Investigation of CD28 Gene Polymorphisms in Patients with Sporadic Breast Cancer in a Chinese Han Population in Northeast China

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Abstract

Background: CD28 is one of a number of costimulatory molecules that play crucial roles in immune regulation and homeostasis. Accumulating evidence indicates that immune factors influence breast carcinogenesis. To clarify the relationships between polymorphisms in the CD28 gene and breast carcinogenesis, a case-control study was conducted in women from Heilongjiang Province in northeast of China.

Methodology/Principal Findings: Our research subjects consisted of 565 female patients with sporadic breast cancer and 605 age- and sex-matched healthy controls. In total, 12 single nucleotide polymorphisms (SNPs) in the CD28 gene were successfully determined using the polymerase chain reaction-restriction fragment length polymorphism (PCR-RFLP) method. The relationship between the CD28 variants and clinical features, including histological grade, tumor size, lymph node metastasis, human epidermal growth factor receptor 2 (C-erbB2), estrogen receptor (ER), progesterone receptor (PR), and tumor protein 53 (P53) status were analyzed. A statistically significant association was observed between rs3116496 and breast cancer risk under different genetic models (additive P = 0.0164, dominant P = 0.0042). Different distributions of the rs3116496 'T' allele were found in patients and controls, which remained significant after correcting the P value for multiple testing using Haploview with 10,000 permutations (corrected P = 0.0384). In addition, significant associations were observed between rs3116487/rs3116494 (D' = 1, r² = 0.99) and clinicopathological features such as C-erbB2 and ER status, in breast cancer patients.

Conclusions/Significance: Our findings indicate that CD28 gene polymorphisms contribute to sporadic breast cancer risk and have a significant association with clinicopathological features in a northeast Chinese Han population.

Introduction

Breast cancer is the most commonly diagnosed female cancer worldwide. It is now estimated as the leading cause of cancer death among women in developing countries [1]. The etiology of breast cancer has not been completely elucidated, but is thought to be multifactorial, with both environmental and genetic factors [2].

The immune system serves as an important natural barrier to cancer development. Innate and adaptive responses are carefully orchestrated through soluble and membrane-bound receptors to eliminate precancerous cells and control neoplastic progression. Avoiding immune destruction has been considered a hallmark of cancer [3,4]. It is therefore of great interest to identify immune genes that influence susceptibility to breast cancer.

Costimulatory signals contribute to immune regulation and homeostasis [5,6,7,8]. CD28, as the best characterized costimulatory molecule, is constitutively expressed by the vast majority of T lymphocytes. Upon interaction with its ligand B7-1 (CD80)/B7-2 (CD86), CD28 mediates signals that promote T lymphocyte differentiation and proliferation, and enhance antibody production of B lymphocytes [9,10,11]. T lymphocytes are the central regulator of the anti-tumor adaptive immune response [12]. Lack of costimulatory signals leads to T lymphocyte tolerance and anergy [6]. It has been demonstrated that deficiencies in both CD28 and inducible costimulator (ICOS) pathways result in complete T lymphocyte tolerance and anergy [13,14]. Both CD4+ and CD8+ memory T cells need CD28 costimulation to achieve maximal expansion and pathogen clearance [15]. The blockade of the CD28-B7 interaction has been used to down-
regulate the activation of the immune system in autoimmune diseases [16]. Cytotoxic T-lymphocyte-associated antigen 4 (CTLA-4), which competes with CD28 for B7 binding, has been widely accepted as a new promising target for cancer immunotherapy. It has been reported that the anti-CTLA-4 monoclonal antibody ipilimumab blocks the activation of immunosuppressive CTLA-4, and thus induces tumor regression [17,18].

SNPs are the most common genetic variations which influence interindividual predisposition to breast carcinogenesis and prognosis [19]. SNP association analysis has provided valuable information about the genetic susceptibility of breast cancer [20]. The human CD28 gene is located in the chromosome 2q33 region. Polymorphisms in the CD28 gene were previously shown to be genetically associated with autoimmune diseases, such as rheumatoid arthritis and Bechet’s disease [21,22]. Several compelling reports characterized the association between this susceptibility loci and cervical cancer risk in different ethnic groups [23,24,25,26,27]. Chen et al. [23] and Ivansson et al. [24] found that the rs3116496 TT genotype was associated with a low risk of cervical cancer in a Chinese Han and a Swedish population, respectively. Conversely, Guzman et al. [26] observed that the rs3116496 TT genotype was associated with an increased cervical cancer risk when combined with the IFN+847AA genotype, in a Brazilian population. Pawlak et al. [25] demonstrated that rs3116496 was not correlated with cervical squamous cell carcinoma, when taking all evaluated patients into consideration.

No case-control study of CD28 gene polymorphisms in sporadic breast cancer has been reported. Given the association between polymorphisms in the CD28 gene region and cervical cancer risk in different populations, as well as the potential role of costimulatory molecules in carcinogenesis, we examined the association between CD28 polymorphisms and breast cancer risk and tumor pathology. Our data provides the first evidence for the involvement of the human CD28 gene in breast cancer.

