Infrequent Mutation in the BRCA2 Gene in Esophageal Squamous Cell Carcinoma

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INTRODUCTION

The BRCA2 gene is located on chromosome 13q (1). Alterations in the BRCA2 gene result in increased risk of breast cancer in both women and men, and a moderately increased risk for a variety of other cancers, including carcinomas of the ovary, pancreas, prostate, colon, and liver (2–7). Thus far only infrequent alterations in BRCA2 have been reported in ESCC (8).3 Not surprisingly, few studies have reported mutation frequencies for all of the coding exons of BRCA2 because of its large size. BRCA2 is thought to be involved in double-strand DNA break repair (9, 10). Several studies have demonstrated that BRCA2 and BRCA1 bind to Rad51, a protein involved in maintaining the integrity of the genome. Rad51 also physically associates with the TP53 tumor suppressor protein. Physical and functional interactions of BRCA2 with these key components of cell cycle control and DNA repair pathways suggest that it likely participates with them in some way to maintain genomic integrity (11). This association is additionally supported by the fact that somatic mutations of TP53 are commonly seen with germ-line mutations of BRCA1 and BRCA2 in breast/ovarian cancer (12, 13).

Esophageal cancer is a very common disease in many areas of China, especially in Shanxi Province (14). In previous studies in Shanxi Province, China, we found frequent LOH on chromosome 13q (15, 16), including chromosome 13q12 where BRCA2 is located (15–17). In the present study we characterized genetic alterations in BRCA2 in ESCC patients by screening the entire BRCA2 gene for mutations using SSCP analysis and DNA sequencing in 56 ESCC patients examined previously for both TP53 mutations and LOH on chromosome 13q (18, 19).

MATERIALS AND METHODS

Patient Selection. Patients presenting in 1995 and 1996 to the Shanxi Cancer Hospital in Taiyuan, Shanxi Province, People’s Republic of China, who were diagnosed with ESCC and considered candidates for curative surgical resection, were identified and recruited to participate in this study. The study was approved by the Institutional Review Boards of the Shanxi Cancer Hospital and the United States National Cancer Institute. A total of 56 patients with ESCC were selected who had a histological diagnosis of ESCC confirmed by pathologists at both the Shanxi Cancer Hospital and the National Cancer Institute. None of the patients had prior therapy, and Shanxi was the ancestral home for all of the patients.

After obtaining informed consent, patients were interviewed to obtain information on demographic and cancer lifestyle risk factors, and a detailed family history of cancer. A total

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3 The abbreviations used are: ESCC, esophageal squamous cell carcinoma; SSCP, single-strand conformation polymorphism; LOH, loss of heterozygosity; UGI, upper gastrointestinal.
of 56 ESCC patients, including 34 males and 22 females, were evaluated. Details on these ESCC patients have been described previously (19). All of the patients were previously evaluated for allelic loss on 13q, including D13S260 and D13S267, which flank BRCA2 (15–17) and mutations in TP53 (exons 4 to 9; Ref. 18). The frequencies of LOH on D13S260 and D13S267 were 57% (17 of 30 informative cases) and 83% (33 of 40 informative cases), respectively (17). Mutations in TP53 were found in 77%, and intragenic allelic loss was observed in 76% (18).

Biological Specimen Collection and Processing. Venous blood (10 ml) was taken from each patient before surgery, and genomic DNA was extracted and purified. Tumor tissue obtained during surgery was fixed in ethanol and embedded in paraffin.

Laser Microdissection and Extraction of DNA. Tumor cells were microdissected under light microscopic visualization using methods described previously (20).

PCR and SSCP Analysis. Mutations in all 26 coding exons of the BRCA2 gene were screened by PCR-SSCP. The 57 pairs of PCR primers used to cover all of the intron/exon boundaries are listed in Table 1. DNA extracted from tumor cells was microdissected from the resection specimen, and genomic DNA extracted from venous blood was used for each patient. PCR reactions and SSCP analyses were conducted using methods described previously (19) except the annealing temperature was adjusted to 55–60°C.

DNA Sequencing. DNA sequencing was performed using methods described previously (19). All of the mutations were confirmed by repeating the procedures outlined above. Subcloning was performed in 1 case (SHE247) with the TOPO Cloning kit (Invitrogen, Carlsbad, CA) according to the manufacturer’s instructions.

