

RESEARCH ARTICLE

Increased Risk of Differentiated Thyroid Carcinoma with Combined Effects of Homologous Recombination Repair Gene Polymorphisms in an Iranian Population

Shima Fayaz¹, Maryam Karimmirza², Shokoofeh Tanhaei², Mozhde Fathi², Peyman Mohammadi Torbati³, Pezhman Fard-Esfahani^{1*}

Abstract

Homologous recombination (HR) repair has a crucial role to play in the prevention of chromosomal instability, and it is clear that defects in some HR repair genes are associated with many cancers. To evaluate the potential effect of some HR repair gene polymorphisms with differentiated thyroid carcinoma (DTC), we assessed *Rad51* (135G>C), *Rad52* (2259C>T), *XRCC2* (R188H) and *XRCC3* (T241M) polymorphisms in Iranian DTC patients and cancer-free controls. In addition, haplotype analysis and gene combination assessment were carried out. Genotyping of *Rad51* (135G>C), *Rad52* (2259C>T) and *XRCC3* (T241M) polymorphisms was determined by PCR-RFLP and PCR-HRM analysis was carried out to evaluate *XRCC2* (R188H). Separately, *Rad51*, *Rad52* and *XRCC2* polymorphisms were not shown to be more significant in patients when compared to controls in crude, sex-adjusted and age-adjusted form. However, results indicated a significant difference in *XRCC3* genotypes for patients when compared to controls (p value: 0.035). The GCTG haplotype demonstrated a significant difference (p value: 0.047). When compared to the wild type, the combined variant form of *Rad52/XRCC2/XRCC3* revealed an elevated risk of DTC (p value: 0.007). It is recommended that *Rad52* 2259C>T, *XRCC2* R188H and *XRCC3* T241M polymorphisms should be simultaneously considered as contributing to a polygenic risk of differentiated thyroid carcinoma.

Keywords: Differentiated thyroid carcinoma - polymorphism - homologous recombination repair genes

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Introduction

Most cancers are initiated by DNA damage accumulation (Lengauer et al., 1998). Several forms of DNA damage such as double strand breaks (DSBs) must be repaired for cells to survive (Tambini et al., 2010). DSBs are produced by replication errors and exogenous agents such as ionizing radiation. DSBs are more difficult to repair than other types of DNA damage because no undamaged template is available (Khanna and Jackson, 2001). At least two pathways of DSBs repair are recognized: homologous recombination (HR) pathway and non homologous end joining (NHEJ) pathway (Paques and Haber, 1999). The repair of DNA damage by HR is the major pathway for the maintenance of genetic stability in all eukaryotes cells (Jackson, 2002; Thompson and Schild, 2002).

Five *Rad51* paralogs (*XRCC2*, *XRCC3*, *Rad51B*, *Rad51C*, *Rad51D*) play essential roles in the HR pathway in the most lethal forms of DNA damage (Suwaki et al., 2011). Additionally, *Rad52* protein has an important role

in homology directed DNA repair by mediating *Rad51* nucleoprotein filament formation on single-stranded DNA (ssDNA) protected by replication protein-A (RPA) and annealing of RPA-coated ssDNA (Honda et al., 2011). Mutations in many HR-related genes lead to accumulation of unrepaired DSBs and are associated with tumorigenesis (Suwaki et al., 2011).

Thyroid carcinoma accounts for <1% of all human cancers but is the most frequent endocrine neoplasia (Schlumberger and Torlontano, 2000). In the Iranian population, thyroid carcinoma is the 7th most common cancer in females, 14th in males and 11th in both sexes (Khayamzadeh et al., 2011). Approximately 98% of thyroid carcinomas are Differentiated Thyroid Carcinoma (DTC). DTC consists of papillary, follicular and Hürthle cell carcinoma (Caron and Clark, 2004). In this study we carried out a case control study in the Iranian population to evaluate the potential effects of *Rad51* (135G>C), *Rad52* (2259C>T), *XRCC2* (R188H) and *XRCC3* (T241M) polymorphisms, separately and together, on individual susceptibility to DTC.

