The Chemokine CCL5 as a Potential Prognostic Factor Predicting Disease Progression in Stage II Breast Cancer Patients

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Abstract

Purpose: The aim of this study was to determine the prognostic value of the chemokine CCL5, considered as a promalignancy factor in breast cancer, in predicting breast cancer progression and to evaluate its ability to strengthen the prognostic significance of other biomarkers.

Experimental Design: The expression of CCL5, alone and in conjunction with estrogen receptor (ER)-α, ER-β, progesterone receptor (PR), and HER-2/*neu* (ErbB2), was determined in breast tumor cells by immunohistochemistry. The study included 142 breast cancer patients, including individuals in whom disease has progressed.

Results: Using Cox proportional hazard models, univariate analysis suggested that, in stage I breast cancer patients, CCL5 was not a significant predictor of disease progression. In contrast, in stage II patients, the expression of CCL5 ($CCL5^+$), the absence of ER-α ($ER\text{-}\alpha^-$), and the lack of PR expression (PR^-) increased significantly the risk for disease progression ($P = 0.0045$, 0.0041, and 0.0107, respectively). The prognostic strength of CCL5, as well as of $ER\text{-}\alpha^-$, improved by combining them together ($CCL5^+/ER\text{-}\alpha^-$: $P = 0.0001$), being highly evident in the stage IIA subgroup [$CCL5^+/ER\text{-}\alpha^-$ ($P = 0.0003$); $ER\text{-}\alpha^-$ ($P = 0.0315$)]. In the stage II group as a whole, the combinations of $CCL5^-/ER\text{-}\alpha^+$ and $CCL5^-/PR^+$ were highly correlated with an improved prognosis. Multivariate analysis indicated that, in stage II patients, ER-α and CCL5 were independent predictors of disease progression.

Conclusions: CCL5 could be considered as a biomarker for disease progression in stage II breast cancer patients, with the $CCL5^+/ER\text{-}\alpha^-$ combination providing improved prediction of disease progression, primarily in the stage IIA subgroup.

Breast cancer is a significant cause of mortality among women in the Western world. The establishment of better diagnostic and prognostic measures in breast cancer requires identification of reliable biomarkers. To date, different studies have investigated the applicability of a variety of molecules as indicators for disease status, among them are steroid receptors (1–7) and HER-2/*neu* (ErbB2; refs. 6–9). Although progress has been made, additional markers for screening may provide new ways to evaluate the status of the disease in a given patient and may provide new therapeutic targets in human breast cancer.

The aim of the present study was to determine the prognostic strength of the CCL5 chemokine alone and to analyze its ability to strengthen the prognostic value of other molecules, which have already been characterized as markers for progression in breast cancer. CCL5 was selected in view of findings, suggesting that it is a potential contributor to breast malignancy. CCL5 belongs to the family of chemokines, primarily identified as potent inducers of leukocyte motility (10, 11). This chemokine was first identified in our published research to be expressed almost exclusively in breast tumor cells and was only minimally detected in adjacent normal breast epithelial cells (12). Moreover, CCL5 was found to be highly expressed in breast cancer patients compared with healthy individuals and was significantly associated with advanced disease course (12). These findings are suggestive of a role for CCL5 in breast malignancy and were supported by the study of Niwa et al. (13), whose results indicated that elevated CCL5 serum levels are correlated with advanced breast cancer. Later clinical studies on CCL5 supported a role for the chemokine in breast cancer, and animal studies indicated that CCL5 has a causative role in breast malignancy (14–17). Finally, various *in vitro* and *in vivo* analyses provided evidence for a variety of tumor-supporting functions that may be exerted by the chemokine in this disease, such as acting on the tumor microenvironment and on the tumor cells themselves to promote tumor-associated functions (15–26).

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The other proteins investigated in this research included conventional markers in this disease [i.e., estrogen receptor (ER)-α, progesterone receptor (PR), and ErbB2; refs. 6–9]. Whereas ER-α and PR are considered as markers for good prognosis (1–7), the expression of ErbB2, documented in 15% to 30% of invasive breast cancers, has been associated with poor prognosis (6–9). We also analyzed the prognostic value of CCL5 together with ER-β, whose role in breast cancer has not yet been fully elucidated (1–3).