Statistical Analysis. All of the statistical analyses were performed using Statistical Analysis Systems (SAS; SAS Corp., Cary, NC). Associations were tested using Fisher’s exact test. All P were two-sided and considered statistically significant if P < 0.05.

RESULTS

Screening the entire coding region of the BRCA2 gene in tumor and blood DNA of 56 ESCC patients identified 8 mutations in 5 cases (5 of 56; 9%). Three cases had germ-line mutations, whereas 2 had only somatic mutations. These mutations are listed in Table 2a, and examples are shown in Figs. 1 and 2. Demographic characteristics and previously determined genetic alterations of TP53, and LOH on D13S260 and D13S267 for these 5 cases are listed in Table 2b. No significant association was seen between alterations (mutations or intragenic allelic loss) in BRCA2 and TP53 (data not shown).

Allelic Loss at Polymorphic Sites in BRCA2. SSCP analysis of BRCA2 exons 2–27 performed in this search for mutations in ESCC samples revealed bandshifts in some samples in exons 2, 10 (primer 10.3), and 11 (primer 11.7; Fig. 3). Direct sequencing of the genomic DNA/PCR products of these exons after SSCP showed the presence of three polymorphic sites (203G>A, N372H, and K1132K) reported previously in the Breast Cancer Information Core database.4 The frequency of allelic loss in tumor DNA at these three polymorphic sites was 20% (10 of 51), 81% (13 of 16), and 64% (16 of 25) for 203G>A, N372H, and K1132K, respectively. Forty-six percent of ESCC cases (26 of 56) were found to have intragenic allelic loss at one or more of these polymorphic sites, including 16 with one, 7 with two, and 3 with loss at all three of the sites. Ten cases lost a wild-type allele at 203G>A; 5 cases lost a wild-type allele and 8 lost a polymorphic allele at N372H; and 8 lost a wild-type allele and 8 lost a polymorphic allele at K1132K (for example, see Fig. 3 for N372H).

Potential Biallelic Inactivation of BRCA2. We found evidence for potential biallelic inactivation of BRCA2 in 4 of 56 (7%) cases (Table 2a). Two cases (SHE138 and SHE437) had a germ-line mutation in one allele and LOH in the other (wild-type) allele. A third case (SHE360) had a germ-line mutation in one allele (at codon 315) and LOH near the mutation position (at codon 372), but we could not determine whether the LOH was in the wild-type or mutant allele. A fourth case (SHE247) had two mutations (one missense and one frameshift) in different exons, but we do not know if these mutations occurred on different alleles. The fifth case (SHE150) also had two mutations, but because one mutation was silent and no other alterations were identified, it is unlikely that biallelic inactivation occurred. In addition, 10 cases without mutation had intragenic allelic loss at either two (n = 7) or all three (n = 3) of the polymorphic sites (data not shown). While it is possible that these losses occurred in different alleles, it seems more likely that these findings were the result of a single large allelic loss rather than multiple discrete events that occurred on different alleles.

Genetic Alterations of BRCA2 and LOH at D13S260 and D13S267. The number of cases with a BRCA2 mutation was too small for meaningful comparison with LOH at microsatellite markers D13S260 or D13S267; however, LOH at D13S267 was significantly associated with allelic loss of at least one of the polymorphisms within BRCA2 (P = 0.004). Furthermore, among the 36 cases informative for both D13S260 and BRCA2, D13S267 showed LOH for all 20 cases with an intragenic BRCA2 allelic loss (sensitivity = 20 of 20 = 100%, specificity = 6 of 16 = 38%). Twenty of the 30 cases with LOH at D13S267 were subsequently found to have intragenic allelic loss in BRCA2 (positive predictive value = 67%). No significant association between LOH at D13S260 and loss at these three polymorphic sites was seen (data not shown).

Genetic Alterations of BRCA2 and Family History. All 3 cases with germ-line mutations had a positive family history of UGI cancer. The frequency of BRCA2 mutations was somewhat higher in patients with a family history of UGI cancer (12%) compared with patients without such a family history (5%), but this difference was not significant (P = 0.36). Also, there was a slightly higher frequency of allelic loss (53%, 18 of 34) at polymorphic sites in patients with a family history of UGI.

4 Internet address: http://www.nchgr.nih.gov/Intramural_research/Lab_transfer/BIC.
cancer compared with patients without such a family history (36%, 8 of 22; \( P = 0.28 \)).

