A functional BRCA1 coding sequence genetic variant contributes to risk of esophageal squamous cell carcinoma

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As a tumor suppressor, breast cancer susceptibility gene 1 (BRCA1) plays a pivotal role in maintaining genomic stability. A functional rs799917 T>C polymorphism located in the BRCA1 coding sequence could influence miR-638-mediated regulation of BRCA1 expression. Therefore, we examined the association between this polymorphism and esophageal squamous cell carcinoma (ESCC) risk as well as its biological function. Genotypes were determined in two independent case-control studies consisted of 1128 ESCC patients and 1150 controls. Odds ratios (ORs) and 95% confidence intervals (CIs) were estimated by logistic regression. The allele-specific regulation on BRCA1 expression by the polymorphism was investigated in vitro and in vivo. We found that the BRCA1 rs799917 CC genotype was significantly associated with increased ESCC risk compared with the TT genotype in both studies (Jinan population: OR = 1.28, 95% CI = 1.04–1.58, P = 0.020; Huaian population: OR = 1.46, 95% CI = 1.17–1.83, P = 0.001). Stratified analyses with pooled data indicated that a multiplicative interaction between rs799917 and smoking or drinking in intensifying ESCC risk was evident (gene–smoking: Pinteraction = 5.8 × 10−5; gene–drinking: Pinteraction = 7.1 × 10−5). In vitro experiments indicate that miR-638 could negatively regulate BRCA1 expression and enhance proliferation of ESCC cells. In vivo BRCA1 messenger RNA expression analyses showed that the rs799917 C allele carriers had significantly decreased BRCA1 expression in both normal and cancerous esophagus tissues compared with T allele carriers, suggesting that lower BRCA1 expression may lead to higher risk for malignant transformation of esophageal cells. These results suggest that BRCA1 functional rs799917 polymorphism is involved in susceptibility to developing ESCC, alone and in a gene–environment interaction manner.

Introduction

As one of the most common and fatal malignant tumors in the world, esophageal squamous cell carcinoma (ESCC) shows a relatively high morbidity in China compared with Western countries (1). Although the entire etiology of ESCC remains to be clarified, epidemiological evidences suggest that tobacco smoking, heavy alcohol drinking, micronutrient deficiency and dietary carcinoma exposure might be main environmental causes of this lethal disease (2,3). Interestingly, only a fraction of exposed individuals develop ESCC, indicating that genetic components may also contribute to esophageal malignant transformation (2–5).

Although 90–95% of breast cancer cases are sporadic, there are 5–10% of all patients whose etiology is mostly inherited (6). Among all cases with the heritable breast cancer, 40–45% of cases are associated to germline mutations of breast cancer susceptibility gene 1 (BRCA1) (6,7). As a nuclear phosphoprotein, BRCA1 plays a pivotal part in maintaining genomic stability and acts as a tumor suppressor (8–13). In human cells, this versatile protein can interact with DNA damage sensing proteins, DNA damage response effectors and cell cycle regulators. Therefore, it functions in multiple DNA repair pathways (particularly homologous recombination, non-homologous end joining and single-strand annealing) and in cell cycle checkpoint regulation (8–13). Mori et al. (14) reported that there is frequent loss of heterozygosity in the region including BRCA1 on chromosome 17q in ESCC, even in tumors at an early stage. This indicates that inactivation of this tumor suppressor gene might be an early event during esophageal carcinogenesis (14). Moreover, the role of BRCA1 in esophagus cancer formation has also been examined in a BRCA1 null mouse model. It has been found that absence of full-length BRCA1 sensitizes mice to oxidative stress and carcinogen-induced tumorigenesis in the esophagus (15).

