Functional polymorphisms in the BRCA1 promoter influence 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: Using 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 Chinese. Each polymorphism was studied independently and in combination by functional assays. Although all four variants significantly altered promoter activity, the c.-2265T allele had 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 Hong Kong Chinese women with breast cancer and age-matched controls and replicated in a large population-based study of Shanghai Chinese, together totalling >3000 participants, showed that carriers of the c.-2265T allele had a reduced risk for breast cancer (combined odds ratio [OR] = 0.80, 95% CI 0.69 to 0.93; p = 0.003) which was more evident among women aged ≥45 years at first diagnosis of breast cancer and without a family history of breast cancer (combined OR = 0.75, 95% CI 0.61 to 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 a family history of breast cancer (OR = 0.64, 95% CI 0.46 to 0.89; p = 0.008).

Conclusion: This comprehensive study of BRCA1 promoter polymorphisms found four variants that 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.

The BRCA1 gene, a tumour 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 and 81% of breast–ovarian cancer families. Women carrying loss-of-function mutations in BRCA1 have been reported to carry an 81% lifetime risk of developing breast cancer. BRCA1 mutations account for only 5–10% of all breast cancers. Besides germline and somatic mutations, promoter hypermethylation is attributed to reduced BRCA1 expression in some cases of breast cancer, including sporadic cases.

Single-nucleotide polymorphisms (SNPs) 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. We hypothesised that potentially functional BRCA1 promoter polymorphisms could alter transcriptional activity, thus affecting susceptibility to develop sporadic breast cancer. To date, no study has comprehensively investigated the BRCA1 promoter SNPs for their functional roles and contribution to risk of developing sporadic breast cancer. Two recent risk association studies have investigated 4 tagging SNPs and 28 SNPs spanning the BRCA1 gene. In the study of Freedman et al., however, the minor allele frequency (MAF) of c.-2613G→C was <0.6% in our Chinese population and thus could not be analysed. To test our hypothesis, polymorphisms located in the BRCA1 promoter were identified from the public dbSNP (http://www.ncbi.nlm.nih.gov/projects/SNP/) database and by resequencing healthy Chinese people 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. Although each polymorphism could affect promoter activity, the contribution was most significant for c.-2265C→T, as supported by computed 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.

METHODS

Identification of BRCA1 promoter polymorphisms

BRCA1 promoter SNPs were identified from the dbSNP database (build no 120) at the start of the study. The BRCA1 genomic reference sequence (GenBank number U57574) was used with the first nucleotide upstream of the translation initiation codon designated as nucleotide -1. To identify DNA variations that may be unique to Chinese
populations, direct sequencing of the promoter region (1.6 kb upstream of the transcription start site at the exon 1a of BRCA1) was also performed on 20 healthy Hong Kong Chinese. A study group of 20 individuals would give at least 87% probability of finding variants of MAF ≥5%.

Identification of putative transcription factor binding elements

A computer-based search for putative transcription factor binding elements harboured by the BRCA1 promoter polymorphisms was performed using the software TFSEARCH (V1.5) (http://www.gzic.jp/research/db/TFSEARCH.html). All putative transcription factors that are unidirectional with respect to the sense strand sequence of the BRCA1 promoter in humans were identified.

Electrophoretic mobility shift assay

Nuclear protein extraction (from human cervical cancer (HeLa) cells) and EMSA were performed as described previously with some modifications. Briefly, double-stranded c.-2804T, c.-2804C, c.-2265T and c.-2265C oligonucleotide probes (supplementary table 1) were 32P-end-labelled and purified by using MicroSpin G-50 columns (GE Healthcare, Piscataway, New Jersey, USA). Binding experiments were conducted by incubating 10 μg nuclear-protein extracts with 0.14 pmol (400 000 counts/min) of probe at room temperature for 30 minutes. The nuclear proteins and various oligonucleotide probes were incubated in a binding buffer containing 10 mmol/l Tris (pH 7.5), 10% glycerol, 5 μg/ml of poly(dI-dC), 10 mg/ml bovine serum albumin and 1% Nonidet P-40. For competition experiments, unlabelled oligonucleotide probes were added to the radiolabelled probe reaction mixture at 25 or 50 times molar excess before incubation. After electrophoresis, gels were dried and subjected to autoradiographic analysis. The shifted band intensity was analysed by Grab-IT image analysis software (UVP Inc., Upland, California, USA), the final 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.-2004C, c.-2265A, c.-2004(ACA)1 and Cc.-2004T, c.-2265C, c.-2004(ACA)1, were amplified using specific primers containing 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 a Hi-Fi Expand PCR kit (Roche Diagnostics, Mannheim, Germany) according to the manufacturer’s recommendations. The amplified products were then cloned into firefly luciferase-reporter pGL3-basic vector (Promega, Madison, Wisconsin, 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 were verified by sequencing. Based on the pGL3-basic/BRCA1-2265C haplotype construct, four other mutant promoter 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.-2004T, c.-2265G, c.-2004(ACA)1 and Tc.-2004C, c.-2265A, c.-2004(ACA)1 (bold letters show the difference with respect to pGL3-basic/BRCA1-2265C).

