

Review

The role of TF- and Tn-antigens in breast cancer metastasis

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Summary. Almost 40 years ago, researchers found that the Thomsen-Friedenreich (TF) and the Thomsen nouvelle (Tn) antigens could be detected in carcinoma, but not in healthy tissue. A short time after that it became clear that TF and Tn are precursor molecules of the MN-blood group antigens. In normal tissue TF and Tn are coated by glycosyl structures, thereby forming the glycoproteins which are known to account for the MN-blood group antigens, but in malignant tissue these molecules are uncovered. TF, which has an additional Galectin-residue compared to Tn, is correlated with a more favourable prognosis for patients. On the contrary, patients with Tn-bearing tissues have a worse prognosis for overall and progression-free survival. It is known that TF and Tn are involved in the adhesion of tumour cells to the endothelium via a mechanism recruiting Galectin-3 and MUC-1, which is the first step in metastasis formation. Furthermore, it became clear that this pathway can be blocked by a growing number of molecules, thereby creating ways of therapeutical intervention.

Key words: Antigen, Breast Cancer, Glycosyl-transferase, Metastasis, Mucin, Vaccination

TF and Tn – looking back to almost 4 decades of research

In 1975 the Springer research group published a manuscript on the role of MN-blood group antigens and their precursors in normal and malignant human breast tissue (Springer et al., 1975). It described that MN-blood group antigens could be reduced to their precursor molecules, the Thomsen-Friedenreich (TF, in the literature often referred to as T-Antigen) and the Thomsen nouvelle (Tn) structures by simple sialic acid depletion. Therefore it was concluded that the MN-blood group was not determined genetically. Although MN-antigens could be found in normal as well as in malignant tissue, their precursors were only found in cancerous breast tissue (Springer and Desai, 1975). Only one year later another study from the Springer group arose, describing depression of anti-TF-antibodies in patients with breast cancer compared to healthy persons and increasing amounts of anti-TF-antibodies after mastectomy (Springer et al., 1976). Additionally a cellular immunity of breast carcinoma patients but not of healthy individuals towards the TF-antigen could be shown *in vitro* and *in vivo*. A delayed-type hypersensitivity reaction (DTHR) to the TF-antigen was observed in most ductal breast carcinoma patients but not in healthy persons (Springer et al., 1979a). In 1979 two more studies appeared, doubting the association of TF-antigen with breast cancer (Klein et al., 1979; Newman et al., 1979). The second study found higher levels of TF-antigen in more differentiated carcinomas than in undifferentiated tissues of the disease. During the same year a first diagnostic test for human breast adenocarcinoma based on a delayed-type skin

hypersensitivity to TF-antigen was propagated (Springer et al., 1979b). In the following a cell-mediated immune response to TF-antigen in breast cancer patients but not in healthy persons could be shown (Springer et al., 1980). In 1984 the Seitz group confirmed these recent findings, using TF as a tool in histochemical detection of metastasis by FITC-labelled Peanut Agglutinin (PNA). They found different staining patterns in normal and malignant tissue and reported a specific PNA-staining in the cytoplasm of tumour cells occurring in lymph node metastasis. They were even able to detect single tumour cells by this type of staining (Seitz et al., 1984). One year later another study was published, in which different DTH-reactions in breast and lung cancer patients were compared to the reactions on the TF-antigen in normal persons. The differences were shown to be statistically significant (Springer et al., 1985). On the basis of all those studies people began to estimate TF as a useful diagnostic tumour marker (Schmitz and Bosse, 1987). In the late 1980s paraffin-embedded sections of primary breast tumours and metastatic lesions were analysed for their TF-expression, and it was noticed that TF levels increased during tumour progression (Wolf et al., 1988). From all these studies arose the idea to use TF- and Tn-molecules immunotherapeutically (Yacyshyn et al., 1995) as vaccines against recurrence of advanced breast cancer. A total of 18 patients with all stages of cancer were tested for therapeutic response and all of them survived at least 5 years postoperatively (Springer et al., 1994). The mechanism of these vaccinations is again based on the DTH-reaction. TF and Tn were more and more estimated for their use in cancer prognosis, diagnosis and immunotherapy, by being involved in cell adhesion and metastasis (Springer, 1995). Additionally, it was found that carcinoma patients had altered serum-TF levels in comparison to healthy test persons, quantified by ELISA (Desai et al., 1995). In 1997 the molecular basis of TF and Tn structures were reviewed in detail by Springer (1997). It was explained that TF and Tn are forms of glycosylations, covalently bound to transmembrane proteins, mostly on aminoacids (aa) Thr, Gln, Leu, Ser, Val and Pro. Thereby Tn is the crudest structure consisting only of a GalNAc-residue (GalNAc- α 1-O-Aa). For TF one more Galectin-residue is bound to Tn (Gal- β 1-Tn). In normal tissue these carbohydrate residues are further covered by N-Ac-Neuraminic acid, thereby creating the MN-blood group antigens. TF and Tn were associated with cell adhesion properties (Paszkiwicz-Gadek et al., 2006), and cancer patients with high Tn-levels had a significantly shorter 5-year survival, a higher TNM-staging and histological grading. Furthermore, a negative or low expression of Tn was regarded as a predictor for a better overall survival (Imai et al., 2001). Additionally Tn and TF immunoassays are highly efficient in the detection of incipient and clinically overt carcinomas, long before they can be visualized by biopsy or mammography (Springer, 1995).

