

Cytokines as prognostic tool in breast carcinoma

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1. ABSTRACT

Serum cytokines are promising biomarkers of cancer staging and outcome prediction, including response to treatment. Serum samples were collected from 200 breast carcinoma patients prior to chemotherapy treatment. Luminex liquid protein chip technology was used to analyze 25 cytokines in serum. Linear regression was used to analyze the relationship of cytokine levels and tumor size. The independent sample T-test and Chi-square test methods were used to analyze the difference of cytokine levels between two groups. IL-12p40, sIL-2R, MMP-2 levels showed linear correlation with tumor size. Eotaxin, IL-10, IL-12p70, IL-7, IL-1ra, IP-10, MCP-1beta, MP-2 and MIP-1beta levels showed significant difference between different lymph node groups, but only Eotaxin, IP-10 and MCP-1 levels had an inverse correlation with the number of positive nodes. Fractalkine, G-CSF, MIP-1alpha, MIP-1beta levels showed significant differences between different ER+ groups. Eotaxin, Fractalkine, IL-6, IL-7, IL-10, MCP-1 and VEGF levels had significant differences between different HER-2 groups. Our study resulted in the identification of a serum cytokine profile with the potential to be clinically applicable to predict disease outcome and in monitoring of efficacy of treatment.

2. INTRODUCTION

The incidence of breast cancer has increased over the past few decades, presumably due to more sensitive diagnostic technologies and increased environmental and physiological stresses experienced in modern life. Prevention and early detection remain the two best strategies towards winning the battle with breast cancer. Currently available methods to detect early stage carcinoma are acceptable, but improved methods to detect even earlier stages will significantly benefit patient prognosis and survival. Upon detection of a breast tumor, the clinical analysis moves towards staging to determine lymph node (LN) involvement, ER and HER-2 status and whether distant metastasis events have occurred.

It is known to all doctors that tumor size has a meaningful sense to patients' prognosis. As tumor size increased, survival decrease regardless lymph node status. But clinical estimation of tumor size is not accurate enough for prognosis prediction, with clinical-pathological agreement is only 54% of cases (1). Even pathologic tumor size can vary because of different assessed ways and the definition of pathologic size, especially for minimal invasive carcinoma (2). There is a discrepancy between microscopic tumor size and macroscopic tumor size (3). So

we need a method that can objectively reflect tumor size or at least help to detect tumor size more accurately.

Lymph node statue is another essential prognostic factor. The 5-year overall survival is reduced by up to 40% in LN+ patients, as compared with their LN- counterparts (4). In addition, an inverse, and nearly linear, relationship exists between tumor burden of LN and breast cancer survival (5). Patients presenting with a higher amount of positive lymph nodes are not only associated with poor overall survival rates but also experience increased incidence of local regional recurrence and distant metastasis (6); therefore, these patients are targeted for more aggressive treatment (7). It is possible that early breast cancer may have a component of regional lymph node metastasis. The value of this evidence-based TNM N staging system has been supported by surveillance, epidemiology and patient outcomes showing that five year survival rates are inversely related to the number of positive nodes, regardless of tumor size (8). In practice, the number of LN+ after axillary node dissection can be quite variable based upon the surgeon's technique, pathologist's expertise and individual anatomy (9, 10). In fact, 13% of the variation in LN yield has been attributed to institution-, provider-, patient-, and tumor-related factors, with the remaining 87% accounted for by inherent biological or the other differences between patients (10). How many axillary lymph nodes should be removed and examined is also disputing, since under-staging is directly associated with under-treatment. Increasing evidence has emerged in the literature suggesting that the lymph node ratio (LNR; defined as the total number of positive lymph nodes with respect to the total number of lymph nodes removed or examined) represents a useful tool to accurately stage solid malignancies, including breast tumors (9, 11). LNR has proven useful in identifying additional subsets of patients within the standard TMN N classification groupings, but it is still not perfect. When the total lymph node involvement is less than 3, the contribution of TMN N classification decreases, and when the LNR is zero, micro-metastasis cannot be fully ruled-out. So is there a method that can objectively reflect lymph node statue or at least help to detect lymph node more accurately?

There have same question in ER and HER-2 detecting. Both the two factors have import meanings for breast cancer patient prognosis and treatment. But different detecting methods can lead different results, and different pathologic doctors may lead different results too. And how can we evaluate the curative effect of endocrine therapy or Trastuzumab therapy, when tumor has been removed?

Recently, comprehensive proteomic analysis has garnered attention in field of cancer research. Compared to genomic and transcriptomic focused analysis, proteomic features appear to be more realistic platforms for identification of cancer-related alteration in molecules and signaling pathways, and could therefore significantly contribute to our understanding of cancerogenic developments. Stage- and tissue-specific proteomic profiles have already proven capable of monitoring dynamic changes in patients; hence they can be used clinically to

follow disease course and response to drug therapy. In addition, the broad temporal and spatial range of protein abundance in proximal body fluids and serum/blood plasma provide a rich source for biomarker identification (12). Given that cytokines and chemokines are serum proteins and often associated with the presence and development of tumors, current research has focused on the use of cytokine concentrations to develop new strategies for diagnosis and/or monitoring of cancer development and treatment (13, 14). This method is speculated to also be useful for monitoring the interaction between the immune system and the tumor. But now most studies only focused on one or two cytokines.

In this study, we employed Luminex multiplex technology to assess the levels of 25 different cytokines simultaneously in breast cancer patients, in an attempt to discover the relationship among change in specific cytokine concentration and tumor size, lymph node metastasis, ER statue, and HER-2 statue. Ultimately, we aimed to identify one or more tumor size and lymph node metastasis biomarker (s) that would be applicable to an improved clinical strategy for accurate staging and to readily identify those breast cancer patients at high risk of lymph node metastasis, even when lymph node status was negative by standard pathology. In addition, we evaluated the differences in the serum cytokine profile between patients with ER+ and ER-, HER-2 over-expression (++, +++) and non-expression (-, +), in order to identify ER and Her-2 related biomarker (s).

3. MATERIALS AND METHODS

3.1. Patient characteristics

Serum samples were collected from 200 pathologically confirmed breast patients from Affiliated 10th Hospital of Tongji University prior to any type of surgical or medical intervention being initiated. All the patients were adult females, and consisted of 100 LN (+) patients and 100 LN (-) patients. Patients were excluded from further analysis based on axillary's lymph node biopsy, primary tumor having broken, chest wall being offended, presence of multicentre tumor or other tissue tumor, breast augmentation or prosthetic implants. All patients signed an informed consent and ethical approval was obtained from Affiliated 10th Hospital of Tongji University ethics committee.