Luciferase reporter assay

HeLa cell lines were used for transient transfection experiments. The HeLa cells were cultured in Dulbecco’s modified Eagle’s medium with high glucose (4.5 mg/ml) (Invitrogen, Carlsbad, California, USA). In total, 8 x 104 cells were cultured in dishes (35 mm diameter) for 24 hours before transfection, then 1 μg of luciferase-reporter plasmid and 0.1 μg 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 normalised 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, North Carolina, USA). The experiments were performed in three sets of triplicates. Results were compared using a non-parametric t test.

Study participants

Hong Kong study participants

The protocol was approved by the Institutional Review Boards of the University of Hong Kong Hospital Authority, and patient consent was obtained for study participation and blood collection.

This was a hospital-based sample collection consisting of 416 women with incident breast cancer, recruited during the period June 2005 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 Elisabeth Hospital and Kwong Wah Hospital). All participants completed face to face interviews and were unselected for family history. Control participants (n = 599) matched for age at 10-year intervals were recruited from outpatients attending the general gynaecological clinic in Queen Mary Hospital who had no personal history of cancer. They were also questioned about any family history of breast and/or ovarian cancer. About 70% of 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 extraction.

Shanghai study participants

The protocol for this study was approved by the relevant ethics committee.

This was a population-based case-control sample set consisting of participants recruited from 1996 to 1998 by the Shanghai Breast Cancer Study group. Detailed study methods and recruitment have been published previously. The study comprised 1459 incident breast cancer cases diagnosed at 25–64 years of age and 1556 age-frequency-matched community controls. The cancer cases were unselected for family history. Blood samples were obtained from 1195 (82%) cases and 1310 (84%) controls who consented to participate in the study and completed the face to face interviews. DNA extraction from the blood samples was performed as described above.
Genotyping assays
The c.-2265C→T, c.-2804T→C and c.-2004A→G polymorphisms in the Hong Kong participants were genotyped by denaturing high-performance liquid chromatography (DHPLC). The reaction was performed as described previously.10 For the Shanghai study participants, the c.-2265C→T was assessed using a TaqMan assay (Applied Biosystems, Foster City, California, USA). Analysis of the c.-1896(ACA)_1→(ACA)_2 polymorphism was performed by fragment length polymorphism analysis, assessed by GenScan and GenoTyper software (Mac OS, Applied Biosystems) as described previously.10 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 (online).

Statistical analysis
Hardy–Weinberg equilibrium was investigated using χ² 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 analysed using the χ² test and logistic regression analysis. Haploview22 was used for linkage disequilibriums thus obtained were used for haplotype case–control association analysis. Haplotype frequencies or haplotypes being analysed minus 1). Two-tailed tests were used, with p<0.05 considered 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 used to compare the risk association OR between the Hong Kong participants were genotyped by DHPLC. The reaction was performed as described previously.19

RESULTS
At the time the study was started, 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 (rs5092986: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 since been included in the dbSNP database. The c.-2613G→C and c.-1884A→G variants were found to be monoallelic in these 20 healthy participants and thus were not included for further study.