Results

Polymorphism Detection

A total of 565 breast cancer patients (Table 1) and 605 healthy controls were involved in this case-control study. SNPs were excluded if they deviated from HWE (P<0.05), which did not occur in this study. 12 SNPs (rs3181097, rs3181100, rs1181388, rs10932017, rs4673259, rs3769684, rs3116487, rs3116494, rs3116496, rs12693993, rs3769686, and rs35593994) were genotyped were excluded, because more than 10% of the data was missing (resulting from the lack of sufficient genomic DNA in the samples).

Linkage Disequilibrium

All SNP detected were located in the chromosome 2q33 region. Linkage disequilibrium (LD) patterns were analyzed using our own material (data from 565 patients and 605 controls) with Haploview 4.1 software. LD was expressed by both D’ and r². The strongest LD was observed between rs3116497 and rs3116494, with D’ = 1 and r² = 0.99. The remaining LDs were presented in the LD plot (Figure S1). Using the methods of Gabriel et al. [28] described in the statistical analysis section, which defines confidence limits for strong LD (upper, 0.85; lower, 0.70), and the confidence interval maximums for strong recombination (upper, 0.85) and for strong LD (upper, at least 0.8), two LD blocks were identified from the maximums for strong recombination (upper, 0.85) and for strong LD (upper, at least 0.8), two LD blocks were identified from the statistical analysis section, which defines confidence limits for strong LD (upper, 0.85; lower, 0.70), and the confidence interval maximums for strong recombination (upper, 0.85). The human CD28 gene region and its flanking sequence. As shown in Figure S2, LD block 1 was composed of rs3181097, rs35593994, rs3181100, rs1181388, rs10932017, rs4673259, and rs3769684, while block 2 was composed of only rs3116487 and rs3116494. In LD block 1, the haplotype A/rs3116487G/rs35593994G/rs3116494T/rs1116301C/rs10932017C/rs4673259C/rs3769684 was the most common haplotype (45.7% of cases and 48.3% of controls). Haplotype G/rs3116487A/rs35593994C/rs3116494C/rs1116301T/rs10932017T/rs4673259T/rs3769684 was less common (21.9% of cases and 20.0% of controls). rs3116487 and rs3116494 (D’ = 1, r² = 0.99) belonged to LD block 2, and they constructed only two haplotypes. The haplotype G/rs3116487A/rs3116494 was the most common haplotype (92.6% of cases and 91.6% of controls), while G/rs3116487G/rs3116494 was less frequently observed (7.4% of cases and 8.4% of controls).
Features of Breast Cancer

Association between CD28 Gene Variants and Clinical Features of Breast Cancer

The clinical features of 565 sporadic breast cancer patients are summarized in Table 1, including tumor type, tumor size, ER, PR, C-erbB2, and P53 status, and lymph node metastasis condition. Tables S1, S2, S3, S4, S5, S6 list the data involved in the clinical features analysis. A significant association was found between rs3116487/rs3116494 (D' = 1, r² = 0.99) and ER status at the single SNP level (allelic P = 0.013, dominant P = 0.0078). Similar to its role in the ER, rs3116487/rs3116494 (D' = 1, r² = 0.99) was also observed to be associated with C-erbB2 status in breast cancer patients (allelic P = 0.0247, dominant P = 0.0198). We further analyzed the association between haplotypes identified and clinical features using Haploview software. LD Block 2 was associated with ER and C-erbB2 status, which was in accordance

Table 2. Genotyping of CD28 gene SNPs in breast cancer patients and controls.

| SNP          | Allele | Case, Control Ratios | P value for model of inheritance | OR (95%CI) |
|--------------|--------|----------------------|----------------------------------|------------|
| rs3181097    | A-G    | 549:581, 628:582     | 2.571                            | 0.1088     |
| rs35593994   | G-A    | 842:288, 939:271     | 3.069                            | 0.0798     |
| rs3181100    | G-C    | 194:936, 214:996     | 0.109                            | 0.7415     |
| rs1181388    | T-C    | 580:550, 636:574     | 0.357                            | 0.5503     |
| rs10932017   | C-T    | 775:355, 830:380     | 0.000                            | 0.9954     |
| rs4673259    | C-T    | 516:569, 610:600     | 0.138                            | 0.7017     |
| rs3769684    | T-C    | 538:592, 580:630     | 0.024                            | 0.8757     |
| rs3116487    | G-C    | 84:1046, 102:1108    | 0.792                            | 0.3734     |
| rs3116494    | G-A    | 84:1046, 103:1107    | 0.925                            | 0.3362     |
| rs12693993   | A-G    | 173:957, 195:1015    | 0.286                            | 0.5925     |
| rs3769686    | A-G    | 111:200, 118:211     | 0.004                            | 0.9495     |

1SNP data was analyzed using Haploview 4.1. Significant values (p<0.05) are in bold.

2P value for multiple testing with 10,000 permutations.

3The number of controls in study cohort was 605.

4Minor allele 'a' and the major 'A' are shown in the table. 'AA', 'Aa', 'aa' represent a given variant for each SNP genotyped.

5The number of cases in study cohort was 565.

6Estimated odds ratio (OR) and 95% confidence interval (CI) above were assessed using logistic regression with Plink 1.07.

