RESEARCH COMMUNICATION

Frequent Germline Mutation in the BRCA2 Gene in Esophageal Squamous Cell Carcinoma Patients from a Low-risk Chinese Population

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Abstract

Background: The incidence of esophageal squamous cell cancer (ESCC) is strikingly variable by geographic area, which reflect different exposures to risk factors, including genetic predisposition. Previous studies of ESCC patients from several high-risk populations suggested that BRCA2 might play a role in the etiology. This study was conducted to screen for mutations of BRCA2 gene in ESCC cases from a low-risk population. Methods: Forty-seven ESCC patients from a low-risk area of Southeast China were screened for mutations in the entire coding region of the BRCA2 gene by direct sequencing. Results: No somatic mutations were observed in tumors. In total, 9 germline missense point mutations, each in one patient, were identified in male sporadic patients, with a mutation frequency of 19%. Of the 9 mutations, 7 were of heterozygous, while the remaining 2 were homozygous. Screening of an additional 94 healthy controls for the 9 mutations identified in ESCC cases showed that there was only 2 (2%) positive individuals, each carrying one of the mutations. Thus the mutation frequency in ESCC cases (19%) was significantly higher than that in healthy controls (OR = 10.9, 95% CI = 2.2-52.8, P = 0.003). No significant associations were observed for germline BRCA2 mutations with age, sex, cigarette smoking, alcohol drinking and family history of cancer. Conclusion: This series of cases from a low-risk Chinese population presented the highest frequency of germline BRCA2 mutations in ESCC reported to date, highlighting possible etiology roles in this population.

Keywords: BRCA2 gene - esophageal squamous cell carcinoma - germline mutation - low-risk population - China

Asian Pacific J Cancer Prev, 12, 1771-1776

Introduction

Esophageal cancer, approximately 90-95% of which is esophageal squamous cell cancer (ESCC) worldwide, remains one of the most common cancers. The incidence of ESCC is strikingly variable by geographic area, with China having the highest rate in the world (Parkin et al. 2005). In different area of China, the ESCC incidence also varies widely, with the age-adjusted incidence ranging from 0.3 to 132.7 per 100,000 population per year (Zou et al., 2007). This geographic variation in ESCC incidence reflects different exposure to risk factors related to lifestyle, environment, and genetic predisposition (Parkin et al., 2005).

Although much effort has been put into the research on ESCC, the etiology of this common cancer is still largely unknown. Proposed ESCC risk factors include tobacco smoking, excessive alcohol consumption, thermal injury, inappropriate diet, and low socioeconomic status (Islami et al., 2009; Kamangar et al., 2009; Enzinger and Mayer, 2003). It is now believed that ESCC has an intricate molecular mechanism, and the significance of genetically determined increased susceptibility has been stressed (Cheung and Liu, 2009). Many candidate genes, such as tumor suppressor genes, oncogenes and apoptotic genes are known to involve in the initiation and promotion of ESCC (Kuwano et al., 2005; McCabe and Dlamini, 2005). BRCA2, encoded by the tumor suppression gene BRCA2, is involved in homologous recombination repair of double-strand DNA breaks (Pellegrini et al. 2002), as well as in cell cycle regulation, transcription activation, and cell proliferation suppression (Marmorstein et al. 2001; Milner et al. 1997; Tian et al. 2005). Inactivation of BRCA2 can result in chromosomal instability (Sharan et al. 1997; Patel et al. 1998), which pave the way to carcinogenesis (Moynahan 2002).

Germline mutations in the BRCA2 gene have been related with increased risk of breast, ovarian, liver,

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prostate, fallopian tube, and pancreatic cancers (Wooster et al., 1995; Katagiri et al., 1996; Narod, 2002; Simard et al., 2003; Lowenfels and Maisonneuve, 2005; Vicus et al. 2010), and malignant melanoma (Casula et al., 2009). Recently, several studies have been focusing on the ESCC patients from high-risk populations of Chinese, Indianan and Iranian, and indicated the important role of BRCA2 mutations in the etiology of the ESCC among these high-risk Asians (Hu et al., 2002; 2004; Akbari et al., 2008; Kaushal et al., 2010). However, no study has focused exclusively on low-risk population.