The TFSEARCH analysis of the c.-2804T→C, c.-2265C→T, c.-2004A→G and c.-1896(ACA)_1→(ACA)_2 polymorphisms showed that c.-2804T→C and c.-2265C→T modified putative transcription factor recognition motifs with predicted score differences of >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 allele, which were divergent at all four positions. Six promoter constructs were created: the wild-type haplotype (named pGL3-basic/BRCA1-2265C), the wild-type haplotype (named pGL3-basic/BRCA1-2265T) and haplotypes in predicted score for the wild-type allele. In contrast, the putative transcription factors recognition sequences affected by c.-2004A→G and c.-1896(ACA)_1→(ACA)_2 generally scored >80, none with predicted score differences >10 between the two alleles.

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

Consistent with the results predicted by TFSEARCH, the EMSA for c.-2265C→T gave the most clear-cut results. Upwardly shifted bands for both alleles could be competed by excess unlabelled oligonucleotides, with a difference noted between the C and T probes. For the radiolabelled C probes (fig 1A, panel a), lanes 3 and 4 (competing with unlabelled C probe) gave stronger band signals than lanes 5 and 6 (competing with unlabelled T probe), indicating stronger competition for the unlabelled T probe. Consistent results were obtained for the radiolabelled T probes (fig 1A, panel b). Lanes 11 and 12 (competing with unlabelled C probe) gave stronger band signals than lanes 9 and 10 (competing with unlabelled T probe), indicating again stronger competition for the unlabelled T probe.

The intensity of the shifted bands was quantified by image analysis software (fig 1B). As the results in panels (a) and (b) were obtained from separate experiments, an arbitrary relative value of 1 was assigned for competition with the unlabelled c.-2265T probe (fig 1A; lanes 5, 6, 9 and 10). For the radiolabelled C probes (fig 1B, panel a), the relative band intensity in lanes 3 and 4 (competing with unlabelled C) was about twice that in lanes 5 and 6 (competing with unlabelled T) for both concentrations (25 and 50 times, respectively) of unlabelled probes. For the radiolabelled T probes (fig 1B, panel b), the relative intensity in lanes 11 and 12 (competing with unlabelled C) was also about twice that in lanes 9 and 10 (competing with unlabelled T). As a band with greater intensity corresponds to weaker competition by the unlabelled probe, these results indicate that c.-2265T has stronger binding to nuclear proteins than does c.-2265C.

For c.-2804T→C, upwardly shifted bands of either labelled probe could be competed by the respective cold probe, but not significantly by the unlabelled probe of the opposite allele, consistent with the opposing predicted putative transcription factor scores ascribed for either allele (supplementary fig 1 online). EMSA for c.-2004A→G, c.-1896(ACA)_1→(ACA)_2 was unable to clarify their level of contribution to promoter activity, in keeping with the given putative transcription factor scores predicted by TFSEARCH (data not shown).

Promoter activity assays
A 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 >94% of Hong Kong cases and controls (supplementary table 3), which were divergent at all four positions. Six promoter constructs were created: the wild-type haplotype (named pGL3-basic/BRCA1-2265C), the wild-type 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 of 1.7, p \( < 0.0001 \), non-parametric t test), supporting the notion that the mutant haplotype harbouring the c.-2265T allele enhances promoter activity (fig 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 \( \rightarrow \) C, c.-2265C \( \rightarrow \) T, c.-2004A \( \rightarrow \) G, c.-1896(ACA) \( \rightarrow \) (ACA) \( _2 \)) could contribute to alteration in promoter activity. Construct IV (T c.-2804 \( \rightarrow \) T c.-2265 \( \rightarrow \) C c.-2004(ACA) \( \rightarrow \) (ACA) \( _2 \) ) showed that the most significant difference (p = 0.002, fold change 1.6), followed closely by construct III (C c.-2804 \( \rightarrow \) A c.-2265 \( \rightarrow \) A(ACA) \( _1 \) ) (p = 0.008, fold change 1.6), whereas the other two constructs contributed to a smaller fold difference in promoter activity (fold change 1.3) (fig 2), although these differences remained 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.

