

## ORIGINAL ARTICLE

**Germline BRCA2 mutations and the risk of esophageal squamous cell carcinoma**

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**The incidence of esophageal squamous cell carcinoma (ESCC) is very high among the Turkmen population of Iran. Family studies suggest a genetic component to the disease. Turkmen are ethnically homogenous and are well suited for genetic studies. A previous study from China suggested that BRCA2 might play a role in the etiology of ESCC. We screened for mutations in the coding region of the BRCA2 gene in the germline DNA of 197 Turkmen patients with ESCC. A nonsense variant, K3326X, was identified in 9 of 197 cases (4.6%) vs 2 of 254 controls (0.8%) (OR = 6.0, 95% CI = 1.3–28;  $P = 0.01$ ). This mutation leads to the loss of the C-terminal domain of the BRCA2 protein, a part of the region of interaction with the FANCD2 protein. We observed nine other BRCA2 variants in single cases only, including two deletions, and seven missense mutations. Six of these were judged to be pathogenic. In total, a suspicious deleterious BRCA2 variant was identified in 15 of 197 ESCC cases (7.6%).** *Oncogene* advance online publication, 27 August 2007; doi:10.1038/sj.onc.1210739

**Keywords:** esophageal squamous cell carcinoma; Turkmen population; BRCA2; K3326X; Fanconi anemia pathway

**Introduction**

The southeastern part of Caspian littoral of Iran, which is inhabited mainly by Turkmen, is the western-most extremity of a region with a high incidence of esophageal cancer that extends eastward to the north

of China (Schottenfeld, 1984). The incidence of esophageal cancer in the Turkmen population is among the highest in the world, with an annual incidence rate of more than 100 per 100 000 (Mahboubi *et al.*, 1973; Saidi *et al.*, 2000). More than 90% of all esophageal cancers in Turkmen are squamous cell carcinomas (Islami *et al.*, 2004). The Turkmen are genetically homogenous and are well suited for genetic studies of esophageal squamous cell carcinoma (ESCC).

In addition to several proposed environmental factors, a number of studies have reported clustering of the disease in families (Pour and Ghadirian, 1974; Ghadirian, 1985). Recently, we reported a strong familial component to ESCC among Turkmen (Akbari *et al.*, 2006). Esophageal cancer was significantly more common in the first-degree relatives of the cases than the relatives of unaffected controls (62% vs 31%,  $P = 2 \times 10^{-8}$ ). We estimated the cumulative risk of esophageal cancer in the first-degree relatives of the cases to be 34% by the age of 75, compared to 14% for the relatives of the controls (hazard ratio = 2.3;  $P = 3 \times 10^{-8}$ ). This suggests that the genetic factors may contribute to the high incidence rate of esophageal cancer among Turkmen.

In a study of Chinese patients with ESCC, a genome-wide scan using polymorphic micro-satellite markers showed a high frequency of loss of heterozygosity on a region of chromosome 13q (Hu *et al.*, 2000). Two of the informative markers in this region (D13S260 and D13S267) are adjacent to BRCA2 (Li *et al.*, 2001). Subsequent studies among high-risk Chinese populations (Hu *et al.*, 2002, 2003, 2004) suggested that BRCA2 mutations may contribute to the development of ESCC, especially in those with a family history of upper GI cancers.

BRCA2 was identified as a breast cancer susceptibility gene in 1995 (Wooster *et al.*, 1995). Germline mutations of BRCA2 also contribute to the development of ovarian (Narod, 2002), prostate (Simard *et al.*, 2003) and pancreatic cancers (Lowenfels and Maisonneuve, 2005). Moreover, biallelic BRCA2 mutations cause Fanconi anemia (FA) (Howlett *et al.*, 2002; Offit *et al.*,

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2003). BRCA2 is mainly involved in homologous recombination repair through control of RAD51 recombinase (Pellegrini *et al.*, 2002) and it interacts with many other proteins involved in various cellular functions, including cell cycle regulation (Marmorstein *et al.*, 2001), transcription regulation (Milner *et al.*, 1997), cytokinesis (Daniels *et al.*, 2004) and control of cell proliferation (Tian *et al.*, 2005).

