European Review for Medical and Pharmacological Sciences 2013; 17: 596-608

Chemokines fluctuate in the progression of primary breast cancer

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Abstract. – BACKGROUND: Many studies have demonstrated that chemokines and their receptors play important roles in breast cancer. However, few of them focus on the concentration change of chemokines along breast cancer evolvement, especially for primary breast cancer.

PURPOSE OF THE STUDY: To investigate the effects of chemokines and their receptors on different stage of primary breast cancer, and to find correlationships between chemokines, between different clinico-pathological characters of patients or between chemokines and different clinico-pathological characters of patients.

PATIENTS AND METHODS: We evaluated and compared the concentration of 10 chemokines and receptors in serum of patients diagnosed as breast benign change, epithelial proliferation (present only or with atypia), in situ carcinoma and invasive carcinoma.

RESULTS: Our oneway ANOVA analysis results showed that in all cases from benign diseases to invasive carcinoma, the concentration of CXCL8, CXCR4 and CXCL12 was significantly different; in benign subgroups (benign change, benign change with proliferation, atypia), the concentration of CCL2 and CCR5 was significantly different; in invasive carcinoma cases, DARC concentration was significantly correlated with the relapse risk of patients.

CONCLUSIONS: The correlation analysis indicated the great crosstalk between chemokines and receptors in the course of primary breast cancer; Ki67 expression was associated with CXCL5 and CXCL7 concentration; tumor size was associated with CXCL8 concentration; and the correlation analysis between clinico-pathological characters of patients showed that pathological diagnosis was correlated with tumor size, relapse risk and Ki67 expression; nuclear grades was correlated with LN metastasis, ER status, PR status and the breast cancer genotype; LN metastasis was correlated with relapse risk. Our findings clearly indicated for the first time that the fluctuations of chemokines and receptors contributed to the evolving of primary breast cancer.

Key Words:
Chemokines, Breast cancer.

Introduction

Chemokines are initially described as regulators of leukocyte trafficking for their ability to stimulate migration of leukocytes during inflammatory processes. However, chemokines and their receptors work in many biological processes, such as embryogenesis, angiogenesis, hematopoiesis, atherosclerosis, and HIV-infection[1,2]. In tumor growth and metastasis, chemokines and their receptors have a multifaceted effects for regulating angiogenesis, tumor cell proliferation and apoptosis, mediating tumor cell metastasis in an organ-specific manner. There are currently four subgroups within the chemokine family: CXC, CC, CX3C, and C chemokine ligands (X represents any amino acid) depending on the positioning of the conserved cysteines in the aminoterminal part of these small inducible proteins[3]. Chemokines bind to their cognate receptors, most of which belong to G Protein-Coupled seven transmembrane Receptors (GPCR)[4], except for some atypical chemokine binders including DARC (Duffy antigen receptor for chemokines), D6, and CCX-CKR (Chemo Centrix-chemokine receptor).

DARC is a typical decoy receptor that binds with angiogenic ELR$^+$ CXC (glutamic acid leucine-arginine$^+$) CXC chemokines, as well as some CC (chemotactic cytokines) chemokines[5,6], but not with ELR$^-$ CXC chemokines and C chemokine[7]. It is reported that DARC plays a negative regulatory role in human breast cancer. Overexpression of DARC protein in breast cancer cells can result in significant inhibition of tumorigenesis and lung metastasis *in vivo*[8].

In our study, we examined by ELISA the serum concentration of 10 chemokines and receptors: CCL2 (MCP-1, MCAF, JE), CXCL5 (ENA-78), CXCL7 (NAP-2), CXCL8 (IL-8), CXCL12 (SDF-1), DARC, CCR2, CCR5, CCR7, CXCR4, which had been indicated to be related with breast carcinoma, to investigate the effects of these chemokines in the whole process of pri-

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mary breast cancer, and to find correlationships between chemokines, between different clinico-pathological characters of patients or between chemokines and different clinico-pathological characters of patients.

Patients and Methods

Samples

148 patients (26-80 years old) with breast mass (1.00-7.00 cm) were enrolled in this prospective study. Blood specimens were obtained between December 2010 and August 2012, from patients who underwent surgery at the Department of Breast Surgery, The International Peace Maternity and Child Health Hospital, Shanghai, China. Blood specimens were obtained during patients' admission examination, by vein puncture collected in sterile screw-capped bottles. The samples were allowed to clot at room temperature and the serum was then separated, labeled and stored in a deep freezer (–80ºC) until testing (classical method). In all 148 cases, 51 benign disease, 26 in situ carcinoma, 71 invasive breast cancer cases. Clinico-pathological data of these patients was summarized in Table I. The study protocol was approved by the Ethics Committee of University Hospital. All subjects gave written informed consent. All the samples were diagnosed by pathologists.

Table I. Clinico-pathological characteristics.

Characteristics (No of cases)		N	%	Mean ± std. error (min-max)
Age (148)				50.39 ± 6.42 (26-80)
Pathological diagnosis (148)				
Benign disease				
	Benign change	18	12.24	
	Benign change with proliferation atypia	17	11.56	
		16	10.20	
In situ carcinoma		26	17.69	
Invasive carcinoma		71	48.30	
Tumor size (127)				2.24 ± 1.59 (1.00-7.00) cm
Nuclear grades (76)				
	I	8	10.53	
	II	43	56.58	
	III	25	32.89	
LN metastasis (71)				1.78 ± 0.47 (0-26)
ER status (82)				
	–	24	29.27	
	+	16	19.51	
	2+	22	26.83	
	3+	20	24.39	
PR status (82)				
	–	33	40.24	
	+	18	21.95	
	2+	22	26.83	
	3+	9	10.98	
HER2 status (81)				
	–	22	27.16	
	+	35	43.21	
	2+	18	22.22	
	3+	6	7.41	
Relapse risk (68)				
	Low risk	5	7.35	
	Medium risk	46	67.65	
	High risk	17	25	
Genotype (63)				
	LA	33	52.38	
	LB	7	11.11	
	HER2	6	9.52	
	NL	6	9.52	
	BL	11	17.46	
Ki67 (68)				0.19 ± 0.03 (0.01-0.96)

ELISA Analysis

The concentration level of chemokines and receptors were determined by using a quantitative immunometric sandwich enzyme linked immunosorbent assay (ELISA) kit (Human immunoassay kit; BioSource International Inc., Camarillo, CA, USA). The OD of the test sample was compared with the standard curve.