DISCUSSION

Somatic mutations in \( BRCA2 \) are very rare in breast cancer and other tumors (4–7, 21). Only one previous study has reported testing all of the coding exons in \( BRCA2 \) in ESCC, and no mutations were detected in those Japanese patients (8). To our knowledge, our report is the first to identify germ-line or somatic mutations in \( BRCA2 \) in ESCC patients. In the present study of 56 ESCC patients from a high-risk population in China, we found that 5 patients (9%) had 8 \( BRCA2 \) mutations. How-

<table>
<thead>
<tr>
<th>Exon</th>
<th>Sense primer (5′–3′)</th>
<th>Antisense primer (5′–3′)</th>
</tr>
</thead>
<tbody>
<tr>
<td>2</td>
<td>CTCAGTCACATAAAGAAATGAC</td>
<td>CAAACTCGTGAGCTAGCTAGGGT</td>
</tr>
<tr>
<td>3</td>
<td>CAATTGGTCTCAGCTAGTTTA</td>
<td>CTAATTCCTATTGTGAGTTC</td>
</tr>
<tr>
<td>4</td>
<td>ACATGCTCAAGAGAAATGGCTACTAG</td>
<td>CTTCCTCAAGGCTACTAG</td>
</tr>
<tr>
<td>5</td>
<td>ATATCCTACAAATGGTCTACACTAC</td>
<td>AAATCATGCTACTAGCTAG</td>
</tr>
<tr>
<td>6</td>
<td>CTCTGAAGCTAATGGTACTAGTAC</td>
<td>TCTTAATCATGCTACTAG</td>
</tr>
<tr>
<td>7</td>
<td>CGTTCGTAGCGTAAATGGTCTACAT</td>
<td>TCTTAATCATGCTACTAG</td>
</tr>
<tr>
<td>8</td>
<td>GTGTCGTAGTACATAATTTGTTCAT</td>
<td>TCTTAATCATGCTACTAG</td>
</tr>
</tbody>
</table>

**Table 1.** Sequence of primers used for PCR-SSCP analysis of \( BRCA2 \)

\( ^a \) Total \( n = 57 \).
### Table 2 Genetic changes and demographics for patients with BRCA2 alterations

#### A. BRCA2 genetic alterations in 5 of 56 ESCC patients

<table>
<thead>
<tr>
<th>Patient ID</th>
<th>Mutation type</th>
<th>Exon</th>
<th>Codon/mucleotide</th>
<th>Base change</th>
<th>Amino acid change</th>
<th>Designation/mutation type</th>
<th>Allelic loss</th>
<th>Biallelic alterations</th>
</tr>
</thead>
<tbody>
<tr>
<td>SHE138</td>
<td>Somatic</td>
<td>4</td>
<td>118/581</td>
<td>G→A</td>
<td>Arg→His</td>
<td>R118H/missense</td>
<td>Yes</td>
<td>Yes</td>
</tr>
<tr>
<td></td>
<td>Somatic</td>
<td>11</td>
<td>1682/5274</td>
<td>T→C</td>
<td>Ser→Ser</td>
<td>S1682S/silent</td>
<td>No</td>
<td>—</td>
</tr>
<tr>
<td>SHE150</td>
<td>Somatic</td>
<td>11</td>
<td>1338/4242</td>
<td>C→T</td>
<td>Gly→Gly</td>
<td>G1338G/silent</td>
<td>No</td>
<td>—</td>
</tr>
<tr>
<td>SHE247</td>
<td>Somatic</td>
<td>3</td>
<td>25–26/after 303</td>
<td>TTAGGA(ccaatg)</td>
<td>CCAATA</td>
<td>stop codon 30</td>
<td>303ins7/frame shift</td>
<td>No</td>
</tr>
<tr>
<td>SHE360</td>
<td>Germline</td>
<td>10</td>
<td>315/1171</td>
<td>C→T</td>
<td>Arg→Cys</td>
<td>R2842C/missense</td>
<td>No</td>
<td>—</td>
</tr>
<tr>
<td>SHE437</td>
<td>Germline</td>
<td>27</td>
<td>3300/10126</td>
<td>C→T</td>
<td>Pro→Ser</td>
<td>P3300S/missense</td>
<td>Yes</td>
<td>Yes</td>
</tr>
</tbody>
</table>

#### B. Demographics and results of TP53 mutation and microsatellite marker LOH testing in ESCC patients with BRCA2 mutations

