RESEARCH ARTICLE

Equivocal Association of RAD51 Polymorphisms with Risk of Esophageal Squamous Cell Carcinoma in a Chinese Population

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Abstract

Aim: To study the contribution of genetic variation in RAD51 to risk of esophageal squamous cell carcinoma (ESCC). Methods: Three single nucleotide polymorphisms (SNPs) in RAD51 (rs1801320, rs4144242 and rs4417527) were genotyped in 316 ESCC patients and 316 healthy controls in Anyang area of China using PCR-RFLP (polymerase chain reaction-restriction fragment length polymorphism). Demographic variables between cases and controls were statistically compared by T test and Chi-square test. Hardy-Weinberg equilibrium was evaluated by the Chi-square test. Odds ratios (ORs) and 95% confidence intervals (CIs) were calculated to measure any association with ESCC. Haplotype frequencies were estimated by Phase 2.1. Result: The genotype frequencies of rs1801320, rs4144242 and rs4417527 in patients with ESCC demonstrated no significant differences from those in control group (P>0.05). When the haplotypes of these three SNPs were constructed and their relationships with ESCC risk investigated, however, CGG was observed to increase the risk (P=0.020, OR=2.289). Conclusions: There was no association between the three SNPs of RAD51 and ESCC susceptibility in our Chinese population. However, the CGG haplotype might be a risk factor.

Keywords: RAD51 - esophageal squamous cell carcinoma - genetic polymorphisms

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Introduction

Esophageal cancer is one of the most common cancers over the world, which causes about 400,000 deaths every year (http://globocan.iarc.fr/). In China, more than 90 percent of esophageal cancer is histologically squamous cell carcinoma (ESCC). Patients with ESCC have a very poor prognosis. ESCC has a marked geographic variation in terms of incidence and mortality. For example, ESCC is prevalent among Asian population, especially in China, which has a 20-fold difference in incidence from western Africa (Parkin et al., 2002) . Even though factors such as smoking, drinking, nutritional deficiency and nitrosamines have been suggested to be responsible for pathogenesis of ESCC (Kollarova et al., 2007), they only affect a small population. The familial aggregation of ESCC has also been reported, which suggests that genetic predisposition may explain the high rate of ESCC (Roberts-Thomson et al., 2005).

RAD51 has been demonstrated to participate in homologous recombination (HR) repair of double-stranded DNA breaks (DSBs) which may cause genomic instability and cancer (Pâques et al., 1999; Shrivastav et al., 2008). It is located on the human chromosome 15q15.1, which contains 10 exons and encodes a protein with 339 amino acids (Lu et al., 2007; Nogueira et al., 2012). RAD51 is highly polymorphic: It has more than 100 SNPs, some of which have been suggested to contribute to cancers the risk of variable (Poplawski et al., 2006; Lu et al., 2007; Nogueira et al., 2012; Gresner et al., 2012). Several studies showed that rs1801320 (the G135C polymorphism) was associated with susceptibility to human cancers, such as breast (Romanowicz- Makowska et al., 2011) , colorectal (Krupa et al., 2011) and head and neck cancer (Sliwinski et al., 2010). There remains no evidence, however, to indicate whether it is associated with ESCC. In our study, rs1801320 as well as two other SNPs, rs4144242 and rs4417527, were detected in order to clarify the role of genetic variants of RAD51 in susceptibility of ESCC.

Materials and Methods

Study subjects

316 patients with ESCC (186 male and 130 female) were recruited from Anyang Tumor Hospital, who were diagnosed by surgical ablation and subsequent pathological examination. The control group consisted of 316 healthy individuals without history of malignant

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Table 1. Primer Sequences of Three Polymorphisms of RAD51 and Their Restriction Enzymes

Position	Primer sequence 5'-3'	PCR products(bp)	Restriction enzyme	Digested products(bp)
Rs1801320	F 5'-CTGGGAACTGCAACTCATC-3' R 5'-CCTCACACACTCACCTCG-3'	183	BstNI	111+72
Rs4144242	F 5'-AGCATGGGCTAATTCATTTTG-3' R 5'-GGGCTAATTCATTTTGTAACCT-3'	195	BgIII	129+66
Rs4417527	F 5'-GCTGGAAATCTTGTTAAACGG-3' R 5'-TTAGCCAGGATGTTCTCGAT-3'	167	NlaIII	103+64

