Original Article

Relationship between esophageal cancer-related gene 2 polymorphism and esophageal squamous cell carcinomas in Kazakhs and Hans of Xinjiang

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Abstract: In recent years, many studies have focused on the novel esophageal cancer-related gene 2 (ECRG2), which might be important in esophageal cancer development. The aim of this study is to investigate the relationship between ECRG2 short tandem repeat (STR) polymorphism and susceptibility to esophageal squamous cell carcinomas (ESCCs) in Kazakhs and Hans in Xinjiang. ECRG2 genotypes were detected by PCR-SSCP in 178 cases of esophageal carcinomas and 153 blood samples from the Kazakh and Han population. In Kazakhs and Hans, the frequencies of ECRG2 STR genotypes TCA3/TCA3, TCA4/TCA4, and TCA3/TCA4 were 47.8%/8.7%, 43.5%/67.9%, and 7.1%/25.0% in esophageal carcinomas with metastasis, respectively; and 14.1%/38%, 47.9%/14.3%, and 44.6%/41.1% in carcinomas without metastasis, respectively. A significant difference was observed between the groups with metastasis and without metastasis (Kazakh: χ² = 13.77, P<0.01; Han: χ² = 26.183, P<0.01). Compared with patients who carried the TCA4/TCA4 genotype, those who carried the TCA3/TCA3 genotype were at an increased risk of ESCC, with the adjusted odds ratios being 4.06 (95% confidence interval (CI), 1.69-9.74) in Kazakhs and 3.25 (95% CI, 1.25-8.45) in Hans. Our findings suggested that subjects who carried the TCA3/TCA3 genotype are at an increased risk of ESCC and metastasis compared with those who carried the TCA4/TCA4 genotype.

Keywords: Esophageal cancer, ECRG2, genetic polymorphism, STR, Kazakh population, Han

Introduction

Esophageal cancer is one of the most common malignant tumors in the world [1, 2]. It ranks eighth among the most common incident tumors and fifth in cancer-related death [3]. Epidemiological studies have found that the incidence of esophageal cancer in different areas varies [4], and its geographic distribution has obvious characteristics [5]. Esophageal cancer is a multifactor, multi-gene mutation accumulation and interaction complex [6, 7]. Epidemiological studies have revealed that the incidence of esophageal squamous cell carcinoma (ESCC) is related to many factors, such as N-nitrosamines [8], which have been shown to be involved in the etiology of ESCC in Linxian [9]. In addition, previous studies have shown many genetic factors, including amplification of C-myc [10], Int-2 [11] and Hst [12], mutation and/or deletion of p53 and Rb; and allelic deletion, in human ESCC and esophageal cancer cells [13, 14]. However, the factors that promote the development of esophageal cancer still need to be determined. Therefore, genetic factors may be important in ESCC incidence.

In recent years, many studies of esophageal cancer have focused on the novel esophageal cancer related genes (ECRG) 1-4, which might be important in its development [15-18]. ECRG2, which is located in chromosome 5q33.1, is closely related to esophageal cancer and has three short tandem repeat (STR) genotypes, TCA3/TCA3, TCA3/TCA4, and TCA4/TCA4 [19]. Bioinformatics analysis has shown that 97% of the amino acid sequences of ECRG2 are similar to a tumor-related KAZAL-type serine protease inhibitor, which may be important in the protection of esophageal mucosal cells [20]. These findings indicate that the ECRG2 STR is a genetic susceptibility factor for ESCC. Moreover, it
has significance for studying the genetic background and early screening of ESCC.

So far, the relationship between ECRG2 STR polymorphism and esophageal cancer risk in the Kazakh and Han populations of Xinjiang has not been studied. Furthermore, the distribution of ECRG2 STR polymorphisms between Kazakh and Han ESCC patients still needed to be investigated to determine significant differences. Therefore, in the present study, we used PCR-SSCP to examine the ECRG2 and 3 STR genotype polymorphisms and their distribution characteristics in 100 Kazakh and 87 Han esophageal cancer tissues and 103 Kazakh and 57 Han blood samples. We explored the relationships between ECRG2 STR polymorphism and esophageal cancer metastasis. Our findings suggested that genetic variants in ECRG2 may serve as candidate markers for Kazakh and Han ESCC susceptibility.

