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Evaluation of the anti-Thomsen–Friedenreich antibodies Nemod-TF1 and Nemod-TF2 as prognostic markers in breast cancer

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Abstract

Purpose The TF (Thomsen–Friedenreich, CD176, Galβ1-3GalNAc) carbohydrate moiety is known as a specific oncofetal carbohydrate epitope present in fetal and neoplastic tissue as well as in stem cells. TF was demonstrated to mediate tumor-promoting features and to be highly immunogenic. The current study aimed to evaluate whether presence of the TF antigen is associated with clinico-pathological parameters and prognosis of early breast cancer (BC).

Methods Primary BC tissue ($n = 226$) was stained for TF using two monoclonal anti-TF antibodies (Nemod-TF1, Nemod-TF2). Staining results were correlated to clinical data including survival.

Results Nemod-TF1 staining was positively correlated to lymph node metastasis ($p = 0.03$) and the presence of tumor-associated MUC1 (TA-MUC1; $p = 0.003$). Further, the presence of the Nemod-TF1 epitope predicted worse prognosis in TA-MUC1 positive (overall survival: $p = 0.026$) as well as in triple negative (overall survival: $p = 0.002$; distant metastasis-free survival: $p = 0.012$) BC.

Conclusions The data presented here further support a role of TF in BC tumor biology. Whether anti-TF directed treatment approaches may gain clinical relevance in those cases determined as triple negative or TA-MUC1 positive remains to be determined.

Keywords Nemod-TF1/2 · CD176 · Breast cancer · Prognosis

Abbreviations

BC	Breast cancer
CIS	Carcinoma in situ
CSC	Cancer stem cell
DFS	Disease-free survival
DMFS	Distant metastasis-free survival
ER	Estrogen receptor
FFPE	Formalin-fixed, paraffin-embedded
IRS	Immunoreactive score
LRFS	Local recurrence-free survival
OS	Overall survival
PR	Progesterone receptor
REMARK	REporting recommendations for tumor MARKer prognostic studies
TA-MUC1	Tumor-associated mucin-1
TF	Thomsen–Friedenreich, CD176, Galβ1-3GalNAc
TNBC	Triple negative BC
WHO	World Health Organization

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Background

The Thomsen–Friedenreich (TF, core-1) antigen is a specific oncofetal carbohydrate (Galβ1-3GalNAc) moiety present on the surface of neoplastic cells of numerous cancer entities [1]. For instance, mucin-like glycoproteins such as MUC1 (CA15-3, CD227), which has developed to be the most widely used breast cancer (BC) serum tumor marker, were demonstrated to abundantly carry the TF antigen [2, 3]. TF is covered by a cryptic glyco-structure in healthy adult tissue, but has been detected to become exclusively demasked during the process of malignant transformation [4]. Accordingly, TF is hardly found in non-neoplastic adult cells but widely present in cancer cells especially in

epithelial descent. For this reason, TF has been hypothesized to be a tumor marker of exceptional specificity [4]. The TF carbohydrate moiety is linked to carrier molecules by α- or β-*O*-glycosylation and has therefore be termed as TFα or TFβ, respectively [5]. As TFβ is absent in tumors of epithelial descent [5], in this manuscript the term TF—unless otherwise stated—refers to TFα.

TF has been found to mediate endothelium adhesion and tumor invasion implying its potential as a marker of cancer cell aggressiveness [6]. A recent study of our group also demonstrated that the apoptotic potential of galectin-1 on BC cells is dependent on the presence of cell surface TF [7]. In addition, the TF glycoepitope is highly immunogenic and thus several attempts have been made to design antibodies targeting TF. These antibodies have already been shown to comprise anti-cancer cell activity in vitro and a mouse BC model [8, 9].

