzymes known as the UDP N-acetylgalactosamine: polypeptide N-acetyl galactosaminyl transferases (GalNAc-Ts) (9), which are responsible for the transfer of the monosaccharide GalNAc from UDP-GalNAc to the hydroxyl group of the serine, threonine or tyrosine residues found in the target protein substrate (10). Interestingly, the genes comprising this family of enzymes show tissue-specific expression (11,12), but also have overlapping substrate specificities, suggestive for partial functional redundancy in their function (11). Several *in vitro* studies have shown that GalNAc-Ts are differentially expressed in cells and tissues during development (9). Aberrant GalNAc-Ts expression patterns have been frequently observed in cancer (13), which warrants further studies on the possible implications of these enzymes in tumor progression and their potential use as therapeutic targets and/or prognostic markers (14).

We have previously identified the GalNAc-T3 (GALNT3) gene as a potential EOC oncogene, highly expressed in advanced disease, as GALNT3 expression was significantly associated with poor outcome (15). We also demonstrated that in EOC cells, GALNT3 might directly alter biosynthesis and/or aberrant glycosylation of different O-glycoproteins that could play an essential role in EOC dissemination (15-17). However, to date, no other studies have comprehensively assessed the implications of other members of the GalNAc-Ts gene family in EOC dissemination. We thus decided to examine the role of some of the GalNAc-Ts that have been reported to play essential roles in the etiology of different cancer types. Five members of this gene family (GALNT2, T4, T6, T9 and T14) have previously displayed significant alterations in their expression in different cancer types (reviewed in ref. 7). *In silico* analysis of the GalNAc-Ts expression profiles from publicly available data were also indicative for overexpression of some of these GalNAc-Ts (including GALNT4, T6, and T14), as well as GALNT3, in ovarian HGSC samples.

The above data prompted us to investigate the expression levels of these five GalNAc-Ts (GALNT2, T4, T6, T9 and T14) in EOC cells and EOC tumor tissues, and to correlate their expression with the corresponding clinicopathological characteristics. To our knowledge this is the first report that comprehensively examines the expression profiles of several GalNAc-Ts in EOC with the perspective of identifying novel prognostic factors for this deadly disease. We show that most of the GalNAc-Ts analyzed (including GALNT2, T6, T9 and T14) displayed altered expression in EOC cells and tumors importantly, our data are indicative for a significant association of two GalNAc-Ts (GALNT6 and T14) with progression-free survival (PFS) values of EOC patients, suggestive for oncogenic functions of these two enzymes in EOC, and their potential use as novel EOC prognostic biomarkers.

Table I. Detailed patients' clinicopathological characteristics.

Variable	Range	N/Total	%
Age (years)	≥65	54/162	33
	<65	108/162	67
Median age	60		
Tissue/tumor type	Normal	18/162	11
	LMP	13/162	8
	High-grade	131/162	81
Grade	3	126/131	96
	2	5/131	4
Stage	II	5/131	4
	III	92/131	70
	IV	34/131	26
Chemotherapy[a]	Platinum+Taxol	120/131	92
	Other	11/131	8
CA125[b] (U/ml)	≥800	59/118	50
	<800	59/118	50
PFS[c] (months)	0-6	53/124	43
	7-24	44/124	35
	>25	27/124	22

[a]All patients were subjected to adjuvant therapy. [b]Extended follow-up, including CA125 values, were available for 118 patients. CA125 values correspond to the baseline serum concentrations of EOC patients. [c]Extended follow-up, including progression-free survival (PFS) values, were available for 124 patients. PFS is defined as the time from surgery to the first observation of disease progression, recurrence or death. The follow-up was available until death or to the date the study was closed. LMP, low-malignant potential.

Materials and methods

Patient cohort. Patients included in this study were operated between January 1998 to December 2015 for advanced ovarian cancer at the CHUQ Hôtel-Dieu Hospital in Quebec City, Canada. Inclusion criteria were: serous papillary carcinoma histology, FIGO stages II, III or IV and chemotherapy received after the surgery (see Table I for detailed clinicopathological characteristics). All tumors were histologically classified according to the criteria defined by the World Health Organization (18). Disease progression was evaluated following the guidelines of the Gynecology Cancer Intergroup (18). PFS was defined as the time from surgery to the first observation of disease progression, recurrence or death. The follow-up was available until death or to the date the study was closed (30 December 2016). Nineteen non-tumoral (control) ovarian samples were derived from women subjected to hysterectomy with oophorectomy due to non-ovarian pathologies.

Ethics statement approval and consent to participate. The study was approved by the Clinical Research Ethics Committee of the Hotel-Dieu de Québec Hospital, and all patients signed

Table II. Dilution and technique used for each antibody in IHC and western blot analyses.

