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Research Article

Assessment of genetic mutations in the *XRCC2* coding region by high resolution melting curve analysis and the risk of differentiated thyroid carcinoma in Iran

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Abstract

Homologous recombination (HR) is the major pathway for repairing double strand breaks (DSBs) in eukaryotes and XRCC2 is an essential component of the HR repair machinery. To evaluate the potential role of mutations in gene repair by HR in individuals susceptible to differentiated thyroid carcinoma (DTC) we used high resolution melting (HRM) analysis, a recently introduced method for detecting mutations, to examine the entire *XRCC2* coding region in an Iranian population. HRM analysis was used to screen for mutations in three *XRCC2* coding regions in 50 patients and 50 controls. There was no variation in the HRM curves obtained from the analysis of exons 1 and 2 in the case and control groups. In exon 3, an $Arg^{188}His$ polymorphism (rs3218536) was detected as a new melting curve group (OR: 1.46; 95%CI: 0.432-4.969; p = 0.38) compared with the normal melting curve. We also found a new $Ser^{150}Arg$ polymorphism in exon 3 of the control group. These findings suggest that genetic variations in the *XRCC2* coding region have no potential effects on susceptibility to DTC. However, further studies with larger populations are required to confirm this conclusion.

Key words: DNA repair, gene polymorphism, mutation analysis.

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Introduction

Thyroid cancer is the most common endocrine malignancy and its incidence has increased in recent years (Leenhardt *et al.*, 2004; Davies and Welch, 2006; Jemal *et al.*, 2008). Differentiated thyroid carcinoma (DTC) is the most common type of all thyroid carcinomas (accounting for ~90% of all cases) and consists of papillary, follicular and Hürthle cell carcinomas, the latter being a subtype of follicular thyroid carcinoma (Hundahl *et al.*, 1998). Risk factors associated with DTC include exposure to various carcinogenic agents, ethnicity and dietary habits, although exposure to ionizing radiation is still the only recognized risk factor (Ron *et al.*, 1995; Xiong *et al.*, 2005; Preston *et al.*, 2007).

Recent studies have proposed that genetic variation in conserved DNA repair systems may influence susceptibility to cancer (Gatzidou *et al.*, 2010). These systems normally ensure the genetic intactness of cell populations such that any alteration in the genes related to these systems could lead to a defect in DNA repair pathways and ultimately affect the cellular genetic stability and susceptibility to cancer. In severe DNA damage, such as double-strand breaks (DSBs), there are two important recombination systems for cell survival, namely, homologous recombination (HR) and non-homologous end joining (NHEJ) (Paques and Haber, 1999; Peterson and Cote, 2004).

In eukaryotes, HR is the major pathway for DSB repair and has an important role in preventing mutations, chromosomal instability and cancer; these functions make HR essential for cell viability and genomic stability (Jackson, 2002; Thompson and Schild, 2002). The HR repair system functions primarily during the S and G2 phases of the cell cycle. Since HR is an error-free pathway of damage tolerance that allows the replication bypass of lesions during a template switch it has a distinct advantage over NHEJ (Jackson, 2002; Thompson and Schild, 2002).

The *RAD51* gene family is the key component of HR and its impairment can lead to extreme sensitivity to certain DNA damaging agents, intense genomic instability and a

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risk of cancer (Sonoda *et al.*, 1998; Cui *et al.*, 1999; Deans *et al.*, 2000, 2003; Takata *et al.*, 2001; Gruver *et al.*, 2005; Siraj *et al.*, 2008). The *RAD51*-like gene family in somatic mammalian cells is composed of *XRCC2*, *XRCC3*, *RAD51L1*, *RAD51L2* and *RAD51L3* (Thacker, 1999). There are indications that XRCC2 has an important role in enhancing the action of RAD51, with a loss of XRCC2 delaying the early steps of HR, including the integration of RAD51 at the site of DNA damage, nucleoprotein filament formation and strand invasion (Sugawara *et al.*, 2003; Wolner *et al.*, 2003). In *XRCC2*-deficient cells, RAD51 responses are reduced by approximately five-fold, and this has led to *XRCC2* being recognized as a repair response-enhancing factor (Tambini *et al.*, 2010).

XRCC2 is a 29,668 kb gene located on human chromosome 7q36.1 and consists of three exons (1 to 3) that contain 38, 82 and 722 bp, respectively. XRCC2 protein is highly conserved among mammalian species (Tambini *et al.*, 2010). Several studies have examined the association between genetic polymorphisms in *XRCC2* and different cancers (Han *et al.*, 2004; Jiao *et al.*, 2008; Curtin *et al.*, 2009). In the present report, we describe the first case-control study of an Iranian population to examine the association between mutations in the entire coding region of the *XRCC2* gene and individual susceptibility to DTC based on high resolution melting (HRM) analysis, a recently introduced method for detecting mutations (Montgomery *et al.*, 2010).

