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PRECLINICAL STUDY

Elevated serum levels of sialyl Lewis X (sLe^X) and inflammatory mediators in patients with breast cancer

Evan N. Cohen[1,2] · Tamer M. Fouad[1,3,6] · Bang-Ning Lee[1,2,7] · Banu K. Arun[3] · Diane Liu[5] · Sanda Tin[1,2] · Angelica M. Gutierrez Barrera[3] · Toshihide Miura[10] · Iwao Kiyokawa[10] · Jun Yamashita[10] · Ricardo H. Alvarez[1,3,8] · Vicente Valero[3] · Wendy A. Woodward[1,4] · Yu Shen[5] · Naoto T. Ueno[1,3] · Massimo Cristofanilli[1,3,9] · James M. Reuben[1,2]

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Abstract

Purpose The carbohydrate sialyl Lewis^X (sLe^X) mediates cell adhesion, is critical in the normal function of immune cells, and is frequently over-expressed on cancer cells. We assessed the association, differential levels, and prognostic value of sLe^X and inflammatory cytokines/chemokines in breast cancer sera.

Methods We retrospectively measured sLe^X and a panel of cytokines/chemokines in the sera of 26 non-invasive ductal carcinoma in situ (DCIS), 154 invasive non-metastatic breast cancer (non-MBC), 63 metastatic breast cancer (MBC) patients, and 43 healthy controls. Differences in sLe^X and inflammatory cytokines among and between patient groups and healthy controls were assessed with nonparametric tests and we performed survival analysis for the prognostic potential of sLe^X using a cut-off of 8 U/mL as previously defined.

Results Median serum sLe^X was significantly higher than controls for invasive breast cancer patients (MBC and non-MBC) but not DCIS. In univariate analysis, we confirmed patients with serum sLe^X > 8 U/mL have a significantly shorter progression-free survival (PFS) ($P = 0.0074$) and overall survival (OS ($P = 0.0003$). Similarly, patients with high serum MCP-1 and IP-10 had shorter OS ($P = 0.001$ and $P < 0.001$, respectively) and PFS ($P = 0.010$ and $P < 0.001$, respectively). sLe^X, MCP-1 and IP-10 remained significant in multivariate survival analysis.

Conclusion Elevated serum sLe^X was associated with invasive cancer but not DCIS. High serum sLe^X levels were associated with inflammatory mediators and may play a role in facilitating local invasion of breast tumor. Furthermore, serum MCP-1, IP-10 and sLe^X may have prognostic value in breast cancer.

Keywords Sialyl Lewis^x (SLE^X) · Inflammatory cytokines · Breast cancer · Serum

Introduction

The regulation of cell adhesion is a critical process throughout the development of metastatic cancer. Both heterotypic and homotypic cancer cell adhesion are mediated in part by specific interactions between cell surface lectins and their cognate carbohydrate ligands presented on glycoproteins and glycolipids [1–3]. Increased sialylation is a common feature in the glycoconjugates of malignant cells [4]. Sialyl Lewis X (sLe^X) is a mucin-associated carbohydrate ligand on cancer cells that binds to its receptor E-selectin on endothelial cells to mediate adhesion between the cancer cells and the endothelium. E-selectin plays a pivotal role in the capture and rolling of cancer cells on the surface of endothelial cells. Surface expression of sLe^X on cancer cells mediates their tethering and rolling along vascular endothelium expressing E-selectin, thereby facilitating migration and dissemination of cancer cells, potentially leading to metastasis. Aberrant expression of sLe^X is associated with tumor formation and metastasis [5] and the level of sLe^X was found to be elevated in the sera of patients with metastatic breast cancers (MBC) [6] and correlated with metastasis [7]. Furthermore,

Evan N. Cohen and Tamer M. Fouad have contributed equally to the work.

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✉ James M. Reuben
jreuben@mdanderson.org

Extended author information available on the last page of the article

overexpression of sLe^X was associated with higher tumor stage [8] poorer prognosis and malignant relapse [9].

Using an immunoassay (CSLEX; Nittobo Medical Co. Ltd., Japan), it is possible to measure sLe^X in serum and it has been reported that the level is associated with clinical stage and response to treatment of breast cancer [10]. Using a cut-off of 8 U/mL for sLe^X, serum sLe^X in combination with CA15-3 (Cancer Antigen-Breast) was found to be more useful than carcinoembryonic antigen (CEA) and tumor marker CA15-3 combined in monitoring breast cancer patients [10]. Others have demonstrated that gene transcripts of sLe^X are significantly increased in estrogen receptor alpha-negative (ER-negative) tumors compared with that of ER-positive ones. In this study, serum sLe^X level had no association with survival, irrespective of ER expression by their tumors. However, high expression of sLe^X in ER-positive tumors is correlated with metastasis to the bone where the sLe^X receptor, E-selectin, is constitutively expressed [11].

Typically, initial adhesion of leukocytes to a site of injury is mediated by E-selectin, the specific receptor for sLe^X [12]. In tumors, E-selectin plays a pivotal role in recruiting leukocytes to the tumor microenvironment where immune-specific tumor lysis can trigger a storm of inflammatory cytokines inducing the over-expression of E-selectin on endothelial cells of blood vessels [13]. sLe^X on the tumor cells interacts with E-selectin on endothelial cells to initiate motility by causing tumor cells to roll on the endothelium. Whereas sLe^X binds to E-selectin to facilitate metastasis, inflammatory cytokines may enhance the metastatic potential of human cancer by upregulating the expression of E-selectin on endothelial cells.

Abnormal expression of sLe^X has attracted much attention because of its function in cancer cell extravasation, mimicking a molecular mechanism involved in leukocyte extravasation [14–16]. In colorectal cancer, the high expression of sLe^X in adenomas was correlated with a high degree of dysplasia [17]. In patients with small cell lung cancer, preoperative serum sLe^X values were associated with pathological stages and survival after surgery [18].

In this study, we evaluated the serum expression of sLe^X and a panel of 39 inflammatory proteins. The primary aims were to compare differential serum levels of sLe^X in patients with non-invasive ductal carcinoma in situ (DCIS), metastatic (MBC) and non-metastatic breast cancer (non-MBC) as well as healthy donors (HD) with the hypothesis that higher serum sLe^X would correlate with higher disease stage, and second to confirm previous publications that serum $sLe^x > 8$U/mL is correlated with decreased survival.

Methods

Patient characteristics

This retrospective study was approved by the MD Anderson Institutional Review Board (IRB) as LAB09-0347 for the evaluation of patients with non-invasive DCIS, invasive breast cancer (MBC), and healthy donors (HD) who have participated in previous diagnostic protocols at MDACC (IRB approved as LAB09-049, 2005-0243, ID02-052, 2006-1072, ID02-458, LAB03-0479, LAB05-0083, ID99-231, LAB08-0231, and LAB08-0199). A request for waiver of informed consent was approved for this retrospective study as it posed no more than minimal risk and would not adversely affect the rights or welfare of the patients. Serum samples were collected pre-treatment or at the start of a new line of therapy. Patients were classified as having hormone receptor (HR)-positive tumors if estrogen receptor (ER) and/or progesterone receptor (PR) were expressed at > 10% by immunohistochemistry (IHC) of the primary tumor. Likewise, patients were categorized as human epidermal growth factor receptor 2 (HER2) enriched if either the primary tumor had a fluorescent in situ hybridization (FISH) HER2:CEP 17 ratio > 2 or III + by IHC if FISH was not performed.

