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Functional polymorphisms in the promoter of *BRCA1* influences transcription and are associated with decreased risk for breast cancer in Chinese women

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Abstract

Background—The *BRCA1* gene is an important breast cancer susceptibility gene. Promoter polymorphisms can alter the binding affinity of transcription factors, changing transcriptional activity and may affect susceptibility to disease.

Methods and Results—By direct sequencing of the *BRCA1* promoter region, we identified four polymorphisms c.-2804T>C (rs799908:T>C), c.-2265C>T (rs11655505:C>T), c.-2004A>G (rs799906:A>G) and c.-1896(ACA)₁/(ACA)₂ (rs8176071:(ACA)₁/(ACA)₂) present in Hong Kong

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Chinese. Each was studied independently and in combination by functional assays. While all four variants significantly altered promoter activity, the c.-2265T allele most clearly provided stronger binding than the C allele and the most common mutant haplotype which contains the c.-2265T allele increased promoter activity by 70%. Risk association first tested in breast cancer cases and age-matched controls of Hong Kong Chinese women and replicated in a large population-based study of Shanghai Chinese, altogether totaling over 3,000 subjects, demonstrated the c.-2265T allele carriers had a reduced risk for breast cancer (combined ORs=0.80, 95%CI=0.69–0.93; p=0.003) which was more evident among women aged ≥ 45 years at first diagnosis of breast cancer and without family history of breast cancer (combined ORs=0.75, 95%CI=0.61–0.91; p=0.004). The most common haplotype containing the c.-2265T allele also showed significant risk association for women aged ≥ 45 years without family history of breast cancer (ORs=0.64, 95%CI=0.46–0.89; p=0.008).

Conclusion—Our comprehensive study of *BRCA1* promoter polymorphisms demonstrated four variants which altered promoter activity, and with the most significant contribution from c.-2265C>T, which could affect susceptibility to breast cancer in the Chinese population. Its significance in other populations remains to be investigated.

Keywords

BRCA1 promoter; polymorphism; transcription activity; breast cancer; Chinese women

INTRODUCTION

The *BRCA1* gene, a tumor suppressor locus in chromosome 17q12–21, is an autosomal dominant gene that plays an important role in breast cancer risk. Germline mutations in *BRCA1* are associated with approximately 20% of familial breast cancers in Caucasian women [1] and 81% of breast-ovarian cancer families [2]. Women carrying loss-of-function mutations in *BRCA1* have been reported to carry a 81% life-time risk of developing breast cancer [3]. *BRCA1* mutations, however account for only 5–10% of all breast cancers [4]. Besides germline and somatic mutations [5], promoter hypermethylation [6,7] was attributed to reduced *BRCA1* expression in some cases of breast cancer, including those from sporadic cases [8,9].

Single nucleotide polymorphism (SNP) in the promoter region can affect promoter activity as nucleotide change may alter the binding affinity of transcriptional factor involved in the regulation of gene expression [10,11]. We hypothesized that potentially functional *BRCA1* promoter polymorphisms could alter transcriptional activity thus affecting susceptibility to develop sporadic breast cancer. So far no study has comprehensively investigated the *BRCA1* promoter SNPs for their functional roles and contribution to risk in developing sporadic breast cancer. Two recent risk association studies have investigated 4 tagging SNPs [12] and 28 SNPs [13] spanning the *BRCA1* gene. In Cox et al's study [12], the tagging SNPs were not located in the promoter region. Only two promoter SNPs, c.-2613G>C (rs799907:G>C) and c.-2004G>A (rs799906:G>A), were included in Freedman et al's study [13]. However, the minor allele frequency (MAF) of c.-2613G>C was <0.6% in our Chinese population, and thus could not be analyzed. To test our hypothesis, polymorphisms located in the *BRCA1* promoter were identified from the public dbSNP database as well as by re-sequencing on healthy Chinese individuals from Hong Kong. Four promoter polymorphisms c.-2804T>C (rs799908:T>C), c.-2265C>T (rs11655505:C>T), c.-2004A>G (rs799906:A>G) and c.-1896(ACA)₁/(ACA)₂ (rs8176071:(ACA)₁/(ACA)₂) were studied by *in-vitro* assays and genotyping. Whilst each polymorphism could affect promoter activity, the contribution was most significant for c.-2265C>T, as supported by *in-silico* prediction of putative transcription factor binding elements, electrophoretic-mobility-shift assay (EMSA) and promoter activity assay. This finding was further supported by genetic association analysis in two independent case-control cohorts of Chinese women.

MATERIALS AND METHODS

Identification of *BRCA1* Promoter Polymorphisms

BRCA1 promoter SNPs were identified from the dbSNP database (build #120) at the time the study was initiated. The *BRCA1* genomic reference sequence (GenBank number U37574) was used with the first nucleotide upstream to translation initiation codon as -1 nucleotide [14]. To identify DNA variations that may be unique to Chinese subjects, direct sequencing of the promoter region (1.6kb upstream to the transcription start site at the exon 1a of *BRCA1* [14]) was also performed on 20 healthy Hong Kong Chinese. Twenty individuals give at least 87% probability of finding variants of MAF $\geq 5\%$.