3.2. Collection and storage of blood serum

Peripheral blood was drawn from patients prior to any type of surgical or medical intervention. Milliliter amounts of peripheral blood were drawn using standard phlebotomy procedures, and collected without anticoagulant. Sera were separated by centrifugation at 3000 g for 10 minutes, immediately aliquoted, snap-frozen, and stored at -80°C until further use. No more than two freeze-thaw cycles were allowed for each sample.

3.3. Serum cytokine analysis by Luminex technology

We used the Luminex-200™ system (Luminex Corp., Austin, TX, USA) to evaluate the serum sample from 200 breast cancer patients (100 LN+ and 100 LN-).

Table 1. The relationship between cytokine levels and tumor size

Cytokines	Points Number	r	p
MMP-2	159	-0.46	0.00
VEGF	156	0.02	0.78
MCP-1	200	-0.08	0.21
EGF	172	0.01	0.88
Eotaxin	124	-0.03	0.77
Fractalkine	26	-0.37	0.06
G-CSF	52	-0.08	0.54
RANTES	143	0.16	0.06
IL-1ra	52	0.04	0.77
IL-1a	16	-0.16	0.54
IL-2	6	-0.50	0.31
sIL-2R	102	-0.40	0.00
IL-4	18	-0.27	0.28
IL-6	14	0.07	0.62
IL-7	18	-0.22	0.37
IL-8	32	0.31	0.08
IL-10	80	-0.13	0.26
IL-12p40	56	0.32	0.02
IL-12p70	20	0.17	0.46
IP-10	200	-0.02	0.76
MIP-1alpha	54	0.00	0.99
MIP-1beta	160	-0.09	0.24
sCD40L	150	0.01	0.22
TGF-alpha	14	0.05	0.85
sTNFR	144	0.03	0.69

Levels of 25 cytokines were assessed, including: EGF, Eotaxin, Fractalkine, G-CSF, IL-10, IL-12p40, IL-12p70, RANTES, IL-1alpha, IL-1ra, IL-2, IL-4, sIL-2R, IL-6, IL-7, IL-8, IP-10, MCP-1, MIP-1beta, sCD40L, TGF-alpha, VEGF, sTNFR, MMP-2. Assays for each of the 25 cytokines were all purchased from Linco Research Corp (St. Charles, MO, USA). Briefly, 25 L of the sample used to generate the standard curve, the pre-diluted control samples, and patient samples were dispensed into the wells. An equal volume of mixed micro-beads was added into each well. The plate was incubated with shaking at room temperature for one hour, washed and re-incubated with another 25 L of detection antibody for 30 min. Following another wash, the samples were re-incubated with 25 L of Streptavidin-Phycoerythrin for 30 min; the plated was then washed twice and the samples were re-suspended with 100 L of PBST and cytokine levels were detected by using the Luminex-200™. Cytokine levels are expressed as median fluorescent intensity (MFI). The concentrations of analysis were quantified from MFI using standard curves generated by Bio-Rad five-parameter curve fitting to the series of known concentrations for each analyze.

3.4. Statistical analysis

Linear regression was used to analyze the relationship of cytokine levels and tumor size. Chi-square test was used to compare the positive ratio, while the Independent-Sample test was used to compare the mean serum cytokines levels among LN+ group verse LN- group, ER+ group verse ER- group, HER-2 over-expression (++, +++) group verse HER-2 non-or low-expression (-, +) group. One-Way Analysis of Variance (ANOVA) was used to compare the mean serum levels among multiple groups. All the statistic procedures were carried out with SPSS 17.0 software (Chicago, IL, USA). A *p*-value of ≤ 0.05 was considered significant.

4. RESULTS

4.1. The relationship between cytokine levels and tumor size

Among all 25 cytokines, IL-12p40 level showed linear correlation with tumor size (table1, Figure 1), and sIL-2R, MMP-2 levels showed negative linear correlation (Table 1, Figure 2, 3). Some other cytokines also had high *r* value, but the number of points is too low and/or the slopes were not significantly different from zero, so we did not think them has linear correlation with tumor size.

4.2. Serum cytokine levels in LN (+) patients versus LN (-) patients

Since some samples may be composed of cytokine levels that are lower than the minimum the system can test, we relied on positive ratio to reflect their status in peripheral blood. Most cytokines' positive ratio in the LN+ group was lower than that of the LN- group, and the differences of Eotaxin, IL-10, IL-12p70, IL-1ra, IL-7 and MIP-1beta had statistically significant (Table 1). At the same time, we identified five specific cytokines (IL-7, IP-10, MCP-1, MIP-1beta and MMP-2) with statistically significant decreases in the LN + group (Table 2, Figure 4). Fractalkine, IL-6, IL-7, MIP-1, sCD40L, TGF-alpha and VEGF also had an observable decrease in the LN+ group, but the differences did not reach statistical significance.

4.3. Serum cytokine levels in different LN+ groups

According to the results above, we selected nine cytokines (Eotaxin, IL-10, IL-12p70, IL-7, MCP-1, IP-10, MIP-1beta, IL-1ra and MMP-2) that had statistically significant differences between LN+ and LN- patients. Based on the number of positive lymph nodes, the 100 LN+ patients were further sub-divided into the following three groups according to numbers of positive lymph nodes: LN+ 1-3, LN+ 4-9 and LN+ ≥ 10 . Hence, we were able to analyze the relationship between cytokine levels and positive lymph node numbers. Table 3 and Figure 5, 6, 7 illustrated the statistically significant decreasing trend of Eotaxin, MCP-1 and IP-10 levels in patients who had more positive lymph nodes. There was a similar tendency observed for MMP-2, MIP-1beta and IL-12p70, and a reverse tendency showed by IL-1ra, IL-7 and IL-10, but the differences were not statistically significant. So finally, we selected Eotaxin, MCP-1 and IP-10 as lymph node metastasis biomarkers.