F, forward primer; R, reverse primer

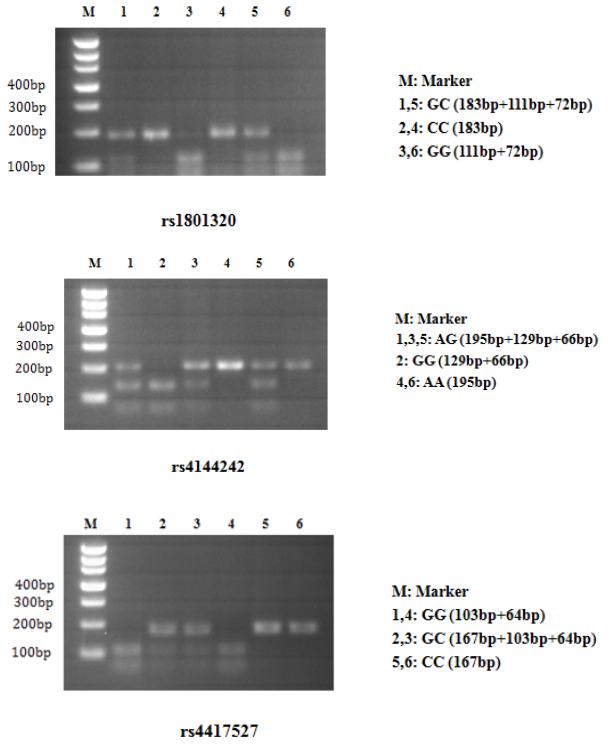

Figure 1. Electrophoresis Patterns of PCR-RFLP for the Three SNPs

tumors or autoimmune disorders, with their gender and age matched with the patient group. The pathological and clinical information of patients was collected from their medical files, and that of the control group collected from investigators. All the individuals participating in this study signed the informed consent and allowed to record their lifestyle characteristics and other personal information. Blood samples (5 ml) were collected.

Genotype detection

Genomic DNA extracted from peripheral blood leukocytes of each subject by proteinase K digestion, phenol-chloroform extraction and ethanol precipitation. Then PCR-RFLP method was used to detect such three SNPs of RAD51. Primers were designed by Oligo.v.7.37 software, as showed in Table 1. The 25ul PCR reaction system contained 100-150ng genomic DNA, 0.5μl of each oligonucleotide primer (10 μM), 1.0μl dNTP (2.5 mM), 2.5 μl 10×PCR buffer (200mM Tris-HCl, 200 mM KCl, 100 mM $(NH_4)_2SO_4$, 20 mM $MgSO_4$) and 1.0 U Easy Taq DNA polymerase. The PCR mixture was incubated as initial denaturation for 5 min at 94.0°C, followed by 35 cycles for 30 s at 94.0°C as denaturation, 30s annealing at each annealing temperature (rs1801320 and rs4417527 at

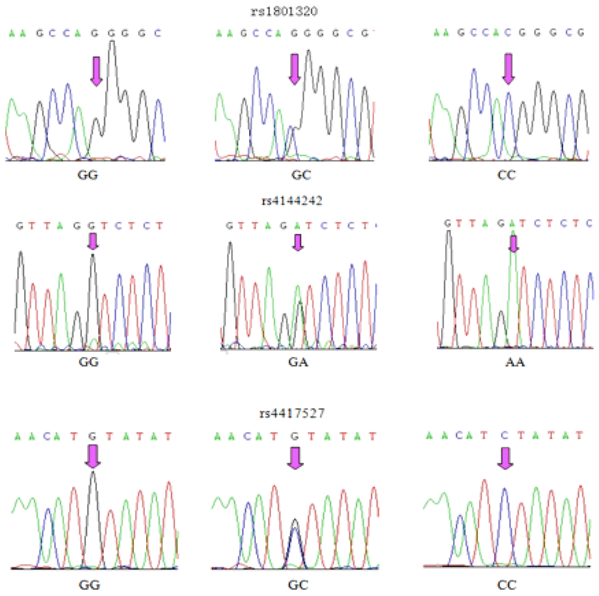

Figure 2. Sequencing Maps of PCR Products for the Three SNPs

60°C while rs4144242 at 56°C) as well as 30s extension at 72.0°C.The final extension was carried out for 7min at 72°C.