Measurement of sLe^X

Sera from 243 patients (26 with DCIS, 154 with non-MBC, 63 with MBC), and 43 HD were assayed for sLe^X using a two-step sandwich enzyme immunoassay method (Nittobo Medical Co., Japan). Briefly, 20 µL of sera were incubated in a 96-well plate coated with anti-sLe^X monoclonal antibody in a buffer solution for 2 h at 37 °C to enable the capture of sLe^X. Thereafter, the plates were washed to remove unbound proteins. Next, horseradish peroxidase-labeled secondary antibody was added to the antibody-sLe^X complex in buffer solution to form a sandwich complex of antibody–antigen–antibody and incubated for 2 h at 37 °C. Again, unbound proteins were washed out before the addition of substrate TMB (3,3,5,5′-tetramethylbenzidine). The absorbance was measured from the result of the enzymatic reaction. Because the strength of absorbance is closely related to the concentration of sLe^X in the patient serum, the sLe^X concentration was estimated using the standard curve.

Serum cytokines and chemokines

A panel of 39 cytokines and chemokines including MCP-1 and IP-10 were measured in a 25 µL sample using the Millipore Milliplex Human Cytokine/Chemokine Panel

I, a multiplex assay kit (Millipore Corp, Billerica, MA), and a Luminex Analyzer 100 (Austin, TX), as previously described [19].

Statistical analysis

The primary objective was to compare the serum level of sLe^X in healthy individuals and patients with ductal carcinoma in situ (DCIS), non-metastatic breast cancer (non-MBC) and metastatic breast cancer (MBC). The secondary objective was to evaluate the prognostic value of sLe^x in patients with primary breast cancer (Stages I-III). The non-parametric Mann–Whitney U tests or Kruskal–Wallis H tests were used to determine the differences in sLe^X, cytokine, and chemokines levels between/or among patient groups and HD. Spearman's correlation was used to determine the correlation between the serum levels of sLe^X and tumor-promoting inflammatory mediators. Overall survival time was calculated from the time of sample to death (event) and, progression-free survival (PFS) time from sample time to progression or death whichever happens the first (event) or last follow-up time (censor). Only patients with Stage I–IV breast cancers (no DCIS) were included in the time-to-event analysis. OS and PFS times were estimated using the Kaplan–Meier method and compared between or among patients' characteristics groups using log-rank test. A predetermined cut-point of 8 U/mL was used for sLe^X, and for cytokines, a cutoff was established at the 95th percentile of the mean of the healthy donors. Cox proportional hazard models are applied to estimate the effect of covariates of interest on OS and PFS times. Stepwise selection method is used to choose the statistically significant covariates that are associated with OS and PFS times. All computations are carried out in SAS 9.3 (SAS Institute Inc., Cary, NC, USA) and Splus 8.2 (TIBCO Software Inc, Palo Alto, CA) with plots generated in R (R Core Team (2017), Vienna, Austria) and Graph Pad Prism (La Jolla, CA).

Results

Patient characteristics

The study included a total of 243 breast cancer patients including 26 who presented with DCIS, 154 with primary, non-metastatic disease and 63 with metastatic disease. The median follow-up period for stage I–IV breast cancer was 74.4 months (95% CI 63.3–79.5 months). In addition, 43 healthy donors volunteered samples. Baseline patient characteristics are shown in Table 1. There were 166 patients with hormone receptor-positive tumors (HR); 90 patients were HER2-enriched; 46 patients had triple-negative breast cancer (TNBC). The five patients that had low or weak-positive hormonal staining were classified TNBC ($n=3$) or HER2 ($n=2$). The clinic stages and subgroups of patients were listed in Table 2.

Table 1 Patient characteristics

	Count	Percentage
Type		
DCIS (0)	26	10.7
Non-MBC (I,II, III)	154	63.4
MBC (IV)	63	25.9
HD	43	
Clinical stage		
DCIS (0)	26	10.7
I	48	19.8
II	47	19.3
III	59	24.3
IV	63	25.9
ER		
Negative	79	32.5
Positive	164	67.5
PR		
Negative	103	42.4
Positive	140	57.6
HR (ER or PR)		
Negative	77	31.7
Positive	166	68.3
Her2		
Negative	153	62.9
Positive	90	37.1
Triple negative		
No	197	81.1
Yes	46	18.9

sLe^X and inflammatory cytokines elevated in breast cancer

Compared with the median serum sLe^X level in HD (1.6 U/mL), the median serum levels of sLe^X of DCIS (2.3 U/mL) non-MBC (3.0 U/mL) and MBC (4.0 U/mL) were significantly higher (all $P<0.05$) (Fig. 1). In confirmation of previous reports that suggested using 8 U/mL as a cut-off for high levels of CSLEX [10], the 95th percentile of HD in the current study's cohort was 9.2 U/mL, confirming 8.0 U/mL as a reasonable cut-off. Although not significant, patients with MBC were more likely than those with non-MBC to have serum sLe^X levels above 8.0 U/mL (33.3% vs. 20.8%, $P=0.08$).

Compared with HD, DCIS or non-MBC patients had significantly higher median levels of epidermal growth factor (EGF), eotaxin (CCL11), fibroblast growth factor (FGF)-2, interferon (IFN)-α, interleukin (IL)-1 receptor

Table 2 Clinical stages and distribution

Levels	CSLEX		*P* value
	High (%)	Low (%)	
Breast cancer type			
HD	3 (7)	40 (93)	0.0106
MBC	21 (33.3)	42 (66.7)	0.1366*
DCIS	5 (19.2)	21 (80.8)	
nMBC	32 (20.8)	122 (79.2)	
Clinical stage			
HD	3 (7)	40 (93)	0.0202
0	5 (19.2)	21 (80.8)	0.1926*
I	8 (16.7)	40 (83.3)	
II	8 (17)	39 (83)	
III	16 (27.1)	43 (72.9)	
MBC	21 (33.3)	42 (66.7)	
Hormone			
Negative	28 (36.4)	49 (63.6)	0.0019
Positive	30 (18.1)	136 (81.9)	
HER2			
Negative	35 (22.9)	118 (77.1)	0.6361
Positive	23 (25.6)	67 (74.4)	
TNBC			
Non-TNBC	42 (21.3)	155 (78.7)	0.0538
TNBC	16 (34.8)	30 (65.2)	

*Excluding HD cases

antagonist (RA), IL-1α, IL-1β, IL-2, IL-4, IL-6, IL-8, monocyte chemotactic protein (MCP)-1, MCP-3, macrophage inflammatory protein (MIP)-1β, and transforming growth factor (TGF)-α and lower growth-regulated oncogene (GRO). Samples from DCIS patients had significantly higher levels of IL-5, IL-9 and IL-12p40 than HD serum samples. MBC patients also had significantly higher serum levels of EGF, eotaxin, interferon gamma response protein (IP)-10, IL-1RA, IL-1β, IL-6, IL-8, IL-10, MCP-1, MCP-3, MIP-1β, sCD40L and tumor necrosis factor (TNF)-α than those of HD. Compared with DCIS or patients with non-MBC, those with MBC had a significantly higher level of IP-10 ($P=0.0001$) and a higher level of MCP-1 ($P<0.0001$) (Fig. 1) as well as higher levels TGF-α and lower IL-2 and TGF-β (not shown). Furthermore, from an analysis of all study samples, there were positive correlations between the serum level of sLe^X and the levels of growth factors EGF, and FGF, pro-inflammatory cytokines IL-1α, IL-1β, IL-1RA, IL-2, IL-2RA, IFN-α2, TGF-α, TNF-β, and chemokines IL-8 (CXCL8), fractalkine (CX3CL1), MIP-1β (CCL4) and MCP-3 (CCL7) and a negative correlation for GRO (CXCL1) (Spearman correlation, $P<0.05$; Table 3, Fig. 2). Additional summary statistics are provided in a supplemental table.