Producing monoclonal antibodies (mAbs) detecting TF appeared to be difficult in the past. In addition, mAbs detecting TF differ regarding specificity and selectivity and only few of them proved to be highly specific and selective for TF in its 'natural' context without showing cross-reactivity to synthetic TF-conjugates. Goletz et al. proposed Nemod-TF1 and Nemod-TF2 to be two of the most thoroughly characterized mAbs detecting TF [10]. Though basically Nemod-TF1 and Nemod-TF2 both detect the same carbohydrate moiety (i.e., TF), their fine specificities are different. Highly avid Nemod-TF2 detects TF but is also cross-reactive to TFβ and to core-2, a carbohydrate structure similar but not equal to TF [10]. Since core-2 has not been found in humans and TFβ is not present in human tissue at a level detectable by IHC, the cross-reactivity of Nemod-TF2 to core-2 does not alter the detection of TF by Nemod-TF2 in human tumors [10]. Nemod-TF1 holds both high affinity and specificity for the α-anomer of TF (herein termed TF), is not cross-reactive to core-2, and even detects very low densities of TF. Therefore, Nemod-TF1 is regarded to be the most specific TF antibody which is seen as a basic requirement in terms of potentially using Nemod-TF1 as a therapeutic antibody in the future [10].

In view of the above, we selected the two highly selective TF antibodies Nemod-TF1 and Nemod-TF2 to investigate whether TF may be related to clinico-pathological parameters or prognosis of BC patients. To the best of our knowledge, no data exist upon Nemod-TF1 or Nemod-TF2 immunoreactivity from a large panel of BC patients. We thus correlated immunoreactivity of both Nemod-TF1 and Nemod-TF2 to clinico-pathological variables including overall survival (OS), local recurrence-free survival (LRFS), distant metastasis-free survival (DMFS), and disease-free survival (DFS).

Methods

Patients and specimen characteristics

Formalin-fixed, paraffin-embedded (FFPE) BC samples from 226 patients who underwent surgery from 1988 to 2000 due to a malignant tumor of the breast at the Department of Obstetrics and Gynecology, Ludwig-Maximilians-University of Munich, Germany, were included in this study (Table 1). Histopathological and clinical data as well as the follow-up were retrieved from patients' charts, the laboratory archive, or from the Munich Cancer Registry. Study endpoints were defined as follows: OS = period of time from the date of surgery until the date of death or the date of last follow-up. Patients alive at the time of last follow-up and patients who died due to a non-BC-related cause were treated as censored cases. LRFS = time a patient survives without developing local recurrence (i.e., ipsilateral breast or axilla); DMFS = time a patient survives without developing distant metastatic spread; DFS = period a patient survives without local or distant evidence of disease [11]. Patients not having experienced an event (i.e., local recurrence [LRFS], distant metastasis [DMFS], either local or distant recurrence/metastasis [DFS]) were treated as censored cases.

Assay methods

Immunohistochemistry (IHC)

In total, 226 cases were investigated by Nemod-TF immunohistochemistry. Data on both antibody stainings were available from 222 cases. Four patient samples were only available for either Nemod-TF2 or Nemod-TF1 staining, respectively. The number of cases eligible for Nemod-TF1 IHC was 224. Nemod-TF2 was IHC was also performed in 224 cases.

IHC of TF and scoring was described by our group before [12, 13]. Tissue samples were fixed in buffered formalin solution (3.7%) immediately after resection and underwent standardized paraffin embedding. Samples were stored under standardized conditions. Tissue slides were cut using a microtome and dewaxed in xylene. Tissue peroxidase was blocked using 3% peroxide in methanol. Following a descending series of alcohols, a washing and a blocking step slides were stained using Nemod-TF1 (mouse IgM, kappa produced by Glycotope, Berlin, Germany) or Nemod-TF2 (mouse IgM, kappa produced by Glycotope, Berlin, Germany) as described before [13, 14]. Antibody dilutions were 1:100 in case of Nemod-TF1 and

Table 1 TF1 and TF2 positivities as correlated to clinico-pathological parameters

	Nemod-TF1			Nemod-TF2		
	Neg.	Pos.	*p*	Neg.	Pos.	*p*
Histology						
NST	75	54	ns	76	52	ns
Non NST	63	32		53	43	
Subtype						
Luminal A	52	47	ns	51	49	ns
Luminal B	50	21		43	28	
Her2 pos.	16	5		12	9	
TNBC	18	11		22	7	
Grading						
G1, G2	63	39	ns	59	42	ns
G3	29	23		30	22	
pT						
pT1	94	57	ns	88	65	ns
pT2-pT4	43	29		40	30	
pN						
pN0	82	38	0.03	74	47	ns
pN1-pN3	50	43		50	42	
CIS fraction in invasive CA						
No	67	38	ns	66	39	ns
Yes	71	48		63	56	
Age (years)						
≤60	90	46	ns	85	52	ns
>60	48	39		43	43	

CIS carcinoma in situ in invasive CA, *ns* not signficant

1:200 for Nemod-TF2. Following another series of washing steps biotinylated anti-IgM secondary antibody was applied and visualized using the Vectastain® Elite ABC-Kit (Vector Laboratories, Peterborough, GB) in combination with DAB as a chromogenic substrate. Slides were counterstained with hemalaun, washed, and mounted.