Antibody	Species	Dilution TMA	Company	Retrieval	Incubation TMA	Dilution WB	Incubation WB
Anti-GALNT2	Rabbit	1:50	Abcam	Microwave	4°C overnight	1:1,000	4°C overnight
Anti-GALNT4	Rabbit	1:100, 1:50, 1:25	Proteintech	Microwave/ pronase	4°C overnight	1:500	4°C overnight
Anti-GALNT6	Mouse	1:75	Abcam	Microwave	4°C overnight	1:1,000	4°C overnight
Anti-GALNT9	Rabbit	1:100	LifeSpan BioScience	Microwave	4°C overnight	1:1,000	4°C overnight
Anti-GALNT14	Rabbit	1:100	Abcam	Microwave	4°C overnight	1:1,000	4°C overnight
Anti-β-actin	Mouse	N/A	Santa Cruz	N/A	N/A	1:2,000	1 h/room temperature

IHC, immunohistochemistry; TMA, tissue microarray; WB, western blotting.

an informed consent for voluntary participation and agreed to report individual patient data.

Cell culture. The EOC cell lines SKOV3 and CaOV3 were purchased from the American Tissue Type Collection; OV-90, OV2008, TOV-112 and TOV-21 cell lines were a kind gift from Dr Anne-Marie Mes-Masson (Montreal University), A2780s and A2780cp cell lines were a kind gift from Dr Benjamin Tsang (Ottawa University), the OVCAR4 cell line was a kind gift from Dr Stephan Gobeil (Laval University), and the two human ovarian surface epithelial (HOSE) cell lines; HOSE 6.3 and 17.1 were a kind gift from Dr Francis Jacob (University Hospital Basel). The cell lines were passaged in different culture media supplemented with 10% fetal bovine serum, and 1% penicillin streptomycin or 50 μg/ml of gentamicin as described previously (15).

Western blotting. Western blot analysis was performed as previously described (15). Briefly, protein lysates of the EOC and HOSE cell lines, were prepared by suspending cell pellets in Laemmli sample buffer containing 5% β-mercaptoethanol. Ovarian tumor tissue and non-tumoral tissues were homogenized and sonicated in RIPA buffer [50 mM Tris (pH 7.4), 150 mM NaCl, 0.5% sodium deoxycholate, 0.1% SDS, 1% Triton X-100] containing protease and phosphatase inhibitors, samples were then incubated on ice for 15 min. Protein samples from cells and tissues were measured using a BCA protein assay kit (Thermo Scientific Pierce, Rockford, IL, USA). BSA standard or samples (20 μl) were transferred to a 96 well plate to which 200 μl working reagent was added (working reagent 50:1 ratio of assay reagents A and B). The plate was incubated for 30 min at 37°C, the plates were then analyzed with a spectrophotometer at 560 nm [iMARK microplate reader (Bio-Rad Hercules, CA, USA)]. After centrifugation at 13,300 rpm for 15 min at 4°C, the supernatant was taken and 20-30 μg of the protein were used for sample preparation. Protein lysates were separated by 6-12% Tris-glycine gel electrophoresis and transferred onto a polyvinylidene difluoride membrane using a semidry apparatus (Bio-Rad Laboratories, Mississauga, ON, Canada). The membrane was blocked with 4% non-fat dry milk in TBST (20 mmol/l Tris-HCl, 0.5 M NaCl and 0.1% Tween-20), and the membranes were incubated overnight with the primary antibody at 4°C or 1 h at RT (for a list of all the antibodies used in this study refer to Table II). After 3x15 min washes with TBST (20 mmol/l Tris-HCl, 0.5 M NaCl and 0.1% Tween-20) at room temperature, the membranes were incubated with horseradish peroxidase-conjugated secondary antibody and detected with ECL solution (Thermo Fisher Scientific, Waltham, MA, USA).

Tissue micro arrays (TMAs) and immunohistochemistry (IHC). TMAs were constructed as previously described (15). Briefly, one representative block of each ovarian tumor and control ovarian tissue was selected for the preparation of the tissue arrays. Three 0.6-mm cores of tumor were taken from each tumor block and placed, 0.4 mm apart, on a recipient paraffin block using a commercial tissue arrayer (MTA-II arrayer) (Beecher Instruments, Silver Spring, MD, USA). The cores were randomly placed on one of three recipient blocks to avoid evaluation biases.