Risk Categories

Patients of invasive carcinoma were stratified into 3 relapse risk groups based on the St. Gallen consensus recommendations published in 2007. The low-risk group included patients with node-negative, tumor size ≤ 2 cm, tumor grade 1, positive ER (estrogen receptor) and/or PR (progesterone receptor) expression, age ≥ 35 years, and peritumoral invasion negative; Patients were stratified into the intermediaterisk group if they possessed at least one of the following features: tumor size > 2 cm, tumor grade 2 or 3, HER2/neu (Human Epidermal Growth Factor Receptor 2) overexpression, peritumoral invasion positive or age < 35 years. High-risk group meant: node-positive 1-3+, and lack of ER and PgR expression or HER2 overexpression or amplification; Or node-positive 4+. HER2/neu staining with 3+ on IHC (Immunohistochemistry) or FISH (Fluorescence In Situ Hybridization) positive was considered positive for overexpression.

Statistical Analysis

Results were mean ± standard error of the mean (SEM) of three independent experiments. Oneway ANOVA analysis and Spearman correlation coefficient analysis were used. *p*-values were two-sided: < 0.05 was considered statistically significant.

Results

All the cases were divided into different groups by pathological diagnosis, pathological diagnosis in benign disease, pathological diagnosis in cancer, ER status, PR status, HER2 status, Ki67 expression, tumor size, nuclear grades, relapse risk, breast cancer genotype, LN metastasis. The mean concentrations of all chemokines and receptors fluctuated from benign change to benign change with proliferation, atypia, in situ carcinoma and invasive carcinoma (Tables II, III) (Figures 1, 2, 3, 4).

Table II. Mean concentrations of chemokines and receptors in all cases.

Pathological diagnosis		DARC (pg/ml)	CXCL8 (pg/ml)	CXCR4 (ng/ml)	CXCL7 (pg/ml)	CCL2 (pg/ml)	CXCL12 (pg/ml)	CCR2 (pg/ml)	CCR5 (pg/ml)	CCR7 (pg/ml)	CXCL5 (ng/ml)
Benign disease	N	49	49	49	49	49	49	49	49	49	49
	Mean ± std. error	1061.72 ± 189.53	896.83 ± 22.60	10.94 ± 0.19	115.73 ± 2.49	1346.97 ± 35.38	1791.48 ± 54.39	1542.49 ± 73.98	1398.58 ± 78.43	885.68 ± 22.49	13.47 ± 0.76
	Median	1069.85	873.48	11.01	119.36	1328.36	1767.31	1440.76	1315.48	885.03	12.01
In situ carcinoma	N	24	24	24	24	24	24	24	24	24	24
	Mean ± std. error	1059.67 ± 33.84	961.75 ± 26.59	11.63 ± 0.12	116.42 ± 3.29	1387.82 ± 47.64	1836.38 ± 56.75	1420.75 ± 68.58	1376.38 ± 104.03	862.38 ± 19.62	11.93 ± 1.02
	Median	1050.29	945.74	12.37	117.20	1335.39	1885.42	1360.37	1320.32	870.55	12.38
Invasive carcinoma	N	68	68	68	68	68	68	67	67	67	68
	Mean ± std. error	1059.32 ± 13.33	877.45 ± 11.23	10.84 ± 0.64	115.79 ± 1.22	1336.45 ± 17.32	1723.58 ± 21.30	1556.57 ± 38.47	1440.57 ± 48.96	859.24 ± 14.97	13.64 ± 0.67
	Median	1053.46	870.87	11.60	114.65	1346.35	1723.78	1643.74	1418.04	862.48	13.64
Total	N	141	141	141	141	141	141	140	140	140	141
	Mean ± std. error	1058.53 ± 10.35	892.49 ± 8.03	10.49 ± 0.24	115.34 ± 1.46	1348.56 ± 14.23	1759.48 ± 15.94	1536.45 ± 31.23	1347.44 ± 22.95	864.05 ± 11.44	1245 ± 0.73
	Median	1061.35	880.43	12.48	118.64	1327.02	1763.56	1606.44	1405.86	869.53	13.47

Table III. Mean concentrations of chemokines and receptors in benign cases.

