

RESEARCH ARTICLE

Association of RAD 51 135 G/C, 172 G/T and XRCC3 Thr241Met Gene Polymorphisms with Increased Risk of Head and Neck Cancer

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Abstract

Homologous recombination repair (HRR) plays an important role in protection against carcinogenic factors. Genes regulating the HRR mechanisms may impair their functions and consequently result in increased cancer susceptibility. RAD 51 and XRCC3 are key regulators of the HRR pathway and genetic variability in these may contribute to the appearance and progression of various cancers including head and neck cancer (HNC). The aim of the present study was to compare the distribution of genotypes of RAD51 (135G/C, 172 G/T) and XRCC3 (Thr241Met) polymorphisms between HNC patients and controls. Each polymorphism was genotyped using the polymerase chain reaction-restriction fragment length polymerase (PCR-RFLP) technique in 200 pathologically confirmed HNC patients along with 150 blood samples from normal, disease free healthy individuals. We observed that homozygous variant CC genotype of RAD51 135G/C was associated with a 2.5 fold increased HNC risk (OR=2.5; 95% CI=0.69-9.53; p<0.02), while second polymorphism of RAD 51 172 G/T, heterozygous variant GT genotype was associated with a 1.68 fold (OR=1.68; 95% CI=1.08-2.61; p<0.02) elevation when compared with controls. In the case of the Thr241Met polymorphism of XRCC3, we observed a 16 fold (OR=16; 95% CI=3.78-69.67; p<0.0002) increased HNC risk in patients compared to controls. These results further suggested that RAD51 (135G/C, 172 G/T) and XRCC3 (Thr241Met) polymorphisms may be effective biomarkers for genetic susceptibility to HNC. Larger studies are needed to confirm our findings and identify the underlying mechanisms.

Keywords: HRR pathway - DSBs - RAD 51 polymorphism - XRCC3 polymorphism - HNC - smoking status

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Introduction

Head and neck squamous cell carcinoma (HNSCC) is the fifth most common cancer worldwide and is associated with low survival and high morbidity when diagnosed in advance stage (Siegel et al., 2011). HNSCC is a major cancer problem in Southern China, Pakistan, Thailand, India and Brazil. Overall prevalence of head and neck squamous cell carcinoma is 12.3 per 100,000 out of total cancers treated in Pakistan (Bray et al., 2012). The main causes of head and neck carcinogenesis are tobacco, alcohol consumption, ultraviolet radiation, reactive oxygen species and genetic susceptibility which include the genes regulating the cell cycle or those involved in DNA repair mechanisms (Sabir et al., 2013). Among these mechanisms, homologous recombinant repair (HRR) constitute key pathway to maintain genomic stability. Homologous recombinant repair supports DNA replication and aids replication restart after fork stalling or breakage (Yin et al., 2012).

The key molecules of HRR pathway are RAD51 and X-ray cross-complementing group 3 (XRCC3) (Areeshi 2013). RAD51 protein polymerizes onto single-stranded DNA (ssDNA) to form a helical nucleoprotein filament (Mimitou et al., 2009). RAD51 is known to play its role in all three stages of HRR pathway and catalyses the invasion of broken ends of the DSB into intact sister chromatid (Zhang et al., 2014). Mutations of RAD51 result in defects in the repair of double-stranded DNA breaks. Loss of RAD51 function would therefore be expected to result in an elevated mutation rate, thus leading to accumulation of DNA damage and, subsequently to increased cancer risk (Shin et al., 2008; Venkitaraman, 2009). The RAD51 SNPs (135 G/C and 172 G/T) present in the 5'UTR have been reported to be associated with altered gene transcription and may be involved in carcinogenesis (Cheng et al., 2014). XRCC3 is the second important member of HRR pathway and takes part in DSB repair as it causes slowing of DNA synthesis and recruit RAD51 at repair sites (Mao et al., 2014). The XRCC3 gene region contains mostly

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intronic polymorphisms, however a few polymorphisms that change amino acid residues have been described in the coding region, but their impact is largely unknown. Many earlier studies have investigated the XRCC3 722C/T (rs861539) polymorphism that leads to the substitution of threonine (Thr) with methionine (Met) at position 241 (Thr241Met) which is associated with increased risk of different cancers (Zhao et al., 2012; Fayaz et al., 2013).