IHC analyses were performed, as previously described (15). Briefly, 4-μm tissue sections were deparaffinised and rehydrated in graded alcohols, then incubated with blocking serum for 20 min. Following treatment with 3% H_2O_2 for 10 min to quench the endogenous peroxidise activity, sections were incubated with the primary antibody overnight at 4°C (for a list of all the antibodies used in this study refer to Table II). Incubation and detection with SignalStain 3,3'-diaminobenzidine (DAB) Substrate kit (IDetect Universal Mouse Kit HRP-DAB; ID Labs, Buffalo, NY, USA) were done according to the manufacturer's instructions. Sections were then counterstained with hematoxylin. Images were acquired using a Leica Confocal Scope (TCS SP5 X; Leica Microsystems, Exton, PA, USA) and analyzed via the Leica Application Suite Software (Leica Microsystems).

Scoring and statistical analysis. Protein expression was scored according to intensity (value of 0 for absence, 1 for low, 2 for moderate, and 3 for high) of staining based on manual visualization. A composite score was defined as the product
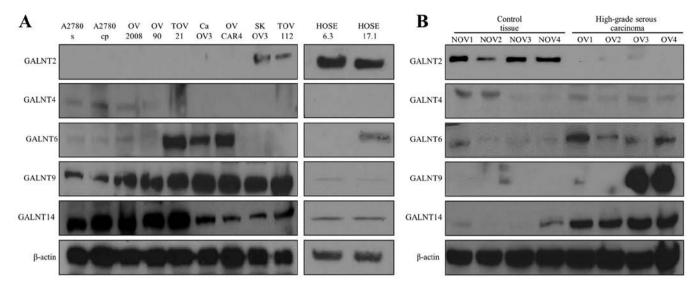
Figure 1. GalNAc-Ts expression in EOC cells and EOC tumors. (A) Western blot analysis of endogenous GALNT2, T4, T6, T9 and T14 protein expression in different EOC cell lines and the HOSE cell lines. (B) Western blot analysis of endogenous GALNT2, T4, T6, T9 and T14 protein expression in 4 HG ovarian samples (OG1-OG4) and 4 non-tumoral ovarian tissue (control tissue) samples (NOV1-NOV4). β-actin was used as a loading control. EOC, epithelial ovarian cancer; HOSE, human ovarian surface epithelial; NOV, normal ovarian; OV, ovarian tumor.

of staining intensity (nuclear, cytoplasmic, or membranous depending on the expected staining). All slides were independently scored in a blinded manner by 2 observers, and the integration was >85%. In case of differences between the 2 scorings, the core was re-evaluated to reach a consensus. The relationship between the protein expression of the listed genes in HGSC and LMP tumors, and control ovarian tissues was evaluated by the Wilcoxon two-sample test. A significant association was considered when p-values were <0.05.

Multiple correspondence analysis (MCA) was used to produce 2-dimensional displays of similarities of the relative expression patterns amongst the five GalNAc-Ts staining intensity in the different patient samples. MCA statistical analyses were carried out using SPSS software, version 13.0. Survival analysis results were visualized using Kaplan-Meier survival curve analysis (SPSS software, version 13.0). A Kaplan-Meier curve and the log-rank test were performed based on PFS values to test the effect of the intensity of the gene (3, 2 vs. 0, 1) on disease progression. The relationship between the GalNAc-Ts staining intensity and PFS was determined by the non-parametric Mantel-Cox log-rank test to compare survival distributions (SPSS software, version 13.0). Bivariate and multivariate analyses, taking into account standard or strongly associated prognostic variables, were performed to identify independent prognostic factors and a statistic test p-value of <0.05 was considered statistically significant.

Results

Analyses of the expression profiles of different members of the GalNAc-Ts family in both ovarian HGSC tumors and EOC cell lines. Initially, we investigated GalNAc-Ts expression profiles from publicly available data generated from samples found in the independently derived Affymetrix GeneChip Human Genome U133 Plus 2.0 [HG-U133_Plus_2] (19). The *in silico* data examined were based on global gene expression analysis of differentially expressed candidate mRNAs

in 21 moderately differentiated (MD) and poorly differentiated (PD) serous ovarian carcinomas (SOC) (MD/PD SC), 13 serous ovarian borderline (SOBT), and seven superficial scrapings from normal ovary (SNO) samples (19). These analyses showed significant correlation and high fold change in the overexpression of several GalNAc-Ts in the SOC samples compared to both the SBOT and SNO samples including: GALNT3, GALNT4, GALNT6, and GALNT14. Based on the above *in silico* analyses, as well as on literature data (as can be seen in Introduction), we decided to further proceed with investigating the expression levels of five GalNAc-Ts (GALNT2, T4, T6, T9 and T14) in EOC specimens by western blotting. The protein expression levels of these GalNAc-Ts were initially analyzed in nine EOC cell lines and in two HOSE cell lines (Fig. 1A). As seen from Fig. 1A, GALNT2 displayed lack of expression in seven EOC cell lines studied and quite a weak expression in SKOV3 and TOV112 EOC cells, compared to its rather strong expression in the two control HOSE cell lines, indicative for a suppression of this enzyme in EOC cells. GALNT4 showed very weak, or lack of expression in all (both EOC and HOSE) cell lines analyzed. GALNT6 expression was highly observed in three EOC cell lines (TOV21, CaOV3 and OVCAR4) while showing no, or weak expression in the two HOSE cell lines (Fig.1A). Finally, both GALNT9 and GALNT14 displayed very high expression in all EOC cell lines and almost lack of expression in the HOSE cell lines (Fig. 1A).