In the current study, we scanned the germline DNA of 197 Turkmen ESCC cases for BRCA2 mutations. We then screened 254 Turkmen controls for the mutations detected in cases to evaluate whether mutations in this gene are associated with elevated ESCC risk in the Turkmen population of Iran.

**Results**

We studied 197 Turkmen cases of ESCC, including 114 men and 83 women. The mean age at diagnosis was 64.0±11 years (range 30–86). A family history of esophageal cancer was reported for 144 (73.1%) of the cases. The control group consisted of 254 Turkmen individuals (113 men and 141 women) from hospital patients who had diagnoses other than cancer. The mean age of controls was 59±12 years.

We identified the K3326X nonsense variant in nine cases (4.6%) and two controls (0.8%) (OR = 6.0, 95% CI = 1.3–28; P = 0.01). None were related to each other. Eight cases and one of the controls had a family history of esophageal cancer (Table 3). The variant was more frequent among familial cases (8/144; 5.5%) than controls (2/254; 0.8%) (OR = 7.4, 95% CI = 1.6–36; P = 0.005). This mutation leads to premature truncation of the BRCA2 protein and the loss of half of the amino acids coded by exon 27 (Mazoyer *et al.*, 1996). The mean age at diagnosis of ESCC for the nine patients with the K3326X variant was 62.3 years, similar to the age of diagnosis in cases without this variant (64.1 years). The pathologic tumor grades of the cases with the K3326X variant were not different from other cases. The K3326X nonsense variant was completely in linkage disequilibrium (LD) with an intronic variant IVS24-16T > C in cases and controls (r<sup>2</sup> = 1.0).

In addition to the K3326X variant, we detected two deletions and seven unique missense mutations. The two deletions (501delCCAATCTCCTGTA and 3734delA) were found in patients who had no family history of esophageal or other cancer (Table 1). Seven patients had a unique missense variant (Y42C, C315S, L1019V, I2490T, T2542M, K2729N and Q3227E). Five of these patients had a family history of esophageal or other cancer. Four of the missense variants (Y42C, C315S, I2490T and K2729N) were judged to be deleterious and two others (L1019V and T2542M) are likely to be benign, based on available evidence, including the impact of the mutation on the BRCA2 protein structure, and the potential interaction with other proteins (see discussion). There is not enough information available to assess the effect of the Q3227E variant on the BRCA2

**Table 1** Germline BRCA2 mutations detected in ESCC patients

Patient ID	Gender	Age at diagnosis	Exon	Base change	Amino-acid change	PSIC score difference <sup>a</sup>	Family history of cancer
P30819	Male	30	3	A353G	T42C	2.76	Paternal grandfather (EC) <sup>b</sup> , two paternal aunt (EC)
P28410	Female	58	3	501 del CCAATCTCCTGTA	Frame shift	NA	No
P28200	Male	73	10	T1171A	C315S	2.31	No
P28416	Male	62	11	C3283G	L1019V	0.36	Mother (EC)
P28285	Male	63	11	3734 del A	Frame shift	NA	No
P30841	Female	73	15	T7697C	I2490T	1.64	16 individuals (EC) <sup>c</sup>
P30844	Male	73	16	C7853T	T2542M	0.71	Half-brother (BT)
P28250	Female	44	18	G8415T	K2729N	1.46	Father (EC), mother (EC)
P30833	Male	39	27	C9907G	Q3227E	1.56	No
Table 3	Table 3	Table 3	27	A10204T	K3326X	NA	Table 3

Abbreviations: EC, esophageal cancer; ESCC, esophageal squamous cell carcinoma; BT, brain tumor; PSIC, position-specific independent counts. <sup>a</sup>PSIC score difference for two amino acids according to alignment data of homologous proteins calculated by the PolyPhen algorithm (<http://genetics.bwh.harvard.edu/pph>). A variant predicted to be probably damaging if the PSIC score difference is greater than 1.7 and possibly damaging if the PSIC score difference is between 1.5 and 1.7 (Sunyaev *et al.*, 2001). <sup>b</sup>Cancer types: EC and BT. <sup>c</sup>Two paternal grandparents, two paternal aunts, one paternal uncle, seven paternal cousins, one brother, one half-brother, one nephew and one maternal aunt.

protein. We evaluated the 254 controls for the presence of these seven missense variants and the 3734delA deletion, but none was detected.

