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# Germline BRCA2 Truncating Mutation in Familial Esophageal Squamous Cell Carcinoma: A Case Controlled Study in China

Authors' Contribution:
Study Design A
Data Collection B
Statistical Analysis C
Data Interpretation D
Manuscript Preparation E
Literature Search F
Funds Collection G

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**Background:** Germline mutations of BRCA2 have been reported in various malignancies. We investigated BRCA2 germline mutations in familial clusters with esophageal squamous cell carcinoma (ESCC).

**Material/Methods:** We screened the DNA of familial ESCC patients for BRCA2 germline mutations with whole gene sequencing. Multiple BRCA2 mutations including one novel splice variant, c.426-2A>G were identified. Other family members, sporadic ESCC patients, and controls were also assessed for the novel mutation.

**Results:** The mutation c.426-2A>G was found in 2 affected ESCC sisters and 7 other family members. The splice variant mutation results in exon 5 skipping with a frame shift leading to a premature stop codon in exon 6 and truncation. Novel mutation tracking ruled out single nucleotide polymorphism (SNP) in 100 chromosomes of healthy individuals.

**Conclusions:** BRCA2 germline mutation in ESCC patients may play a role in genetic susceptibility to familial ESCC. Genetic analysis of BRCA2 in patients with familial ESCC could provide opportunities for targeted therapies.

**MeSH Keywords:** Carcinoma, Squamous Cell • Esophageal Neoplasms • Genes, BRCA2 • Protein Isoforms

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## Background

Esophageal cancer has a high mortality rate with overall 5-year survival of 15% to 25% which declines to 5% in advanced stages [1]. Esophageal cancer has 2 major histologic subtypes with different epidemiology, pathogenesis, and clinicopathological features. Esophageal squamous cell carcinoma (ESCC) is, worldwide, the more common subtype with a distinct geographic distribution that is predominantly prevalent in esophageal cancer belt stretched from northern and western China towards central Asia and northern Iran [2]. In contrast, adenocarcinoma is more frequently seen in western countries. While adenocarcinoma is thought to develop in the setting of gastroesophageal reflux disease and Barrett's esophagus, major risk factors for the development of ESCC include tobacco smoking, alcohol intake, diet (lack of fresh fruits and vegetable, hot beverages) and exposure to certain chemical factors such as polycyclic aromatic hydrocarbons (PAH) [3,4].

There is growing evidence in the literature that supports an important role for genetic and epigenetic predisposition in the carcinogenesis of ESCC, in addition to the environmental factors, particularly in the areas with a high-risk population [5–10]. Yet, the exact molecular biology of ESCC carcinogenesis has not yet been clearly identified. Environmental insults such as smoking and excessive alcohol consumption can cause DNA alterations and in the absence of intact DNA repair mechanism; this will lead to disrupt of the normal cell cycle which promotes tumorigenesis [11]. The role of genetic aberrations and subsequent impairment of DNA repair and cell cycle control has been shown in upper aerodigestive SCC [12].

BRCA2 is a gene that exerts an important role in DNA repair. It is involved in homologous recombination repair of double-strand DNA breaks. It is located in chromosome 13 at 13q12.3 and encodes a protein with 3418 amino acids [13]. In addition to breast cancer, as was originally identified, germline mutations in the BRCA2 gene have been associated with increased risk of various other cancers such as ovarian cancer, melanoma, and cancers of the pancreas and liver [14–18]. In the current study, we evaluated family clusters with ESCC for possible germline mutations in BRCA2 and identified a novel BRCA2 variant in a family, one with multiple primary cancers, as well as several other mutations with unknown clinical significance and polymorphisms in BRCA2 gene.

## Material and Methods

In this cohort, we retrospectively identified and enrolled 5 family clusters with familial ESCC (ESCC diagnosed in at least 2 first-degree relatives), and 50 patients with sporadic ESCC with no familial history of esophageal cancers as a control group. Informed consents were obtained in accordance with institutional ethics committee guidelines. The institutional ethics committee approved the protocols of the study and patients were enrolled in our hospital. The study has been reported in line with the STROCSS (Strengthening the Reporting of Cohort Studies in Surgery) criteria [19]. A blood sample of 3 mL was obtained from each individual. For all samples, DNA was extracted and purified using standard methods.

Five families with affected probands were analyzed for BRCA2 mutations in germline DNA by whole gene sequencing in accordance to previously published protocol [20]. Patient's nucleotide sequence was compared with reference sequences: BRCA2 GeneBank U43746. Large genomic deletion detection of BRCA2 gene was performed using multiplex ligation-dependent probe amplification (MLPA) assay.