Survival analysis

Progression-free survival (PFS) and overall survival (OS) were assessed in stage I–IV breast cancer patients based on sLe^X stratification at 8 U/mL. The median follow-up time was 74.4 months and the median PFS and OS were not reached. For MBC patients, the median PFS was 8.5 months (95% CI 6.1–18.2) and median OS was 26.7 months (95% CI 13.8–not reached). In univariate analysis, clinical pathological features including clinical stage, ER/PR negativity, TNBC status (but not HER2 alone) and sLe^X status were all associated with decreased PFS and OS. Patients with a serum sLe^X level above the predetermined level of 8 U/mL have a significantly shorter PFS ($P=0.0074$) and OS ($P=0.0003$) (Fig. 3 top). Five-year OS is 84.9% in patients with serum $sLe^X<8$ U/mL group compared to 58.6% patients with $sLe^X\geq 8$ U/mL.

The primary end-point of the study was to determine the prognostic utility of CSLEX with a pre-defined cut-off of 8 U/mL in primary breast cancer (Stage I–III). The study included 154 non-MBC patients of whom 25 were diagnosed with inflammatory breast cancer (IBC). The full cohort of non-MBC patients (including IBC) with serum $sLe^X\geq 8.0$ U/mL had a trend towards shorter PFS ($P=0.07$) and significantly shorter OS ($P=0.0047$) (Fig. 3 bottom) compared to patients with serum $sLe^X<8.0$ U/mL. Excluding inflammatory breast cancer (not shown) there were 129 patients with non-MBC with an median follow-up of 81.1 months (range 6 to 135 months). There were three deaths, all in patients with serum $sLe^X\geq 8.0$ U/mL (log-rank $P=0.0003$).

To characterize additional systemic inflammatory factors in breast cancer associated with breast cancer survival, univariate Cox models of continuous variables indicates that higher CSLEX and the chemokine IP-10 are associated with significantly shorter PFS time with hazard ratios of 1.004 and 1.002, respectively ($P=0.0012$ and $P<0.0001$, respectively). In addition, higher CSLEX, IP10 and MCP1 are significantly associated with a shorter OS time with hazard ratios of 1.007, 1.002 and 1.0003, respectively ($P<0.0001$, $P<0.0001$ and $P=0.002$, respectively, not shown).

Multicovariate Cox models indicate that adjusted for the presence of metastasis (MBC vs. non-MBC) and hormonal receptor status (positive vs. negative), sLe^X (≥ 8 U/mL vs. <8 U/mL) was a significant factor associated with OS (HR 2.985, 95% CI 1.563–5.698, $P=0.0009$), as well as MCP-1 (HR 1.001, $P=0.0003$) and IP-10 (HR 1.001, $P=0.0357$, Table 4). Furthermore, adjusting for metastatic disease, sLe^X (≥ 8 U/mL vs. <8U/mL) was associated with shorter PFS time (HR 1.653, 95% CI 0.977–2.795, $P=0.0609$), as well as IP-10 (HR 1.001, $P=0.0017$, Table 5).

Dichotomizing MCP-1, patients with serum MCP-1≥ 668 pg/mL, the 95th percentile for HD, have a significantly shorter OS ($P=0.0014$) and PFS ($P=0.0095$)

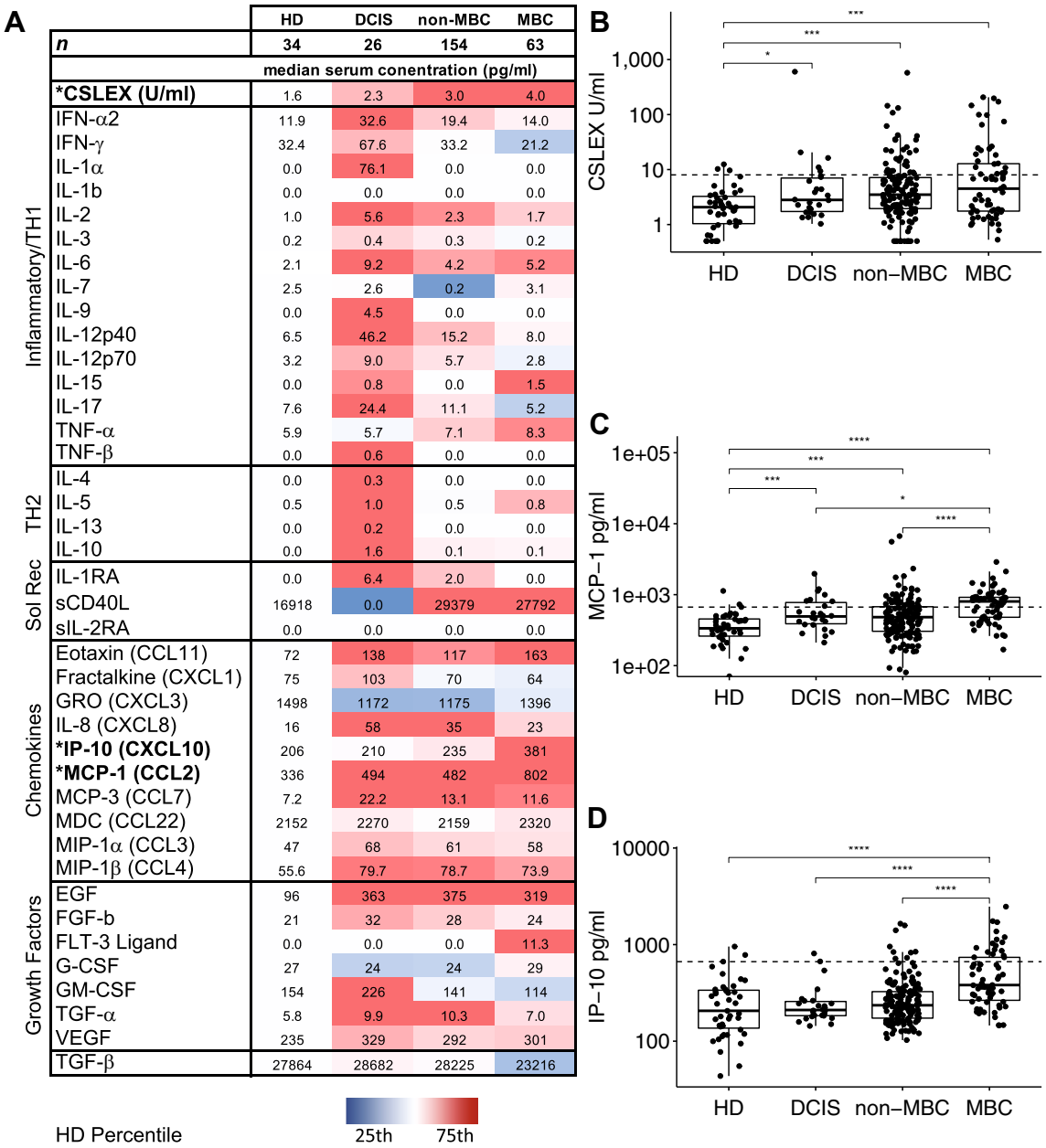

Fig. 1 Levels of serum sLe^X, IP-10, and MCP-1 in the study groups. The heatmap **a** shows median values for each study group. Factors selected for survival analysis are in bold. The color code scale is set to the distribution of HD values for each target protein. Cytokines are loosely grouped into generally inflammatory and T-helper 1 (TH1) cellular immunity-related cytokines, TH2 humoral immunity-related cytokines, soluble cytokine receptors (Sol Rec), chemokines, and growth factors. **b** Compared to HD, median sLe^X levels of DCIS (2.3 U/mL), non-MBC (2.9 U/mL), and MBC (4.0 U/mL) were significantly higher (all $P<0.05$). MBC patients had a significantly higher levels of MCP-1 than DCIS ($P=0.019$) or non-MBC ($P<0.0001$, **c**); and higher levels of IP-10 than non-MBC, DCIS and HD ($P<0.0001$, **d**). Dotted line shows cut-off for survival analysis

(Fig. 4 top). Patients with serum IP-10 > 666 pg/mL, likewise the 95th percentile for HD, have significantly shorter OS and PFS (both $P<0.0001$, Fig. 4 middle). Combining serum sLe^X, MCP-1 and IP-10 into a single prognostic factor, patients with positive serum levels for all three had significantly worse OS and PFS compared to patients that were negative for all 3 or positive for any of the three (both $P<0.0001$, Fig. 4 bottom).