Previously, Nicoloso et al. (16) showed that miR-638 can negatively regulate BRCA1 expression in breast cancer cells through targeting coding sequence (CDS) but not 3′-untranslated region of BRCA1. This observation has been validated in human bronchial epithelial cells using chemical carcinogens [i.e. benzo(a)pyrene (BaP)]-induced cell transformation (17), highlighting the essential involvements of both miR-638 and BRCA1 in tumorigenesis of epithelial cells. Remarkably, an rs799917 T>C single nucleotide polymorphism (SNP) located in the BRCA1 CDS could impact interaction between BRCA1 messenger RNA (mRNA) and miR-638 (16). The rs799917 T allele has been associated with a significantly weaker miR-638-dependent BRCA1 reduction (16), which suggests that cells with rs799917 T allele would express higher BRCA1 compared with C allele carriers. Based on the aforementioned findings, we hypothesized that the BRCA1 rs799917 genetic polymorphism may influence miR-638-mediated regulation of BRCA1 expression during malignant transformation of esophageal epithelial cells, and thus, ESCC risk in Chinese populations. To test this hypothesis, we conducted a large case–control study of ESCC in two independent case–control studies from Jinan city (Shandong Province, China) and Huaian city (Jiangsu Province, China). To validate the biological function of this SNP, we not only investigated allelic regulation of miR-638 on BRCA1 in ESCC cells but also examined the association between its genotypes and BRCA1 mRNA expression levels in normal and cancerous esophageal tissues.

Materials and methods

Study subjects

This study consisted of two case–control sets: (i) Jinan study: 540 ESCC patients from Shandong Cancer Hospital, Shandong Academy of Medical Sciences (Jinan, Shandong Province, China) and 550 sex- and age-matched (±5 years) controls. Patients were recruited between June 2009 and April 2012 at Shandong Cancer Hospital. Controls were randomly selected from a pool of 4500 individuals from a community-based cancer screening program for early detection of cancer conducted in Jinan city during the same time period as the patients were recruited. (ii) Huaian study: 588 ESCC patients from Huaian No. 2 Hospital (Huaian, Jiangsu Province, China) and 600 sex- and age-matched controls. Patients were consecutively recruited between January 2009 and February 2012 at Huaian No. 2 Hospital. Controls were cancer-free.

Abbreviations: BaP, benzo(a)pyrene; BRCA1, breast cancer susceptibility gene 1; CDS, coding sequence; CI, confidence interval; ESCC, esophageal squamous cell carcinoma; mRNA, messenger RNA; OR, odds ratio; SNP, single-nucleotide polymorphism.

*These authors contributed equally to this work.
individuals selected from a community-based cancer screening program (3600 individuals) for early detection of cancer conducted in Huaian city during the same time period as the patients were recruited. The diagnosis of all patients was histologically confirmed. Individuals who smoked one cigarette per day for over 1 year were considered as smokers. Subjects were considered as alcohol drinkers, if they drank at least once per week. A total of 29 esophageal normal tissues adjacent to the tumors and 29 paired ESCC tissues were obtained from surgically removed specimens of patients in Huaian No. 2 Hospital. The normal tissues were sampled at least 2 cm away from the margin of the tumor.

Part of the two case–control studies and the tissue samples have been reported previously (18,19). All subjects were ethnic Han Chinese. At recruitment, the informed consent was obtained from each subject. This study was approved by the institutional review board.

**Genetic polymorphism genotyping**

**BRCA1 rs799917 T>C genotypes** were determined using PCR-based restriction fragment length polymorphism. During genotyping, the primers used for amplifying DNA segments with the SNP site (the mismatch base is underlined) were 5′-AACACAGTGGAAGAAAG-3′ and 5′-CTGCATTTCCCGAATTGAAAAC-3′. The PCR was performed with a 25 μl reaction mixture containing 0.1 μmol/l of each primer, 0.2 μmol/l of deoxynucleoside triphosphate, 1.0 U of Taq DNA polymerase (TaKaRa), 1x reaction buffer and 1.5 μmol/l of MgCl₂. The PCR profile consisted of an initial melting step of 2 min at 95°C, followed by 35 cycles of 30 s at 94°C, 30 s at 58°C, 30 s at 72°C and a final elongation step of 10 min at 72°C. Restriction enzyme Mspl (New England Biolabs) was utilized to distinguish the rs799917 T>C genotypes. A 15% random sample was reciprocally tested by different personnel, and the reproducibility was 99.8%. In addition, a 5% random sample was also examined by Sanger sequencing, and the reproducibility was 100%.

