Effect of Genetic Variants in Two Chemokine Decoy Receptor Genes, DARC and CCBP2, on Metastatic Potential of Breast Cancer

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Abstract

The inhibitory effect of two chemokine decoy receptors (CDRs), DARC and D6, on breast cancer metastasis is mainly due to their ability to sequester pro-malignant chemokines. We hypothesized that genetic variants in the DARC and CCBP2 (encoding D6) genes may be associated with breast cancer progression. In the present study, we evaluated the genetic contributions of DARC and CCBP2 to metastatic potential, indicated by lymph node metastasis (LNM). Ten single-nucleotide polymorphisms (SNPs) (potentially functional SNPs and block-based tagging SNPs) in DARC and CCBP2 were genotyped in 785 breast cancer patients who had negative lymph nodes and 678 patients with positive lymph nodes. Two non-synonymous SNPs, rs12075 (G42D) in DARC and rs2228468 (S373Y) in CCBP2, were observed to be associated with LNM in univariate analysis and remained significant after adjustment for conventional clinical risk factors, with odds ratios (ORs) of 0.54 (95% confidence interval [CI], 0.37 to 0.79) and 0.78 (95% CI, 0.62 to 0.98), respectively. Additional functional experiments revealed that both of these significant SNPs could affect metastasis of breast cancer in xenograft models by differentially altering the chemokine sequestration ability of their corresponding proteins. Furthermore, heterozygous GD genotype of G42D on human erythrocytes had a significantly stronger chemokine sequestration ability than homozygous GG of G42D ex vivo. Our data suggest that the genetic variants in the CDR genes are probably associated with the varied metastatic potential of breast cancer. The underlying mechanism, though it needs to be further investigated, may be that CDR variants could affect the chemokine sequestration ability of CDR proteins.

Introduction

The major cause of treatment failure and mortality from breast cancer is metastasis to distant organs [1]. Because breast cancer is a highly heterogeneous disease, it is a major challenge to predict the outcome of an individual patient [2]. Intensive research is currently underway to identify the genetic determinants that have a high diagnostic power for prognosis prediction, which would help identify patients who have a high risk of metastasis and to tailor therapeutic measures.

Chemokine and chemokine receptors have been highlighted for their vital roles in breast cancer progression and metastasis, involving proliferation, invasion, migration, senescence, angiogenesis, and regulation of host immune response [3,4]. It is evident that CCL2 (MCP-1), CCL5 (RANTES), and CXCL8 (IL-8) have pro-malignant activity that is associated with breast cancer progression and more advanced disease [5–8]. Furthermore, functional single-nucleotide polymorphisms (SNPs) located in the promoter region of CCL2 and CXCL8, and thus regulating transcription of these chemokines, have been associated with clinical breast cancer outcome [9–11].

Chemokines are not only mediated by transcriptional activity but also by posttranslational regulation. A chemokine decoy receptor (CDR), also called an atypical chemokine binder, is a new subgroup of chemokine receptors incapable of transmitting their signals through the classic G-protein-mediated pathways. They act as scavengers by efficiently internalizing their cognate chemokine ligands. The CDR group consists of at least three members: Duffy antigen receptor for chemokine (DARC), D6 (coded by CCBP2) and ChemoCentryx chemokine receptor (CCX-CKR) [12–14]. Previous research in our laboratory has demonstrated that DARC, D6, and CCX-CKR play inhibitory roles in breast cancer growth and metastasis, mainly by sequestration of the pro-malignant chemokines [15–17]. There are some potentially functional non-synonymous SNP in DARC and CCBP2 but no non-synonymous ones were found in CCX-CKR, therefore we did not investigate the CCX-CKR variation in present study.

Lymphatic and hematogenous dissemination are two common ways for breast cancer cells to spread. DARC is widely expressed...
on erythrocytes and vascular endothelial cells [18] while D6 is mainly expressed on lymphatic endothelial cells [19]. DARC and D6 present on blood and lymphatic vessels and on erythrocytes in the circulation serve as a systemic barrier to metastasis. Given the broad distribution of CDRs within the body, their inhibitory effects on cancer progression and metastasis, and the potential influence of genetic variants on gene expression and protein activity, we hypothesized that breast cancer patients carrying certain CDR genotypes may be more susceptible to tumor spread. To test our hypothesis, we investigated the relationship between lymph node metastasis (LNM) and ten genetic variations in DARC and CCBP2 in a cohort of patients with primary breast cancer. The biological mechanism was subsequently examined.

Materials and Methods

Ethics statement

All participants provided their written consent to participate in this study. This study was approved by the Science and Ethics Committee of the Shanghai Cancer Center and conforms to the principles outlined in the Declaration of Helsinki (IRB number: 050432-4-10087A).

All animal work was conducted in accordance with the National Institutes of Health ‘Guide for the Care and Use of Laboratory Animals’. The study protocol was approved by the Shanghai Medical Experimental Animal Care Committee.