Over the last decade, many studies have revealed association of 135G/C, 172 G/T and Thr241Met polymorphism with occurrence of various cancers. The vast majority of these results concerned breast cancer, especially in conjunction with BRCA2 mutation (Vral et al., 2011; Zhou et al., 2011). Increasing number of reports supporting significant role of inefficiency of HRR in tumorigenesis has led us to consider SNP variability of RAD51 and XRCC3 as potential risk factor. In this study the objective was to assess the possible affects of the RAD51 135G/C (rs1801320), 172 G/T (rs1801321) and XRCC3 Thr241Met (rs861539) gene polymorphisms on development and course of head and neck cancer.

Materials and Methods

Blood sample collection

This study involved pathologically verified head and neck cancer patients. Blood samples of head and neck cancer patients were collected from NORI (Nuclear Medicine, Oncology and Radiotherapy Institute), Islamabad. A total of 200 patients blood samples along with 150, age and sex matched, healthy and disease free individuals without prior history of any disease were used as controls. The observed mean age of patients and controls was 45 (± 16.35) and 43 (± 32.23) years respectively. Non-significant difference was observed in case of age ($p=0.75$) and gender ($p=0.61$) between cancer cases and controls. However, smoking status showed ($p<0.0001$) a statistically significant difference in cases when compared with controls. Blood samples of HNC patients and controls were collected in 5ml sterile EDTA containing blood vacutainers. Blood samples were obtained with informed consent of patients according to approved procedures by the ethical committee of respective hospital and department. All the samples were stored in the refrigerator at 4°C for further processing.

DNA typing

For germ-line mutation screening, DNA was isolated from the blood by phenol organic method as described by Mahjabeen et al., 2011 with minor alterations and stored at -20°C for further processing. PCR cycle conditions for RAD51 and XRCC3 were: one cycle at 94°C for 5min; 35 cycles of 94°C for 30sec, 55°C for 30sec and 72°C for 30sec and a final extension at 72°C for 10min. Pairs of PCR primer sequences and restriction enzymes for each DNA product are all listed in Table 1.

Table 1. RFLP Details for RAD51 and XRCC3 Polymorphisms.

Gene (polymorphisms)	PCR Product	Polymorphism effect on restriction site	Polymorphism patterns after digestion
RAD51 (135G/C)	157 bp	G > C, Abolish one site for MvaI enzyme	G/G: 86, 71 bp; G/C: 157, 86, 71 bp; C/C: 157 bp
RAD51 (172 G/T)	131bp	G > T, Abolish one site for NgoMIV enzyme	G/G: 110 and 21bp; G/T: 131, 110 and 21bp; C/C: 131 bp
XRCC3 (Thr241Met)	315 bp	C > T, Creates one NlaIII	C/C: 22, 293 bp; C/T: 22, 105, 188, 293 bp; T/T: 22, 105, 188 bp

157bp PCR product of RAD51 was digested overnight with 3units of restriction enzyme MvaI. The homozygous G/G genotype produced 86 and 71bp fragments, heterozygous G/C genotype three fragments: 157, 86 and 51bp and the homozygous C/C genotype produced one 157bp fragment. The 131bp PCR product of RAD51 was digested overnight with 3units of the restriction enzyme NgoMIV. Homozygous G/G genotype (lacking the restriction site for the enzyme) produces two fragment: 110 and 21bp, heterozygous G/T genotype (containing the restriction site for enzyme) three fragments: 131, 110 and 21bp and the homozygous T/T genotype produced 131bp fragments. In case of XRCC3 315bp PCR product was digested overnight with 3units of the restriction enzyme NlaIII. The homozygous C/C genotype produced 22 and 293bp fragments, heterozygous C/T genotype four fragments: 22, 105, 188 and 293bp and the homozygous T/T genotype produced three fragments: 22, 105 and 188bp. ≥ 10 of digested products were loaded onto a 4% agarose gel containing ethidium bromide for electrophoresis.