Identification of putative transcription factor binding elements

In-silico search for putative transcription factor binding elements harbored by the *BRCA1* promoter polymorphisms was done using the software TFSEARCH (V1.3) [15] (<http://www.cbrc.jp/research/db/TFSEARCH.html>). All putative transcription factors which are unidirectional with respect to the sense strand sequence of the *BRCA1* promoter in humans were identified.

Electrophoretic mobility shift assay (EMSA)

Nuclear proteins extraction from HeLa cells and EMSA were performed as described previously with some modifications as follows [16]. Briefly, double-stranded c.-2804T, c.-2804C, c.-2265T and c.-2265C oligonucleotide probes (Supplementary Table 1) were ³²P-end-labeled and purified by using MicroSpin G-50 columns (GE Healthcare, Piscataway, NJ, USA). Binding experiments were conducted by incubating 10 µg nuclear-protein extracts with 0.14 pmol (400,000 cpm) of probe at room temperature for 30 minutes. The nuclear proteins and various oligonucleotide probes were incubated in a binding buffer containing 10 mM Tris (pH 7.5), 10% glycerol, 5 µg/mL of poly (dI-dC), 10mg/mL BSA, and 1% Nonidet P-40. For competition experiments, unlabeled oligonucleotide probes were added to the radio-labeled probe reaction mixture at 25× or 50× molar excess before incubation. After electrophoresis, gels were dried and subjected to autoradiographic analysis. The shifted band intensity was analyzed by Grab-IT image analysis software (UVP Inc., Upland, CA, USA), measurement being averaged from results of two experiments.

Promoter activity assay

Cloning of the *BRCA1* promoter—Genomic DNA of individuals homozygous for the two most common promoter haplotypes, Tc-2804Cc.-2265Ac.-2004(ACA)₁c.-1896 and Cc.-2804Tc.-2265Gc.-2004(ACA)₂c.-1896, were amplified using specific primers which contained the *XhoI* and *HindIII* restriction site linker at the 5' end of the forward and reverse primers respectively (Supplementary Table 1). Amplification was carried out using Hi-Fi Expand PCR kit according to manufacturer's recommendations (Roche Diagnostics, Mannheim, Germany). The amplified products were then cloned into firefly luciferase-reporter pGL3-basic vector (Promega, Madison, WI, USA). These two constructs were named pGL3-basic/*BRCA1*-2265C (the wild-type haplotype) and pGL3-basic/*BRCA1*-2265T (the most common mutant haplotype), and verified by sequencing. Based on the pGL3-basic/*BRCA1*-2265C haplotype construct, four other promoter mutant constructs for which each polymorphism in turn was replaced by the mutant allele, were created by PCR site-directed mutagenesis and cloned into pGL3-basic vectors. These four additional haplotypes are as follows: Cc.-2804Cc.-2265Ac.-2004(ACA)₁c.-1896, Tc.-2804Tc.-2265Ac.-2004(ACA)₁c.-1896, Tc.-2804Cc.-2265Gc.-2004(ACA)₁c.-1896 and Tc.-2804Cc.-2265Ac.-2004(**ACA**)₂c.-1896 (bolded letters showing the difference with respect to pGL3-basic/*BRCA1*-2265C).

Luciferase reporter assay—Human cervical cancer (HeLa) cell lines were used for transient transfection experiments. The HeLa cells were cultured in DMEM with high glucose (4.5mg/mL) (Invitrogen). A total of 8×10^4 cells were cultured in 35 mm diameter dishes for 24 hours prior to transfection. One μ g of Luciferase-reporter plasmid and 0.1 μ g of Renilla plasmid were transfected into the cell lines using Lipofectamine 2000™ (Invitrogen). After transient transfection for 24 hours, the cells were harvested and the activity of the promoter constructs assayed using the Dual-Luciferase Reporter Assay System (Promega). To determine promoter activity, firefly luciferase expression levels were normalized against Renilla luciferase levels. The luciferase expression levels of pGL3-basic/BRCA1-2265T and each of the four mutant constructs were compared with that of pGL3-basic/BRCA1-2265C which was assigned a relative value of 1. The pGL3-basic plasmid was used as negative control. Light emission measurements were carried out using an Infinite 200 reader (Tecan, Durham, NC, USA). The experiments were performed in three sets of triplicates. Results were compared using a non-parametric *t*-test.