Study subjects

The candidates for this study were from consecutive female patients at the Shanghai Cancer Hospital (between Jul.2006 and Dec. 2008) with pathologically confirmed operable primary invasive breast cancer. Subjects were identified as genetically unrelated Han Chinese from the Shanghai City and its surrounding regions. All patients underwent mastectomy or lumpectomy plus level I/II axillary lymph node dissection or sentinel node biopsy. Patient characteristics and tumor features were extracted from clinical documents. All data were eventually integrated into a computerized database established by our department, as described elsewhere [20]. The patients were excluded from this study if they had received neoadjuvant treatment or had bilateral breast cancer, ductal carcinoma in situ, or any history of other cancers. The histological type of the primary tumors was evaluated according to the WHO classification, and the Ellis & Elston system was used for histologic grading. The estrogen receptor (ER), progesterone receptor (PR), and human epidermal growth factor receptor-2 (HER2) statuses were determined by immunohistochemical staining, as previously described [21]. Routine pathologic examination of the lymph nodes was done with hematoxylin and eosin (H&E) staining; patients who had only micro-metastasis but no evidence of macro-metastasis were also excluded from the study. Finally, there were a total of 1,463 cases included, 785 with no evidence of macro-metastasis were also excluded from the study. All participants provided their written consent to participate in this study. This study was approved by the Science and Ethics Committee of the Shanghai Cancer Hospital and conforms to the principles outlined in the Declaration of Helsinki (IRB number: 050432−4−10087A).

Cell lines

The human breast cancer cell line MDA-MB-231 was obtained from the American Type Culture Collection (ATCC). Liquid nitrogen cell stocks were made upon receipt and stored until the start of each study. Morphology and doubling times were also recorded regularly to ensure maintenance of the phenotype. Cells were used for no more than 6 months after being thawed (most experiments were finished within approximately 4 months). Thus, all of the cell lines have been tested and authenticated by ATCC and maintained in our laboratory for fewer than 6 months, during which all experiments were conducted. Cells were routinely cultured in Leibovitz’s L-15 medium supplemented with 10% fetal bovine serum at 37° in a humidified 5% CO₂ atmosphere.

Plasmid constructs

DARC and D6 expression vectors were constructed using the pcDNA3.1 (+) plasmid (Invitrogen, USA). The full-length human cDNA for DARC and CCBP2 were amplified using the primers listed in Table S1. The fragment of DARC with the 42G allele of rs12075 was cloned between KpnI and XbaI sites of the vector to generate a ‘pcDNA3.1-DARC-42G’ construct. The fragment of CCBP2 with the 373S allele of rs2284682 was cloned between KpnI and EcoRI sites of the vector to generate a ‘pcDNA3.1-D6-373S’ construct. A site-directed mutagenesis kit (Stratagene, USA) was used to generate the ‘pcDNA3.1-DARC-42D’ and ‘pcDNA3.1-D6-373Y’ constructs, respectively. Both constructs were confirmed by sequencing.

Generation of stable transfectants

MDA-MB-231 cells were transfected with the same dose of plasmids or plasmid mixtures (1:1) for transient transfection, respectively. Stable transfectants were selected by G418 (Invitrogen, USA) and identified by RT-PCR, real-time PCR, and western blot. The procedure of generation of stable transfectants using plasmid mixture and selection by G418 has been described elsewhere [22]. We screened and selected the transfectants expressing similarly high levels of DARC and/or D6 for further experiments.

Cell proliferation was done by using Cell Counting Kit-8 (Dojindo). Invasion experiments were conducted with a Matrigel invasion chamber (BD Labware). Flow cytometry analysis of DNA content was done to assess the cell cycle phase distribution. Due to limited number of words, the descriptions of DNA/RNA preparation, transient transfection, RT-PCR, real-time PCR, western blot, immunohistochemistry, and enzyme-linked immunoabsorbent assay (ELISA), are supplied in Text S1.

Animal experiments

Four- to six-week-old athymic female BALB/c nu/nu mice were used in this study. A cohort of seventy nude mice was divided into seven groups of ten mice each. Cells (2×10⁶) were inoculated into the anesthetized mice in 100 μl of culture medium. The tumorigenicity of the cell lines was determined by injection into the cleared mammary fat pad of the mice. The volume of tumors was calculated by the following formula: volume = 0.5×length×width². The mice were examined weekly for tumor appearance and growth. For ethical reasons, the primary tumors were surgically removed while mice were under Ketaset-Rompun anesthesia at five-weeks postinoculation. Mice were then maintained to allow further growth of lung metastases. Mice were sacrificed and autopsied at 12 weeks after initial tumor cell inoculation. DARC and D6 expression levels in the xenografts were assessed by western blot. The lung tissues were cut into 5 μm slices, and one in every ten sections was stained with H&E to evaluate the formation of lung metastasis.