Materials and Methods

Study subjects

A sample size calculation (Kasiulevicious *et al.*, 2006) indicated that the minimum sample size for this case-control study was ~45 for each group (controls and cases). In addition, the minimum odds ratio (OR) for significance was 2.5 ($p < 0.05$). The probability of having a mutant allele in the *XRCC2* coding region in control individuals was estimated as 0.35. Based on these preliminary calculations, our study population consisted of 50 patients with histopathologically confirmed DTC and 50 controls. Informed consent was obtained from all participants before commencement of the study and the study was approved by the ethics committee of Tehran University of Medical Science. The DTC patients were recruited from the Research Institute for Nuclear Medicine of Shariati Hospital, Tehran, Iran. The control population was matched for age (≤ 50 and > 50 year) and sex, with no previous or concurrent malignant disease. The controls were recruited from volunteers at two academic centers in Tehran. Individuals with a history of other cancers, radiation exposure, alcohol consumption or smoking were excluded from the study.

DNA extraction

5 mL of peripheral blood was collected from each subject into tubes containing 1 mL of EDTA (1 g/dL) and stored at -20 °C until used. Whole blood DNA was extracted by a salting out procedure (Miller *et al.*, 1988).

Primer design and assay conditions for PCR-HRM

One primer pair for exon 1 (Ex1), three primer pairs for exon 2 (Ex2) and three primer pairs for exon 3 (Ex3) were used in this work (Table 1). PCR-HRM was done in a Rotor-Gene 6000 real-time rotary analyzer (Corbett Life Sciences). The reactions were prepared in 10 μL volumes in 0.1 mL strip tubes of a 72-well rotor. For symmetric PCR-HRM, the amount of DNA in each reaction was adjusted to 50 ng. Each reaction contained 5 μL of 2 x HRM PCR Master Mix (Type-it HRM PCR kit, QIAGEN), 0.7 μL (10 μM) of primer mix, 1.5 μL (50 ng) of DNA and DNA RNase-free water to a final volume of 10 μL.

The PCR cycling profile is summarized in Table 1. After amplification, HRM analysis data were collected from 65 °C to 85 °C, with each step raised by 0.05 °C, followed by a waiting time of 10 s. Samples from the top, middle and bottom of each melting curve group and suspicious melting curves were sequenced. Any new mutation detected by PCR-HRM was screened for in the other samples by using the Allele Refractory Mutation System (ARMS). The ARMS primers and fragment lengths are listed in Table 1.

Statistical analysis

Hardy-Weinberg equilibrium of the *XRCC2* alleles in the control population was assessed using the Chi square test (χ^2). The homogeneity of age distribution between the controls and cases was assessed with an independent sample *t*-test and Levene's test. Allelic and genotypic frequencies were compared across groups using the Chi square test. The odds ratio (OR) and the corresponding 95% confidence intervals (CIs) between DTC and a detected polymorphism were calculated using logistic regression. All analyses were done with SPSS v13 software.

Results

This study included 50 DTC patients and 50 cancer-free controls. The two groups were matched for sex and age (≤ 50 and > 50 years). The mean age (± SEM) in the cases and controls was 38.4 ± 2.1 and 36.2 ± 1.2 years, respectively. Age and sex were not significantly different between the two groups. The general characteristics of both groups are shown in Table 2.

HRM analysis results

HRM analysis showed no variations in the melt phase analysis curves for the Ex1, Ex2a, Ex2b, Ex2c, Ex3a and Ex3c fragments in the case and control groups. A new melting curve group was detected in the Ex3b fragment compared to the normal melting curves retrieved from the wild type genotype group (Figure 1). To confirm the existence of

Table 1 - (A) Primers for the *XRCC2* coding regions and the PCR conditions used before HRM analysis. (B) Primers used in PCR-ARMS.