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Table 3. Alleles of the genotyped SNPs in CD28 gene.

| SNP          | Allele | Case, Control Ratios | Chi-square | P value |
|--------------|--------|----------------------|------------|---------|
| rs3181097    | A-G    | 549:581, 628:582     | 2.571      | 0.1088  |
| rs35593994   | G-A    | 842:288, 939:271     | 3.069      | 0.0798  |
| rs3181100    | G-C    | 194:936, 214:996     | 0.109      | 0.7415  |
| rs1181388    | T-C    | 580:550, 636:574     | 0.357      | 0.5503  |
| rs10932017   | C-T    | 775:355, 830:380     | 0.000      | 0.9954  |
| rs4673259    | C-T    | 516:569, 610:600     | 0.138      | 0.7017  |
| rs3769684    | T-C    | 538:592, 580:630     | 0.024      | 0.8757  |
| rs3116487    | G-C    | 84:1046, 102:1108    | 0.792      | 0.3734  |
| rs3116494    | G-A    | 84:1046, 103:1107    | 0.925      | 0.3362  |
| rs12693993   | A-G    | 173:957, 195:1015    | 0.286      | 0.5925  |
| rs3769686    | A-G    | 111:200, 118:211     | 0.004      | 0.9495  |

1SNP data was analyzed using Haploview 4.1. Significant values (p<0.05) are in bold.

2P value for multiple testing with 10,000 permutations.

3The number of controls in study cohort was 605.

4Minor allele 'a' and the major 'A' are shown in the table. 'AA', 'Aa', 'aa' represent a given variant for each SNP genotyped.

5The number of cases in study cohort was 565.

6Estimated odds ratio (OR) and 95% confidence interval (CI) above were assessed using logistic regression with Plink 1.07.

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For SNP rs3119686, no GG genotype was detected in this study and the frequency of G allele is only 1.75%. Thus, only allelic P value for rs3119686 was calculated between cases and controls in Table 3. As shown in Table 3, a higher prevalence of 'C' alleles was observed (P = 0.0046) in breast cancer patients than in healthy controls, even after correcting the P value for multiple testing with 10,000 permutations (P = 0.0384). Although there was a trend for a higher proportion of rs3116494 G A genotype (AA vs. Aa vs. aa) respectively. Significant values (P < 0.05) are in bold.

The distributions of the CD28 genotypes are shown in Table 2. The odds ratios and 95% CIs in Table 2 are presented for the dominant genetic model identified by a logistic regression analysis using Plink 1.07 software. At the single SNP level, a trend in genotype distribution was observed for rs3116494 (GG vs. AA, additive P = 0.0164, dominant P = 0.0042). Although there was also a trend for a higher proportion of rs35593994 genotype (AA+AG) in the dominant genetic model in patients with breast cancer compared with controls, the trend did not achieve statistical significance (P = 0.0989). Moreover, the rs3116487 GG and rs3116494 GG genotypes had a lower prevalence in breast cancer patients than in healthy controls, under the recessive genetic model. For SNP rs3119686, no GG genotype was detected in this study and the frequency of G allele is only 1.75%. Thus, only allelic P value for rs3119686 was calculated between cases and controls in Table 3. As shown in Table 3, a higher prevalence of 'C' alleles was observed (P = 0.0046) in breast cancer patients than in healthy controls, even after correcting the P value for multiple testing with 10,000 permutations (P = 0.0384). Although there was a trend for a higher proportion of rs3116494 G A genotype (AA vs. Aa vs. aa) respectively. Significant values (P < 0.05) are in bold.
with the results at the single SNP level. Additionally, a moderate association was found between LD Block 1 of rs3116495-Grs3559399-Crs3181100-Crs1181388-Crs10932017-Trs4673259-Trs3769684 haplotype and ER status (P = 0.0309). The G rs3116495-Grs3559399-Crs3181100-Crs1181388-Crs10932017-Trs4673259-Crs3769684 haplotype had a higher prevalence in C-erbB2 positive patients compared to C-erbB2 negative ones (P = 0.0242). The distribution of other haplotypes did not differ between patients and controls. No statistically significant relationships were observed in regard to histological grade (data not shown), lymph node metastasis, or PR and P53 status.

**Discussion**

The etiology and pathogenesis of breast cancer depend on multiple factors. Understanding of the patient's genetic background helps to optimize the approaches for breast cancer prevention and treatment. Previous studies have extensively examined the association of CTLA-4 polymorphisms and malignancies [29,30]. As a homolog of CTLA-4, CD28 may also contribute to the development of cancer. In this case-control study, we genotyped SNPs that completely span the CD28 gene region, and we classified the association between CD28 gene variants and breast cancer in a Chinese Han population in Northeast China.

There was a difference in the rs3116496 polymorphism of the CD28 gene between breast cancer patients and controls in a Chinese Han population. The rs3116496 CC+CT genotype and ‘C’ allele frequencies were higher in breast cancer patients compared with controls, suggesting that the rs3116496 CC+CT genotype and ‘C’ allele may contribute to breast carcinogenesis. The rs3116496 SNP is located at position IVS3+17 of the third intron of the CD28 gene. It is difficult to explain the exact biological function of intronic SNPs. However, the potential explanations may be as follows. The intronic SNP rs3116496 is located near the splice acceptor site [23,26], where mutations may induce aberrant splicing due to disruption of the splice site [31]. This polymorphism probably alters the expression of the CD28 protein by influencing mRNA splicing, and eventually, leads to immune alteration.