Using Cox proportional hazard models, univariate and multivariate analyses were done to assess the applicability of the different proteins, alone or together, as biomarkers for breast cancer progression. By immunohistochemistry, we have determined the expression of the different proteins in breast tumor cells, in a total of 142 breast carcinoma patients, and correlated their expression with the clinical status of the patients. The study included patients who remained disease-free in the course of a follow-up that was carried out for at least 5 years after diagnosis, whereas others relapsed with local tumor or distant metastasis or died of breast cancer. To improve the prognostic strength of our investigations, all the analyses were done separately on stage I patients and on stage II patients.

The results of the present research suggest that, in patients diagnosed in stage I of disease, CCL5 does not have a significant value in predicting disease progression. In contrast, in stage II patients, CCL5 expression by breast tumor cells may be considered as a potential marker for prediction of disease progression, and its combined analysis with the lack of ER-α improves the prognostic value of each of these two proteins, especially in patients diagnosed at stage IIA of disease. Moreover, our findings suggest that a favorable outcome for stage II patients may be predicted in individuals who are negative for CCL5 expression, however, positive for ER-α or PR.

Overall, our investigation calls for multicenter studies that will further determine and possibly validate the prognostic value of CCL5, alone and together with ER-α, for better identification of breast cancer patients who are at risk for progression and for assessment of its therapeutic implications in this group of patients.

Materials and Methods

Patients. The characteristics of the study patients are presented in Table 1. Staging was done according to the guidelines of the Cancer Staging Manual of the American Joint Committee on Cancer at the time of diagnosis (dating at least 5 years ago; see below). The study included 142 female breast cancer patients, 53 diagnosed at stage I and 89 at stage II of disease. In addition, the stage II group was divided into two subgroups: stage IIA (62 patients, including individuals diagnosed as T_1N_1 or T_2N_0) and stage IIB (27 patients, including individuals diagnosed as T_2N_1).

All the study patients have undergone lumpectomy. After treatment, the clinical status of the patients was followed for 5 to 22 years (excluding patients who died of breast cancer before study closure). Each group (stages I and II) included "disease-free" patients, defined as those remaining free of breast disease throughout the entire follow-up period, and "progressed" patients, defined as those who relapsed with a local tumor or metastasis or who died of breast cancer. The average time of follow-up and the incidence of progressed patients were similar in the two stages (51% in stage I and 62% in stage II).

Immunohistochemistry. Tissues from malignant breast lesions were formalin fixed and embedded in paraffin. All the tissues were obtained from the Oncology Department, Tel Aviv Sourasky Medical

Table 1. The characteristics of breast cancer patients included in the study

		Stage I, *N* = 53	Stage II, *N* = 89
Age (y)	Range	32-80	33-78
Menopause	Premenopause	7 (13%)	23 (26%)
	Postmenopause	45 (85%)	58 (65%)
	Unknown	1 (2%)	8 (9%)
Histologic type	Invasive ductal carcinoma	44 (83%)	73 (82%)
	Invasive lobular carcinoma	4 (8%)	12 (13%)
	Other	5 (9%)	4 (5%)
Progression	Disease free	26 (49%)	34 (38%)
	Progressed	27 (51%)	55 (62%)
Node status	0	53 (100%)	30 (34%)
	1-4		41 (46%)
	>4		18 (20%)
Tumor size (cm)	T_1 (<2)	53 (100%)	32 (36%)
	T_2 (2< × <5)		57 (64%)
Radiotherapy	Yes	46 (87%)	73 (82%)
	No	6 (11%)	13 (15%)
	Unknown	1 (2%)	3 (3%)
Hormone therapy	Yes	33 (62%)	55 (62%)
	No	19 (36%)	32 (36%)
	Unknown	1 (2%)	2 (2%)
Chemotherapy	Yes	4 (8%)	53 (60%)
	No	49 (92%)	34 (38%)
	Unknown	0 (0%)	2 (2%)

Center (Tel Aviv, Israel) with the approval of the institutional Helsinki Committee. Serial sections (5-μm thick) were prepared from the blocks and processed as described previously (12, 18).