The exons 4–6 of BRCA2 were next scanned by a specific primer to investigate the novel variation detected by whole gene sequencing in family members. Polymerase chain reaction (PCR) was carried out in a volume of 20 µL with approximately 100 ng of genomic DNA, 1x PCR buffer, 2 mM $MgCl_2$, 200 µmol mix, 10 pmol of both primer (forward: 5'-AAATACACGGTTTCCAGCAG-3', reverse: -GAAACAAACTCCCACATACCAC-3') and 1 unit Taq polymerase (GenetBio, South Korea). An initial denaturation of 94°C for 5 minutes was followed by 30 cycles (94°C for 30 seconds, 61°C for 30 seconds, 72°C for 30 seconds) and a final extension of 72°C for 5 minutes. The amplified products were visualized by electrophoresis in ethidium bromide stained 2% agarose gel; 10 µl of the PCR reaction product was subjected to digestion with 1 unit of AvaII (New England Biolabs) at 37°C for 6 hours. The results were analyzed in 2.5% agarose gel.

For RNA analysis, whole blood was collected in EDTA tubes. RNA quality and quantity were evaluated at an absorbance of 260 and 280 nm using an ultraviolet spectrophotometer. First strand cDNA was synthesized from 1 µg total RNA using oligo(dt)18 as amplification of cDNA was conducted with flanking exonic primers designed by oligo software. RT-PCR was performed by the specific primer (forward: TCTTCGCACAGTGAAAACTA, reverse: TTCAGATGCTTCTTCATTT) in a final volume of 20 µL that contained 2 µL of cDNA. Samples were denatured at 94°C for 30 seconds, 58°C for 30 seconds, and 72°C for 30 seconds and extension step at 72°C for 5 minutes. The fragment size was 304 bp. RT-PCR products were confirmed by direct sequencing (Macrogen).

## Results

The whole gene mutation analysis of patients with familial ESCC is summarized in Tables 1–5 (for patients 1–5 respectively). Our analysis revealed one novel mutation which is located in the





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**Table 1.** Summary of mutations in the BRCA2 gene in patient #1 with familial esophageal cancer.

| Exon number | Nucleotide changes | Amino acid changes | Mutation type |
|---|---|---|---|
| 9 | c.793+53delT | – | IVS, UV |
| 10 | c.1114C>A | His372Asn | Missense |
| | c.1796T>C | Phe599Ser | Missense |
| | c.1909+12delT | – | IVS, UV |
| 11 | c.3396A>G | – | Synonymous |
| | c.6841+80delTTAA | – | IVS, UV |
| 14 | c.7242A>G | – | Synonymous |
| 17 | c.7806–14T>C | – | IVS, UV |
| 22 | c.8755–66T>C | – | IVS, UV |

IVS – intervening sequence (intron); UV – unclassified variant.

**Table 2.** Summary of mutations in the BRCA2 gene in patient #2 with familial esophageal cancer.

| Exon number | Nucleotide changes | Amino acid changes | Mutation type |
|---|---|---|---|
| 9 | c.793+53delT | – | IVS, UV |
| 10 | c.1114C>A | His372Asn | Missense |
| | c.1796T>C | Phe599Ser | Missense |
| | c.1909+12delT | – | IVS, UV |
| 11 | c.3396A>G | – | Synonymous |
| | c.5634C>T | – | Synonymous |
| | c.6841+80delTTAA | – | IVS, UV |
| 14 | c.7242A>G | – | Synonymous |
| 17 | c.7806–14T>C | – | IVS, UV |
| 22 | c.8755–66T>C | – | IVS, UV |

IVS – intervening sequence (intron); UV – unclassified variant.

**Table 3.** Summary of mutations in the BRCA2 gene in patient #3 with familial esophageal cancer.

| Exon number | Nucleotide changes | Amino acid changes | Mutation type |
|---|---|---|---|
| 9 | c.793+53delT | – | IVS, UV |
| 10 | c.1114C>A | His372Asn | Missense |
| | c.1796T>C | Phe599Ser | Missense |
| | c.1909+12delT | – | IVS, UV |
| 11 | c.3396A>G | – | Synonymous |
| | c.6841+80delTTAA | – | IVS, UV |
| 14 | c.7242A>G | – | Synonymous |
| 17 | c.7806–14T>C | – | IVS, UV |
| 22 | c.8755–66T>C | – | IVS, UV |

IVS – intervening sequence (intron); UV – unclassified variant.