¹Department of Biochemistry, Pasteur Institute of Iran, ²Department of Biochemistry and Genetics, Payam-e-Noor University of Tehran, ³Department of Pathology, Labbafi-Nezhad Hospital, Shahid Beheshti Medical University, Tehran, Iran *For correspondence: fard-esfahani@pasteur.ac.ir

Materials and Methods

Participants

The study population consisted of patients with histopathologically confirmed DTC, and cancer-free controls. Individuals with a prior history of other cancers, alcohol consumption or history of smoking were excluded from the study. The sample size for assessment of each polymorphism is shown in Table 1. The anonymity of both patients and control population was guaranteed, and all studies were conducted with the written informed consent of all individuals involved, which was obtained prior to blood samples being taken. The DTC patients were recruited from the Research Institute for Nuclear Medicine of Shariati hospital in Tehran, Iran between September 2008 – September 2009. The Controls were recruited from volunteers at two academic centers in Tehran, Iran.

DNA extraction and genotyping

5ml of peripheral blood was collected into tubes containing 1ml EDTA (1g/dl) and stored at -20°C until use. DNA was extracted from the whole blood by salting out procedure (Miller *et al.*, 1988). The genotyping of *Rad51* (135G>C), *Rad52* (2259C>T) and *XRCC3* (T241M) polymorphisms was determined by PCR-RFLP. The primers are shown in Table 2.

PCR was performed in 25 μ l reactions containing 60-250 ng of genomic DNA, 10 μ M of each primer, 1.5 mM MgCl₂, 0.2 mM dNTPs, 1 \times PCR buffer, and 1 unit of Smart Taq DNA polymerase. Thermal cycling was performed as follows: initial activation at 95°C for 2min, followed by 35 amplification cycles consisting of denaturation at 95°C for 30s, annealing at 63°C (*Rad51*), 61°C (*Rad52*) and 61°C (*XRCC3*) for 30s, extension at 72°C for 45s and a final extension at 72°C for 7 min. Each PCR product: *Rad51* 135G>C, *Rad52* 2259C>T and *XRCC3* T241M, was digested overnight at 37°C by *BstNI*, *HaeIII* and *NlaIII* (Fermentas, Switzerland), respectively. Restriction products were subjected to electrophoresis in 3% agarose gel with ethidium bromide (0.5 μ g/ml) for visualization under ultraviolet light. The expected products for each genotype are shown in Table 2.

The genotyping of *XRCC2* R188H was determined by PCR-HRM analysis. PCR-HRM was performed in 0.1ml strip tubes of 72-well rotor in Rotor-Gene™ 6000 real-time rotary analyzer (Corbett Life Sciences) with Type-it HRM PCR kit, QIAGEN. The PCR-HRM profile was obtained by the method previously explained in the literature (Fayaz *et al.*, 2012). The PCR annealing temperature is mentioned in Table 2.

Retrieved Melting curves of PCR-HRM were analyzed; Heterozygote and variant groups were identified according to their melting transition to the wild-type group. To confirm that genotyping of samples exists in each of the three melting curve groups, some of the PCR products of the three afore-mentioned groups were digested with *SexA1* as follows: 10 μ l of each PCR product was digested with 2 U of *SexA1*, overnight at 37°C, and Restriction products were electrophoresed in 3.5% agarose gel with ethidium bromide for visualization under ultraviolet light. Restriction patterns after *SexA1* digestion are referred to

in Table 2.

Statistical analysis

Hardy–Weinberg equilibrium for *Rad51* (135G>C), *Rad52* (2259C>T), *XRCC2* (R188H) and *XRCC3* (T241M) alleles in control groups was carried out by Chi-square test. Evaluation the differences in the genotype, haplotype and allele frequency in patients and controls were analyzed using Chi-square test. The association between polymorphism and DTC risk in each, were analyzed by calculating the crude, age and sex adjusted odds ratio (OR) and the corresponding 95% confidence intervals (CIs) using unconditional multiple logistic regression. In haplotype analysis the most common haplotypes among controls were used as reference in the logistic regression model. The p values reported in the study are based on a two-sided probability test with a significance level of 0.05. All analyses were performed with SPSS v13 software.

Results

Characteristics of subjects

The study comprised DTC cases and controls with no previous or current malignant disease. All individuals claimed to have had no previous exposure to ionizing radiation sources. General characteristics for both groups in each genotype are listed in Table 1. Age and sex were not statistically different between DTC patients and controls (Table 1). The frequencies of all polymorphisms in the control population were in agreement with the Hardy Weinberg expectations.

HRM analysis

In HRM analysis, three groups of melting curves were retrieved from the individuals studied. The *XRCC2* Arg188His mutation was easily distinguished in the normalized melting curves and the normalized difference curves. Heterozygote and homozygous mutation was identified with a T_m shift when compared with the wild-type (Figure 1A). In the normalized difference curves, the melting profile of heterozygotes was chosen as the horizontal base line, and the relative differences in the melting of all other samples were plotted relative to the baseline (Figure 1B).