Alternative splicing in CD28 pre-mRNA gives rise to different mRNA isoforms, leading to soluble and membrane-bound isoforms of expressed CD28 protein [32,33,34]. Balance of these putative polypeptides, with different physiologic roles, is important to immune homeostasis. It is likely that intronic SNPs in the CD28 gene, e.g. rs3116496, lead to the unbalanced expression of various CD28 protein isoforms by aberrant splicing, which leads to altered immune function. In line with this hypothesis, a report comparing sCD28 expression of each rs3116496 genotype in rheumatoid arthritis patients found higher sCD28 levels in TT carriers than in those with the TC genotype [21]. Further studies of the potential mechanisms underlying intronic polymorphisms, on CD28 pre-mRNA splicing, will therefore be interesting.

rs3116496 is probably only one marker in linkage disequilibrium with other true susceptible variants. The relationship between the CD28 rs3116496 polymorphisms and susceptibility to various diseases, including cancer, has been previously investigated. Bechet’s disease [22], rheumatoid arthritis [21], and cervical cancer [23,24,25,26,27] are all known to be associated with the rs3116496 polymorphism. It was reported that the rs3116496 TT genotype was associated with a low risk of cervical cancer in a Chinese Han and in a Swedish population, respectively [23,24]. Guzman et al. [26] observed that the rs3116496 TT genotype, combined with IFN+847AA, increased cervical cancer risk in a Brazilian population. Pawlak et al. [25] demonstrated that this SNP was not associated with cervical squamous cell carcinoma. These conflicting observations in cervical cancer, among different ethnic groups, are interesting. Genetic background and environmental factors are different among populations. Every population shares their own linkage disequilibrium pattern. Accordingly, a functional SNP may be in linkage disequilibrium with distinct markers in different ethnic groups. Thus, rs3116496 may be only a marker linked to the real functional variant in the studied population, with no biological function itself at all. Nevertheless, we cannot deny the possibility

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**Table 4. Haplotype of the CD28 gene SNPs between cases and controls.**

| Block 1 | Haplotype | Freq. | Case, control ratios | Chi Square | P value | Permutation P valuea |
|---------|-----------|-------|----------------------|------------|---------|----------------------|
| GAGCTCC | 0.469 | 510:1:609.9, 577:9:620.1 | 1.689 | 0.1937 | 0.8582 |
| GACCTTT | 0.208 | 243:0:877.0, 238:4:959.6 | 1.140 | 0.2857 | 0.9570 |
| GGGCCTT | 0.133 | 145:7:974.3, 163:7:1034.3 | 0.216 | 0.6424 | 0.9998 |
| GGCCCTT | 0.070 | 71:6:1048.4, 90:6:1107.4 | 1.227 | 0.2680 | 0.9463 |
| GGCTCTC | 0.014 | 17.3:1102.7, 16:2:1181.8 | 0.151 | 0.6976 | 0.9999 |
| GGCTCTC | 0.014 | 110:1109.0, 21.4:1176.6 | 2.714 | 0.0995 | 0.7081 |
| GGCCTCC | 0.012 | 142:1105.8, 14.5:1183.5 | 0.016 | 0.8987 | 1.0000 |
| Block 2 | CA | 0.921 | 1046:84.0, 1107:101.0 | 0.689 | 0.4065 | 0.9906 |
| CA | 0.079 | 84:0:1046.0, 101:0:1107.0 | 0.689 | 0.4065 | 0.9906 |

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a Blocks were constructed according to the values of D’ and r² calculated from our own data. SNPs with the minor allele frequency less than 5% were excluded in the haplotype construction. The definition of the LD blocks were based on the method of Gabriel et al. with confidence limits for strong LD (upper, 0.85; lower, 0.70) and the confidence interval maximums for strong recombination (upper, 0.85) and for strong LD (upper, at least 0.8) in informative comparisons.

b P values for haplotypes in the two blocks were permuted 10,000 times by Haploview 4.1 program.

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that functional rs3116496, together with other uncovered functional variants in linkage disequilibrium, synergistically influence disease risk. Epidemiological studies with ethnically diverse populations need to evaluate this hypothesis.

There was a trend toward higher proportion of rs35593994 ‘A’ allele and genotype (AA+AG) in the dominant genetic model, in patients with breast cancer, compared with controls. Non-coding SNPs may disrupt transcription factor binding sites, splice sites, and other functional sites at the transcription level. The rs35593994 SNP is located in the promoter region of the CD28 gene. A search for a TFBS (transcription factor binding site) using the TRANSFAC program [35] revealed that rs35593994 G/A alleles may influence gene transcription. Compared with rs35593994 G’, rs35593994 ‘A’ may promote transcription of the CD28 gene by the presence of a binding site available for the transcriptional repressor) [36]. The immune alteration generated by this potentially functional SNP probably contributes to disease risk. However, the association between this SNP and breast cancer risk did not achieve statistical significance in our study.

