Two E-selectin ligands, BST-2 and LGALS3BP, predict metastasis and poor survival of ER-negative breast cancer

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Abstract. Distant metastases account for the majority of cancer-related deaths in breast cancer. The rate and site of metastasis differ between estrogen receptor (ER)-negative and ER-positive tumours, and metastatic fate can be very diverse even within the ER-negative group. Characterisation of new pro-metastatic markers may help to identify patients with higher risk and improve their care accordingly. Selectin ligands aberrantly expressed by cancer cells promote metastasis by enabling interaction between circulating tumour cells and endothelial cells in distant organs. These ligands consist in carbohydrate molecules, such as sialyl-Lewis x antigen (sLex), borne by glycoproteins or glycolipids on the cancer cell surface. We have previously demonstrated that the molecular scaffold presenting sLex to selectins (e.g. glycolipid vs. glycoproteins) was crucial for these interactions to occur. Moreover, we reported that detection of sLe⁰ alone in breast carcinomas was only of limited prognostic value. However, since sLex was found to be carried by several glycoproteins in cancer cells, we hypothesized that the combination of the carbohydrate with its carriers could be more relevant than each marker independently. In this study, we addressed this question by analysing sLex expression together with two glycoproteins (BST-2 and LGALS3BP), shown to interact with E-selectin in a carbohydrate-dependent manner, in a cohort of 249 invasive breast cancers. We found both glycoproteins to be associated with distant metastasis risk and poorer survival. Importantly, concomitant high expression of BST-2 with sLe⁰ defined a subgroup of patients with ER-negative tumours displaying higher risks of liver and brain metastasis and a 3-fold decreased survival rate.

Introduction

The 5-year overall survival of breast cancer patients with distant metastasis is 23.4% (http://seer.cancer.gov/ for details see ref. 1) and once detected, metastatic breast cancer is incurable. Distant metastases arise from circulating cancer cells that migrate from the blood stream to colonise a distant organ (extravasation) (2). Specific inhibition of cancer cell extravasation would therefore potentially lead to a decrease in the incidence of metastasis. Thus, delineating the molecular mechanisms involved in this process is one of the crucial challenges for breast cancer treatment.

One such mechanism is the interaction between sialyl-Lewis x (sLe⁰) antigen expressed by cancer cells and the selectins expressed by endothelial or circulating immune cells (3). There is a compelling body of evidence that sLe⁰/selectin interaction is involved in metastatic progression in several types of solid tumours including gastric (4), lung (5) and prostate (6) cancers. However, the relationship in breast cancer metastasis remains controversial (7).

We have previously reported that sLe⁰ was over-expressed in estrogen receptor (ER)-negative breast tumours compared to ER-positive ones. Although ER-negative cancers are known to typically be of higher grade and intrinsically more aggressive and more often develop metastatic foci, we found no evidence that sLe⁰ was correlated with metastatic behaviour either in the ER-negative or the ER-positive group (7).

This lack of association led us to question the context of expression of sLe⁰ in breast cancer cells, asking whether the scaffold carrying sLe⁰ (e.g. glycosphingolipids or specific glycoproteins) influenced its role in metastasis. Indeed, we reported that rolling on endothelial cells was more efficient when sLe⁰ was carried by glycoproteins rather than glycolipids (7). However, sLe⁰ was found to be carried by several membrane bound glycoproteins in a breast cancer cell line that...
displayed selectin-dependent rolling on endothelial lining. We identified two of these glycoproteins as tetherin, also known as bone marrow stromal antigen 2 (BST-2), and the galectin-3-binding protein (also named MAC-2BP) encoded by the LGALS3BP gene (7). Subsequently, Shirure et al have shown that MAC-2BP carrying sLeα was functionally involved in ZR-75-1 rolling on HUVECs cultured as monolayer (8).

In the present study, we assessed the expression of sLeα, BST-2 and LGALS3BP in a cohort of 249 invasive breast cancer patients. We investigated whether the combination of sLeα with one or other of its known carriers has a bearing on the prognostic value of sLeα.

Materials and methods

**Human samples.** The samples used in the study were provided by the King's Health Partners Cancer Biobank under their generic ethics approval from National Research Ethics Service, Committee East of England-Cambridge East, reference 12/EE/0493. Under this approval the Biobank are also able to provide pseudo-anonymised samples for research without informed consent if they were collected before September 2006. All tissue samples used in the study were collected prior to Human Tissue Act 2004.