Results of treatment by *SexA1* restriction enzyme on samples from each group was completely in concordance with data obtained by RFLP and data from HRM analysis (data not shown).

Association analysis

Logistic regression analysis of the *Rad51* (135G>C), *Rad52* (2259C>T) and *XRCC2* (R188H) polymorphisms showed no separately significant difference between patients and controls in crude, sex- adjusted and age-adjusted form, (Table 1). However, the results obtained indicated a significant difference in *XRCC3* (CT+TT/CC) genotypes and slightly significant involvement of *XRCC3* T241M (C>T) mutant allele to the wild type for patients to controls; p value: 0.035, OR: 1.58 (95%CI: 1.03-2.42) and p value: 0.062, OR: 1.37 (95%CI: 0.48.03-1.90), respectively (Table 1).

Haplotype analysis

Haplotype analysis was performed for the *Rad51* 135G>C, *Rad52* 2259C>T, *XRCC3* T241M (C>T) and *XRCC2* R188H (G>A) polymorphisms. Eight haplotypes were predicted: the most common haplotype among controls was GCCG, which was used as the reference, four haplotypes with one variant allele and three haplotypes with the coexistence of two variant alleles were analyzed to the reference haplotype; Only GCTG haplotype differed significantly between patients and controls: p value: 0.047, OR: 1.55 (95%CI:1.00-2.41).

Gene combination

To assess the combined effect of these polymorphisms, we conducted gene combination analysis. Binary logistic regression evaluation showed that the combined variant genotype of *Rad52* (CT+TT) and *XRCC3* (CT+TT) has a slightly higher significant difference compared to the combined *Rad52* (CC) and *XRCC3* (CT+TT) to the wild type of them (CC/CC); p value: 0.058, p value: 0.066.

The combined *Rad52* (CC)/ *XRCC2* (GG)/ *XRCC3* (CT+TT) demonstrated an increased risk of DTC in

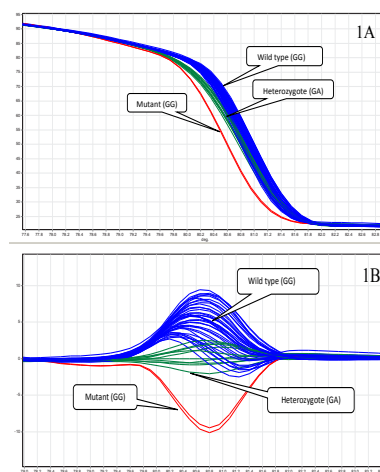


Figure 1. A) HRM Analysis of Arg188His XRCC2 Gene in Patients and Controls. Three types of HRM curves were obtained: blue curves represent wild type Arg/Arg (G/G), green curves represent heterozygote Arg/His (G/A) and red curves represent variant form His/His (A/A); **B) Different Normalized Curves of HRM Analysis.** The melting profile of heterozygotes is the horizontal base line, and the relative differences in the melting of all other samples were plotted relative to the baseline

Table 1. Demographical Details and Distribution of Genotypes and Alleles Frequencies among DTC Patients and Controls