Material and methods

Study subjects

In Kazakhs, ECRG2 genotypes were detected by PCR-SSCP in 94 esophageal carcinomas and 100 blood samples for control. All patients received surgical treatment at the Department of Pathology of Yili Friendship Hospital and the First Affiliated Hospital of School of Medicine, Shihezi University. All cases were referred by two senior pathologists, with 52 being male and 42 being female. A total of 17 cases were well differentiated squamous cell carcinomas, 68 were moderately differentiated, and 9 were poorly differentiated. Lymph node metastases were found in 23 cases. Moreover, we collected from a same aged group of Kazakh people without a tumor history through physical, blood samples from 100 cases, to be used as controls. In addition, 84 Han patients diagnosed with histologically confirmed ESCC and 53 normal cases were also randomly recruited for this study by multistage cluster sampling.

DNA extraction and identification of ECRG2 STR

Using standard procedures, DNA samples were extracted from blood or tissues of subjects. PCR-based SSCP analysis was used to scan the fourth exon of ECRG2 for selecting variations. Based on the exon 4 flanking DNA sequences, PCR primers were designed to amplify a 235 bp fragment, and they were 5’-CT-TGTG CTA ATG AAT CTT GTG AAC TGT G-3’ (forward) and 5’-AAA CTT TCT CCA TTC AGT CAA GAT TAC-3’ (reverse). PCR was performed in a GeneAmp 2400 Thermal Cycler (Perkin-Elmer, Norwalk, CT) with a 25 µL reaction mixture containing about 100 ng DNA, 200 pmol primer, 200 pmol dNTP, 1.5 mM Mg²⁺, 2 U Platinum Pfx DNA Polymerase, and 1× reaction buffer (Promega, Madison, WI). Thermal cycles were 95°C for 2 min, 35 cycles of 95°C for 30 s, 52°C for 30 s, and 72°C for 1 min, followed by extension at 72°C for 7 min.

The PCR products were introduced into the mobile phase at an injection volume of 5 µL using the autosampler on a WAVE DNA Fragment Analysis System (Transgenomic, Omaha, NE) using a method described by Gross et al. [21]. Each PCR product was not denatured and kept 50°C. The variations determined by DH-PLC were further confirmed by DNA sequencing. PCR product of each variation was cloned in T-vector (Promega) and sequenced on an ABI 377 automated sequencer (Applied Biosystems, Foster City, CA) by using M13 primers.

The polymorphisms of the ECRG2 STR among cases and controls were detected by PCR-based SSCP analysis, as described above.

Statistical analysis

Statistical evaluation was performed using χ² tests. Statistical significance was considered P<0.05. The association between the ECRG2 TCA polymorphism and the risk of esophageal cancer were measured using odds ratios (ORs) and 95% confidence intervals (CIs), which were calculated using unconditional logistic regression. The ORs were adjusted for age and gender. Compared with the TCA4/TCA4 genotype, the ORs for TCA3/TCA4 and TCA3/TCA3 were computed. All analyses were done using the Statistical Analysis System.

Results

Distribution of age and gender among cases and controls in Kazakhs and Hans

A total of 278 subjects, including 94 Kazakh patients with ESCC, 100 normal controls, 84
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Table 1. General demographic characteristics of cases and control