**Tissue microarray (TMA) construction and analysis.** Formalin-fixed paraffin wax embedded tissue from consecutive cases of invasive breast carcinoma dating from 1990 to 1995 (n=400) were selected from the King’s Health Partners Cancer Biobank. Clinical data, including date and site of metastatic recurrence, were collected and validated for each sample. From each block 0.6-mm diameter cores were marked on haematoxylin and eosin-stained slides and the invasive cancer subsequently sampled using the Beecher TMArrayer (Wisconsin, MA, USA) and placed into replicate, 100 core TMA blocks. Sections from two duplicate TMA blocks were cut at 3 µm and dried overnight at 37°C. Sections were baked for 2 h at 60°C, blocked using 10% bovine serum albumin in PBS and stained with anti-sLeα monoclonal antibody (mAb) HECA-452 (7) (BD Biosciences, Oxford, UK) at a concentration of 0.2 µg.ml⁻¹, anti-BST-2 polyclonal antibody (9) (Novus Biologicals) at a concentration of 1 µg.ml⁻¹, anti-LGALS3PB mAb 6B7 (Novus Biologicals, Cambridge, UK) at a concentration of 0.2 µg.ml⁻¹, or anti-BST-2 polyclonal antibody (9) (Novus Biologicals) at a concentration of 0.2 µg.ml⁻¹. The staining was performed using a Ventana Ultra automat (Roche, Rotkreuz, Switzerland) running on Program U33. Primary antibodies were incubated for 32 min on slides, without heating, followed with appropriate secondary and tertiary reagents (UltraView universal DAB Detection kit; Dako, Ely, UK). Staining conditions were optimised for each antibody using a panel of pellets of breast cancer cell lines that were formalin-fixed and paraffin-embedded (Fig. 1). This panel included the ZR-75-1 for which both BST-2 and LGALS3BP staining were positive, according to the fact that these proteins were immuno-precipitated from this particular cell line (7). The staining of biopsies was independently scored by two observers (S.J. and S.E.P., breast pathologist). Score was calculated as the core average of the product IxP, where I is the intensity of the staining (0, 1, 2 or 3) and P the percentage (0-100%) of stained tumour cells (see Fig. 2 for illustration). Cases where there was disagreement between the observers were reviewed and consensus reached on a multiheaded microscope. Tumours were classified as ‘low’ when ≤ to the median and ‘high’ otherwise. Statistical analyses were carried on using Prism5 (Graph-Pad software).

Depending on technical issues (e.g. cores damaged or lost during process, quality of the tissue present in the cores) unbiased observation could only be performed for about 350 samples for each marker. To avoid any cohort bias, we chose to include in the statistical analysis only the samples for which observation could have been made for each of the three markers. When cross-referenced, averaged scores for each of the targets were validated for 249 tumours out of the 400 initially included. The details of clinical and biological features of this cohort is given in Table I. Overall, this cohort fits the known statistics of the pathology regarding the rate of expression biomarkers (10) or the survival rates (1), thus excluding bias from the cohort selection.

### Table I. Clinical and biological features of the cohort of 249 patients.

![Table I](image-url)
HER2 expression status was only available for 134 (53%) of the samples rendering difficult to specifically address the triple-negative group (17 samples, 12.7%) within this cohort. We thus decided to stratify our cohort based on the sole ER expression for subsequent analysis.

Results

Staining and scoring of biopsies. For all three antibodies, the observed staining was mainly cytoplasmic with membrane signal observable when the cells were strongly stained (Fig. 2). Variation of staining intensities allowed to classify each sample according to the level of expression of each marker (11). The frequency distributions of the scores were distinct when comparing the three markers (Fig. 3A) and quite different from the Gaussian-type frequency distribution commonly observed when measuring gene expression (Fig. 3B). Due to the asymmetrical score distributions, we chose to use median to discriminate between ‘Low’ and ‘High’ scores and compare group of tumours of the same size if possible. Median scores were 2.5 for sLex and 200 for LGALS3BP. Median was also 200 for the BST-2 group, but the very homogeneous staining in this group resulted in 91 tumours being scored 200, making the use of the median inappropriate to split the series in two equal groups. After comparing the survival of the patients according to the 25 and 75% percentile scores (Fig. 3C) we chose to use the highest quartile (score >202.5) to define our ‘High’ BST-2 group. Although we identified both proteins as carriers of sLe\(^{\alpha}\) in a breast cancer cell line (ZR-75-1), scores of sLe\(^{\alpha}\) did not significantly correlate with either BST-2 (Pearson r\(^{2}\)=0.017, p=0.042) or LGALS3BP (Pearson r\(^{2}\)=0.002, p=0.445). This suggests that in breast tumours, the occurrence of the high expression of either carrier is independent of the occurrence of high sLe\(^{\alpha}\) expression and vice versa.