The slides were deparaffinized in alcohol and treated with hyaluronidase and 3% H_2O_2. Nonspecific binding was blocked by normal goat serum, and staining was done overnight at 4°C by antibodies having well-determined specificities, as indicated by published articles, using immunohistochemistry and Western blotting (see below). For detection of CCL5, monoclonal mouse $IgG2_b$ recognizing human CCL5 by Western blotting (data not shown) was used (50 μg/mL; PeproTech 500-M75, Rocky Hill, NJ). The binding specificity of the antibodies against CCL5 was verified in our laboratory compared with an isotype-matched antibody and in published studies (12). In addition, the following antibodies were used: monoclonal mouse IgG1 anti-ER-α (1D5; 10 μg/mL; Zymed, South San Francisco, CA), polyclonal goat anti-ER-β (SC-6820; 20 μg/mL; Santa Cruz Biotechnology, Santa Cruz, CA) (for antibodies against ER-α and ER-β: refs. 27–30), monoclonal mouse IgG1 anti-PR (1A6 antibody; 1:40 dilution; according to the manufacturer's instructions; Novocastra Laboratories, Newcastle, United Kingdom; refs. 28–30), and monoclonal mouse IgG1 anti-ErbB2 (3B5; 0.5-1 μg/mL; Oncogene, Boston, MA; refs. 31, 32). The antibodies against ER-α, PR, and ErbB2 were all IgG1 and therefore served as internal controls for each other's specificity: The ER-α and PR specimens showed nuclear staining but often with different patterns, and the ErbB2 specimens showed membrane and/or cytoplasmic staining. The staining of all biopsies was negatively controlled by omission of the antibodies and by buffer substitution. Antigen retrieval by microwave was used for staining by antibodies against ER-α, ER-β, PR, and ErbB2; however, it was not used for staining by antibodies to CCL5.

The sections were washed thoroughly in PBS and stained by biotinylated anti-broad spectrum second antibody (Histostain kit, Zymed) according to the manufacturer's instructions. They were then stained with horseradish peroxidase–streptavidin (Histostain kit), counterstained, and processed as described previously (18). The staining pattern of the tested proteins in tumor cells was evaluated on the whole section area in a blind manner by an expert pathologist and by two researchers and is summarized in Table 2.

Statistical analysis. Cox proportional hazard models were used to assess the effect of each marker on time to recurrence of the disease, addressing the problem of censored data. Cox models were constructed as follows: first univariate model for each marker separately and then multivariate models to account for possible colinearity between markers (as shown in Tables 3 and 4, respectively). Possible coexpression between proteins was examined using the χ^2 or Fisher exact tests as applicable. Survival analysis according to the Kaplan-Meier method was carried out to estimate the disease-free time function according to protein expression. The SAS for Windows version 9.1 was used for all statistical analyses.

Results

Patterns of protein expression by immunohistochemistry. The current study included 142 female breast cancer patients, diagnosed at stages I or II of disease, whose clinical characteristics are provided in Table 1 and in Materials and Methods. The biopsy sections from the primary breast tumors of these patients were stained with specific antibodies to CCL5, ER-α, ER-β, PR, and ErbB2. Figure 1 displays representative examples of the staining patterns of each of these proteins in the examined breast tumor cells. In line with the previously described patterns of their expression, CCL5 staining was mainly cytoplasmatic, whereas ER-α, ER-β, and PR were primarily nuclear. ErbB2 staining in the tumor cells ranged between a membranous staining (graded 3+) and weak cytoplasmic expression (graded 1+).

Table 2. The incidence of expression of CCL5, ER-α, ER-β, PR, and ErbB2 in breast tumor cells as detected by immunohistochemistry in biopsies of breast cancer patients diagnosed in stages I or II of disease

	Disease free	Progressed	Progressed/total (%)
Stage I			
$CCL5^+$	11	12	12/23 (52)
$CCL5^-$	15	15	15/30 (50)
ER-α^+	14	14	14/28 (50)
ER-α^-	12	13	13/25 (52)
ER-β^+	21	22	22/43 (51)
ER-β^-	4	4	4/8 (50)
PR^+	16	13	13/29 (45)
PR^-	10	14	14/24 (58)
$ErbB2^+$	4	11	11/15 (73)
$ErbB2^-$	22	16	16/38 (42)
Stage II			
$CCL5^+$	9	29	29/38 (76)
$CCL5^-$	25	26	26/51 (51)
ER-α^+	27	28	28/55 (51)
ER-α^-	7	27	27/34 (79)
ER-β^+	25	37	37/62 (60)
ER-β-	8	18	18/26 (69)
PR^+	24	24	24/48 (50)
PR^-	10	31	31/41 (76)
$ErbB2^+$	7	15	15/22 (68)
$ErbB2^-$	27	40	40/67 (60)

Protein expression in stage I breast cancer patients. The data in Table 2 depict the distribution of expression of each of the tested proteins in patients diagnosed in stage I of disease. Univariate analysis done with Cox proportional hazard models has shown that the distribution of most of the proteins, including CCL5, was not indicative of disease progression (Table 3); however, the expression of ErbB2 was on the borderline of statistical significance ($P = 0.0563$).