Ovarian cancer tissue served as positive control for Nemod-TF1 and Nemod-TF2 staining as explained elsewhere [12], while replacement of the primary antibody with mouse IgM was performed as negative control.

The immunoreactivity of Nemod-TF1 and Nemod-TF2 was examined by two independent observers by consensus. Samples were assessed by applying an established semi-quantitative immunoreactive score (IRS) [15–17]. The IR score quantifies immunoreactivity by multiplication of staining intensity (graded as 0 = no, 1 = weak, 2 = moderate, and 3 = strong staining) and percentage of positively stained cells (0 = no staining, 1 = ≤ 10% of the cells, 2 = 11–50% of the cells, 3 = 51–80% of the cells, and 4 = ≥ 81% of the cells). A Leitz (Wetzlar, Germany) microscope was employed, and representative images were taken by a CCD color camera (JVC, Japan). Since both Nemod-TF1 and Nemod-TF2 positivity rates were found to be quite low; the threshold was set at an IR score of 0 with cases scored as IRS higher than 0 counted as positive. TA-MUC1 staining on the study panel has been published by our group before [18].

Statistical analysis methods

This study has been carried out according to the REMARK (REporting recommendations for tumor MARKer prognostic studies) criteria [19]. We used the IBM statistic package SPSS (version 24) and Microsoft Excel 2010 to test data for statistical significance and to plot graphs. Chi-square test was performed to test data for statistical independence. Fisher's exact test was used where numbers in each group were insufficient for Chi-square test. Survival analysis was done by applying the log rank test and data are presented as Kaplan–Meier survival curves. Observations with $p < 0.05$ were considered as statistically significant.

Results

Study cohort

A breast tumor smaller than 2 cm in size was diagnosed in 68.0% of cases (*n* (data available) = 225, *n* (pT1) = 153

(68.0%), *n* (pT2) = 66 (29.3), *n* (pT3) = 1 (0.4%), *n* (pT4) = 5 (2.2%)), and about half of all patients were diagnosed for cancer without lymph node metastasis (*n* (data available) = 215; *n* (pN0) = 122 (56.7%)), respectively. About one-third of tumors was classified as high grade (*n* (G3) = 53 (34.2%)). A significant number of cases also displayed a DCIS/LCIS fraction within the invasive carcinomas. Information on intrinsic subtypes was available from 222 cases and subtypes were distributed as follows: *n* (Luminal A) = 100 (45.0%), *n* (Luminal B) = 71 (32.0%), *n* (Her2 positive) = 21 (9.5%), *n* (TNBC) = 30 (13.5%). Study endpoints were OS, DMFS, DFS, and LRFS.

Mean OS was 12.2 years (95% CI 11.6–12.8 years), mean follow-up was 9.8 years (95% CI 9.27–10.3 years), and 49 cancer-related deaths were documented. Another 23 women died due to non-cancer-related reasons and were treated as censored cases. Mean DMFS was 13.0 years (*n* (events) = 34), mean DFS was 11.4 years (*n* (events) = 55), and mean LRFS was 13.4 years (*n* (events) = 32). Mean patient age was 58.3 ± 13.3 years. Further patients' characteristics are listed in Table 1.

TF with respect to clinico-pathological parameters

Both Nemod-TF1 and Nemod-TF2 showed immunopositivity in about 40% of cases (Nemod-TF1: 38.4%, Nemod-TF2: 42.4%; Fig. 1). Correlation analysis revealed a strong concordance between Nemod-TF1 and Nemod-TF2 staining ($p < 0.001$) which was independent of the intrinsic BC subtype (i.e., Luminal A/B, Her2 positive, TNBC), respectively. Co-expression of both Nemod-TF epitopes was observed in 63 out of 222 cases ('double positive', 28.4%), while the absence of both epitopes was found in 105 cases ('double negative,' 47.3%) samples. The presence of neither Nemod-TF1 nor Nemod-TF2 epitope was related to histopathological parameters like histology, histologic subtype, pT stage, the presence of an in situ component, or tumor grade (Table 1). TF immunostaining was not associated with patient age. Those cases that presented with lymph node metastasis at initial diagnosis expressed TF significantly more often than lymph node-negative tumors did (fraction of Nemod-TF1 positive in pN+: 46.2% vs. fraction of Nemod-TF1 positive in pN0: 31.7%; $p = 0.03$). Nemod-TF1 was inversely correlated to anthracycline-containing systemic treatment ($p = 0.048$) while Nemod-TF2 was positively correlated with radiotherapy ($p = 0.047$; Table 2).