Figure 1  (A) EMSA using 28bp double-stranded oligonucleotides (probes) of the BRCA1 c.-2265C \( \rightarrow \) T SNP. Lanes 1 and 7: negative controls (free probe, without nuclear proteins). Nuclear proteins could bind to radiolabelled c.-2265C probe (lane 2) and to radiolabelled c.-2265T probe (lane 8). Competitive binding assay: addition of increasing molar excess (25 and 50 times, respectively) of non-radiolabelled probes to radiolabelled (a) c.-2265C and (b) c.-2265T probes. Lanes 3, 4, 11, 12, competition with non-radiolabelled C probe; lanes 5, 6, 9, 10, competition with non-radiolabelled T probe. White arrows indicate upwardly shifted bands of the protein-DNA complexes. (B) Relative intensity of the shifted bands in the competition assay shown in (A) for (a) the labelled c.-2265C probe and (b) the labelled c.-2265T probe (panel b). Lane numbers as before. Bars are standard deviation.

Genetic association study of BRCA1 promoter variants in Hong Kong Chinese

To confirm whether these in vitro findings could be shown in vivo, a genetic association study was carried out on 380 cases and 390 controls recruited in Hong Kong. Genotyping was performed for each of the four polymorphisms (c.-2804T \( \rightarrow \) C, c.-2265C \( \rightarrow \) T, c.-2004A \( \rightarrow \) G, c.-1896(ACA) \( \rightarrow \) (ACA) \( _2 \) ). All genotypes were in Hardy–Weinberg equilibrium for both cases and controls. Interestingly, only the c.-2265C \( \rightarrow \) 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 to 0.88) and allele distribution (p = 0.023; OR = 0.79, 95% CI 0.64 to 0.97) (table 1). For c.-2804T \( \rightarrow \) C, significant association was noted only for minor allele carrier genotype (p = 0.036; OR = 0.72, 95% CI 0.53 to 0.98) and allele distribution (p = 0.038; OR = 0.80, 95% CI 0.65 to 0.99), whereas for c.-2004A \( \rightarrow \) G, significant association was noted for the minor allele carrier genotype only (p = 0.052; OR = 0.72, 95% CI 0.55 to 0.97). The c.-1896(ACA) \( \rightarrow \) (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 ($D^2 > 0.90$ and $r^2 > 0.85$) (supplementary table 4). Frequency estimates of the haplotypes identified two predominant haplotypes occurring in $94\%$ of our Hong Kong studied participants (supplementary table 3). Analysis of the overall haplotype distribution using PLINK showed significant