4.4. Serum cytokine levels in different ER groups

For the same reason, we selected independent sample T-test and Chi-square test methods to analysis the difference between ER- group and ER+ group (Table 4 and Figure 8). In all 25 cytokines, we found that most cytokine positive ratio or concentrations showed no significant difference in ER- group and ER+ group. But Fractalkine positive ratio significant increased in ER+ group than ER- group, while G-CSF positive ratio showed inverse trends. MIP-1alpha levels significant decreased in ER+ group than ER- group and sCD40L levels significant increased in ER+ group ($p < 0.05$).

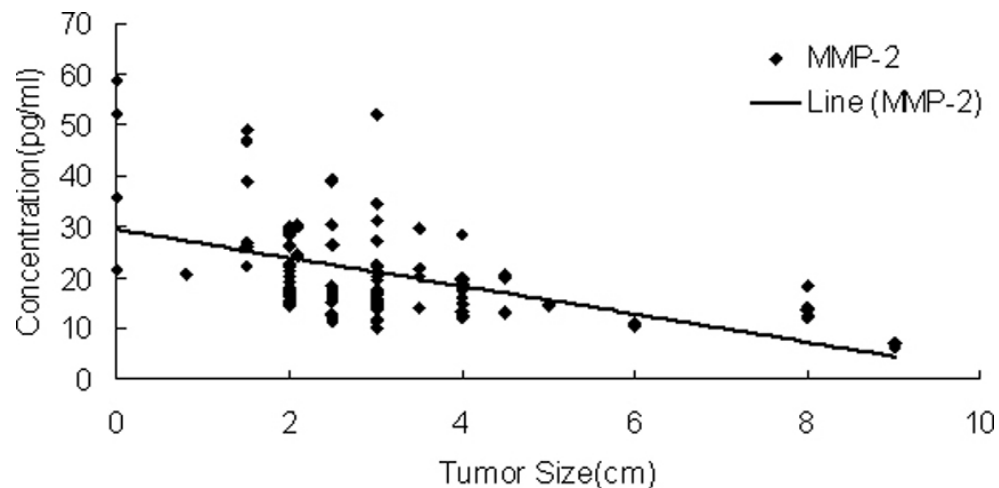


Figure 1. MMP-2 levels and tumor size (n=159), and MMP-2 levels showed negative linear correlation ($r=-0.46$, $p=0.00$).

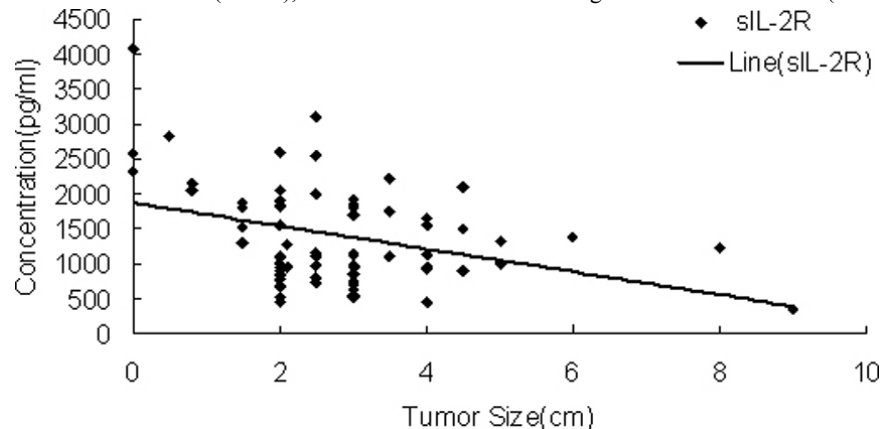


Figure 2. sIL-2R levels and tumor size (n=102), and sIL-2R, MMP-2 levels showed negative linear correlation ($r=-0.40$, $p=0.00$).

4.5. Serum cytokine levels in different HER-2 groups

The positive ratios of five cytokines (Eotaxin, Fractalkine, IL-6, IL-7 and IL-10) were found to significant different between two groups. VEGF level was decreased in HER-2 over-expression group as compared to that found in HER-2 non- or low-expression group, while MCP-1 level in HER-2 over-expression groups was increased, and they were all statistically significant (Table 5 and Figure 9).

5. DISCUSSION

Tumor size, Axillary lymph node status, ER and HER-2 status are important factors that are known to affect overall survival of breast cancer patients. Unfortunately, accurate staging of these factors' status, especially for the exact number of positive axillary lymph nodes, remain a clinical challenge. Although the TNM breast cancer staging system and LNR can provide considerable prognostic information, they are also subject to some limitations; in particular, when the total lymph nodes removed and evaluated after axillary node dissection is too few, the prognostic contribution weakens dramatically (15). Therefore, in this study we sought to identify some serum markers whose levels altered are capable of reflecting

patient tumor size, tumor burden of the lymph nodes, and ER, HER-2 status.

Here, we have shown that many cytokines levels changed could reflect patient tumor burden and receptors status. IL-12p40, sIL-2R, MMP-2 levels showed linear correlation with tumor size, when tumor size became bigger IL-12p40 levels would increase and sIL-2R, MMP-2 levels would decrease. LN+ patients present with decreased levels of pro-inflammatory mediators, including Eotaxin, IL-10, IL-12p70, IL-7, MCP-1, IP-10, MIP-1beta, IL-1ra and MMP-2 in serum, as compared to LN- patients. More importantly, three cytokines (Eotaxin, IP-10 and MCP-1) decreased even more dramatically when patients had higher numbers of positive lymph nodes. By analyzing the relationship between cytokine levels and ER statue, there were four cytokines related to. Fractalkine, G-CSF and MIP-1alpha in ER- group had higher levels than ER+ group, but sCD40L had opposite tendency. As referred to the relationship between HER-2 statue and cytokine level, we also discovered that many cytokine levels change could reflect HER-2 status, especially MCP-1 which had tight relationship with patient tumor burden in lymph nodes. Finally, we selected Eotaxin, IP-10 and MCP-1 as the most

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Table 2. Cytokine levels in LN+ patients and LN- patients