The I3412V variant was identified frequently among both cases (12/197; 6.1%) and controls (20/245; 8.2%) ( $P=0.8$ ). Other silent variants and intronic polymorphisms are listed in Table 2.

In summary, we identified a deleterious mutation in 15 of 197 ESCC cases (7.6%). The frequency of BRCA2 deleterious mutations among ESCC cases (7.6%) was significantly higher than controls (2/254; 0.8%) ( $OR=10.4$ , 95%  $CI=2.3-46$ ;  $P=0.0002$ ). There was no synergistic effect between BRCA2 mutations and any of the conventional environmental risk factors, such as smoking and alcohol drinking (data not shown).

## Discussion

We screened the entire coding region of BRCA2 in the germline DNA of 197 Turkmen patients with ESCC. Two deletions were identified which are predicted to be deleterious. These include a deletion of 13 nucleotides at position 501–513, which results in frameshift translation and a premature ochre stop codon (UAA) at codon 91. The second deletion found was a single nucleotide deletion at position 3734 in BRCA2 that leads to a

premature amber stop codon (UAG) at codon 1176. This truncated BRCA2 protein is missing domains that are crucial for DNA repair function. It is interesting that, although these are typical of deleterious BRCA2 mutations, neither of these patients had a family history of breast or ovarian cancer (Table 1).

We found the nonsense variant K3326X in nine of our ESCC cases, eight of whom had a family history of esophageal cancer. The association between this variant and ESCC risk was statistically significant ( $OR=6.0$ , 95%  $CI=1.3-28$ ;  $P=0.01$ ). The pathogenicity of this variant regarding breast cancer risk has been questioned (Mazoyer *et al.*, 1996); however, we believe that our data support that it is predisposing for ESCC. Martin *et al.* (2005) reported the K3326X variant to be more prevalent among patients with familial pancreatic cancer than those in controls ( $OR=4.8$ , 95%  $CI=1.3-19$ ;  $P<0.01$ ). The allele frequency of the K3326X variant has been reported to be 0% in Asian, 0% in Sub-Saharan African and 1.7% European populations (International HapMap Consortium, 2005). In our study, the frequency among ESCC patients from a Turkmen population (which is believed to have a genetic background more similar to East Asians than those of HapMap populations) was 4.6% and among controls was 0.8%. None of our cases and controls who were carriers of this variant had any other deleterious mutation in BRCA2; however, the IVS24-16T>C and

**Table 2** Germline BRCA2 polymorphisms detected in 197 Turkmen ESCC patients

No	Base change	Amino-acid change	Number of heterozygote patients (%)	Number of homozygote patients (%)
1	5'UTRG203A	NA	31 (15.7)	8 (4.1)
2	IVS 4+67 A>C	NA	30 (15.2)	1 (0.5)
3	A3624G	L1132L	1 (0.5)	0 (0.0)
4	T4035C	V1269V	1 (0.5)	0 (0.0)
5	IVS 12+34 C>T	NA	1 (0.5)	0 (0.0)
6	A7470G	S2414S	22 (11.2)	10 (5.1)
7	IVS17-14 T>C	NA	30 (15.2)	16 (8.1)
8	IVS 19+82 G>A	NA	4 (2.0)	0 (0.0)
9	C9132A	T2968T	11 (5.6)	0 (0.0)
10	IVS24-16 T>C	NA	9 (4.6)	0 (0.0)
11	C10251T	D3341D	1 (0.5)	0 (0.0)
12	G10338A	R3370R	1 (0.5)	0 (0.0)
13	A10462G	I3412V	12 (6.1)	0 (0.0)

Abbreviations: ESCC, esophageal squamous cell carcinoma; NA, not applicable.

