Relevance of βGal–βGalNAc-containing glycans and the enzymes involved in their synthesis for invasion and survival in breast cancer patients

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Abstract To study the influence of glycosylation on breast cancer progression by analyses on glycan, mRNA, and protein level. For detection of glycan structures, we performed lectin histochemistry with five lectins of different specificity (UEA-1, HPA, GNA, PNA, and PHA-L) on a tissue microarray with >400 breast cancer samples. For comparison, mRNA expression of glycosylation enzymes involved in the synthesis of HPA and PNA binding glycostructures (GALNT family members and C1GALT1) was analyzed in microarray data of 194 carcinomas. Additionally, C1GALT1 protein expression was analyzed by Western blot analysis in 106 tumors. Correlations with clinical and histological parameters including recurrence-free (RFS) and overall survival (OAS) were calculated. Positive binding of four lectins (HPA, GNA, PNA, and PHA-L) correlated significantly with parameters involved in tumor metastasis, namely lymphangiosis, vascular invasion, lymph node involvement, and presence of disseminated tumor cells in bone marrow. HPA and PNA binding also showed a negative prognostic impact in our cohort. Correspondingly, high expression of C1GALT1, GALNT1, GALNT8, or GALNT14 mRNA and C1GALT1 protein correlated significantly with shorter OAS. Notably, combined overexpression of C1GALT1/GALNT1 or C1GALT1/GALNT8 mRNA was associated with a significantly reduced OAS (HR 3.15 and 2.73) and RFS (HR 2.01 and 1.94), pointing to an additive influence of these enzymes. This prognostic impact retained significance in multivariate analysis including classical prognostic markers. Our data indicate that glycan structures containing βGal–βGalNAc residues and the enzymes involved in their

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synthesis play a role in breast cancer progression, at least partly by their promoting influence on haematogenic and lymphatic spread.

Keywords Breast cancer · Glycosylation · Lectin · C1GALT1 · GALNT enzymes · Prognosis

Introduction

Many proteins involved in breast cancer progression, especially cell adhesion molecules and secreted proteins, are heavily glycosylated. Since glycosylation has deep impact on the function of these proteins, the study of glycans has come into focus recently, and understanding the role of glycan alterations in breast cancer cells might give the opportunity for new prognostic markers and therapeutic strategies.

Four main groups of glycans can be differentiated: *O*-glycans, *N*-glycans, glycosaminoglycans (GAG), and glycolipids. In *O*-glycosylation, a GalNAc residue is added to OH-groups of serine or threonine, whereas in *N*-glycans, a GlcNAc is added to an asparagine residue within a consensus peptide sequence. In a recent study based on mRNA expression data from 194 breast cancer patients, we found 25 glycosylation genes with independent prognostic impact in this cohort. Some of them could be validated in a second cohort [14] and were predictive of specific metastatic patterns, i.e., bone or brain metastasis. This observation emphasizes the important role of glycosylation in breast cancer progression.

In contrast to the study of glycosylation genes, a comprehensive analysis of glycans themselves in cancer tissues is technically unfeasible at the current state, since antibodies directed against specific carbohydrate structures are rare. In order to obtain some information about the glycosylation pattern of tumor cells, we therefore applied the well-known method of lectin histochemistry on a tissue microarray (TMA) including >400 breast cancer samples and 72 lymph node metastases. Lectins are carbohydrate-binding proteins of plant, microbial, or animal origin which bind to specific glycan structures [19]. Using five lectins with different specificities, we examined if specific glycan structures play a role in breast cancer progression.

Based on our finding which revealed a prognostic impact of PNA and HPA, we further analyzed whether enzymes involved in synthesis of their ligands, namely GALNT family members and C1GALT1, correlate with clinical and histological tumor parameters. Our results point to an influence of βGal–βGalNAc -containing *O*-glycans on lymphatic invasion and metastasis in breast cancer, and a strong prognostic impact of enzymes involved in their synthesis.

Methods

For lectin staining, a TMA containing tissue samples from 411 breast cancer patients treated at University Medical Center Hamburg-Eppendorf between 1999 and 2006 as well as 72 corresponding lymph node metastases was investigated. The diagnosis including evaluation of vascular invasion and lymphangiosis had been performed by pathologists, and routine immunohistochemical determination of estrogen receptor (ER) and progesterone receptor (PR) status had been performed.

For TMA generation, 0.6 mm cores were taken from invasive tumors. All cases are well characterized regarding follow-up information, presence of DTCs in bone marrow [16] and lymph node involvement. Patient data and tumor characteristics are described in Table 1. Due to loss of tissue spots during the staining procedure or lack of tumor cells in the spot, the number of evaluable primary tumors differed for the five lectins, ranging from 182 to 293. As control, large overview sections of 26 carcinomas which had been positive for at least one lectin on the TMA were analyzed.

Informed consent for the scientific use of tissue materials, which was approved by the local ethics committee (Ethik-Kommission der Ärztekammer Hamburg, #OB/V/03), was obtained from all patients. The study was performed in accordance to the principles of the declaration of Helsinki and REMARK criteria [13].

Lectin histochemistry and immunohistochemistry

Lectin histochemistry was performed as described [25] using the following biotinylated lectins: *Phaseolus vulgaris* leukoagglutinin (PHA-L; Vector B-115), *Arachis hypogaea* (PNA; Sigma L6135), *Galanthus nivalis* (GNA; Vector B-1245), *Helix pomatia* (HPA; Sigma L6512), and *Ulex europaeus* (UEA-I; Sigma L8262).

Formalin-fixed paraffin-embedded tissue sections were deparaffinized and treated with 0.1 % trypsin (Sigma; St Louis, MO, USA) for 15 min at 37 °C. Then they were incubated with the biotinylated lectins at 0.1 µg/ml^{-1} for 60 min, followed by an avidin-alkaline phosphatase complex (Vectastain ABC AP, Vector Laboratories, Inc., Burlingham, CA, U.S.A.). Control sections were incubated in the same way, omitting the lectins.

Stained TMA sections were digitalized using a Mirax Midi slide scanner (Zeiss, Germany) and evaluated using the Panoramic Viewer program (3DHistech, Budapest, Hungary). Staining intensity was evaluated by two investigators (US, KML) using the following categories: 0: no staining in tumor cells; (1) minimal staining in few tumor cells; (2) weak staining; (3) weak to moderate staining; (4) moderate staining; (5) moderate to strong staining; (6)

Table 1 Cohort characteristics of breast cancer patients analyzed by lectin histochemistry[b]