As ErbB2 was found to be the sole protein that had the potential to predict progression in stage I patients, we also examined the distribution and statistical significance of the $CCL5^+/ErbB2^+$ combination by univariate analysis and found that it had no significant prognostic value for disease progression for this group (Table 3).

Protein expression in stage II breast cancer patients. When compared with the group of stage I breast cancer patients, a different pattern of protein expression was noted in patients diagnosed at stage II of disease (Tables 2 and 3). Three of the tested proteins, CCL5, ER-α, and PR yielded significant values for prediction of progression using univariate analysis in Cox proportional hazard models. As shown in Table 3, CCL5 expression highly increased the risk for disease progression ($P = 0.0045$), a finding that was also shown by Kaplan-Meier plots, which showed the distribution of disease-free patients according to the presence or absence of CCL5 expression (Fig. 2A).

An inverse relationship was obtained with ER-α and PR: the absence of ER-α expression significantly increased the risk for disease progression ($P = 0.0041$) as did the absence of PR

expression ($P = 0.0107$; Table 3). The contribution of the lack of hormone receptor expression to progression of the disease in our study is in line with the current and accepted views on ER-α and PR in breast cancer (1–7). With regard to ErbB2, its incidence of expression was in line with published studies denoting its overexpression in 15% to 30% of breast cancer patients (22 of the 89 patients were positive to ErbB2; Table 2; refs. 6–9). Nevertheless, in contrast to other studies associating ErbB2 amplification with poor outcome in patients with nodal involvement (6–9), this protein did not have a significant predictive value for disease progression in this group of patients in our study (Table 3). In addition to ErbB2, ER-β also did not have prognostic significance in this group of patients. Taken together, then, these results indicate that the presence of CCL5 and the absence of ER-α or PR are predictive markers for disease progression in stage II breast cancer patients.

Table 3. Univariate analyses of CCL5, ER-α, ER-β, PR, and ErbB2 expression in breast cancer patients using Cox proportional hazard models

	P	HR	95% limits
Stage I			
CCL5	0.9086	1.046	0.489, 2.237
ER-α	0.6311	0.831	0.389, 1.772
ER-β	0.6745	0.814	0.312, 2.126
PR	0.5044	0.772	0.362, 1.648
ErbB2	0.0563	2.123	0.980, 4.599
$CCL5^+/ErbB2^+$	0.3856	1.496	0.602, 3.715
Stage II			
CCL5	0.0045	2.218	1.280, 3.844
ER-α	0.0041	0.458	0.269, 0.780
ER-β	0.9156	0.971	0.568, 1.661
PR	0.0107	0.499	0.292, 0.851
ErbB2	0.2307	1.441	0.793, 2.618
$CCL5^+/ER\text{-}\alpha^-$	0.0001	3.455	1.838, 6.491
$CCL5^+/PR^-$	0.0253	2.016	1.091, 3.727
$CCL5^+/ErbB2^+$	0.0140	2.310	1.184, 4.506
$CCL5^-/ER\text{-}\alpha^+$	0.0029	0.387	0.207, 0.722
$CCL5^-/PR^+$	0.0005	0.277	0.135, 0.569
Stage IIA			
CCL5	0.0610	1.982	0.969, 4.055
ER-α	0.0315	0.470	0.236, 0.935
PR	0.0260	0.458	0.230, 0.910
$CCL5^+/ER\text{-}\alpha^-$	0.0003	5.680	2.203, 14.64
$CCL5^+/PR^-$	0.0523	2.425	0.991, 5.932
$CCL5^-/ER\text{-}\alpha^+$	0.0488	0.481	0.233, 0.996
$CCL5^-/PR^+$	0.0092	0.345	0.155, 0.769
Stage IIB			
CCL5	0.1758	2.013	0.731, 5.543
ER-α	0.1739	0.546	0.228, 1.306
PR	0.4496	0.715	0.299, 1.708
$CCL5^+/ER\text{-}\alpha^-$	0.1715	1.870	0.762, 4.587
$CCL5^+/PR^-$	0.5642	1.299	0.534, 3.162
$CCL5^-/ER\text{-}\alpha^+$	0.1109	0.304	0.070, 1.314
$CCL5^-/PR^+$	0.0917	0.177	0.024, 1.325

NOTE: The analysis includes 53 patients at stage I of disease and 89 patients at stage II of disease. Stage II patients were further divided to stage IIA (62 patients) and stage IIB (27 patients).
Abbreviation: HR, hazard ratio.