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**Table 4.** Summary of mutations in the BRCA2 gene in patient #4 with familial esophageal cancer.

| Exon number | Nucleotide changes | Amino acid changes | Mutation type |
|---|---|---|---|
| 5 | c.426–2A>G* | –** | Splice-site |
| 9 | c.793+53delT | – | IVS, UV |
| 10 | c.1114C>A | His372Asn | Missense |
| | c.1796T>C | Phe599Ser | Missense |
| | c.1909+12delT | – | IVS, UV |
| 11 | c.3396A>G | – | Synonymous |
| | c.6841+80delTTAA | – | IVS, UV |
| 14 | c.7242A>G | – | Synonymous |
| 17 | c.7806–14T>C | – | IVS, UV |
| 22 | c.8755–66T>C | – | IVS, UV |

*Novel mutation; ** splice site mutation leads to downstream frameshift with a premature stop codon. IVS – intervening sequence (intron); UV – unclassified variant.

**Table 5.** Summary of mutations in the BRCA2 gene in patient #5 with familial esophageal cancer.

| Exon number | Nucleotide changes | Amino acid changes | Mutation type |
|---|---|---|---|
| 9 | c.793+53delT | – | IVS, UV |
| 10 | c.1114C>A | His372Asn | Missense |
| | c.1796T>C | Phe599Ser | Missense |
| | c.1909+12delT | – | IVS, UV |
| 11 | c.3396A>G | – | Synonymous |
| | c.6841+80delTTAA | – | IVS, UV |
| 14 | c.7242A>G | – | Synonymous |
| 17 | c.7806–14T>C | – | IVS, UV |
| 22 | c.8755–66T>C | – | IVS, UV |

IVS – intervening sequence (intron); UV – unclassified variant.

intronic sequence of BRCA2 in one patient with familial ESCC. Familial pedigree is shown in Figure 1. The sequence of the region of interest was submitted to splice site predictor program (NNSPLICE version 9) which confirmed c.426-2A>G mutation at the splice site, as heterozygote in Patient Number 4.

To characterize the impact of the novel mutation on splicing site, RNA was isolated from peripheral blood and cDNA was synthesized with specific primers spanning the exons that involved variation. cDNA analysis revealed an unexpectedly small product size in electrophoresis. Comparison of mutant RNA with normal RNA showed a smaller size in the amplified fragment. Direct sequencing of the amplified fragment revealed aberrant RNA splicing effect in exon 5, which predicted to result in skipping of exon 5 in the splicing process and premature truncation of protein during translation (Figure 2). Sequencing revealed that mutation at the splice site leads to exon 5 skipping and disrupted the coding reading frame in exon 6, which in turn created a frameshift with a premature stop codon in exon 6 and subsequent truncation of the in BRCA2 protein. We then investigated the same mutation in other family members of this proband. The proband was a female diagnosed with ESCC at age 53 years. Her symptoms included weight loss and dysphagia, and an upper endoscopy revealed her tumor at 35 cm and a biopsy proved ESCC. She underwent neoadjuvant chemoradiation and subsequent esophagectomy and pathology revealed a complete pathologic response with no nodal involvement. Two years after diagnosis of ESCC, she was diagnosed with invasive ductal carcinoma of the left breast and underwent left mastectomy with sentinel lymph node biopsy which showed no nodal involvement. Her tumor was estrogen receptor positive, progesterone receptor positive, and human epidermal growth factor receptor 2 (HER2) positive. Six months after her diagnosis of ESCC, her sister was also diagnosed with





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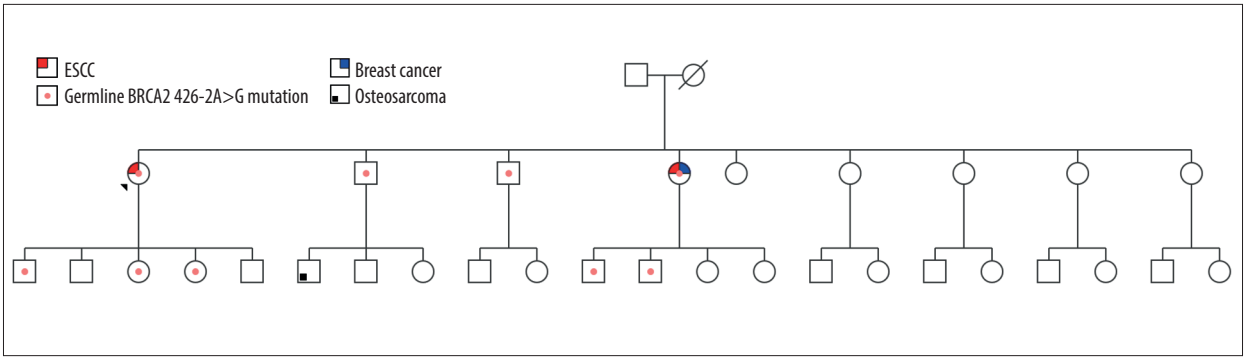


**Figure 1.** Pedigree of 2 patients with familial ESCC who shared the truncating mutation 426-2A>G. " + "shows family members who carry this mutation and " – "represents patients without this mutation.