	UEA1[a]	HPA[a]	GNA[a]	PNA[a]	PHA-L[a]
n	268	218	261	283	182
Age at diagnosis (year)					
Mean (median)	56.3 (56.9)	57.9 (59.8)	57.1 (57.5)	56.9 (57.5)	56.1 (56.6)
Histological type					
Ductal	191 (71.3)	152 (69.7)	185 (70.9)	210 (74.2)	130 (73.9)
Lobular	48 (17.9)	38 (17.4)	45 (17.2)	40 (14.1)	27 (15.3)
Ductulolobular	12 (4.5)	14 (6.4)	12 (4.6)	14 (4.9)	11 (6.3)
Others/unknown	17 (6.3)	14 (6.4)	19 (7.3)	19 (6.7)	8 (4.4)
Stage					
T1	132 (49.3)	106 (48.6)	132 (50.6)	147 (51.9)	81 (46.0)
T2	106 (39.6)	84 (38.5)	104 (39.8)	107 (37.8)	79 (44.9)
T3	18 (6.7)	14 (6.4)	14 (5.4)	15 (5.3)	9 (5.1)
T4	10 (3.7)	12 (5.5)	9 (3.4)	12 (4.2)	6 (3.4)
Nodal involvement					
Negative	150 (56.0)	125 (57.3)	147 (56.3)	168 (59.4)	93 (52.8)
Positive	116 (43.3)	91 (41.7)	112 (42.9)	113 (39.9)	81 (46.0)
Bone marrow status					
No tumor cells	195 (72.8)	160 (73.4)	195 (74.7)	209 (73.9)	129 (73.3)
1–2 tumor cells	72 (26.9)	58 (36.6)	65 (24.9)	74 (26.1)	46 (26.1)
Grading					
G1	10 (3.7)	16 (7.3)	13 (5.0)	16 (5.7)	5 (2.8)
G2	147 (54.9)	125 (57.3)	140 (53.6)	148 (52.3)	96 (54.5)
G3	105 (39.2)	72 (33.0)	103 (39.5)	113 (39.9)	73 (41.5)
ER status					
Negative	62 (23.1)	45 (20.6)	60 (23.0)	67 (23.7)	44 (25.0)
Positive	206 (76.9)	173 (79.4)	201 (77.0)	216 (76.3)	132 (77.0)
PR status					
Negative	89 (33.2)	73 (33.3)	90 (34.5)	96 (33.9)	62 (35.2)
Positive	179 (66.8)	145 (66.5)	171 (65.5)	187 (66.1)	114 (64.8)
Lymphangiosis					
Positive	21 (7.8)	18 (8.3)	20 (7.7)	21 (7.4)	14 (8.0)
Negative	186 (69.4)	154 (70.6)	184 (70.5)	205 (72.4)	125 (71.0)
Vascular invasion					
Positive	27 (10.1)	22 (10.1)	27 (10.3)	28 (9.9)	21 (11.9)
Negative	213 (79.5)	174 (79.8)	207 (79.3)	225 (79.5)	136 (77.3)
Type of surgery					
Breast conserving surgery	186 (69.4)	152 (69.7)	177 (67.8)	204 (69.6)	116 (65.9)
Mastectomy	76 (28.4)	61 (28.0)	78 (29.9)	83 (28.3)	56 (31.8)
Adj. Chemotherapy					
Yes	181 (67.5)	141 (64.7)	172 (65.9)	188 (66.4)	119 (67.6)
No	81 (30.2)	71 (32.6)	83 (31.8)	88 (31.1)	52 (29.5)
Radiotherapy					
Yes	218 (81.3)	173 (79.4)	208 (79.7)	222 (78.4)	135 (76.7)
No	44 (16.4)	40 (18.3)	48 (18.4)	54 (19.1)	37 (21.0)
Hormone therapy					
Yes	191 (71.3)	158 (72.5)	188 (72.0)	202 (71.4)	125 (71.0)
No	68 (25.4)	51 (23.4)	65 (24.9)	71 (25.1)	43 (24.4)
Recurrence					
No	194 (72.4)	165 (75.7)	191 (73.2)	207 (73.1)	121 (68.8)

Table 1 continued[b]

	UEA1[a]	HPA[a]	GNA[a]	PNA[a]	PHA-L[a]
Yes	58 (21.6)	41 (18.8)	59 (22.6)	63 (22.3)	47 (26.7)
Died of disease					
No	221 (82.5)	183 (83.9)	222 (85.1)	230 (81.3)	
Yes	43 (16.0)	33 (15.1)	36 (13.8)	50 (17.7)	

[a] Missing values to 100 %: unknown (percentages in brackets)

[b] Primary carcinomas

strong staining. No discrimination between membraneous and cytoplasmic staining was performed, and positive staining in stromal cells was not evaluated. For correlations with clinical and histological characteristics, two groups of negative and minimal staining (0–1) and weak to strong lectin binding (2–6) were compared.

In order to visualize vascular and lymphatic invasion, CD31 and podoplanin (clone D2-40) immunohistochemistry in large paraffin sections of selected cases ($n = 10$) was performed with the following antibodies: monoclonal mouse anti-human CD31, endothelial, clone JC70A (1:60; Dako, Glostrup, Denmark), and anti-D2-40 monoclonal antibody (1:40; Abcam, Cambridge, UK). For antigen retrieval, sections were first incubated for 20 min in hot pH6 citrate buffer (D2-40) or pH9 target retrieval solution (Dako; CD31) before overnight incubation at 4 °C with the primary antibodies. Detection was performed with Vectastain Elite ABC Kit—Peroxidase (Mouse IgG; Vector Lab.) and the DAB Peroxidase Substrate Kit (Vector Lab.).

Analysis of mRNA expression data

In order to investigate the mRNA expression of GalNT1-14 and C1GALT1, microarray data (Affymetrix HG-U133A) from an independent cohort of 194 mammary carcinomas from our clinic were analyzed. The clinical and histological characteristics of this cohort as well as technical details have been described [14] and are given in Supplementary Table S1. All microarray data have been submitted to Gene Expression Omnibus (GEO) under the following accession numbers: GSE26971 (samples GSM663775-GSM663852), GSE31519 (samples GSM782523-GSM782529), GSE31519 (samples GSM782554-GSM782568), and GSE46184 (samples GSM1125783-GSM112856 [14].

Western blot analysis

For validation of C1GALT1 mRNA data, Western blot analysis with 106 tumors from the same cohort was performed. Tissue samples of around 100 mg were homogenized in RIPA buffer using a Precellys Homogenisator (Peqlab, Erlangen, Germany). Only samples with a tumor cell content of >50 % as shown in H&E-stained cryo-cut

sections were used for protein extraction. SDS-PAGE of 20 μg proteins per sample and transfer to Immobilon membranes were performed as described [12]. C1GALT1 was detected by a monoclonal antibody (F31; Santa Cruz, Heidelberg, Germany) diluted 1:200, with MDA-MB231 cell extracts as positive control. As control for protein loading, the membranes were reprobed with anti-GAPDH antibody (FL-335; Santa Cruz). Band intensities were quantified by densitometry (Imaging Densitometer GS-700, BioRad, Munich, Germany) and calculated as percent intensity of the control sample.

Statistics

Statistical analysis was conducted by using SPSS software Version 21 (IBM SPSS Statistics, Armonk, NY, USA). Correlations between the lectin staining results were examined using two-sided Pearson tests. For further analysis, all tumors were divided into two groups representing negative/minimal (score 0–1) and low, moderate, and high staining intensity (score 2–6) for each lectin. χ^2-tests were used to examine correlations between lectin staining and clinicopathologic factors. Survival was analyzed by Kaplan–Meier analysis and Log-Rank Tests. Cox regression models were calculated for uni- and multivariate analysis. Probability values less than 0.05 were regarded as statistically significant.