**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 participants

<table>
<thead>
<tr>
<th>Polymorphism</th>
<th>Genotype/allele</th>
<th>Case (n (%))</th>
<th>Control (n (%))</th>
<th>p Value</th>
<th>OR (95% CI)*</th>
</tr>
</thead>
<tbody>
<tr>
<td>c.-2804T→C</td>
<td>TT</td>
<td>133 (37.50)</td>
<td>112 (30.10)</td>
<td>0.094†</td>
<td>1.00 (RV)</td>
</tr>
<tr>
<td></td>
<td>TC</td>
<td>169 (47.60)</td>
<td>192 (51.60)</td>
<td>0.071</td>
<td>0.74 (0.54 to 1.03)</td>
</tr>
<tr>
<td></td>
<td>CC</td>
<td>53 (14.90)</td>
<td>68 (18.30)</td>
<td>0.059</td>
<td>0.65 (0.42 to 1.02)</td>
</tr>
<tr>
<td></td>
<td>TC/CC</td>
<td>222 (62.50)</td>
<td>260 (69.90)</td>
<td>0.036</td>
<td>0.72 (0.53 to 0.98)</td>
</tr>
<tr>
<td></td>
<td>T allele</td>
<td>435 (61.30)</td>
<td>416 (55.90)</td>
<td>0.100</td>
<td>0.90 (RV)</td>
</tr>
<tr>
<td></td>
<td>C allele</td>
<td>275 (38.70)</td>
<td>328 (44.10)</td>
<td>0.038</td>
<td>0.80 (0.65 to 0.99)</td>
</tr>
<tr>
<td></td>
<td>HWE p value;‡</td>
<td>0.95</td>
<td>0.37</td>
<td></td>
<td></td>
</tr>
<tr>
<td>c.-2265C→T</td>
<td>CC</td>
<td>143 (38.90)</td>
<td>109 (29.10)</td>
<td>0.018†</td>
<td>1.00 (RV)</td>
</tr>
<tr>
<td></td>
<td>CT</td>
<td>164 (44.60)</td>
<td>197 (52.50)</td>
<td>0.006</td>
<td>0.63 (0.46 to 0.88)</td>
</tr>
<tr>
<td></td>
<td>TT</td>
<td>61 (16.60)</td>
<td>69 (18.40)</td>
<td>0.068</td>
<td>0.67 (0.44 to 1.03)</td>
</tr>
<tr>
<td></td>
<td>CT/TT</td>
<td>225 (61.10)</td>
<td>266 (70.90)</td>
<td>0.005</td>
<td>0.64 (0.47 to 0.88)</td>
</tr>
<tr>
<td></td>
<td>C allele</td>
<td>450 (61.10)</td>
<td>415 (55.30)</td>
<td>0.001†</td>
<td>1.00 (RV)</td>
</tr>
<tr>
<td></td>
<td>T allele</td>
<td>286 (38.90)</td>
<td>335 (44.70)</td>
<td>0.023</td>
<td>0.79 (0.64 to 0.97)</td>
</tr>
<tr>
<td></td>
<td>HWE p value;‡</td>
<td>0.23</td>
<td>0.22</td>
<td></td>
<td></td>
</tr>
<tr>
<td>c.-2004 A→G</td>
<td>AA</td>
<td>130 (36.70)</td>
<td>113 (30.30)</td>
<td>0.099†</td>
<td>1.00 (RV)</td>
</tr>
<tr>
<td></td>
<td>AG</td>
<td>171 (46.60)</td>
<td>196 (52.30)</td>
<td>0.061†</td>
<td>0.71 (0.52 to 0.99)</td>
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<tr>
<td></td>
<td>GG</td>
<td>58 (15.80)</td>
<td>66 (17.60)</td>
<td>0.135</td>
<td>0.72 (0.47 to 1.11)</td>
</tr>
<tr>
<td></td>
<td>AG/GG</td>
<td>229 (62.40)</td>
<td>262 (69.90)</td>
<td>0.032†</td>
<td>0.72 (0.53 to 0.97)</td>
</tr>
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<td>A allele</td>
<td>447 (60.90)</td>
<td>422 (56.30)</td>
<td>0.001</td>
<td>1.00 (RV)</td>
</tr>
<tr>
<td></td>
<td>G allele</td>
<td>287 (39.10)</td>
<td>328 (43.70)</td>
<td>0.070</td>
<td>0.83 (0.67 to 1.02)</td>
</tr>
<tr>
<td></td>
<td>HWE p value;‡</td>
<td>0.68</td>
<td>0.23</td>
<td></td>
<td></td>
</tr>
<tr>
<td>c.-1896 (ACA)$_1$→(ACA)$_2$</td>
<td>(ACA)$_1$</td>
<td>126 (33.60)</td>
<td>118 (30.30)</td>
<td>0.538†</td>
<td>1.00 (RV)</td>
</tr>
<tr>
<td></td>
<td>(ACA)$_2$</td>
<td>185 (49.30)</td>
<td>207 (53.20)</td>
<td>0.276</td>
<td>0.84 (0.61 to 1.15)</td>
</tr>
<tr>
<td></td>
<td>(ACA)$_1$</td>
<td>64 (17.10)</td>
<td>64 (16.50)</td>
<td>0.764</td>
<td>0.84 (0.61 to 1.44)</td>
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<tr>
<td></td>
<td>(ACA)$_2$</td>
<td>249 (66.40)</td>
<td>271 (69.70)</td>
<td>0.333</td>
<td>0.86 (0.63 to 1.17)</td>
</tr>
<tr>
<td></td>
<td>(ACA)$_1$ allele</td>
<td>437 (58.30)</td>
<td>443 (56.90)</td>
<td>1.00</td>
<td>0.71 (0.53 to 1.11)</td>
</tr>
<tr>
<td></td>
<td>(ACA)$_2$ allele</td>
<td>313 (41.70)</td>
<td>335 (43.10)</td>
<td>0.600</td>
<td>0.95 (0.77 to 1.16)</td>
</tr>
<tr>
<td></td>
<td>HWE p value;‡</td>
<td>0.78</td>
<td>0.09</td>
<td></td>
<td></td>
</tr>
</tbody>
</table>

HWE, Hardy–Weinberg equilibrium; RV, reference value.