The PCR product was digested by restriction enzymes at 37°C for overnight and subsequently analyzed on 2% agarose gel stained with ethidium bromide and photographed under UV light. The used restriction enzymes and their digested product length were illustrated in Table 1. The products of the three SNPs offer enzyme digestion were showed in Figure 1. To make sure the 3 polymorphisms exist, several PCR products of each genotype were selected and sequenced as showed in Figure 2.

Statistical analysis

Statistical analyses were performed by SPSS software (version 16.0 for Windows, Chicago, USA). Chi-square test was used to assess the consistency of the genotype distributions of the three polymorphisms with Hardy-Weinberg equilibrium. Between cases and controls, the distribution of age was compared by t test, as well as smoking, alcohol drinking, history of esophageal cancer and genotype frequencies by chi-square test. PHASE 2.1 software was used to construct haplotypes for such three SNPs and estimate haplotype frequencies. Association with ESCC was measured by odds ratio (OR) and 95% confidence interval (95%CI) adjusted by drinking and history of esophageal cancer, which were calculated by conditional multivariate logistic regression. Differences

Table 2. Characteristics of Cases and Controls

Variables	Cases n (%)	Controls n (%)	*P* value
Mean age(M±SD)	59.60±8.34	59.42±7.98	0.785*
Gender			
Male	186(58.86)	186(58.86)	-
Female	130(41.14)	130(41.14)	
Smoking			
No	170(53.80)	204(64.56)	0.006**
Yes	146(46.20)	112(35.44)	
Drinking			
No	226(71.52)	258(81.65)	0.003**
Yes	90(28.48)	58(18.35)	
EC history			
No	214(67.72)	262(82.91)	0.000**
Yes	102(32.28)	54(17.09)	

*T-test; **Chi-square test

Table 3. Association Between RAD51 Gene Polymorphisms and Risk of ESCC

Variables	Cases n(%)	Controls n(%)	*P* value	OR(95% CI)*
RS1801320				
GG	206	216		
GC	100	92	0.447	1.143(0.810-1.613)
CC	10	8	0.575	1.307(0.512-3.337)
Rs4144242				
GG	246	226		
AG	64	84	0.071	0.718(0.501-1.029)
AA	6	6	0.924	-
Rs4417527				
GG	146	122		
GC	146	164	0.086	0.752(0.543-1.041)
CC	24	30	0.19	0.666(0.363-1.223)

*Chi-square test

Table 4. Distribution of the Estimated Haplotype Frequencies for RAD51 Gene in Cases and Controls

Haplotypes	Rs1801320	Rs4144242	Rs4417527	Cases (%)*	Controls (%)*
a	G	G	G	62.4	61.48
b	G	G	C	17.59	18.42
c	C	A	C	10.62	12.15
d	C	G	G	6.17	2.62
e	C	G	C	1.82	2.29
f	G	A	C	0.66	2.27
g	C	A	G	0.38	0.03
h	G	A	G	0.36	0.74

*Calculated by PHASE 2.1 software

were considered to be statistically significant only when the two-sided *p*-value was less than 0.05.

Results

Distribution of general characteristics

A total of 632 subjects (316 cases and 316 controls) participated in this study. The baseline characteristics were summarized in Table 2. All of the genotype distributions of each polymorphisms followed the Hardy–Weinberg equilibrium equation ($P>0.05$). No significant differences were observed in gender and age between cases and controls evaluated by t-test. Smoking and alcohol drinking individuals in patient group were more than control group, and we found the differences between the two groups in terms of smoking and drinking were significant ($P=0.006$, $P=0.003$, respectively). EC history was also significantly different between the two groups, which had a *P* value similar to zero. However, when taking into multiple-factor regression, only the differences of alcohol drinking status and ESCC history between the two groups were statistically significant ($P=0.001$, P≈0, respectively) .