Results

Lectin staining indicates involvement of certain glycan structures in lymphangiosis and vascular invasion

Since the results of our prior RNA analysis revealed a significant prognostic value of enzymes involved in *O*- and *N*-glycosylation, we performed lectin histochemistry using 5 lectins with different specificity (see Table 2) using TMA paraffin sections including 411 breast cancer samples.

Representative staining results and distribution of scoring values are shown in Fig. 1. Positive results were observed for UEA-1 in 10.5 % of the samples ($n = 29/277$

Table 2 Correlations of UEA-I, HPA, GNA, PNA, and PHA-L lectin staining with clinical and histological parameters

Lectin	UEA-I	HPA	GNA	PNA	PHA-L
Full name	*Ulex europaeus* agglutinin	*Helix pomatia* agglutinin	*Galanthus nivalis* lectin	Peanut agglutinin	*Phaseolus vulgaris* lectin
n	268	218	261	283	176
Preferred binding specificity	a-linked fucose	a-GalNAc (Tn antigen)	a-1,3 mannose	Gal-ß(1,3)-GalNAc (T antigen, core 1)	complex glycans with terminal Gal, GlCNac, Man
Chi-square test (p)[a]					
Lymphangiosis	0.921	**0.001**	0.857	**0.005**	0.143
Vascular invasion	0.094	0.171	**<0.001**	**0.023**	**0.021**
Lymph node involvement	0.261	**0.025**	0.497	**0.003**	**0.020**
DTC in bone marrow	0.840	**0.023**	0.188	0.487	0.203
FIGO stage	**0.021**	0.184	0.144	0.172	**<0.001**
Grading	0.187	0.376	0.478	0.910	**0.025**
Kaplan–Meier analysis (log-rank; p)[b]					
Recurrence-free survival	0.702	**0.013**[b]	0.273	0.086	0.961
Overall survival	0.752	0.073[b]	0.917	**0.026**	0.549

[a] For Chi-square tests and log-rank analysis, the following groups were compared: UEA-I, negative/minimal ($n = 240$) versus positive binding ($n = 28$); for HPA, negative/minimal ($n = 136$) versus positive binding ($n = 82$); GNA, negative/minimal ($n = 206$) versus positive binding ($n = 55$); PNA, negative/minimal ($n = 189$) versus positive binding ($n = 94$), PHA-L, negative/minimal ($n = 63$) versus positive binding ($n = 113$)

[b] For Kaplan–Meier analysis, the same groups were compared except for HPA: negative results ($n = 88$) versus any positive binding ($n = 130$). Significant results ($p < 0.05$) are shown in bold

evaluable tumors), for HPA in 38.1 % ($n = 86/226$), for GNA in 20.7 % ($n = 56/270$), for PNA in 33.1 % ($n = 97/293$), and for PHA-L in 64.5 % of the tumors ($n = 118/182$). The staining pattern was mostly membranous for HPA, UEA-I, and PHA-L, membranous and cytoplasmic for PNA, and mostly cytoplasmic with a granular pattern for GNA. By Pearson tests, significant associations ($p < 0.01$) were found between the results for UEA-I and GNA ($\rho = 0.209$; $p = 0.001$), HPA and PNA ($\rho = 0.209$; $p = 0.003$), and GNA and PNA ($\rho = 0.236$; $p < 0.001$; not shown). Lectin staining in large sections of 26 tumors which had been positive for at least one lectin largely confirmed the TMA-based results indicating rather homogeneous staining patterns within the tumors (not shown).

Regarding histological and clinical tumor parameters, we found significant positive correlations of GNA, PNA, and PHA-L binding to vascular invasion. In addition, HPA and PNA binding were associated with lymphangiosis, and HPA, PNA, and PHA-L binding correlated to lymph node involvement (Table 2, Supplementary Table S2; Fig. 2h–j). In Fig. 2a–g, some examples of lectin-positive tumors showing invasion into CD31-positive blood vessels and/or podoplanin-positive lymphatic vessels are shown.

Regarding DTCs in bone marrow, only HPA binding showed a significant association. UEA-I and PHA-L correlated with advanced stage, and PHA-L positivity was significantly associated with high grading and a negative PR status (Table 2 and S2). No significant correlation with age, histological subtype, and ER status was found (not shown).

## PNA and HPA binding is associated with poor survival in breast cancer patients

Using long-term follow-up information for our cohort, PNA binding was significantly associated with shorter OAS ($p = 0.026$; HR 1.97, 95 % CI 1.07–3.60), whereas its association with RFS did not reach significance ($p = 0.086$; Fig. 3a). In addition, HPA binding was weakly associated with shorter RFS ($p = 0.059$; HR 1.80, 95 % CI 0.97–3.35), but not with OAS. The prognostic significance of HPA was more clear cut if completely negative tumors were compared to those showing any positivity (score 1–6). Here HPA staining correlated significantly with RFS ($p = 0.013$; HR 2.49, 95 % CI 1.17–4.92), and was weakly associated with shorter OAS ($p = 0.073$; R 1.99, 95 % CI
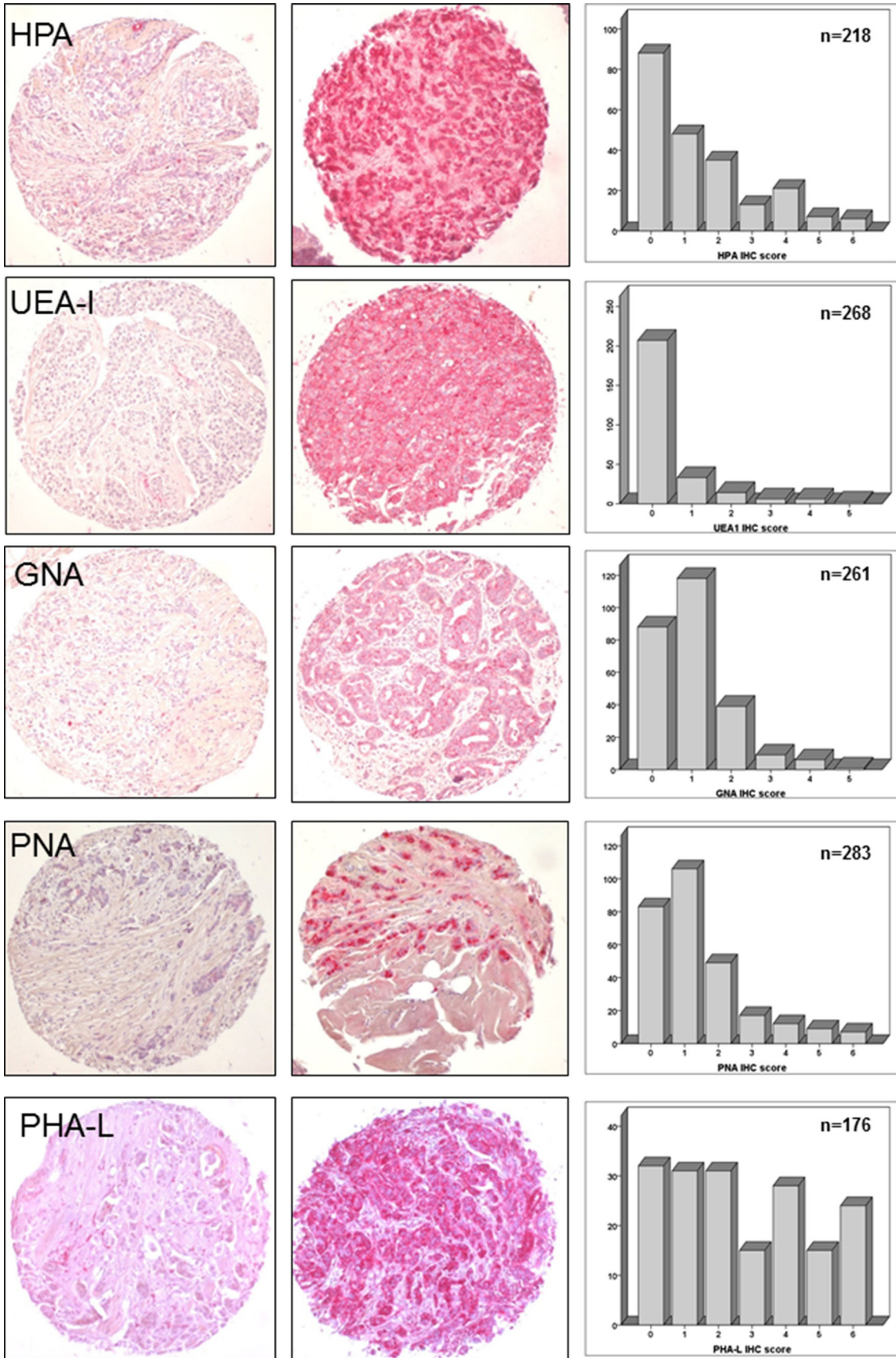
◄**Fig. 1** Lectin histochemistry on breast cancer samples. Representative HPA, UEA-I, GNA, PNA, and PHA-L binding results showing examples with negative/minimal staining (*left row*) or positive staining (*right row*). On the right side, the distribution of IHC scoring results ranging from 0 (negative) to 6 (strong lectin staining) is shown

