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CXCR4 as a Predictive Marker for Locally Advanced Breast Cancer Post-Neoadjuvant Therapy[1]

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Background. **CXCR4 is a G-protein coupled receptor that has been linked with metastasis in several cancers, including breast cancer. We recently demonstrated that high CXCR4 levels in primary tumors of patients with breast cancer had a prognostic significance. We hypothesize that patients whose tumors had a low CXCR4 overexpression level following neoadjuvant chemotherapy will have a lower recurrence rate than those whose tumors remained high.**

Methods. **Seventeen locally advanced breast cancer (LABC) patients were accrued, and tumor specimens were obtained before and after neoadjuvant therapy. CXCR4 levels were quantified by Western blots against 1 μg of protein from HeLa cells. The primary end-point was cancer recurrence. Statistical tests utilized include Kaplan-Meier survival analysis and log-rank test. A P value ≤ 0.05 was considered significant.**

Results. **We previously defined low CXCR4 overexpression as ≤6-fold elevation and high overexpression as > 6-fold elevation over HeLa cells. Of 17 LABC tumors evaluated, 10 (59%) remained in the low group, 5 (29%) reduced from high to low overexpression, and 2 (12%) maintained a high overexpression after neoadjuvant therapy. With a median follow-up of 28 mo, patients whose tumors maintained a high CXCR4 overexpression level after neoadjuvant therapy had a significantly higher rate of cancer recurrence ($P = 0.0068$).**

Conclusions. **CXCR4 was a predictive molecular marker of response to neoadjuvant chemotherapy for patients with LABC. Patients whose tumors had a persistently high CXCR4 overexpression level after neoadjuvant therapy are at a significant risk for recurrence, and therefore, should be targeted for more intensive and/or novel therapy.**

Key Words: **chemokine receptor CXCR4; locally advanced breast cancer; predictive marker.**

INTRODUCTION

Locally advanced breast cancer (LABC) refers to primary breast cancers that are stages IIB or III based on the TNM staging system [1]. Compared with lower-staged breast cancers, LABC has a higher risk of recurrence and metastasis, and a worse overall survival. Neoadjuvant chemotherapy is the preferred initial treatment for patients with LABC. While neoadjuvant therapy has not shown to improve overall survival, it does offer several advantages [2]. Treatment can lead inoperable tumors to become operable [3], complete pathologic response can occur in up to 33% of patients, which portends a favorable outcome [4], and response to treatment can be a predictor of survival [5]. In addition, the neoadjuvant approach has a theoretical advantage of allowing for the genetic profiling of tumors with subsequent design of target-specific therapeutic regimen [6].

Genetic profiling of primary breast tumors, however, requires good molecular markers that have reliable prognostic and/or predictive value, of which there are few. One such common pathway regulator appears to be eukaryotic initiation factor 4E (eIF4E). It can serve as a strong prognostic factor for both node-negative [7] and node-positive [8] patients. Furthermore, we have shown that a reduction of eIF4E overexpression level following neoadjuvant therapy was predictive of an improved disease-free survival [9].

Another biomarker that may be of significance is CXCR4. CXCR4 is a seven-transmembrane chemokine receptor that has been implicated in the invasion and

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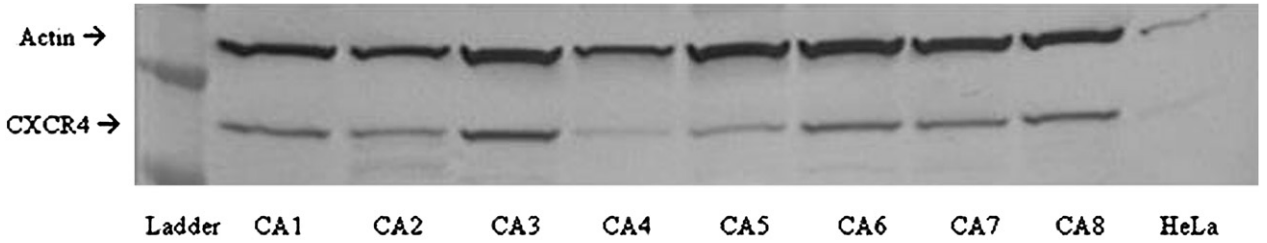

FIG. 1. Western Blot for CXCR4. Western blot for CXCR4 using protein extracted from breast cancer specimens. Lanes 2 through 9 show a varying degree of CXCR4 overexpression. Lane 10 is a positive control of HeLa cells, which are known to overexpress CXCR4.

metastasis of several cancers, including breast. Muller *et al.* found that CXR4 is involved in the pathway that activates actin polymerization and pseudopodia formation in breast cancer cells [10]. They demonstrated that organs that are common targets for breast cancer metastasis (i.e., brain, bone) have an increased amount of stromal derived factor-1 (SDF-1), the CXCR4 receptor ligand.