Isolation of human erythrocytes and erythrocytes chemokine sequestration assay

Within two hours of the whole blood collection, erythrocytes were purified using a technique as previously described [23]. Briefly, whole blood from patients with early-stage primary breast cancer was obtained via venipuncture and collected in K₂-EDTA tubes (BD, Biosciences, USA). About 1 ml of the fresh whole blood...
was passed over a 5 ml syringe packed with a 2 ml column containing a mixture of microcrystalline cellulose (type 50) and cellulose (1:1 by weight; Sigma-Aldrich, USA). The column was washed with 9 ml of sterile phosphate-buffered saline (PBS). The eluent was centrifuged at 1000 g for 4 minutes at 4°C. The erythrocyte pellet was washed 3 times with cool, sterile PBS. The erythrocyte concentrates were stored at 4°C in 50 ml polypropylene tubes [BD, Biosciences, USA]. MDA-MB-231 cells were routinely cultured for 24 hours before the erythrocytes chemokine sequestration experiments. Freshly isolated erythrocytes from breast cancer patients with known genotypes were immediately counted manually. Conditioned media from the MDA-MB-231 cells were then incubated with 2×10⁷ erythrocytes or PBS at 37°C for 1 hour. Erythrocytes were pelleted by centrifugation. The supernatant was collected and frozen at −80°C.

**Statistical analysis**

A Student’s test or Mann-Whitney test was used to compare continuous variables between the two groups. Tests of association were conducted using the Pearson’s $\chi^2$ test. The odds ratio (OR) and its 95% confidence intervals (CIs) were also determined. The significance levels of single locus association results were corrected using a false discovery rate (FDR) [24]. In FDR, as soon as one voxel in this ascending list of p-value sorted voxels is found that does not meet with the correction criterion, then all subsequent voxels are assumed to belong to the falsely claimed active voxels. Multivariate logistic regression was used to study the association between a single locus and the risk of LNM by adjusting for clinicopathological factors (method: forward stepwise, likelihood ratio). A $P$-value of <0.05 (two-sided) was considered statistically significant. Statistical analysis was performed using Stata/SE version 10.0 (StataCorp, USA) and SPSS Software version 12.0 (SPSS, USA).

**Results**

Subject characteristics

The clinicopathological characteristics of the breast cancer patients included in this study are summarized in Table 1, which shows that the mean age in the group with LN+ was about two years lower than that in the group without LN- ($P<0.001$). Patients with LN+ had a larger tumor size ($P<0.001$), a higher frequency of poorly differentiated tumor ($P<0.001$), and a higher proportion of hormone receptor negative tumor ($P<0.001$) and HER2 positive tumor ($P<0.001$) compared to those without LNM.

**Selection of genetic variants and genotyping**

Because of the limited data available on Chinese population, DARC genetic variants in the HapMap database, we screened polymorphisms across the DARC genetic region and its flanking sequences (from approximately 1.0 kb upstream to 0.5 kb downstream of DARC) by directly sequencing the PCR products of genomic DNAs from the blood samples of 24 patients with sporadic breast cancer. This sub-sample was randomly selected from the total sample pool of our study. The primers used for identifying DARC SNPs are listed in Table S2. As a result, two polymorphisms, rs3027012, with a minor allele frequency (MAF) of 0.021 in 5’-flanking region, and rs12075 (G42D) with a MAF of 0.062 in exon 2, were identified (Figure S1A). SNP rs2814778, which was identified in the other ethnic samples previously, was not found in our sub-sample of 24 subjects (21, 22). Considering the potential biological function of rs2814778 (also recorded as T-46C, a mutation in DARC promoter disrupting a GATA consensus binding site), it was also selected for further genotyping within a much larger sample.

For CCBP2, SNPs were surveyed in the region spanning 59.3 kb from 1.0 kb upstream to 0.5 kb downstream of the transcribed sequence of CCBP2 in the NCBI-bSNP and the International HapMap websites. The HapMap database of the Han Chinese population (HapMap Data Rel 27/phase II+III) was used. Tagging-SNPs (tSNPs) were selected using the pairwise method under the restriction of MAFs >0.05 and $r^2 ≥0.8$, with the aim of identifying a set of tSNPs that efficiently captures all known common variants and that is likely to tag most unknown variants. In all, ten tSNPs were identified (rs9815043, rs4682859, rs17317763, rs7653015, and rs4683335). Each of these six SNPs was located in an intron and was thus excluded from further genotyping within a much larger sample.