In the present study, we recruited 47 ESCC patients from a low-risk population from Shanghai, China where the age-adjusted annual incidence rate is 9.2 and 3.0 per 100,000 in males and in females, respectively (Zou et al., 2007), and performed BRCA2 mutation detection by sequencing all coding exons of the gene. Additionally, we screened 94 healthy volunteers from the same geographic area for the BRCA2 mutations identified in ESCC cases to evaluate the potential association of germline BRCA2 mutation with ESCC risk

Materials and Methods

Subjects

A total of 47 patients with ESCC, who underwent surgery at Changzheng Hospital, Shanghai, China from April 2007 to May 2008, were recruited into this study. All patients were ethnic Han Chinese, and Shanghai was the ancestral home for them. None of the patients had received chemotherapy or radiation therapy before surgery. Final diagnoses of ESCC were pathologically confirmed from the specimens obtained at surgery. Of 47 patients whose mean age was 60.9 (range 34-76) years, 78.7% (37 cases) were male. Tumors and matched adjacent normal esophagus tissues were collected from each patients. All samples were snap frozen in liquid nitrogen within 30 minutes after surgical removal and then stored at -80°C

Table 1. Sequences of Primers Used for Mutation Analysis of the BRCA2 Gene

Exon	Forward primer (5' to 3') †	Reverse primer (5' to 3') ‡
2	CCGTTCCAGGAGATGGGACTGA	TGCTAGTCAAGGGGCCAGTTTCC
3	TGATCTTTAACTGTTCTGGGTCACAA	TGGCAAATTTATCAAAGGAGGGATGA
4	GGGGGTAATCAGCAAACTGAAAAACCT	CCAGCCAATTCAACATCACAAGAACA
5	AAAATACACGGTTTCCAGCAGC	CTCCCACATACCACTGGGGG
6	GGGATTTGCTTTGTTTTATTTTAGTCC	TCAGGATCCACCTCAGCTCC
7	ACCCCCAGTGGTATGTGGGA	TGCTTGACACCACTGGACTACCA
8	CCTCACAGCATCATCTGACTTTCCA	CAGAGAGACAGCAGAGTTTCACAGGA
9	CTACTACTATATGTGCATTGAGAG	CCTGTAGTTCAACTAAACAGAGG
10.1	CAGGAGAAGGGGTGACTGACCG	CCATTCACAGGCCAAAGACGG
10.2	TGAAGTGGAACCAAATGATACTGATCC	CCCTGAAATGAAGAAGCCACTGGA
10.3	TGCCACGTATTTCTAGCCTACCA	GCATTTGCTTCAAACTGGGCTGA
10.4	GCCAGCCACCACCACACAGA	TTGAGTGACCTGATTCTAAACACTGG
11.1	TTTAGTGAATGTGATTGATGGTAC	TCTGGGATTGAAAGTCAGTATC
11.2	TCTTGGCTGCAGCATGTCACC	TGGTAGAGTTCTTGAAAATGGGTTCG
11.3	CGTTGAGCTGTTGCCACCTGA	TTGGACCTAAGAGTCCTGCCCA
11.4	CGAACCCATTTTCAAGAACTCTACCA	AAATCCTGCTTGGAAAATAACATCTG
11.5	GAAACTGAGCAAGCCTCAGTCAA	GCCATGAGCAGAATAAAAGCCCC
11.6	TTCTGAGGAATGCAGAGATGCTGA	TCATTTTTACTTGAATCACTGCCATCA
11.7	ACTGCTGCCAGTAGAAATTCTC	TGCTCCGTTTTAGTAGCAGTTAA
11.8	TTTGGAAGTTGCGAAAGCTCA	GGGGCAGCTGTGATCTCAATGG
11.9	AGTGACCTTCCAGGGACAACC	CAAGTTGCAGGACTTTTTGCTG
11.10	CATTGAGATCACAGCTGCCCCA	CCTCATCAGAATGGTAGGAATAGCTG
11.11	GAAGGAATATTTGATGGTCAACCAGA	CAACCTGCCATAATTTTCGTTTGGC
11.12	CCCTGCAAAAATAAAAATGCAGCCA	TTTCAGAAAACACTTGTCTTGCGT
11.13	AGGGAAGCTTCATAAGTCAGTC	TTTCTAAAATGGAAACTTGCTTTCC
11.14	TGTGGTAAATTCATCTGCTTTCTCTGG	GGTGAAGCCTGTTCTTTTCCCA
11.15	TGAAGGTGGTTCTTCAGAAAATAATCA	TCAAACCATACTCCCCCAAACTGA
12	GGTCTATAGACTTTTGAGAAATA	CATACCTATAGAGGGAGAACAG
13	TGTATTTACAGTAACATGGATATTC	TGTTAACTTCTTAACGTTAGTGTC
14	TGAGGGTCTGCAACAAAGGCA	CCAAAGGGGGAAAACCATCAGG
15	CTGGCCAGGGGTTGTGCTTTTT	ACAAAGCCATTTGTAGATACTAG
16	CGGGGTGGAAAAGGTACAGCA	CCCCAGGACAAACAGCACTTTTCA
17	CAGAGAATAGTTGTAGTTGTTGAA	AGTCACAGACTACACAGAAACC
18	CTCAGTTATTCAGTGACTTGTTTA	ATCTAAGAAATTGAGCATCCTTAG
19	CTGTCTTACTAATCTTCCTAAGAC	AGTTAATTGTATCAGGCCAGGCA
20	GGCCTGATACAATTAACTTGAATG	CAAAGTCTCTAAGACTTTGTTCTC
21	CCCTTCTTTGGGTGTTTTATGCTTGG	CCACCACACTCGTCTGGCACA
22	CATTAACCACACCCTTAAGATGAGC	TCTGATGATGGACGCCAAATACTCA
23	GCAAAATCCACTACTAATGCCCACA	TCCACCTCAGAACAAGATGGCTGA
24	TGAGTATTTGGCGTCCATCATCAGA	TTTGCCAACTGGTAGCTCCAAC
25	GGAAAACCTGAGCTTTCGCCA	CCCCATTCCCCCATCTCCTG
26	TCTGTTCCCCTCTCCCTATCAGC	TGTTTGGAAAGTGTGCACCCAGA
27.1	GGAGTTAGGGGAGGGAGACTGTGTG	TGAACCAGACAAAAGAGCTTGGG
27.2	TCTCCGGCTGCACAGAAGGC	GCCCGATACACAAACGCTGAGG