Tumors expressing the Gatipotuzumab (formerly known as PankoMabGEX) epitope (= TA-MUC1) at the tumor cell membrane stained positive for Nemod-TF1 more often than those classified as negative for TA-MUC1 (fraction of Nemod-TF1 positive in TA-MUC1 positive: 44.9% vs. fraction of Nemod-TF1 positive in TA-MUC1 negative: 23.5%; $p = 0.003$). The presence of the Nemod-TF1 epitope was inversely correlated to ki67 ($p = 0.013$). Immunoreactivity of Nemod-TF2 was significantly higher in ER-positive (fraction of Nemod-TF2 positive in ER positive: 46.0% vs. fraction of Nemod-TF2 positive in ER negative: 20.7%; $p = 0.011$) and PR-positive cancers (fraction of Nemod-TF2 positive in PR positive: 46.9% vs. fraction of Nemod-TF2 positive in PR negative: 31.7%; $p = 0.049$). Finally, Nemod-TF2 was positively correlated to membrane staining of TA-MUC1, too (fraction of Nemod-TF2 positive in TA-MUC1 positive: 49.0% vs. fraction of Nemod-TF2 positive in TA-MUC1 negative: 27.5%; $p = 0.003$) (Table 3).

Nemod-TF1 immunoreactivity predicts shortened distant metastasis-free and OS in triple negative BC patients

BC samples were stratified as either Nemod-TF1 positive vs. negative or Nemod-TF2 positive vs. negative, respectively. Neither immunoreactivity of Nemod-TF1 nor immunoreactivity of Nemod-TF2 was associated with OS, LRFS, DMFS, or DFS regarding the non-stratified study cohort. Then, immunopositivity of both anti-TF antibodies was tested for its ability to predict prognosis in subgroups of the cohort. Staining of Nemod-TF1 was related to shortened OS in cases expressing the carbohydrate

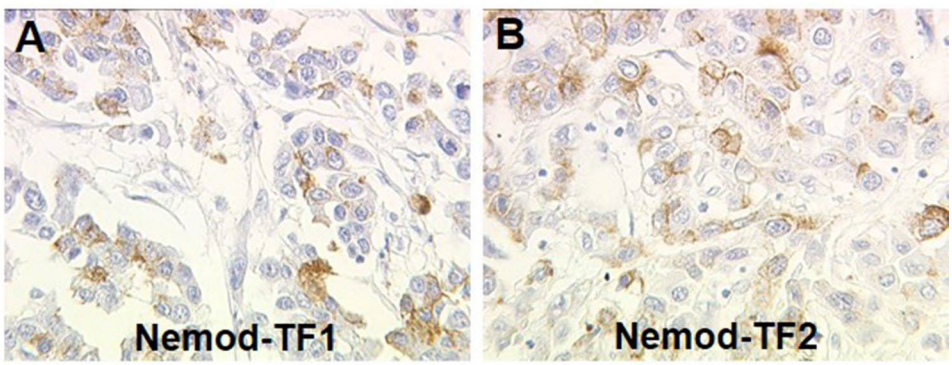

Fig. 1 Staining of TF by Nemod-TF1 and Nemod-TF2 in BC tissue. Representative photomicrographs of Nemod-TF1 and Nemod-TF2 immunostaining are presented (25× lens)