A treatment of cancer cells with a TF-antagonist was also shown to inhibit tumour cell adhesion by 60-80% (Khaldoyanidi et al., 2003). Later, TF was shown to be expressed on disseminated tumour cells (DTCs) in bone marrow and the expression seemed to be correlated to MUC-1, which was therefore regarded to be a carrier of TF on DTCs (Schindlbeck et al., 2005), and it was found that the expression of TF might have prognostic significance and could help to improve the biological understanding of metastases (Schindlbeck et al., 2007). Changes to MUC-1 found in breast cancer concern the number of glycosylated sites and the composition of carbohydrate chains added to MUC-1 (Taylor-Papadimitriou et al., 1999), which are based on Core1 and not on Core2 structures like in healthy breast tissue (Springer, 1997). One reason for these altered glycosylation patterns could be the different expression of glycosyltransferases in malignant tissue, as for example ST3Gal1 is markedly increased in breast cancer tissue and this upregulated expression was correlated to invasiveness and the expression of SM3 epitope (Burchell et al., 1999). The altered glycosylation seems to affect the behaviour of tumour cells concerning, first of all, cell adhesion properties. It was shown that MUC-1 transfected cells show reduced cell-cell adhesion (Ligtenberg et al., 1992) and reduced adhesion to extracellular matrix (Wesseling et al., 1995). But adhesion could also be enhanced by interactions with beta-catenin (Yamamoto et al., 1997), can be a ligand for ICAM1 to endothelial cells (Regimbald et al., 1996), enhance antigen presentation to T-cells by lectin interactions (Bohm et al., 1997) and may be involved in recruiting macrophages into the tumour site (Nath et al., 1999). A big drawback of the use of monoclonal antibodies against different glycosylation structures of MUC-1 is that the antibodies frequently have different binding affinities (Reddish et al., 1997; Avichezer et al., 1998), so that the results of different groups can hardly be compared. As Galectin-3 is also highly expressed in metastatic breast cancer, an interaction of Galectin-3 with MUC-1 via TF was assumed, which promotes cancer cell adhesion to the endothelium and therefore promotes cancer metastasis (Yu et al., 2007). Yet TF was shown to be a marker for immunomagnetic enrichment of tumour cells, thereby increasing DTC-detection rate and allowing a larger cohort of patients to be analysed and a characterization of DTCs (Schindlbeck et al., 2008). A new finding is, that neoadjuvant chemotherapeutic regimens could stimulate the immune response to tumour associated carbohydrate antigens like TF and Tn, and that high levels of anti-TF-antibody before surgery are correlated to a better prognosis concerning overall survival of stage II breast cancer patients (Kurtenkov et al., 2005). Additionally a sophisticated method for breast cancer detection could be the examination of nipple aspirate fluid: if it contains high levels of TF and Tn compared to healthy breast tissue, the probability for cancer increases tremendously

(Kumar et al., 2005).