Exon number	Primer (5' to 3')	Amplicon size (bp)	PCR conditions
A			
Exon1 (Ex1)	F: TATTGCGCATGCTCCCGCC R: CCAATCCCCCGGGTTCCC	277	95 °C, 5 min; 45 cycles (95 °C, 10 s; 64 °C, 30 s; 72 °C, 16 s); 72 °C, 5 min
Exon2a (Ex2a)	F: GGTATAAATATAGATGTCTAGG R: GGTTCTATTTCTTTCAG	118	95 °C, 5 min; 45 cycles (95 °C, 10 s; 52 °C, 30 s; 72 °C, 16 s); 72 °C, 5 min
Exon2b (Ex2b)	F: CCTTCTCTCTTCTTTTATAAGC R: ACAGGTGAATCTTCATCAGC	99	95 °C, 5 min; 45 cycles (95 °C, 10 s; 57 °C, 30 s; 72 °C, 16 s); 72 °C, 5 min
Exon2c (Ex2c)	F: GACTTGAAGGTAGAAGTTCC R: CTTGTGAGGAGTATGTGTATAC	164	95 °C, 5 min; 45 cycles (95 °C, 10 s; 56 °C, 30 s; 72 °C, 16 s); 72 °C, 5 min
Exon3a (Ex3a)	F: CTTTCACATTCCAGTAAGTGTC R: GTAAAGTGTAAGAAGTAAGTGGG	379	95 °C, 5 min; 45 cycles (95 °C, 10 s; 57 °C, 30 s; 72 °C, 16 s); 72 °C, 5 min
Exon3b (Ex3b)	F: TCAAATACTGCCTGGGAAG R: CTGCCATGCCTTACAGAG	373	95 °C, 5 min; 45 cycles (95 °C, 10 s; 57 °C, 30 s; 72 °C, 16 s); 72 °C, 5 min
Exon3c (Ex3c)	F: CAAACTATAATGCAGAAAGCC R: GAGCCATGATTGTGCCAC	376	95 °C, 5 min; 45 cycles (95 °C, 10 s; 64 °C, 30 s; 72 °C, 16 s); 72 °C, 5 min
B			
Exon3b (Ex3b)-F	TCAAATACTGCCTGGGAAG		
Exon3b (Ex3b)-R	CTGCCATGCCTTACAGAG	273, 265	
ARMs C (wild)	CCTTTTGATTTTGGATAGC		95 °C, 2 min; 30 cycles (95 °C, 10 s; 58 °C, 30 s; 72 °C, 16 s); 72 °C, 4 min
Exon3b (Ex3b)-F	TCAAATACTGCCTGGGAAG		
Exon3b (Ex3b)-R	CTGCCATGCCTTACAGAG	273, 147	
ARMs G (mutant)	TCCAGTAAAAAGCTGACAGC		

F- forward, R- reverse.