† Tagged with M13 primer of 5'-actgtaaaacgacggccagt-3'; ‡ Tagged with M13 primer of 5'-accaggaaacagctatgacc-3'

until use. All tissue samples were reviewed by pathologists (Y.S. and J.Z) from the H & E stained slides to confirm to be tumor tissue or normal. The percentage of tumor cells for tumor sample was estimated and micro-dissection was performed for 16 cases to make sure every tumor sample represented greater than 80% of tumor cells before DNA extraction. Information on age at diagnosis, sex, cigarette smoking, alcohol drinking, and family history of cancer was obtained using a structured questionnaire through in-person interviews. Blood samples from 94 age- and sex-matched healthy controls from the same geographic area were also collected for screening the mutations identified in cases. Written informed consent was obtained from each participant, and the study protocol was approved by the ethics review committee of the Institutional Review Board of the hospital.

Mutation analysis of BRCA2

Genomic DNA from tumor tissue and adjacent normal tissue for ESCC cases and blood leukocytes for controls was isolated by proteinase K digestion and phenol/chloroform extraction. Dye terminator DNA sequencing method with ABI 3730xl Genetic Analyzer was used for screening mutations of BRCA2 gene. Forty-four pairs of PCR primers (Table 1) were designed to cover all 26 coding exons including intron/exon boundaries. Each primer is tagged with M13 primer as uniformed sequencing primer for individual PCR product to facilitate sequencing process. All PCR products were sequenced in both directions. Sequence traces were analyzed after assembling and quality calling with SeqScape2.5 sequence analysis software.

Array comparative genomic hybridization (aCGH) analysis

Two tumor samples carrying homozygous mutation were further analyzed by aCGH using the Agilent Human Genome Microarray Kit 244K (Hu-244A, Agilent Technologies, Massy, France) to examine the copy number at BRCA2 locus, according to the standard Agilent protocol (Protocol v4.0, June 2006; http://www. agilent. com). The resolution limit of this platform is about 6.4 kb. Scanning was done with Agilent Autofocus Dynamic Scanner (G2565BA, Agilent Technologies). Image analysis was performed using the Feature Extraction software version 9.5 (Agilent Technologies). The CGH Analytics software version 3.5.14 (Agilent Technologies) was used to demonstrate the copy number at BRCA2 locus.