Cytokine	Group	Total number	Number positive	$\bar{X} \pm S$ (pg/ml)	χ	P	T	P
MMP-2	LN-	100	83	23.44 \pm 5.62	1.10	0.29	0.69	0.04
	LN+	100	76	19.93 \pm 6.35				
VEGF	LN-	100	78	104.71 \pm 62.15	0.00	1.00	1.33	0.18
	LN+	100	78	88.80 \pm 85.63				
MCP-1	LN-	100	700	179.75 \pm 59.42	0.00	1.00	3.00	0.00
	LN+	100	700	153.82 \pm 62.46				
EGF	LN-	100	86	134.92 \pm 81.07	0.00	1.00	0.67	0.50
	LN+	100	86	142.14 \pm 57.60				
Eotaxin	LN-	100	74	71.23 \pm 35.45	11.23	0.00	0.16	0.88
	LN+	100	50	72.32 \pm 42.62				
Fractalkine	LN-	100	14	266.70 \pm 374.32	0.04	0.83	1.57	0.13
	LN+	100	12	95.43 \pm 45.84				
G-CSF	LN-	100	28	129.80 \pm 11.77	0.23	0.63	0.28	0.78
	LN+	100	24	139.98 \pm 144.02				
RANTES	LN-	100	69	60.97 \pm 1.90	0.39	0.53	1.73	0.11
	LN+	100	74	53.15 \pm 5.32				
IL-1ra	LN-	100	36	91.07 \pm 121.21	9.38	0.00	1.51	0.14
	LN+	100	16	44.71 \pm 27.70				
IL-1a	LN-	100	8	75.27 \pm 81.07	0.00	1.00	1.51	0.14
	LN+	100	8	36.00 \pm 17.23				
IL-2	LN-	100	4	16.37 \pm 0.00	0.00	1.00	1.34	0.26
	LN+	100	2	13.85 \pm 0.00	0.17	0.68		
sIL-2R	LN-	100	48	1425.73 \pm 779.25	0.50	0.48	1.25	0.21
	LN+	100	54	1183.71 \pm 484.07				
IL-4	LN-	100	8	71.86 \pm 35.65	0.06	0.80	0.80	0.44
	LN+	100	10	405.94 \pm 116.25				
IL-6	LN-	100	10	39.95 \pm 22.89	1.92	0.17	0.33	0.75
	LN+	100	4	36.08 \pm 6.26				
IL-7	LN-	100	14	29.41 \pm 8.09	0.43	0.03	3.12	0.01
	LN+	100	4	16.33 \pm 2.80				
IL-8	LN-	100	16	23.70 \pm 5.24	0.00	1.00	0.29	0.77
	LN+	100	16	23.03 \pm 7.70				
IL-10	LN-	100	52	103.92 \pm 128.85	11.02	0.00	1.48	0.14
	LN+	100	28	150.49 \pm 143.541				
IL-12p40	LN-	100	24	455.06 \pm 370.23	1.53	0.22	1.00	0.32
	LN+	100	16	542.81 \pm 289.08				
IL-12p70	LN-	100	14	41.30 \pm 35.492	6.43	0.01	0.07	0.95
	LN+	100	3	40.17 \pm 33.91				
IP-10	LN-	100	100	223.56 \pm 119.62	0.00	1.00	3.90	0.01
	LN+	100	100	170.46 \pm 64.77				
MIP-1alpha	LN-	100	24	71.37 \pm 76.10	0.63	0.43	1.33	0.19
	LN+	100	30	47.58 \pm 55.29				
MIP-1beta	LN-	100	88	123.46 \pm 97.293	7.03	0.00	3.91	0.00
	LN+	100	72	75.30 \pm 41.97				
sCD40L	LN-	100	78	31565.57 \pm 12970.00	0.67	0.41	0.33	0.74
	LN+	100	72	32346.61 \pm 15898.00				
TGF-alpha	LN-	100	6	33.08 \pm 8.91	0.08	0.78	1.45	0.17
	LN+	100	8	24.47 \pm 12.30				
sTNFR	LN-	100	71	221.58 \pm 66.70	1.81	0.18	0.54	0.46
	LN+	100	61	207.73 \pm 33.49				

∴ no applicable

accurate and informative lymph node metastasis biomarkers. When the three cytokines serum concentrations were lower, breast cancer patients were experiencing a higher tumor burden in lymph nodes and, ultimately, had a worse prognosis. At same time we also found IL-12p40, sIL-2R, MMP-2 levels related to tumor size; Fractalkine, G-CSF, MIP-1alpha and sCD40L related to ER statue; and Eotaxin, Fractalkine, IL-6, IL-7, IL-10, VEGF and MCP-1 levels related to HER-2 statue. Taken together, these overall differences in serum cytokine profiles may be indicative of an endogenous immune response to the cancer state.

As early as 1983, Virchow hypothesized that there is functional relationship between inflammation and cancer (16). Today, the causal relationship between

inflammation, innate immunity and cancer is more widely accepted (17). The mediator and cellular effectors of inflammation are important constituents of the local environment of tumors. The inflammation that occurs in the tumor microenvironment can support proliferation and survival of malignant cells, promote angiogenesis and metastasis, subversion of the adaptive immune responses, and alter responses of hormones and chemotherapeutic agents (18). Thus, an increasing amount of research has focused on attempting to use cytokines in serum to improve methods of diagnosis and treatment and to monitor curative effect. Zai *et al.* employed Luminex technology to compare serum cytokine profiles of breast cancer patients to healthy controls, node-positive patients to node-negative, and pre- and post-vaccination serum of breast cancer patients vaccinated with a HER2/neu E75 peptide vaccine (19).

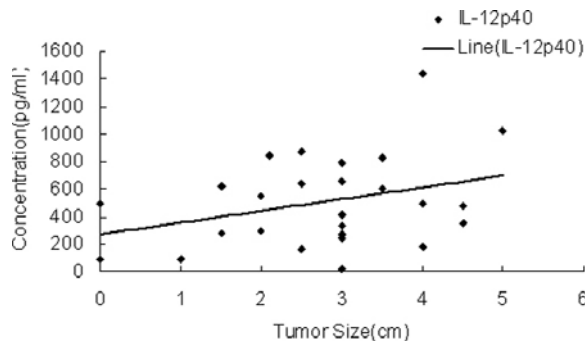


Figure 3. IL-12p40 levels and tumor size (n=56), and IL-12p40 level showed linear correlation with tumor size ($r=0.32$, $p=0.02$).