*Test of genotype and allele distribution in cases and controls by $\chi^2$ test (df = 1).
†Test of overall genotype distribution in cases and controls by $\chi^2$ test (df = 2).
‡p Values for HWE were calculated by $\chi^2$ test, df = 1.
Significant p values are in **bold** type.
differences in frequency and composition of haplotypes between cases and controls (p = 0.001). Risk association analysis comparing the two predominant haplotypes alone showed that the association was significant for participants aged ≥45 years 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, three marker analyses, in which each polymorphism was excluded in turn, were performed to identify the contribution of each of the polymorphisms to risk susceptibility. Analysis of all cases showed that 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 participants aged ≥45 years 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 polymorphisms contribute in some way towards risk association.

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

Our findings consistently support c.-2265C→T contributing most towards breast-cancer risk, with genotype data of Hong Kong participants showing that women with the c.-2265CT/TT genotype had a significantly reduced breast-cancer risk (p = 0.005; OR = 0.64, 95% CI 0.47 to 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 of harbouring germline mutations in high-penetrance breast-cancer susceptibility genes. As cases had not been previously screened for BRCA1 and BRCA2 mutations, stratified analyses according to age at cancer diagnosis and a family history of breast cancer were also performed to minimise potential confounding influence of possible mutations in our analysis. 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 to 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 the results in the Hong Kong study, a tendency towards a reduced risk was found 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 to 0.97) and among women aged ≥45 years at first diagnosis and without a family history of breast cancer (p = 0.039, OR = 0.79, 95% CI 0.63 to 0.99) (table 3), using logistic regression analysis with adjustment for confounding factors as previously reported.

Stratified analysis was performed between the Hong Kong and Shanghai populations. A test of heterogeneity comparing the reduced odds ratio did not show significant differences (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 to 0.98) relative to the c.-2265CC genotype, which was again stronger among older women (aged ≥45 years at first diagnosis) without a family history of breast cancer (combined OR = 0.75, 95% CI 0.61 to 0.91) (table 3).

DISCUSSION

Although some studies have reported promising associations of cancer risk with the 5’ flanking region and promoter polymorphisms of several important genes such as oestrogen receptor-α, IGFBP-3 and MIMP-2 to date 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. A consistent decrease in BRCA1 expression has been found in tumour samples and epigenetic effects such as aberrant cytosine methylation, histone hypoacetylation and chromatin condensation have been suggested as possible mechanisms in downregulating BRCA1 expression.

We report, for the first time to our knowledge, a comprehensive study of BRCA1 promoter polymorphisms and show that the common genetic variants c.-2004T→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 computer-based prediction of putative transcription factor binding sites, the contribution of c.-2265C→T was most significantly shown by EMSA, which showed stronger binding for the T allele, whereas promoter activity assay showed that the most common mutant haplotype, containing the c.-2265T allele, increased promoter activity by 70%. A genetic association study supported this finding, showing that carriers of the T allele had a reduced risk of...

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

<table>
<thead>
<tr>
<th>c.-2804</th>
<th>c.-2265</th>
<th>c.-2004A</th>
<th>c.-1896 (ACA)1</th>
<th>Cases, n (%)</th>
<th>Controls*, n (%)</th>
<th>p Value†</th>
<th>OR (95% CI)‡</th>
</tr>
</thead>
<tbody>
<tr>
<td>T</td>
<td>C</td>
<td>A</td>
<td>[ACA]1</td>
<td>394 (57.8)</td>
<td>398 (54.21)</td>
<td>RV</td>
<td>RV</td>
</tr>
<tr>
<td>C</td>
<td>T</td>
<td>G</td>
<td>[ACA]2</td>
<td>253 (37.1)</td>
<td>311 (42.4)</td>
<td>0.076</td>
<td>0.82 (0.66 to 1.02)</td>
</tr>
<tr>
<td>Participants without a family history of breast cancer (n = 614, n = 712): T</td>
<td>C</td>
<td>A</td>
<td>[ACA]1</td>
<td>360 (58.6)</td>
<td>388 (54.5)</td>
<td>RV</td>
<td>RV</td>
</tr>
<tr>
<td>C</td>
<td>T</td>
<td>G</td>
<td>[ACA]2</td>
<td>224 (36.5)</td>
<td>299 (42.0)</td>
<td>0.062</td>
<td>0.81 (0.64 to 1.01)</td>
</tr>
<tr>
<td>Participants ≥45 years at first diagnosis and without a family history of breast cancer (n = 312, n = 322): T</td>
<td>C</td>
<td>A</td>
<td>[ACA]1</td>
<td>189 (60.6)</td>
<td>169 (52.5)</td>
<td>RV</td>
<td>RV</td>
</tr>
<tr>
<td>C</td>
<td>T</td>
<td>G</td>
<td>[ACA]2</td>
<td>105 (33.7)</td>
<td>146 (45.3)</td>
<td>0.008</td>
<td>0.64 (0.46 to 0.89)</td>
</tr>
</tbody>
</table>