Table 1. Clinicopathological characteristics of the breast cancer patients.

| Characteristics | Number (%) | $P$ |
|-----------------|------------|-----|
| Age (years) mean±SD | 50±10.5 | 48.6±9.7 | <0.001 |
| Tumor size ≤2 cm | 484 (61.7) | 268 (39.5) | <0.001* |
| 2–5 cm | 262 (33.4) | 316 (46.6) | |
| >5 cm | 30 (3.8) | 75 (11.1) | |
| Unknown | 9 (1.1) | 19 (2.8) | |
| ER Positive | 638 (81.3) | 471 (69.5) | <0.001* |
| Negative | 138 (17.6) | 188 (27.7) | |
| Unknown | 9 (1.1) | 19 (2.8) | |
| PR Positive | 592 (75.5) | 439 (64.8) | <0.001* |
| Negative | 184 (23.4) | 220 (32.4) | |
| Unknown | 9 (1.1) | 19 (2.8) | |
| HER2 Positive | 166 (21.2) | 272 (40.1) | <0.001* |
| Negative | 610 (77.7) | 387 (57.1) | |
| Unknown | 9 (1.1) | 19 (2.8) | |
| Grade I | 125 (15.9) | 42 (6.2) | <0.001* |
| II | 493 (62.9) | 376 (55.5) | |
| III | 158 (20.1) | 241 (35.5) | |
| Unknown | 9 (1.1) | 19 (2.8) | |
| Pathology IDC | 699 (89.1) | 601 (88.7) | 0.408 |
| ILC | 65 (8.3) | 51 (7.5) | |
| Others | 21 (2.6) | 26 (3.8) | |

SD, standard deviation; LN-, positive lymph node; LN+, negative lymph node; IDC, invasive ductal carcinoma; ILC, invasive lobular carcinoma; ER, estrogen receptor; PR, progesterone receptor; HER2, human epidermal growth factor receptor-2.

*based on a two-sided $\chi^2$ test excluding the missing values.

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changes or alternative splicing) were chosen for genotyping whenever possible. Two potentially functional polymorphisms with MAFs >0.01 were used; they consisted of the SNP rs2228468 (Ser373Tyr, S373Y) and rs1366046 (in the 3′-untranslated region [UTR]) Figure S1B. The plot of pairwise linkage disequilibrium (LD) among selected variants in the CCBP2 gene is shown in Figure S1C.

In total, ten SNPs (three in DARC and seven in CCBP2) were selected for further genotyping. Genotyping work was performed using the 12-plex SNPStream system and was done by the Chinese National Human Genome Center in Shanghai. The sequences of the primers and probes for each SNP are listed in Table S3. In addition, 10% of the samples were randomly selected for re-genotyping for each of the ten SNPs and the results were 100% concordant.

Association between LNM and SNPs in CDRs

Among the 10 SNPs selected for genotyping, no variation was observed for rs2814778 and therefore this SNP was excluded from the analysis. The remaining nine SNPs were successfully genotyped with genotyping call rates ranging from 95.0% to 99.7%.

There were significant associations between LNM and four of the studied SNPs, i.e., rs3027012 and rs12075 in DARC, and rs2228468 and rs1366046 in CCBP2, with the minor alleles having lower frequencies in the LNM patients compared with those without LNM (Table 2). After correction of multiple comparisons by FDR, the non-synonymous SNP, rs12075 (G42D), maintained a significant association with LNM (\(P = 4.0 \times 10^{-5}\)), and another non-synonymous SNP, rs2228468 (S373Y), remained borderline statistically significantly associated (\(P = 0.052\)). Compared with the G-allele, the A-allele of rs12075 could decrease the possibility of LNM by 50% (OR = 0.50, 95% CI, 0.35–0.69). Similar to results in the allelic genotype analysis, the data from genotype analysis indicated that the genotypes of rs3027012 and rs12075 in DARC and of rs2228468 in CCBP2 were significantly correlated with LNM in a dominant model (\(P = 0.035\), \(P = 3.36 \times 10^{-5}\), and \(P = 0.027\), respectively, Table 2), but not in a recessive model (data not shown).

Finally, we predicted the overall value of the four crude significant SNPs in DARC and CCBP2 for the risk of LNM. Traditional variables of LNM also were chosen for the regression model. We found that rs12075 in DARC and rs2228468 in CCBP2 were independent risk factors for LNM, with ORs (95% CI) of 0.54 (0.37–0.79) and 0.78 (0.62–0.98), respectively (Table 3).

Significant SNPs in DARC and CCBP2 do not have differential biological effects on gene expression in vitro

Although the two SNPs in DARC and CCBP2 were associated with LNM in our epidemiological study, the precise mechanism is still unclear. We therefore examined whether the DARC-42G and DARC-42D alleles or the CCBP2-373S and CCBP2-373Y alleles had differential effects on DARC and D6 expression, respectively. No difference in expression was found between DARC-42G and DARC-42D or between CCBP2-373S and CCBP2-373Y in breast cancer cells, respectively (RT-PCR in Figure S2A, real-time PCR in Figure S2B, and western blot in S2C). No interaction effect of these two SNPs on gene expression was observed either.

We also generated stable transfectants of DARC. In this procedure, we aimed to obtain stable transfectants that expressed similarly high DARC despite having different alleles (42G and 42D). Similarly, we generated stable transfectants of D6 with different alleles (373S and 373Y). Because we had demonstrated that both of these SNPs (G42D and S373Y) had no effect on the differential expression of genes, the similar expression between DARC-42D and DARC-42G (or CCBP2-373Y and CCBP2-373S) in stable transfectants indicated that there was a comparable number of vector copies integrated into the genome of the MDA-MB-231 cells. Individual clones expressing similarly high levels of DARC and/or D6 were selected for further experiments.

