Glycodelin Expression in Correlation to Grading, Nodal Involvement and Steroid Receptor Expression in Human Breast Cancer Patients

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Abstract. Background: Glycodelin (Gd) is an endometrially secreted glycoprotein, found in high quantities during early pregnancy. It shapes local immune responses in order to allow implantation and invasion of the semiallogeneic blastocyst into the maternal endometrium. It exists in various glycosylation isoforms, that influence their staining behaviour. Materials and Methods: A polyclonal anti-Gd IgG-antibody specific for the core amino acid sequence of Gd was used to stain 121 paraffin-embedded breast cancer tissue blocks. We correlated Gd expression to known prognostic markers such as grading, nodal involvement and steroid-receptor positivity. Results: Gd was expressed in invasive lobular and ductal breast cancer and its expression was significantly reduced upon dedifferentiation. A non significant increase in Gd staining intensity was noted upon axillary lymph node involvement steroid receptor positivity. Conclusion: These results indicate that Gd may be used as an additional marker for the differentiation of breast cancer tissue, yet indicating an increased tendency towards lymph node metastasis.

Breast cancer is the most common malignant tumor in women worldwide. While genetic and epigenetic aberrations initiate carcinogenesis, there is growing evidence that the tumor micromilieu shapes and promotes progression to cancer and helps in establishing locally invasive and metastatic disease (1). Many immunologically active components are found at the tumor–stroma interface and help to shape this microenvironment (2). Among those substances we and others described glycodelin (Gd), a secreted immunosuppressive glycoprotein of the human genital tract, in breast tissue (3-5).

Gd is known to have immunomodulatory effects by suppressing the reactivity of stimulated T-lymphocytes, inhibiting NK and B-cells and maintaining a tolerogenic phenotype in dendritic cells (DC) in vitro (6, 7). Gd expression in breast cancer is also associated with less aggressive behavior and better differentiation in vitro and in vivo (3, 8). In breast cancer cell lines, Gd-expressing cells showed a phenotype of more differentiated organized glandular epithelium (5).

Taken together, these data imply that Gd might contribute towards tumor progression in an early phase of carcinogenesis in which an organized local immune evasion is provided by the expression of Gd, while antigenicity and intracellular signaling are still intact (9).

Gd exists in different glycosylation patterns and with various isoforms that influence their staining behavior (10-12). Because the structure of a specific breast cancer glycodorm remains elusive, we used a polyclonal, protein-specific antibody to stain for Gd. The immunoreactivity of glycodelin (Gd) was evaluated in 121 invasive breast cancer specimens to investigate the capability of Gd as a marker of differentiation.

Materials and Methods

Specimens. Breast cancer tissue blocks were formalin-fixed and embedded in paraffin. Specimens were obtained from patients undergoing surgery at the department of obstetrics and gynecology Klinikum – Innenstadt of the Ludwig Maximilians University, Munich. All specimens were histologically classified as lobular or ductal breast cancer by a gynecological pathologist and none of the patients had chemotherapy prior to surgery or metastatic disease. We excluded all specimens that contained ductal carcinoma in situ (DCIS), as virtually all DCIS stain positive for Gd (3).
Tissue blocks of 121 breast cancer patients fulfilled these criteria. The mean age of patients was 62.2 (standard deviation (SD) 9.8) years, with 85% being postmenopausal. Nine out of the 83 ductal carcinoma were graded as G1, 40 as G2 and 34 specimens as G3. In lobular carcinoma (n = 38), 15 were graded as G1, 16 as G2, 3 as G3 (Table IA). Grading was performed according to criteria published by Elston and Ellis (13). If one or more of the criteria was not assessable, the specimen was classified as Gx. Nodal involvement, as well as steroid receptor status was equally distributed among the study population. As part of the clinical work-up ER-α and PR-A isoforms were routinely evaluated. All receptor-positive breast cancer specimens were positive for ER-α and PR-A. (ER-α and PR-A positive: 48 ductal, 20 lobular; ER-α and PR-A negative: 35 ductal, 18 lobular). In total, ductal carcinoma had a Gd staining intensity that ranged from 0-12 with a mean of 8.92 (standard error of mean (SEM) 3.63). In lobular breast cancer, staining intensity ranged from 3-12, with a mean of 8.63 (SEM 3.09) (Table IB).

Immunohistochemistry. Gd immunostaining was performed in paraffin-embedded sections (4 μm) of breast cancer tissue as previously published (3). Sections were first incubated in 3% methanol/H_{2}O_{2} (30 min) to inhibit endogenous peroxidase activity, subsequently washed in phosphate-buffered saline (PBS, pH 7.4) for 5 min and treated with 1.5% goat serum for 20 min at 22°C to reduce non-specific background staining. In order to release aldehyde bonds, sections were boiled for 5 min in pH 6.0 buffer containing 2% Na-citrate and citric acid and afterwards carefully cooled to 22°C in tap water. The primary polyclonal antibody (clone UCI-2), which is specific for the core amino acid sequence of Gd, was from Bioscience AG (Berlin, Germany) and used at a 1:200 dilution. Specific binding of the antibody to Gd was analyzed by Western blot analysis. Incubation with the primary antibody was performed overnight at 4°C. Sections were then incubated with biotinylated secondary anti-mouse antibody (1:200) for 1 h and prepared with avidin-biotin peroxidase complex (1:25) according to manufacturer’s instructions for 45 min (Vectastain Elite ABC Kit, Vector Laboratories, Burlingame CA, USA). Immunolabeling was finally stained by incubating the slides with 1 mg/ml diaminobenzidine/H_{2}O_{2} (Dako, Hamburg, Germany) for 3 min and subsequently washing them in tap water for 10 min. Counterstaining was performed by staining with hemalaun for 1 min before sections were cover-slipped. In controls the primary antibody was replaced with pre-immune rabbit serum. Positive (decidual tissue) and negative (human trophoblast tissue) controls were always included.