**Table 3** Demographic information of cases and controls with the K3326X nonsense variant in BRCA2

ID	Group	Gender	Age at diagnosis	Family history of esophageal cancer
P28218	Control	Female	76	Maternal first cousin
P28309	Case	Female	70	Mother, paternal uncle, maternal aunt, maternal first cousin
P28326	Case	Male	42	Maternal uncle
P28350	Case	Male	37	Father, paternal first cousin
P28482	Case	Male	67	Paternal uncle
P28488	Case	Female	79	Brother
P28534	Control	Male	68	No
P28547	Case	Female	53	Father, brother, paternal uncle
P30812	Case	Female	73	No
P30850	Case	Female	78	Paternal aunt, maternal first cousin
P30854	Case	Male	62	Mother, sister

K3326X variants which are 3816 bp away from each other were in complete LD, suggesting that the carriers of K3326X in the Turkmen population share a common ancestor.

The BRCA2 variant K3326X results from mutation of adenosine to thymine at position 10 204 of exon 27 in the BRCA2 mRNA sequence, which leads to the ochre stop codon (UAA) and the truncation of 93 amino acids from the C terminus of the protein. Studies of mouse models suggest that the C terminus of BRCA2 is important in interacting with RAD51 (Mizuta *et al.*, 1997). Mouse cells with a BRCA2 exon 27 deletion showed hypersensitivity to ionizing radiation (Morimatsu *et al.*, 1998) and reduced life span (Donoho *et al.*, 2003). Mice with a homozygous deletion of exon 27 had increased susceptibility for various types of solid tumors, but not mammary tumors (McAllister *et al.*, 2002). This is consistent with the hypothesis that in humans, K3326X predisposes to malignant tumors of the pancreas and esophagus, but not to breast cancer.

Howlett *et al.* (2002) reported biallelic inactivation of BRCA2 in a FA cell line which contained the K3326X variant as well as the frameshift mutation 3033de-IAAAC in exon 11. FA is an autosomal recessive disorder characterized by cancer susceptibility and cellular hypersensitivity to DNA cross-linking agents (Joenje and Patel, 2001). BRCA2/FANCD1 is actually a member of a group of at least 13 different proteins which constitute the FA pathway (Taniguchi and D'Andrea, 2006; Reid *et al.*, 2007). Eight of these proteins (FANCA, B, C, E, F, G, L and M) form a nuclear complex, known as the FA core complex, which is required for monoubiquitination of the FANCD2 protein in response to DNA damage (Taniguchi and D'Andrea, 2006). Interaction of monoubiquitinated FANCD2 and BRCA2 in the C terminus of BRCA2 is essential for activation of RAD51 and for loading onto the damaged DNA (Wang *et al.*, 2004; Atanassov *et al.*, 2005). Cells that express truncated BRCA2 protein, which lacks the C-terminal exon 27 coding region, do not show co-localization of FANCD2, BRCA2 and RAD51 on chromatin and are hypersensitive to cross-linking agents like mitomycin C (Wang *et al.*, 2004; Atanassov *et al.*, 2005). Loss of the interaction between monoubiquitinated FANCD2 and BRCA2 on chromatin and failure to load BRCA2 onto damaged chromatin is characteristic of all subtypes of FA cells (Wang *et al.*, 2004). FANCD2 knockout mice showed susceptibility to epithelial cancers (Houghtaling *et al.*, 2003) which is comparable to that reported in mice with truncated BRCA2 lacking the exon 27 coding region (McAllister *et al.*, 2002). This suggests that the functions of FANCD2 and the C terminus of BRCA2 are closely related (Houghtaling *et al.*, 2003).

According to available evidence, four of the missense variants observed here (Y42C, C315S, I2490T and K2729N) seem to be pathogenic and two (L1019V and T2542M) seem to be benign. There is insufficient evidence to judge the effect of the Q3227E variant.

The Y42C variant is located very close to the binding site of PALB2/FANCN (residues 10–40) in the BRCA2

protein (Xia *et al.*, 2006). By binding to BRCA2, PALB2 promotes the stability of BRCA2 intranuclear localization that is essential for its homologous recombination repair function (Xia *et al.*, 2006). Moreover, the BRCA2 transcription activation core sequence (Milner *et al.*, 1997) which is also the binding site for EMSY protein (Hughes-Davies *et al.*, 2003) overlaps with the location of this variant. Because the side chains of tyrosine and cysteine are completely different, this variant might affect the structure and binding of BRCA2 with other proteins. Furthermore, tyrosine at codon 42 of BRCA2 is highly conserved in vertebrate species.