<table>
<thead>
<tr>
<th>Gender</th>
<th>Kazakh Population</th>
<th>Han Population</th>
<th>P*</th>
<th>Kazakh Population</th>
<th>Han Population</th>
<th>P*</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>Case (n=94)</td>
<td>Controls (n=100)</td>
<td>0.605</td>
<td>Case (n=84)</td>
<td>Controls (n=53)</td>
<td>0.721</td>
</tr>
<tr>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Male</td>
<td>52 (55.3)</td>
<td>59 (59.0)</td>
<td>0.605</td>
<td>47 (56.0)</td>
<td>28 (37.7)</td>
<td>0.721</td>
</tr>
<tr>
<td>Female</td>
<td>42 (44.7)</td>
<td>41.1</td>
<td></td>
<td>37 (44.0)</td>
<td>25 (62.3)</td>
<td></td>
</tr>
<tr>
<td>Mean age ± SD (years)</td>
<td>55±9.63</td>
<td>50±12.78</td>
<td>0.011</td>
<td>61±7.53</td>
<td>47±10.90</td>
<td>&lt;0.01</td>
</tr>
<tr>
<td>&lt;50</td>
<td>23</td>
<td>47</td>
<td></td>
<td>4</td>
<td>30</td>
<td>&lt;0.01</td>
</tr>
<tr>
<td>50-60</td>
<td>42</td>
<td>32</td>
<td></td>
<td>30</td>
<td>16</td>
<td></td>
</tr>
<tr>
<td>60-70</td>
<td>22</td>
<td>14</td>
<td></td>
<td>41</td>
<td>6</td>
<td></td>
</tr>
<tr>
<td>&gt;70</td>
<td>7</td>
<td>7</td>
<td></td>
<td>9</td>
<td>1</td>
<td></td>
</tr>
</tbody>
</table>

*P for χ² test for comparison with controls.

Han patients with ESCC, and 53 normal controls, were analyzed for STR in ECRG2 (Table 1). The demographic variables of the subjects are shown in Table 1. The distributions of gender among subjects were not statistically different (P>0.05), suggesting that frequency matching was adequate. However, the age distributions of cases and controls were different (P<0.05) (Table 1). The average age of incidence of esophageal cancer in Kazakh patients is five years younger than Han patients (Table 1).

PCR amplification and genotyping

In our study, the ECRG2 gene PCR products of agarose gel electrophoresis were 235 bp DNA fragments (Figure 1). Three PCR products were obtained by acrylamide gel electrophoresis. The two front bands were 235 bp fragments and represent the genotype of the homozygous TCA3/TCA3. The bands toward the rear were 238 bp fragments that represented the homozygous genotype TCA4/TCA4. The TCA3/TCA4 is a heterozygous genotype that has two kinds of bands (Figure 2). This finding proved that ECRG2 has three STR genotypes, TCA3/TCA3, TCA3/TCA4, and TCA4/TCA4.

Genotype frequency distribution of ECRG2 TCA STR among ESCC cases and controls

We found three STR genotypes that were identified in exon 4, namely, TCA3/TCA3, TCA3/TCA4, and TCA4/TCA4. To investigate the relationship between the ECRG2 TCA STR genotype and ESCC incidence, we compared the distribution and allele frequency of the ECRG2 TCA STR genotype between Kazakhs, Hans, and a the combined groups (Table 2). Among Kazakhs with ESCC, the distribution of ECRG2 STR genotypes (TCA4/TCA4, 56.0%; TCA3/TCA4, 34.0%; and TCA3/TCA3, 10.0%) and controls did not deviate from the Hardy-Weinberg equilibrium (P<0.01). The frequencies of the three genotypes (30.9%, 46.8%, and 22.3%) among ESCC patients significantly differed from controls (P<0.01), with the TCA3/TCA3 and TCA3/TCA4 genotypes being more prevalent. Compared with the TCA3/TCA4 genotype, subjects that were homozygous for the TCA3/TCA3 genotype...
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were at an increased risk of developing ESCC: Kazaks (adjusted OR, 4.06; 95% CI, 1.69-9.74), Hans (adjusted OR, 3.25; 95% CI, 1.25-8.45), and total samples (adjusted OR, 3.91; 95% CI, 2.06-7.40). By contrast, subjects that were heterozygous for the TCA4/TCA4 genotype did not have a significant association with risk of cancer.

**ECRG2 gene sequence diagram**

DNA samples were extracted from blood and tumor tissue of ESCC patients and exhibited single bands after PCR amplification. SSCP analysis and DNA sequencing revealed STR in the noncoding region of the exon 4 of ECRG2, and the genotypes of ECRG2 STR were readily discerned. Three size variations were observed: TCA3/TCA3, TCA3/TCA4, and TCA4/TCA4 (Figure 3). The genotypes of the ECRG2 STR in DNA samples from blood and tumor were the same for the same individuals.