Study Subjects

Hong Kong Study Subjects—This was a hospital-based sample collection consisting of a total of 416 incident breast cancer subjects, recruited during the period June 2003 to March 2004, from patients attending follow-up surgical and oncology outpatient clinics at three major public hospitals in Hong Kong Island (Queen Mary Hospital) and Kowloon (Queen Elizabeth Hospital and Kwong Wah Hospital). All subjects completed in-person interviews and were unselected for family history. The protocol was approved by the Institutional Review Boards of the University of Hong Kong/Hospital Authority. Patient consent was obtained for study participation and blood collection. Control subjects (n=399) matched for age at 10 year intervals were recruited from outpatients attending the general gynecological clinic in Queen Mary Hospital ascertained to have no personal history of cancer. They were also asked regarding family history of breast and/or ovarian cancer. About 70% cases and controls interviewed agreed to participate in this project. Blood samples were obtained from 380 (91.3%) cases and 390 (97.7%) controls which were subsequently used for DNA extraction by proteinase K digestion followed by conventional phenol/chloroform/ethanol method

Shanghai Study Subjects—This was a population-based case-control sample set consisting of subjects recruited from 1996 to 1998 by the Shanghai Breast Cancer Study group. Detailed study methods and recruitment have been published previously [17,18]. Briefly, this study included 1,459 incident breast cancer cases diagnosed at an age between 25–64 years and 1,556 age-frequency-matched community controls. The cancer cases were unselected for family history. Blood samples were obtained from 1193 (82%) cases and 1310 (84%) controls who consented to participate in the study and completed the in-person interviews. The protocol was approved by the relevant ethics committee. DNA extraction from the blood samples were performed as described above.

Genotyping Assays

The c.-2265C>T, c.-2804T>C and c.-2004A>G polymorphisms were genotyped by denaturing high-performance liquid-chromatography (DHPLC) in the Hong Kong subjects. The reaction was performed as described previously [19]. For the Shanghai study participants, the c.-2265C>T was assessed using a TaqMan assay (Applied Biosystems, Foster City, CA, USA). Analysis of c.-1896(ACA)₁>(ACA)₂ polymorphism was performed by fragment length genotyping method analyzed by GeneScan and GenoTyper (Mac OS, Applied Biosystems) as described previously [20]. Known homozygous and heterozygous genotype control samples were included in each genotyping assay and 5% of test samples for each 96-well plate reaction were duplicated, with 100 % agreement in genotype obtained. The primers and probes sequences are listed in Supplementary Table 1.

Statistical analysis

Hardy-Weinberg equilibrium (HWE) was examined using a Chi-square test (degree of freedom (df)=1). Odds ratio (OR) and 95% confidence interval (CI) were used to measure the strength of association. Genotype and haplotype distribution between cases and controls were analyzed using a Chi-square test (where df = number of genotypes or haplotypes being analyzed minus one). Two-tailed tests with p-value<0.05 were considered as statistically significant. For the Shanghai Breast Cancer Study population, additional adjustments were made in logistic regressions for body mass index, waist-to-hip ratio, physical activity, education menopausal status, menarche and age-at-first-live-birth. Mantel-Hanzel test was applied to compare the Hong Kong and Shanghai groups' risk association ORs. Study subjects with all polymorphisms successfully genotyped were used for haplotype analysis. The program PLINK (v.1.03) [21] was used for computational reconstruction of haplotypes. Haplotype frequencies thus obtained were used for haplotype case-control association analysis. Haploview [22] was used for linkage disequilibrium (LD) analysis to obtain D' and r² values. SPSS for Windows (version 15, SPSS Inc., Chicago, Illinois, USA) was used to perform non-parametric t-test, Chi-square test, and logistic regression analysis.

RESULTS

At the time the study was initiated, four promoter SNPs within the studied *BRCA1* promoter region were identified from the dbSNP database (build 120): c.-2804T>C, c.-2613G>C, c.-2004A>G, c.-1884A>G (rs3092986:A>G). Direct sequencing of the *BRCA1* promoter region in 20 healthy Hong Kong Chinese identified two previously unreported polymorphisms, the c.-2265C>T and the c.-1896(ACA)₁/(ACA)₂ variants, which have subsequently been included in the dbSNP database. The c.-2613G>C and c.-1884A>G variants on the other hand, were found to be monoallelic in these 20 healthy subjects, and were thus not included for further study..

In-silico analysis of c.-2804T>C, c.-2265C>T, c.-2004A>G and c.-1896(ACA)₁/(ACA)₂ by TFSEARCH [15] showed that c.-2804T>C and c.-2265C>T polymorphisms modified putative transcription factor recognition motifs with predicted score differences ≥10 between the two alleles (Supplementary Table 2). The transcription factors GATA-X (GATA-1,2,3) and Oct-1 had higher predicted scores for the mutant (minor) allele of c.-2265C>T. For c.-2804T>C, whilst Elk-1 had higher predicted scores for the mutant allele, an opposing transcription factor USF had higher predicted score for the wild-type allele. On the other hand, the putative transcription factors recognition sequences affected by c.-2004A>G and c.-1896(ACA)₁/(ACA)₂, were generally scored below 80, none with predicted score differences ≥10 between the two alleles..

Electrophoretic mobility shift (EMSA)

To investigate whether the mutant alleles of each polymorphism would modify binding affinity to nuclear protein, EMSA assay was performed by incubating HeLa nuclear-protein extract with double-stranded oligonucleotide probes containing either alleles.