Pathological diagnosis		DARC (pg/ml)	CXCL8 (pg/ml)	CXCR4 (ng/ml)	CXCL7 (pg/ml)	CCL2 (pg/ml)	CXCL12 (pg/ml)	CCR2 (pg/ml)	CCR5 (pg/ml)	CCR7 (pg/ml)	CXCL5 (ng/ml)
Benign change	N	17	17	17	17	17	17	17	17	17	17
	mean±std. error	1038.43 ± 21.56	884.64 ± 32.59	10.76 ± 043	119.68 ± 3.36	1373.37 ± 47.30	1749.98 ± 74.70	1535.43 ± 100.74	1283.63 ± 89.75	887.31 ± 36.65	11.39 ± 0.36
	Median	1038.69	857.28	10.73	122.49	1326.03	1753.57	1428.42	1228.59	875.93	10.49
Benign change with proliferation	N	17	17	17	17	17	17	17	17	17	17
	mean ± std. error	1082.48 ± 47.27	906.49 ± 60.39	11.48 ± 1.48	108.65 ± 9.63	1467.31 ± 50.38	1811.82 ± 129.18	1738.48 ± 129.34	1758.64 ± 114.38	872.49 ± 31.31	15.85 ± 1.83
	Median	1076.19	901.58	10.82	108.84	1464.28	1824.32	1759.74	1750.55	850.69	17.93
Atypia	N	15	15	15	15	15	15	15	15	15	15
	mean ± std. error	1073.58 ± 41.49	902.49 ± 29.93	11.69 ± 2.39	116.49 ± 12.59	1201.57 ± 18.20	1885.56 ± 116.41	1359.47 ± 180.11	1208.49 ± 145.39	908.47 ± 30.49	17.43 ± 1.54
	Median	1124.49	887.86	18.49	116.29	1201.87	1849.92	1337.49	1229.74	901.29	10.64
Total	N	49	49	49	49	49	49	49	49	49	49
	mean ± std. error	1061.72 ± 189.53	896.83 ± 22.60	10.94 ± 0.19	115.73 ± 12.49	1346.97 ± 35.38	1791.48 ± 54.39	1542.49 ± 73.98	1398.58 ± 78.43	885.68 ± 22.49	13.47 ± 0.76
	Median	1069.85	873.48	11.01	119.36	1328.36	1767.31	1440.76	1315.48	885.03	12.01

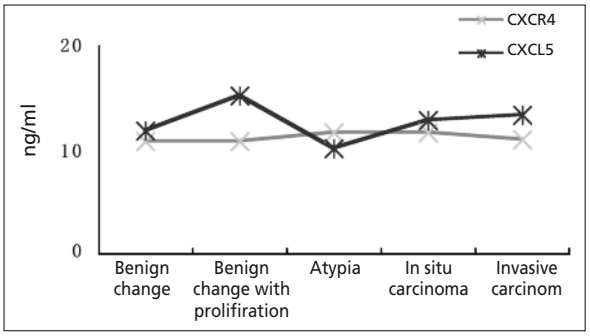

Figure 1. Means of CXCR4, CXCL5 in all diseases.

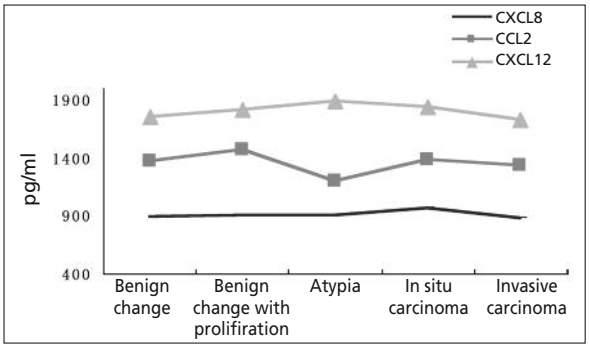

Figure 2. Means of CXCL8, CCL2, CXCL12 in all diseases.

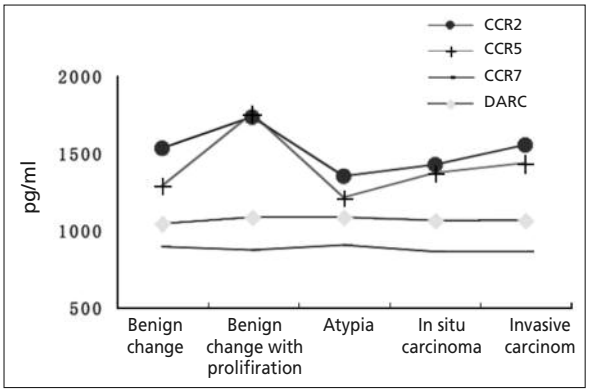

Figure 3. Means of CCR2, CCR5, CCR7, DARC in all diseases.

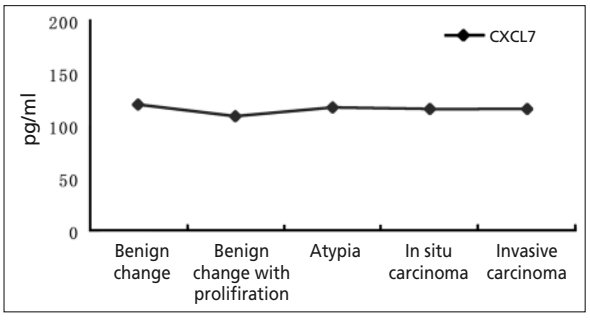

Figure 4. Means of CXCL8, CXCL7, CCL2, CXCL12 in all diseases.

In all cases, the mean concentrations of CXCL8, CXCR4 and CXCL12 in benign disease, in situ carcinoma and invasive carcinoma were significantly different: in benign disease, the mean concentration of CXCL8 was 896.83±22.60 pg/ml, in in situ carcinoma, the concentration was 961.75±26.59 pg/ml, in invasive carcinoma, the concentration was 877.45±11.23 pg/ml (p = 0.006); the mean concentrations of CXCR4 in different groups were 10.94±0.19 ng/ml, 11.63±0.12 ng/ml and 10.84±0.64 ng/ml respectively (p = 0.025); The mean concentrations of CXCL12 were 1791.48±54.39 pg/ml, 1836.38±56.75 pg/ml and 1723.58±21.30 pg/ml respectively (p = 0.029) (Tables II, IV).