Table 3 Non-parametric correlation between sLe^X and inflammatory cytokines

	Correlation with sLe^X	
	Spearman's rho	*P*
IL-2	0.17490	0.0030
TGF-a	0.17156	0.0036
IL-8	0.16728	0.0046
Fractalkine	0.16558	0.0050
MCP-3	0.16121	0.0063
GRO	−0.15812	0.0074
TNF-b	0.15055	0.0108
MIP1-b	0.14824	0.0121
IL-1a	0.14273	0.0157
IL-1ra	0.14117	0.0169
IL-1b	0.12844	0.0299
IFN-a2	0.12497	0.0346
EGF	0.12027	0.0421
FGF	0.1180	0.0462
sIL-2Ra	0.11787	0.0464

Discussion

sLe^x is a tumor-associated carbohydrate that is used clinically for the management of lung, gastric and colorectal cancers in Japan [10]. Previous studies showed serum $sLe^x > 8$U/mL was a useful clinical marker in breast cancer patients. Here, we report that serum sLe^X levels tended to increase with advanced breast cancer stage and higher levels are correlated with decreased survival. Specifically, we found that MBC patients are more likely than non-MBC patients to have elevated serum sLe^X levels > 8 U/mL. In an earlier study, the expression of sLe^X in paraffin-embedded tissue containing tumor was found to be highest in the primary tumor of patients with invasive mammary carcinoma that had already metastasized to the axillary lymph nodes [8]. Nevertheless, expression of sLe^X was reduced in lymph node metastasis compared to the primary tumor. One possible explanation that could account for this difference between primary and metastatic tumor is the ability of natural killer (NK) cells to attack tumor cells that express high levels of sLe^X [5] and could have broad implications for

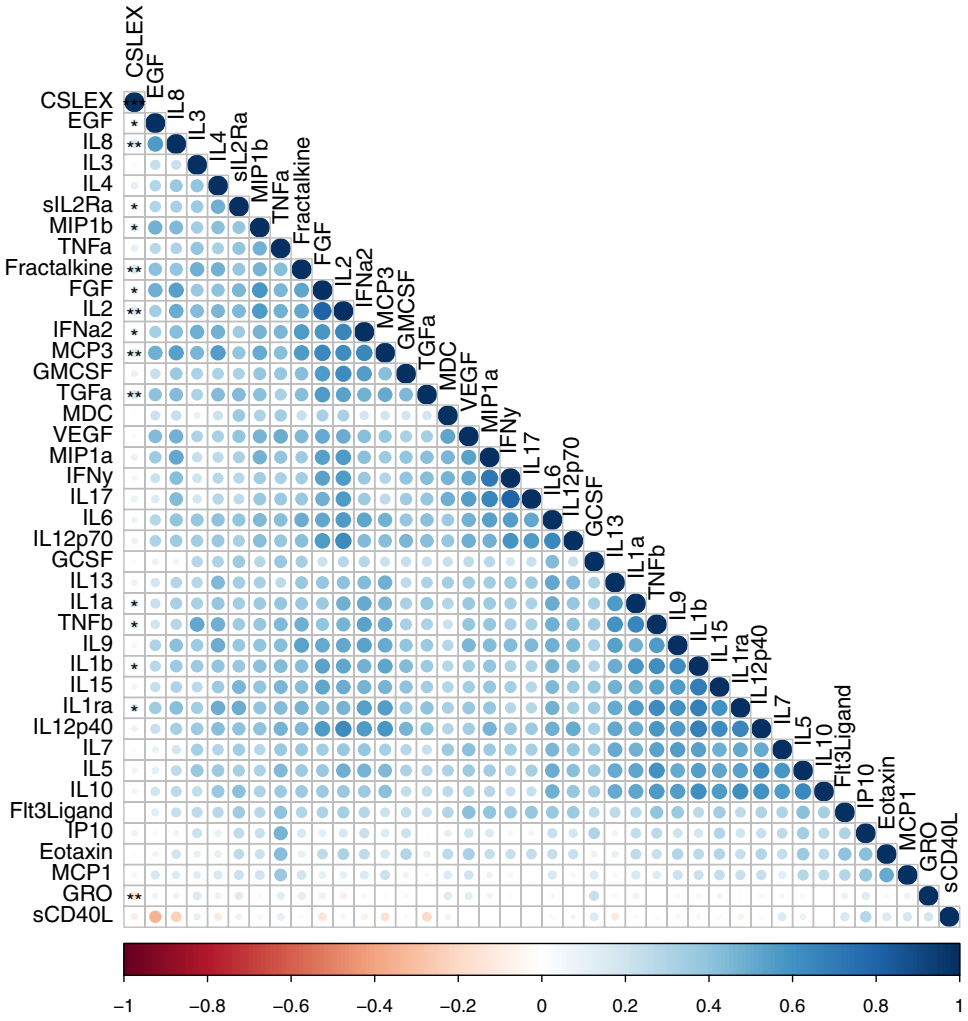

Fig. 2 Non-parametric correlation between sLeX and inflammatory cytokines. The correlation matrix shows Spearman correlation hierarchical clustering of sLe^X (CSLEX assay, first column) with inflammatory cytokines in all samples. Color shows strength of correlation and stars in the CSLEX column indicate significance level of correlation with sLe^X: $^*P < 0.05$, $^{**}P < 0.01$, $^{***}P < 0.001$

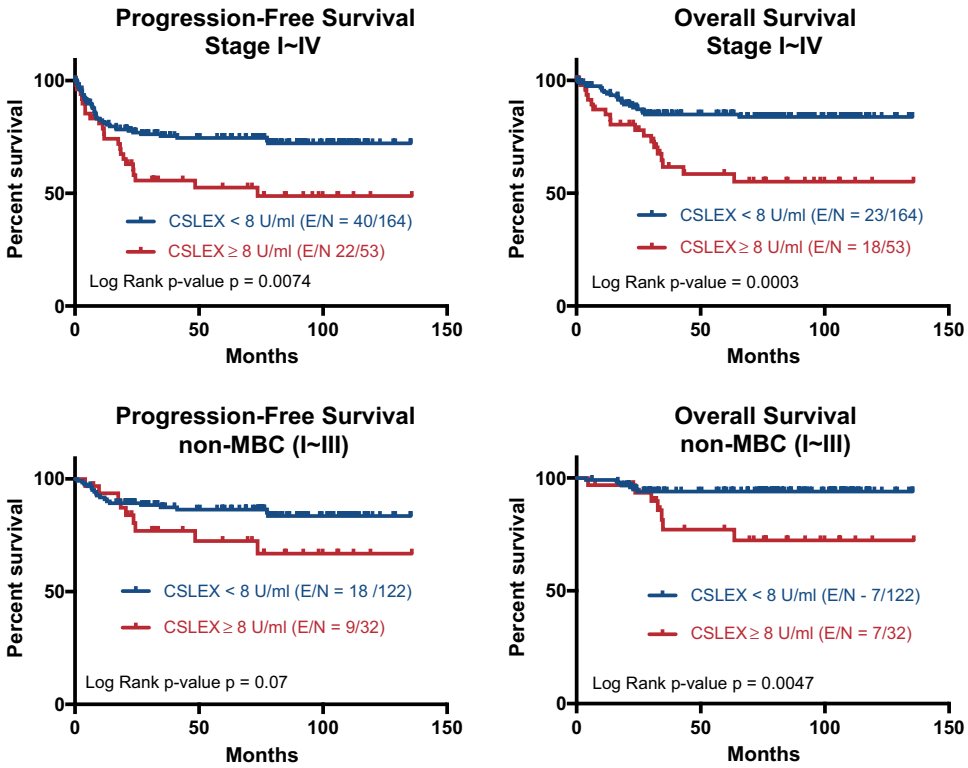

Fig. 3 Elevated sLe^X associated with decreased survival. Top: Retrospective analysis of patients with stage I–IV breast cancer shows patients with high serum sLe^X (above 8 U/mL, $n=53$) have a significantly shorter PFS ($P=0.007$) or OS ($P<0.001$) than those with low serum sLe^X ($n=164$). Bottom: In primary breast cancer (stages I–III including IBC), patients with high sLe^X ($n=32$) have a trend towards shorter PFS ($P=0.07$) and significantly reduced OS ($P=0.0047$) compared to patients with low sLe^X ($n=122$)