Table 4. Multivariate analyses of CCL5, ER-α, ER-β, PR, and ErbB2 expression in breast cancer patients using Cox proportional hazard models

	P	HR	95% limits
Stage I			
CCL5	0.761	0.872	0.362, 2.101
ER-α	0.907	1.055	0.426, 2.614
ER-β	0.784	1.152	0.419, 3.17
PR	0.919	0.954	0.388, 2.346
ErbB2	0.063	2.276	0.957, 5.417
Stage II			
CCL5	0.005	2.256	1.282, 3.969
ER-α	0.049	0.537	0.289, 0.996
ER-β	0.443	0.785	0.423, 1.456
PR	0.550	0.827	0.443, 1.543
ErbB2	0.968	0.987	0.518, 1.879

Furthermore, univariate analysis of the $CCL5^+/ER\text{-}\alpha^-$ combination as well as of the $CCL5^+/PR^-$ combination provided significant statistical values for disease progression ($P = 0.0001$ and 0.0253, respectively; Table 3) as was also indicated by the Kaplan-Meier plots shown in Figs. 2B and 2C. Importantly, combining $CCL5^+$ with $ER\text{-}\alpha^-$ improved the prognostic value not only of CCL5 but also of ER-α in predicting disease progression: whereas the statistical significance of $CCL5^+$ alone or of $ER\text{-}\alpha^-$ alone was $P = 0.0045$ and 0.0041, respectively, the combination of $CCL5^+/ER\text{-}\alpha^-$ improved the statistical value for prediction of breast cancer progression to $P = 0.0001$ (see also the Kaplan-Meier plot in Fig. 2B). In addition, although ErbB2 was not predictive of disease progression in our stage II patients, the combination of $CCL5^+/ErbB2^+$ showed an elevated risk for progression in that group ($P = 0.0140$; Fig. 2D; Table 3).

In view of the improved predicting value of the $CCL5^+/ER\text{-}\alpha^-$ combination in stage II breast cancer patients compared with $CCL5^+$ or $ER\text{-}\alpha^-$ alone, further investigation of this observation was done. To this end, the group of stage II patients was divided into two subgroups, stage IIA and stage IIB, according to tumor size and lymph node involvement (for details, see Materials and Methods). Of the two subgroups, that of stage IIA has yielded a similar pattern to the stage II group as a whole, providing evidence to the major advantage of the $CCL5^+/ER\text{-}\alpha^-$ combination ($P = 0.0003$) over $ER\text{-}\alpha^-$ alone ($P = 0.0315$) in predicting disease progression (Table 3). These results indicate that the $CCL5^+/ER\text{-}\alpha^-$ combination has a highly significant value in this respect in the stage IIA breast cancer patients.

To analyze the possibility that the prognostic protective value of the presence of ER-α and PR will take effect when there was no CCL5 expression, we determined the statistical significance of specific combinations, in which the absence of CCL5 expression was combined with presence of ER-α and PR. First, this analysis was done for the group of stage II patients as a whole. As shown in Table 3 and Fig. 2E, the combination $CCL5^-/ER\text{-}\alpha^+$ was predictive for improved prognosis ($P = 0.0029$) as was the combination of $CCL5^-/PR^+$ ($P = 0.0005$; Fig. 2F; Table 3). More specifically, these combinations were

predictive for a favorable outcome in the subgroup of stage IIA patients [$CCL5^-/ER\text{-}\alpha^+$ combination ($P = 0.0488$); $CCL5^-/PR^+$ combination ($P = 0.0092$)].

To conclude, these results indicate that positive expression of CCL5 and the absence of ER-α and PR are predictors of progression and that the combination of $CCL5^+/ER\text{-}\alpha^-$ is advantageous for predicting progression primarily in stage IIA patients. In contrast, a favorable outcome was indicated by the absence of CCL5, combined with the presence of ER-α or with PR in breast tumor cells.

Dependence between variables. We did stepwise multivariate analyses using Cox proportional hazard models to determine the dependence between the different proteins that were found to be of significance for disease progression. The results indicated that none of the proteins had an independent prognostic significance in stage I patients, whereas the absence of ER-α and the expression of CCL5 had an independent significance in predicting disease progression for stage II patients ($P = 0.049$ and 0.005, respectively). The other studied proteins, ER-β, PR, and ErbB2, did not have any independent predictive value for disease progression at this stage of disease.