Consistent with the *in-silico* results predicted by TFSEARCH, EMSA assay for c.-2265C>T gave the most clear-cut results. Up-shifted bands observed for both alleles could be competed by excess unlabeled oligonucleotides, with a difference noted between the C and T probes. For the radio-labeled C probes (Figure 1A, panel a), lanes 3 and 4 (competing with unlabeled C probe) gave stronger band signals than lanes 5 and 6 (competing with unlabeled T probe), indicating stronger competition for the unlabeled T probe. Consistent results were obtained for the radio-labeled T probes (Figure 1A, panel b). Lanes 11 and 12 (competing with unlabeled

C probe) gave stronger band signals than lanes 9 and 10 (competing with unlabeled T probe), indicating again stronger competition for the unlabeled T probe.

The intensity of the shifted bands were quantified by image analysis software and illustrated in Figure 1B. As panel (a) and panel (b) were performed in separate experiments, an arbitrary relative value of 1 was assigned for competition with unlabeled c.-2265T probe (lanes 5, 6, 9, and 10 of Figure 1A). For the radio-labeled C probes (Figure 1B, panel a), the relative band intensity in lanes 3 and 4 (competing with unlabeled C) was about 2-fold higher than that in lanes 5 and 6 (competing with unlabeled T) for both 25x and 50x competition respectively. For the radio-labeled T probes (Figure 1B, panel b), the relative intensity in lanes 11 and 12 (competing with unlabeled C) was also about 2-fold higher than that in lanes 9 and 10 (competing with unlabeled T). Since a band with greater intensity corresponds to weaker competition of the unlabeled probe, these results indicate that c.-2265T has stronger binding than c.-2265C with nuclear proteins.

For c.-2804T>C, up-shifted bands of either labeled probe could be competed by the respective cold probe, but not significantly by the unlabeled probe of the opposite allele, consistent with the opposing predicted putative transcription factor scores ascribed for either allele (Supplementary Figure 1). EMSA assay for c.-2004A>G, c.-1896(ACA)₁>(ACA)₂ were unable to clarify their level of contribution to promoter activity, in keeping with the given putative transcription factors predicted by *in-silico* search. (data not shown).

Promoter activity assays

Luciferase assay was performed to investigate whether each of these four polymorphisms c.-2804T>C, c.-2265C>T, c.-2004A>G and c.-1896(ACA)₁/(ACA)₂ could alter *BRCA1* promoter activity. Frequency estimates of haplotypes using PLINK had identified two predominant haplotypes occurring in more than 94% of Hong Kong cases and controls (Supplementary Table 3). These two haplotypes were divergent at all four positions. Six promoter constructs were created: the wild-type haplotype (named pGL3-basic/*BRCA1*-2265C), the predominant mutant haplotype (named pGL3-basic/*BRCA1*-2265T), and haplotypes in which one of the polymorphisms in turn was replaced by the mutant allele.

With the wild-type haplotype as reference, the transcriptional activity of pGL3-basic/*BRCA1*-2265T was significantly higher (fold change=1.7, $p<0.0001$, non-parametric t-test), supporting that the mutant haplotype harboring the c.-2265T allele enhances promoter activity (Figure 2).

Promoter constructs testing for the contribution of one polymorphism at a time, showed significant difference in promoter activity for each ($p<0.05$), suggesting that each of the four polymorphisms c.-2804T>C, c.-2265C>T, c.-2004A>G, c.-1896(ACA)₁>(ACA)₂ could contribute to alteration in promoter activity. Construct IV (T^{c.-2804}T^{c.-2265}A^{c.-2004}(ACA)₁^{c.-1896}) showed the most significant difference ($p=0.002$, fold change=1.6), followed closely by construct III (C^{c.-2804}C^{c.-2265}A^{c.-2004}(ACA)₁^{c.-1896}) ($p=0.008$, fold change=1.6), whilst the other two constructs contributed to a smaller fold difference in promoter activity (fold change=1.3) (Figure 2), although these differences remain significant. The higher promoter activity of the pGL3-basic/*BRCA1*-2265T corresponds to the higher binding affinity of the c.-2265T probe to nuclear protein in EMSA assay.

Genetic association study of *BRCA1* promoter variants in Hong Kong Chinese

To confirm whether these *in-vitro* findings could be demonstrated *in-vivo*, genetic association study was first evaluated on 380 cases and 390 controls recruited in Hong Kong. Genotyping was performed for each of the four polymorphisms (c.-2804T>C, c.-2265C>T, c.-2004A>G,

c.-1896(ACA)₁>(ACA)₂). All genotypes were in HWE for both cases and controls. Interestingly, only the c.-2265C>T variant showed significant association for overall genotype ($p=0.018$), minor allele carrier genotype ($p=0.005$; OR=0.64, 95% CI=0.47–0.88) as well as allele distribution ($p=0.023$; OR=0.79, 95% CI= 0.64–0.97) (Table 1). For c.-2804T>C, significant association was only noted for minor allele carrier genotype ($p=0.036$; OR=0.72, 95% CI=0.53–0.98) and allele distribution ($p=0.038$; OR=0.80, 95% CI=0.65–0.99), whilst for c.-2004A>G significant association was noted for the minor allele carrier genotype only ($p=0.032$; OR=0.72, 95% CI=0.53–0.97). The c.-1896(ACA)₁>(ACA)₂ polymorphism showed no significant genotype nor allelic association.