**Relationship between genotype distribution and metastasis of esophageal cancer**

The frequencies of the three genotypes (TCA3/TCA3, 58.8%; TCA4/TCA4, 7.8%; and TCA3/TCA4, 33.3%) among ESCC patients with metastasis significantly differed from controls (P<0.01). The result suggested that subjects carrying the TCA3/TCA3 genotype are at an increased risk of metastasis compared with those carrying the TCA4/TCA4 genotype (Table 3).

**Relationship between genotype frequency of ECRG2 TCA STR and differentiation of esophageal cancer**

No statistical difference was observed between the frequencies of the three genotypes and differentiation in Kazaks with esophageal cancer (χ²=7.497, P=0.112), Hans with esophageal cancer (χ²=3.296, P=0.510), and total samples (χ²=1.739, P=0.784) (Table 4).

**Discussion**

ECRG2 is expressed in many tissues, such as fetal liver, lung, brain, heart, stomach, spleen, colon, and kidney [22-26]. The study of Kai showed that ECRG2 STR polymorphism TCA3/TCA3 in exon 4 is the most prevalent polymorphism found in pancreatic adenocarcinoma and chronic pancreatitis [27]. However, its biologic significance has not been fully understood in Kazaks in Xinjiang. Therefore, we studied the relationship between ECRG2 polymorphism and ESCC in Kazaks and Hans in Xinjiang.

Research by Helena showed that the male-to-female incidence rate ratios in esophageal cancer vary considerably according to histology, age, and race. The highest M: Fratios were seen in 50-59 year old patients [28] However, our research data showed that the distributions of gender between ESCC and control subjects had no statistical difference. Nonetheless, the average age of incidence of esophageal cancer in Kazaks is five years earlier than in Han patients; therefore, the hypothesis that environmental and genetic factors affect the pathogenesis of esophageal cancer of Kazaks and Hans in Xinjiang is plausible.

According to Yue’s study, exon 4 of ECRG2 has STRs, whose genotypes are TCA3/TCA3, TCA3/
Table 2. Genotype frequency of ECRG2 TCA STR among controls and cases with ESCC

<table>
<thead>
<tr>
<th>Genotype</th>
<th>Kazak*</th>
<th>Han**</th>
<th>Total***</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>Cases n (%)</td>
<td>Controls n (%)</td>
<td>OR (95%)</td>
</tr>
<tr>
<td>TCA4/TCA4</td>
<td>29 (30.9)</td>
<td>56 (56.0)</td>
<td>1.00</td>
</tr>
<tr>
<td>TCA3/TCA4</td>
<td>44 (46.8)</td>
<td>34 (34.0)</td>
<td>2.50 (1.33-4.71)</td>
</tr>
<tr>
<td>TCA3/TCA3</td>
<td>21 (22.3)</td>
<td>10 (10.0)</td>
<td>4.06 (1.69-9.74)</td>
</tr>
</tbody>
</table>

*χ²=13.589, P<0.01; **χ²=6.100, P=0.047; ***χ²=20.24, P<0.01.
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The data showed that the risk of carrying TCA3/TCA3 genotype in patients suffering from esophageal cancer was higher than for those with the TCA4/TCA4 genotype [26]. Our research explored the STRs in ECRG2 exon 4 in Han and Kazakh esophageal carcinoma tissues and normal blood samples through PCR-SSCP applications. The frequencies of the three genotypes among ESCC patients significantly differed from controls, with the TCA3/TCA3 and TCA3/TCA4 genotypes being more prevalent. Compared with the TCA3/TCA4 genotype, subjects that were homozygous for the TCA3/TCA3 genotype were at an increased risk of developing ESCC. Our results are consistent with Yue’s study and further confirmed that the ECRG2 polymorphism is related to ESCCs [26].

Yue’s study showed that the genotypes of the ECRG2 STR in DNA samples from blood, tumor, and normal tissues adjacent to the tumor were identical in the same individuals [26]. Moreover, we extracted DNA samples from blood and tumor tissue of ESCC patients and the genotypes of the ECRG2 STR in the DNA samples were the same for the same individuals. Our result further confirmed that ECRG2 polymorphism is related to ESCC. Huang et al. suggested that ECRG2 inhibits the aggressiveness of cancer cells, possibly through the down-regulation of uPA/plasmin activity [29]. Our study showed that subjects carrying the TCA3/TCA3 genotype are at an increased risk of metastasis compared to those carrying the TCA4/TCA4 genotype. This finding indicated that TCA3/TCA3 is a risk factor for metastasis. We hypothesized that aside from environmental factors, genetic factors may influence the activity of the ECRG2 gene.