Table 4 Multivariate Cox regression model on overall survival

Parameter	*P* value	Hazard ratio	95% HR CI	
Stage				
MBC versus non-MBC	<0.0001	8.787	4.314	17.895
Hormone				
Positive versus negative	0.012	0.429	0.222	0.83
CSLEX_High				
High versus low	0.0009	2.985	1.563	5.698
MCP-1	0.0003	1.001	1	1.001
IP-10	0.0357	1.001	1	1.002

Table 5 Multivariate Cox regression model on progression-free survival

Parameter	*P* value	Hazard ratio	95% HR CI	
Stage				
MBC versus non-MBC	<0.0001	7.661	4.371	13.428
CSLEX				
High versus low	0.0609	1.653	0.977	2.795
IP-10	0.0017	1.001	1	1.002

immunotherapy. It should be noted that among the primary cytokines regulating NK, serum IL-2 but neither IL-15 nor IL-12p70 were correlated with serum sLe^X in the current study. However, despite our observation that the median serum sLe^X level tended to increase with the stage of disease severity, sLe^X was not significantly different by breast cancer stage.

We also observed that patients with a high serum sLe^X have a significantly shorter survival (both PFS and OS) compared with patients with lower serum sLe^X. Others have also assessed the prognostic value of sLe^X expression in non-metastatic disease with a median follow-up of 140 months and found that the expression of sLe^X antigen in breast cancer is not associated with breast cancer survival [20].

Cytokines and chemokines are known to be involved in tumor growth, metastasis, and progression of disease [21]. In particular, TNF-α, IL-1β or interferon-gamma (IFN-γ) are potential activators of expression of E-selectin, P-selectin, intercellular adhesion molecule 2 (ICAM-2) or vascular cell adhesion molecule on endothelial cells [22]. Further, the pro-inflammatory cytokine, IL-6 in serum is a negative prognosticator in breast tumor patients [23] and is predictive of inferior survival in MBC patients [24]. Moreover, the serum IL-8 level increases significantly with more advanced stages of disease and is associated with accelerated clinical progression, the presence of lymph node and liver metastasis [25] and represents a potential target for inhibiting breast cancer stem cells [26]. In our study, patients with DCIS, non-MBC, or MBC have significantly higher serum levels of IL-1RA, IL-1β, IL-6, and IL-8 than those of HD. Numerous studies have linked the increased serum levels of

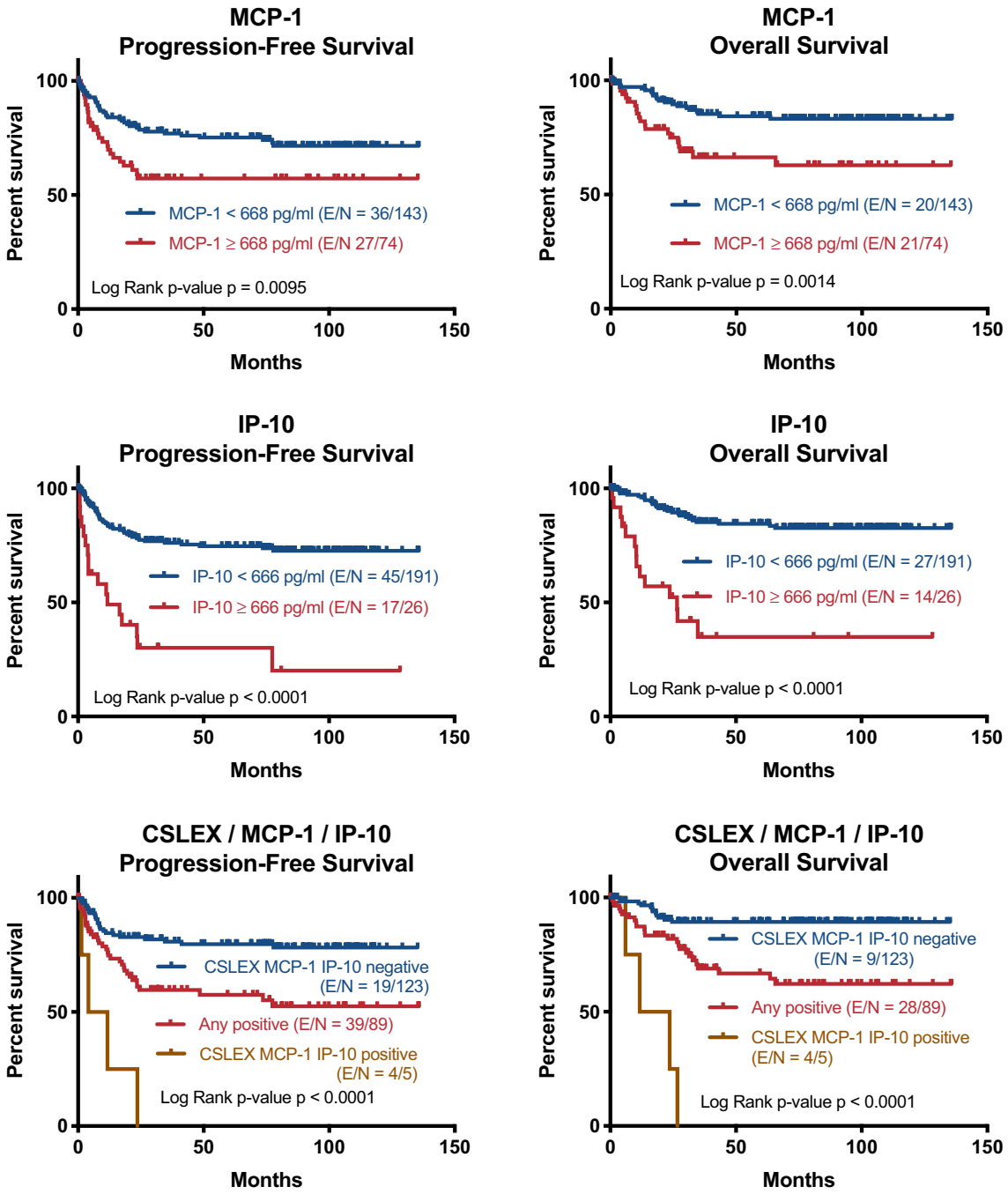

Fig. 4 Serum MCP-1 and IP-10 levels are predictive of PFS and OS in breast cancer. Top: patients with a serum MCP-1 level above the 95 percentile of serum level of HD (668 pg/mL) have a significantly shorter PFS ($P=0.0095$) and OS ($P=0.0014$), respectively. MCP-1 high $n=74$, MCP-1 low $n=143$. Middle: patients with a serum IP-10 level above the 95 percentile of serum level of HD (666 pg/mL) have a significantly shorter PFS ($P<0.0001$) and OS ($P<0.0001$), respectively. IP-10 high $n=26$, IP-10 low $n=191$. Bottom: There is a significant survival advantage to low levels of sLe^X, MCP-1 and IP-10. All low $n=123$, any high $n=89$, all high $n=5$

inflammatory cytokines to breast cancer progression [27]. High levels of serum IL-1β correlated with disease recurrence in breast cancer patients [28], and patients with higher levels of serum IL-6 were found to be less responsive to chemo-endocrine treatment than those with lower levels of IL-6 [29]. In the current study, we found that there were positive correlations between the serum level of sLe^X and inflammatory cytokines (IL-1β, IL-1RA, and IL-2, sIL-2Ra, IFN-α) and chemokines (IL-8 (CXCL8), Fractalkine (CXCL1), MCP-3 (CCL7) and MIP-1β (CCL4)). Although

this study only measured serum levels with no quantification of tumor-stromal levels, it is possible that by working in concert sLe^X, inflammatory mediators may facilitate local invasion of breast tumor.