0.93–4.29; Fig. 3a). This prognostic value retained its significance for RFS in multivariate Cox regression analysis, whereas PNA staining lost its statistical significance (Supplementary Table S3). For UEA-I, GNA, and PHA-L binding, no association with RFS or OAS was found.


Fig. 2 Association of lectin staining with vascular and lymphatic invasion of mammary carcinomas. **a–c**, poorly differentiated carcinoma showing positive PNA binding (**a**), invasion into CD31-positive capillaries (**b**), and into Podoplanin (PDPN)-positive lymphatic vessels (**c**; arrows). **d–e**, poorly differentiated carcinoma showing PNA binding (**d**) and invasion into PDPN-positive lymphatic vessels (**e**). **f–g**, poorly differentiated carcinoma showing positive HPA staining (**f**) and invasion into a larger lymphatic duct (**g**). **h–j**, correlation of GNA and PNA positivity with vascular invasion (**h**) and correlation of HPA and PNA staining with lymphangiosis (**j**) as documented in pathological reports. *p* values after Chi square tests are given
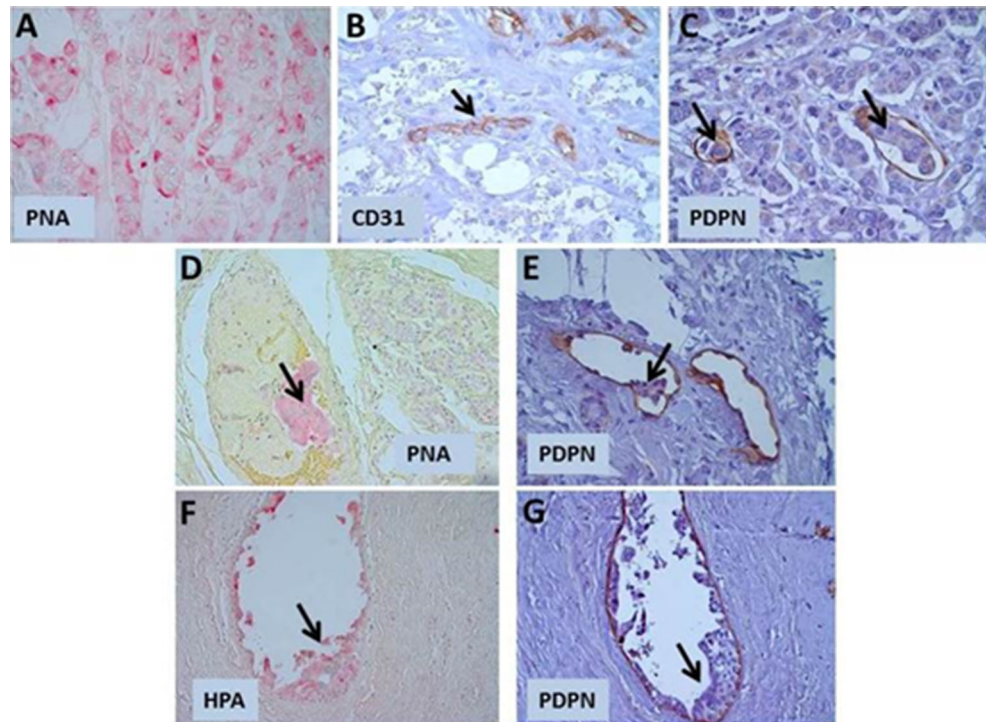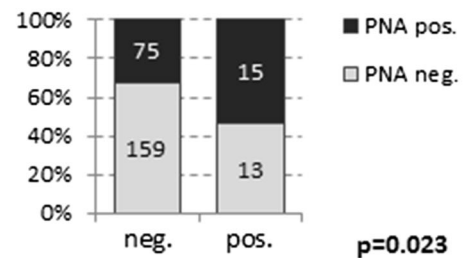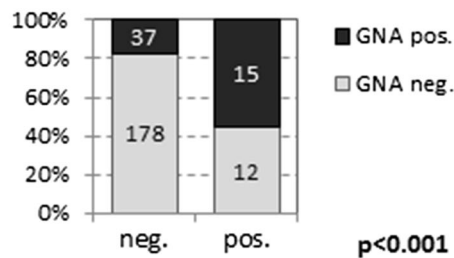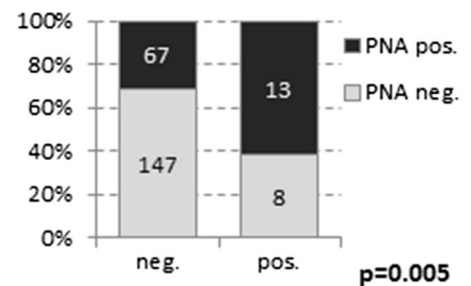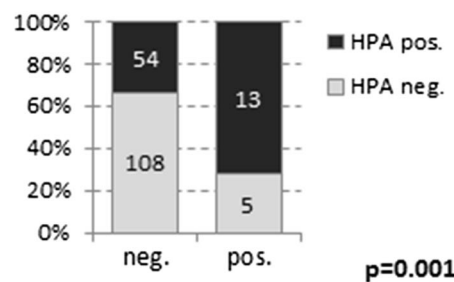
Fig. 3 Kaplan–Meier analysis of RFS and OAS based on HPA and PNA lectin binding. **a** all primary tumors. **b** ER-negative carcinomas. For HPA staining, the dashed line represents lectin-negative tumors (score 0), the *solid line* lectin-positive cases (score 1–6). For PNA, negative/minimal binding (score 0–1; *dashed line*) was compared to clearly positive staining results (score 2–6; *solid line*). *p* values after log-rank tests are shown