Two independent observers, including a gynecological pathologist, assessed the specimens using the semi-quantitative immunoreactivity score (IRS) described in detail by Remmele et al., (14), which is routinely used for assessing hormone receptor positivity in breast cancer. The IRS was calculated by multiplication of optical staining intensity (graded as 0=no, 1=weak, 2=moderate and 3=strong staining) and the percentage of positive 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). Evaluation of each specimen was performed without having any knowledge of the pathological diagnosis. For all sections, we assessed the mean optical density and the quantity of pixels which had a positive reaction for glycodelin using KSRun software (imaging system KS400, release 3.0; Zeiss, Vision GmbH, Germany).

<table>
<thead>
<tr>
<th>Histology</th>
<th>Min.</th>
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<th>Mean</th>
<th>SD</th>
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<tr>
<td>Lobular</td>
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<td>12</td>
<td>8.63</td>
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</tr>
<tr>
<td>Ductal</td>
<td>0</td>
<td>12</td>
<td>8.92</td>
<td>3.74</td>
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Statistics. The SPSS/PC software package, version 16.0 (SPSS GmbH, Munich, Germany), was used for collection, processing and statistical analysis of all data. We performed statistical analysis using the non-parametrical Mann-Whitney U-test and in cases of 3 or more groups its extension, the Kruskal-Wallis one-way analysis of variance by ranks. P-values resulted from two-sided statistical tests and p≤0.05 was considered to be significant.

Results

Gd was expressed both in lobular and in ductal breast carcinoma. Although there was a tendency towards increased staining in ductal carcinoma, no significant differences were found (median IRS for ductal carcinoma: 8.92 versus median IRS=8.63 lobular carcinoma) (Table IA). Figure 1 shows a staining example of a Gd positive (IRS:8) lobular G3 breast cancer specimen and Figure 2 two magnifications of a Gd-positive (IRS:9) ductal G1 specimen. There was no significant difference between tumors of patients with positive axillary lymph nodes and those without (p=0.16). Median IRS for tumors without nodal involvement was 8.54 (n=61), while for those with affected lymph nodes, it was 9.12 (n=60) (Figure 3).

Gd expression was increased nonsignificantly in those specimens that also stained positive for steroid receptors (p=0.93). Median IRS of all hormone receptor-negative tissue sections was 8.70 (n=53) compared to hormone receptor-positive specimens that had a median IRS of 8.93 (n=68) (Figure 4).
Gd expression in lobular and ductal carcinoma cells was reduced with dedifferentiation and reached statistical significance when comparing G1 to G3 \((p=0.038)\) (Figure 5).

**Discussion**

Gd expression was analyzed in breast cancer tissue without indications of carcinoma *in situ*. Glycosylation seems to play a pivotal functional role in Gd and influences staining behavior (15). We found a non-significant increase in glycosylated Gd protein expression in hormone receptor-positive (ER-α and PR-A) specimens and those breast cancer tissues that corresponded to axillary lymph node involvement. However, tumors lost their Gd-positivity upon dedifferentiation. Kamarrainen *et al.* (4) were able to describe Gd expression in 21/35 ductal carcinomas, 9/9 tubular carcinomas, 9/9 mucinous carcinomas, 3/3 mixed ductal/tubular carcinomas and 7/11 lobular carcinomas. In this study, however, DCIS was not excluded from the analyses. DCIS, however, was later found to uniformly stain positive for Gd (3). Accordingly, we limited our study population to invasive breast cancer without any fraction of DCIS. In accordance to the study population used by Kamarrainen *et al.*, we did not find any correlation between Gd expression and nodal involvement or hormone receptor positivity with this polyclonal, amino acid-specific antibody (4).

There is a current debate on the status of grading in the assessment of breast cancer and additional markers to specify intact intracellular signaling are necessary (16). Grading of our specimens was performed by a gynecological pathologist according to the most commonly used international criteria defined by Elston and Ellis (13). One of the central aspects of tumor progression is the establishment of a favorable tumor-immunomicromilieu (17). While all breast cancer specimens showed a considerably high staining intensity, we saw a significant decrease in Gd-positivity upon dedifferentiation, notably between G1 and G3 tumors. Generating specific immunomodulatory factors is dependent on an intact and to some degree regulated intracellular proteinbiosynthesis and signaling (9). This might not be the case in dedifferentiated tissues. Furthermore, strategies that “edit” the local immune response seem to be employed rather early in tumor progression (17). The known immunomodulatory effects of Gd make it a likely candidate to play a role in this immunoediting processes taking place in early breast cancer progression. The known immunological actions of Gd...
encompass direct inhibition of NK and T-cells and modulation of DCs towards a tolerogenic phenotype (7, 18, 19). DCs in particular are central regulators of immunity and are known to shape immunological tumor-stroma interactions (20).

Recently, Hautala et al. described that Gd expressing breast cancer cell lines showed a reduced proliferative behavior which corresponds to a higher degree of differentiation and expression of tumor suppressor genes (8). In other gynecological malignancies, such as serous ovarian cancer, correlation with dedifferentiation and survival has been described by Mandelin et al. (21). These observations underpin our finding of a correlation between grading and Gd expression. Breast cancer is also known for its tendency for early micro-metastatic spreading. We have described earlier that Gd is an efficient inhibitor of E-selectin-mediated cell adhesion in vitro, which might in turn foster tumor spread even in well-differentiated types of cancer (22).

Taken together, the presented findings promote Gd as an additional marker for differentiation, with intriguing capabilities in shaping the tumor micromilieu even in well differentiated tumors.

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References


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