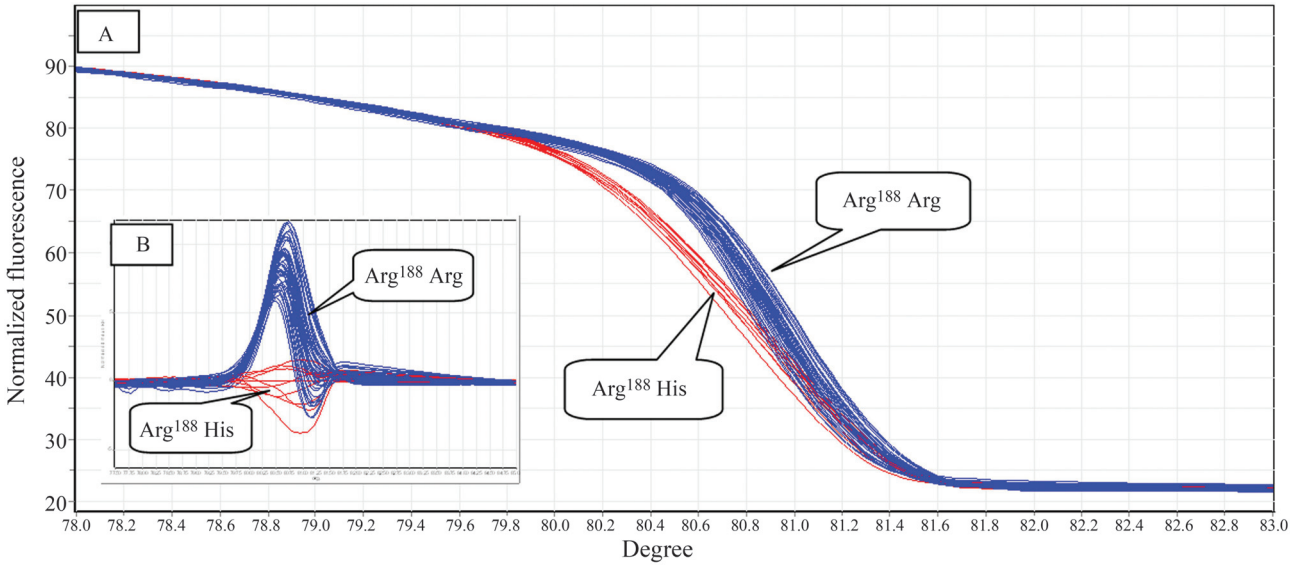

Figure 1 - (A) HRM curves for exon 3b (Ex3b) of the *XRCC2* gene in patients and controls. The Arg^{188}His genotype (red curves) was detected as a new melting curve group in a few patients and controls compared with the normal melting curves obtained for the wild type (Arg^{188}) genotype (blue curves). (B) Difference graph of the HRM curves.

a single nucleotide polymorphism (SNP) in the Ex3b fragment, the samples in this group were subjected to DNA sequencing and a previously reported polymorphism (Arg^{188}His, rs3218536) was detected in all of them. No His/His alleles were found in the population. Table 2 summarizes the distributions of the allelic and genotypic frequencies in the case and control groups.

Another suspicious melting curve was identified in the Ex3b fragment and DNA sequencing confirmed a novel missense mutation (AGC → AGG, Ser^{150}Arg) in one sam-

Table 2 - General characteristics of the case and control groups and the allelic and genotypic ($Arg^{188}His$) frequencies of *XRCC2*.

Variable	Case n (%)	Control n (%)	p	OR (95% CI)
Age (years)				
≤ 50	44 (88%)	44 (88%)	0.383	
> 50	6 (12%)	6 (12%)		
Sex				
Male	12 (24%)	12 (24%)	1.000	
Female	38 (76%)	38 (76%)		
Genotype ($Arg^{188}His$)				
GG	43 (86%)	45 (90%)	0.38[a]	1.46
GA	7 (14%)	5 (10%)		(0.432-4.969)[b]
Allele frequency				
G	93 (0.93%)	95 (0.95%)	0.39[a]	1.43
A	7 (0.07%)	5 (0.05%)		(0.438-4.666)[c]

[a]One tailed chi-squared analysis comparing genotype distributions and allelic frequencies between cases and controls.
[b]OR (95%CI) GA to GG genotype ($Arg^{188}His$) of *XRCC2* for case *vs.* control groups.
[c]OR (95%CI) A to G allele ($Arg^{188}His$) of *XRCC2* for case *vs.* control groups.

ple of the control group. No mutation was detected in the other samples screened for this mutation using ARMS.

The difference between the $Arg^{188}His$ and Arg^{188} genotypic frequencies was not significant and there was no significant association between DTC and the G and A allelic frequencies (Table 2).

Discussion

In this study, we used HRM analysis to screen for genetic mutations in the entire coding region of the *XRCC2* gene and examined the association between these mutations and DTC in a subpopulation from Tehran, Iran. HRM analysis is an innovative technique for genotyping that is based on DNA melting analysis such that any alteration in genotype leads to variation in the HRM curves when compared to the wild type (Montgomery *et al.*, 2010). To our knowledge, this is the first time that the entire coding region of the *XRCC2* gene has been analyzed. Two polymorphisms, CGC → CAC ($Arg^{188}His$, rs3218536) and AGC → AGG ($Ser^{150}Arg$), were identified in Ex3b of the *XRCC2* gene. To detect the $Arg^{188}His$ polymorphism we distinguished between wild homozygote (G::C) and heterozygote (A::T, G::C) samples based on a discernible melting transition (Figure 1A).

There was no significant association between DTC and the $Arg^{188}His$ polymorphism and its genotypic frequency in the individuals studied (Table 2). Bastos *et al.* (2009) and Garcia-Quispes *et al.* (2011) also found no significant involvement of the *XRCC2* $Arg^{188}His$ polymorphism in thyroid cancer in 109 (OR: 0.8, 95%CI: 0.4-1.6) and 402 (OR: 1.12, 95%CI: 0.80-1.59) patients, respectively. There are currently no additional data on the relationship between the *XRCC2* $Arg^{188}His$ polymorphism and thyroid cancer.

A recent meta-analysis by Yu *et al.* (2010) reported that there was no evidence of a significant association between *XRCC2* $Arg^{188}His$ and the risk of breast cancer in any genetic model. A meta-analysis of three case-control studies in the United Kingdom and a family-based study in the United States found no association between a putatively functional $Arg^{188}His$ SNP and colorectal cancer (Curtin *et al.*, 2009). Similarly, a meta-analysis of 16 studies compiled by the International Ovarian Cancer Association Consortium showed there was also no association between $Arg^{188}His$ and the risk of ovarian cancer (Pearce *et al.*, 2009). However, the *XRCC2* $Arg^{188}His$ polymorphism may be a genetic modifier for smoking-related pancreatic cancer (Jiao *et al.*, 2008).