A All tumors

B ER-negative tumors
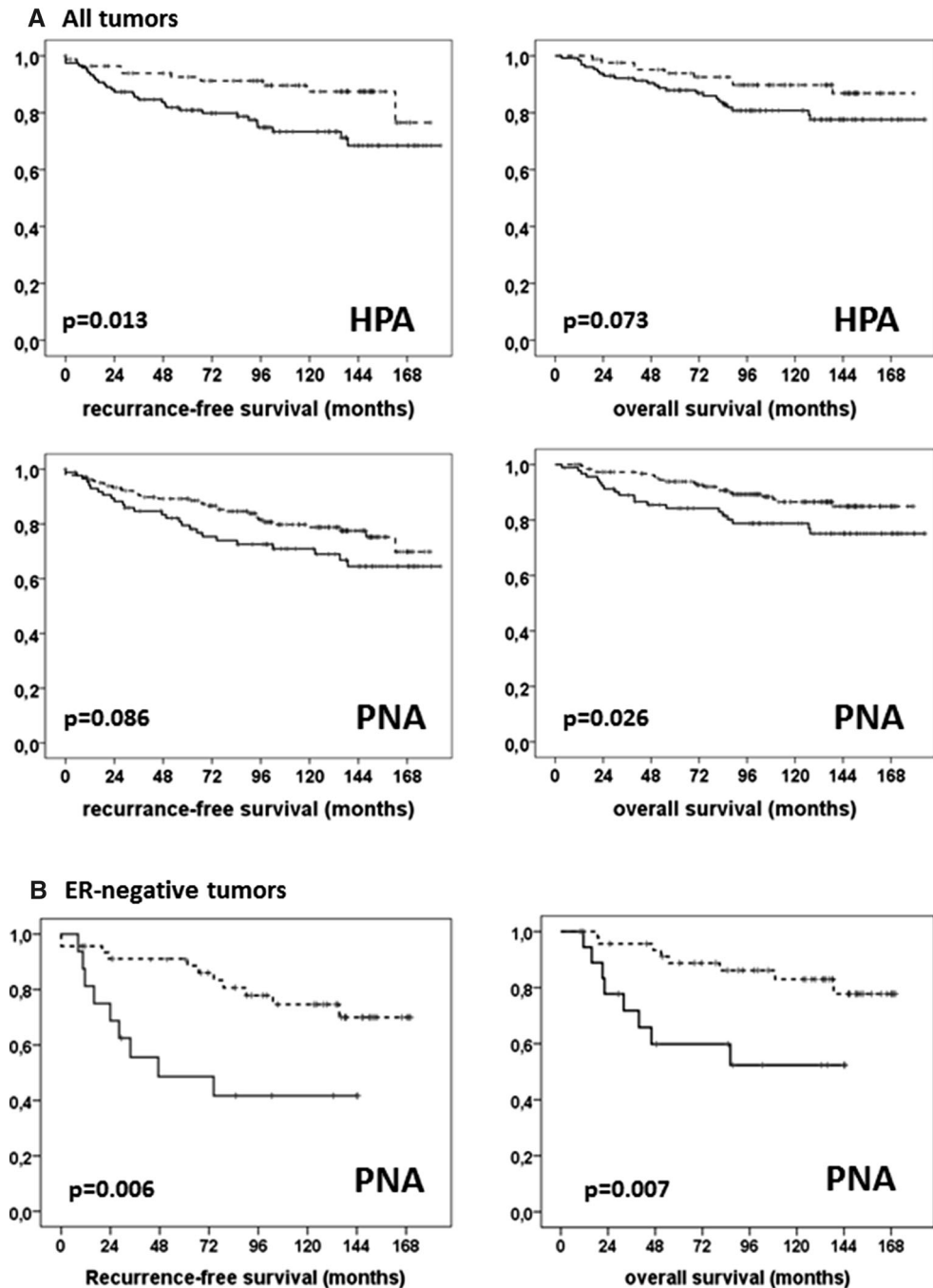
In order to compare different molecular subtypes, we performed a stratified analysis of ER-positive and ER-negative carcinomas. By this approach, PNA binding turned out as strong prognostic indicator in ER-negative, but not ER-positive tumors (Fig. 3b). PNA staining was associated with significantly reduced OAS ($p = 0.007$; HR 3.58, 95 % CI 1.34–9.61) and RFS ($p = 0.006$; HR 3.26, 95 % CI 1.34–7.93) in this subgroup. In contrast, HPA binding predominantly correlated with shorter RFS in ER-positive carcinomas ($p = 0.046$; not shown). UEA-I, GNA,

and PHA-L binding was not associated with survival in these subgroups.

For 72 carcinomas, the TMA included not only primary tumors, but also lymph node metastases. The number of evaluable metastases differed from 38 (PHA-L) to 62 (UEA-I). Compared to primary tumors, the percentage of lymph node metastases showing positive lectin binding (score 2–6) was slightly higher: 18 % (metastases) versus 10 % (primary tumors) UEA-I-positive; 46 versus 38 % HPA-positive; 32 versus 21 % GNA-positive; 35 versus

33 % PNA-positive; 84 versus 65 % PHA-L-positive (not shown). In several cases, primary tumors and corresponding lymph node metastases could be compared for lectin binding. After blinded evaluation, identical results (score 0–6) or a change of only one scoring category was found in most tumors (Supplementary Fig. S1). Thus, the glycan patterns of the tumors seem to be generally stable during metastatic progression.

Enzymes involved in the synthesis of HPA or PNA binding glycostructures are prognostic in breast cancer patients

Based on the prognostic relevance of HPA and PNA, we investigated if a similar impact can be found for glycosylation enzymes involved in the synthesis of their corresponding ligand structures. HPA mainly recognizes terminal *N*-acetyl galactosaminyl (GalNAc) residues, and GalNAc binding to serine or threonine residues in the Golgi is performed by members of the UDP-GalNAc:polypeptide *N*-acetylgalactosaminyltransferase (GALNT) family of 14 enzymes (GALNT1–GALNT14) with different, but overlapping substrate specificities and patterns of expression. Addition of galactose to this GalNAc residue in beta 1,3-linkage by a glycoprotein-*N*-acetylgalactosamine 3-beta-galactosyltransferase (C1GALT1) results in formation of a suitable PNA ligand (Fig. 4a) which can be further elongated by additional sugar residues.