In addition, to investigate whether these two SNPs could modulate the proliferation and invasion of MDA-MB-231 cells in vitro, we assessed the growth and invasion of DARC-42D and DARC-42G (or CCBP2-373Y and CCBP2-373S) transfectants. However, neither DARC-42G nor CCBP2-373S had an effect on the proliferation and invasion of cells compared to their wild-type counterpart. Moreover, no obvious changes in the cell cycle distribution could be seen between wild-type allele and variant allele for either DARC or CCBP2.

Significant SNPs in DARC and CCBP2 alter the chemokine sequestration ability of their corresponding proteins in vitro

Previous research in our lab has demonstrated that high-expression of DARC or D6 in human breast cancer cells induces inhibition of tumorigenesis and metastasis by clearing pro-malignant chemokines. We therefore examined whether the variants G42D and S373Y had any differential effects on chemokine sequestration ability. Similar to our previous results, the CXCL1, CXCL8, CCL2, and CCL5 levels were significantly lower in both the 231-DARC-42G and 231-DARC-42D transfectants compared with the controls (\(P<0.05\)). Similarly, the CCL2, CCL17, and CCL22 levels were significantly lower in both the 231-D6-373S and 231-D6-373Y transfectants compared with the control cells (\(P<0.05\)). Regarding the effect of G42D, the 231-DARC-42D transfectant had an additional 30–35% decrease in the CCL2 level relative to the 231-DARC-42G transfectant (\(P=0.031\), \(P=0.028\), and \(P=0.027\), respectively). Regarding S373Y, the 231-D6-373Y transfectant had an additional 16.4% decrease in the CCL2 level relative to the 231-D6-373S transfectant, with a borderline \(P\)-value of 0.069 (Table 4).

Having observed differential chemokine sequestration ability of each SNP, we further investigated the joint effect of rs12075 and rs2228468. These two SNPs had a synergistic effect on altering the chemokine sequestration capabilities of their CDR proteins. For instance, 231-DARC-42D and 231-D6-373Y decreased the CCL2 levels by 53% and 33%, respectively. When the 2 SNPs work together, the theoretical clearance rate for synthetic effect should be 69% (calculated by the formula: [53%+(1–53%)×33%]). Actually, the combination of the two proteins harboring the two SNPs (231-DARC-42D-D6-373Y) reduced the CCL2 level by 80%, much higher than 69%. A similar trend was observed in the CCL5 levels (Table 4).

Significant SNPs in DARC and CCBP2 have differential effects on inhibition of tumor growth, angiogenesis, and lung metastasis by interfering with chemokine sequestration ability in vivo