Table 5. Association Between RAD51 Haplotypes and ESCC

Variables	Cases n (%) n=316	Controls n (%) n=316	*P* value*	OR (95% CI)*
a=GGG				
-/-	41	45		
-/a	155	153	0.664	1.112(0.689-1.794)
a/a	120	118	0.662	1.116(0.681-1.828)
b=GGC				
-/-	209	211		
-/b	103	93	0.519	1.118(0.796-1.570)
b/b	4	12	0.052	-
c=CAC				
-/-	253	238		
-/c	59	78	0.08	0.712(0.486-1.042)
c/c	4	0	0.053	-
d=CGG				
-/-	280	301		
-/d	34	15	0.02	2.289(0.954-5.490)
d/d	2	2	0.943	-
e=CGC				
-/-	304	302		
-/e	12	14	0.689	1.087(0.712-1.659)
e/e	0	0	-	-
f=GAC				
-/-	312	304		
-/f	4	10	0.102	-
f/f	0	2	0.153	-
g=CAG				
-/-	314	316		
-/g	2	0	0.157	-
g/g	0	0	-	-
h=GAG				
-/-	314	311		
-/h	2	5	0.254	-
h/h	0	0	-	-

*Calculated by unconditional multivariate logistic regression, adjusted by drinking status

Association of Genotype of SNP polymorphisms and risk of ESCC

The genotype distribution of rs1801320, rs4144242 and rs4417527 of RAD51 were shown in Table 3. However, the analysis of the population revealed no substantial difference between cases and controls in such three SNPs.

Association analysis of haplotypes with the risk of ESCC

In the analysis of association of genotypes with the risk of ESCC, no considerable difference was found in genotype distribution in rs1801320, rs4144242 and rs4417527. Furthermore, 8 haplotypes were found by using PHASE 2.1software. The distribution of different haplotype frequencies in cases and controls was shown in Table 4. The association of every haplotype and risk of

ESCC was analyed by using Chi-square test and the date were displayed in Table 5. Taken together the haplotype GGG was observed to have the most frequency, and a significant difference in haplotype -/CGG between cases and controls, which increased the risk of ESCC (OR=2.289, 95%CI=0.954-5.490, *P* =0.020).

Discussion

Esophageal squamous cell carcinoma (ESCC), one of the most lethally malignant diseases, is quite common in East Asian countries. In China, it leads to the fifth morbidity of cancers, and ranks the fourth in cancer-related mortality (http://globocan.iarc.fr/). Despite of recent advances of early diagnosis and treatment, the clinical outcome and overall survival remains poor. More studies are still needed to further understand the molecular mechanisms of the development of ESCC. Anyang, located in Henan Province of China, is one of the cities with the highest incidence and mortality of ESCC in China (Cheng et al., 2011). Every patient was matched with a control individual in terms of the gender and age (*P*=0.785). By chi-square test, alcohol drinking, smoking and ESCC history were all found to be significantly different between the patients and controls. When taking into multiple-factor regression, the differences of alcohol drinking status and ESCC history between the two groups were statistically significant (*P*=0.001, OR=1.879; P≈0, OR=2.305, respectively), which indicated that the two factors may increase the risk of ESCC by about two-fold, respectively.

