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Elevated Levels of Chemokine Receptor CXCR4 in HER-2 Negative Breast Cancer Specimens Predict Recurrence

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Introduction. **CXCR4 is a chemokine receptor that has recently been implicated to play a pivotal role in breast cancer growth and metastasis. In animal models, reduction of CXCR4 expression significantly abrogated metastatic disease and prolonged survival. In human breast cancers, CXCR4 overexpression may portend a worse clinical course. Recent data suggest that HER-2 up-regulates CXCR4, but whether this is applicable in the clinical setting is not known. In this study, we evaluated the role of CXCR4 overexpression in breast cancer and determined whether it can serve as a potential marker of tumor recurrence in HER-2 negative tumors.**

Methods. **One hundred three patients with stages I to III breast cancers and 6 benign breast tissues were prospectively accrued and analyzed. Study homogeneity was maintained by standardized treatment, surveillance, and compliance protocols. CXCR4 levels were detected using Western blots and results were quantified against 1 μg of HeLa cells (positive controls). HER-2 expression was evaluated using the Hercep program, (Dako Corp., Carpinteria, CA) with a positive result defined as ≥2. CXCR4 expression was defined as low (<6.6-fold) or high (≥6.6-fold). Primary endpoints were cancer recurrence and death. Statistical analysis performed included Spearman's correlation, independent samples *t*-test, Kaplan-Meier survival analysis, and log-rank test.**

Results. **All 103 cancer specimens had CXCR4 overexpression (mean 6.6 ± 4.7), while none of the 6 benign breast tissues had detectable level of CXCR4. There were 36 HER-2 (+) tumors and 67 HER-2 (−) tumors. There was no statistical significance in mean CXCR4 overexpression between HER-2 (+) [5.6] and HER-2 (−) [6.6] cancers ($P = 0.3$; independent samples *t*-test). Recurrences occurred in 18 of 103 patients (17%); 10 occurred in HER-2 (+) tumors, and 8 occurred in HER-2 (−) patients. CXCR4 expression level was not predictive of cancer recurrence ($P = 0.80$) or overall survival ($P = 0.70$) in the HER-2 (+) group. However, among HER-2 negative tumors, 7 of 8 recurrences occurred in the high CXCR4 group ($P = 0.037$). There was no correlation between the degree of CXCR4 overexpression with tumor size ($r = 0.13$, $P = 0.22$), nodal status ($r = 0.019$, $P = 0.4$), ER/PR status ($r = 0.12$, $P = 0.29$), and HER-2 status ($r = 0.091$, $P = 0.36$).**

Conclusions. **CXCR4 overexpression was observed in all 103 breast cancer specimens but was undetectable in benign breast tissues. CXCR4 overexpression does not correlate with tumor size, nodal status, ER/PR status, and HER-2 status. High CXCR4 overexpression had a significant impact on disease-free survival in HER-2 negative breast cancer patients and may help identify a subset of HER-2 negative breast cancers that have a more aggressive biological behavior.**

Key Words: **breast cancer; CXCR4; HER-2; cancer recurrence.**

INTRODUCTION

In 2005, 211,240 women in United States were diagnosed with breast cancer. It is the most common malignancy and the second leading cause of cancer death in women [1]. Our understanding of crucial molecular defects that give rise to cancers has ushered in a new era of molecularly targeted therapy. In operable breast cancer, trastuzumab (Herceptin), a humanized monoclonal antibody directed against the extracellular domain of HER-2, significantly reduces the relative risk

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of cancer recurrence by approximately 50%, a degree that has not been observed since the introduction of tamoxifen for hormone-receptor-positive tumors [2, 3]. However, this effective therapy is available only for those whose tumors are HER-2 positive, and this represents only 20% to 30% of all breast cancers. Although HER-2 negative tumors are perceived to carry a better prognosis than HER-2 positive tumors, there is a subset of patients whose tumors behave aggressively. The molecular mechanism(s) to account for this is poorly understood.

The vast majority of breast cancer related deaths are due to the development and subsequent progression of systemic disease. The development of metastases is a complex process involving multiple molecular factors [4, 5]. Many of these factors have characteristics common to lymphocyte homing and organogenesis, one of which is the chemokine receptor CXCR4 [6–8]. CXCR4 is a G protein-coupled seven-transmembrane receptor that has been linked to invasion and metastasis in a variety of cancers, including breast cancer [9]. Although multiple studies on CXCR4 have recently yielded promising results, CXR4's clinical significance has not been well elucidated.

In this study, we used a large prospective database of invasive breast cancer patients to evaluate the role of CXCR4 overexpression in cancer specimens to predict outcome. We believe that CXCR4 plays a pivotal role in predicting a worse outcome in a subset of patients with HER-2 negative tumors. A better understanding of its role may help identify a group of high risk patients who may be candidates for more intensive treatment and/or for novel targeted therapy.