Haplotype analysis of the BRCA1 promoter region

Linkage Disequilibrium analysis of the four BRCA1 promoter polymorphisms showed that they were in strong and complete LD ($D'>0.90$ and $r^2>0.85$) (Supplementary Table 4). Frequency estimates of the haplotypes identified two predominant haplotypes occurring in more than 94% of our Hong Kong studied subjects (Supplementary Table 3). Analysis of the overall haplotype distribution using PLINK showed significant difference in frequency and composition of haplotypes between cases and controls ($p=0.001$). Risk association analysis comparing the two predominant haplotypes alone showed the association was significant for subjects aged ≥ 45 yrs at diagnosis without a family history of breast cancer ($p=0.008$, OR=0.64, 95% CI=0.46 to 0.89) (Table 2). As all four polymorphisms were in strong LD, to identify the contribution of each of the polymorphisms to risk susceptibility, three marker analyses in which each polymorphism was left out in turn was performed. Analysis of all cases showed the exclusion of c.-2265C>T resulted in loss of haplotype association, suggesting that this SNP contributed most towards risk association (Supplementary Table 5). Risk association remained significant for analysis of subjects aged ≥ 45 yrs at diagnosis without a family history of breast cancer, regardless of which polymorphism was excluded from the analyses. This suggests that consistent with our promoter activity assay findings, all four polymorphism contribute in some way towards risk association.

Risk association analysis of the c.-2265C>T SNP in Hong Kong and Shanghai Subjects

Our findings thus consistently support c.-2265C>T contributing most towards breast cancer risk, with genotype data of Hong Kong subjects showing women with c.-2265CT/TT genotype had a significantly reduced breast cancer risk ($p=0.005$; OR=0.64, 95% CI=0.47–0.88) (Table 3), the T allele acting dominantly.

Early-onset breast cancers, and/or those women with a strong family history of breast cancer, have a higher likelihood to harbor germline mutations in high penetrant breast cancer susceptibility genes. As cases had not been previously screened for *BRCA1* and *BRCA2* mutations, to minimize potential confounding influence of possible mutations in our analysis, stratified analyses according to age at cancer diagnosis and family history of breast cancer were also performed. As shown in Table 3, the association was more pronounced among women diagnosed at a later age (≥ 45 years) without a family history of breast cancer ($p=0.006$; OR=0.51, 95% CI=0.32–0.81).

To confirm the positive association identified for the c.-2265C>T SNP in the Hong Kong cohort, we replicated it on an independent large sample set recruited by the Shanghai Breast Cancer Study Group. Similar to what was observed in the Hong Kong study, a tendency towards a reduced risk was observed for carriers with the c.-2265CT/TT genotypes, which became significant among women without a family history of breast cancer ($p=0.019$, OR=0.81, 95% CI=0.69–0.97) and among women of age ≥ 45 yrs at first diagnosis and without a family history of breast cancer ($p=0.039$, OR=0.79, 95% CI=0.63–0.99) (Table 3) by logistic regression analysis with adjustment for confounding factors as previously reported [18,23]

Stratified analysis was performed between the Hong Kong and Shanghai populations. Test of heterogeneity comparing the reduced odds ratio did not demonstrate significant difference ($p=0.11$) between the two populations, enabling combined analysis of these two datasets (Table 3). The c.-2265CT/TT genotypes had reduced cancer risk with combined OR=0.80 (95% CI=0.69–0.93) relative to the c.-2265CC genotype, which was again stronger among older women (aged ≥ 45 yrs at first diagnosis) without a family history of breast cancer (combined OR=0.75, 95% CI=0.61–0.91) (Table 3).

DISCUSSION

Whilst studies have reported promising associations of cancer risk with 5' flanking region and promoter polymorphisms of several important genes such as estrogen receptor-alpha [24], *IGFBP-3* [25] and *MMP-2* [11], so far there has been no comprehensive investigation of *BRCA1* promoter polymorphisms with breast cancer risk. Changes in transcriptional regulation of *BRCA1* are likely to play an important role in the initiation or progression of sporadic breast cancer. Consistent decrease in *BRCA1* expression has been observed in tumor samples, and epigenetic effects such as aberrant cytosine methylation [6,7], histone hypoacetylation and chromatin condensation [26] as possible mechanisms down-regulating *BRCA1* expression have been demonstrated.

We report for the first time a comprehensive study of *BRCA1* promoter polymorphisms and show that the common genetic variants c.-2804T>C, c.-2265C>T, c.-2004A>G and c.-1896 (ACA)₁/(ACA)₂, could affect the binding affinity of nuclear protein and alter promoter activity. As supported by *in-silico* prediction of putative transcription factor binding sites, the contribution of c.-2265C>T was most significantly demonstrated by EMSA assay which showed stronger binding for the T allele, whilst promoter activity assay showed that the most common mutant haplotype which contained the c.-2265T allele increased promoter activity by 70%. Genetic association study supported this finding demonstrating that carriers of the T allele were associated with a reduced risk of breast cancer. This association was stronger among older women, particularly those without a family history of breast cancer. Such a pattern of association is expected since germ-line mutations in high penetrant breast cancer susceptibility genes are less likely to be found in late-onset cases and cases without a family history of breast cancer [27]. Moreover, this finding in Hong Kong Chinese was also replicated in an independently conducted population-based case-control study of Shanghai Chinese, which minimizes the possibility of type I error. Meta-analysis of both datasets by Mantel-Hanzen test showed no significant difference between reduced odds ratios between the two populations, allowing for combined odds ratio analysis, thus totaling 1484 cases and 1574 controls.