Further analyses of the protein expression levels of the selected GalNAc-Ts in four ovarian HGSC tumor samples and four control ovarian tissue samples were quite confirmatory to the data obtained with the EOC and HOSE (control) cell lines (Fig. 1B). Indeed, GALNT2 showed a relatively high expression in all control samples, while no expression was observed in the HGSCs, while GALNT4 showed an analogous pattern of rather weak or no expression between control tissues and HGSC tumors. Correspondingly, GALNT6 showed a relatively high expression in the HGSCs tumor samples compared
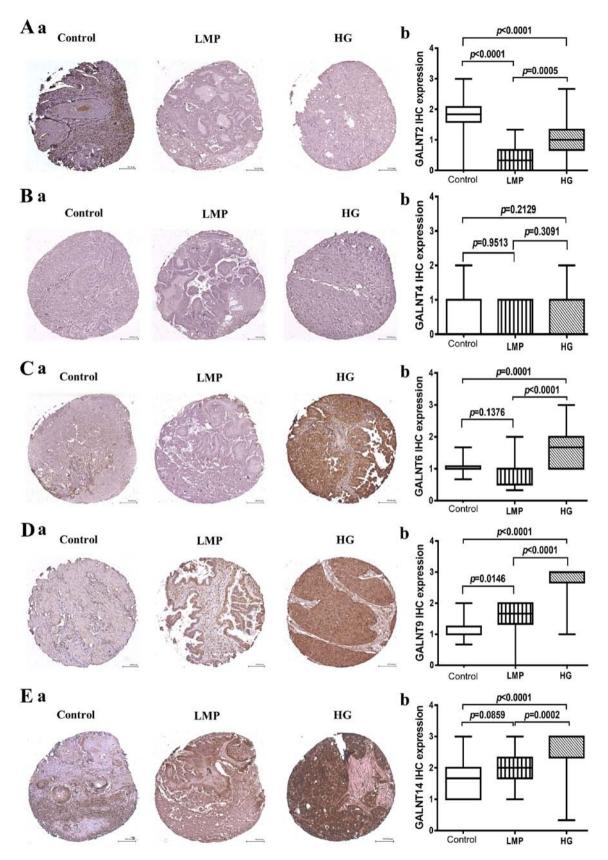
Figure 2. GalNAc-Ts protein expression in HG samples compared to LMP and non-tumoral ovarian samples. (A) a, GALNT2 staining patterns in representative cores in control ovarian tissues, LMP tumors and HG tumors. b, Box-plot presentation of GALNT2 protein expression levels in control ovarian tissues, LMP tumors and HG tumors. (B) a, GALNT4 staining patterns in representative cores in control ovarian tissues, LMP tumors and HG tumors. b, Box-plot presentation of GALNT4 protein expression levels in control ovarian tissues, LMP tumors and HG tumors. (C) a, GALNT6 staining patterns in representative cores in control ovarian tissues, LMP tumors and HG tumors. b, Box-plot presentation of GALNT6 protein expression levels in control ovarian tissues, LMP tumors and HG tumors. (D) a, GALNT9 staining patterns in representative cores in control ovarian tissues, LMP tumors and HG tumors. b, Box-plot presentation of GALNT9 protein expression levels in control ovarian tissues, LMP tumors and HG tumors. (E) a, GALNT14 staining patterns in representative cores in control ovarian tissues, LMP tumors and HG tumors. b, Box-plot presentation of GALNT14 protein expression levels in control ovarian tissues, LMP tumors and HG tumors. All p-values were derived from log-rank tests. Error bars denote standard deviation of each mean calculation. HG, high-grade; LMP, low-malignant potential.
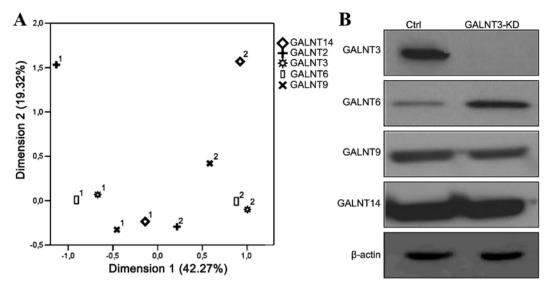
Figure 3. (A) Multiple correspondence analyses (MCA) of the relationship of the GalNAc-Ts expression in the HG ovarian tissue samples. Two major groups, separated by the first and second components, are observed. The first group includes the genes GALNT6 and T9 showing a somewhat overlapping expression pattern in the cohort of patients examined in the study. The second group indicates the inverse correlation observed between GALNT2 and GALNT14. For each analysis, the percentage of variance explained by the first two dimensions is indicated in parenthesis. (B) Western blot analysis of GALNT3, T6, T9 and T14 expression in control (Ctrl) and GALNT3 knockdown (KD) A2780s clones. β-actin was used as a loading control. HG, high-grade.