Table 2 Local and systemic treatment of patients studied

	Data available			Nemod-TF1			Nemod-TF2		
	Nemod-TF1	Nemod-TF2		Neg.	Pos.		Neg.	Pos.	
Type of breast surgery	185	185	Breast conserving	88	49	ns	84	55	ns
			Mastectomy	28	20		25	21	
ALNE	175	175	Performed	113	62		106	69	
Radiation	137	139	Yes	87	43	ns	81	51	0.047
			No	6	1		7	0	
Systemic treatment	105	106	No	40	26	ns	40	27	ns
			Yes	27	12		25	14	
Anthracycline-containing CHT	104	105	No	47	34	0.048	47	35	ns
			Yes	19	4		17	6	
Platinum-containing CHT	104	105	No	64	37	ns	62	40	ns
			Yes	2	1		2	1	
Taxane-containing CHT	104	105	No	55	35	ns	52	39	ns
			Yes	11	3		12	2	
Endocrine treatment	105	106	No	22	9	ns	22	10	ns
			Yes	45	29		43	31	

ALNE axillary lymphonodectomy, *ns* not significant, *neg.* negative, *pos.* positive, *CHT* chemotherapy

Table 3 Nemod-TF1 and Nemod-TF2 positivities as correlated to cancer biomarkers

	Nemod-TF1			Nemod-TF2		
	Neg.	Pos.	*p*	Neg.	Pos.	*p*
ER						
Negative	19	10	ns	23	6	0.011
Positive	105	68		94	80	
PR						
Negative	39	21	ns	41	19	0.049
Positive	74	53		68	60	
Her2						
Negative	98	62	ns	97	62	ns
Positive	16	3		11	7	
ki67						
Negative	52	48	0.013	52	49	ns
Positive	51	21		44	28	
Gatipotuzumab^{mem}						
Negative	52	16	0.003	50	19	0.003
Positive	86	70		79	76	
Gatipotuzumab^{cyt}						
Negative	94	52	ns	87	58	ns
Positive	44	34		42	37	

ns not significant

epitope TA-MUC1 at the cell surface (as detected by Gatipotuzumab) (OS: $p = 0.026$, Fig. 2c). Regarding DMFS ($p = 0.057$, Fig. 2a) and DFS ($p = 0.067$, Supp. Fig. 1a), this observation was of borderline significance. DMFS and OS of patients diagnosed for triple negative BC were significantly shortened in case of expressing the Nemod-TF1 epitope (DMFS: $p = 0.012$, Fig. 2b; OS: $p = 0.002$, Fig. 2d). This association failed to be significant ($p = 0.091$, Supp. Fig. 1b) for DFS. Multivariate models were built to test whether Nemod-TF1 might evolve to be an independent prognosticator in the subgroups mentioned above. Nemod-TF1 proved to be an independent predictor for OS and DMFS in TNBC (Table 4). Since data on systemic treatment were available from 17 TNBC patients