MATERIALS AND METHODS

Approval to use our tissue bank and database was obtained from our institutional Internal Review Board (IRB). Complete clinicopathologic data and tissue specimens from 103 patients with stages I to III breast cancers were examined. Treatment and surveillance protocols were standardized to ensure study homogeneity. Compliance with treatment and surveillance protocol was 95% and 99%, respectively. Surgical treatment consisted of either a modified radical mastectomy or breast conservation therapy (BCT, lumpectomy with tumor-free margin, axillary lymph node dissection, and breast irradiation; a subset of patients who had T1 lesion underwent sentinel node biopsy, followed by a complete axillary lymph node dissection for those who had positive sentinel nodes). Adjuvant axillary irradiation, systemic chemotherapy, and antiestrogen therapy were offered and administered as indicated per current standard of care. Surveillance protocol consisted of a history and physical examination every 3 mo for 3 y, every 6 mo in years 4 and 5, and annually thereafter. Annual chest X-ray, mammogram, complete blood count, and liver function test were obtained. Any additional radiological and/or histological evaluation was performed based on the patient's examination and history. Clinical data were accrued and recorded prospectively and included age at diagnosis, comorbid conditions, stage of disease, treatment protocol, surveillance protocol compliance, and study endpoints. Study primary endpoints were cancer recurrence and cancer-related death.

Tissue Procurement

A cancer specimen of at least 100 mg was obtained from the tumor core at the time of surgery from each patient. The specimen was verified by the study pathologist to be an invasive mammary carcinoma. It was then immediately frozen in liquid nitrogen and stored at −70°C.

Assay for CXCR4

Specimen assay for CXCR4 expression was performed using Western blot analysis. In brief, a protein lysate from each breast specimen was prepared using a 10 mg portion of tumor tissue cut into tiny pieces, suspended in 0.5 mL RIPA buffer (150 mmol/L NaCl; 1% NP-40; 0.5% DOC; 0.1% SDS; 50 mmol/L Tris [pH 8.0]; 0.1 mmol/L PMSF), and mechanically homogenized using a Savant Bio 101 Fastprep FP120 system (Savant Instruments, Inc., Holbrook, NY). The lysate was then centrifuged at 10,000 *g* for 10 min (at 4°C), and total protein content was determined using a standard BCA (bicinchoninic acid) copper reduction assay kit (Pierce, Rockford, IL). An equal amount of protein lysate from each specimen as well as benign control breast tissue (20 μg diluted in 1:10 RIPA) was loaded onto and separated by using 4% to 20% denaturing gel Tris HCL polyacrylamide gel electrophoresis. Electroblotting onto a nylon membrane (Immobilon PVDF; Millipore, Bedford, MA) was performed, and the membranes were blocked with 5% nonfat milk for 1 h. Primary incubation of the membrane was performed using polyclonal goat x human anti-CXCR4 antibody (Fusin SC6190; Santa Cruz Biotechnology, Inc., Santa Cruz, CA). Secondary incubation of the membrane was performed using a bovine/goat horseradish peroxidase conjugate. Blot development was accomplished using Opti 4CN (4-chloro-1-naphthol; Bio-Rad Laboratories, Hercules, CA). Quantification of CXCR4 protein expression was accomplished using the Biophotonics system (Biophotonics Corp., Ann Arbor, MI). The blots were scanned and the band intensity was evaluated using the Intelligent Quantifier software (Bio Image, Ann Arbor, MI). Since benign breast tissues have undetectable level of CXCR4, 1 μg total protein from cell lysate of HeLa (ATCC no.CCL-2.2) cell lines (known to overexpress CXCR4) was used, to which all cancer specimens were compared. Band intensity from tumor samples was compared against 1 μg HeLa cells. Quantification of CXCR4 level in each cancer specimen was expressed as x-fold elevated over known concentration of HeLa cells. This process was repeated three times for each specimen and the results were averaged. Protein extracts from the following cells obtained from American Type Culture Collection (ATCC) were used as controls: (1) CRL6509 cells (normal rat kidney) served as a negative control, (2) HeLa (human cervical cancer) and HL60 (human acute myeloid leukemia) cells served as positive controls.

Assay for HER-2 Expression

The tumor's HER-2 status is defined as two or more, using the Hercep program (Dako Corp., Carpinteria, CA).

Estrogen and Progesterone Receptor Status

Estrogen receptor (ER) and progesterone receptor (PR) status was determined using immunohistochemical methods. Slides were stained and evaluated using the Dako Autostriker and the automated cellular imaging system. Activity greater than ten percent was considered positive.

Statistical Analysis

Statistical analyses were performed using MedCalc software (Microsoft, Inc.). Level of CXCR4 overexpression, tumor size, tumor grade, nodal, HER-2, ER and PR statuses were correlated using the samples *t*-test and Spearman rank correlation. Survival analysis was