Two serum chemokines that did not significantly correlate with sLe^X, MCP-1 and IP-10, showed independent prognostic value. Chemokines play a major role in recruiting leukocytes to the site of the tumor microenvironment. Monocyte chemoattractant protein-1 (MCP-1), also known as CCL2, is a ligand for CCR2 and CCR4. MCP-1 is a key member involved in the migration and infiltration of monocyte/macrophages from the bloodstream into the tumor microenvironment and has been shown to have properties that promote tumor progression [30, 31] as well as to augment immune surveillance through recruitment of γδ T lymphocytes [32]. Others have found that MCP-1 expression was significantly associated with the number of tumor-associated macrophages and high expression levels of MCP-1 and VEGF were a significant indicator of early relapse [33]. In our study, sera of patients with DCIS, non-MBC, or MBC have significantly higher median levels of MCP-1, MCP-3, and MIP-1β than those of HD. Compared with DCIS or non-MBC patients, serum from MBC patients have a significantly higher level of MCP-1. Since breast tumor sites are enriched with inflammatory constituents and cytokines such as IL-1 and IFN-γ, potent stimulators of MCP-1, it is not surprising that serum level of MCP-1 is increased in MBC patients and further suggests a convergence of tumor-promoting effects at the tumor site.

Similar to the results with serum sLe^X, patients with elevated serum MCP-1 levels above the 95 percentile of serum level of HD have a significantly shorter OS ($P = 0.0014$) and PFS ($P = 0.0095$). This is consistent with previous reports that showed the expression of MCP-1 in extracts of primary tumors and infiltrating macrophages from patients with breast cancer was of a significant prognostic value for relapse-free survival [33] and was significantly correlated with high grade tumor [34] and suggests that serum evaluation can also be useful.

IP-10 (interferon-gamma inducible protein 10 kDa), also known as CXCL10, is an ELR negative CXC ligand for CXCR3 induced by IFN-γ and LPS in a variety of cells including monocytes, T cells, keratinocytes, endothelial cells and fibroblasts. Like most chemokines, it has pleiotropic effects targeting numerous inflammatory pathways including cell adhesion through induction of integrins, natural killer and T cell migration, and stimulation of monocytes, but does not attract neutrophils [35]. IP-10 (CXCL10) has been associated with osteolytic bone metastases [36] and although it inhibits both angiogenesis and bone marrow formation, high levels have been associated with advanced cancers [37] although high tumor levels have been positively associated with a doubling of overall survival in ovarian cancer [38]. In circulation, breast cancer patients have been found to have significantly higher CXCL10 than healthy controls [39].

For IP-10, we observed a small but significant hazard associated with overall survival and progression-free survival, both cases showing hazard ratios of non-transformed serum concentrations of 1.001. However, the range of IP-10 concentrations observed was quite large (44 pg/mL to 2474 pg/mL), so a one-unit change is relatively small. Therefore, the hazard associated with a change on the order of the difference observed between mean non-MBC IP-10 (302 pg/mL) and MBC IP-10 (558 pg/mL) would be more substantive 1.292 (1.001^{256}) and the hazard associated with a 1 mg/mL difference would be 2.7 or 2.8. For univariate analysis, we used the 95th percentile of healthy donors IP-10 serum levels at 666 pg/mL to establish a cutoff. This is slightly higher than most other publications but of the same order of magnitude. For example Toiyama used ROC analysis to establish a cut-off of 199 pg/mL in colorectal cancer [40] and Berres optimized survival analysis in hepatitis C with a cut-off of 220 pg/mL [41], whereas Piro used ROC analysis to set a cut-off of 2958 pg/mL in pancreatic cancer [42]. As such, IP-10 serum concentrations above 200–2000 pg/mL are consistently related to poor survival in the literature, consistent with data presented here.

Patients with inflammatory breast cancer made up a disproportionate share of the non-MBC patients in this study. These patients have inferior prognosis in primary [43] and metastatic [44] disease. Interestingly, a reduced tissue expression of sLe^x has been proposed as a possible feature of IBC [45]. This may skew some results but was part of the initial study design. The association between inflammatory breast cancer and inflammatory serum proteins will be evaluated in further studies.

In addition to an oversampling of IBC patients, other study limitations include small sample size and patient heterogeneity and the limitation to a single institution. About half of the samples from patients with metastatic disease were collected post chemotherapy, which may alter the inflammatory profile. Because of the heterogeneity in sample times, survival times were calculated from the time of sample collection rather initial diagnosis and maybe shorter and not directly comparable to outcomes in the literature. Due to the small sample size for subgroup analyses along with the highly skewed distributions for cytokine expression with a subset of very high values, we used non-parametric Mann–Whitney U test to test for differences in cytokine levels between groups. This can report significantly different distributions in two populations with the same median [46] as observed for IL-1β, IL-1RA, sIL-2Ra. Although each of these soluble factors all have medians of 0 for MBC and HD, there is a tail of MBC patient samples with very high levels. It is worth noting that although the levels are significantly higher in MBC samples, this represents a minority of the

population. Finally, recent studies have found that it may be possible to engineer IgG-based CSLEX ELISA assays that are less susceptible to the steric hindrance associated with the current IgM-based assays [47].

In conclusion, we found that serum sLe^X levels tended to increase with severity of stage in breast cancer. Moreover, our study reporting commensurate increases in the levels of circulating sLe^X and inflammatory mediators with severity of disease confirms previous reports that reported these factors separately [6, 9, 10, 33, 34, 39]. Both serum MCP-1, IP-10 and sLe^X may have prognostic value in breast cancer.

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Compliance with ethical standards

Conflict of interest MC has received research Grants from Nittobi Medical, Co. TM, IK and JK are employed by Nittobi Medical, Co. All other authors declare no conflicts of interest.

Ethical approval 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 project was approved by the Institutional Review Board of The University of Texas MD Anderson Cancer Center.