Statistical analysis

The t-test was used to compare the age between patients with and without germline BRCA2 mutation. Exact $\chi 2$ test was used for the association analysis between mutation and sex, cigarette smoking, alcohol drinking, and family history of cancer. The association between germline BRCA2 mutation and ESCC risk was estimated by computing the odd ratio (OR) and its 95% confidence interval (CI) from univariate logistic regression analysis. The statistical significance of the multiplicative interaction terms between mutation and age (≤60 years, >60 years), sex, cigarette smoking, alcohol drinking, and family history of cancer on ESCC risk was tested using the likelihood ratio test, comparing logistic regression models with and without the appropriate interaction term. A P-value of < 0.05 was considered statistically significant, and all of the tests were two-tailed. Statistical analyses were conducted using the Stata 10.1 software (Stata Corporation, College Station, TX).

Results

Entire BRCA2 coding region was screened for mutation in tumor tissue and matched adjacent normal tissue of the 47 ESCC patients. In total, 9 missense point mutations, each in one patient, were identified in tumors, with a mutation frequency of 19% (Table 2). Figure 1 shows an example of the sequence traces of the M408T mutation. Three of the mutations, C315S, Y828 and M1149, have been reported in previous studies (Hu et al. 2002; Hu et al. 2004; Akbari et al. 2008) or in Breast Cancer Information Core (BIC) database (http://research.nhgri.nih.gov/bic). The remaining 6 mutations, Q147R, M408T, H523R, D1864N, M1936V, and G2508S, are novel according to our knowledge. Of the 9 mutations, 7 were of heterozygous mutation, while 2 mutations, C315S and Y828H, were of homozygous. Sequencing results from matched adjacent normal tissues showed that all the 9 mutations also existed in adjacent normal

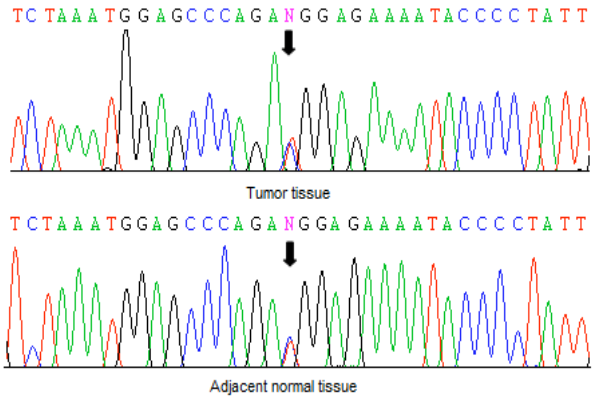

Figure 1. Sequence Traces for the M408T Mutation in Tumors and Matched Normal Adjacent Tissue. Mutation is indicated with arrow

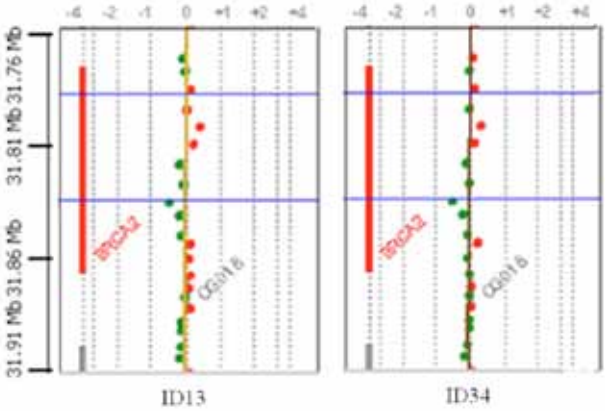

Figure 2. Array Comparative Genomic Hybridization (aCGH) Analysis on Copy Number of BRCA2 Gene in Tumor Tissue for Patients ID13 and ID34

Table 2. Demographics and Germline Mutation and cSNP Status in BRCA2 Gene among ESCC Patients