**Dual luciferase reporter assay**

The BRCA1 rs799917T and rs799917C allelic reporter constructs were prepared by amplifying the BRCA1 CDS in DNA from subjects homozygous for the rs799917TT or rs799917CC genotype, using primers of 5′-CCGGAGCTCAAGAGCACAGCGATAC-3′ and 5′-CCGGCTCAGAGGCTCCTTTGAGATAC-3′, which include the Sox1 and Sox10 restriction sites (italicized sequences). The PCR products were digested with Sox1 and Sox10 restriction enzymes (TaKaRa) and ligated, respectively, into an appropriately digested pGL3-Basic vector (Promega). The constructs were designated as pGL3b-rs799917T and pGL3b-rs799917C, respectively.

**KYSE-30 and KYSE-150 ESCC cells** were plated in 12-well plates (10⁴ cells per well) and kept under indicated conditions. At indicated time point, the cells were trypsinized and collected. The number of viable cells was determined by Trypan blue exclusion and directly counted using a hemocytometer. Data represent means ± SD from three independent replicates.

**Results**

There are no statistically significant differences between patients and control subjects for Jinan case–control study and Huaian case–control study in terms of median age and sex distributions (all P > 0.05), indicating that the frequency matching was adequate (Table 1). However, more smokers were observed among ESCC cases compared...
with controls in both case–control studies (Jinan study: 65.5% versus 51.8%, \( P < 0.001 \); Huaian study: 74.3% versus 33.8%, \( P < 0.001 \)). Moreover, we found that there are more individuals who drink alcohol among ESCC patients than among normal controls in these two studies (Jinan study 55.6% versus 45.6%, \( P = 0.001 \); Huaian study: 56.8% versus 40.3%, \( P < 0.001 \)).

The genotype frequencies of \( BRCA1 \) rs799917 T>C genetic polymorphism are summarized in Table II. The frequency for the rs799917 T allele was 0.372 and 0.339 among healthy controls from Jinan and Huaian population, and 0.319 and 0.355 among ESCC patients from Jinan and Huaian population. All observed genotype frequencies in both controls and patients conform to Hardy–Weinberg equilibrium. Distributions of these \( BRCA1 \) genotypes were then compared among ESCC cases and controls. The frequencies of \( BRCA1 \) rs799917 TT, CT and CC genotypes among cases were significantly different from those among controls in Jinan population (\( \chi^2 = 10.47, \ P = 0.005, \ df = 2 \)), with the CC variant being more frequent among cases than among controls (44.8% versus 40.4%, \( P = 0.020 \); Table II). Similarly, the frequencies of \( BRCA1 \) rs799917 TT, CT and CC genotypes among cases were significantly different from those among controls in Huaian population (\( \chi^2 = 6.20, \ P = 0.045, \ df = 2 \)), with the CC homozygotes being over-represented among cases compared with controls (40.8% versus 37.0%, \( P = 0.001 \); Table II).

Unconditional logistic regression analysis was used to estimate associations between genotypes of \( BRCA1 \) rs799917 T>C polymorphism and risk of ESCC (Table II). The \( BRCA1 \) rs799917 C allele was shown to be risk allele. Individuals having the rs799917 CC genotype had an OR of 1.28 (\( P = 0.020 \)) or 1.46 (\( P = 0.001 \)) for developing ESCC in Jinan or Huaian population, respectively, compared with individuals having the rs799917 TT genotype. In Jinan population, the rs799917 heterozygous CT genotype had a 1.76-fold increased risk for ESCC compared with the rs799917 TT genotype (\( P = 0.007 \)). However, logistic regression analyses revealed that individuals with the rs799917 CT genotype were not significantly associated with ESCC risk in Huaian population (\( P = 0.118 \); Table II). In the pooled analyses, we found that the rs799917 CT or CC genotype carriers had a 1.48- or 1.34-fold increased risk to develop ESCC compared with the TT genotype carriers (trend test, \( P = 6.9 \times 10^{-4} \); Table II). All ORs were adjusted for sex, age, smoking and alcohol drinking status.