Carcinogenesis is a complexed process in which environment and genetic factors are involved. Up to date, many genes have been proved to be associated with high risk of cancer, such as P53, XRCC and PTEN. The RAD51 recombinase is an essential factor for homologous recombination and repair of DNA double strand breaks (DSBs). DSBs are the most serious factor leading to DNA lesions which can be resulted from collapsed replication forks, or through programmed events during meiotic recombination and V (D)J recombination. DSBs also occur with respect to the action of exogenous agents such as ionizing radiation and chemical agents (Khanna et al., 2001; O'Driscoll et al., 2006). Eukaryotic cells repair DSBs primarily by two mechanisms: non-homologous end-joining (NHEJ) and homologous recombination (HR), of which HR plays a major role (Shrivastav et al., 2008). The RAD51 protein is primarily involved in HR and crucial for the stability of genome and normal cell cycle. The RAD51 knock out in cells of chicken and mouse is lethal (Tsuzuki et al., 1996; Lim et al., 1996; Sonoda et al., 1998). It was well believed to interact with XRCC2, XRCC3, BRCA1, BRCA2 and other proteins to form a complex which is essential for the repair of DSBs (Thacker 2005). HR initiates with the hydrolyzation of the broken end by 5'-3' exonuclease, resulting in a long 3' ssDNA tail. The tail is bound by Replication Protein A (RPA), which is subsequently replaced by RAD51 with the help of other proteins such as RAD52, BRCA2 and XRCC3 (Schild et al., 2010; Pasaje et al., 2011). The RAD51 protein binds to DNA at the site of a break and envelopes it in a protein sheath, which is an essential step in the initiation of the repair process (http://ghr.nlm.nih.gov/gene=RAD51). The resulting Rad51 nucleoprotein filament searches for and invades a homologous sequence, a process facilitated by Rad54. The Srs2 helicase dissociates Rad51 from the ssDNA, allowing the extension of normal basepairing of the invading and complementary donor strands and the following strand by DNA polymerase. The extended strand dissociates and anneals with the processed end of the non-invading strand on the opposite side of the DSB in a process called synthesis-dependent strand annealing (SDSA), or both ends may invade to produce a double-Holliday junction which yields crossover or non-crossover recombinants. Once intermediates are resolved, the remaining ssDNA gaps and nicks are repaired by DNA polymerase and DNA ligase (West, 2003; Shrivastav et al., 2008). In this regard, the RAD51 gene may be associated with risk of carcinogenesis. The mutation of this gene may reverse the risk of cancers. Many documents have reported the association of RAD51 genetic factors with human cancers (Poplawski et al., 2006; Lu et al., 2007; Gresner et al., 2012; Nogueira et al., 2012).

The rs1801320 polymorphism was reported to be associated with many human cancers such as breast cancer and colorectal cancer (Krupa et al., 2011; Romanowicz-Makowska et al., 2011). However, it has never been mentioned to be related to esophageal cancer. In our study, three SNPs, rs1801320, rs4144242 and 4417527, were detected to assess their association with the risk of ESCC. Beyond our expectations, we found that the genotype frequency of three polymorphisms of the RAD51 gene were not associated with esophageal cancer susceptibility in Anyang area.

It has been suggested that the haplotype analysis might be more useful than that of the single SNP for identifying cancer risk (Humar et al., 2002).We hence studied the haplotypes of the three SNPs and their relationship with ESCC. On the basis of statistical analysis, we found that individuals carrying the haplotype CGG had a 2.3-fold increase with respect to the risk of ESCC (*P*=0.020, OR=2.289). This suggested that there might be interactions between three SNPs, and CGG haplotype is a dangerous factor for ESCC.

In conclusion, to our knowledge, this is the first report to investigate the relationship between RAD51 gene and ESCC in China. We found that there was no association between the three SNPs of RAD51 and ESCC susceptibility, but the haplotype CGG might be a dangerous factor for ESCC. To be well understood the potential influence of the genetic variations, further studies with enlarged samples and of different regions should be done. The functional aspects of these polymorphisms in tumor tissue can lead to a better understanding of tumor biology and behavior, and provide theory gist for prevention and treatment of tumor.

References

Cheng XL, Ning T, Xu CQ, et al (2011). Haplotype analysis of CTLA4 gene and risk of esophageal squamous cell carcinoma in Anyang area of China. *Hepatogastroenterology*, **58**, 432-7.

Gresner P, Gromadzinska J, Polanska K, et al (2012). Genetic variability of Xrcc3 and Rad51 modulates the risk of head and neck cancer. *Gene*, **504**, 166-74.

Humar B, Graziano F, Cascinu S, et al (2002). Association of CDH1 haplotypes with susceptibility to sporadic diffuse gastric cancer. *Oncogene*, **21**, 8192-5.

Khanna KK, Jackson SP (2001). DNA double-strand breaks: signaling, repair and the cancer connection. *Nat Genet*, **27**, 247-54.