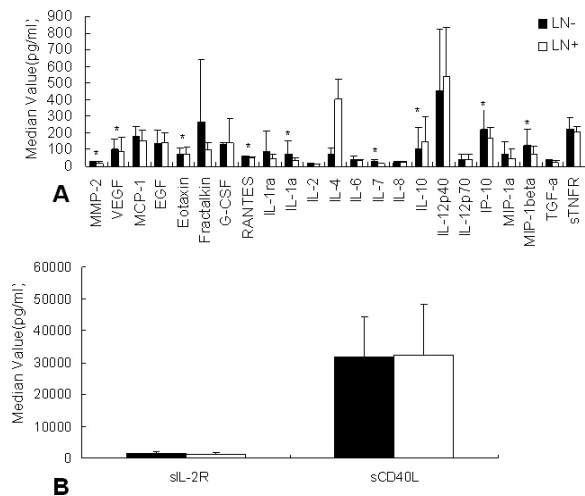


Figure 4. Cytokine levels in LN+ group (n=100) and LN- group (n=100). Results are expressed as median values in pg/ml.*indicates the difference of the positive value between the two groups was statistically significant ($p<0.05$).

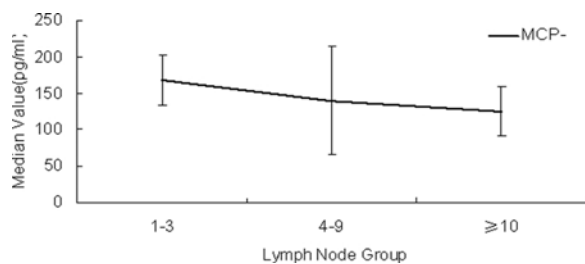


Figure 5. MCP-1 levels in different lymph node groups ($F=3.79$, $p=0.03$). Results were expressed as median values in pg/ml.

Their study found that, of all 22 cytokines examined, MCP-1, Eotaxin, RANTES and GM-CSF levels were significantly higher in breast cancer patients than in healthy controls ($p<0.009$) and IL-1 and IL-4 levels were significantly lower in breast cancer patients ($p<0.015$); IL-6, MIP-1beta and G-CSF levels were decreased in node-positive patients as opposed to node-negative patients

($p<0.05$); MCP-1, Eotaxin and IL-13 were significantly elevated in post-vaccination patients, with MCP-1 demonstrating the most robust response ($p=0.003$). It is important to note, however, that there are some critical differences between their study and ours. Zai *et al.* collected samples after patients completed a standard course of surgery, chemotherapy and/or radiation therapy. In contrast, when patients were enrolled in our study, no detectable tumor burden existed in their bodies, and no treatment had been yet initiated; we chose to target these patients for study since treatment is presumed to change cytokine levels in serum due to the fact that the immune status of patient has changed. On the other hand, Brain *et al.* assayed 55 serum biomarkers of locally advanced breast cancer patients by Luminex assay, and demonstrated that serum biomarker profiles may offer predictive power concerning treatment response and outcome in the neoadjuvant setting (20).

In our study, we first selected IL-12p40, sIL-2R, MMP-2 whose levels change could be used as tumor size biomarkers. As we known tumor growing will lead microenvironment changing, and the later will lead cytokine levels changing no matter in microenvironment and in serum. Some other studies analyzed the relationship of cytokines and tumor size (21, 22). But because the grouping methods and detection means different, the results were not unification, and most studies often focused on one or two cytokines. MMP-2 is proteolytic enzymes involved in the extracellular matrix turnover, and considered as possible tumor markers in breast cancer patients. In our study we found lower level of MMP-2 in serum correlated with bigger tumor size, the same as Paula *et al.* study (23). One thing must keep in mind that the tumor tissue expression of MMP-2 had an inverse correlation with proMMP-2–TIMP-2 complex levels in the serum, while many studies have shown that serum levels and activity of proMMP-2 in breast cancer patients show a positive association with TNM stage of the disease (24). Our results demonstrated again that cytokine levels change could be used to assess tumor size and further more may be used to evaluate the curative effect of neoadjuvant chemotherapy.

The relationship of cytokine levels and lymph node is the focal point in our study. We demonstrated that Eotaxin, MCP-1 and IP-10 can be used as lymph node metastasis markers, for them had highly significant association with tumor burden in lymph node. Other cytokines also had significant differences between LN- group and LN+ group, but they could not show further association with the number of positive lymph nodes, so we did not see them as lymph node metastasis biomarkers. MCP-1, as its full name of monocyte chemotactic protein-1 suggests, is a chemokine ligand that is associated with aggressive behavior in breast, papillary thyroid and prostate cancer (20, 25). Breast cancer cells, other stroma and immune cells can secrete MCP-1. In fact, several studies have demonstrated that MCP-1 and RANTES, and other cytokines, can interact to promote breast tumor cell metastasis properties (26). In addition, MCP-1 and RANTES are active participants in the tumor microenvironment, influencing factors such as tumor-

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Table 3. Cytokine levels in different LN+ groups (pg/ml, -X±S)

Cytokine	Group	Number	Positive Number	$\bar{X} \pm S$ pg/ml	F	P
Eotaxin	1-3	58	30	105.56±42.05	4.87	0.01
	4-9	14	10	71.52±17.68		
	>10	28	10	65.50±42.17		
IL-10	1-3	58	12	96.22±94.57	1.67	0.21
	4-9	14	6	171.15±241.1		
	>10	28	10	203.22±106.22		
IL-12p70	1-3	58	4	49.24±39.85	0.83	0.414
	4-9	14	0	0±0		
	>10	28	2	22.05±0		
IL-1ra	1-3	58	6	29.48±18.38	3.36	0.08
	4-9	14	0			
	>10	28	10	53.85±29.04		
IL-7	1-3	58	0	0±0		
	4-9	14	0	0±0		
	>10	28	4	16.33±2.80		
IP-10	1-3	58	58	185.16±57.33	3.37	0.02
	4-9	14	14	173.63±71.02		
	>10	28	28	127.92±23.27		
MCP-1	1-3	58	58	167.58±73.94	3.79	0.03
	4-9	14	14	139.51±33.94		
	>10	28	28	125.42±34.56		
	1-3	58	42	83.38±44.54	2.79	0.07
	4-9	14	12	75.79±9.75		
	>10	28	18	56.13±38.06		
	1-3	58	41	21.64±7.53	0.71	0.59
	4-9	14	14	20.12±6.89		
	>10	28	21	19.666.59		
MIP-1beta	1-3	58	42	83.38±44.54	2.79	0.07
	4-9	14	12	75.79±9.75		
	>10	28	18	56.13±38.06		
MMP-2	1-3	58	41	21.64±7.53	0.71	0.59
	4-9	14	14	20.12±6.89		
	>10	28	21	19.666.59		