Variable		DTC n (%)	Control n (%)	p value ^a , Crude OR (95% CI)	p value, Sex Adj. OR (95%CI) ^d	p value, Age Adj. OR (95% CI) ^d
Rad51 135G>C		n=151	n=196			
Age, years	≤50	125(82.8%)	153 (78.1)	0.27		
	>50	26(17.2%)	43 (21.9%)			
Sex	Male	33(21.9%)	50 (25.5%)	0.43		
	Female	118(78.1%)	146 (74.5%)			
Genotype	GG	126(83.4%)	162 (82.7%)			
(Rad51 135G>C)	GC	25(16.6%)	34 (17.3%)	0.84, 0.95 (0.54-1.67) ^b	0.10, 0.96 (0.54-1.69) ^b	0.96, 0.95 (0.54-1.66) ^b
Allele frequency	C	25 (8.3%)	34 (8.7%)	0.85, 0.95(0.55-1.63) ^c	0.10, 0.96(0.56-1.66) ^c	0.97, 0.95(0.56-1.63) ^c
Rad52 2259C>T		n=168	n= 190			
Age, years	≤50	140(83.33%)	145(76.31%)	0.1		
	>50	28(16.66%)	45(23.68%)			
Sex	Male	39(23.21%)	47(24.73%)	0.74		
	Female	129(76.78%)	143(75.26%)			
Genotype	CC	75(44.64%)	87(45.79%)			
	CT	86(51.19%)	91(47.89%)			
	TT	7 (4.17%)	12 (6.32%)			
	CT+TT	93(55.36%)	103(54.21%)	0.83, 1.04(0.69-1.59) ^b	0.90, 1.0(0.69-1.6) ^b	0.87, 1.06(0.70-1.61) ^b
Allele frequency	T	100(46.51%)	115(53.48%)	0.88, 0.98(0.71-1.34) ^c	0.95, 0.98(0.71-1.35) ^c	0.99, 0.99(0.72-1.36) ^c
XRCC2 Arg188His		n=171	n=204			
Age, years	≤50	142 (83%)	159 (78%)	0.24		
	>50	29 (17%)	45 (22%)			
Sex	Male	38 (22%)	50 (24.5%)	0.63		
	Female	133 (78%)	154 (75.5%)			
Genotype	GG	141 (82.4%)	170 (83.3%)			
	GA	28 (16.4%)	34 (16.7%)			
	AA	2 (1.2%)	0 (0%)			
	GA+AA	30 (17.6%)	34 (16.7%)	0.89, 1.06 (0.62-1.82) ^b	0.89, 1.08(0.63-1.85) ^b	0.92, 1.06 (0.62-1.83) ^b
Allele frequency	A	32 (9.4%)	34 (8.3%)	0.62, 1.13 (0.68-1.88) ^c	0.68, 1.15 (0.69-1.91) ^c	0.70, 1.14 (0.69-1.89) ^c
XRCC3 Thr241Met		n=161	n=182			
Age, years	≤50	28 (17.4%)	39 (21.4%)	0.35		
	>50	133 (82.6%)	143 (78.6%)			
Sex	Male	35 (21.7%)	44 (24.2%)	0.59		
	Female	126 (78.3%)	138 (75.8%)			
Genotype	CC	71 (44.1%)	101 (55.5%)			
	CT	76 (47.2%)	68 (37.4%)			
	TT	14 (8.7%)	13 (7.1%)			
	CT+TT	90 (55.9%)	81 (44.5%)	0.03, 1.58(1.03-2.42) ^b	0.05, 1.58(1.03-2.41) ^b	0.05, 1.577(1.03-2.42) ^b
Allele frequency	T	104	94	0.06, 1.37(0.98-1.91) ^c	0.08, 1.37(0.98-1.91) ^c	0.08, 1.37(0.98-1.90) ^c

^aTwo sided chi-squared analysis in case to control; ^bOR (95%CI) variant+heterozygous to the wild type genotype in case to control; ^cOR (95%CI) mutant to the wild type allele in case to control; ^dSex and Age Adjusted OR (95% CI) were estimated by Mantel-Haenzel statistics

Table 2. Primer Sequences, PCR Products and Restriction Patterns after Enzyme Digestion for Each of Rad51 135G>C, Rad52 2259C>T, XRCC2 R188H and XRCC3 T241M Polymorphisms

Polymorphism	Primer sequences 5' to 3'	Annealing Temperature	PCR product (bp)	No. of restriction enzyme site in PCR product (restriction site)	Restriction patterns after enzyme digestion
<i>Rad51</i> 135G>C	F: GGAATGTTCTCAGTGTAGAG R: TTCTTCTGATGAGCTCGAGG	63 °c	157 bp	G creates one <i>Bst</i> NI site. (5'-CC↓WGG-3')	G/G: 86, 71 bp ; G/C: 157, 86, 71 bp; C/C: 157 bp
<i>Rad52</i> 2259C>T	F: GTTTGCCCTGACTGGAGTT R: GTTCAGACTGGTCCCATC	61 °c	286 bp	C creates two <i>Hae</i> III site. C to T creates one <i>Hae</i> III site. (5'-GG↓CC-3')	C/C: 162, 65, 58 bp; C/T: 162, 125, 65, 58 bp; T/T: 162, 125 bp
<i>XRCC2</i> R188H (G>A)	F: GGAATGTTCTCAGTGTAGAG R: TTCTTCTGATGAGCTCGAGG	59 °c	104 bp	A creates one <i>SexA</i> I site. (5'-A↓CCWGGT-3')	G/G: 104 bp ; G/A: 104, 61, 42 bp; G/G: 61, 42 bp
<i>XRCC3</i> T241M (C>T)	F: ATGGCTGCCCTGGTGTCA R: CAATCTGGCTAAAAATAGG	61 °c	211 bp	C to T creates one <i>Nla</i> III site. (5'-CAITG↓-3')	C/C: 211 bp; C/T: 211, 108, 103 bp; T/T: 108, 103 bp

comparison to individuals carrying wild type genes (CC/GG/TT); p value: 0.029, OR: 2.204 (95%CI:1.08-4.51). Furthermore, the combined variant form of *Rad52*/*XRCC2*/*XRCC3* compared to the wild type, revealed an elevated risk of DTC; p value: 0.007, OR:5.04 (95%CI:1.45-17.58).