ID	Sex	Age	FH	Alc	Cig	Mutation		cSNP
						Tumor	Adjacent	
1	M	59	No	Yes	Yes	WT	WT	I3412V
2	F	59	No	No	No	WT	WT	N372H
3	M	57	No	No	Yes	WT	WT	N372H
4	M	70	Yes	No	No	WT	WT	N289H; N991D
5	F	54	No	No	No	WT	WT	N289H; N991D
6	F	51	No	No	No	WT	WT	N372H
7	M	56	No	No	No	WT	WT	N289H; N991D
8	F	73	No	No	No	WT	WT	WT
9	M	74	No	Yes	Yes	Q147R[1]	Q147R[1]	WT
10	M	66	No	No	Yes	D1864N[1]	D1864N[1]	N372H
11	M	53	No	No	No	WT	WT	N372H
12	M	62	No	No	No	WT	WT	N289H; N991D
13	M	63	No	No	Yes	Y828H[2]	Y828H[1]	WT
14	M	65	No	No	No	WT	WT	N372H
15	M	61	No	No	Yes	WT	WT	WT
16	M	61	No	No	Yes	H523R[1]	H523R[1]	N372H
17	M	68	No	No	No	M1936V[1]	M1936V[1]	N372H
18	M	42	No	Yes	Yes	WT	WT	WT
19	M	70	No	No	No	WT	WT	N372H
20	M	68	No	No	Yes	WT	WT	N372H
21	M	66	No	No	Yes	WT	WT	N372H
22	F	72	No	No	No	WT	WT	N372H; I3412V
23	M	63	No	Yes	Yes	WT	WT	WT
24	M	62	No	No	No	WT	WT	WT
25	M	61	No	No	Yes	WT	WT	I3412V
26	M	50	No	No	Yes	WT	WT	WT
27	M	64	No	No	No	WT	WT	N372H
28	M	53	Yes	No	Yes	WT	WT	N372H
29	M	60	No	No	Yes	M1149V[1]	M1149V[1]	N372H
30	F	63	No	No	No	WT	WT	WT
31	M	67	No	No	No	WT	WT	WT
32	M	52	No	No	Yes	WT	WT	N372H
33	M	53	No	No	Yes	WT	WT	N289H
34	M	44	No	No	Yes	C315S[2]	C315S[1]	WT
35	F	58	No	No	No	WT	WT	N372H
36	M	72	No	No	No	WT	WT	WT
37	M	75	No	No	Yes	WT	WT	WT
38	M	34	No	No	No	G2508S[1]	G2508S[1]	WT
39	M	61	No	No	No	WT	WT	N372H; I3412V
40	F	64	No	No	No	WT	WT	I3412V
41	M	76	No	No	No	M408T[1]	M408T[1]	WT
42	M	64	No	Yes	Yes	WT	WT	N289H; N991D; I3412V
43	M	62	No	No	Yes	WT	WT	N372H
44	M	56	No	No	No	WT	WT	N372H
45	F	63	No	No	No	WT	WT	N372H
46	M	60	No	No	Yes	WT	WT	N372H
47	F	54	No	No	No	WT	WT	N372H; N991D

ID, identity; FH, family history; Alc, alcohol consumption; Cig, cigarette consumption; cSNP, coding single nucleotide polymorphism; M, male; F, female; WT, wild type; [1]heterozygous; [2]homozygous

tissues and were of heterozygote. Thus, in this dataset, no somatic mutation was observed and all the 9 mutations were germline origin. Further aCGH analysis on the tumor sample of the 2 patients (ID13 and ID34, Table 2), who had homozygous mutation in tumor but heterozygous mutation in matched adjacent normal tissue, demonstrated that there were two copies of the gene in each tumor (Figure 2). In addition to the 9 germline mutations, 4 missense coding single nucleotide polymorphisms (cSNP) were identified among multiple patients (Table 2).

The mean age of the 9 patients with the germline BRCA2 mutation was 60.7 years, almost same to the age of patients without mutation (60.9 years). All the germline mutations were observed in male patients, however, no statistically significant associations were observed of mutations with sex as well as with cigarette smoking, alcohol drinking and family history of cancer (data not shown).

Screening 94 healthy controls for the 9 mutations identified in ESCC cases showed that there was only 2 individuals each carrying one (H523R or C315S) of the mutations. The mutation frequency in ESCC cases (19%) was significantly higher than that in healthy controls (2%, P = 0.001). Based on this dataset, individuals with germline BRCA2 mutation had a 10.9-fold (95% CI = 2.2-52.8, P = 0.003) increased risk of developing ESCC. The interaction between germline BRCA2 mutation and age (≤ 60 years, > 60 years), sex, cigarette smoking, alcohol drinking or family history of cancer in relation to ESCC risk was not statistically significant (data not shown).