In the clinical features analysis, associations were found between rs3116487/rs3116494 (D’ = 1, r² = 0.99) and the status of C-erbB2 and ER in breast patients. Similar to rs3116496, rs3116487 was located in intron 1 and rs3116494 was located in intron 2. They could contribute to splicing events which play roles in CD28 signaling and T lymphocyte activation. It has been well established that routine clinical management of breast cancer depends on clinicopathological factors, such as ER and C-erbB2 expression. Steroid hormone receptors play an important role in disease-free (DFS) and overall survival (OS) of breast cancer patients, and are considered predictive markers of endocrine therapy [37]. Breast cancer patients with a complete lack of ER expression do not benefit from endocrine therapies [38,39]. C-erbB2 expression is accepted as a significant prognostic factor for DFS and OS. Overexpression of C-erbB2 in breast cancer patients is associated with a worse prognosis and a higher recurrence rate [39,40]. C.rs3116487/rs3116494 had a higher prevalence in ER positive subgroups, and a lower prevalence in C-erbB2 positive ones. C.rs3116487/rs3116494 was probably a marker for good prognosis in breast cancer patients. The exact biological function of these SNPs on the expression of ER and C-erbB2 in breast cancer is not known. From the evidence of this case-control study we can just conclude that the two SNPs may be potential markers to predict prognosis of breast cancer patients.

In the haplotype analysis, LD among polymorphic loci was assessed using Haploview software. Only two LD blocks were identified. LD block 1 G.rs311809/G.rs35593994/C.rs311100/C.rs311103 rs311809/rs35593994/C.rs311100/T.rs3769684 haplotype was associated with ER status, while block 2 G.rs311809/G.rs35593994/C.rs311100/T.rs3769684 haplotype had a higher prevalence in the C-erbB2 positive subgroup. LD Block 2 was associated with ER and C-erbB2 status, in accordance with the results at the single SNP level. These results indicated that rs3116487/rs3116494 (D’ = 1, r² = 0.99) might be meaningful in the pathology of breast cancer, as well as potential markers to predict prognosis of breast cancer patients.

Taken together, the current study shows that polymorphisms of the CD28 gene region may affect susceptibility to sporadic breast cancer risk in Chinese Han women. This study provides the first evidence for the involvement of the human CD28 gene in breast cancer. The clinical features analysis in this study revealed an association between CD28 gene polymorphisms and prognostic factors in breast cancer, including ER and C-erbB2 status. However, the basic functions of CD28 gene mutations remain to be further elucidated.

Materials and Methods

Ethics Statement

This study was conducted in Heilongjiang Province in northeast of China. Before the research was conducted, ethical board approval from the Third Affiliated Hospital of Harbin Medical University was obtained, and all of the volunteers provided written informed consent.

Subjects

All sporadic breast cancer patients were recruited from the Third Affiliated Hospital of Harbin Medical University, Heilongjiang Province, China. Patients’ clinical information included tumor type, tumor size, lymph node metastasis, human epidermal growth factor receptor 2 (C-erbB2), estrogen receptor (ER), progesterone receptor (PR) and tumor protein 53 (P53) status (Table 1). Our study consisted of 565 patients (mean age 49.6±10.3 years). All patients were diagnosed based on surgical and pathological findings. For comparison, 605 healthy controls (mean age 49.0±9.8 years) were recruited randomly from community volunteers. The healthy controls had no history of personal or familial malignancy or autoimmune disorders, and were frequency matched to patients by age.

Preparation of Genomic DNA

Lymphocytes were separated from 5 ml of anticoagulated whole blood by centrifugation. Genomic DNA was extracted using the Universal Genomic DNA Extraction Kit Ver. 3.0 (TaKaRa, Japan), following the manufacturer’s protocol.

SNP Selection

SNPs were selected using the HapMap database. Thirteen haplotype-tagging SNPs [rs1879877, rs3181097, rs3181100, rs2140148, rs1181388, rs10932017, rs4673259, rs3769684, rs3116486 (replaced by rs3116487 in this study with r² = 1), rs3116494, rs3116496, rs12693993, and rs3769686] from the CD28 gene were selected from the HapMap database using Haplovew software 4.1, with pair-wise r²<0.8 for each SNP pair and minor allele frequencies >5%. In addition to the SNPs mentioned above, the SNP rs35593994, on which the HapMap database has no information, was selected in this study because it has been reported to be potentially functional. All selected SNPs were validated using extensive database searches (http://www.ensemble.org,http://ncbi.nlm.nih.gov/SNP). In total, 14 SNPs were selected to span the entire CD28 gene region.

Genotyping

All 14 SNPs were genotyped using the polymerase chain reaction-restriction fragment length polymorphism (PCR-RFLP) assay. The accuracy of genotyping results were confirmed using direct sequencing in random samples. The primers and restriction enzymes for CD28 PCR-RFLP genotyping are shown in Table S7. The polymorphic regions were amplified by PCR using a T-Gradient Thermoblock PCR System (BioRad, USA) in a 25 µl-reaction solution containing 0.3 µg genomic DNA, 2.5 µl 10× PCR buffer (Mg²⁺ plus), 2.0 µl dNTP mixture (TaKaRa, Japan), 2.5 µl TaqDNA polymerase (TaKaRa, Japan), and 0.2 µl of each primer (10 umol/L) (Invitrogen, China). The PCR products of each SNP were digested with restriction enzymes (NEB, USA), following the manufacturer’s instructions (Table S7), and isolated using 2% agarose gel; Table S8 provided the information on the digestion results.
rs4673259, rs3769684, rs3116487, rs12693993, rs3769686, and rs35593994 and 3% (for rs1879877, rs1181388, rs3116494, and rs3116496) agarose gel electrophoresis.