to control tissues, and again, GALNT9 and GALNT14 displayed the strongest overexpression levels in HGSCs, relative to the controls (Fig. 1B).

IHC analysis of the expression patterns of five GalNAc-Ts in numerous EOC tumors using TMAs. We next proceeded with evaluating the GalNAc-Ts expression levels by IHC in TMAs comprising triplicate cores of 131 HGSCs and 13 LMP tumors, and including 18 control ovarian tissue specimens. Table I shows the major clinical characteristics of these patients for whom extensive follow-up clinical data were available. The age ranged from 38 to 91 years (median: 60 years). HGSC tumors were grade 3 [126 patients (96%) and grade 2 (5 patients (4%)]. Grade 3 tumors included stage III (70%) and stage IV (26%) tumors, while grade 2 tumors included stage II (4%). Most patients (92%) received standard treatment of cytoreductive surgery followed by platinum/ taxane chemotherapy regimens. The median baseline CA125 was ~800. Forty-three percent of the patients had a progression or a recurrence within the first 6 months of follow-up; for 35% of the patients the PFS interval was in the range of 7 to 24 months, and 22% of the patients displayed PFS values >25 months. The mean score for expression was defined by the extent and intensity of immunohistochemistry staining for GALNT2, T4, T6, T9 and T14 (Fig. 2). As expected, GALNT2 displayed highly significant expression (strong cytoplasmic granular staining) in control tissues compared to negative staining in both LMP (p<0.0001) and HGSC tumors (p<0.0001) (Fig. 2A). As previously, GALNT4 showed negative expression in all cases (tumor samples and control tissues), despite repeated attempts with various retrieval systems, antibody concentration, incubating time, or signal enhancements systems (Fig. 2B). GALNT6 showed a strong diffuse cytoplasmic staining which was exclusively present in HGSCs compared to control tissues (p=0.0001) and LMP tumors (p<0.0001) (Fig. 2C). A similar expression pattern

was also observed for GALNT9 and GALNT14, as both these GalNAc-Ts were significantly overexpressed in HGSC tumors, when compared to control tissues (p<0.0001), and (p<0.0001) respectively (Fig. 2D and E); however, GALNT9 also displayed significant overexpression in LMP tumors compared to control samples (p=0.0146) (Fig. 2D), while no significant difference in GALNT14 protein expression was observed between LMP tumors and control tissues (p=0.0859) (Fig. 2E).