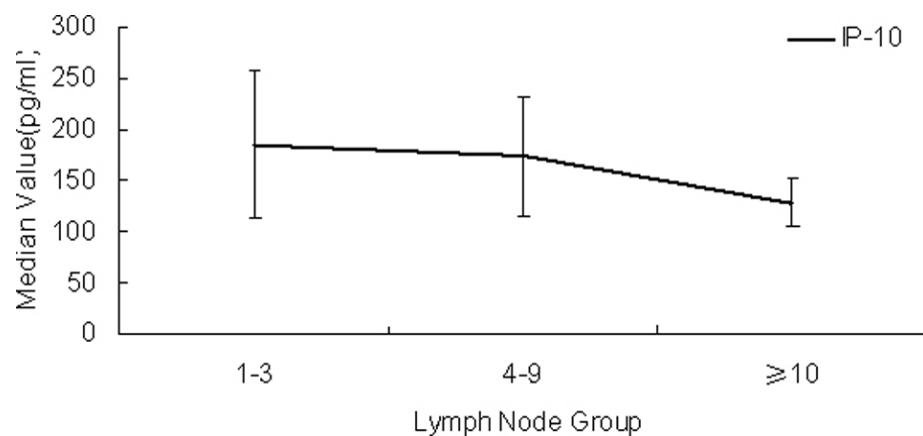


Figure 6. IP-10 levels in different lymph node groups (F=3.37, p=0.02) Results were expressed as median values in pg/ml.

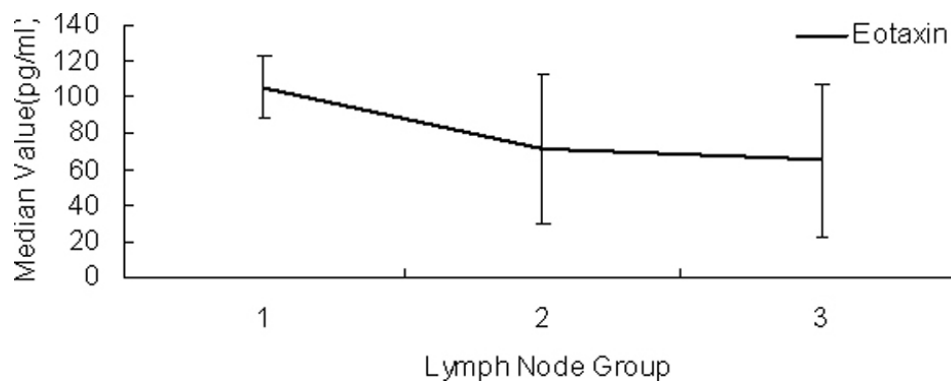


Figure 7. Eotaxin levels in different lymph node groups (F=4.87, p=0.01). Results were expressed as median values in pg/ml.

Table 4. Cytokine levels in ER- group and ER+ group (pg/ml, -X±S)

Cytokine	Group	Total number	Number positive	$\bar{X} \pm S$ (pg/ml)	Z	P	T	P
MMP-2	ER-	72	61	21.26±8.67	0.51	0.48	0.04	0.91
	ER+	122	98	21.53±8.53				
VEGF	ER-	72	58	83.84±64.27	0.15	0.69	1.87	0.06
	ER+	122	94	107.22±80.51				
MCP-1	ER-	72	72	162.08±57.94	1	1	0.92	0.36
	ER+	122	122	170.65±65.48				
EGF	ER-	72	66	138.40±60.26	2.71	0.10	0.06	0.95
	ER+	122	100	137.74±75.54				
Eotaxin	ER-	72	46	71.47±43.06	0.27	0.60	0.6	0.56
	ER+	122	72	75.64±33.34				
Fractalkine	ER-	72	0	0.00±0.00	15.93	0.00	1	1
	ER+	122	26	187.65±285.25				
G-CSF	ER-	72	20	62.89±35.80	0.10	0.75	3.27	0.00
	ER+	122	30	160.62±129.76				
RANTES	ER-	72	53	55.81±6.71	0.03	0.85	1.73	0.11
	ER+	122	87	61.07±9.62				
IL-1ra	ER-	72	16	118.56±154.60	0.88	0.35	1	1
	ER+	122	36	58.25±65.61				
IL-1alpha	ER-	72	8	33.51±12.14	0.71	0.40	1	1
	ER+	122	8	77.77±80.51				
IL-2	ER-	72	0	0.00±0.00	2.20	0.14	1	1
	ER+	122	6	15.53±1.30				
sIL-2R	ER-	72	31	1342.21±692.30	2.78	0.09	0.65	0.52
	ER+	122	69	1349.67±587.64				
IL-4	ER-	72	8	70.5±37.77	0.18	0.67	0.86	0.41
	ER+	122	10	107.02±115.36				
IL-6	ER-	72	4	25.66±7.91	0.16	0.69	1.73	0.11
	ER+	122	10	44.12±20.17				
IL-7	ER-	72	4	26.65±3.76	0.25	0.26	0.03	0.97
	ER+	122	14	26.46±10.29				
IL-8	ER-	72	8	26.03±9.90	1.83	0.18	1.36	0.18
	ER+	122	24	22.48±4.87				
IL-10	ER-	72	24	126.61±143.87	1.82	0.18	0.16	0.87
	ER+	122	54	121.26±133.41				
IL-12p40	ER-	72	26	506.25±384.78	2.39	0.12	0.02	0.98
	ER+	122	30	504.29±272.13				
IL-12p70	ER-	72	8	33.30±31.23	0.00	0.92	0.81	0.43
	ER+	122	13	46.07±36.33				
IP-10	ER-	72	72	187.92±91.94	1	1	1.2	0.23
	ER+	122	122	205.77±104.81				
MIP-1alpha	ER-	72	22	35.64±38.99	0.23	0.63	2.16	0.04
	ER+	122	32	73.63±75.91				
MIP-1beta	ER-	72	58	92.67±59.39	0.00	0.97	1.11	0.27
	ER+	122	98	107.69±92.00				
sCD40L	ER-	72	56	35854.11±13392.00	0.49	0.48	2.72	0.01
	ER+	122	88	29165.69±14927.00				
TGF-alpha	ER-	72	4	33.24±12.14	0.16	0.69	1.05	0.31
	ER+	122	10	26.13±11.17				
sTNFR	ER-	72	37	232.56±66.71	0.23	0.64	0.81	0.41
	ER+	122	95	210.99±27.77				