References

1. Orr FW, Wang HH, Lafrenie RM, Scherbarth S, Nance DM (2000) Interactions between cancer cells and the endothelium in metastasis. J Pathol 190(3):310–329. https://doi.org/10.1002/(SICI)1096-9896(200002)190:3%3c310:AID-PATH525%3e3.0.CO;2-P
2. Daniotti JL, Lardone RD, Vilcaes AA (2015) Dysregulated expression of glycolipids in tumor cells: from negative modulator of anti-tumor immunity to promising targets for developing therapeutic agents. Front Oncol 5:300. https://doi.org/10.3389/fonc.2015.00300
3. Kannagi R, Izawa M, Koike T, Miyazaki K, Kimura N (2004) Carbohydrate-mediated cell adhesion in cancer metastasis and angiogenesis. Cancer Sci 95(5):377–384
4. Zhang Z, Wuhrer M, Holst S (2018) Serum sialylation changes in cancer. Glycoconj J 35(2):139–160. https://doi.org/10.1007/s10719-018-9820-0
5. Ohyama C, Tsuboi S, Fukuda M (1999) Dual roles of sialyl Lewis X oligosaccharides in tumor metastasis and rejection by natural killer cells. EMBO J 18(6):1516–1525. https://doi.org/10.1093/emboj/18.6.1516
6. Matsuura N, Narita T, Mitsuoka C, Kimura N, Kannagi R, Imai T, Funahashi H, Takagi H (1997) Increased level of circulating adhesion molecules in the sera of breast cancer patients with distant metastases. Jpn J Clin Oncol 27(3):135–139
7. Fujita T, Murayama K, Hanamura T, Okada T, Ito T, Harada M, Komatsu A, Koyama H, Kanai T, Maeno K, Mochizuki Y, Hama Y, Ito K, Amano J, Fujimori M (2011) CSLEX (Sialyl Lewis X) is a useful tumor marker for monitoring of breast cancer patients. Jpn J Clin Oncol 41(3):394–399. https://doi.org/10.1093/jjco/hyq190
8. Jeschke U, Mylonas I, Shabani N, Kunert-Keil C, Schindlbeck C, Gerber B, Friese K (2005) Expression of sialyl lewis X, sialyl Lewis A, E-cadherin and cathepsin-D in human breast cancer: immunohistochemical analysis in mammary carcinoma in situ, invasive carcinomas and their lymph node metastasis. Anticancer Res 25(3A):1615–1622
9. Nakagoe T, Fukushima K, Itoyanagi N, Ikuta Y, Oka T, Nagayasu T, Ayabe H, Hara S, Ishikawa H, Minami H (2002) Expression of ABH/Lewis-related antigens as prognostic factors in patients with breast cancer. J Cancer Res Clin Oncol 128(5):257–264. https://doi.org/10.1007/s00432-002-0334-5
10. Kurebayashi J, Nomura T, Hirono M, Okubo S, Udagawa K, Shiiki S, Ikeda M, Nakashima K, Tanaka K, Sonoo H (2006) Combined measurement of serum sialyl Lewis X with serum CA15-3 in breast cancer patients. Jpn J Clin Oncol 36(3):150–153. https://doi.org/10.1093/jjco/hyi235
11. Julien S, Ivetic A, Grigoriadis A, QiZe D, Burford B, Sproviero D, Picco G, Gillett C, Papp SL, Schaffer L, Tutt A, Taylor-Papadimitriou J, Pinder SE, Burchell JM (2011) Selectin ligand sialyl-Lewis x antigen drives metastasis of hormone-dependent breast cancers. Can Res 71(24):7683–7693. https://doi.org/10.1158/0008-5472.CAN-11-1139
12. Silva Z, Tong Z, Cabral MG, Martins C, Castro R, Reis C, Trindade H, Konstantopoulos K, Videira PA (2011) Sialyl Lewisx-dependent binding of human monocyte-derived dendritic cells to selectins. Biochem Biophys Res Commun 409(3):459–464. https://doi.org/10.1016/j.bbrc.2011.05.026
13. Narita T, Kawakami-Kimura N, Matsuura N, Hosono J, Kannagi R (1995) Corticosteroids and medroxyprogesterone acetate inhibit the induction of E-selectin on the vascular endothelium by MDA-MB-231 breast cancer cells. Anticancer Res 15(6B):2523–2527
14. Barthel SR, Gavino JD, Descheny L, Dimitroff CJ (2007) Targeting selectins and selectin ligands in inflammation and cancer. Expert Opin Therap Targets 11(11):1473–1491. https://doi.org/10.1517/14728222.11.11.1473
15. Laubli H, Borsig L (2010) Selectins as mediators of lung metastasis. Cancer Microenviron 3(1):97–105. https://doi.org/10.1007/s12307-010-0043-6
16. Laubli H, Borsig L (2010) Selectins promote tumor metastasis. Semin Cancer Biol 20(3):169–177. https://doi.org/10.1016/j.semcancer.2010.04.005
17. Portela SV, Martin CV, Romay LM, Cuevas E, Martin EG, Briera AF (2011) sLea and sLex expression in colorectal cancer: implications for tumourigenesis and disease prognosis. Histol Histopathol 26(10):1305–1316
18. Iwata T, Nishiyama N, Nagano K, Izumi N, Tsukioka T, Chung K, Hanada S, Inoue K, Kaji M, Suehiro S (2012) Preoperative serum value of sialyl Lewis X predicts pathological nodal extension and survival in patients with surgically treated small cell lung cancer. J Surg Oncol 105(8):818–824. https://doi.org/10.1002/jso.23002
19. Ferrajoli A, Lee BN, Schlette EJ, O'Brien SM, Gao H, Wen S, Wierda WG, Estrov Z, Faderl S, Cohen EN, Li C, Reuben JM, Keating MJ (2008) Lenalidomide induces complete and partial remissions in patients with relapsed and refractory chronic lymphocytic leukemia. Blood 111(11):5291–5297. https://doi.org/10.1182/blood-2007-12-130120