The above results, as well as data from our previous study (15) are indicative for the simultaneous overexpression of several GalNAc-Ts in EOC cell lines and tumor tissues (including GALNT3, T6, T9 and T14). Since a considerable degree of redundancy between the different members of the GalNAc-Ts gene family has been frequently observed (20), we decided to apply the MCA approach in order to more deeply investigate the extent of overlapping expression of these GalNAc-Ts among the EOC tumor samples included in this study. Two quantitative variables are included in the analysis based on staining intensity: i) for positive staining in patient samples, and ii) for negative staining in patient samples. The two dimensions of the MCA explained 42.27% (dimension 1) and 19.32% (dimension 2) of the total data variability, respectively (Fig. 3A). These analyses were indicative for a strong and a highly overlapping relationship between GALNT3 and GALNT6, as additionally, some overlapping relationship between GALNT6 and GALNT9 cannot be excluded (Fig. 3A). GALNT14 showed the highest diversity between staining 1 and 2, with no observed overlap with the other 4 genes (Fig. 3A). GALNT2 confirmed our previous data showing a complete inverse relationship to the other 4 genes, as GALNT2 staining expression 1 and 2 showed a high inverse correlation to expression pattern observed for GALNT14, and overall differential expression to those of GALNT3, T6 and T9 (Fig. 3A). The MCA-suggested GALNT3/T6 redundancy was further confirmed by examining protein
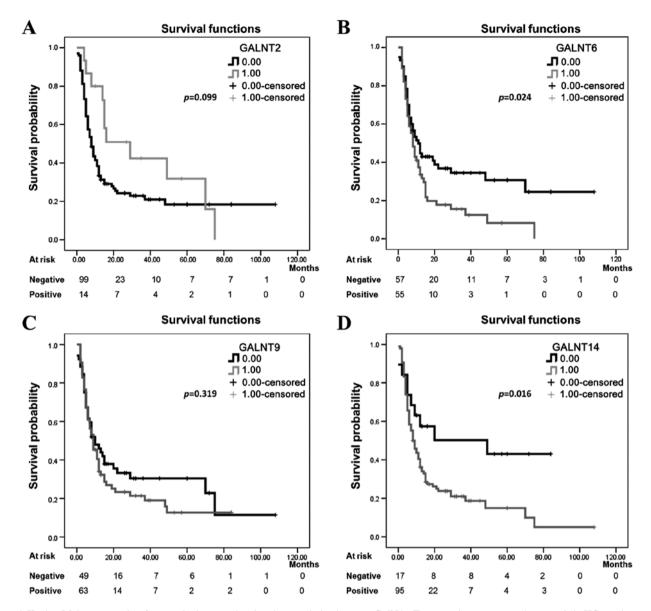
Figure 4. Kaplan-Meier progression-free survival curves showing the association between GalNAc-Ts expression patterns and prognosis in HG ovarian cancer. Kaplan-Meier survival curve analysis of HG cases for progression-free survival (PFS), presented as survival probability, of patients whose tumors show a defined staining pattern. For evaluation of GalNAc-Ts staining, HG ovarian cancer subjects were divided into two groups, those with positive staining (3 or 2 gene staining intensity) defined here as 1,00 on the Kaplan-Meier curves and those with negative staining (1 or 0 gene staining intensity) defined here as 0.00 on the Kaplan-Meier curves. The staining pattern for (A) GALNT2 protein, negative (n=101), and positive (n=15). (B) GALNT6 protein, negative (n=60), and positive (n=56). (C) GALNT9 protein, negative (n=52), and positive (n=64). (D) GALNT14 protein, negative (n=19), and positive (n=97). All p-values were derived from log-rank tests. HG, high-grade.

expression profiles of GALNT6, T9 and T14 in the GALNT3 knockdown (KD) clone, previously generated in the EOC cell line A2780s (15). As shown in Fig. 3B, we observed a clear upregulation in GALNT6 protein expression in the GALNT3 KD clone, while no expression alterations were observed for both GALNT6 and T14.

Association of GalNAc-Ts expression with clinicopathological data. We further proceeded to investigate the prognostic values of the four differentially expressed GalNAc-Ts (T2, T6, T9 and T14) in EOC patients, as we evaluated the relationship between the TMA immunostaining of the 4 GalNAc-Ts and the patient PFS data using Kaplan-Meier survival curve analyses. PFS follow-up data were available for 124 patients (Table I). The analyses were based on the staining

intensities: low (includes staining levels of <2) and high (includes staining levels of >2) (Fig. 4). No significant differences were observed between GALNT2 and T9 expression levels and the patient PFS values (log-rank=2.715, p=0.099) and (log-rank=0.995, p=0.319) respectively (Fig. 4A and C), which suggests that staining intensities for both GALNT2 and T9 in pre-treatment surgical EOC specimens are not predictive of PFS. However, there was a significant association between PFS and the expression of the two GalNAc-Ts T6 and T14 (log-rank=5.119, p=0.024) and (log-rank=5.770, p=0.016) respectively (Fig. 4B and D).

Bivariate and multivariate analyses to predict PFS were also performed on all the 4 genes. Multivariate analyses taking into account standard or strongly associated prognostic variables (age, grade, stage and initial CA125) were performed

Table III. Cox regression analysis to predict progression-free survival (PFS).

Marker	Value	Frequency N (%)	Event n (%)	Crude			Adjusted		
				HR	95% CI	P-value	HR[a]	95% CI[b]	P-value
GALNT2	Negative	101 (87.1%)	78 (77.2%)	1.0			1.0		
	Positive	15 (12.9%)	11 (73.3%)	0.599	0.317-1.130	0.113	0.339	0.103-1.119	0.076
GALNT6	Negative	60 (51.7%)	40 (66.7%)	1.0			1.0		
	Positive	56 (48.3%)	49 (87.5%)	1.596	1.047-2.434	**0.030**	1.672	1.043-2.682	**0.033**
GALNT9	Negative	52 (44.8%)	37 (71.2%)	1.0			1.0		
	Positive	64 (55.2%)	52 (81.3%)	1.232	0.807-1.880	0.334	1.470	0.939-2.300	0.092
GALNT14	Negative	20 (17.2%)	11 (55.0%)	1.0			1.0		
	Positive	96 (82.8%)	78 (81.3%)	2.185	1.117-4.275	**0.022**	2.163	1.032-4.534	**0.041**