**Statistical Analysis**

The deviation from Hardy-Weinberg equilibrium (HWE) was determined using a goodness-of-fit chi-squared test to compare the observed genotype frequencies with the expected frequencies, from the healthy controls. The polymorphisms were excluded if they deviated from HWE, or if missing data comprised more than 10% of the total data. Using different models of inheritance (additive, dominant, recessive), the genotype frequencies of the subjects were analyzed using the chi-squared test and Fisher’s exact test. Estimated odds ratio (OR) and 95% confidence interval (CI) were assessed using logistic regression with Plikn software. To determine the significance with corrections for multiple testing biases, we ran 10,000 permutations using Haploview to determine the P value.

All data were analyzed using SPSS (version 17.0), Plikn (version 1.07) (http://pngu.mgh.harvard.edu/~purcell/plink), and Haploview (version 4.1) (http://www.broad.mit.edu/mpg/haploview/). The threshold for significance was P<0.05, and the relative risks associated with haplotypes were estimated as odds ratios (ORs), with 95% confidence intervals (CIs).

Haploview patterns were generated using the algorithm described in Haploview [28], which constructs haplotypes based on the D’ measure of linkage disequilibrium and a LOD score as a measure of significance. The pairwise linkage disequilibrium for a genotyped SNP was confirmed using r^2 values [41,42]. SNPs with a minor allele frequency of less than 5% were excluded in our haplotype constructions. Haploview block definitions were based on the method of Gabriel et al. [28], with confidence limits for strong LD (upper, 0.85; lower, 0.70), and confidence interval maximums for strong recombination (upper, 0.85) and strong LD (upper, at least 0.8) in informative comparisons.

**Supporting Information**

**Figure S1 Haplotype architecture in CD28.** Haplotype LD block structure across the CD28 gene was generated from 565 breast cancer patients and 605 healthy individuals in a Chinese Han population using Haploview software. (A) LD prime chart from Haploview that summarizes the LD patterns is shown. The numbers in each box represent the LD value between the adjacent SNPs. (B) r^2 prime chart from Haploview that summarizes the r^2 patterns is shown. The numbers in each box represent the r^2 value between the adjacent SNPs. The physical representations of the SNP positions in (A, B) have been colored to represent the LD between the adjacent SNPs, according to the standard Haploview software color scheme: LOD>2 and D’=1, red; LOD=2 and D’<1, shades of pink/red; LOD<2 and D’=1, blue; LOD<2 and D’<1, white. The definitions of the LD blocks were based on the method of Gabriel et al. with confidence limits for strong LD (upper, 0.85; lower, 0.70), and confidence interval maximums for strong recombination (upper, 0.85) and strong LD (upper, at least 0.8), in informative comparisons. In order to keep the display unchallenged, D prime values of 1.0 were never shown (the box is empty). The strongest LD shown above was between rs3116487 and rs3116494 (D’=1, r^2=0.99). Two LD blocks were identified from the CD28 gene are presented. Block 1 covered 14 kb, and block 2 about 5 kb. SNP rs3116986, with minor allele frequency of only 1.75%, was excluded from the haplotype analysis.

(FIG)

**Figure S2 Distribution of haplotypes in the case group, the control group and the whole group.** Haplotypes with frequency more than 1% are shown in the case group, the control group, and the whole group. Seven haplotypes in block 1 and two haplotypes in block 2 are shown above. LD block 1- A_3116487C_35593994C_3116494T_44673259C_3769684 and LD block 2- C_3116494T_3116496A_3116494 are the most common haplotypes in each subgroup.

(FIG)

**Table S1 Relationship between ER status in breast cancer patients and variants detected in the CD28 gene.**

1ER information of 484 breast cancer patients was available in the study with 282 (49.91%) positive and 202 (35.75%) negative ones.

2The P values were accessed using Plikn and SPSS software under an additive model (AA vs. Aa vs. aa), dominant model (aa+Aa vs. AA), and recessive model (aa vs. Aa+AA) respectively. Significant values (P<0.05) are in bold.

(DOC)

**Table S2 Relationship between PR status in breast cancer patients and variants detected in the CD28 gene.**

1PR information of 492 breast cancer patients was available in the study with 346 (61.24%) positive and 136 (24.07%) negative ones.

2The P values were accessed using Plikn and SPSS software under an additive model (AA vs. Aa vs. aa), dominant model (aa+Aa vs. AA), and recessive model (aa vs. Aa+AA) respectively. Significant values (P<0.05) are in bold.

(DOC)

**Table S3 Relationship between C-erbB2 status in breast cancer patients and variants detected in the CD28 gene.**

1C-erbB2 information of 479 breast cancer patients was available in the study with 187 (33.10%) positive and 292 (51.68%) negative ones.