Coexpression of the tested proteins. In addition to the above analyses, we looked at the possibility that several of the proteins that were investigated in this study are coexpressed in the different stages of disease. The χ^2 and Fisher exact tests (as applicable) found coexpression between CCL5 and ER-β ($P = 0.027$), between ER-α and PR ($P < 0.001$), and also between PR and ErbB2 in stage I patients. A similar analysis of the coexpression of the different proteins in stage II patients revealed coexpression between ER-α and PR ($P < 0.001$) and between ER-β and ErbB2 ($P = 0.01$). These results provide evidence to complex associations between the expression patterns of the proteins analyzed in the present study.

Discussion

The ongoing and intensive search for biomarkers to provide better prognosis in breast cancer has focused recently on the prognostic value of hormone receptors and ErbB2 (1–9). In the present study, we sought to elucidate the prognostic value of the chemokine CCL5 alone and in combination with other markers in an attempt to identify breast cancer patients who may be at risk for disease progression.

Our focus on CCL5 was based on previous findings, suggesting that this chemokine contributes to breast malignancy (see above). Taken together with our earlier demonstration of CCL5 being highly expressed in advanced stages of disease (12), we now hypothesized that CCL5 may be a

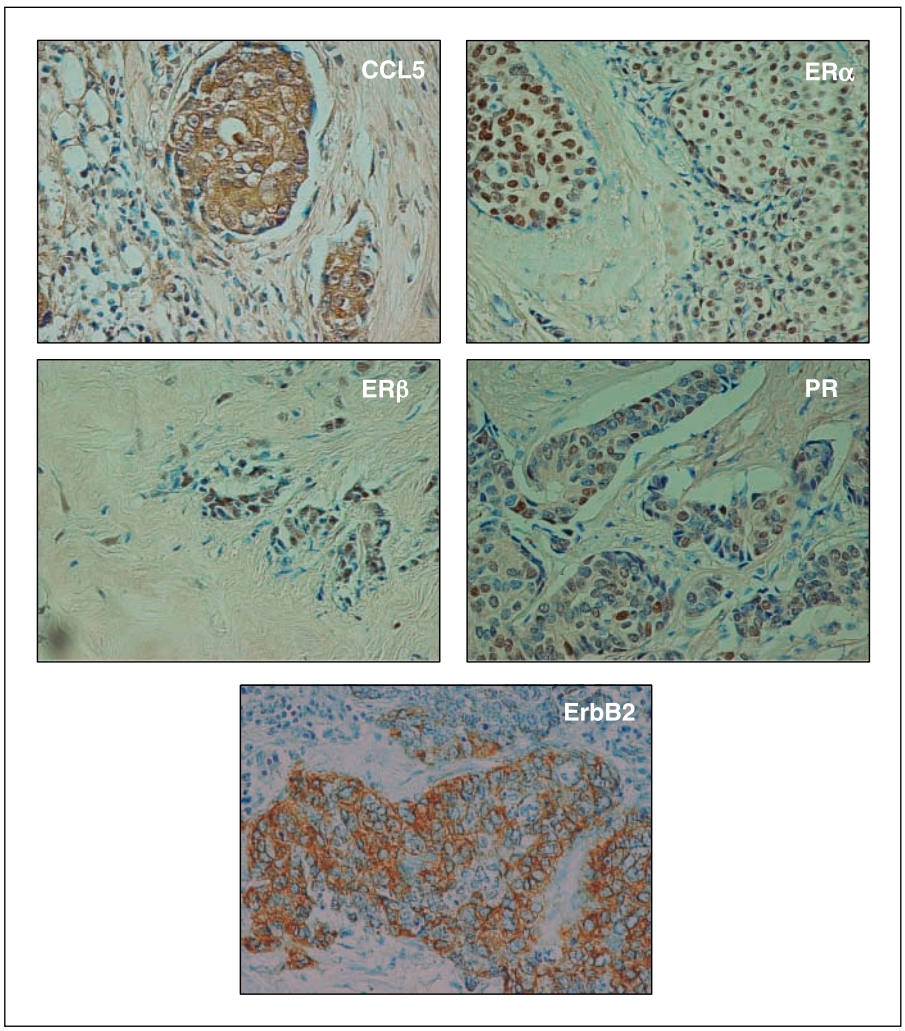

Fig. 1. Representative examples of the expression of CCL5 (*A*), ER-α (*B*), ER-β (*C*), PR (*D*), and ErbB2 (*E*) in breast tumor cells. The expression of the proteins was determined by immunohistochemistry using specific antibodies against each of the proteins.