The effects of the two significant SNPs on tumorigenicity and metastasis potential in vivo were further investigated by using an orthotopic xenograft tumor model in nude mice. Significantly higher DARC and D6 expression levels were observed in tumors formed by DARC and/or D6 high-expressing transfectants compared with the controls (Figure S3). The xenografts with either DARC or D6 high expression levels had slower growth than the controls (Figure 1A and 1B). The mean tumor volume was
| Gene | SNP | Allele Location | Number (%) | OR (95%CI) | \( p^* \) | \( p^{**} \) | OR (95%CI) | \( p^{**} \) |
|------|-----|----------------|------------|-------------|---------|---------|-------------|---------|
| **LN−** | **LN+** |
| DARC | rs3027012 | C | S' near region | 1523 (98.4) | 1338 (99.3) | Reference | 0.036 | 0.108 |
| | | T | | 25 (1.6) | 10 (0.7) | 0.46 (0.22–0.95) | |
| | | CC | | 749 (96.8) | 664 (98.5) | Reference | Reference |
| | | TC | | 25 (3.2) | 10 (1.5) | 0.45 (0.22–0.95) | 0.035 |
| | | TT | | 0 (0) | 0 (0) | N.A. | N.A. |
| | | TC+TT | | 250 (3.2) | 10 (1.5) | 0.45 (0.22–0.95) | 0.035 |
| DARC | rs12075 | G | exon 2 (G42D) | 1412 (92.4) | 1253 (96.1) | Reference | 4.7 × 10^{-5} | 4.0 × 10^{-4} |
| | | A | | 116 (7.6) | 51 (3.9) | 0.50 (0.35–0.69) | |
| | | GG | | 649 (48.9) | 601 (92.2) | Reference | Reference |
| | | GA | | 114 (14.9) | 51 (7.8) | 0.48 (0.34–0.68) | 4.25 × 10^{-5} |
| | | AA | | 92 (12.3) | 51 (7.8) | 1.11 (0.59–2.11) | |
| | | GA+AA | | 208 (15.1) | 102 (13.8) | 0.48 (0.34–0.68) | 3.36 × 10^{-5} |
| CCBP2 | rs4682857 | C | intron 1 | 1300 (83.8) | 1098 (84.1) | Reference | 0.82 | N.S. |
| | | G | | 252 (16.2) | 208 (15.9) | 0.98 (0.80–1.19) | |
| | | CC | | 544 (70.1) | 464 (71.1) | Reference | Reference |
| | | CG | | 212 (27.3) | 170 (26.0) | 0.94 (0.74–1.19) | 0.61 |
| | | GG | | 20 (2.6) | 19 (2.9) | 1.11 (0.59–2.11) | 0.74 |
| | | CG+GG | | 232 (29.9) | 189 (28.9) | 0.96 (0.76–1.20) | 0.69 |
| CCBP2 | rs4682859 | G | intron 2 | 948 (63.6) | 837 (64.9) | Reference | 0.94 | N.S. |
| | | A | | 542 (36.4) | 453 (35.1) | 0.95 (0.81–1.11) | |
| | | GG | | 295 (39.6) | 262 (40.6) | Reference | Reference |
| | | GA | | 358 (48.1) | 313 (48.5) | 0.98 (0.79–1.23) | 0.89 |
| | | AA | | 92 (12.3) | 51 (7.8) | 0.86 (0.60–1.22) | 0.39 |
| | | GA+AA | | 450 (60.4) | 383 (54.9) | 0.96 (0.77–1.20) | 0.70 |
| CCBP2 | rs4683342 | C | intron 2 | 969 (63.1) | 852 (64.9) | Reference | 0.82 | N.S. |
| | | G | | 567 (36.9) | 460 (35.1) | 0.92 (0.79–1.08) | |
| | | CC | | 299 (38.9) | 269 (41.0) | Reference | Reference |
| | | CG | | 371 (48.3) | 314 (47.9) | 0.94 (0.75–1.18) | 0.59 |
| | | GG | | 98 (12.8) | 70 (10.9) | 0.93 (0.59–1.71) | 0.28 |
| | | CG+GG | | 469 (61.1) | 387 (59.0) | 0.92 (0.74–1.13) | 0.43 |
| CCBP2 | rs9815043 | G | intron 2 | 1073 (69.1) | 950 (71.1) | Reference | 0.25 | N.S. |
| | | A | | 479 (30.9) | 386 (28.9) | 0.91 (0.78–1.07) | |
| | | GG | | 361 (46.5) | 325 (48.7) | Reference | Reference |
| | | GA | | 351 (45.2) | 300 (44.9) | 0.95 (0.77–1.18) | 0.64 |
| | | AA | | 64 (8.2) | 43 (6.4) | 0.75 (0.49–1.13) | 0.17 |
| | | GA+AA | | 415 (53.5) | 343 (51.3) | 0.92 (0.75–1.13) | 0.42 |
| CCBP2 | rs3732859 | A | exon 3 | 1477 (94.3) | 1284 (95.3) | Reference | 0.30 | N.S. |
| | | G | | 89 (5.7) | 64 (4.7) | 0.83 (0.59–1.15) | |
| | | AA | | 696 (48.9) | 611 (90.7) | Reference | Reference |
| | | GA | | 85 (10.9) | 62 (9.2) | 0.83 (0.59–1.17) | 0.29 |
| | | GG | | 2 (0.3) | 1 (0.1) | 0.57 (0.05–6.30) | 0.65 |
| | | GA+AA | | 87 (11.1) | 63 (9.3) | 0.82 (0.59–1.16) | 0.27 |
| CCBP2 | rs2228468 | G | exon 3 (S373Y) | 1076 (69.0) | 993 (73.2) | Reference | 0.0116 | 0.052 |
| | | T | | 484 (31.0) | 363 (26.8) | 0.81 (0.69–0.95) | |
| | | GG | | 363 (46.5) | 355 (52.4) | Reference | Reference |
| | | TG | | 350 (44.9) | 283 (41.7) | 0.83 (0.67–1.02) | 0.082 |
| | | TT | | 67 (8.6) | 40 (5.9) | 0.61 (0.40–0.93) | 0.021 |
| | | TG+TT | | 417 (53.5) | 323 (47.6) | 0.79 (0.64–0.97) | 0.027 |

Table 2. Allele and genotype frequencies of polymorphisms in DARC and CCBP2 in relation to lymph node metastasis.
Comparison between two alleles of SNP G42D in CCL2, CCL17, and CCL2.