2The P values were accessed using Plikn and SPSS software under an additive model (AA vs. Aa vs. aa), dominant model (aa+Aa vs. AA), and recessive model (aa vs. Aa+AA) respectively. Significant values (P<0.05) are in bold.

(DOC)

**Table S4 Relationship between P53 status in breast cancer patients and variants detected in the CD28 gene.**

1P53 information of 467 breast cancer patients was available in the study with 146 (25.84%) positive and 321 (56.81%) negative ones.

2The P values were accessed using Plikn and SPSS software under an additive model (AA vs. Aa vs. aa), dominant model (aa+Aa vs. AA), and recessive model (aa vs. Aa+AA) respectively. Significant values (P<0.05) are in bold.

(DOC)

**Table S5 Relationship between lymph node involvement status in breast cancer patients and variants detected in the CD28 gene.**

1LN lymph node. 2LN involvement information of 543 breast cancer patients was available in the study with 245 (45.59%) positive and 308 (56.87%) negative ones.

3The P values were accessed using Plikn and SPSS software under an additive model (AA vs. Aa vs. aa), dominant model (aa+Aa vs. AA), and recessive model (aa vs. Aa+AA) respectively. Significant values (P<0.05) are in bold.

(DOC)

**Table S6 Association between tumor size and polymorphisms.**

1466 tumor size information in detail of breast cancer patients were available. 1Tumor with the diameter less than 2 cm (0<tumor size<2), number = 187.

2Tumor with the diameter of 2 to 5 cm (2<tumor size<5), number = 252.

3Tumor with the diameter more than 5 (tumor size>5), number = 27.

(DOC)
Table S7  Genotyping information for 14 selected SNPs.

1NEB Cutter, names of restriction enzymes purchased from the NEB biolabs company.

Author Contributions
Conceived and designed the experiments: DL, DP. Performed the experiments: SC QZ FX LS ZF WY. Analyzed the data: QZ SC. Contributed reagents/materials/analysis tools: DL YL. Wrote the paper: SC QZ.