The risk of ESCC associated with the \( BRCA1 \) rs799917 genotypes was further examined by stratifying for age, sex, tobacco smoking and alcohol drinking status using the combined data of two Chinese case–control studies (Table III). In stratified analyses with age, rs799917 CT genotype was associated with increased risk in subjects aged 57 years or younger (\( P = 2.9 \times 10^{-5} \)) compared with the TT genotype. However, this genotype was not associated with ESCC risk in the group aged older than 57 years (\( P = 0.092 \)). There was a significantly multiplicative gene–age interaction (\( P_{interaction} = 0.037 \)). Compared with the \( BRCA1 \) rs799917 TT genotype, an increased risk of ESCC was associated with CT and CC genotypes for both males and females (all \( P < 0.05 \)). As expected, no significant gene–sex interaction was observed (\( P_{interaction} = 0.987 \)).

Interestingly, stratified analyses of \( BRCA1 \) rs799917 polymorphism with smoking and drinking status showed significant gene–environment interactions (gene–smoking: \( P_{interaction} = 5.8 \times 10^{-5} \); gene–drinking: \( P_{interaction} = 7.1 \times 10^{-5} \)). In smokers, compared with the rs799917 TT carriers, subjects with CT or CC genotype showed a 2.07- or 1.79-fold increased risk to develop ESCC (\( P = 9.7 \times 10^{-5} \) or \( P = 6.6 \times 10^{-4} \)). However, no significantly increased risk for non-smokers with CT or CC genotype was observed compared with TT non-smoking carriers. Similarly, drinkers with rs799917 CT or CC genotype showed significantly and consistently elevated risks to develop ESCC compared with TT carriers who drink (\( P = 4.0 \times 10^{-5} \) or \( P = 2.7 \times 10^{-10} \)).

### Table II. Genotype frequencies of \( BRCA1 \) rs799917 polymorphism among cases and controls with association with ESCC risk.

<table>
<thead>
<tr>
<th>Genotypes</th>
<th>Cases</th>
<th>Controls</th>
<th>( OR^a ) (95% CI)</th>
<th>( P )</th>
</tr>
</thead>
<tbody>
<tr>
<td>Jinan</td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>study</td>
<td>TT</td>
<td>46 (8.5)</td>
<td>81 (14.7)</td>
<td>1.00 (reference)</td>
</tr>
<tr>
<td></td>
<td>CT</td>
<td>252 (46.7)</td>
<td>247 (44.9)</td>
<td>1.76 (1.16–2.66)</td>
</tr>
<tr>
<td></td>
<td>CC</td>
<td>242 (44.8)</td>
<td>222 (40.4)</td>
<td>1.28 (1.04–1.58)</td>
</tr>
<tr>
<td></td>
<td>( P_{trend} )</td>
<td></td>
<td></td>
<td>0.008</td>
</tr>
<tr>
<td>Huaian</td>
<td>TT</td>
<td>70 (11.9)</td>
<td>101 (16.8)</td>
<td>1.00 (reference)</td>
</tr>
<tr>
<td>study</td>
<td>CT</td>
<td>278 (47.3)</td>
<td>277 (46.2)</td>
<td>1.34 (0.93–1.92)</td>
</tr>
<tr>
<td></td>
<td>CC</td>
<td>240 (40.8)</td>
<td>222 (37.0)</td>
<td>1.46 (1.17–1.83)</td>
</tr>
<tr>
<td></td>
<td>( P_{trend} )</td>
<td></td>
<td></td>
<td>0.028</td>
</tr>
<tr>
<td>Total</td>
<td>TT</td>
<td>116 (10.3)</td>
<td>182 (15.8)</td>
<td>1.00 (reference)</td>
</tr>
<tr>
<td></td>
<td>CT</td>
<td>530 (47.0)</td>
<td>524 (45.6)</td>
<td>1.48 (1.13–1.94)</td>
</tr>
<tr>
<td></td>
<td>CC</td>
<td>482 (42.7)</td>
<td>444 (38.6)</td>
<td>1.34 (1.16–1.55)</td>
</tr>
<tr>
<td></td>
<td>( P_{trend} )</td>
<td></td>
<td></td>
<td>6.9 \times 10^{-4}</td>
</tr>
</tbody>
</table>