Correlation with clinical features. We first analysed the association of each of the three markers with several histological and clinical features of the tumours (Table II). As previously reported (12), sLe\(^{\alpha}\) high expression correlated with ER-negativity, lymph node (LN) involvement, and high grade. We also observed a correlation with progesterone receptor (PR) negativity. When considering the entire cohort, BST-2 and LGALS3BP did not correlate with any of the tested features.
Both BST-2 and LGALS3BP high expression correlated with earlier time of metastasis and poor prognosis. Kaplan-Meier analyses were performed for each marker individually after ER stratification (Fig. 4). sLe\textsuperscript{x} on its own did not associate with either distant metastasis-free survival (DMFS) or breast cancer specific survival (BCSS), as we have previously reported. However, strikingly, both BST-2 and LGALS3BP high expression predicted earlier development of distant metastasis and shorter patient survival in the ER-negative group, but not in the ER-positive subset of patients (Fig. 4). While none of the markers were associated with DM when analysing the cohort as a whole, both BST-2 and LGALS3BP correlated with DM within the ER-negative group (p=0.0270 and p=0.0353 Fisher’s exact test, respectively). This suggests that both glycoproteins are involved in ER-negative tumour progression independently of sLe\textsuperscript{x} expression.

BST-2 significantly alters the organotropism of ER-negative metastasis. We previously reported that ‘very-high’ (i.e. score of >60) expression of sLe\textsuperscript{x} in ER-positive tumours drove metastasis to the bone (12). In the present study metastatic ER-positive tumours with very-high sLe\textsuperscript{x} similarly colonised bone more frequently than those with low sLe\textsuperscript{x} expression (87.5 and 52.6%, respectively).

ER-negative tumours colonised distant organs at various frequencies: lung (8.9%), liver (12.5%), bone (14.3%), brain (17.9%) and other distant organs (23.2%) such as distant lymph nodes (contra-lateral or mediastinal), pleura or skin. These frequencies were compared between groups displaying high or low expression of sLe\textsuperscript{x}, BST-2 and LGALS3BP (Fig. 5). Taken independently, variation in occurrence of metastasis for each site was not found to be significantly associated to the expression of any of the tested markers. However, high expression of
BST-2 seemed to generally affect the distribution of metastasis across the board. High expression of BST-2 was associated with increased metastasis in liver (2.8-fold) and brain (1.4-fold) while metastasis was decreased in lung (1.3-fold) and bone (1.9-fold). By comparison, the two other markers had limited effects. High sLex expression seemed to follow the same trend as high BST-2, especially for liver and lung metastasis. High expression of LGALS3BP tended to be associated with an overall higher frequency of metastasis without affecting the tropism. As a result, we found that expression of BST-2 significantly altered the pattern of metastasis when compared to the two other markers (Chi-square test, p=0.0246).

Combined expression of BST-2 and sLe^x further improves BST-2 prognostic value. Although we did not find sLe^x correlation with its carriers in tumours, there is, nonetheless, a discrete fraction of patients whose tumours displayed high sLe^x expression together with high expression of BST-2 (10.6% of patients) and/or LGALS3BP (24.4%). Due to the documented function of sLe^x carried by these proteins as E-selectin ligand (7,8), we investigated the prognostic value of these combinations of markers in our series.

We analysed metastasis-free and breast cancer specific survival of patients within groups displaying high sLe^x expression together with high expression of one or the other of its carriers (BST-2 or LGALS3BP) compared to the rest of the patients (Fig. 6).

This stratification did not bear any significance for the patients with ER-positive tumours. In the ER-negative group, the combination of sLe^x with LGALS3BP did not improve, and
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Actually reduced, the prognostic value of LGALS3BP. This suggests that the LGALS3BP function in ER-negative breast cancer metastasis is independent of sLeα expression. This was surprising since sLeα bearing LGALS3BP has been shown to be involved in the rolling of the ZR-75-1 breast cancer cell line on endothelial cells (8). However, LGALS3BP, as a secreted protein, has been linked to cancer metastasis through interactions with other proteins such as Tem1 (13) or galectins 1 and 3 (14-16). These multiple mechanisms may explain why LGALS3BP on its own bears some prognostic value, as previously reported (17), while the sLeα/LGALS3BP does not.