Statistical analysis

The chi-square test was used to compare the distribution of categorical variables such as the RAD51 and XRCC3 genotype, age, sex etc. Adjusted and stratified odds ratios (OR) and 95% confidence intervals (CI) were estimated by unconditional logistic regression. All the analysis was performed using statistical software GraphPad PRISM version 5.04 and SPSS.

Results

The purpose of the present study was to elucidate the role for HRR pathway gene such as RAD51 and X-ray cross-complementing group 3 (XRCC3) as a high-risk HNC genes using of PCR-RFLP method. Three polymorphisms RAD51 135G/C (rs1801320), 172 G/T (rs1801321) and XRCC3 Thr241Met (rs861539) gene were selected for this study (Figure 1). The genotypic distributions of RAD51 (135G/C, 172 G/T) and XRCC3 (Thr241Met) gene polymorphisms in the controls was in Hardy-Weinberg equilibrium. The genotypic frequency distribution of RAD51 (135G/C, 172 G/T) and XRCC3 (Thr241Met) between cancer cases and controls is given in Table 2. In case first polymorphism 135G/C of RAD51, our results signified that homozygous variant CC genotype had 2.5 folds increased HNC risk (OR=2.5; 95% CI=0.69-9.53; $p<0.02$) in patients compared to controls. Similarly variant allele C carrier (GC+CC) genotypes were found to be associated with 1.6 folds increased risk of HNC (OR=1.6, 95%CI=1.02-2.52; $p<0.03$) in patients compared to controls. For second selected polymorphism of RAD51 (172 G/T), heterozygous (GT) and homozygous variant (TT) allele genotypes were at 1.68 folds (OR=1.68; 95%CI=1.08-2.61; $p<0.02$) and 16 folds (OR=16; 95%

CI=3.78-69.67; $p<0.0002$) increased HNC risk in patients compared to controls, respectively. Similarly, our results showed that variant allele T genotype (GT+TT) was at 2.7

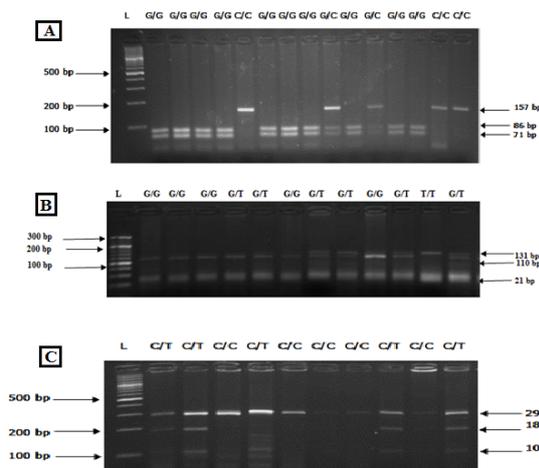


Figure 1. (A) Banding Pattern of RAD51 135G/C Polymorphism After Enzyme Digestion. (G/G=Wild type, G/C=Heterozygous, C/C=Variant and L=Ladder). **(B)** Banding pattern of RAD51 172 G/T polymorphism after enzyme digestion (G/G=Wild type, G/T=Heterozygous, T/T=Variant and L=Ladder). **(C)** Banding pattern for XRCC3 after enzyme digestion (C/C=Wild, C/T=Heterozygous, T/T=Variant and L=Ladder)

Table 2. RAD51 (135G/C), RAD51 (172 G/T) XRCC3 (Thr241Met) Genotypes and Allele Percentage for HNC Patients and Controls