Previous studies had largely focused on association of *BRCA1* coding SNPs. Dunning et al found homozygotes of Arg356 to be inversely associated with breast cancer risk [28] which however could not be replicated by Cox et al [12] or Freedman et al [13]. Cox et al, identified one haplotype associated with a slight increased risk [12] but the functional variant(s) responsible for this association remains unknown, and promoter SNPs were not included for analysis. Freedman et al's multiethnic cohort haplotype analysis found no significant association between the common variants of *BRCA1* and breast cancer risk. Heterogeneity of *BRCA1* haplotypes was noted among the ethnic groups tested, with the haplotypes of Japanese and Native Hawaiian populations showing relatively lower diversity and being different from those of African American, Latino, and Caucasian populations [13]. Indeed one of Freedman et al's promoter SNPs, namely the c.-2613G>C variant was monoallelic in the African-Americans, which we also found to be monoallelic in 90 healthy Hong Kong Chinese women (unpublished data). Two other SNPs (rs1546585 and rs2175957) included by Freedman are located much further upstream at the 5' of *BRCA1* (18609bp and 8805bp respectively) and unlikely play an important role in promoter activity, and were hence not included in our study.

As Thakur and Croce [29] had demonstrated that substantial high level of promoter activity was maintained up to -1582bp, our study had included up to 1.6 kb of the promoter sequence for analysis.

It is noted that another gene, “*NBR2*”, is present adjacent to *BRCA1* in a head-to-head position [30]. Indeed the 3' end of the *BRCA1* promoter overlaps with 188 bp of the *NBR2* promoter, with the remaining 5' portion of the *BRCA1* promoter corresponding to complementary sequence of exon 1 to partial intron 1 of *NBR2*. The c.-2265C>T SNP corresponds to 589 nucleotides downstream intron 1 of *NBR2* and thus is less likely to give rise to a functional effect on *NBR2* expression/translation.

In-silico TF-SEARCH had identified OCT-1, GATA-1, GATA-2, GATA-3, USF and Elk-1 as potential transcription factors whose binding sites spanned over *BRCA1* promoter polymorphisms and therefore could alter their binding affinity. Co-transfection of vectors expressing these putative factors with pGL3-basic/*BRCA1*-2265T or pGL3-basic/*BRCA1*-2265C was however unable to show significant change in the promoter activity assay (data not shown) and the identity of the transcriptional factors remains to be investigated.

Although it cannot entirely be ruled out that the observed functional effect could be attributed to other variants of *BRCA1* not analyzed here, given our promoter assay results and the strong LD of the polymorphisms in this study, c.-2265C>T is a most justifiable tagging SNP.

The risk-reduction of 20–25% is consistent with that of low-penetrant gene effect. As this SNP (with minor allele T frequency over 35%) is relatively common in the Chinese population, its use as a predictive marker for reduced cancer risk is limited. However, as the most common mutant haplotype (which contains the c.-2265T allele) increased promoter activity by 70%, the functional consequences of such higher gene expression in *BRCA1*, a tumor-suppressor gene, may indeed confer an important protective role.

In summary, we are the first to show that the common genetic variants c.-2804T>C, c.-2265C>T, c.-2004A>G and c.-1896(ACA)₁/(ACA)₂, can affect the binding affinity of nuclear proteins and alter promoter activity, with the effect of c.-2265C>T being most significant. Our genetic association analysis of two independent Chinese cohorts totaling over 3,000 subjects support these findings by demonstrating that this *BRCA1* promoter SNP was significantly associated with reduced breast cancer risk. Together with gene expression regulation by epigenetic mechanisms, promoter polymorphisms may indeed make an important contribution towards breast cancer development.

Supplementary Material

Refer to Web version on PubMed Central for supplementary material.

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A

Lane No.	1	2	3	4	5	6	7	8	9	10	11	12
Labeled c.-2265C probe	+	+	+	+	+	+	-	-	-	-	-	-
Labeled c.-2265T probe	-	-	-	-	-	-	+	+	+	+	+	+
HeLa nuclear extract	-	+	+	+	+	+	-	+	+	+	+	+
25x unlabeled c.-2265C	-	-	+	-	-	-	-	-	-	-	+	-
50x unlabeled c.-2265C	-	-	-	+	-	-	-	-	-	-	-	+
25x unlabeled c.-2265T	-	-	-	-	+	-	-	-	+	-	-	-
50x unlabeled c.-2265T	-	-	-	-	-	+	-	-	-	+	-	-

B

Figure 1.
(A) EMSA assay using 28bp double-stranded oligonucleotides (probes) of the *BRCA1* c.-2265C>T SNP. Negative controls: (lanes 1 and 7) free probe, without nuclear proteins.