In order to study the prognostic impact of these enzymes, we analyzed cDNA microarray data from 194 patients. Table 3 shows the expression levels of all GALNT family members present on the Affymetrix chip (GALNT5, 9 and 13 were not represented there) and C1GALT1. By Cox regression analysis, high expression (>median) of GALNT1, GALNT8, GALNT14, and C1GALT1 was associated with a significantly reduced overall survival, whereas GALNT11 was associated with longer OAS. In addition, longer RFS was significantly associated with high GALNT8 and low GALNT11 expression (Table 3).

Since C1GALT1 was the most significant predictor of OAS (Fig. 4c, top; Table 3), we verified its prognostic impact on protein level in 106 carcinomas from the same cohort. After Western blot analysis, the resulting C1GALT1 protein expression values correlated significantly with the corresponding mRNA data ($r = 0.589$; $p < 0.00001$). In addition, high C1GALT1 protein expression was significantly associated with shorter RFS ($p = 0.028$), whereas its correlation with OAS did not reach statistical significance ($p = 0.208$) (Fig. 4b).

Further analysis revealed correlations with histological or clinical tumor characteristics (Supplementary Table S4): Both C1GALT1 mRNA and protein overexpression were significantly associated with nodal involvement (mRNA: $p = 0.011$/protein: $p = 0.031$), and a negative estrogen receptor ($p < 0.001$/$p = 0.010$) and progesterone receptor status ($p = 0.016$/$p = 0.002$).

Combined analysis of GALNT and C1GALT1 expression levels reveals additive effects on metastasis and survival

Since PNA binding was an adverse prognostic indicator and PNA ligands arise from the activity of at least one GALNT family member and C1GALT1, we presumed that combined analysis of two relevant enzymes (Fig. 4a) might enhance their prognostic impact. Therefore, we combined C1GALT1 mRNA results with GALNT1, GALNT8, or GALNT14 data, leading to four groups for each combination (C1GALT1^{low}/GALNT^{low}; C1GALT1^{low}/GALNT^{high}; C1GALT1^{high}/GALNT^{low}; C1GALT1^{high}/GALNT^{high}). By survival analysis, it could be shown that high expression of two enzymes (C1GALT1/GALNT1 or C1GALT1/GALNT8) had a strong adverse prognostic impact which exceeded the influence of only one enzyme (Table 3; Fig. 4c). In contrast, combination with GALNT14 expression did not increase the prognostic significance of C1GALNT1 (Table 3).

In multivariate Cox regression analyses including stage, nodal involvement, histological grading, and ER status, the C1GALT1^{high}/GALNT1^{high} combination retained its prognostic significance for overall survival and recurrence-free survival with hazard ratios of 2.67 and 1.96, respectively (Table 4). Similarly, the combination C1GALT1^{high}/GALNT8^{high} was an independent predictor of shorter OAS and RFS with hazard ratios of 2.36 and 1.78.

Discussion

Cell surface glycans are involved in various biological functions, e.g., cell proliferation, differentiation, adhesion, and signal-transduction [28]. In cancer, alterations in glycosylation can provide invasiveness and the potential to metastasize [1]. Due to technical limitations, experimental data exist for only few glycan structures: Lewis y antigen significantly enhanced the Human epididymis protein 4 (HE4)-mediated invasion and metastasis of ovarian cancer cells [34]. In addition, presence of sialyl-Lewis on colon cancer cells increased xenograft growth and angiogenesis [24], and removal of fucose moieties by fucosidase treatment of breast cancer cells decreased their invasive potential [33]. The importance of glycosylation processes is also underlined by the high amount of genes involved in glycan synthesis and modification. In a recent study on glycosylation genes in breast cancer tissue [14], we identified 25 genes with independent prognostic value in this tumor entity.
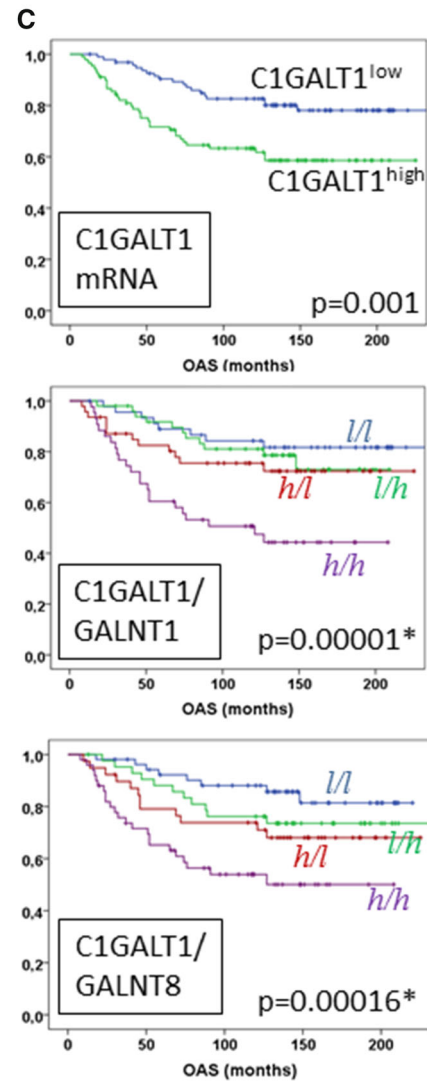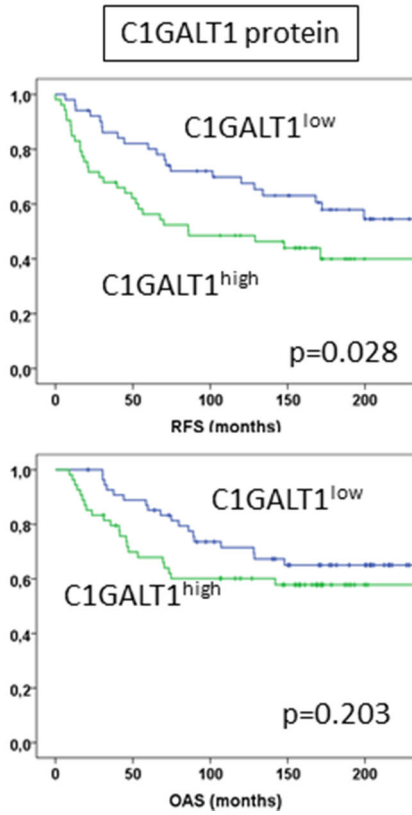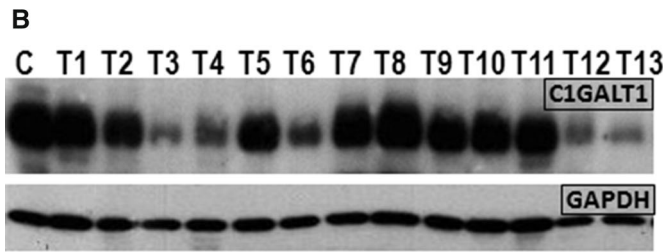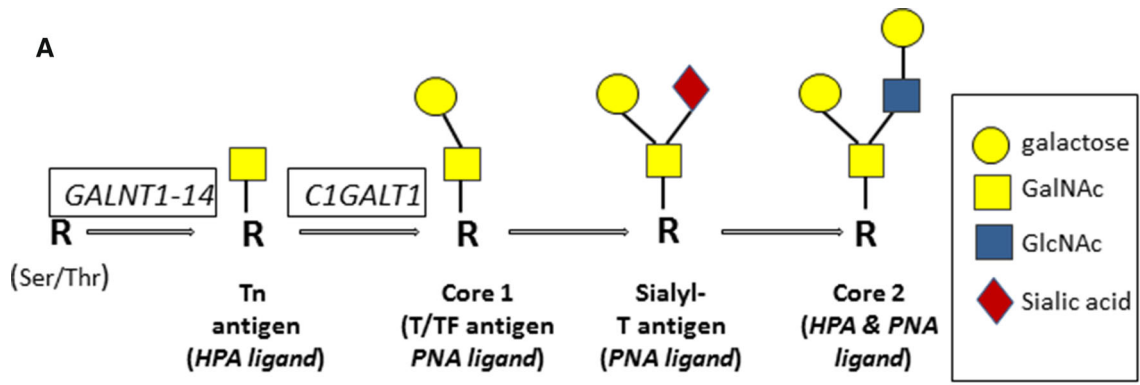
◄**Fig. 4** Prognostic influence of C1GALT1 and GALNT family members. **a** Representation of the first steps of O-glycan biosynthesis relevant to this study and the role of GALNT family members and C1GALT1 in generation of lectin ligands. **b** Correlation of C1GALT1 protein expression with recurrence-free survival and overall survival in breast cancer patients ($n = 106$). On top, representative Western blot analysis of thirteen breast cancer patients (T1–T13) and the control cell line (**c**) are shown. **c** Kaplan–Meier analysis showing the prognostic value of C1GALT1 mRNA expression (</> median) as well as C1GALT1/GALNT1 and C1GALT1/GALNT8 combinations in breast cancer patients ($n = 194$). For combination of two genes, four different groups were compared using the median expression levels as cut-off: low expression of both genes (*l/l*), C1GALT1[low]/GALNT[high] (*l/h*), C1GALT1[high]/GALNT[low] (*h/l*), and high expression of both genes (*h/h*). *p* values after Log-rank tests are shown (*for combinations, tumors with high expression of both genes were compared to the rest)