In benign disease cases, the mean concentrations of CCL2 (chemokine ligand 2) and CCR5 (chemokine receptor 5) in subgroups of benign change, benign change with proliferation and atypia were significantly different: the mean concentrations of CCL2 were 1373.37 ± 47.30 pg/ml, 1467.31 ± 50.38 pg/ml and 1201.57 ± 18.20 pg/ml respectively (p = 0.013); the mean concentrations of CCR5 were 1283.63±89.75 pg/ml, 1758.64 ± 114.38 pg/ml and 1208.49 ± 145.39 pg/ml (p = 0.016) (Tables III, IV) (Figures 5, 6).

In cancer cases, including in situ carcinoma and invasive carcinoma, the mean concentrations of CXCL8, CXCR4 and CXCL12 were significantly different: for in situ carcinoma, the mean concentration of CXCL8 was 961.75±26.59 pg/ml, for invasive carcinoma, the concentration was 877.45±11.23 pg/ml (p = 0.002); the mean concentrations of CXCR4 in different groups were 11.63±0.12 ng/ml and 10.84±0.64 ng/ml respectively (p = 0.006); the mean concentrations of CXCL12 were 1836.38±56.75 pg/ml and 1723.58±21.30 pg/ml respectively (p = 0.017) (Tables III, IV) (Figures 7, 8, 9).

Oneway ANOVA analysis showed the concentration of DARC was significantly different among different relapse risk groups, LN metastasis groups and PR status groups (p = 0.012, p = 0.012, p = 0.017 respectively). In all cases, CXCL5 concentration was significantly different according to tumor size; and also the concentration of DARC, CXCL8 and CCR2 according to LN metastasis (Table IV). But the correlation analysis showed that only the correlation coefficient between DARC and relapse risk was statisticly significant (p = 0.048) (Table Va). In addition, the correlation analysis indicated that Ki67 expression was associated with CXCL5 and

Table IV. Significance of oneway ANOVA analysis.

	ER status (N)	PR status (N)	HER2 status (N)	Ki67 (N)	Pathological diagnosis in all cases (N)	Pathological diagnosis in benign disease (N)	Pathological diagnosis in cancer (N)	Tumor size (N)	Nuclear grades (N)	Relapse risk (N)	Genotype (N)	LN metastasis (N)
DARC	0.065 (79)	0.017 (79)*	0.083 (79)	0.253 (63)	0.964 (141)	0.505 (49)	0.976 (92)	0.979 (127)	0.256 (74)	0.012 (66)*	0.117 (61)	0.012 (69)*
CXCL8	0.721 (79)	0.394 (79)	0.227 (79)	0.854 (63)	0.006 (141)*	0.936 (49)	0.002 (92)*	0.785 (127)	0.143 (74)	0.426 (66)	0.583 (61)	0.048 (69)*
CXCR4	0.484 (79)	0.723 (79)	0.684 (79)	0.643 (63)	0.025 (141)*	0.679 (49)	0.006 (92)*	0.836 (127)	0.906 (74)	0.368 (66)	0.755 (61)	0.527 (69)
CXCL7	0.952 (79)	0.412 (79)	0.678 (79)	0.368 (63)	0.854 (141)	0.443 (49)	0.735 (92)	0.978 (127)	0.323 (74)	0.605 (66)	0.224 (61)	0.573 (69)
CCL2	0.357 (79)	0.847 (79)	0.411 (79)	0.929 (63)	0.466 (141)	0.013 (49)*	0.207 (92)	0.927 (127)	0.057 (74)	0.718 (66)	0.345 (61)	0.796 (69)
CXCL12	0.553 (79)	0.249 (79)	0.615 (79)	0.623 (63)	0.029 (141)*	0.633 (49)	0.017 (92)*	0.912 (127)	0.698 (74)	0.453 (66)	0.456 (61)	0.555 (69)
CCR2	0.581 (78)	0.416 (78)	0.343 (78)	0.276 (62)	0.233 (140)	0.234 (49)	0.073 (91)	0.956 (126)	0.543 (73)	0.137 (65)	0.822 (60)	0.027 (68)*
CCR5	0.857 (78)	0.658 (78)	0.057 (78)	0.456 (62)	0.748 (140)	0.016 (49)*	0.505 (91)	0.947 (126)	0.914 (73)	0.064 (65)	0.356 (60)	0.062 (68)
CCR7	0.315 (78)	0.395 (78)	0.130 (78)	0.323 (62)	0.344 (140)	0.915 (49)	0.914 (91)	0.138 (126)	0.435 (73)	0.102 (65)	0.427 (60)	0.574 (68)
CXCL5	0.599 (79)	0.562 (79)	0.198 (79)	0.705 (63)	0.655 (140)	0.054 (49)	0.608 (92)	0.000 (126)*	0.627 (74)	0.423 (66)	0.218 (61)	0.981 (69)

*Statistically different, $p < 0.05$.

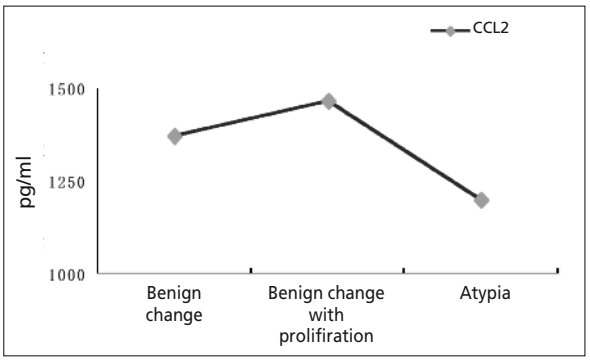

Figure 5. Means of CCL2 in benign diseases.