Polymorphisms	Patients	Controls	OR; 95%CI	p-value
RAD51;135G>C				
GG	120	106	Ref	
GC	70	41	1.43 (0.90 to 2.27)	0.1
CC	10	3	2.5 (0.69-9.53)	<0.02
GC+CC	80	44	1.6 (1.02-2.52)	<0.03
G	310	253	Ref	
C	90	67	1.0 (0.66-1.55)	0.2
RAD51;172 G>T				
GG	83	99	Ref	
GT	90	49	1.68 (1.08-2.61)	<0.02
TT	27	2	16 (3.78-69.67)	<0.0002
GT+TT	117	51	2.7 (1.76-4.24)	<0.0001
G	256	247	Ref	
T	114	53	2.4 (1.56-3.75)	<0.0001
XRCC3,722C>T				
CC	101	95	Ref	
CT	79	51	1.26 (0.82-1.96)	0.29
TT	20	4	4.05 (1.36-12.12)	<0.01
CT+TT	100	55	1.72 (1.12-2.66)	<0.01
C	281	241	Ref	
T	121	59	2.36 (1.53-3.64)	<0.0001

Table 3. Association between RAD51 (135G/C), (172 G/T), XRCC3 (Thr241Met) Polymorphism Genotypes and Smoking Status for HNC Patients and Controls

Polymorphism/genotype	Smokers				Non smokers			
	Patients	Controls	OR 95%CI	p-value	Patients	Controls	OR 95%CI	p-value
RAD51;135G>C								
GG	76	44	Ref		44	62	Ref	
GC	45	26	2.47(0.25-0.90)	<0.02	25	15	0.8 (0.38-1.72)	0.5
CC	6	01	2.27 (0.26-19.31)	<0.04	04	02	2.3 (0.41-12.91)	0.34
RAD51;172 G>T								
GG	63	48	Ref		20	51	Ref	
GT	61	23	0.96 (0.51-1.82)	0.91	29	26	1.41 (0.72-2.74)	0.31
TT	19	1	7.90 (1.03-60.75)	<0.04	08	02	4.88(1.00-23.38)	<0.04
XRCC3,722C>T								
CC	68	47	Ref		33	48	Ref	
CT	58	22	0.98 (0.51-1.81)	0.89	21	29	0.76 (0.40-1.45)	0.41
TT	14	02	2.75 (0.60-12.55)	<0.01	06	02	3.54 (0.69-18.01)	0.12

folds increased HNC risk (OR=2.7; 95%CI=1.76-4.24; $p<0.0001$) in patients compared to controls. In case of Thr241Met polymorphism of XRCC3, variant genotype (TT) was at 4.05 folds increased HNC risk (OR=4.05; 95% CI=1.36-12.12; $p<0.01$) in patients with smoking status. In a similar fashion, variant allele T genotype (CT+TT) was at 1.72 folds increased HNC risk (OR=1.72; 95%CI=1.12-2.66; $p<0.01$).

To investigate whether 135G/C (RAD51), 172 G/T (RAD51) and Thr241Met (XRCC3) polymorphisms interact with cancer risk modifier such as smoking, a stratified analysis was performed for which the data is summarized in Table 3. In case of 135G/C genotype of RAD51, heterozygous genotype (GC) was at 2.47 folds increased HNC risk (OR=2.47; 95%CI=0.25-0.90; $p<0.02$) in patients with smoking status compared to controls. Similarly, homozygous variant genotype (CC) of 135G/C polymorphism was also at 2.27 folds increased HNC risk (OR=2.27; 95% CI=0.26-19.31; $p<0.04$) in patients with smoking status. However in case of non smokers no significant difference was observed in all genotypes (GG, GC and CC) of 135G/C (RAD51) between cancer patients and controls. For, second selected polymorphism of RAD51, 172 G/T, homozygous variant genotype (TT) was at 7.09 folds increased HNC risk (OR=7.09; 95% CI=1.03-60.75; $p<0.04$) in patients with smoking status compared to controls. Similar trend was observed in case of Thr241Met polymorphism of XRCC3 in which homozygous variant genotype (TT) was at 2.75 folds increased HNC risk (OR=2.75; 95%CI=0.60-12.55; $p<0.01$) in patients with smoking status compared to

Table 4. Association between RAD51 (135G/C), (172 G/T), XRCC3 (Thr241Met) Polymorphism Genotypes and Area of Cancer of HNC Patients

Polymorphism /Genotype	Oral Cavity	Pharynx	Larynx	p-value
RAD51;135G>C				
GG	50	30	40	Ref
GC	38	17	15	<0.05
CC	2	3	5	0.06
RAD51;172 G>T				
GG	42	25	16	Ref
GT	41	19	30	<0.04
TT	7	6	14	0.07
XRCC3,722C>T				
CC	49	24	28	Ref
CT	34	18	27	<0.02
TT	7	8	5	<0.01

controls.