The role of TF in breast cancer metastasis

As has already been described above, Thomsen-Friedenreich-antigen is found only in malignant but not in healthy tissue samples (Demichelis et al., 2010), and is demonstrable in all metastatic breast lesions. Breast cancer patients show immunity towards TF-antigen *in vivo* and *in vitro*, and 85% of breast cancer patients have positive DTH-reactions against TF (Springer et al., 1980). Therefore, TF is used in the histochemical detection of breast cancer micrometastasis by visualization via Peanut-Agglutinin (PNA), in which even single malignant cells are detectable and 91% of ductal mammary carcinoma and 75% of metastases show a positive staining (Seitz et al., 1984). DEAE-cellulose column chromatography from various tissue samples yielded a signal especially for breast and liver metastatic carcinoma for the N-blood group precursor TF (Otsuka et al., 1991). Furthermore, it was shown that patients with high levels of the lymphocyte activation marker CD69 have a prolonged survival rate after ASI with vaccines containing TF or sTn, and that the effect of the vaccination in surviving patients is an increase in CD69+ cells, while non-B-lymphocyte HLA-DR+ cells decrease, resulting in a survival advantage (Yacyshyn et al., 1995).

The major role of TF in tumorigenesis is the mediation of tumour cell adhesion to the endothelium, in which Gal-3 is also involved. It is known that TF-antigens are capable of mobilizing Gal-3 to the surface of endothelial cells, priming them for harbouring metastatic cells (Glinsky et al., 2001). In the highly metastatic breast cancer cell line MDA-MB435 enhanced levels of both TF and Gal-3 were found. These levels were much lower in the non-metastatic cell line MDA-MB468. The MDA-MB435 cells were also found to have a greater ability to adhere to endothelial cells. When these metastatic cells adhere *in vitro* to an endothelial cell layer, they acquire the ability to adhere homotypically, leading to a formation of cell aggregates. Treatment of those cells with a synthetic TF-antigen antagonist (lactulosyl-l-leucine) abolishes MDA-MB435 adhesion to 60-80%, caused by a redistribution of gal-3 to sites of heterotypic intracellular contacts. These special adhesion properties created by the interaction of Gal-3 and TF are therefore a main feature of metastasis formation (Khaldoyanidi et al., 2003). Furthermore, blocking of Gal-3 carbohydrate recognition reduces metastasis-associated cell adhesion. Gal-3 antagonists inhibit interaction with TF, and hence rolling and heterotypic adhesion of cancer cells to endothelial cells and homotypic tumour cell aggregation (Zou et al., 2005), which are the two main features of metastasis formation (Glinsky et al., 2003). From these observations it can be concluded that the metastatic arrest in target organs is not a consequence of mechanical trapping, but well-coordinated by TF/Gal-

3 (Glinskii et al., 2005). It was found that, the transmembrane protein MUC-1, which is overexpressed and aberrantly glycosylated in breast cancer tissue is a ligand for Gal-3. Recombinant Gal-3 increases the adhesion of MUC-1 expressing breast cancer cells to HUVECs, and of breast epithelial cells (HCA 1.7), expressing TF. This binding is abolished when TF is cleaved. An interaction of TF with MUC-1 and Gal-3 could only be induced after sialidase treatment. Therefore the interaction of Gal-3 with MUC-1 via TF is a central principle of metastatic cell adhesion and hints towards the importance of glycosylation in cancer progression (Yu et al., 2007). As the interaction between Gal-3 and TF is especially important in early stages of metastasis formation, an inhibition of this interaction could reduce metastatic progression. Therefore the peptide G3-012, which has a high affinity for Gal-3, inhibiting Gal-3/TF as well as Gal-3/Gal-3 binding, reduces cellular adhesion, resulting in diminished lung colonization in mice (Newton-Northup et al., 2013). Thus, metastasis-prone tissue is activated by structures like TF, which are expressed on circulating glycoproteins and neoplastic cells, and is marked by an increased Gal-3 expression. Thereby cancer cells, which dissolved from the primary tumour, circulating in the blood stream are slowed down, arrested and begin rolling along the endothelium. This mechanism can be blocked by an anti-TF-antibody (Glinsky et al., 2004). As Mucin expression varies in amount and localization in different cancer cells, glycosylation creates novel structures, but as metastasis and primary tumour show similarities in their carbohydrate structures and Mucin expression, it can be regarded as an important marker for tumour classification (Gunkel et al., 2005). Disseminated tumour cells (DTCs) for example are shown to have prognostic significance in tumour development, and a co-staining of cytokeratins 8, 18 and 19, which are routinely used for cancer cell detection in the APAAP-staining (Kurec et al., 1988; Noack et al., 2000). TF results in a co-expression of 96-98%, so that TF can be regarded as a marker for DTC-detection. It was further shown that a positive TF-staining of bone marrow samples harbouring DTCs correlated with a strong expression of MUC-1 (Schindlbeck et al., 2005). TF was also used for enrichment of DTCs from bone marrow, solving the problem of a high number of surrounding cells. It was additionally used for detection of circulating tumour cells (CTCs). A comparison of bone marrow and blood from the same patient could help for future monitoring and characterization of DTCs/CTCs, increasing their value as diagnostic and therapeutic targets (Schindlbeck et al., 2008). The use of TF as an immunotherapeutic target was shown by Heimburg et al. (2006). They used JAA-F11, a monoclonal antibody, which was discovered in 1920 and is specific for TF, and noticed a survival advantage of TF-tumours by cytotoxicity, blocking of cell adhesion and, in consequence, inhibition of metastasis. In a mouse model, all metastatic stages involving cell adhesion are