CXCL7 concentration ($p = 0.027$, $p = 0.042$); tumor size was associated with CXCL8 concentration ($p = 0.028$) (Table Vb).

The correlation analysis showed the great correlationship between chemokines and receptors. The concentration of DARC in serum was associated with that of CXCR4, CXCL7 and CCR7 ($p = 0.000$, $p = 0.003$, $p = 0.036$ respectively); CXCL8 with CXCR4, CXCL7 and CXCL12 ($p = 0.005$, $p = 0.042$, $p = 0.000$ respectively); CXCL7 with DARC, CXCL8, CXCR4, CXCL12, CCR5 and CCR7 ($p = 0.003$, $p = 0.042$, $p= 0.007$, $p = 0.003$, $p = 0.043$, $p = 0.003$ respectively); CCR5 with CCR2 and CXCL7 ($p = 0.000$, $p = 0.043$ respectively); CCR7 with DARC, CXCR4, CXCL7 and CXCL12 ($p = 0.036$, $p = 0.007$, $p = 0.003$, $p = 0.043$ respectively); CXCL12 with CXCL8, CXCL7 and CCR7 ($p = 0.000$, $p = 0.003$, $p = 0.043$ respectively); CCR2 with CCR5 ($p = 0.000$) (Tables Va, Vb, Vc).

The correlationship between clinico-pathological characters of patients was also assessed through the correlation analysis, the outcome showed that: pathological diagnosis was correlated with tumor size ($p = 0.000$), relapse risk ($p = 0.025$) and Ki67 expression ($p = 0.025$); nuclear grades with LN metastasis($p = 0.017$), ER status($p = 0.034$), PR status ($p = 0.030$) and the breast cancer genotype ($p = 0.043$); LN metastasis with relapse risk ($p = 0.000$); ER status with PR status ($p = 0.000$) and breast cancer genotype ($p = 0.000$); PR status with ER status ($p = 0.000$) and breast cancer genotype ($p = 0.000$); relapse risk with LN metastasis ($p = 0.000$); breast cancer genotype with ER status ($p = 0.000$) and PR status ($p = 0.000$) (Table Va).

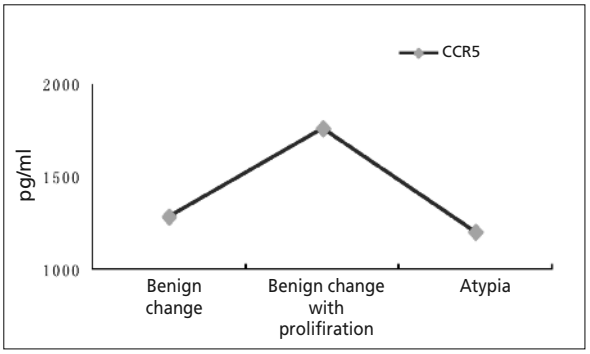

Figure 6. Means of CCR5 in benign diseases.

Discussion

Chemokines and receptors play multifaceted roles in tumorienesis and progression, however, which chemokine or receptor effects during different period of primary breast cancer has not been well known.

In 1998, a successful model was established for obtaining knowledge on the molecular and biological alterations that may contribute to the tumorigenic mechanisms through the neoplastic transformation of HBEC (Human Breast Epithelial Cell) *in vitro*[9]. In 2000, Waldman et al[10] found the chromosomal alterations in ductal carcinomas in situ and their in situ recurrences though Comparative Genomic Hybridization (CGH), and the differences in genetic changes between Infiltrating Lobular Carcinoma (ILC) and Infiltrating Ductal Carcinoma (IDC) was found[11]. Many efforts[9] had contributed to the pattern of multi stage pathological and molecular-biological progression for primary breast cancer: HBEC → benign change → epithelial proliferation atypia → *in situ* carcinoma → invasive carcinoma, which based on the mechanisms as follow: genetic predisposition and differentiation status and prior immortalization; alterations in telomerase activity and differential expression of cell cycle dependent genes as well as others recently isolated through differential cloning such as H-ferritin, and a calcium binding protein; epigenetic and genetic mechanisms microsatellite instability in specific loci on chromosomes 11, 13, and 16 with the progression of cell transformation; the loss of function of functional role of specific genes; tumor suppressor or senescence genes such as chromosomes 11 or 17[9].

To investigate the effects of chemokines and receptors on the different stage of primary breast cancer, we enrolled 148 cases which pathology diagnosis included benign change, epithelial proliferation, atypia, in situ carcinoma and invasive