We also correlated the genotypes of selected polymorphisms (135G/C, 172 G/T and Thr241Met) with different areas of HNC such as oral cavity, larynx and pharynx as shown in Table 4. The heterozygous variant genotype of 135 G/C, 172 G/T and Thr241Met polymorphism was found significantly higher ($p < 0.005$, $p < 0.04$ and $p < 0.02$ respectively) in all three areas of HNC. No significant difference was observed in case of homozygous variant genotype of 135 G/C and 172 G/T polymorphisms. However, the observed difference was significant ($p < 0.01$) in case of homozygous variant genotype of Thr241Met polymorphism for different areas of head and cancer.

Discussion

Genomic instability is one of the main contributors of carcinogenesis. The fidelity of cell division depends on this genomic stability. The period of cell division is particularly critical in terms of exposure to adverse rearrangement in the genome, induced by DNA DSBs (Mucha et al., 2012). Homologous recombination process is essential for maintaining above mentioned genome integrity for the reason of high-intensity activities during S and G2 cell cycle and inefficient repair of HRR pathway may lead to genetic instability and ultimately to carcinogenesis (Vral et al., 2011).

The RAD51 protein is a core component of DNA double-strand break repair by HRR pathway. The cells, which are deficient in this gene product, are defective in homologous recombination and demonstrate genomic instability (Moynahan and Jasin, 2010). The variations of RAD51 repair gene can contribute in protein biosynthesis level. A G to C substitution at position 135 and G to T substitution at position 172 of the RAD51 gene (5'-untranslated region) have been described as single nucleotide polymorphisms (SNPs). Both polymorphisms are located in the regulatory element of RAD51 promoter and are suggested to be associated with messenger RNA stability and expression (Shin et al., 2008; Fayaz et al., 2013). XRCC3 protein is another important protein, structurally and functionally related to RAD51, which plays an important role in the homologous recombination repair system (Krupa et al., 2011). A number of epidemiological studies have evaluated the association between RAD51 135G/C, 172 G/T and XRCC3 Thr241Met polymorphisms with pathogenesis of different cancer (Wang et al., 2013; Mao et al., 2014; Zhao et al., 2014). However, limited numbers of studies are available for above mentioned polymorphisms and HNC patients with inconclusive results.

In the present work we analysed the role of 135G/C, 172 G/T and Thr241Met single nucleotide polymorphisms (SNPs) in the homologous recombination repair genes and risk of head and neck cancer. Our results showed that non coding-region variant in RAD 51 (135G>C) polymorphism is associated with 2.5 folds increased HNC risk (OR=2.5; 95%CI=0.69-9.53; $p < 0.02$). In addition, assessment of variant allele C carrier (GC+CC) genotypes distribution revealed a fairly significant involvement