blocked by JAA-F11, so that survival time is extended and lung metastases are inhibited. The great advantage of this antibody is that it has no uptake in other organs, and improves patient prognosis dependent on the level of TF-expression (Rittenhouse-Olson, 2007). Thereby a clinical utility for this antibody could be taken into consideration (Almogren et al., 2012).

As TF-mediated pathways can be easily blocked it might play an important role in upcoming cancer therapeutics and is furthermore a potent antigen for tumour targeting (Ravn et al., 2007).

Another interesting fact is that the TF-antigen seems to be co-expressed to a rather high percentage with CD44, which is known to be a marker for cancer initiating cells, and it was even hypothesized that CD44 might be a carrier molecule for TF (Lin et al., 2011).

It was recently published, that the JAA-F11-antibody also inhibited the growth rate of human breast cancer cell lines and might therefore get an interesting tool for therapeutic means (Ferguson et al., 2014). TF was also used for detection of disseminated tumour cells from bone marrow samples of breast cancer patients and gave rather good results in a model system, with high recovery rates (Andergassen et al., 2013) and also was applied successfully in patient samples (Andergassen et al., 2014).

The role of Tn in breast cancer metastasis

Already thirty years ago it became clear that MN-blood group antigens were uncovered in malignant tissue, creating TF and Tn antigens, which are immunoreactive, causing autoimmune responses. Their expression was found to be closely linked to carcinoma differentiation and the molecules were associated with cell adhesion properties (Springer, 1984). Tn and sTn, a molecule differing from Tn, only by the addition of a sialic-acid residue, are regarded to be good tumour markers for screening and classification. Some antibodies against these two molecules were tested for their specificity by ELISA. It was shown that anti-Tn B72.3, which was prepared from a metastatic breast adenocarcinoma had the same reactivity as anti-sTn TKH1/2 antibody. From those experiments it was concluded that the anti-Tn B72.3 antibody also reacted with sTn, which is present in adenocarcinomas but not in normal tissue. Therefore it could also be regarded as a tumour marker for screening and classification (Kjeldsen et al., 1988). The epitope sTn is also a target for active specific immunotherapy, a strong predictor for poor prognosis, playing a functional role in metastasis formation. In a trial sTn displayed minimal toxicity in 12 metastatic breast cancer patients, all patients developed cytotoxic antibodies of IgG- or IgM-type against sTn, but the clinical use had to be clarified further (MacLean et al., 1993). In 1995 the use of the Tn- associated molecule 83D4, which is expressed in a cell bound form as well as a soluble antigen, as a marker for malignancy was analysed. It mostly stained malignant effusions, to a