The research of Song showed that ECRG2 is a significant inhibitor of cancer growth, as shown in in vivo experiments using intratumoral Ad-ECRG2 administration [30]. No evident toxicity was observed in an animal model during the study. This study concluded that ECRG2 is a potential molecular target in cancer treatment. However, further study needs to investigate ECRG2 gene expression in a larger number of esophageal tissue samples and test the inhibitory effect of the ECRG2 gene on migration and invasion in vitro and in vivo.

In conclusion, our study demonstrated for the first time, a significant association between

Figure 3. ECRG2 gene PCR products by acrylamide gel electrophoresis.
ECRG2 polymorphisms and esophageal cancer

Table 3. Relationship between the genotype distribution and metastasis of EC

<table>
<thead>
<tr>
<th>Genotype</th>
<th>Hazak*</th>
<th>Han**</th>
<th>Total***</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>Metastatic</td>
<td>Non-metastatic</td>
<td>Metastatic</td>
</tr>
<tr>
<td>TCA3/TCA3</td>
<td>N (%)</td>
<td>N (%)</td>
<td>N (%)</td>
</tr>
<tr>
<td>11 (47.8)</td>
<td>10 (14.1)</td>
<td>19 (67.9)</td>
<td>8 (14.3)</td>
</tr>
<tr>
<td>TCA4/TCA4</td>
<td>2 (8.7)</td>
<td>27 (38.0)</td>
<td>2 (7.1)</td>
</tr>
<tr>
<td>TCA3/TCA4</td>
<td>10 (43.5)</td>
<td>34 (47.9)</td>
<td>7 (25.0)</td>
</tr>
<tr>
<td>Total</td>
<td>23 (100)</td>
<td>71 (100)</td>
<td>28 (100)</td>
</tr>
</tbody>
</table>

*χ²=13.589, P<0.01; **χ²=26.283, P<0.01; ***χ²=40.74, P<0.01.

Table 4. Relationship between the genotype frequency of ECRG2 TCA STR and the differentiation of esophageal cancer (EC)

<table>
<thead>
<tr>
<th>Genotype</th>
<th>TCA3/TCA3</th>
<th>TCA4/TCA4</th>
<th>TCA3/TCA4</th>
</tr>
</thead>
<tbody>
<tr>
<td>Kazakh EC*</td>
<td>Well differentiated</td>
<td>4</td>
<td>1</td>
</tr>
<tr>
<td></td>
<td>Intermediate differentiation</td>
<td>16</td>
<td>24</td>
</tr>
<tr>
<td></td>
<td>Poorly differentiated</td>
<td>1</td>
<td>4</td>
</tr>
<tr>
<td>Han EC**</td>
<td>Well differentiated</td>
<td>6</td>
<td>9</td>
</tr>
<tr>
<td></td>
<td>Intermediate differentiation</td>
<td>13</td>
<td>12</td>
</tr>
<tr>
<td></td>
<td>Poorly differentiated</td>
<td>8</td>
<td>6</td>
</tr>
<tr>
<td>Total*** l</td>
<td>Well differentiated</td>
<td>10</td>
<td>10</td>
</tr>
<tr>
<td></td>
<td>Intermediate differentiation</td>
<td>29</td>
<td>36</td>
</tr>
<tr>
<td></td>
<td>Poorly differentiated</td>
<td>9</td>
<td>10</td>
</tr>
</tbody>
</table>

*χ²=7.497, P=0.112; **χ²=3.296, P=0.510; ***χ²=1.739, P=0.784.

STR genetic polymorphisms and ESCC in Kazakh and Han populations in Xinjiang. Our findings raise the possibility that the influence of the ECRG2 gene polymorphism on the risk of esophageal cancer may be more pronounced in high-risk populations. Further studies from other regions would be helpful to confirm the role of ECRG2 as a high-risk allele in esophageal cancer.

Acknowledgements

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Disclosure of conflict of interest

None.

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References


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