In contrast, the sLeα/BST-2 combination retained (and even increased) statistical significance in analysis for both metastasis and patient survival, despite the fact the sLeα/BST-2 group was reduced to 8 patients. Computation of Hazard Ratio showed an increase of risk when sLeα was combined to BST-2, for both BCSS (from 3.654 to 4.566) and DMFS (from 3.961 to 7.070), indicating that the patients displaying both markers concomitantly were more rapidly at risk than the others (Table III). By contrast, the same comparison performed using LGALS3BP with sLeα did not show any increase. The combination of BST-2 with sLeα identified a sub-group (14%) of ER-negative patients with an 80% risk of metastasis at 5 years, twice the level in the patients with tumours not over-expressing both markers simultaneously. The patients with ER-negative carcinomas expressing both sLeα and BST-2 had only a 20% survival at 10 years, about three times less than other patients with ER-negative cancers. This may be due to the metastatic tropism associated with BST-2, possibly enforced by sLeα, since brain and liver metastasis are more rapidly lethal than bone and lung metastasis (18).

By comparison, stratification based on the high expression of both BST-2 and LGALS3BP did not result in a better prediction of metastasis or survival than each of the markers taken individually (Fig. 7).

Influence of treatment of ER-negative patients on survival analyses. Detailed review of the adjuvant therapy given to the ER-negative groups revealed that half of the patients (28) received chemotherapy drugs, while the other half (29) was only treated with Tamoxifen as it was routinely performed during the nineties (see Table IV for details). We found no
differences in DMFS or BCSS when comparing these two arms, thus excluding any treatment bias in the results reported above. We further explored the effect of each protocol (no chemotherapy vs. chemotherapy) on the aggressive High BST-2/sLex sub-population. The results seemed to indicate that the patients belonging to the poor prognosis group fared better when treated with chemotherapy compared to those treated only with Tamoxifen (Fig. 8). Albeit statistically significant, this analysis was performed with a very limited number of samples and should be considered with appropriate reserve.

Discussion

In this study, we retrospectively assessed the prognostic significance of two membrane bound glycoproteins, BST-2 and LGALS3BP, in breast cancer metastasis and survival. Both proteins were chosen based on the fact that they were found to be E-selectin ligands, when carrying the carbohydrate determinant known as sialyl-Lewis x (sLex) antigen, and potentially involved in blood borne metastasis. We previously investigated the expression of sLex in breast cancer and found this glycan overexpressed in ER-negative breast cancers (7). To detect sLex expression we chose the widely used HECA-452 monoclonal antibody that, in our hands, was able to recognize sLex carried by multiple glycoproteins and/or glycolipids (7). HECA-452 binding is therefore non-discriminative of the carrier of the glycan. This is why we wanted to examine the relevance of sLex together with two of its described protein carriers in our
statistical analyses. Since carbohydrate structures, or glycans, result from the combined expression of several synthesizing enzymes (glycosyltransferases), one of the most efficient way to assess their expression in tumour sample is to use specific probes (monoclonal antibodies or lectins) to detect them on tissue sections. Thus, to analyse both proteins and glycans from the same material, we chose to assess the expression of sLex and its putative carriers by immunohistochemistry. Another aim of our study was to investigate the possible correlation of our markers or combinations of markers with the organotropism of the metastases. To achieve this, we exploited a series of formalin-fixed paraffin-embedded samples archived by the King's Health Partners Cancer Biobank, which consist of 400 patient biopsies all associated with the extensive clinical history (e.g. date and site of metastasis and treatment protocols) of the corresponding patients. From this series, we managed to validate scoring for all three markers for 249 samples. Although this cohort was of modest size, the quality of the associated clinical data made it suitable for the purpose of our study.

Of note, we found that both BST-2 and LGALS3BP glycoproteins were indeed associated with the development of subsequent distant metastasis and also patient survival, albeit only in the patients with ER-negative tumours. Conversely, the expression of these proteins was not dependent on the ER-status of the tumours. This suggests that both proteins act on the pathway of metastasis in partnership with other factors that are potentially specific to ER-negative cancer cells.