lesser extent suspect effusions and only very little staining was seen in apparently benign effusions. Therefore it can be regarded as a marker for the differentiation of malign and benign cells (Beuzelin-Yvraut et al., 1995). Furthermore Tn was found to be associated with a shortened 5-year disease-free survival, increasing TNM-stages, positive lymph node status and higher histological grading (Wang et al., 1997). The localization of Tn-Antigen and GSI-A4 lectins was visualized by Konska et al. in 1998 by VVA-B4 and monoclonal antibody 83D4 respectively. They found a positive membrane labelling already in benign breast tumours, concluding that modifications in glycosylation are the first sign of an upcoming disease. In grade 1 tumours the staining already extends to the apical and the basal part of the cell membrane and in grade 2 and 3 tumours a cytoplasmic staining is also seen. But in grade 3 tumours staining begins to diminish, presumably due to a loss of glycosyl-transferases or reduced protein synthesis in general. As these antigens are mostly localized to the cellular surface, they are thought to play a role in tumour cell adhesion between normal and malignant cells. These adhesive properties were examined by Kishikawa et al. (1999) by *in vitro* adhesion tests. They used a breast cancer cell line (ZR75-30) and a normal epithelial cell line (HLB100) and specific as well as nonspecific antibodies against TF and Tn antigen. They found out, that adhesion was specifically mediated by TF and Tn and was temperature dependent, so that cell adhesion, mediated by these two molecules is an important step in metastasis formation. Ten years later a new study arose, confirming these results (Danussi et al., 2009). A highly specific antibody against Tn was grown in mice, immunized with synthetically produced Tn antigen. This monoclonal antibody (2154F12A4) recognized Tn on MCF7 cells, when there was no pre-incubation step of the cells with purified Tn. In MCF-tumour bearing mice this QDot800-labelled antibody bound to primary tumour lesions and lymph node metastases, and was able to inhibit cancer cell adhesion to lymphatic endothelium. A future use of this antibody could be in the inhibition of lymph node metastases. A further interesting therapeutic approach could be the monoclonal anti-Tn-antibody MLS 128, described by Morita et al. (2009). This antibody binds three consecutive Tn's, and significantly inhibits colon and breast cancer cell growth. It causes down regulation of insulin-like growth factor-I receptor and epidermal growth factor receptor, suggesting a regulation of growth factor receptors. Another study from 2006 showed a correlation between glycoproteins binding Vicia villosa agglutinin (VVA) and Tn. The expression of VVA-binding molecules seems to be correlated to tumour stage, lymphatic invasion and lymph node metastasis. It was shown that VVA-binding was nearly abolished by a pre-incubation with Tn. It was concluded that atypical MUC-1, presenting Tn-antigen, is implicated in lymph node metastasis (Kawaguchi et al., 2006). The detection of Tn by aqueous quantum dots (AQD) could help to

differentiate generally between cancerous and normal breast cancer by a rather high percentage of certainty (Au et al., 2014).

Recent research demonstrates that high levels of Tn, or a Tn upregulation are dependent on a relocation of glycosyltransferases (N-acetylgalactosamine-transferases) from the Golgi towards the endoplasmic reticulum (ER). As Tn stimulates cell adhesion to the extracellular matrix (ECM), cell migration and invasiveness, it is present especially in the lamellipodia of migrating cells. The high density of glycosylation thereby depends on the lectin-domain of glycosyltransferases, and an inhibition of this enzymatic domain inhibits cell migration and metastasis formation (Gill et al., 2013), and could therefore be a target for therapeutical intervention.

Tn was also found to be a basis for further glycosylation which in turn helps the tumours to escape from the immune system (Madsen et al., 2013).

Cancer immunotherapy by MUC-1, TF and Tn

As mucin1 with its different glycosylation patterns could have immunogenic effects, and it was shown that it interacts with CTL (Jerome et al., 1991) and the TCR (Finn et al., 1995), the idea arose to use it for cancer vaccination.

As in breast cancer an increase in truncated O-linked glycans, like Tn and TF, is found on MUC-1 protein, due to an upregulation of sialyltransferases ST3Gal1 and ST3GalNac1 (Marcos et al., 2004; Julien et al., 2006; Sewell et al., 2006), the cellular localization of the differentially glycosylated mucin varies with increasing malignancy (Wesseling et al., 1995; Rahn et al., 2001). It spreads all over the cell surface, as cell polarity is lost.