Alignment of the protein sequence of human XRCC2 with that of other species revealed an $Arg^{188}His$ substitution in *Gallus gallus* and *Pongo abelii* (Figure 2). Furthermore, Arg^{188} is not present at the active site of three XRCC2 proteins. This finding suggests that the $Arg^{188}His$ polymorphism generated in homologous recombination repair pathway may not have severe effects on XRCC2 function and may not influence the susceptibility to cancer in humans.

We detected a novel missense mutation (AGC → AGG, $Ser^{150}Arg$) in the *XRCC2* gene in one sample of the control group. In this G::C conversion, bases switched strands but the GC content was conserved and the melting transitions of heterozygote, wild and variant homozygotes

Homo sapiens (human)	142	SLCLLILDSLSAFYWIDRVNGGESVNLQESTLRKCSQCLEKLVNDYRLVL	191
Pan troglodytes (chimpanzee)	142	SLCLLILDSLSAFYWIDRVNGGESVNLQESTLRKCSQCLEKLVNDYRLVL	191
Canis lupus familiaris (dog)	138	SLCLLILDSLSAFYWIDRVNGGESVNLQEATLKKCAQFLEKLVNEYRLVL	187
Bos taurus (ox)	142	SVCLLILDSLSAFYWIDRVNGGESVNLQESTLRKCSQLLEKLVNEYRLVL	191
Mus musculus (mouse)	142	SLCLLIVDSLSSFYWIDRVSGGESVALQESTLQKCSQLLERLVTEYRLLL	191
Rattus norvegicus (rat)	142	SLCLLIVDSMSSFYWIDRVSGGESVSLQESTLQKCSQLLERLVTEYRLLL	191
Gallus gallus (chicken)	243	SLCLLILDSISAFYWIDRSNGGESLNSQEMNLKKCANFLEKLVREHHLVL	292
Danio rerio (zebrafish)	150	TLGLLVIDSISAFYWTDRFNGGESASCQEANLRKCAELLDRLRRNYGIVI	199
Macaca mulatta (rhesus monkey)	142	SLCLLILDSLSAFYWIDRVNGGESVNLQESTLRKCSQCLEKLVNDYRLVL	191
Pongo abelii (orangutan)	142	SLCLLILDSLSAFYWIDRVNGGESVNLQESTLRKCSQCLEKLVNDYHLVL	191

Figure 2 - Multiple protein sequence alignment of a selected region of *Homo sapiens* XRCC2 with that of other species. Highlighted amino acids represent Ser^{150} and Arg^{188} of *H. sapiens* XRCC2 compared with other species.

were not clearly distinguishable (Cai *et al.*, 2010). We therefore used PCR-ARMS to detect this SNP in other samples. Ser^{150}, which is involved in the formation of the ATP binding site in XRCC2, is highly conserved among species (Figure 2). Consequently, an Arg substitution at this position (replacing a neutral amino acid by a positively charged one) would most probably affect the formation of the ATP binding site. However, protein expression studies are needed to confirm this hypothesis.

The major limitation of this study is the sample size, which may be due to lesser probability of detecting any rare mutations in smaller sample size. On the other hand, for detected SNP, the larger sample size would make the results more significant. In this regard, the coexistence of other members of the *Rad51* gene family with an impaired function alongside $Arg^{188}His$ should be considered since these proteins are also involved in the HR repair system. For example, Bastos *et al.* (2009) showed that the coexistence of three or more variant alleles in the *RAD51* and *XRCC3* genes was associated with a significantly higher risk of DTC (OR = 2.9, p = 0.036; four variant alleles: adjusted OR = 8.0, p = 0.006), while no associations were observed for polymorphisms in *XRCC3* alone (Bastos *et al.*, 2009; Garcia-Quispes *et al.*, 2011).

In conclusion, our findings indicate that there is no association between polymorphisms in the *XRCC2* coding region and the risk of DTC. However, further studies with a larger population are needed to confirm this conclusion. In addition, it would be prudent for other members of the *Rad51* gene family also to be screened for SNPs in their coding region. Finally, HRM analysis was found to be very useful for genotyping, although it was unable to distinguish between G::C and A::T conversions because of the low melting curve transition. Other complementary methods are needed to overcome this limitation.

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