Discussion

Screening for germline mutation of the BRCA2 gene has been carried out in several high-risk ESCC populations from Northwest China, Northeast India and Turkmen of Iran, reporting the mutation rates of 3% to 9% for familiar and sporadic ESCC cases incorporated (Hu et al. 2002; Hu et al. 2004; Akbari et al. 2008; Kaushal et al. 2010). In the present study, 19% (9/47) of ESCC patients from a low-risk area of southeast China were identified carrying germline BRCA2 mutation and all the mutations were in male sporadic patients. Consistent with previous reports in ESCC and other cancers (Hu et al. 2002; Hu et al. 2004; Teng et al. 1996), no somatic mutations in BRCA2 were observed in the present dataset. To our knowledge, the germline mutation frequency in BRCA2 in our series is the highest among published data in ESCC among both familial and sporadic patients. Moreover, individuals with germline BRCA2 mutation had a 10.9-fold increased risk of developing ESCC. Our data suggest that germline mutation in the BRCA2 gene may have a distinct risk effect on ESCC susceptibility in the low-risk Chinese population. Considering that ESCC is one of the most common cancers, and as much as 10.9-fold risk estimate, this risk factor could have a sizable impact on public health.

Most of the 9 germline mutations are located in BRCA2 functional domains, and may have high potential to be deleterious. The mutation G2508S located in the BRCA2 domain (residues 2393–2952) binding to MAGE-D1 protein, a synergistic suppressor of cell proliferation (Yang et al. 2002). Amino acid change from G to S, resulting in switch of amino acid property to hydrophile, might disturb the binding of BRCA2 to MAGE-D1 and other proteins. Mutations of C315S and M408T were both located in the region implicated in BRCA2-P/CAF complex formation (residues 290–453) which shows histone acetyltransferase activity that may responsible for the transcription regulatory function of BRCA2 (Fuks et

al. 1998). The mutations M1149V, D1846N, and M1936V located in the link of repeat1/2, the repeat6, and the link of repeat6/7, respectively, of the conserved domain of eight BRC repeats, which is critical for binding to RAD51, a key protein in DNA recombination repair (Pellegrini et al. 2002). Thus, these mutations might influence the BRCA2 function in positioning RAD51 at the site of DNA repair or in removing RAD51 from DNA once DNA repair has been completed (Pellegrini et al. 2002). Functional studies are warranted to clarify whether these germline missence mutations have significant pathogenic effect, and if so, how they might play a role in the development of ESCC.

It has been reported that patients carrying heterozygous germline BRCA2 mutations demonstrate highly penetrant breast and ovarian cancer phenotypes, and that the tumors arising in these patients often exhibit loss of heterozygosity (LOH) at the wild type allele (Greenberg 2006). In the present study, LOH at C315S and Y828H sites was observed by comparing tumor tissue and matched normal tissue in 2 patients. Further aCGH analysis demonstrated that there remained two copies of the gene in the 2 tumors, indicating the substitution of wild type alleles by corresponding mutant alleles. Although the mechanism of this substitution is unclear, the genetic aberration in these two samples may lead to bi-allelic inactivation of BRCA2. In addition, the co-existence of one mutation and one functional cSNP (4 patients) or of two functional cSNPs (8 patients, Table 2) may also contribute to the bi-allelic inactivation of BRCA2. Further studies, therefore, should be performed to examine the potential role of the cSNPs in BRCA2 in the etiology of ESCC and other cancers.

Our study had the advantage of being based on micro-dissected tumor sample containing greater than 80% of tumor cells, and sequencing all coding exons of the BRCA2 gene in both directions. Therefore, it is unlikely that potential mutations in BRCA2 gene were left out. One limitation of our study is the limited sample size (n = 47), and thus the finding by chance of the relatively higher germline mutation frequency in BRCA2 gene cannot be excluded. Larger studies are needed to validate our findings. Secondly, only two patients with family history of cancer were included in our series. Since the germline BRCA2 mutation frequency has been observed to be higher in patients with cancer family history than in patients without in some (Hu et al. 2002; Hu et al. 2004; Kaushal et al. 2010) although not all (Akbari et al. 2008) ESCC studies, further studies should address the role of germline BRCA2 mutation in familiar ESCC development in the low-risk population. However, given that the germline mutations in BRCA2 would be passed on to next generations, it is reasonable to assume a higher germline mutation frequency in familiar ESCC cases of the population. Finally, the functional significance of the BRCA2 mutations identified in the present study was not further investigated.

In summary, this case series from a low-risk area of Southeast China presented the highest germline BRCA2 mutation frequency in ESCC reported to date, and it highlights the additional implications of the germline mutation of BRCA2 in the ESCC etiology of this population.

Acknowledgements

The authors declare that they have no conflicts of interest.

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