associated macrophage activity, angiogenesis, and metastasis (27). However, their tumor-promoting functions do not fully overlap, and each of the two cytokines “specializes” in unique pro-malignancy activities. For example, it is mainly MCP-1 that promotes angiogenic processes, leading to increased vascularity at the tumor site. On the other hand, RANTES is considered mainly as an invasion-promoting factor, since it was found to potently induce breast tumor cell migration, and to up-regulate the expression of matrix metalloproteinases (MMP) by the tumor cells and by cells of the tumor microenvironment (26). Breast cancer cells can express IP-10 (CXCL10) and its cognate receptor CXCR3 (28). Ras in breast cancer cells has been shown to promote IP-10 expression and down-regulate CXCR3, especially the CXCR3-B splice variant which results in promotion of tumor cell proliferation and invasion (29). Despite these studies of RANTES, MCP-1

and IP-10 in the tumor microenvironment, no consensus exists as to the cellular origin of the three found in serum. Moreover, the current belief is that both the immune system and the tumor microenvironment components may contribute to circulating levels. Our results suggest conformity to the above conclusion.

Our study also analyzed the relationship of estrogen receptor (ER) status and cytokine change in sera, and found four cytokines (Fractalkine, G-CSF, MIP-1alpha and sCD40L) has highly significant association. ER positive is an indication of hormone dependent and endocrine therapy (30), and lack of this receptor has consistently been associated with poorer prognosis (31). Several reports have demonstrated a direct down regulation of cytokines by ER in different organs (32-34), had screened 17 cytokines in breast cancer tissue and found that

Table 5. The levels of 25 cytokines measured in HER-2- [non- or low-expression (-, +)] patients and HER-2+ [over-expression (++, +++)] patients

Cytokine	Group	Number	Positive number	$\bar{X} \pm S$ (pg/ml)	Z	P	T	P
MMP-2	HER-2-	144	127	20.53±5.11	0.84	0.36	0.75	0.62
	HER-2+	46	32	21.67±6.32				
VEGF	HER-2-	144	112	103.49±78.70	0.00	0.94	2.11	0.04
	HER-2+	46	36	74.47±43.08				
MCP-1	HER-2-	144	144	158.81±51.74			2.11	0.04
	HER-2+	46	46	179.36±72.56				
EGF	HER-2-	144	126	140.68±70.74	1.69	0.19	0.57	0.57
	HER-2+	46	36	133.16±68.29				
Eotaxin	HER-2-	144	94	72.29±35.35	6.03	0.01	1.25	0.21
	HER-2+	46	20	83.85±47.62				
Fractalkine	HER-2-	144	18	91.73±100.63	0.92	0.00	30.18	0.00
	HER-2+	46	6	467.97±494.76				
G-CSF	HER-2-	144	30	114.760±70.05	2.97	0.08	0.84	0.41
	HER-2+	46	16	145.18±176.22				
RANTES	HER-2-	144	32	54.37±6.71	0.69	0.41	1.2	0.23
	HER-2+	46	111	57.60±3.21				
IL-1ra	HER-2-	144	32	63.37±68.35	4.31	0.04	1.43	0.16
	HER-2+	46	18	107.38±148.49				
IL-1a	HER-2-	144	10	70.54±72.62	0.98	0.32	1.31	0.21
	HER-2+	46	6	30.80±13.08				
IL-2	HER-2-	144	6	15.53±1.30	0.85	0.36		
	HER-2+	46	0	0.00±0.00				
sIL-2R	HER-2-	144	81	1258.04±423.67	0.29	0.59	1.18	0.28
	HER-2+	46	21	1342.21±708.55				
IL-4	HER-2-	144	14	87.57±101.78	0.04	0.83	0.28	0.78
	HER-2+	46	4	102.07±10.78				
IL-6	HER-2-	144	0	0.0±0.00	8.92	0.00		
	HER-2+	46	4	25.66±8.91				
IL-7	HER-2-	144	0	0.0±0.00	15.36	0.00	0.38	0.71
	HER-2+	46	6	24.01±5.01				
IL-8	HER-2-	144	22	24.56±7.30	0.01	0.91	1.11	0.28
	HER-2+	46	8	21.60±2.88				
IL-10	HER-2-	144	66	116.42±140.87	7.46	0.00	0.86	0.39
	HER-2+	46	10	156.62±111.42				
IL-12p40	HER-2-	144	42	498.82±350.36	0.03	0.87	0.21	0.83
	HER-2+	46	14	521.32±249.81				
IL-12p70	HER-2-	144	14	49.48±37.77	0.04	0.84	1.67	0.12
	HER-2+	46	4	17.35±3.03				
IP-10	HER-2-	144	144	191.72±90.58			1.47	0.14
	HER-2+	46	46	216.70±126.73				
MIP-1alpha	HER-2-	144	40	58.12±70.17	0.00	0.97	0.51	0.61
	HER-2+	46	12	47.04±49.39				
MIP-1beta	HER-2-	144	114	101.88±86.13	0.08	0.77	0.56	0.58
	HER-2+	46	38	93.33±65.08				
sCD40L	HER-2-	144	104	30445.98±14705.00	1.48	0.22	0.47	0.73
	HER-2+	46	38	30400.22±14899.00				
TGF-alpha	HER-2-	144	12	25.56±10.19	0.33	0.56		
	HER-2+	46	2	43.75±0.00				
sTNFR	HER-2-	144	86	177.68±52.06	2.26	0.13	0.37	0.72
	HER-2+	46	21	199.02±52.46				

multiple cytokines were over-expressed in ER negative breast carcinoma, at same time they confirmed that ER-tumor were generally of higher grade than ER+ ones. There are big difference between our results and theirs. The main reason may lay in the sample selection: ours are serum and theirs are tumor tissue. This proves again that cytokines in serum come from tumor cells and immunity cells.

In analysis relationship between HER-2 statue and cytokine levels, we found most cytokines levels increased in HER-2 over-expression group than HER-2 negative or low-expression group, and the differences of Eotaxin, Fractalkine, IL-6, IL-7 and IL-10 were significantly. But we only had 44 patients in HER-2 over-expression group and 144 patients in another group, so the result needs further confirmed. Even

though we demonstrated that HER-2 statue would alter cytokine levels in serum and might used as biomarkers to monitor Trastuzumab therapy. The results coincided with other reports (35, 36).