Nuclear proteins could bind to radiolabeled c.-2265C probe (lane 2) as well as to radiolabeled c.-2265T probe (lane 8). Competitive binding assay: addition of increasing molar excess (25×, 50× respectively) of non-radio-labeled probes to radio-labeled c.-2265C probe (panel a) and to radiolabeled c.-2265T probe (panel b). Competition with non-radiolabeled C probe (lanes 3 and 4, 11 and 12). Competition with non-radiolabeled T probe (lanes 5 and 6, 9 and 10). White arrows indicate up-shifted bands of the protein-DNA complexes.

(B) Relative intensity of the shifted bands in the competition assay shown in (A) for the labeled c.-2265C probe (panel a) and the labeled c.-2265T probe (panel b). The lane number corresponds to that in (A). Bars: standard deviation.

Haplotype constructs*	c.-2804 T>C	c.-2265 C>T	c.-2004 A>G	c.-1896 (ACA) ₁ >(ACA) ₂	Fold change	p-value	Remarks
I	T	C	A	(ACA) ₁	1		wild type haplotype (pGL3-basic/BRCA1-2265C)
II	C	T	G	(ACA) ₂	1.7	<0.0001	pGL3-basic/BRCA1-2265T
III	<u>C</u>	C	A	(ACA) ₁	1.6	0.008	replaced -2804T by C in the wild type haplotype
IV	T	<u>T</u>	A	(ACA) ₁	1.6	0.002	replaced -2265C by T in the wild type haplotype
V	T	C	<u>G</u>	(ACA) ₁	1.3	0.008	replaced -2004A by G in the wild type haplotype
VI	T	C	A	<u>(ACA)₂</u>	1.3	0.039	replaced -1896ACA1 by ACA2 in the wild type haplotype

Figure 2.

Luciferase assay results of the wild type (pGL3-basic/BRCA1-2265C) and mutant *BRCA1* promoter haplotype constructs in HeLa cells. Results are the means from three independent triplicate experiments. The luciferase activity readings of mutant haplotype constructs were normalized against that of pGL3-basic/BRCA1-2265C which was assigned an arbitrary relative value of 1. Luciferase levels were significantly higher for each mutant when compared with pGL3-basic/BRCA1-2265C ($p < 0.05$, by non-parametric t-test) Bars: standard deviation. Construct I and II are the wild-type and the predominant mutant haplotypes respectively, found in Hong Kong Chinese. Construct III to VI were created by site-directed mutagenesis. (Bolded and underlined nucleotides indicate the difference compared to the wild type construct).

Table 1

Analysis of *BRCA1* promoter polymorphisms c.-2804T>C, c.-2265C>T, c.-2004A>G, and c.-1896(ACA)₁>(ACA)₂ for breast cancer risk association in Hong Kong Chinese subjects

Polymorphism	Genotype/Allele	N	Case (%)	Control (%)	N	p-value	OR (95% CI) *
c.-2804T>C	TT	133	37.50%	30.10%	112	0.094 [#]	1.00 (reference)
	TC	169	47.60%	51.60%	192	0.071	0.74 (0.54–1.03)
	CC	53	14.90%	18.30%	68	0.059	0.65 (0.42–1.02)
	TC/CC	222	62.50%	69.90%	260	0.036	0.72 (0.53–0.98)
	T allele	435	61.30%	55.90%	416		1.00 (reference)
	C allele	275	38.70%	44.10%	328	0.038	0.80 (0.65–0.99)
	HWE [†]	p=0.95			p=0.37		
c.-2265C>T	CC	143	38.90%	29.10%	109	0.018[#]	1.00 (reference)
	CT	164	44.60%	52.50%	197	0.006	0.63 (0.46–0.88)
	TT	61	16.60%	18.40%	69	0.068	0.67 (0.44–1.03)
	CT/TT	225	61.10%	70.90%	266	0.005	0.64 (0.47–0.88)
	C allele	450	61.10%	55.30%	415		1.00 (reference)
	T allele	286	38.90%	44.70%	335	0.023	0.79 (0.64–0.97)
	HWE [†]	p=0.23			p=0.22		
c.-2004 A>G	AA	138	37.60%	30.10%	113	0.099 [#]	1.00 (reference)
	AG	171	46.60%	52.30%	196	0.041	0.71 (0.52–0.99)
	GG	58	15.80%	17.60%	66	0.135	0.72 (0.47–1.11)
	AG/GG	229	62.40%	69.90%	262	0.032	0.72 (0.53–0.97)
	A allele	447	60.90%	56.30%	422		1.00 (reference)
	G allele	287	39.10%	43.70%	328	0.070	0.83 (0.67–1.02)
	HWE [†]	p=0.68			p=0.23		
c.-1896 (ACA) ₁ >(ACA) ₂	(ACA) ₁ (ACA) ₁	126	33.60%	30.30%	118	0.538 [#]	1.00 (reference)
	(ACA) ₁ (ACA) ₂	185	49.30%	53.20%	207	0.276	0.84 (0.61–1.15)
	(ACA) ₂ (ACA) ₂	64	17.10%	16.50%	64	0.764	0.94 (0.61–1.44)
	(ACA) ₁ (ACA) ₂ /(ACA) ₂ (ACA) ₂	249	66.40%	69.70%	271	0.333	0.86(0.63–1.17)
	(ACA) ₁ allele	437	58.30%	56.90%	443		1.00 (reference)
	(ACA) ₂ allele	313	41.70%	43.10%	335	0.600	0.95 (0.77–1.16)
	HWE [†]	p=0.78			p=0.09		

[#] Test of overall genotype distribution in cases and controls by Chi-square test (df=2).