The missense variant C315S, which was identified in only one ESCC patient in our study, was reported in a total of 4 out of 246 patients from all three studies that scanned the BRCA2 gene in Chinese ESCC patients (Hu *et al.*, 2002, 2003, 2004). Variant C315S is located in the region implicated in BRCA2-P/CAF complex formation (residues 290–453) which shows histone acetyltransferase activity (Fuks *et al.*, 1998) that maybe responsible for the transcription regulatory function of BRCA2.

Two additional missense variants (I2490T and K2729) result in amino acid changes in the same domain of the BRCA2 protein and may have a similar effect on the protein function. Structural crystallography of BRCA2 and DSS1 shows that the isoleucine at codon 2490 and the lysine at codon 2729 of BRCA2 are involved in  $\alpha$ -helix and  $\beta$ -sheet structures of oligosaccharide-binding (OB) fold 1 (Yang *et al.*, 2002). OB1 is also a site of interaction with FANCG protein—a proposed regulator for unloading of RAD51 from BRCA2 to the damaged DNA (Hussain *et al.*, 2003). Both of these amino acids are also located in the binding domain of BRCA2 to MAGE-D1 protein (residues 2393–2952) a synergistic suppressor of cell proliferation (Tian *et al.*, 2005). Both variants have been reported in FA patients with biallelic BRCA2 mutations, in combination with 5301insA and S2835X, respectively, (Howlett *et al.*, 2002; Offit *et al.*, 2003).

Out of the 38 previously identified missense variants in the BRC motifs region in exon 11, we only identified the I1019V variant in a single patient from a total of 451 cases and controls. This exemplifies the importance of the eight BRC motifs, which are highly conserved in the genome and nucleotide changes in this area are rare. The BRC motifs are binding site of BRCA2 to RAD51 (Pellegrini *et al.*, 2002). Although isoleucine found at codon 1019 in the first BRC motif, a valine is found in this position in five of the other seven motifs (Bork *et al.*, 1996). Because these two amino acids are structurally similar and both are hydrophobic, their substitution at this position seems to be benign. Structural crystallography of BRCA2 in the region of the T2542M variant shows that threonine at codon 2542 does not have a special role in the secondary structure of BRCA2 (Yang *et al.*, 2002). Furthermore, threonine at this codon is not conserved in different species and even methionine is located at this position in the homologous BRCA2 protein in rat.

In total, we identified deleterious BRCA2 mutations in 15 ESCC patients. Three mutations (I2490T, K2729N

and K3326X), which were seen in 11 patients, are located in domains which bind BRCA2 to other proteins in the FA pathway. Moreover, these three specific mutations have been seen in FA patients with biallelic BRCA2 mutations. Patients with FA are at increased risk of esophageal cancer. A review of 1301 FA patients showed the occurrence of 80 solid tumors among 68 patients, nine of which were esophageal cancer and 26 were classified head, neck or upper esophageal cancer (Alter *et al.*, 2005). Rosenberg *et al.* (2003) studied 145 FA patients and found 2 esophageal cancers and 6 head and neck carcinoma out of 18 solid tumors present in this group. The observed to expected incidence ratio was 2362 (95% CI=265–8530) for esophageal cancer. Therefore, it is tempting to speculate that the BRCA2 mutations, specifically those we identified in our cases (I2490T, K2729N and K3326X), increase the risk for development of ESCC by a mechanism related to FA pathway interruption. This may be a different mechanism from that predisposes carriers of other BRCA2 mutations to breast or ovarian cancer. Esophageal epithelial cells are exposed to exogenous carcinogens, some of which produce inter-strand DNA links which cannot be repaired in cells with defective HRR. Some other carcinogens cause double-strand DNA breaks repaired by error-prone non-homologous end joining pathway in absence of intact HRR. Inappropriate repair of damaged DNA results in loss of genome integrity and chromosomal instability that eventually leads to cancer development in esophageal epithelial tissue. Defective HRR in esophageal epithelial cells can result from the mutation of the wild-type copy of BRCA2 in cells which already have a germline BRCA2 mutation, thus leaving no copy of BRCA2 protein capable of binding to FANCD2 and FNACG proteins. Interruption of BRCA2 interaction with these FA pathway genes results in disruption of the FA pathway and consequently causes ineffective HRR.