These altered glycosylation patterns which are characteristic for tumour cells were then used in approaches towards tumour vaccination (Beatson et al., 2010). There are three main forms of vaccination: first passive immunization via monoclonal antibodies (Bioportfolio, 2008). The antibody SM3 (Burchell et al., 1987) was shown to be specific for tumour associated MUC-1 (Burchell et al., 1999) and helps to delay tumour growth (Graham et al., 1996). Bi-specific antibodies help furthermore, to enhance cytotoxicity *in vitro* (Kodama et al., 2002), but clinical trials in this concern are still disappointing (trials, 2010). The second type of vaccination is the active immunization, for example by administering a mannan-coupled MUC-1, which already seemed to be hopeful in phase III trials (Butts et al., 2005; Apostolopoulos et al., 2006). A big advantage of this method is that the necessary glycopeptides can be produced synthetically to high purity and specificity (Becker et al., 2006; Liakatos and Kunz, 2007) and can be coupled to immunostimulants, producing very strong reactions in the mouse model (Gaidzik et al., 2013). Also, a Tn-coupled MUC-1 could produce T-cell specific responses (Julien et al., 2009), but even higher effects might be achieved by targeting all sTn carrying

proteins, not only MUC-1. However there seem to appear immunosuppressive effects (Slovin et al., 2007). The third possibility is to use dendritic cells, pulsing them with tumour peptides (Loveland et al., 2006) or cell lysates (Bohnenkamp et al., 2004) or even fusing them with tumour cells, generating dendritic cells, which present tumour antigens (Avigan, 2004; Avigan et al., 2004). That approach results in a nonspecific activation of the immune system (Grimshaw et al., 2008). Additionally, a vaccination with viral vectors (Acres et al., 1993), cDNA or naked DNA (Graham et al., 1996), or peptides (Ding et al., 1993) could be possible, using exosomes as delivery mechanism (Cho et al., 2005).

Conclusion

Glycosylation is a posttranslational modification of proteins and lipids, which is frequently altered in cancerous tissue, thus creating an interesting topic in medical research. The on hand review article deals with TF- and Tn-Antigens, which are precursor molecules of the MN-blood group antigens, generated by sialic acid depletion, and are only found in tissue with cancer characteristics. Of these glycosyl structures, which are bound to transmembrane proteins like MUC-1, Tn is the most crudest, TF has the structure of Tn with one more galectin residue added. The two molecules are further covered with carbohydrate residues, forming the MN-blood group antigens. TF and Tn are associated with cell adhesion properties, and as cell adhesion and migration are crucial steps during metastasis formation, they play a role in tumorigenesis. TF in terms mediates tumour cell adhesion to the endothelium, facilitating metastasis formation in interaction with Galectin and Mucin, Tn stimulates cell adhesion to the ECM and furthermore cell migration and invasiveness and is present in the lamellipodia of migrating cells. An upregulation of TF and Tn, which is generated by changes in the glycosyltransferases, is thus correlated with tumour progression, higher TNM-staging, and reduced survival respectively. Therefore both molecules are used in tumour diagnosis, for example by a PNA-staining of TF, as well as in tumour therapy. For diagnostic purposes TF and Tn could be detected for example already from nipple aspiration fluid. Furthermore TF can be used for DTC detection and enrichment from bone marrow of breast cancer patients, and for the detection of micrometastases by PNA-staining. Additionally TF is coexpressed with CD44 and is thereby a marker for cancer initiating cells. A Tn-staining of benign tumours is, on the other hand already a sign for an upcoming malignant disease. In terms of tumour treatment regimen, it was found, that a neoadjuvant treatment seems to stimulate immune reactions against TF/Tn, so that high levels of anti-TF/Tn antibodies before surgery are correlated with a better prognosis for overall survival. Furthermore, TF-specific antibodies like JAA-F11 inhibits metastasis formation, while various Tn-specific antibodies inhibit lymph node affection and

cancer cell growth. Therefore it could be summarized, that aberrant glycosylation could be a promising target for tumour diagnosis and therapy, and is clearly worth further research.

Conflict of interest: None declared.

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TF, TN and breast cancer

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TF, TN and breast cancer

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