Table Va. Correlation analysis 1

		Pathological diagnosis	Tumor size	Nuclear grades	LN metastasis	ER status	PR status	HER2 status	Relapse risk	Genotype	Ki67	DARC
Pathological diagnosis	Pearson correlation	1	-.437**	.068	.027	-.003	-.065	-.044	.316*	-.011	.282*	-.002
	Sig. (2-tailed)		.000	.634	.854	.971	.596	.738	.025	.945	.025	.984
	N	148	127	76	71	82	82	82	68	63	68	141
Tumor size	Pearson correlation	-.437**	1	.195	.234	-.237	-.190	.076	.182	.072	-.024	.015
	Sig. (2-tailed)	.000		.164	.080	.077	.161	.568	.206	.656	.875	.918
	N	127	127	76	71	81	81	81	68	63	68	127
Nuclear grades	Pearson correlation	.068	.195	1	.323*	-.298*	-.297*	.034	.186	.314*	.165	.064
	Sig. (2-tailed)	.634	.164		.017	.034	.030	.792	.198	.043	.242	.675
	N	76	76	76	71	76	76	76	68	62	68	74
LN metastasis	Pearson correlation	.027	.234	.323*	1	-.086	-.056	-.038	.563**	.148	-.177	.257
	Sig. (2-tailed)	.854	.080	.017		.545	.697	.787	.000	.345	.215	.064
	N	71	71	71	71	71	71	71	68	62	68	69
ER status	Pearson correlation	-.003	-.237	-.298*	-.086	1	.783**	-.235	-.183	-.823**	-.175	-.083
	Sig. (2-tailed)	.971	.077	.034	.545		.000	.058	.195	.000	.157	.544
	N	82	81	76	71	82	82	82	68	63	68	79
PR status	Pearson correlation	-.065	-.190	-.297*	-.056	.783**	1	-.162	-.163	-.662**	-.242	-.133
	Sig. (2-tailed)	.596	.161	.030	.697	.000		.196	.256	.000	.054	.312
	N	82	81	76	71	82	82	68	63	68	68	79
HER2 status	Pearson correlation	-.044	.076	.034	-.038	-.235	-.162	1	.238	.084	.163	.148
	Sig. (2-tailed)	.738	.568	.792	.787	.058	.196		.094	.576	.194	.276
	N	82	81	76	71	82	82	82	68	63	68	79
Relapse risk	Pearson correlation	.316*	.182	.186	.563**	-.183	-.163	.238	1	.096	-.036	.304*
	Sig. (2-tailed)	.025	.206	.198	.000	.195	.256	.094		.563	.793	.048
	N	68	68	68	68	68	68	68	68	63	67	66
genotype	Pearson correlation	-.011	.072	.314*	.148	-.823**	-.662**	.084	.096	1	.205	.179
	Sig. (2-tailed)	.945	.656	.043	.345	.000	.000	.576	.563		.158	.216
	N	63	63	62	62	63	63	63	63	63	63	61
Ki67	Pearson correlation	.282*	-.024	.165	-.177	-.175	-.242	.163	-.036	.205	1	.066
	Sig. (2-tailed)	.025	.875	.242	.215	.157	.054	.194	.793	.158		.649
	N	68	68	68	68	68	68	68	67	63	68	63
DARC	Pearson correlation	-.002	.015	.064	.257	-.083	-.133	.148	.304*	.179	.066	1
	Sig. (2-tailed)	.984	.918	.675	.064	.544	.312	.276	.048	.216	.649	
	N	141	127	74	69	79	79	79	66	61	63	141

*Significantly different, $p < 0.05$; **Significantly different, $p < 0.01$.

Table Vb. Correlation analysis 2

		Pathological diagnosis	Tumor size	Nuclear grades	LN metastasis	ER status	PR status	HER2 status	Relapse risk	Genotype	Ki67	DARC
CXCR4	Pearson correlation	.004	.176	-.067	.075	.195	.192	.151	.196	-.054	-.265	.476**
	Sig. (2-tailed)	.977	.336	.768	.683	.281	.292	.413	.369	.781	.192	.000
	N	141	127	74	69	79	79	79	66	61	63	141
CXCL7	Pearson correlation	-.105	-.128	-.108	.126	.016	.116	-.008	.117	.207	-.397*	.428**
	Sig. (2-tailed)	.457	.512	.617	.538	.955	.533	.963	.605	.347	.042	.003
	N	141	127	74	69	79	79	79	66	61	63	141
CCL2	Pearson correlation	-.093	.153	-.242	-.033	.071	.117	.175	.094	.015	.051	.044
	Sig. (2-tailed)	.357	.245	.095	.784	.607	.399	.198	.525	.942	.667	.643
	N	141	127	74	69	79	79	79	66	61	63	141
CXCL12	Pearson correlation	-.178	.035	-.097	.087	.183	.197	.108	-.122	-.093	-.175	.102
	Sig. (2-tailed)	.095	.783	.534	.554	.175	.136	.427	.418	.503	.198	.325
	N	141	127	74	69	79	79	79	66	61	63	141
CCR2	Pearson correlation	-.007	.063	-.003	-.056	-.110	-.173	-.194	-.186	.026	.228	-.038
	Sig. (2-tailed)	.964	.639	.982	.694	.423	.195	.152	.254	.858	.106	.732
	N	140	126	73	68	78	78	78	65	60	62	140
CCR5	Pearson correlation	.062	.169	-.045	-.155	.032	.035	-.191	-.299	-.145	.219	.042
	Sig. (2-tailed)	.556	.246	.778	.283	.787	.822	.168	.053	.296	.134	.709
	N	140	126	73	68	78	78	78	65	60	62	140
CCR7	Pearson correlation	-.141	-.092	.204	.158	.165	.038	-.023	.303	-.134	.173	.225*
	Sig. (2-tailed)	.225	.493	.197	.308	.228	.791	.872	.058	.345	.215	.036
	N	140	126	73	68	78	78	78	65	60	62	140
CXCL5	Pearson correlation	.089	-.041	.153	-.038	-.009	-.024	-.057	-.052	-.139	.295*	-.002
	Sig. (2-tailed)	.421	.826	.334	.827	.956	.848	.667	.728	.337	.027	.997
	N	141	127	74	69	79	79	79	66	61	63	141
CXCL8	Pearson correlation	-.115	.295*	-.226	-.053	.083	.163	.039	-.198	-.021	-.172	.017
	Sig. (2-tailed)	.267	.028	.125	.724	.534	.213	.773	.197	.883	.194	.865
	N	141	127	74	69	79	79	79	66	61	63	141

*Significantly different, $p < 0.05$; **Significantly different, $p < 0.01$.