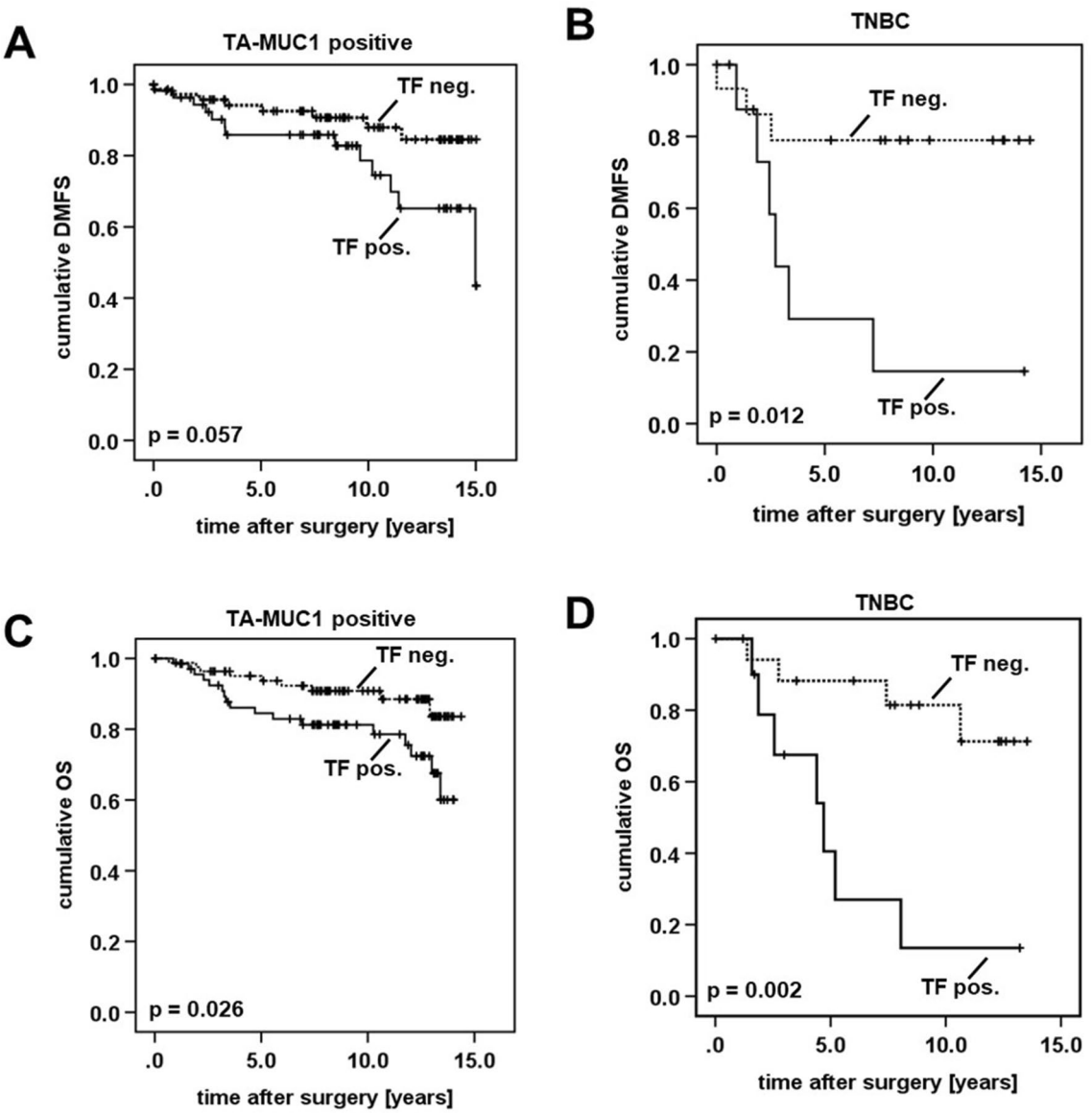

Fig. 2 Presence of the Nemod-TF1 epitope predicts shortened metastasis-free (DMFS) and OS in subgroup analysis. TF as detected by Nemod-TF1 was found to be predictive for shortened OS in a subgroup of breast cancers expressing the Gatipotuzumab epitope TA-MUC1 at the cell surface (TA-MUC1^{mem}) (**c**). Regarding DMFS, this association was of borderline significance only (**a**). In addition, expression of the Nemod-TF1 epitope was predictive for shortened DMFS (**b**) and OS (**d**) in triple negative breast cancer

only, just type of breast surgery, tumor size, lymph node status, patient age, and Nemod-TF1 staining were included in the multivariate model.

On the opposite, though not reaching statistical significance, the presence of TF, as detected by Nemod-TF1, seemed to be associated with favorable DMFS in Her2-positive cases ($p = 0.057$; Supplementary Fig. 2).

Discussion

This study detected immunoreactivity of Nemod-TF1 to predict prognosis in BC cases classified as either positive for the Gatipotuzumab epitope TA-MUC1 (membrane staining) or as triple negative.

Table 4 Multivariate Cox regression analysis in TNBC

	Exp(B)	*p*	95.0% CI for exp(B)	
			Lower	Upper
Distant metastasis-free survival				
pT (T1 vs. T2/3/4)	0.588	0.544	0.105	3.275
pN (neg. vs. pos.)	13.486	0.010	1.886	96.427
Age (≤60 years vs. > 60 years)	0.173	0.136	0.017	1.736
Breast surgery (con. vs. ME)	1.010	0.934	0.796	1.282
Nemod-TF1 (neg. vs. pos.)	7.604	0.038	1.123	51.506
Overall survival				
pT (T1 vs. T2/3/4)	0.342	0.243	0.056	2.074
pN (neg. vs. pos.)	6.915	0.025	1.276	37.487
Age (≤60 years vs. > 60 years)	0.068	0.025	0.007	0.719
Breast surgery (con. vs. ME)	1.022	0.049	1.000	1.045
Nemod-TF1 (neg. vs. pos.)	11.929	0.002	2.500	56.916