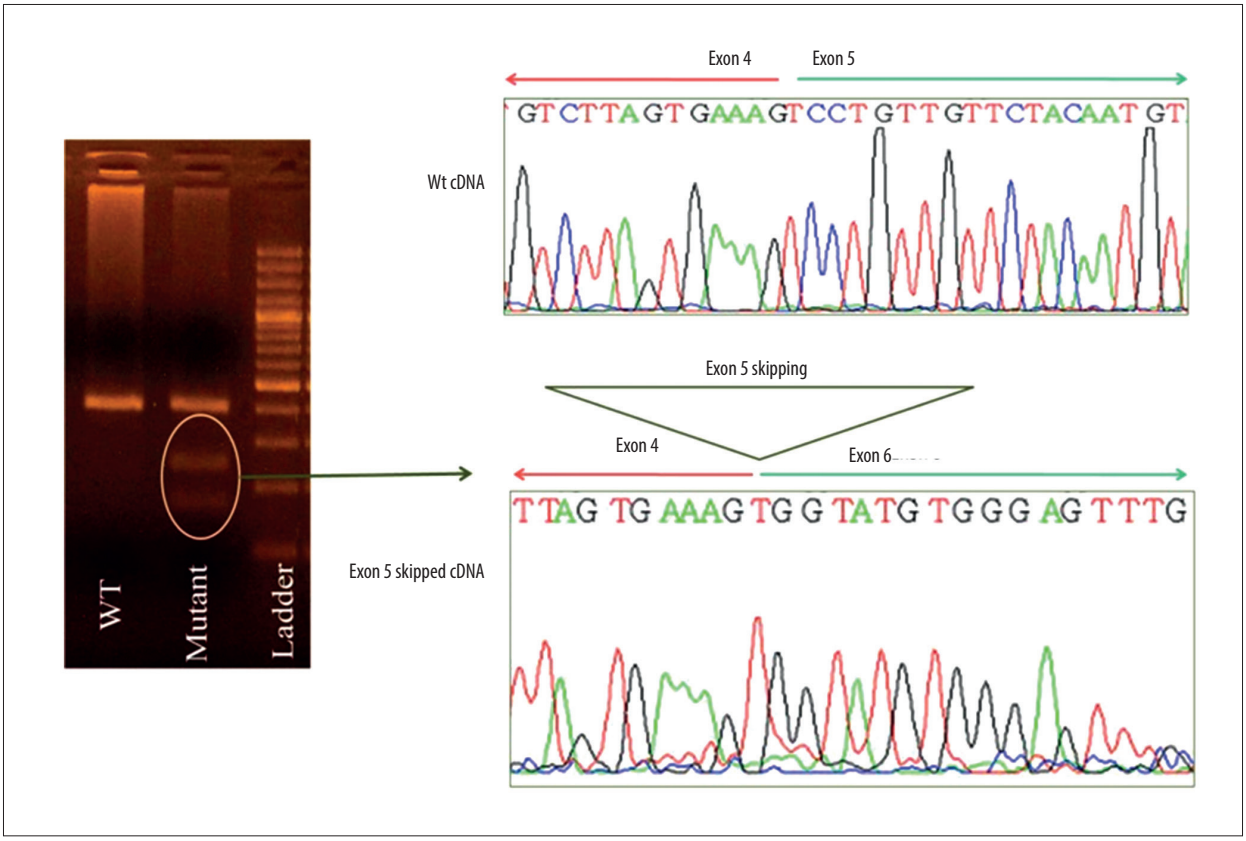


**Figure 2.** Analysis of cDNA of Patient Number 4 with 426-2A>G mutation. Sequencing revealed skipping of exon 5 and a premature stop codon at the exon 6.

ESCC with symptoms of dysphagia, at the age of 48 years old. She also underwent neoadjuvant chemotherapy and esophagectomy; her tumor also showed a complete pathologic response with no nodal involvement. Mutation analysis revealed the same mutation in her as well. Screening of other family members revealed that other sisters were negative for this mutation while brothers carried the mutation (Figure 1). Members of the family have been under surveillance for ESCC and breast cancer, and to date have not been diagnosed with any cancers. We also screened the DNA of 50 ESCC patients with no family history of esophageal cancer, and the mutation was not identified in any other person.