One such factor could be the sLe^x antigen, which correlates with ER negativity (7). We found that combining sLe^x and LGALS3BP high expression to stratify patients did not improve the prognostic significance of LGALS3BP. This suggests that the pro-metastatic function of LGALS3BP is not primarily due the ability of this protein to be recognised by E-selectin. Indeed, LGALS3BP (MAC-2BP) was experimentally shown to be involved in pro-metastatic interactions (15,16).
independently of sLex expression or selectin involvement. One previous study also reported LGALS3BP to be associated with poor survival in node-negative breast cancer patients (17). On the contrary, another large study showed no correlation of the cytosolic expression of LGALS3BP with prognosis in breast cancer (19). However, the authors of this study argued that the cytosolic, immature, form of LGALS3BP may not be appropriate to predict prognosis due to the possible lack of biological activity. While implication of LGALS3BP in tumour progression and metastasis is now well documented in a number of cancers, it is also clear that the galectins/galectin binding protein/ligand networks involved are intricate and plastic (20). The present study confirms the clinical relevance of LGALS3BP by showing its association with occurrence of metastasis in ER-negative breast cancer, but dismisses sLex antigen as a mediator of this effect.

On the contrary, high-sLex/high-BST-2 combined expression was a better predictor of distant metastasis and survival than BST-2 alone. High expression of BST-2 mRNA was previously associated with tumour aggressiveness and decreased survival in breast cancer by others (21). Cai et al also reported BST-2 protein expression to be associated with breast cancer bone metastasis in a smaller cohort (n=50) of breast samples (22). Experimental data, based on cell lines and in vivo models, have hinted that BST-2 was involved in increased proliferation and reduced apoptosis (23), as well as increased migration, invasion and metastasis (21,22,24). The data we are presenting imply that the role of BST-2 in metastasis is specific to patients with ER-negative tumours, and may be further enhanced by sLex expression. The reason why BST-2 appears to specifically influence the tropism of ER-negative breast cancers is unclear. It could be related to the described function of BST-2 as an organiser of membrane microdomains (i.e. lipid rafts) (25). Indeed, by acting on the organization of the cell membrane, oligomers of BST-2 may promote the function of other molecules, specifically expressed in ER-negative cancer cells compared to ER-positive ones, which are involved in brain or liver metastasis. Identifying such partners of BST-2

Figure 7. Correlation of combined BST-2/LGALS3BP expression with distant metastasis-free survival (DMFS) and breast cancer-specific survival (BCSS) according to ER status. Number of patients included is indicated next to each DMFS curve. p-values were calculated using Grehan-Breslow-Wilcoxon test (Prism5).

Figure 8. Effect of adjuvant treatment protocols (no chemotherapy/black lines vs. chemotherapy/grey lines) on distant metastasis-free survival (DMFS) and breast cancer-specific survival (BCSS) of ER-negative patients, according to combined BST-2/sLex high expression (Plain lines). Number of patients included is indicated next to each DMFS curve.
would thus warrant further investigations. Clustering of BST-2 around the lipid rafts would also create patches of BST-2 associated glycans, including sLeα (25). Such organization would indeed enhance the ability of BST-2 borne sLeα to be recognized by selectin by improving the avidity of the interaction. Whether these putative mechanisms actually participate in the metastatic process in breast cancer would require further experimental demonstration.

The prognostic value of combined expression of BST-2 and LGALS3BP was not improved compared to each marker individually. Thus, the synergistic effect of sLeα and BST-2 expression on prognosis is specific of these particular two markers, and not random. Importantly, due to the complex biosynthesis of glycans, such a combination of markers could not have been easily detected using gene profiling strategies on its own. Supporting this, one very recent study has demonstrated that integrated analysis of glycosylation genes and their glycan products indeed resulted in significant prognostic data (26). In the same line, our present study demonstrates the potential of the widely available technique of immunohistochemistry to investigate combinations of glycans together with relevant protein carriers as prognosis markers.

The combination of BST-2 expression and sLeα positivity identified a small subset of patients that fared significantly worse than the other ER-negative breast cancer patients. Although sub-stratification ended up producing small groups of patients, the statistical analysis retained a high degree of significance. Whilst patients with ER-negative tumours may receive adjuvant chemotherapy, depending on other biological and patient variables, it is clear that not all of these women do equally badly (27). Based on new biomarkers, such as our BST-2/sLeα combination, one may therefore consider improving ER-negative patients follow-up and treatment protocols with possibly more aggressive therapy. On the other hand, such biomarkers, presumably involved in selectin-mediated metastasis (28), could also serve as molecular target for tailored therapy (29). In that regard, we believe both BST-2 and LGALS3BP could be considered as putative targets to treat ER-negative metastatic breast cancer.

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