In order to confirm the role of altered glycosylation for breast cancer progression, we performed lectin histochemistry with five lectins of different specificity in a large cohort of breast cancer patients. A special feature of this cohort was the availability of information regarding metastasis-related parameters, namely lymphatic invasion (lymphangiosis), lymph node involvement, vascular invasion, and the presence of DTCs in bone marrow. Unexpectedly, four of five lectins (HPA, GNA, PNA, and PHA-

L) correlated significantly with one or more of these parameters, suggesting that glycosylation promotes metastasis by its positive influence on vascular or lymphatic invasion.

Glycosylation of adhesion proteins strongly influences their adhesive properties. Since cell adhesion to components of the extracellular matrix, endothelial and/or lymphendothelial cells is a prerequisite for cell migration, invasion, and haematogenic or lymphogenic spread, glycosylation might also influence the metastatic potential of tumor cells. The differences in results for the five lectins show that specific glycan structures play different roles in this process.

Ulex europaeus lectin (UEA-I) binding indicating α-linked fucose residues has been shown to be associated with short RFS in a smaller breast cancer cohort [2], but not in our patients. In contrast, HPA staining correlated significantly with lymphangiosis, positive lymph node involvement, the presence of DTCs in bone marrow and shorter RFS in our cohort. HPA ligands (Tn antigens) are carried by a number of important cell surface proteins, i.e., CD44, MUC1, or Integrin α6 [18, 29]. In prior studies, HPA binding correlated with a metastatic phenotype of breast cancer cells [21] and inhibited tumor cell interactions with endothelial cells [27]. Regarding the prognostic

Table 3 mRNA expression and prognostic value of GALNT family members and C1GALT1 in mammary carcinomas

	Affymetrix no.	Median expression	Range	Cox regression analysis (univariate)			
				RFS[a]	HR (95 % CI)	OAS[a]	HR (95% CI)
GALNT1	201722_s_at	1645.39	553–3437	0.523	1.17 (0.73–1.87)	**0.018**	**1.96 (1.22–3.43)**
GALNT1	201723_s_at	1435.36	586–3769	0.081	1.52 (0.95–2.45)	0.054	1.71 (0.99–2.96)
GALNT2	217787_s_at	282.43	13–855	0.422	1.21 (0.76–1.95)	0.151	1.49 (0.87–2.55)
GALNT2	217788_s_at	1198.37	431–3697	0.909	1.03 (0.64–1.64)	0.246	0.73 (0.42–1.25)
GALNT3	203397_s_at	495.97	2–4286	0.373	1.24 (0.77–1.98)	0.159	1.48 (0.86–2.53)
GALNT4	220442_at	91.27	7–372	0.978	1.01 (0.63–1.61)	0.769	0.92 (0.54–1.58)
GALNT6	219956_at	1018.76	55–8065	0.293	0.78 (0.48–1.25)	0.795	0.93 (0.55–1.59)
GALNT7	218313_s_at	1659.3	275–9299	0.998	1.00 (0.63–1.60)	0.223	0.72 (0.42–1.23)
GALNT8	220929_at	73.26	13–329	**0.015**	**1.82 (1.13–2.95)**	**0.023**	**1.90 (1.09–3.30)**
GALNT10	207357s_at	446.54	39–1419	0.594	0.88 (0.55–1.41)	0.231	0.72 (0.42–1.23)
GALNTIO	212256_at	922.32	153–2738	0.557	0.87 (0.54–1.39)	0.136	0.66 (0.39–1.14)
GALNTIO	201724_s_at	985.45	322–3179	0.680	0.91 (0.57–1.45)	0.302	1.33 (0.78–2.27)
GALNT11	219013_at	741.09	146–1627	0.181	0.73 (0.45–1.16)	**0.017**	**0.51 (0.29–0.89)**
GALNT12	218885_s_at	103.35	4–668	0.881	1.04 (0.65–1.66)	0.160	1.47 (0.86–2.53)
GALNT14	219271_at	59.48	6–1810	0.087	1.51 (0.94–2.43)	**0.045**	**1.75 (1.01–3.03)**
C1GALT1	219439_at	173.91	46–2089	0.092	1.50 (9.36–2.40)	**0.002**	**2.43 (1.39–4.25)**
C1GALT1/GALNT1[b]				**0.009**	**2.01 (1.19–3.38)**	**0.00003**	**3.15 (1.83–5.41)**
C1GALT1/GALNT8[b]				**0.011**	**1.94 (1.16–3.24)**	**0.00029**	**2.73 (1.58–4.69)**
C1GALT1/GALNT14[b]				**0.027**	**1.76 (1.07–2.89)**	**0.007**	**2.10 (1.22–3.61)**

[a] *p* values after univariate Cox regression analysis are given. Significant results are shown in bold