There are several limitations in this study that must keep in mind when interpreting our findings. First, our study was performed as a single-institution study. Second, we chose to not rely on “healthy” controls. Third, the median time of follow-up was only 2 months, so we did not analyze the relationship between cytokine levels and distant metastasis because of withstand long-term analysis. However, our cohort of 200 patients was a sufficiently large sample size, and lent power to our analysis and confidence to the results.

Cytokines related prognosis factor in breast carcinoma

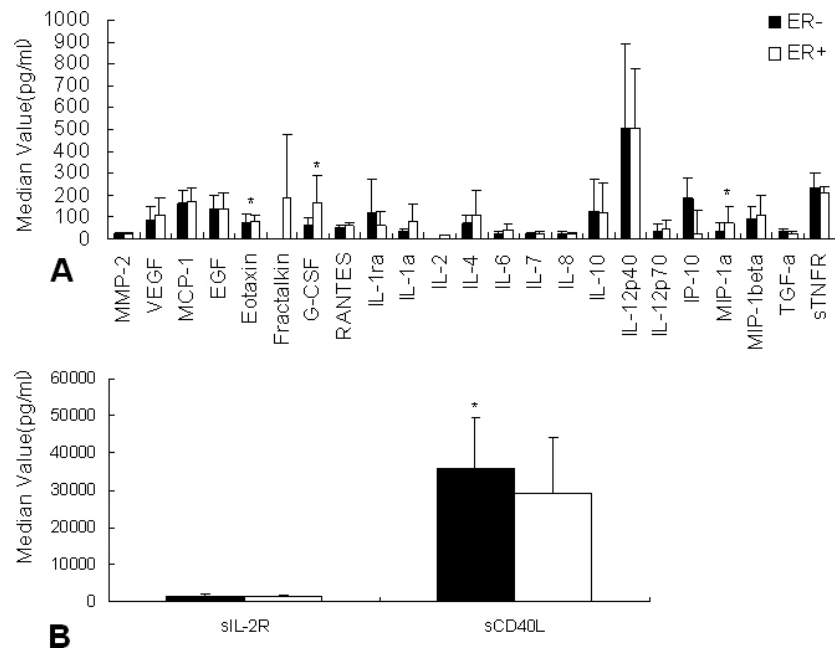


Figure 8. Cytokine levels in ER+ group (n=122) and LN- group (n=72). Results were expressed as median values in pg/ml.* indicates the difference of the positive ratio between the two groups was statistically significant (p<0.05).

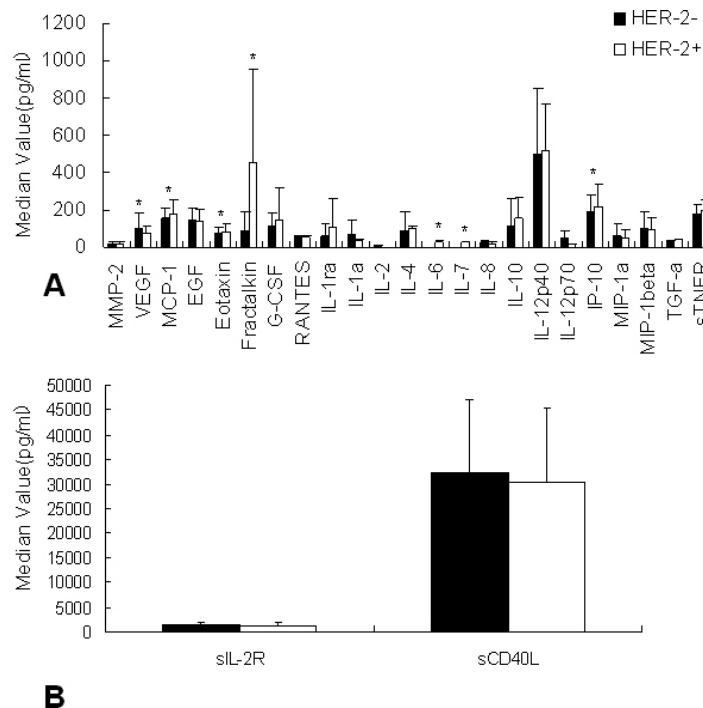


Figure 9. Levels of 25 cytokines in the HER-2+ [over-expression (++, +++)] group and HER-2- [non-or low-expression (-, +)] group. Results were expressed as median values in pg/ml. * indicates the difference of positive ratio among the two groups with statistical significance (P<0.05).

In summary, we have shown in a large patient cohort that cytokine levels are related with breast cancer patient clinical prognosis factors, especially for Eotaxin, MCP-1 and IP-10, which could be used as lymph node metastasis biomarker. Higher levels of the three cytokines

correlated with lower lymph node staging and good prognosis. In addition, our study confirmed that serum cytokines can be used to evaluate a patient's anti-tumor immunity state, improve diagnosis and tumor staging,

select individual's treatment and to monitor curative effects.

6. CONCLUSION

Our relatively large study demonstrated the usefulness of Luminex array approach in finding serum biomarkers that may be specific for breast cancer diagnosis and staging, through our short follow-up time limits the predictive power. As we increase our knowledge about these cytokine involved, we believe that serum cytokine will be used for early detecting, correctly staging, monitoring treatment and reasonable prognosis, finally leads to a more personalized approach to cancer treatment.

7. ACKNOWLEDGEMENT

Mingli Lv and Xiaoping Zhang are contributed equally to the manuscript. ML contributed to samples collection, Luminex assays, results interpretation and drafting the manuscript. HC, FY, DL and JW assisted the transfer of patient samples. XF, MS carried out the statistical analyze. ZL conceived the study and design. The authors declare that they have no competing interests.

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Abbreviations: IL: interleukin; EGF: epidermal growth factor; TFG-alpha: transforming growth factor alpha; INF-gamma: interferon -gamma; G-CSF: granulocyte colony-stimulating factor; TNF-alpha: tumor necrosis factor alpha; sCD40L:CD40 soluble ligand; MIP: monocyte inflammatory protein; IP-10: interferon induced protein-10; MCP-1: monocyte chemoattractant protein; VEGF: vascular endothelial cell growth factor.

Key Words: Cytokine, Breast Cancer, Lymph Node, ER, HER-2

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