References
1. Jemal A, Bray F, Center MM, Ferlay J, Ward E, et al. (2011) Global cancer statistics. CA Cancer J Clin 61: 69–90.
2. Song M, Lee KM, Kang D (2011) Breast cancer prevention based on gene-environment interaction. Mol Carcinog 50: 280–290.
3. Hanahan D, Weinberg RA (2011) Hallmarks of cancer: the next generation. Cell 144: 646–674.
4. Grivennikov SI, Greten FR, Karin M (2010) Immunity, inflammation, and cancer. Cell 140: 883–899.
5. Boyman O, Letourneau S, Krieg C, Sprent J (2009) Homeostatic proliferation and survival of naive and memory T cells. Eur J Immunol 39: 2088–2094.
6. Chen L (2006) Co-inhibitory molecules of the B7-CD28 family in the control of T-cell immunity. Nat Rev Immunol 6: 539–549.
7. Croft M (2003) Co-stimulatory members of the TNFR family: keys to effective T-cell immunity? Nat Rev Immunol 3: 609–620.
8. Greenwald RJ, Freeman GJ, Sharpe AH (2005) The B7 family revisited. Annu Rev Immunol 23: 515–546.
9. Rudd CE, Taylor A, Schneider H (2009) CD28 and CTLA-4 coreceptor expression and signal transduction. Immun Mol Rev 229: 12–26.
10. Collins M, Ling V, Carreno BM (2005) The B7 family of immune-regulatory ligands. Genome Biol 6: 223.
11. Kim HJ, Chung JH, Kang S, Kim SK, Cho BS, et al. (2011) Association of CTLA4, CD28 and ICOS gene polymorphisms with clinicopathologic characteristics of childhood IgA nephropathy in Korean population. J Genet 90: 151–153.
12. Lesterehuis WJ, Haanen JB, Punt CJ (2011) Cancer immunotherapy-revisited. Nat Rev Drug Discov 10: 591–600.
13. Hutloff A, Dittrich AM, Beier KC, Eljaschewitsch B, Kraft R, et al. (1999) ICOS is an inducible T-cell co-stimulator structurally and functionally related to CD28. Nature 397: 263–266.
14. Yoshida SK, Whoriskey JS, Khare SD, Sarmiento U, Guo J, et al. (1999) T-cell co-stimulation through B7RP-1 and ICOS. Nature 402: 827–832.
15. Boesteanu AC, Katsikis PD (2009) Memory T cells need CD28 costimulation to remember. Semin Immunol 21: 69–77.
16. Koreva A, Dunzota A, Korczak-Kowalska G (2007) The role of the CD28 molecule in immunological tolerance. Postepy Hig Med Dosw (Online) 61: 74–82.
17. Lens M, Ferrucci PF, Testori A (2008) Anti-CTLA4 monoclonal antibody Ipilimumab in the treatment of metastatic melanoma: recent findings. Recent Pat Anticancer Drug Discov 3: 105–113.
18. Pandolfi F, Cianci R, Pagliari D, Casciano F, Bagala C, et al. (2011) The immune response to tumors as a tool toward immunotherapy. Clin Dev Immunol 2011: 694704.
19. Faule D, Amodeo V, Cesarini LR, Rizzo S, Bazan V, et al. (2012) Breast cancer genome-wide association studies: there is strength in numbers. Oncogene 31: 2121–2128.
20. Zhang B, Beegly-Fadiel A, Long J, Zheng W (2011) Genetic variants associated with breast cancer risk: comprehensive research synopsis, meta-analysis, and epidemiological evidence. Lancet Oncol 12: 477–486.
21. Leozma-Lozano IY, Padilla-Martinez JJ, Leyva-Torres PD, Parra-Rojas I, Ramirez-Duenas MG, et al. (2011) Association of CD28 IVS3+17/T polymorphism with soluble CD28 in rheumatoid arthritis. Dis Markers 30: 23–29.
22. Gunesacar R, Erken E, Boukurt B, Ozer HT, Dinikci S, et al. (2007) Analysis of CD28 and CTLA-4 gene polymorphisms in Turkish patients with Behcet’s disease. Int J Immunogenet 34: 45–49.
23. Chen X, Li H, Qiao Y, Yu D, Guo H, et al. (2011) Association of CD28 gene polymorphism with cervical cancer risk in a Chinese population. Int J Immunogenet 38: 51–54.
24. Ivansson EL, Juko-Pecirep I, Gyllesten UB (2010) Interaction of immunological genes on chromosome 2q33 and IFNG in susceptibility to cervical cancer. Gynecol Oncol 116: 544–548.
25. Pavlak E, Karabon L, Wlaska-Polina J, Jedynek A, Jonkisz A, et al. (2010) Influence of CTLA-4/CD28/ICOS gene polymorphisms on the susceptibility to cervical squamous cell carcinoma and stage of differentiation in the Polish population. Hum Immunol 71: 195–200.
26. Guzman VB, Vambahare A, Gonzalez-Primo A, Silva JD, Carvalho CR, et al. (2008) New approach reveals CD28 and IFNG gene interaction in the susceptibility to cervical cancer. Hum Mol Genet 17: 1838–1844.
27. Suwalska K, Pavlak E, Karabon L, Tomkiewicz A, Dobosz T, et al. (2008) Association studies of CTLA-4, CD28, and ICOS gene polymorphisms with B-cell chronic lymphocytic leukemia in the Polish population. Hum Immunol 69: 195–201.
28. Gabriel SB, Schaffner SF, Nguyen H, Moore JM, Roy J, et al. (2002) The structure of haplotype blocks in the human genome. Science 296: 2225–2229.
29. Ghaideri A (2011) CTLA4 gene variants in autoimmunity and cancer: a comparative review. Iran J Immunol 8: 127–149.
30. Sun T, Hu Z, Shen H, Lin D (2009) Genetic polymorphisms in cytotoxic T-lymphocyte antigen 4 and cancer: the dialectical nature of subtle human immune dysregulation. Cancer Res 69: 6011–6014.
31. Baralle D, Baralle M (2003) Splicing in action: assessing disease causing sequence changes. J Med Genet 42: 737–746.
32. Deshpande M, Venuprasad K, Parah PB, Saha B, Mitra D (2002) A novel CD28 mRNA variant and simultaneous presence of various CD28 mRNA isoforms in human T lymphocytes. Hum Immunol 63: 20–23.
33. Magistrelli G, Jeannin P, Elson G, Gauchart JP, Nguyen TN, et al. (1999) Identification of three alternatively spliced variants of human CD28 mRNA. Biochem Biophys Res Commun 259: 34–37.
34. Hanawa H, Ma Y, Mikolajczak SA, Charles ML, Yoshida T, et al. (2002) A novel costimulatory signaling in human T lymphocytes by a splice variant of CD28. Blood 99: 2139–2145.
35. Wingender E, Chen X, Hohl R, Karas H, Liebich I, et al. (2000) TRANSFAC: an integrated system for gene expression regulation. Nucleic Acids Res 28: 316–319.
36. Rathinam C, Klein C (2012) Retraction: transcriptional repressor gfi1 integrates cytokine-receptor signals controlling B-cell differentiation. PLoS One 7.
37. Elledge RM, Green S, Pugh R, Allred DC, Clark GM, et al. (2000) Estrogen receptor (ER) and progesterone receptor (PgR), by ligand-binding assay compared with ER, PgR and P52, by immune-histochemistry in predicting response to tamoxifen in metastatic breast cancer: a Southwest Oncology Group Study. Int J Cancer 89: 111–117.
38. Cazzaniga M, Bonammi B (2012) Breast cancer chemoprevention: old and new approaches. J Biomed Biotechnol 2012: 983620.
39. Itrinsiege FK, Thike AA, Cheok PY, Tse GM, Lai PC, et al. (2010) Hormone receptor and c-ERBB2 status in distant metastatic and locally recurrent breast cancer. Pathologic correlations and clinical significance. Am J Clin Pathol 133: 416–429.
40. Jarvinen TA, Liu ET (2003) HER-2/neu and topoisomerase IIalpha in breast cancer. Breast Cancer Res Treat 78: 299–311.
41. Hill WG, Wein BS (1994) Maximum-likelihood estimation of gene location by linkage disequilibrium. Am J Hum Genet 54: 705–714.
42. Devlin B, Risch N (1995) A comparison of linkage disequilibrium measures for linkage disequilibrium. Am J Hum Genet 57: 539–541.