[a]Adjusted for age, stage, grade, and baseline CA125. The hazards models were applied to adjust for the baseline patients' characteristics. [b]The confidence interval was based on the arithmetic mean of the gene expression values of the selected cases from this study. HR, hazard ratio; CI, confidence interval. Bold, statistically significant.

to identify independent prognostic factors. Multivariate analyses showed a significant association for both GALNT6 and GALNT14 and the higher risk of progression (HR, 1.672; CI, 1.043-2.682; p=0.033) and (HR, 2.163; CI, 1.032-4.534; p=0.041) respectively (Table III), but not for GALNT2 and GALNT9 (HR, 0.339; CI, 0.103-1.119; p=0.076) and (HR, 1.470; CI, 0.939-2.300; p=0.092) respectively (Table III). Taken together, our findings indicate that GALNT6 and T14 may represent useful markers to predict PFS of women with high grade serous EOC.

Discussion

Accumulating data are indicative for the important role of glycosylation perturbations in carcinogenesis, including also the abnormal expression of glycans, exclusively involved in embryonic development under normal conditions (21). Glycan alterations can be associated with cancer cell signaling and communication, tumor cell dissociation, migration and invasion, cell-matrix interactions, tumor angiogenesis, immune modulation and metastasis formation (22,23), and can serve as important biomarkers and/or therapeutic targets (24).

Altered expression of glycans in cancer is frequently attributed to aberrant expression of different members of the GalNAc-Ts family in malignant tumors compared to non-tumoral tissue (7,14). The deregulation in the expression of the different GalNAc-Ts allows them to play diverse roles in carcinogenesis (14). We have previously reported that one member of these GalNAc-Ts (GALNT3) represents a potential EOC oncogene, as its expression significantly correlated with shorter PFS intervals in EOC patients with advanced disease (15); however, data concerning the implication of other members of the GalNAc-Ts gene family in EOC dissemination are rather scarce. Indeed, only one study was suggestive for a possible role of GALNT14 in mediating the malignant behavior of EOC cells (25). Otherwise, some of the most extensively studied GalNAc-Ts in cancer include GALNT2, T4, T6, T9

and T14 (7), which represent the transferases included in the present study. Thus, concerning GALNT2, only one study is suggestive for a possible oncogenic role of this transferase in oral cancer (26), while other studies in different cancer types, including neuroblastoma, liver and gastric cancer, are strongly supportive for a role of GALNT2 in suppressing tumorigenesis (27-29). GALNT4 has also displayed discrepant roles in different cancers, as an implication of GALNT4 in breast carcinogenesis has been repeatedly demonstrated (30-32); however low GALNT4 expression was associated with poor PFS and overall survival of clear cell renal cell carcinoma patients (33). GALNT6 has been extensively studied for its implication in the malignant transformation and metastasis of epithelial cancers (34-37), and especially in breast cancer (38-40), where GALNT6 has been suggested as a novel marker for breast cancer detection and potential therapeutic target (41-44). Similarly to GALNT2 and T4, literature data for the role of GALNT9 in carcinogenesis is rather contradictory, since a protective role of GALNT9 was suggested in neuroblastoma and breast cancer dissemination (45,46), while a recent report was indicative for an oncogenic function of this enzyme in colorectal cancer (47). Interestingly, GALNT9 was among the genes identified as potentially hypomethylated and overexpressed in advanced EOC as reported in our previous study (48). Finally, GALNT14 has been characterized as an oncogene in breast and lung cancer (49-51); moreover, recent data are strongly indicative for a role of this transferase in mediating chemotherapy resistance in different cancer types (52-58).

These reports are indicative for either protective or tumorigenic roles of the different GalNAc-Ts in cancer, suggesting a highly complex and rather specific O-glycosylation pattern of glycoproteins in the different cancer types. In this study we initially analyzed the expression levels of the selected five members of the polypeptide GalNAc-Ts family in EOC cell lines and EOC tumor samples (HGSCs) by western blotting. Similar, if not identical patterns of expression were observed

for these GalNAc-Ts in both EOC cell lines and EOC tumors, as GALNT2 displayed very low or lack of expression in all EOC specimens studied, compared to control samples (including HOSE cells and non-tumoral ovarian tissues), while the expression levels of GALNT6, T9 and T14 were significantly higher in EOC cells and EOC tumors, as compared to very low/lack of expression in the corresponding controls (Fig. 1). Only GALNT4 showed very subtle, or absence of any expression in all EOC and control specimens analyzed, indicative for no implications of this enzyme in EOC progression (Fig. 1). These observations were further confirmed by performing IHC analysis of the protein expression levels in numerous EOC tumors and control tissues, using TMAs. Indeed, GALNT6, T9 and T14 exhibited very strong staining in HGSC tissues and very weak/no staining in the LMP tumors and control tissues, while GALNT2 displayed a rather inverse staining pattern indicative for a subtle or no expression in both HGSC and LMP tumors, compared to relatively strong expression in control tissues (Fig. 2). Again, lack of any staining was observed for GALNT4 in all (EOC tumor and control) tissue samples analyzed (Fig. 2). With the exception of GALNT4, our data are rather in accordance with previous studies for the roles of these GalNAc-Ts in different carcinoma types as summarized above, and suggest for a possible correlation of GALNT6, T9 and T14 expression with EOC progression, while a putative protective role of GALNT2 in EOC dissemination cannot be excluded.