Table Vc. Correlation analysis 3

		CXCR4	CXCL7	CCL2	CXCL12	CCR2	CCR5	CCR7	CXCL5	CXCL8C
CXCR4	Pearson correlation	1	.374**	.041	.245	-.265	-.122	.388**	-.034	.415**
	Sig. (2-tailed)		.007	.777	.084	.079	.408	.007	.814	.005
	N	141	141	141	141	140	140	140	141	141
CXCL7	Pearson correlation	.374**	1	.244	.412**	-.216	-.303*	.458**	-.014	.286*
	Sig. (2-tailed)	.007		.078	.003	.152	.043	.003	.938	.042
	N	141	141	141	141	140	140	140	141	141
CCL2	Pearson correlation	.041	.244	1	.015	.022	.007	-.047	-.002	.165
	Sig. (2-tailed)	.777	.078		.903	.847	.959	.682	.983	.118
	N	141	141	141	141	140	140	140	141	141
CXCL12	Pearson correlation	.245	.412**	.015	1	-.155	-.054	.219*	-.036	.632**
	Sig. (2-tailed)	.084	.003	.903		.169	.621	.043	.749	.000
	N	141	141	141	141	140	140	140	141	141
CCR2	Pearson correlation	-.265	-.216	.022	-.155	1	.713**	-.077	.021	-.082
	Sig. (2-tailed)	.079	.152	.847	.169		.000	.459	.856	.423
	N	140	140	140	140	140	140	140	140	140
CCR5	Pearson correlation	-.122	-.303*	.007	-.054	.713**	1	.015	.026	.121
	Sig. (2-tailed)	.408	.043	.959	.621	.000		.918	.839	.274
	N	140	140	140	140	140	140	140	140	140
CCR7	Pearson correlation	.388**	.458**	-.047	.219*	-.077	.015	1	-.013	-.014
	Sig. (2-tailed)	.007	.003	.682	.043	.459	.918		.886	.885
	N	140	140	140	140	140	140	140	140	140
CXCL5	Pearson correlation	-.034	-.014	-.002	-.036	.021	.026	-.013	1	-.037
	Sig. (2-tailed)	.814	.938	.983	.749	.856	.839	.886		.772
	N	141	141	141	141	140	140	140	141	141
CXCL8	Pearson correlation	.415**	.286*	.165	.632**	-.082	.121	-.014	-.037	1
	Sig. (2-tailed)	.005	.042	.118	.000	.423	.274	.885	.772	
	N	141	141	141	141	140	140	140	141	141

*Significantly different, $p < 0.05$; **Significantly different, $p < 0.01$.

carcinoma. We detected the concentration of chemokines and receptors by ELISA in the serum of patients. Our findings demonstrated that there were several chemokines and receptors played important roles during different stage of primary breast cancer. The oneway ANOVA analysis showed the significantly different concentration of CCL2 and CCR5 during the period from benign change to epithelial proliferation and atypia. Both CCL2 and CCR5 concentration curves went up from benign change to epithelial proliferation, and went down from epithelial proliferation to atypia (Figures 5, 6), suggested the function of CCL2 and CCR5 on the process of breast epithelial cells' proliferation. The concentrations of CXCL12, CXCL8 and CXCR4 during the period from in situ carcinoma to invasive carcinoma were also significantly different. The curves of CXCR4, CXCL12 and CXCL8 all went down (Figures 7, 8, 9) mean that *in situ* carcinoma cells' invasive behavior was less affected by CXCL12, CXCL8 and CXCR4.

CCL2 is a cc chemokine, many studies strongly support the possibility that CCL2 expression is associated with advanced disease course and progression in breast cancer[12,13]. CCR5 is a member of CC chemokine receptor family. It is identified as the receptor for CCL3, CCL4 (MIP1-Macrophage Inflammatory protein-1β) and CCL5. CCL5-CCR5 interaction is reported to provide cancer cells with a proliferative advantage[14]. However, the function of these chemokines in benign disease has not been well investigated. Our study demonstrated that the concentration of CCL2 and CCR5 increased during the period from benign change to benign change with proliferation, that is to say, CCL2 and CCR5 promoted not only malignant process, but also benign process before atypia.

The interaction of CXCR4 with its unique ligand: CXCL12 triggered pleiotropic activity outside the immune system[15,16], including cardiac and neuronal development, stem cell motility, neovascularization and tumorigenesis[17-21]. CXCL12 was recently reported to bind also a second receptor CXCR7, resulting in tumor angiogenesis and development[22,23]. CXCR4 expressing tumors preferentially spread to tissues that highly express CXCL12, including lung, liver, lymph nodes and bone marrow. The mechanisms of these functions include hypoxia, MMP-13 and VEGF up-regulating CXCR4 expression[24-27].

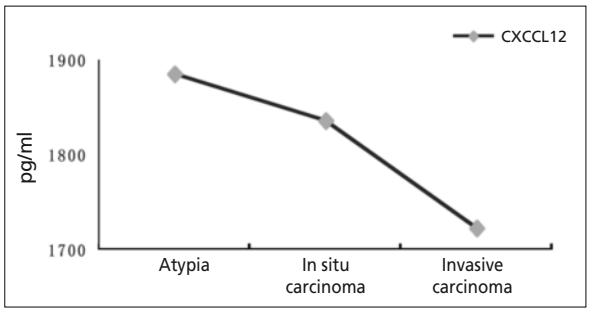

Figure 8. Means of CXCL12 in atypia and cancer.