Table 4.

| Table 4. Chemokine levels in the supernatant of the cells detected by ELISA after 24-hour incubation. |
|-----------------|-----------------|-----------------|-----------------|-----------------|-----------------|-----------------|-----------------|-----------------|-----------------|
| Concentration (pg/ml) | MDA-MB-231 | 231-vector | 231-DARC-42D | 231-DARC-42G | P* | 231-D6-373S | 231-D6-373Y | P* | 231-DARC-42D |
| CXCL1            | 2447.0±93.3   | 2413.0±89.9   | 1795.0±80.0   | 1755.0±88.1   | 0.753 | N.D.      | N.D.      | N.D.      | N.D.          |
| CXCL8            | 568.3±18.8    | 560.0±17.3    | 271.0±21.9    | 177.7±18.2    | 0.031 | N.D.      | N.D.      | N.D.      | N.D.          |
| CCL2             | 280.3±11.6    | 274.3±11.6    | 183.3±13.0    | 127.7±10.1    | 0.028 | 219.0±9.5 | 183.3±10.9 | 0.069 | 54.9±6.3      |
| CCL5             | 250.4±1.4     | 25.7±1.3      | 18.2±1.2      | 12.6±1.1      | 0.028 | 25.4±1.4 | 24.5±1.2  | 0.665 | 84.1±1.0      |
| CCL17            | 238.3±7.3     | 238.7±7.9     | N.D.          | N.D.          | -    | 135.0±8.7 | 111.7±9.3 | 0.140 | N.D.          |
| CCL22            | 425.6±13.2    | 428.7±13.9    | N.D.          | N.D.          | -    | 326.7±11.7 | 303.3±13.0 | 0.253 | N.D.          |

*Comparison between two alleles of SNP G42D in DARC.
†Comparison between two alleles of SNP S373Y in CCBP2 (coding D6).
‡Comparison between two alleles of SNP S373Y in CCBP2 (coding D6). Columns, mean of three independent experiments with standard error. N.D., not detected.
SNPs in CDR Genes Affect Breast Cancer Metastasis

A  
B  

|     | MDA-MB-231 | 231-vect | 231-DARC-42G | 231-DARC-42D | 231-D6-373S | 231-D6-373Y |
|-----|-------------|----------|---------------|---------------|--------------|--------------|
| mouse CCL2 | 44.5±2.0    | 44.7±1.5 | 27.7±1.5 *   | 21.7±1.2 *   | 36.7±1.3    | 32.5±1.2 *   |
| mouse CCL5 | 12.7±0.7    | 12.8±0.9 | 9.3±0.7       | 6.5±0.6 *    | 8.8±0.7     | 9.0±0.6      |

D  

E  

F  

G  

CD34 (×200)  
Lung Metastasis (×100)

Microvessel density  
Metastasis number per lung
microvessels in the DARC and/or D6 high-expressing tumors than in the control tumors. In particular, the number of microvessels in the tumors formed by 231-DARC-42D was nearly 70% of the number formed by 231-DARC-42G. However, there was no difference in the microvessel counts between the tumors formed by 231-D6-373S and 231-D6-373Y. Tumors harboring both DARC and D6 had a synergistic effect on the inhibition of angiogenesis (Figure 1E and 1F).

The effects of the significant SNPs on ability to metastasize in vivo were also investigated. The incidences of lung metastasis were 10/10, 10/10, 6/10, 4/10, 5/10, 6/10, 2/10 for mice injected with MDA-MB-231, 231-vect, 231-DARC-42G, 231-DARC-42D, 231-D6-373S, 231-D6-373Y, and 231-DARC-42D-D6-373Y, respectively. Furthermore, mice injected with DARC and/or D6 high-expressing transfectants had significant fewer lung metastasis nodules than those with control cells. Similarly, the number of lung metastasis nodules in the mice injected with 231-DARC-42D was approximately 73% of the number of nodules in the mice injected with 231-DARC-42G (P=0.037). There was not a significant difference in the numbers of lung metastasis nodules between the mice injected with 231-D6-373Y and those injected with 231-D6-373S. But a synergistic role in the inhibition of lung metastasis was also observed (Figure 1E and 1G).

Variation G42D in DARC influences the chemokine sequestration ability of erythrocytes ex vivo

DARC protein is an abundant receptor expressed on erythrocytes. The effect of rs12075 (G42D) on the chemokine sequestration ability of different genotype erythrocytes was examined ex vivo. The CCL2, CCL5, and CXCL8 levels were significantly lower in the supernatant of the conditioned media that had been incubated with the erythrocytes of the 42GD heterozygote than those incubated with the erythrocytes of the 42GG homozygote (P=0.007, P=0.004, and P=0.008, Figure 2).

Discussion

The present study was based on the hypothesis that SNPs in the genes encoding CDRs that determine pro-malignant chemokine concentrations should also be associated with breast cancer metastasis. Lymph nodes are often the first sites where breast cancer cells spread [25]. It is well established that LNM is an independent indicator of aggressive behavior of primary breast cancer [26]. Here we chose ‘lymph node involvement’ as the surrogate for ‘phenotype of metastasis’, and 10 SNPs in DARC and CCBP2 were analyzed in a cohort of Han Chinese breast cancer patients to test our hypothesis. After a multivariate analysis, with adjustment for classic LNM risk factors, significant associations between two polymorphisms, rs12075 in DARC and rs2228468 in CCBP2, and the risk of LNM were found. Functional assays revealed that the DARC encoded by the DARC-42D allele had increased chemokine-sequestration ability compared with that encoded by the DARC-42G allele. Moreover, DARC-42D had an enhanced inhibitory effect on tumor growth and metastasis in xenograft models. The synergistic effect of chemokine sequestration ability was observed when DARC and D6 were combined. The results in our current study consistently suggest that rs12075 in DARC and rs2228468 in CCBP2 are two important genetic determinants for breast cancer metastasis potential, mainly by regulating the pro-malignant chemokine levels in the tumor microenvironment.