Kollarova H, Machova L, Horakova D, Janoutova G, Janout V (2007). Epidemiology of esophageal cancer--an overview article. *Biomed Pap Med Fac Univ Palacky Olomouc Czech Repub*, **151**, 17-20.

Krupa R, Sliwinski T, Wisniewska-Jarosinska M, et al (2011). Polymorphisms in RAD51, XRCC2 and XRCC3 gene of the homologous recombination repair in colorectalcancer—a case control study. *Mol Biol Rep*, **38**, 2849-54.

Lim DS, Hasty P (1996). A mutation in mouse rad51 results in an early embryonic lethal that is suppressed by a mutation in p53. *Mol Cell Biol,* **16**, 7133-43.

Lu J, Wang LE, Xiong P, et al (2007). 172G>T variant in the 5' untranslated region of DNA repair gene RAD51 reduces risk of squamous cell carcinoma of the head and neck and interacts with a P53 codon 72 variant. *Carcinogenesis*, **28**, 988-94.

Nogueira A, Catarino R, Faustino I, et al (2012). Role of the RAD51 G172T polymorphism in the clinical outcome of cervical cancer patients under concomitant chemoradiotherapy. *Gene*, **504**, 279-83.

O'Driscoll M, Jeggo PA (2006). The role of double-strand break repair - insights from human genetics. *Nat Rev Genet*, **7**, 45-54.

Pâques F, Haber JE (1999). Multiple pathways of recombination induced by double-strand breaks in Saccharomyces cerevisiae. *Microbiol Mol Biol Rev,* **63**, 349-404.

Parkin DM, Bray F, Ferlay J, Pisani P (2005). Global cancer statistics, 2002. *CA Cancer J Clin*, **55**, 74-108.

Pasaje CF, Kim JH, Park BL, et al (2011). Lack of association of RAD51 genetic variations with hepatitis B virus clearance and occurrence of hepatocellular carcinoma in a Korean population. *J Med Virol*, **83**, 1892-9.

Poplawski T, Arabski M, Kozirowska D, et al (2006). DNA damage and repair in gastric cancer--a correlation with the hOGG1 and RAD51 genes polymorphisms. *Mutat Res*, **601**, 83-91.

Roberts-Thomson IC, Butler WJ (2005). Polymorphism and squamous cell cancer of the esophagus. *J Gastroenterol Hepatol*, **20**, 486-7.

Romanowicz-Makowska H, Smolarz B, Zadrozny M, et al (2011). Single nucleotide polymorphisms in the homologous recombination repair genes and breast cancer risk in Polish women. *Tohoku J Exp Med*, **224**, 201-8.

Schild D, Wiese C (2010). Overexpression of RAD51 suppresses recombination defects: a possible mechanism to reverse genomic instability. *Nucleic Acids Res*, **38**, 1061-1070.

Shrivastav M, De Haro LP, Nickoloff JA (2008). Regulation of DNA double-strand break repair pathway choice. C*ell Res*, **18**, 134-47.

Shrivastav M, De Haro LP, Nickoloff JA (2008). Regulation of DNA double-strand break repair pathway choice. *Cell Res*, **18**, 134-47.

Shrivastav M, De Haro LP, Nickoloff JA (2008). Regulation of DNA double-strand break repair pathway choice. *Cell Res*, **18**, 134-47.

Sliwinski T, Walczak A, Przybylowska K, et al (2010). Polymorphisms of the XRCC3 C722T and the RAD51 G135C genes and the risk of head and neck cancer in a Polish population. *Exp Mol Pathol*, **89**, 358-66.

Sonoda E, Sasaki MS, Buerstedde JM, et al (1998). Rad51-deficient vertebrate cells accumulate chromosomal breaks prior to cell death. *EMBO J*, **17**, 598-608.

Thacker J (2005). The RAD51 gene family, genetic instability and cancer. *Cancer Lett,* **219**, 125-35.

Tsuzuki T, Fujii Y, Sakumi K, et al (1996). Targeted disruption of the Rad51 gene leads to lethality in embryonic mice. *Proc Natl Acad Sci USA*, **93**, 6236-40.

West SC (2003). Molecular views of recombination proteins and their control. *Nat Rev Mol Cell Biol*, **4**, 435-45.