* Test of genotype and allele distribution in cases and controls by Chi-square test (df=1).

[†] p-values for HWE were calculated by Chi-square test, df=1. Statistically significant p-values bolded.

Table 2
Haplotype analysis of the four *BRCA1* promoter polymorphisms in relation to breast cancer risk in Hong Kong Chinese.

c.-2804 T>C	c.-2265 C>T	c.-2004 A>G	c.-1896 (ACA) ₁ >(ACA) ₂	No.	Cases ^d %	No.	Controls ^d %	p [*]	OR (95% CI) [*]
All Subjects (Number of alleles analyzed in cases and controls: 682 and 734 respectively) [#]									
T	C	A	(ACA) ₁	394	57.8%	398	54.21%	Ref	Ref
C	T	G	(ACA) ₂	253	37.1%	311	42.4%	0.076	0.82 (0.66 to 1.02)
Subjects without family history of breast cancer (Number of alleles analyzed in cases and controls: 614 and 712 respectively) [#]									
T	C	A	(ACA) ₁	360	58.6%	388	54.5%	Ref	Ref
C	T	G	(ACA) ₂	224	36.5%	299	42.0%	0.062	0.81 (0.64 to 1.01)
Subjects ≥ 45 yrs at first diagnosis and without family history of breast cancer (Number of alleles analyzed in cases and controls: 312 and 322 respectively) [#]									
T	C	A	(ACA) ₁	189	60.6%	169	52.5%	Ref	Ref
C	T	G	(ACA) ₂	105	33.7%	146	45.3%	0.008	0.64 (0.46 to 0.89)

[#] Haplotype analysis was performed on those subjects in which all polymorphisms had been successfully genotyped. Haplotypes were estimated by PLINK (v1.03) .

^{*} p-values were obtained using Chi-square test, two-tailed and df=1. Ref=reference.

^d Cancer cases and control subjects that had been genotyped for all four promoter SNPs.

Table 3

Association of breast cancer risk with the c.-2265C>T SNP in the *BRCA1* gene, on subjects recruited in the Hong Kong Chinese and in the Shanghai Breast Cancer Study.

c.-2265C>T	Hong Kong			Shanghai			Test of heterogeneity		Combined analysis ^{††}	
	Cases (%)	Controls (%)	p-value [*]	OR (95% CI) [*]	Cases (%)	Controls (%)	p-value ^{**}	OR (95% CI) ^{**}	p-value	OR (95% CI)
All subjects ^a										
(368 cases, 375 controls)										
(1109 cases, 1185 controls)										
CC	143 (38.9%)	109 (29.1%)		1.00 (reference)	469 (42.3%)	457 (38.6%)		1.00 (reference)		1.00 (reference)
CT/TT	225 (61.1%)	266 (70.9%)	0.005	0.64 (0.47–0.88)	640 (57.7%)	728 (61.4%)	0.054	0.85 (0.71–1.00)	0.11	0.80 (0.69–0.93)
HWE [‡]	p=0.23	p=0.22			p=0.07	p=0.55				
Subjects without family history of breast cancer										
(333 cases, 363 controls)										
(1070 cases, 1156 controls)										
CC	129 (38.7%)	107 (29.5%)		1.00 (reference)	459 (42.9%)	442 (38.2%)		1.00 (reference)		1.00 (reference)
CT/TT	204 (61.3%)	256 (70.5%)	0.01	0.66 (0.48–0.91)	611 (57.1%)	714 (61.8%)	0.019	0.81 (0.69–0.97)	0.23	0.78 (0.68–0.91)
Subjects ≥ 45 yrs at first diagnosis and without family history of breast cancer										
(166 cases, 166 controls)										
(637 cases, 678 controls)										
CC	71 (42.8%)	46 (27.7%)		1.00 (reference)	281 (44.1%)	265 (39.1%)		1.00 (reference)		1.00 (reference)
CT/TT	95 (57.2%)	120 (72.3%)	0.006	0.51 (0.32–0.81)	356 (55.9%)	413 (60.9%)	0.039	0.79 (0.63–0.99)	0.08	0.75 (0.61–0.91)

* p-values were obtained by Chi-square test, df=1.

** Logistic regression analysis adjusted for age, education, body mass index, waist-to-hip ratio, physical activity, menopausal status, age at menarche, and age at first live birth.

‡ p-values for HWE were calculated by Chi-square test, df=1.

†† Combined analysis was performed using Mantel-Haenszel test.

^a Overall cases and control subjects successfully genotyped.