RV, reference value.
*Cancer cases and control participants that had been genotyped for all four promoter single-nucleotide polymorphisms.
†p values were obtained using χ² test, two-tailed and df = 1
‡Haplotype analysis was performed on those participants in which all polymorphisms had been successfully genotyped.

Haplotypes were estimated by PLINK (v1.03).
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 as germline mutations in high-penetration breast-cancer susceptibility genes are less likely to be found in late-onset cases and in cases without a family history of breast cancer. Moreover, this finding in Hong Kong Chinese was also replicated in an independently conducted population-based case-control study of Shanghai Chinese, which minimises the possibility of type I error. Meta-analysis of both datasets by the Mantel–Hanzel test showed no significant difference in reduced ORs between the two populations, allowing for combined OR analysis, giving a total of 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, but this was not replicated by Cox et al or Freedman et al. Cox et al identified one haplotype associated with a slightly increased risk but the functional variant(s) responsible for this association remains unknown and promoter SNPs were not included in the analysis. The multiethnic cohort haplotype analysis performed by Freedman et al 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 than in Caucasian populations. Indeed one of the promoter SNPs (c.-2265C→T) may indeed confer an important protective role.

Although we cannot entirely exclude that the observed functional effect could be attributed to other variants of BRCA1 not analysed 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 a low-penetration gene effect. As this SNP (with minor allele T frequency >38%) 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 tumour-suppressor gene, may indeed confer an important protective role.

### Table 3 Association of breast-cancer risk with the c.-2265C→T SNP in the BRCA1 gene, on participants recruited in the Hong Kong Chinese and in the Shanghai Breast Cancer Study

<table>
<thead>
<tr>
<th></th>
<th>Hong Kong</th>
<th></th>
<th>Shanghai</th>
<th></th>
<th>TOH</th>
<th>Combined analysis</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>n</td>
<td>(%)</td>
<td>p Value*</td>
<td>OR (95% CI)*</td>
<td>n</td>
<td>(%)</td>
</tr>
<tr>
<td>c.-2265C→T</td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Cases</td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>n</td>
<td>166</td>
<td>166</td>
<td>356</td>
<td>356</td>
<td>637</td>
<td>678</td>
</tr>
<tr>
<td>CC</td>
<td>164 (99.4)</td>
<td>356</td>
<td>0.51</td>
<td>356</td>
<td>637</td>
<td>678</td>
</tr>
<tr>
<td>CT/TT</td>
<td>41.6</td>
<td>0.32 to 0.81</td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Controls</td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>n</td>
<td>166</td>
<td>166</td>
<td>356</td>
<td>356</td>
<td>637</td>
<td>678</td>
</tr>
<tr>
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<td>637</td>
<td>678</td>
</tr>
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<td>CT/TT</td>
<td>41.6</td>
<td>0.32 to 0.81</td>
<td></td>
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<tr>
<td>p Value</td>
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<td></td>
<td></td>
</tr>
<tr>
<td>OR (95% CI)</td>
<td></td>
<td></td>
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<td></td>
</tr>
</tbody>
</table>

HWE, Hardy–Weinberg equilibrium; RV, reference value; TOH, test of heterogeneity.

*p Values were obtained by χ² 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.

‡Combined analysis was performed using Mantel–Hanzel test.

Overall cases and control participants successfully genotyped.

p Values for HWE were calculated by χ² square test, df = 1.
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 totalling >3000 participants support these findings by showing 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.

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


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