<table>
<thead>
<tr>
<th>Patient ID</th>
<th>Age/sex</th>
<th>Family history of cancer</th>
<th>TP53 mutation in exons 4–9</th>
<th>Intragenic allelic loss in R72P of TP53</th>
<th>LOH at D13S260/267</th>
</tr>
</thead>
<tbody>
<tr>
<td>SHE 138</td>
<td>55/F</td>
<td>EC (mother)</td>
<td>12bp del (codon 174)</td>
<td>Retention</td>
<td>Loss/homozygous</td>
</tr>
<tr>
<td>SHE 150</td>
<td>57/M</td>
<td>EC (mother), cervical (paternal aunt)</td>
<td>No</td>
<td>Homozygous (Pro/Pro)</td>
<td>Retention/retenion</td>
</tr>
<tr>
<td>SHE 247</td>
<td>45/M</td>
<td>No</td>
<td>2bp del (codon 69)</td>
<td>Homozygous (Arg/Arg)</td>
<td>Homozygous/homozygous</td>
</tr>
<tr>
<td>SHE 360</td>
<td>55/M</td>
<td>EC (father), BC (brother)</td>
<td>No</td>
<td>Loss (Arg allele)</td>
<td>Loss/loss</td>
</tr>
<tr>
<td>SHE 437</td>
<td>47/F</td>
<td>2 EC (father and mother)</td>
<td>18bp del (Codon 134)</td>
<td>Retention</td>
<td>Loss/loss</td>
</tr>
</tbody>
</table>

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* Not reported in the BIC as of February 2001.
* Includes complete family history of cancer in first, second, and third degree relatives; EC, esophageal cancer; BC, body of stomach cancer.
* Polymorphism at codon 72, Arg→Pro, in exon 4 of TP53.
ever, none of the 56 tumors showed classic Knudsen two-hit inactivation with clear cut functionally inactivating mutations. Two cases showed LOH with missense mutations of unknown significance. Thus, we conclude that \textit{BRCA2} is not the target of LOH on chromosome 13q. Because we did not evaluate \textit{BRCA2} mRNA or protein levels, we do not know if function was altered in the cases with either biallelic or single allele changes. At present there are no compelling clinical or experimental data that we are aware of indicating that \textit{BRCA2} haplo-insufficiency contributes to tumorigenesis (22). The three germ-line mutations we saw included one not reported previously, whereas the three polymorphisms we observed have all been reported before. Distinguishing between mutations and polymorphisms in these patients is complicated by the fact that previous studies of these alterations in Chinese populations have not been reported. The overall significance of our findings is not known and may represent either biallelic inactivation of \textit{BRCA2} in a small percentage of ESCC cases, or simply missense changes with no functional consequence. Whereas functional studies will be required to determine whether \textit{BRCA2} has any role in ESCC, it is apparent from our results here that \textit{BRCA2} is not frequently inactivated by the traditional two-hit mechanism. In summary, we showed for the first time that mutations in the \textit{BRCA2} gene

![Fig. 1 Somatic mutation of BRCA2 gene in case 247. A, SSCP gel, sequencing result shows that bands 1 and 3 are strands of the mutant allele, bands 2 and 4 are strands of the wild-type allele in the tumor. B, sequencing gel demonstrates somatic mutation with 7-bp (ccatg) insertion after codon 25 of BRCA2 resulting in a reading frameshift in the tumor.](image)

![Fig. 2 Germline mutation and wild-type allelic loss in the tumor in case 437. A, SSCP gel shows an abnormal migration pattern in germ-line DNA; sequencing result demonstrates that bands 1 and 3 are strands of the mutant allele and bands 2 and 4 are strands of the wild-type allele in germ-line DNA. B, sequencing gel shows a missense mutation, C → T, resulting amino acid change of Pro → Ser at codon 3300 (P3300S).](image)

![Fig. 3 Polymorphism and allelic loss at exon 11 (primer 11.7) of BRCA2. A, SSCP gel for 9 cases. B, sequencing demonstrates that bands 1 and 3 are strands of the wild-type allele (His, H), and bands 2 and 4 are strands of the polymorphic allele (Asn, N) at codon 372 (N372H). Genotype of case 459 is heterozygous and shows loss of the polymorphic allele in tumor. N, germ-line DNA; t, tumor DNA.](image)
do occur in ESCC patients but at low frequency. Moreover, the functional significance of these predominantly missense mutations remains to be determined. Evidence for classic biallelic inactivation was not seen. The putative target tumor suppressor gene corresponding to the high rate of chromosome 13q allelic loss remains unknown.

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