We recently showed that elevated CXCR4 expression in primary breast cancer specimens following neoadjuvant therapy for patients with LABC can serve as an important prognostic marker for outcome, independent of nodal status, tumor size, ER/PR status, and HER-2 status [11]. What has not been studied, however, is whether CXCR4 response to neoadjuvant therapy has a predictive value for patients with LABC. This study was initiated to determine if a significant reduction in CXCR4 overexpression level following neoadjuvant therapy was predictive of patient outcome for patients with LABC.

MATERIALS AND METHODS

Approval to perform this study was obtained from our Institutional Review Board (IRB). At least 100 mg of tumor specimen from 17 patients with locally advanced breast cancer (LABC) was obtained before and after neoadjuvant therapy. Tumor specimens were immediately frozen and stored in liquid nitrogen.

Study homogeneity was obtained by standardizing all treatment and surveillance protocols. Standard treatment protocols for neoadjuvant chemotherapy, radiation therapy, and biologic therapy were offered to all patients. The majority of patients received four cycles of adriamycin and cyclophosphamide. Definitive surgeries included either breast conservation therapy (BCT, lumpectomy with tumor-free margin, axillary lymph node dissection, and breast irradiation) or a modified radical mastectomy.

Surveillance protocol consisted of a history and physical examination every 3 mo for 3 y, every 6 mo in y 4 and 5, and annually thereafter. Annual chest X-rays, mammograms, complete blood counts, and liver function tests were obtained. Any additional biopsies or imaging were dictated by history and/or physical findings. Clinical data were accrued and recorded prospectively and included age at diagnosis, co-morbid conditions, stage of disease, treatment protocol, surveillance protocol compliance, cancer recurrence, and death. Compliance with treatment and surveillance protocols was over 90%. Patients were staged according to AJCC 6th Edition [1].

Specimen assays for CXCR4 expression were performed using Western blot analysis, as previously described [11, 12]. Protein lysate from each breast specimen was prepared using a 10-mg portion of tumor tissue cut in small pieces and suspended in a 0.5 mL RIPA buffer and homogenized using a Savant Bio 101 Fastprep FP120 system (Savant Instruments, Inc., Holbrook, NY). A standard BCA copper reduction assay kit (Pierce, Rockford, IL) was used to determine total protein content. Twenty μg of specimen protein lysate or benign control breast tissue was run on a 4% to 20% denaturing gel and electroblotted onto a nylon membrane (Immobilon PVDF; Millipore, Bedford, MA). Membranes were incubated with polyclonal goat $\times$ human anti-CXCR4 antibody (Fusin SC6190; Santa Cruz, CA). Secondary incubation was done with a bovine $\times$ goat horseradish peroxidase conjugate. Opti 4CN (4-chloro-1-naphthol; Bio-Rad Laboratories, Hercules, CA) was used for blot development and quantification of CXCR4 protein expression was done with the Biophotonics system (Biophotonics Corp., Ann Arbor, MI). After scanning the blots, band intensity was evaluated with the Intelligent Quantifier software (Bio Image, Ann Arbor, MI). Benign breast tissues contain undetectable levels of CXCR4 by this method, so 1 μg of total protein from cell lysate of HeLa (ATCC #CCL-2.2) cell line was used and compared with all cancer specimens. Band intensity of the tumor samples was compared to 1 μg of HeLa cells. Quantification of CXCR4 levels was expressed as x-fold elevated over known concentration of HeLa cells. This was repeated three times for each specimen and the results were averaged.

Statistical analysis was performed using MedCalc software (Microsoft, Inc., Redmond, WA, USA). Analyses included survival analysis using the Kaplan-Meier method, log-rank test, and independent t-test. A P value ≤ 0.05 was considered statistically significant.

RESULTS

Seventeen patients with LABC were accrued for this study. The median age at diagnosis was 57 y, and the median follow-up time was 28 mo. Figure 1 is a representative Western blot analysis for CXCR4 expression. Note that CXCR4 overexpression was observed in varying degrees in breast cancer specimens (lanes CA1-CA8). HeLa cells, known to overexpress CXCR4, were used as positive controls (HeLa lane).

TABLE 1

Neoadjuvant Therapy Regimens

Patient	Neoadjuvant therapy regimen
a, d, e, f, g, h, k, m, n, o, q	AC
i, j, l	Docetaxel-based
b	CMF
c	Anastrozole
p	Capecitabine, zoledronic acid

AC = doxorubicin (adriamycin) and cyclophosphamide; CMF = cyclophosphamide, methotrexate, fluorouracil.