Con breast conserving, *ME* mastectomy

Immunopositivity of either Nemod-TF1 or Nemod-TF2 was present in about 40% of BC samples. In line with this, others have demonstrated a similar fraction of TF-positive cases in different cancer entities and report comparable staining patterns [4, 20, 21]. Since TF has been hypothesized to be an oncofetal carbohydrate tumor marker expressed on cancer stem cells (CSC) or cell populations directly descending from them, such a distribution pattern may be rather expected [4]. Thus, to get a representative impression of Nemod-TF1/2 staining, IHC analysis presented here was performed on slides cut from tumor paraffine blocks rather than from core biopsies of tissue microarrays. We observed a strong positive correlation between Nemod-TF1 and Nemod-TF2 staining ($p < 0.001$). Although there was a positive correlation of the two antibodies, they differed regarding their correlation to clinico-pathological parameters and prognosis prediction. We hypothesize this to be due to the different fine specificity and affinity of the two as explained in the "Background" section.

TF was shown to be linked to CSC markers (e.g., CD44) by *O*-glycosylation [22]. Supporting its features as a CSC marker, TF has been highlighted to be abundantly present on circulating tumor cells [23] and to promote metastatic spread [8]. Gunkel et al. used A78-G/A7 to stain tissue samples of mucinous BC and their corresponding lymph node metastasis for TF. They found a strong co-incidence of TF expression in primary mucinous BC samples and lymph node metastasis [24]. We also report a positive correlation of TF expression and the presence of lymph node metastasis. These results may imply that the presence of the TF antigen in the primary breast tumor may facilitate metastatic spread to axillary lymph nodes or distant sites during early carcinogenesis. A biomarker reliably predicting lymph node positivity in patients diagnosed for primary BC may become of particular importance in terms of differentiating those patients with high vs. low risk for cancer spread to lymph nodes. While lymph node dissection may not be necessary in patients with low risk for positive lymph nodes, it may remain to be an indispensable part of surgical treatment of patients at high risk. Hence, a biomarker predicting lymph node status may assist to safely de-escalate surgical radicality. About half of all cases with positive axillary lymph nodes expressed the Nemod-TF1 epitope (fraction of Nemod-TF1 positive in pN+: 46.2%). However, 38 (31.7%) out of 120 patients diagnosed as lymph node negative were found to express the epitope of Nemod-TF1, too. As a consequence, though expression of the Nemod-TF1 epitope significantly correlates with lymph node positivity, it seems to too unspecific to predict the lymph node status as a single marker. Thus, to clear whether a biomarker set including Nemod-TF1 may assist to prognosticate cancer spread to axillary lymph nodes, a specifically designed and powered trial would need to be set up.

Up to now, several glycoproteins in particular those classified as mucin-like glycoproteins have been identified to act as TF carrier molecules [4, 22]. We performed correlation analysis thus to screen for potential candidates. Statistical correlation in our study sample confirms that membranous TA-MUC1 (as detected by Gatipotuzumab) carries TF and exposes it on the cell surface [25]. In line with this, canonical MUC1 has already been demonstrated to get glyco-modified by the TF moiety in breast and gastric cancer [26, 27]. MUC1 itself holds kinase-like activities [28], may directly modulate downstream kinase signaling [29], and has been demonstrated to regulate multiple tumor biologic features [29]. Interestingly, the presence of TF predicted shortened DDFS and OS especially in the TA-MUC1-positive subgroup, supporting that the oncogenic activity of TF may somehow be dependent on expression of TA-MUC1 at the cell membrane. Insight analysis apart from statistical correlation is indispensable in order to prove this.

TF has been described in multiple types of cancer. However, whether the presence of TF may be related to patients' prognosis remains to be contradictory. Though the majority of studies published on this topic report TF to be associated with unfavorable prognosis [30–33], others found TF to be a positive predictor [23, 34]. Despite appearing inconsistent or even conflicting, both statements may be interpreted by TF exerting different biological effects in BC, i.e., alteration of the host anti-tumor immune response and direct regulation of cell signaling. In detail, since TF—which has been named the 'hidden tumor antigen' [10]—is covered by a "glycocap" in non-neoplastic tissue and becomes exposed only during malignant transformation [4], it may be recognized as a foreign, 'non-self' antigen by the host's immune system. Therefore, TF might facilitate a kind of host anti-tumor