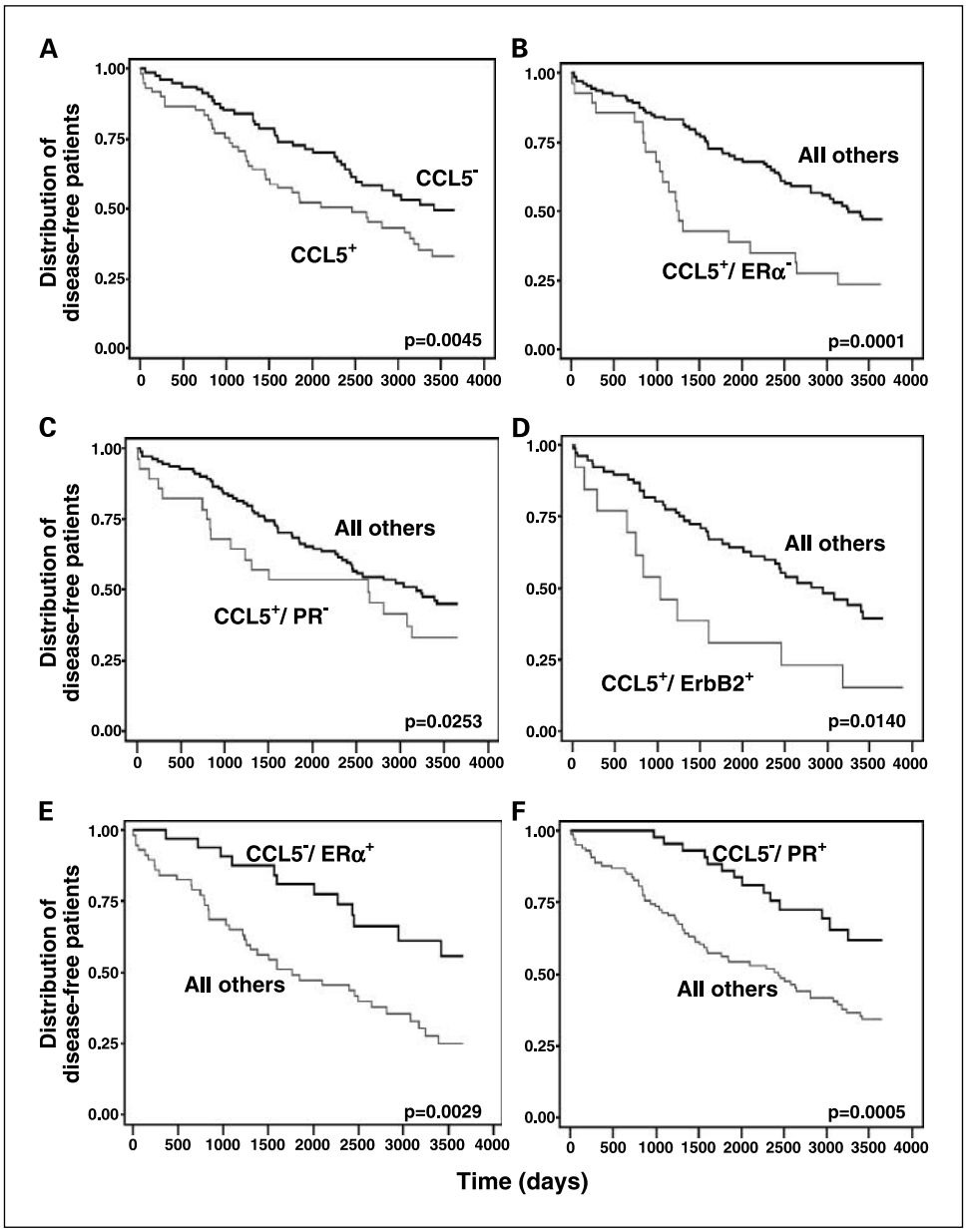

Fig. 2. Kaplan-Meier step graphs of CCL5, ER-α, ER-β, PR, and ErbB2 expression in patients diagnosed in stage II of breast cancer (a total of 89 patients). *P*s were calculated by Cox proportional hazard models. *A,* $CCL5^+$ versus $CCL5^-$. *B,* the combination of $CCL5^+/ER\text{-}\alpha^-$ versus all the other combinations of these two markers when joined together ($CCL5^+/ER\text{-}\alpha^+$, $CCL5^-/ER\text{-}\alpha^-$, and $CCL5^-/ER\text{-}\alpha^+$). *C,* the combination of $CCL5^+/PR^-$ versus all the other combinations of these two markers when joined together ($CCL5^+/PR^+$, $CCL5^-/PR^-$, and $CCL5^-/PR^+$). *D,* the combination of $CCL5^+/ErbB2^+$ versus all the other combinations of these two markers when joined together ($CCL5^+/ErbB2^-$, $CCL5^-/ErbB2^+$, and $CCL5^-/ErbB2^-$). *E,* the combination of $CCL5^-/ER\text{-}\alpha^+$ versus all the other combinations of these two markers when joined together ($CCL5^-/ER\text{-}\alpha^-$, $CCL5^+/ER\text{-}\alpha^+$, and $CCL5^+/ER\text{-}\alpha^-$). *F,* the combination of $CCL5^-/PR^+$ versus all the other combinations of these two markers when joined together ($CCL5^-/PR^-$, $CCL5^+/PR^+$, and $CCL5^+/PR^-$).

powerful biomarker for breast cancer progression and determined its prognostic significance in prediction of breast cancer progression.

To this end, we have characterized CCL5 when solely analyzed and have determined its ability to strengthen the prognostic value of other biomarkers as well. Our current findings showed that CCL5 may be used as a prognostic marker for disease progression in stage II breast cancer patients and that its combination with the lack of ER-α expression improves the prognostic value of each of the two proteins. Of importance, our analysis indicates that the $CCL5^+/ER\text{-}\alpha^-$ combination has a major advantage over $ER\text{-}\alpha^-$ alone in the subgroup of stage IIA patients, suggesting that these patients can considerably benefit from the use of the $CCL5^+/ER\text{-}\alpha^-$ combination for prediction of disease progression.

Our findings also indicated that the $CCL5^-/ER\text{-}\alpha^+$ and $CCL5^-/PR^+$ combinations may be predictive of a favorable outcome, suggesting that the protective properties of the hormone receptors become evident when CCL5 is absent. These results imply that, when the chemokine is expressed by breast tumor cells, it actually dominates the potential protective role of ER-α and PR. Subsequently, it is possible that, in $CCL5^+$ patients, endocrine therapy may be ineffective. Only when CCL5 is absent, the protective effects of ER-α and PR may come into effect; therefore, the $CCL5^-/ER\text{-}\alpha^+$ and $CCL5^-/PR^+$ combinations are indicative of favorable prognosis.