## Discussion

In this study, we report a novel BRCA2 germline mutation at the splicing site c.426-2A>G in familial ESCC and concomitant breast cancer in one of the patients. This mutation leads to a premature stop codon and affects the splicing process. RT-PCR





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analysis of the proband revealed exon skipping and was predicted to result in a truncated protein. Alteration in exon-intron junctions will impact mRNA splicing fidelity [21].

RAD51 is a protein that is essential for DNA recombination. RAD51-BRCA2 complex and the interaction between the 2 molecules is crucial for their role in DNA repair [22]. The binding site for RAD51 is an 8 conserved sequence motifs in BRCA2, called BRC repeats (located between residues 990 and 2100) [23]. The truncated BRCA2 protein lacks the crucial BRC domain for binding to RAD51 and the RAD51-BRCA2 complex will not be formed to participate in DNA repair process [24]. Lack of functional BRCA2, similar to RAD51, leads to chromosome breakage and genome instability during cell division [25].

Hu et al. reported loss of heterozygosity (LOH) in chromosome 13 of patients with ESCC [26] and later, for the first time, reported germline (Cys315Ser, Pro3300Ser, and Arg118His) and somatic mutations of BRCA2 in patients with familial as well as sporadic esophageal cancer. Nevertheless, they showed that BRCA2 was not a target for LOH [27]. They next focused on familial ESCC in a high-risk area in China and reported 12% germline mutations, including 3 novel ones, and concluded that germline mutation in BRCA2 was associated with genetic susceptibility to familial esophageal squamous cell carcinoma. They also showed segregation of C315S mutation in the siblings of the proband in their study [28]. On the other hand, Zhong et al. reported germline mutations in sporadic ESCC from an area with low risk of esophageal cancer in China [29]. In another study from a high-risk area in India, Kaushal et al. reported 2 germline mutations in 3 familial ESCC patients while none of the sporadic ESCC patients or healthy controls had any germline mutations [6]. Akbari et al. studied germline mutations in the coding regions of BRCA2 in a cohort of patients with Turkmen ethnic background in northern Iran. They reported 2 novel deletions which lead to a premature stop codon and translates to a truncated protein. They also detected k3326X mutation in 8 familial ESCC patients, as the most frequent mutation associated with genetic susceptibility to familial ESCC [5]. The product of this mutation was a truncated protein. A robust association between k3326X mutation and upper aerodigestive tract cancers was demonstrated by Delahaye-Sourdeix et al. in another report, studying multiple patient populations from different continents [30].

In the familial ESCC patients we studied, a similar pattern of genetic alterations was observed. His372Asn is a common polymorphism (rs144848) that was found in all the studied familial ESCC patients, and it has been shown to be associated with increased risk of non-Hodgkin lymphoma in a meta-analysis [31]. Phe599Ser is another single nucleotide variant which was observed in all the familial ESCC tested and has not been widely studied but has been considered to likely be pathogenic [32]. Patients with familial ESCC also shared multiple unclassified variant intervening sequences (IVS). Further studies are required to better understand the clinical significance of these unclassified variants.

Multiple other primary cancers have been reported in patients with esophageal cancer [33–35]. Gastric cancer, colon cancer, and head and neck cancer are the most common malignancies associated with esophageal cancer as a metachronous or synchronous tumor. It is hypothesized that a history of smoking and the concept of field cancerization plays an important role in the development of multiple primary cancer. Based on this theory, exposure of the epithelium (head and neck and gastrointestinal tract) to the carcinogens, such as tobacco and alcohol, promotes carcinogenesis in multiple sites [33]. Breast cancer as a second primary tumor in patients with ESCC, however, it has a lower prevalence in patients with multiple primary cancers. Our findings support the hypothesis that genetic susceptibility might play an important role in metachronous familial ESCC and breast cancer.

## Conclusions

Our findings have potentially important clinical implications. Genetic analysis of BRCA2 in patients with familial ESCC should be considered and could identify family members at higher risk, both for esophageal cancer and future risk for breast cancer. Moreover, genetic alterations of BRCA2 have potential therapeutic significance with recent advancements in PARP inhibitors in BRCA2 related tumors [36]. Screening for BRCA2 mutations in familial esophageal cancer patients could provide opportunities for targeted therapies.

### Conflicts of interest

None.





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