immune response enabling the host to initiate a more effective anti-tumor immune defense [23]. As a consequence, those cancer cells that expose TF on their surface may get eliminated more easily than those cancer cells not expressing TF. This seems to be especially attractive in terms of developing anti-cancer, i.e., anti-TF-directed vaccines or therapeutic antibodies for personalized oncology. Bearing in mind its immunogenic, 'non-self' characteristics, the presence of the TF epitope may be causally related to favorable DDFS in Her2-positive BC patients by augmenting a host anti-tumor immune action. Though this hypothesis seems to be conclusive and our analysis demonstrated survival curves of TF-positive vs. TF-negative patients to be clearly separated in Her2-positive BC, this did not reach statistical significance—potentially due to the small number of patients in this subgroup. Finally, it needs to be mentioned that this was a retrospective study and that patients were treated between 1988 and 2000, i.e., before the trastuzumab era. Whether our results can be reproduced in patients non-naïve for trastuzumab remains to be analyzed.

On the other side, antibodies targeting TF effectively blocked cell proliferation and extended survival of mice with metastatic BC [8, 9, 35], hence supporting the hypothesis that TF itself may comprise pro-tumorigenic actions and may thus be associated with worse prognosis. This seems to turn especially obvious in those BC cases either positive for the Gatipotuzumab epitope TA-MUC1 at the cell membrane or classified as triple negative. TA-MUC1 functions as a TF carrier molecule and—as speculated above—might be involved in mediating TF oncogenic activity. To gain mechanistic insights, functional studies using the Nemod-TF1 antibody would be required. Regarding TNBC, the presence of Nemod-TF1 was prognostic for shortened OS and DMFS within both uni- and multivariate analysis. However, results need to be interpreted with care since herein only relatively few TNBC cases were studied. Larger trials are needed to validate these results and to verify whether TF may turn out to be a targetable epitope in BC. Especially in case of TNBC, there is an unmet clinical need to develop effective therapies to target molecules that—like TF—are widely expressed in TNBC and that potentially hold oncogenic activity in this molecular subtype.

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Author contributions SH participated in design and coordination of the study, significantly participated in experimental assays and analysis as well as in statistical analysis, and wrote the manuscript. ME participated in IHC assays and analysis and helped to draft the manuscript. LB participated in data collection and analysis. JZ participated in experimental assays. DM supervised IHC as a gynecologic pathologist and participated in IHC analysis. CS participated in the design of the study and carefully reviewed the manuscript for important intellectual content. CK optimized Nemod-TF staining and participated in immunohistochemistry. SSH participated in cell culture experiment design and conduction. FS helped to draft the manuscript and revised the manuscript for important intellectual content. UJ conceived and coordinated the study, participated in study design, and approved the final version of the manuscript. ND conceived and coordinated the study, participated in study design, and approved the final version of the manuscript.

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Compliance with ethical standards

Conflict of interest All authors read the manuscript and agree to the publication of the manuscript. SH reports grants from the following organizations/companies: FöFoLe LMU Munich Medical Faculty, FERRING, Novartis Oncology, Astra Zeneca, Apceth, Heuer Stiftung, Deutsche Forschungsgemeinschaft. She further reports personal fees from Roche and non-financial support from Addex. ND held honorary speeches for Roche, AstraZeneca, Mentor, Omniamed, TEVA, and MSD. All the support listed here has been received outside the submitted work. Remaining authors have no competing interests to declare.

Ethical approval This article does not contain any studies with animals performed by any of the authors. All procedures performed in studies involving human participants were in accordance with the ethical standards of the institutional and/or national research committee and with the 1964 Helsinki declaration and its later amendments or comparable ethical standards. The tissue samples were retrieved from the archive of Obstetrics and Gynecology, Ludwig-Maximilians-University, Munich, Germany. The study was approved by the Ethics Committee of the Ludwig-Maximilians-University, Munich, Germany (approval number: 18-166). The tumor tissue re-used for our analysis had initially been collected for histopathological diagnostics. At the time, the tissue was examined for the current study all diagnostic procedures had already been fully completed and thus the tissue was classified as "leftover" material. Clinical data and tissue specimens were irreversibly anonymized prior to inclusion in the study. Researchers were blinded from patient data during experimental and statistical analysis.

Informed consent As per declaration of our ethics committee [Ludwig-Maximilians-University, Munich, Germany (approval number: 18-166)], no written informed consent of the participants is needed given the circumstances described above.

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