\( ^a \)Data were calculated by logistic regression with adjustment for sex, age, smoking and drinking status.

### Table III. Risk of ESCC associated with \( BRCA1 \) rs799917 genotypes by age, sex, smoking and drinking status

<table>
<thead>
<tr>
<th>Variable</th>
<th>( BRCA1 ) rs799917 T&gt;C</th>
<th>( BRCA1 ) rs799917 T&gt;C</th>
<th>( P_{interaction}^b )</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>TT^a</td>
<td>CT^a</td>
<td>OR^a (95% CI)</td>
</tr>
<tr>
<td>Age (year)</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>( \leq 57 )</td>
<td>55/95</td>
<td>250/287</td>
<td>1.43 (0.97–2.12)</td>
</tr>
<tr>
<td>( &gt;57 )</td>
<td>61/87</td>
<td>280/237</td>
<td>1.54 (1.05–2.27)</td>
</tr>
<tr>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Sex</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Male</td>
<td>89/138</td>
<td>392/399</td>
<td>1.44 (1.06–1.96)</td>
</tr>
<tr>
<td>Female</td>
<td>27/44</td>
<td>138/125</td>
<td>1.93 (1.34–3.58)</td>
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<tr>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Smoking status</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Non-smoker</td>
<td>47/93</td>
<td>135/261</td>
<td>1.00 (0.66–1.51)</td>
</tr>
<tr>
<td>Smoker</td>
<td>69/89</td>
<td>395/263</td>
<td>2.07 (1.43–2.97)</td>
</tr>
<tr>
<td>Alcohol drinking</td>
<td>No</td>
<td>80/107</td>
<td>253/304</td>
</tr>
<tr>
<td></td>
<td>Yes</td>
<td>36/75</td>
<td>277/220</td>
</tr>
</tbody>
</table>

\( ^a \) Number of patients with genotype/number of control subjects with genotype.

\( ^b \) Data were calculated by logistic regression, adjusted for sex, age, smoking and drinking status, where it was appropriate.

\( ^c \) \( P \) values for gene–environment interaction were calculated using the multiplicative interaction term in SPSS software.
However, we did not find any association between rs799917 CT or CC genotype and ESCC among non-drinkers (both $P > 0.05$; Table III).