Moreover, Kaplan-Meier survival curves and consecutive Cox-regression analyses revealed that the expression levels of two of the GalNAc-Ts analyzed (GALNT6 and GALNT14) were significantly related with poor PFS in the studied cohort of HG serous EOC patients (Fig. 4B and D and Table III), which suggests the putative use of these two transferases as novel prognostic serous EOC biomarkers. These results, as well as data from our previous study (15), are indicative for the significant inverse association of three members of the GalNAc-Ts gene family (GALNT3, T6 and T14) with disease progression in HGSC patients.

Furthermore, the simultaneous overexpression of four GalNAc-Ts (GALNT3, T6, T9 and T14) in advanced EOC raises the question about the specific and/or redundant functions of the members of this gene family in normal and pathological conditions, including EOC. Notably, it is known that the different GalNAc-Ts do have partially overlapping but distinct substrate specificities, which may result in these GalNAc-Ts having partial functional redundancy (59). So far, twenty members of the human GalNAc-Ts gene family have been identified (20), as such an abundance of GalNAc-Ts could provide substantial biosynthetic back-up. Although different GalNAc-Ts are differentially expressed within tissues, between cells, within a single tissue, and in different patterns at different stages in the development and differentiation, it is now becoming clear that a subset of the GalNAc-Ts display both distinct and overlapping substrate specificities (11,13). Some of the mammalian GalNAc-transferase isoforms have been grouped into subfamilies based on their high homology (11,20). One such example is the human subfamily comprised of GALNT3 and GALNT6, displaying 65% homology in their coding sequence, although this homology does not provide complete functional redundancy (59). Interestingly, GALNT6

displayed quite similar oncogenic functions in breast cancer (modulating aberrant O-glycosylation and MUC1 stabilization) (39), as those found by us for GALNT3 in EOC (15). Increased GALNT3 and T6 co-expression has been detected in pancreatic (60) and renal (61) carcinomas, suggestive for GALNT3/T6 complementary correlations. As shown (60,61), the upregulation of GALNT3 within the malignant transformation and progression of these cancers could partly depend on that of GALNT6 in both synergistic and compensatory ways. Moreover, not a separate GALNT3 or GALNT6 KD, but only double GALNT3/GALNT6 KD was shown to inhibit TGFβ-induced epithelial-to-mesenchymal transition (EMT) in prostate cancer cells (35). Similarly, our western blotting and consecutive MCA analyses described herein (Fig. 3) are strongly suggestive for a possible functional overlap and redundancy of GALNT3 and T6 in EOC. Based on the above considerations we can suggest that different GalNAc-Ts perform redundant and/or overlapping functions in disease progression of women with HGSC. We believe that these relationships need to be examined further, both *in vitro* and *in vivo* to better understand how these transferases function together in initiating the biosynthesis of specific target glycoproteins, and if they actually demonstrate compensatory methods that may enhance their implication in disease progression.

In conclusion, we have shown that four members of the GalNAc-Ts gene family are differentially expressed in EOC specimens, as GALNT6, T9 and T14 were significantly overexpressed in both EOC cell lines and HGSCs, while GALNT2 displayed an inversed expression pattern indicative for very weak or no expression in both EOC cells and EOC tumors, compared to relatively strong expression in HOSE cells and control tissues. Importantly, GALNT6 and GALNT14 expression significantly correlated with poor prognosis of EOC patients with advanced disease. These data and our previously published data are indicative for a possible implication of different members of the GalNAc-Ts gene family (including GALNT2, T3, T6, T9 and T14) in modulating EOC progression, as GALNT6 and GALNT14, together with the previously characterized GALNT3, could represent novel prognostic EOC biomarkers. Moreover, our results are suggestive for overlapping functions of some GalNAc-Ts, and especially GALNT3 and GALNT6, in EOC, in conformity with GALNT3/T6 functional redundancy described in other cancer types. Further functional studies are warranted to more completely elucidate *in vitro* and *in vivo* the individual and/or synergistic implications of the members of the GalNAc-Ts gene family in ovarian tumorigenesis.

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