of RAD 51(135G/C) variant allele (C) on individual susceptibility towards HNC ($p < 0.03$). Similar results has earlier been observed in different cancer e.g, endometrial cancer (Michalska et al., 2014), breast cancer (Wang et al., 2001) and colorectal cancer (Romanowicz-makowska et al., 2012). Phenotypic effects of above mentioned polymorphism are yet to be defined. Presumably, 135G/C variation can be responsible for mRNA stability which modulates interactions with regulatory elements (Mucha et al., 2012). Increased transcription for G allele in 135G/C polymorphism has been demonstrated in *in vitro* studies (Hasselbach et al., 2005). In order to further elaborate the involvement of RAD51 in head and neck carcinogenesis, we screened second important polymorphism (172 G/T) of RAD51 in same study cohort. Our results showed that homozygous variant genotype (TT) in RAD51 polymorphism is associated with 16 folds higher HNC risk (OR=16; 95%CI=3.78-69.67; $p < 0.0002$). Moreover, variant allele T (GT+TT) showed significant involvement towards HNC ($p < 0.0001$). Similar results have already been observed in case of breast (Lee et al., 2005), ovarian cancers (Auranen et al., 2005), and squamous cell carcinoma of head and neck (Lu et al., 2007). RAD51 G172T polymorphism at the 5'-UTR region can have an important role on protein expression and stability. Since it is located in the 5'-UTR region of the RAD51 gene, this genetic variation could affect mRNA stability or translational efficiency, leading to altered polypeptide product levels and alteration in the function of the final product of RAD51 protein (Hasselbach et al., 2005; Lu et al., 2007).

One step forward to further clarify the effect of gene-gene and gene-environment interactions, we selected functional polymorphisms (Thr241Met) of XRCC3 gene which is core components of RAD51 in DSBs. After RFLP analysis, we observed that homozygous variant genotype (TT) of Thr241Met was associated with 4 folds increased HNC risk (OR=4.05; 95% CI=1.36-12.12; $p < 0.01$). In addition, assessment of variant allele T carrier (CT+TT) genotypes distribution revealed a fairly significant involvement of XRCC3 (Thr241Met) variant allele (T) on individual susceptibility towards HNC ($p < 0.01$). Similar trend has already been observed in different cancers such as lung cancer (Tian et al., 2013), breast cancer (Mao et al., 2014), gliomas (Liang et al., 2013) and colorectal cancer (Wang et al., 2013). The Thr241Met (T241M) amino acid substitution due to a C18067T transition at exon 7 is the most frequent polymorphism in XRCC3, which may affect the coding enzyme's function and/or its interaction with other proteins involved in DNA repair. The variant allele (241Met) is associated with relatively high DNA adduct levels in lymphocyte DNA, indicating relatively low DNA repair capacity (Matullo et al., 2001).

In present study, the data on gene-environment interactions illustrated that smokers have higher incidence of heterozygous ($p < 0.02$) and homozygous ($p < 0.04$) variant genotype of 135G/C polymorphism when compared to healthy controls and nonsmokers. In case of RAD 51 172 G<T polymorphism, homozygous variant genotype (TT) was found significantly higher in smoker patients compared to smoker controls category ($p < 0.04$).

In addition to this, XRCC3 Thr241Met polymorphism also showed similar trend and homozygous variant allele ($p < 0.01$) was significantly higher in smokers compared to non smoker controls. A possible reason for this effect is that impaired HRR pathway activity conferred by this polymorphism may result in inefficient repair of DNA damage, which is determined by the level of carcinogenic exposure. Such DNA damage may lead to tumorigenesis if left unrepaired. Smokers usually are exposed to a larger biologically available dose of reactive intermediates from tobacco carcinogens compared with non smokers and, hence, may suffer greater DNA damage that cannot be repaired efficiently (Wang et al., 2003). Tobacco smoke contains procarcinogenic compounds that are metabolized into reactive intermediates and cause DNA damage. The DNA damage caused by these intermediates may require homologous recombination and, consequently, the help of XRCC3.

This study investigated several gene-gene interactions in the context of a general relationship between selected homologous recombination genes. RAD51 135G/C and 172 G/T polymorphisms elucidated a significant risk with HNC, and this risk was enhanced in combination with XRCC3 Thr241Met polymorphism. We consider that direct functional studies on these DSB repair genes would reveal more information on gene-gene interactions and post translational variations. Conflicting evidence of different studies on the association of HNC with HR repair genes may be failure to consider the possibility of gene-gene interaction, or to population-specific differences and ethnic variations. To summarize, we have demonstrated that investigated polymorphisms 135G/C, 172 G/T of RAD51 and Thr241Met of XRCC3 should be simultaneously considered as contributing to the polygenic risk of HNC. Larger studies, as well as functional studies in homologous recombination genes may further validate our claims.

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