Initially, DARC was introduced as the antigen of the Duffy blood group system that consists of two variants, Fyα and Fyβ, which are identified by the Gly42Asp polymorphism (rs12075, G42D) [27,28]. Recently, this non-synonymous SNP was identified as a major determinant of circulating CCL2 concentration in a genome-wide association study [29]. But no data have been presented describing an association between SNPs in CCBP2 and cancer initiation or progression. The biological function of the two non-synonymous SNPs (rs12075 in DARC and rs2228468 in CCBP2) in the regulation of cancer metastasis is largely unknown,

![Figure 2](https://example.com/figure2.png)
and we proposed that the mechanism by which the two SNPs respectively alter DARC and D6 function may be mediated by amino acid substitution. In the functional assays, DARC-42D led to a higher ability of sequestering the pro-malignant chemokines than DARC-42G in tumor cell lines in vitro and in xenograft tumors in vivo. Furthermore, human erythrocytes carrying the DARC-42GD genotype in an ex vivo model showed significantly stronger chemokine sequestration ability than their counterparts carrying the DARC-42GG genotype. Additionally, microvessel density in DARC-42D expressing tumors is also lower than that in DARC-42G expressing tumors. Metastasis is a multi-step and complex process. Our experimental data suggest that DARC isoforms in the tumor microenvironment could reduce the levels of pro-malignant chemokines (such as CXCL2, CCL5, and CXCL8) in different degrees, have differential effects on tumor growth and vascularization, and contribute to differential potential of metastasis. Taken together, the change in DARC function due to the non-synonymous SNP rs12075 may interfere with the metastatic potential of breast cancer via altered chemokine sequestration ability. Another non-synonymous SNP in CCBP2, rs2228468, showed limited differential effects on chemokine sequestration ability and metastatic potential. The biological function of this SNP should be further elucidated. Notably, it was evident that the combination of DARC and D6 displayed a synergistic effect on clearing CCL2 and CCL5. One reasonable explanation is that DARC and D6 have overlapping functions in the sequestration of CCL2 and CCL5, binding to these chemokines with high affinity [30,31]. Therefore, DARC and D6 can act together to clear more pro-malignant chemokines. Alternatively, since CCL2 is central to the inflammatory process and it could upregulate the release of CCL5 from endogenous pre-made vesicles in breast cancer cells, it is reasonable that the mouse tumors expressing both DARC and D6 significantly reduced mouse CCL2, thus resulting in significantly lower mouse CCL5 level detected in those tumors. Therefore, it is expected that the patients carrying certain multiple genotypes of CDR genes might have a critically modified chemokine network in the tumor microenvironment, thus being far less susceptible to breast cancer metastasis.

Our study has several limitations. First, LNM could be due to later stage at diagnosis and does not absolutely represent aggressiveness or potential for metastasis; there could be metastasis without lymphatic invasion. In this study, we chose LNM as the surrogate of a metastasis phenotype; this choice of endpoint for metastasis should be further justified. Second, this study has a relatively small sample size. The promising association between SNPs and the metastatic potential of breast cancer needs to be replicated in other large-scale, independent population sets. Third, immune cell infiltration in xenograft tumors generated from stable transfectants expressing different isoforms of DARC or D6 remains to be assessed. Despite of these limitations, our data suggest that rs12075 and rs2228468 act as indicators of altered metastatic potential of breast cancer in Han Chinese patients.

In summary, our study shows an association between rs12075 in DARC and rs2228468 in CCBP2 and susceptibility to breast cancer metastasis. The two SNPs, which cause amino acid substitutions, might lead to differential chemokine sequestration ability, which may be the underlying mechanism that confers LNM risk. Although further investigation on detailed mechanism is still needed, our findings probably support the hypothesis that genetic polymorphisms in the genes encoding CDRs could mediate metastatic risk.

**Supporting Information**

**Text S1** Supplemental Materials and Methods.

(PPT)

**Table S1** Primers for discovering SNPs in *DARC*.

(DOC)

**Table S2** Primers for the plasmid constructs, RT-PCR and real-time PCR.

(DOC)

**Table S3** Primers and probes for the SNP Stream platform.

(DOC)

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**Author Contributions**

Conceived and designed the experiments: CY KDY ZLO ZMS. Performed the experiments: CY KDY WHX AXC LF. Analyzed the data: CY KDY. Contributed reagents/materials/analysis tools: ZMS. Wrote the paper: CY KDY WHX AXC LF ZLO ZMS.
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