If correct, this model could have implications for the treatment of ESCC. If HRR is defective in ESCC cells of patients with BRCA2 mutations, we expect that ESCC tumors in these patients might be more sensitive to chemotherapeutic agents like cis-platin (which makes inter-strand DNA links which require HRR for their repair) than that of radiation which produces double-strand breaks repaired by non-homologous end joining pathway (Houghtaling *et al.*, 2005). We hope by determining the role of other FA genes in the development of ESCC in future studies we can provide more evidence for this model.

## Materials and methods

### Study subjects

This study is a part of an ongoing case-control study of UGI cancers (Islami *et al.*, 2004) in the city of Gonbad, the second largest city of Golestan province, in northeastern Iran. The Digestive Disease Research Center (DDRC), Tehran University of Medical Sciences is running this project in collaboration with the National Cancer Institute and the

International Agency for Research on Cancer. In total, 197 Turkmen with ESCC and available blood samples were enrolled in this study. The diagnosis of ESCC was confirmed for all cases by upper gastrointestinal endoscopy and pathologic evaluation of tumor biopsies. Cases and controls were collected between 1 August 2001 and 15 December 2005. The control group consisted of 254 Turkmen patients from the central city hospital (Khatam hospital) who had diagnoses other than cancer. The study protocol was approved by the ethics committee of the DDRC of Tehran University of Medical Sciences.

### Scanning of BRCA2 nucleotide changes

A variety of methods were employed to detect the presence of BRCA2 nucleotide changes. Exons 10 and 11 of BRCA2 were scanned by the protein-truncation test (PTT). Primer sequences used to amplify overlapping fragments were obtained from the Breast Cancer Information Core (BIC) (<http://research.nhgri.nih.gov/projects/bic>). PTT was performed by the TNT rabbit reticulocyte lysate system (Promega, Madison, WI, USA), with [35S]-methionine (New England Nuclear) for protein detection. All mutant bands detected by PTT were confirmed by direct sequencing.

Because most deleterious mutations in exons 10 and 11 of BRCA2 are truncating mutations, PTT is a standard method for mutation detection; however, structural crystallography of BRC motifs in exon 11 suggested that some missense variants in these regions could be deleterious (Pellegrini *et al.*, 2002). As of 31 August 2006, 38 missense variants have been reported in the BIC database in the BRC motifs in exon 11, including the C315S variant (T1171A) in exon 10 which has been reported previously in a few families with ESCC (Hu *et al.*, 2002, 2003, 2004) were genotyped in all cases and controls using iPLEX chemistry on a MALDI-TOF MassARRAY system (Sequenom Inc., San Diego, CA, USA). The mutations detected in cases were also genotyped in controls by MassARRAY. All of the procedures were performed according to the manufacturer's standard protocol. The average genotype call rate for this study was 98.4%.

The remaining exons of BRCA2, exons 2–9 and 12–27, were scanned for finding mutations by the Denaturing High-Performance Liquid Chromatography (DHPLC) method (Varian's Helix system). Primer sequences used to amplify each amplicon were obtained from the BIC. To enhance heteroduplex formation, the PCR products were denatured at 95°C for 5 min and slowly cooled to room temperature on the bench top. Products were automatically loaded on a column and eluted with a linear acetonitrile gradient in triethylamine acetate buffer (pH 7) at a constant flow rate of 0.5 ml min<sup>-1</sup>. Under these conditions, double-stranded DNA fragments were eluted for 5 min. The optimized column temperature against a positive control used for each amplicon was varied between 53 and 58°C. Eluted DNA fragments were detected by a UV detector. All mutations detected by DHPLC were confirmed by direct sequencing. However, direct sequencing was applied for mutation detection of exons 2–9 and 12–27 on one third (60/197) of the cases. The BigDye Terminator Cycle Sequencing kit was employed on ABI PRISM 3130XL Genetic Analyzers (Applied Biosystems Inc., Foster City, CA, USA) for all sequencing.

### Statistical analyses

The permutation version of the exact test was done for Hardy-Weinberg Equilibrium testing. Fisher's exact test was employed for comparing the frequency of genotypes between

cases and controls. Two-sided *P*-values were reported. All analyses were done by SAS 9.1.3 software.

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