20. Sozzani P, Arisio R, Porpiglia M, Benedetto C (2008) Is Sialyl Lewis x antigen expression a prognostic factor in patients with breast cancer? Int J Surg Pathol 16(4):365–374. https://doi.org/10.1177/1066896908324668
21. Culig Z (2011) Cytokine disbalance in common human cancers. Biochim Biophys Acta 1813(2):308–314. https://doi.org/10.1016/j.bbamcr.2010.12.010
22. Walzog B, Gaehtgens P (2000) Adhesion molecules: the path to a new understanding of acute inflammation. News Physiol Sci 15:107–113
23. Knupfer H, Preiss R (2007) Significance of interleukin-6 (IL-6) in breast cancer (review). Breast Cancer Res Treat 102(2):129–135. https://doi.org/10.1007/s10549-006-9328-3
24. Salgado R, Junius S, Benoy I, Van Dam P, Vermeulen P, Van Marck E, Huget P, Dirix LY (2003) Circulating interleukin-6 predicts survival in patients with metastatic breast cancer. Int J Cancer 103(5):642–646. https://doi.org/10.1002/ijc.10833
25. Benoy IH, Salgado R, Van Dam P, Geboers K, Van Marck E, Scharpe S, Vermeulen PB, Dirix LY (2004) Increased serum interleukin-8 in patients with early and metastatic breast cancer correlates with early dissemination and survival. Clin Cancer Res 10(21):7157–7162. https://doi.org/10.1158/1078-0432.CCR-04-0812
26. Singh JK, Simoes BM, Howell SJ, Farnie G, Clarke RB (2013) Recent advances reveal IL-8 signaling as a potential key to targeting breast cancer stem cells. Breast Cancer Res 15(4):210. https://doi.org/10.1186/bcr3436
27. Goldberg JE, Schwertfeger KL (2010) Proinflammatory cytokines in breast cancer: mechanisms of action and potential targets for therapeutics. Curr Drug Targets 11(9):1133–1146
28. Nicolini A, Carpi A, Rossi G (2006) Cytokines in breast cancer. Cytokine Growth Factor Rev 17(5):325–337. https://doi.org/10.1016/j.cytogfr.2006.07.002
29. Zhang GJ, Adachi I (1999) Serum interleukin-6 levels correlate to tumor progression and prognosis in metastatic breast carcinoma. Anticancer Res 19(2B):1427–1432
30. Soria G, Yaal-Hahoshen N, Azenshtein E, Shina S, Leider-Trejo L, Ryvo L, Cohen-Hillel E, Shtabsky A, Ehrlich M, Meshel T, Keydar I, Ben-Baruch A (2008) Concomitant expression of the chemokines RANTES and MCP-1 in human breast cancer: a basis for tumor-promoting interactions. Cytokine 44(1):191–200. https://doi.org/10.1016/j.cyto.2008.08.002
31. Soria G, Ben-Baruch A (2008) The inflammatory chemokines CCL2 and CCL5 in breast cancer. Cancer Lett 267(2):271–285. https://doi.org/10.1016/j.canlet.2008.03.018
32. Lança T, Costa MF, Gonçalves-Sousa N, Rei M, Grosso AR, Penido C, Silva-Santos B (2013) Protective role of the inflammatory CCR32/CCL2 chemokine pathway through recruitment of type 1 cytotoxic γδ T lymphocytes to tumor beds. J Immunol 190(12):6673–6680. https://doi.org/10.4049/jimmunol.1300434
33. Ueno T, Toi M, Saji H, Muta M, Bando H, Kuroi K, Koike M, Inadera H, Matsushima K (2000) Significance of macrophage chemoattractant protein-1 in macrophage recruitment, angiogenesis, and survival in human breast cancer. Clin Cancer Res 6(8):3282–3289
34. Chavey C, Bibeau F, Gourgou-Bourgade S, Burlinchon S, Boissiere F, Laune D, Roques S, Lazennec G (2007) Oestrogen receptor negative breast cancers exhibit high cytokine content. Breast Cancer Res 9(1):R15. https://doi.org/10.1186/bcr1648
35. Tokunaga R, Zhang W, Naseem M, Puccini A, Berger MD, Soni S, McSkane M, Baba H, Lenz HJ (2018) CXCL9, CXCL10, CXCL11/CXCR35 axis for immune activation—A target for novel cancer therapy. Cancer Treat Rev 63:40–47. https://doi.org/10.1016/j.ctrv.2017.11.007
36. Lee JH, Kim HN, Kim KO, Jin WJ, Lee S, Kim HH, Ha H, Lee ZH (2012) CXCL10 promotes osteolytic bone metastasis by enhancing cancer outgrowth and osteoclastogenesis. Can Res 72(13):3175–3186. https://doi.org/10.1158/0008-5472.CAN-12-0481
37. Liu M, Guo S, Stiles JK (2011) The emerging role of CXCL10 in cancer (Review). Oncol Lett 2(4):583–589. https://doi.org/10.3892/ol.2011.300
38. Bronger H, Singer J, Windmuller C, Reuning U, Zech D, Delbridge C, Dorn J, Kiechle M, Schmalfeldt B, Schmitt M, Avril S (2016) CXCL9 and CXCL10 predict survival and are regulated by cyclooxygenase inhibition in advanced serous ovarian cancer. Br J Cancer 115(5):553–563. https://doi.org/10.1038/bjc.2016.172
39. Jafarzadeh A, Fooladseresht H, Nemati M, Assadollahi Z, Sheikhi A, Ghaderi A (2016) Higher circulating levels of chemokine CXCL10 in patients with breast cancer: evaluation of the influences of tumor stage and chemokine gene polymorphism. Cancer Biomark 16(4):545–554. https://doi.org/10.3233/CBM-160596
40. Toiyama Y, Fujikawa H, Kawamura M, Matsushita K, Saigusa S, Tanaka K, Inoue Y, Uchida K, Mohri Y, Kusunoki M (2012) Evaluation of CXCL10 as a novel serum marker for predicting liver metastasis and prognosis in colorectal cancer. Int J Oncol 40(2):560–566. https://doi.org/10.3892/ijo.2011.1247
41. Berres ML, Trautwein C, Schmeding M, Eurich D, Tacke F, Bahra M, Neuhaus P, Neumann UP, Wasmuth HE (2011) Serum chemokine CXC ligand 10 (CXCL10) predicts fibrosis progression after liver transplantation for hepatitis C infection. Hepatology 53(2):596–603. https://doi.org/10.1002/hep.24098
42. Piro G, Simionato F, Carbone C, Frizziero M, Malleo G, Zanini S, Casolino R, Santoro R, Mina MM, Zecchetto C, Merz V, Scarpa A, Bassi C, Tortora G, Melisi D (2017) A circulating TH2 cytokines profile predicts survival in patients with resectable pancreatic adenocarcinoma. Oncoimmunology 6(9):e1322242. https://doi.org/10.1080/2162402X.2017.1322242
43. Dawood S, Ueno NT, Valero V, Woodward WA, Buchholz TA, Hortobagyi GN, Gonzalez-Angulo AM, Cristofanilli M (2012) Identifying factors that impact survival among women with inflammatory breast cancer. Ann Oncol 23(4):870–875. https://doi.org/10.1093/annonc/mdr319
44. Fouad TM, Barrera AMG, Reuben JM, Lucci A, Woodward WA, Stauder MC, Lim B, DeSnyder SM, Arun B, Gildy B, Valero V, Hortobagyi GN, Ueno NT (2017) Inflammatory breast cancer: a proposed conceptual shift in the UICC-AJCC TNM staging system. Lancet Oncol 18(4):e228–e232. https://doi.org/10.1016/S1470-2045(17)30192-4
45. Alpaugh ML, Tomlinson JS, Ye Y, Barsky SH (2002) Relationship of sialyl-Lewis(x/a) underexpression and E-cadherin overexpression in the lymphovascular embolus of inflammatory breast carcinoma. Am J Pathol 161(2):619–628. https://doi.org/10.1016/S0002-9440(10)64217-4
46. Conroy RM (2012) What hypotheses do "nonparametric" two-group tests actually test? Stata J 12(2):182–190. https://doi.org/10.1177/1536867x1201200202
47. Yamashita J, Kobayashi I, Tatematsu K, Sezutsu H, Noda K, Ishihara H (2016) Sandwich elisa using a mouse/human chimeric CSLEX-1 antibody. Clin Chem 62(11):1516–1523. https://doi.org/10.1373/clinchem.2016.260968

Affiliations

Evan N. Cohen[1,2] · Tamer M. Fouad[1,3,6] · Bang-Ning Lee[1,2,7] · Banu K. Arun[3] · Diane Liu[5] · Sanda Tin[1,2] · Angelica M. Gutierrez Barrera[3] · Toshihide Miura[10] · Iwao Kiyokawa[10] · Jun Yamashita[10] · Ricardo H. Alvarez[1,3,8] · Vicente Valero[3] · Wendy A. Woodward[1,4] · Yu Shen[5] · Naoto T. Ueno[1,3] · Massimo Cristofanilli[1,3,9] · James M. Reuben[1,2]

1 Morgan Welch Inflammatory Breast Cancer Research Program and Clinic, The University of Texas MD Anderson Cancer Center, 1220 Holcombe Blvd, Houston, TX 77030, USA

2 Department of Hematopathology, The University of Texas MD Anderson Cancer Center, 1515 Holcombe Blvd, Houston, TX 77030, USA

3 Department of Breast Medical Oncology, The University of Texas MD Anderson Cancer Center, 1515 Holcombe, Unit 3552, Houston, TX 77030, USA

4 Department of Radiation Oncology, The University of Texas MD Anderson Cancer Center, 1515 Holcombe, Unit 76468, Houston, TX 77030, USA

5 Department of Biostatistics, The University of Texas MD Anderson Cancer Center, 1400 Pressler Street, Houston, TX 77030, USA

6 Department of Medical Oncology, The National Cancer Institute, Cairo University, Kasr El-Aini Road, Cairo 11796, Egypt

7 Present Address: Michael E. DeBakey Veterans Affairs Medical Center, Conroe, TX, USA

8 Present Address: Cancer Treatment Centers of America, Newnan, GA, USA

9 Present Address: Feinberg School of Medicine, Northwestern Univeristy, Chicago, IL, USA

10 Nittobo Medical Co., Ltd., 1, Shiojima Fukuhara, Fukuyama, Koriyama, Fukushima, Japan