Discussion

HR genes are involved in the repair of bulky DNA adducts damage. The role of *Rad51* 135G>C, *Rad52* 2259C>T, *XRCC2* R188H and *XRCC3* T241M polymorphisms in different cancers were assessed in several case-control studies. In this study we evaluated the association of afore-mentioned polymorphisms separately, and for the first time, assessment of combined genotypes with each other, on individual susceptibility in Iranian DTC patients. Our results showed coding-region variant in *XRCC3* (Thr241Met) polymorphism associated with 1.58 fold (95%CI: 1.031-2.422) elevated risk of DTC. In addition, assessment of allele frequency distribution revealed a fairly significant involvement of *XRCC3* T241M variant allele (T) on individual susceptibility toward DTC (p value: 0.058). The results are similar to earlier reports with regard to differentiated thyroid cancer in Caucasian Portuguese (Bastos et al., 2009) and a non-Hispanic white population (Sturgis et al., 2005). They found *XRCC3* variant allele was associated with a twofold increased risk of thyroid cancer. Other Studies on carcinoma of the bladder (Matullo et al., 2001), hepatocellular carcinoma (Long et al., 2008), breast cancer (Romanowicz-Makowska et al., 2012) and sporadic melanoma (Winsey et al., 2000), are in agreement with our result. *XRCC2* and *XRCC3* proteins are structurally and functionally related to *Rad51*, which plays an important role in the homologous recombination repair system (Krupa et al., 2011). *Rad52* protein also has an important role in homology-directed DNA repair by mediating *Rad51* nucleoprotein filament formation on single-stranded DNA (ssDNA) protected by replication protein-A (RPA) and annealing of RPA-coated ssDNA in HR repair system (Honda et al., 2011).

In our study, *Rad51* 135G>C, *Rad52* 2259C>T and *XRCC2* R188H were not separately associated with a significant increase of DTC (Table 1). Likewise, other studies in the Portuguese (Bastos et al., 2009) and Spanish population (Garcia-Quispes et al., 2011) did not reveal an association of *XRCC2* R188H and thyroid cancer risk. In the Saudi Arabian population however, *Rad52* 2259C>T was associated with papillary thyroid cancer risk (Siraj et al., 2008).

Nevertheless, assessment of the combined genotypes showed significant differences between DTC patients and controls: a combination of *Rad52* 2259C>T and *XRCC3* T241M (C>T), (variant or heterozygote type, TT+CT/TT+CT) compared to wild type (CC/CC) revealed an almost significant involvement (p value: 0.058). Another notable significant risk was elevated 5.04 fold when a combination of *Rad52* 2259C>T, *XRCC2* and *XRCC3* genotypes, in variant or heterozygote type (TT+CT/AA+GA/TT+CT), was compared to the wild type of (CC/GG/CC) (p value: 0.007). In haplotype, analysis observed that coexistence of the mutant allele of *XRCC3* T241M beside the wild alleles of *Rad51* 135G>C, *Rad52* 2259C>T and *XRCC2* R188H leads to a significantly higher risk for DTC (p value: 0.047).

This study enabled us to investigate several gene-gene interactions in the context of a general relationship between selected homologous recombination genes. *XRCC3* T241M polymorphism elucidated a significant risk with DTC, and the risk was enhanced in combination with *Rad52* 2259C>T and *XRCC2* R188H polymorphisms. 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 DTC with HR repair genes may be due to failure to consider the possibility of gene-gene interaction, or to population-specific differences and ethnic variation.

To summarize, we have demonstrated that investigated polymorphisms *Rad52* 2259C>T, *XRCC2* R188H and T241M of *XRCC3* should be simultaneously considered as contributing to the polygenic risk of differentiated thyroid carcinoma. Larger studies, as well as functional studies in homologous recombination genes are required to validate our outcome.

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