Because rs799917 T-to-C change could lead to increased affinity between miR-638 and BRCA1 mRNA and decreased BRCA1 expression in other cancer cells ($16,17$), we examined whether there is an allele-specific effect of rs799917 SNP on BRCA1 expression in both ESCC cells (in vitro) and esophagus tissues (in vivo) by miR-638. Relative luciferase expression assays indicated that miR-638 can regulate BRCA1 3′UTR region in an allele-specific way, with significant lower luciferase activities in cancer cells transfected with BRCA1 rs799917C allelic reporter constructs compared with cells expressing rs799917T allelic reporter constructs ($P = 0.001$ in KYSE-30 cells and $P = 0.031$ in KYSE-150 cells; Figure 1). However, no such differences can be found in ESCC cells without transfection with miR-638 mimics (Figure 1). To reveal the potential role of miR-638 in ESCC development, we investigated its impact on ESCC cell growth. Overexpressed miR-638 may lead to faster proliferation of both ESCC cell lines compared with cells transfected with control NC-RNAs (Figure 2). These results demonstrated that miR-638 might act as an onco-miR during malignant transformation of esophageal cells. As shown in Figure 1, we found that subjects with the rs799917 CC genotype had significantly lower BRCA1 mRNA levels (mean ± standard error) than those with the CT genotypes in normal and cancerous esophagus tissues [normal tissues: 0.022 ± 0.005 (n = 11) versus 0.034 ± 0.004 (n = 14), $P < 0.05$; ESCC tissues: 0.022 ± 0.003 (n = 11) versus 0.044 ± 0.007 (n = 14), $P < 0.05$]. Similar results were observed when the BRCA1 mRNA levels were compared as a function of rs799917 CC and CT genotypes [normal tissues: 0.022 ± 0.005 (n = 11) versus 0.036 ± 0.011 (n = 4), $P < 0.05$; ESCC tissues: 0.022 ± 0.003 (n = 11) versus 0.073 ± 0.007 (n = 4), $P < 0.01$; Figure 3).

Discussion

In this study, we investigated the association between a BRCA1 functional rs799917 SNP and risk of developing ESCC in a case–control design. To the best of our knowledge, this is the first case–control study to examine the role of BRCA1 rs799917 polymorphism in development of ESCC. We found significantly increased ESCC risk among carriers of BRCA1 rs799917 CT or CC genotype compared with those with TT genotype in Chinese. In vitro experiments indicate that miR-638 could negatively regulate BRCA1 expression and enhance
proliferation of ESCC cells. In the genotype–phenotype correlation analyses of 29 human ESCC and paired esophagus tissue samples, rs799917 CC was associated with a statistically significant decrease of BRCA1 mRNA expression. These results are consistent to functional relevance of rs799917 polymorphism, miR-638 and BRCA1 in malignant transformation of human epithelia cells (16,17). Our observations also support the hypothesis that genetic variants influencing micro RNA-mediated regulation of tumor suppressor genes or oncogenes may explain a proportion of ESCC genetic basis.

As a tumor suppressor, BRCA1 protein causes growth arrest for repairing DNA double-strand breaks and remove bulky DNA adducts through homologous recombination repair and non-homologous end joining in response to DNA damage and other forms of cellular stress (i.e. chemical carcinogens) (8–13). Accumulated evidences indicate that miR-638 can downregulate BRCA1 expression through directly binding to its BRCA1 CDS target site and promote BaP-induced tumorigenesis (16,17). The rs799917 T>C polymorphism in the miR-638 target site of BRCA1 CDS has been shown to depress the expression of BRCA1 when there is elevated expression of miR-638 induced by chemical carcinogens such as BaP (16,17). These are consistent with our findings that rs799917 could intensify ESCC susceptibility interacting with tobacco smoking or alcohol drinking. Tobacco smoke contains high levels of polycyclic aromatic hydrocarbons (a well-established group of chemical carcinogens) including BaP (26), which can stimulate increased miR-638 expression and decline more BRCA1 expression in esophageal epithelia cells carrying rs799917 C allele. This can explain why there was more evident association between rs799917 C allele and ESCC risk among smokers but not in non-smokers. Additionally, metabolism of alcohol can directly result in production of reactive oxygen species and reactive nitrogen species, which could induce DNA damage through formatting of DNA adducts (27). Because BRCA1 protein plays an essential role in removing DNA adducts (26), it could be expected that carriers of rs799917 C allele would have less BRCA1 expression and poor ability to repair DNA damages caused by ethanol.

Conclusion

In summary, this study suggested that functional BRCA1 rs799917 SNP was associated with a significantly increased risk of ESCC in Chinese populations. The association between the SNP and ESCC risk is especially noteworthy in individuals who smoke or drink.

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References


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