CXCL8 is a strong inducer of angiogenesis and it mediates endothelial cell chemotaxis and proliferation *in vitro* and angiogenic activity *in vivo*[28,29]. Activation by VEGF in endothelial cells can lead to the up-regulation of bcl-2 (B-cell lymphoma 2), an anti-apoptotic molecule that promotes CXCL8 expression[30]. In addition, CXCL8 exerts its angiogenic activity in part by the up-regulation of MMP2 and MMP9 (Matrix Metalloproteinase)[31,32]. In our study, we demonstrated that tumor size was associated with CXCL8 concentration.

We found that CXCL8 concentration from atypia to in situ carcinoma and invasive carcinoma made "^" shape (Figure 9): from atypia to in situ carcinoma, it went up, which meant that CXCL8 contributed to the breast atypia cells' canceration; from in situ carcinoma to invasive carcinoma, it went down, and also the concentration

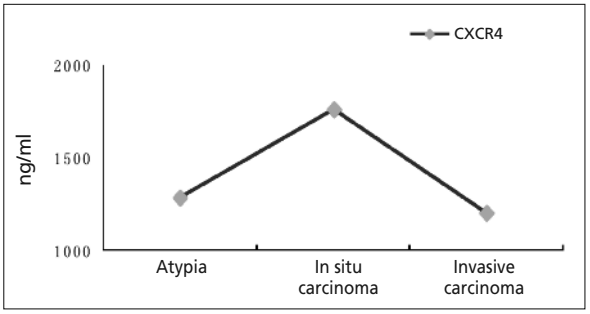

Figure 7. Means of CXCR4 in atypia and cancer.

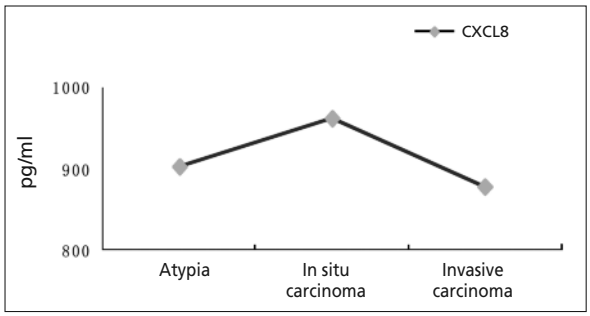

Figure 9. Means of CXCL8 in atypia and cancer.

of CXCR4 and CXCL12, which meant that at the time point of tumor cells passing through the basement membrane of breast duct, they were down-regulated. Therefore, the secretion of chemokines and receptors was undulating in the whole process of primary breast cancer.

All invasive carcinoma cases were divided into 3 groups according to relapse risk, which based on the St. Gallen Consensus Recommendations published in 2007: low-risk group, intermediaterisk group and high-risk group. Correlation analysis showed that the concentration of DARC was significantly correlated with relapse risk (p = 0.041) (Table Va).

Although DARC is known as a receptor for the malaria parasites *plasmodium vivax* and *knowlesi*[33], it is also identified a typical decoy receptor binds with angiogenic CXC chemokines, as well as some CC chemokines. Unlike other chemokine receptors, ligand binding to DARC does not induce G protein-coupled signal transduction nor Ca^{2+}-flux[6]. DARC is proposed to angiostatic factor and to limit tumor metastasis. Many studies have demonstrated the function of DARC in tumorigenesis and tumor metastasis: Addison et al[34] had studied the effects of DARC on lung cancer cells and showed that DARC induced tumor necrosis. In addition, the antimetastatic function of DARC was also derived from the interaction between DARC and the tumor suppressor gene KAI1 (Kangai 1)[35].

The correlation analysis showed the great correlationship between chemokines and receptors, which indicated the great crosstalk among chemokines and receptors in the process of tumorigenesis of primary breast cancer.

Chemokines are thought to facilitate carcinogenesis by providing a prolonged inflammation microenvironment for tumor cells. It includes indirect function by influencing angiogenesis, tumor-leukocyte interactions, as well as direct function by influencing tumor transformation, survival and growth, invasion and metastasis. However, solid tumors contain not only tumor cells, but also various types of stromal cells, such as fibroblasts and endothelial cells. Moreover, tumors are infiltrated by inflammatory cells, including neutrophils, macrophages and lymphocytes. All of these contribute to the whole microenvironment for tumor cells to develop. Tumor derived chemokines further determine the influx of leukocytes into the tumor[36], attracted neutrophils and macrophages favor tumor progression by secreting specific cytokines and matrix degrading enzymes and growth factors, respectively[37-39]. Our finding showed the great correlation between chemokines. It is reported that the expression of CCL5 and its principle receptor CCR5 displayed a significant positive correlation, indicating a strong affinity between the ligand and receptor[40].

For all invasive carcinomas, genotype was identified based on immunohisto-chemistry staining of ER, PR, and HER2[41]. Therefore, it is a natural result that ER and PR status were correlated with breast cancer genotype. It is reported that the expression of ER and PR at any level correlated with low nuclear or tumour grades[42,43], nuclear grades were found to be significantly related to the presence or absence of both the ER and PR[44]. All of these results are in line with our findings that nuclear grades of invasive carcinoma was correlated with ER status and PR status.

Chemokines and receptors play important roles in both malignant and benign breast disease, however, the concentration of chemokines and receptors fluctuates along the progression of disease.

Conclusions

Our study demonstrated that the concentration of CCL2 and CCR5 increased during the period from benign change to benign change with proliferation, to some extent, it is more important because of its possible prevention function, since prevention of breast cancer is better than therapy after the onset of breast cancer. In addition, our outcome identified that for invasive cancer patients, DARC concentration in serum before operation strongly indicated patient's relapse risk, which implied a more individual and appropriate therapy plan for each patient.

Acknowledgements

This research was supported by National Natural Science Foundation of China (81001172).

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