Overall, CCL5, either alone or together with the lack of ER-α, emerged as a potential novel biomarker that could be used for prediction of progression in stage II breast cancer patients (primarily in patients diagnosed in stage IIA). It is important to note the differences between patients diagnosed in stages I and II of disease, as indicated by the fact that the markers that were associated with disease progression were different for the two groups of patients. Accordingly, CCL5 was significantly

associated with progression in stage II but not stage I patients. This observation is in line with the conclusions of our earlier study that has also shown a more significant relevance of CCL5 to stage II as indicated by the fact that CCL5 was more prevalent in stage II patients than in stage I patients (12).

We would like to note that, in contrast to our published study, the current investigation is advantageous as it includes an additional variable, being the criteria of disease progression. This approach allows us to better analyze the prognostic value of CCL5 in breast cancer and to determine its ability to predict progression in this disease.

Accordingly, differences were observed between our two studies in the incidence of CCL5 expression, as a sole factor, in stages I versus II of disease. In the present study, the incidence or CCL5 expression was not markedly different between patients diagnosed at stage I or II of disease (43% in both stages), whereas in our previous investigation, the incidence of CCL5 expression was significantly higher in stage II patients than in stage I patients (83% versus 55%, respectively). Because in our published study (12) we did not include the criteria of progression of disease, it is difficult to assess the incidence of progression in stage I versus stage II patients in that study. Therefore, the possibility exists that the group of stage II patients included relatively more patients who have progressed compared with the stage I group, giving rise to elevated incidence of CCL5 expression in stage II of disease compared with stage I. In contrast, in the current study, the two groups of patients, at stages I and II, had a similar incidence of individuals whose disease has progressed. This may explain the observation that, in the current study, no marked differences have been observed in the incidence of CCL5 expression between the two stages of breast carcinoma.

To conclude, the findings presented herein show the prognostic value of CCL5 in predicting the progression of stage II breast cancer patients to local relapse, metastasis, or death. Our observations further support the importance of CCL5 as a prognostic factor when combined with ER-α and propose their joint roles as potential prognostic markers for disease progression in this group of patients, more specifically in patients belonging to the stage IIA subgroup.

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