[b] High expression of both genes versus others

Table 4 Multivariate Cox regression analysis including FIGO stage, nodal involvement, histological grading, ER status and C1GALT1/GALNT combinations (microarray data)

Characteristics		Hazard ratio	95 % confidence interval	*p* value
Overall survival				
C1GALT1/GALNT1 combination	C1GALT1 high/ GALNT1 high versus others	2.67	1.43–4.99	**0.002**
FIGO stage	FIGO III versus others	2.07	1.19–3.59	**0.010**
Nodal status	Positive versus negative	1.60	0.87–2.93	0.132
Grading	G3 versus G1/G2	1.46	0.88–2.43	0.146
ER status	Positive versus negative	0.77	0.39–1.53	0.461
C1GALT1/GALNT8 combination	C1GALT1 high/ GALNT8 high versus others	2.36	1.29–4.30	**0.005**
FIGO stage	FIGO III versus others	2.01	1.19–3.40	**0.009**
Nodal status	Positive versus negative	1.75	0.98–3.13	0.057
Grading	G3 versus G1/G2	1.35	0.84–2.18	0.216
ER status	Positive versus negative	0.72	0.38–1.39	0.327
Recurrence-free survival				
C1GALT1/GALNT1 combination	C1GALT1 high/ GALNT1 high versus others	1.96	1.04–3.68	**0.036**
FIGO stage	FIGO III versus others	1.39	0.87–2.24	0.169
Nodal status	Positive versus negative	1.71	1.01–2.89	**0.045**
Grading	G3 versus G1/G2	1.47	0.96–2.27	0.080
ER status	Positive versus negative	1.01	0.52–1.95	0.980
C1GALT1/GALNT8 combination	C1GALT1 high/ GALNT8 high versus others	1.78	1.01–3.13	**0.047**
FIGO stage	FIGO III versus others	1.37	0.86–2.20	0.186
Nodal status	Positive versus negative	1.75	1.04–2.94	**0.034**
Grading	G3 versus G1/G2	1.35	0.89–2.05	0.155
ER status	Positive versus negative	0.97	0.52–1.81	0.917

significance of HPA in breast cancer, conflicting results were reported [4, 5, 26] which might be due to experimental details. In our cohort, the prognostic significance of HPA was dependent on the cut-off used for evaluation of the staining results.

In contrast to HPA, the role of GNA (*Galanthus nivalis*) binding to breast cancer cells has not been investigated so far. GNA detects a high-mannose tree of *N*-glycans, and GNA binding results in cell death and antitumor activity in vitro [32]. In our breast cancer cohort, GNA binding showed significant correlations with vascular invasion and advanced FIGO stage, but was not associated with survival in these patients.

Similar to GNA, little is known about the role of PHA-L binding in cancer, although it was associated with advanced malignant potential in breast and colon tumours [3, 15]. ß-1,6-branched oligosaccharides as detected by PHA-L binding had an independent prognostic impact in colorectal carcinoma [22], and in our recent study, cytoplasmic PHA-L staining was associated with poor prognosis in prostate cancer patients [9]. Our present results indicate that although total PHA-L binding correlates with vascular invasion and lymph node involvement, it did not show any prognostic impact in breast cancer, indicating that PHA-L

binding structures are not sufficient for promotion of metastasis.

The most promising lectin was PNA that mainly recognizes βGal–βGalNAc structures on glycoproteins [8]. In lung adenocarcinoma, PNA staining correlated with nodal involvement [23]. In our present cohort, PNA showed significant associations with lymphangiosis, vascular invasion, lymph node involvement, and shorter OAS. Interestingly, high expression of the TF antigen which represents a major PNA ligand has not only been shown in node-positive breast cancer [30], but also on DTCs in bone marrow, suggesting that it might be used as a target for antibody-based therapy [20]. In stratified analysis, the negative prognostic impact of PNA binding in our cohort turned out to be due to a strong correlation with shorter RFS and OAS in ER-negative, but not in ER-positive patients. Similar to our study of glycosylation enzymes [14], these results point to molecular subtype-specific influences of glycosylation on breast cancer metastasis.

For comparison with lectin staining, we analyzed the expression of enzymes involved in the synthesis of HPA and PNA ligands, eleven GALNT family members and C1GALT1, in a second breast cancer cohort from our clinic (for the TMA cohort, no mRNA expression data or frozen

tissue were available). Interestingly, C1GALT1 and three GALNT enzymes (GALNT-1, -8, -14) were associated with an unfavorable prognosis, and the highest risk for early recurrence or death was found for patients with high expression of both C1GALT1 and GALNT-1 or -8, pointing to an additive effect of these enzymes. For C1GALT1, we could demonstrate a strong correlation of mRNA and protein expression levels indicating transcriptional regulation of this enzyme. Interestingly, these genes were not among those genes with prognostic significance identified in a prior study using the same dataset [14] which obviously derives from different statistical approaches (Cox regression analysis with continuous expression values vs. Log-rank tests using two groups of low/high expression of the respective gene). Obviously, continuous increase of expression values for C1GALT1, GALNT1, GALNT8, and GALNT14 did not have a significant impact on mortality, whereas differentiation between low and high expression levels (</> median) of these genes resulted in significant differences in patient outcome, pointing to non-linear associations with recurrence or mortality.

To our knowledge, this is the first report of a prognostic influence of C1GALT1 in breast cancer. In experimental systems, activity of this enzyme showed an oncogenic effect: it enhanced proliferation and invasive potential of hepatocarcinoma cells via glycosylation of MET and integrin β1 [11, 31] and promoted invasion of colon carcinoma cells via FGFR2 glycosylation [7]. Which C1GALT1 substrate proteins are responsible for the clear prognostic effect of this enzyme in breast cancer should be investigated in further studies.

Regarding GALNT family members, GALNT14 increases proliferation, motility, and invasion of MCF7 breast cancer cells [6]. In addition, GALNT6 was shown to transform mammary epithelial cells in vitro [17], but had no prognostic impact. GALNT1 plays a role in bladder cancer through aberrant glycosylation of integrin α3 [10]. To date, no other studies concerning the prognostic influence of these enzymes in cancer patients have been published.

In contrast to PNA binding which is dependent on the activity of at least one GALNT and C1GALT1, the combined prognostic effect of these enzymes is more clear cut and not restricted to ER-negative tumors. Obviously, expression of these two glycosylation enzymes must not necessarily correlate with the presence of a PNA ligand. The activity of additional glycosylation enzymes (i.e., sialyl transferases) leading to larger, more complex O-glycans might alter these structures in a way that retains their biological properties but modifies lectin binding. Thus, the poor prognosis of GALNT^{high}/C1GALT1^{high} tumors indicates that these genes are involved in the synthesis of glycan structures which play an important role in

tumor progression, but are not always PNA binding. Unfortunately, a direct comparison of enzyme expression and lectin binding in the same tumors could not be performed in our study.

Taken together, our data indicate that glycan structures containing βGal–βGalNAc residues play a role in breast cancer progression, at least partly by their positive influence on vascular and lymphatic invasion. Therefore, these glycans and the enzymes involved in their synthesis might represent promising new targets for breast cancer therapy.

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