Original Article

Association of RB1 rs9568036 and CDKN1A rs1801270 Polymorphisms with Retinoblastoma Susceptibility

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Abstract

Purpose: To investigate the association of polymorphisms (rs9568036 and rs1801270) in the *RB1* and *P21* genes with susceptibility to retinoblastoma (RB).

Methods: This case–control study was designed with 50 patients with RB and 50 controls. Polymerase chain reaction was performed to amplify the intron 17 of *RB1* rs9568036 and exon 2 of *P21* rs1801270. Then, all the amplified fragments were subjected to directional sequencing, and finally, the association between genotypes and the development of RB risk and invasion was studied.

Results: A statistically significant difference in genotypic or allele frequencies of single-nucleotide polymorphisms (SNPs) (rs1801270 and rs9568036) was found between Iranian RB patients and the controls ($P > 0.05$). However, the frequency of genotype *RB1* rs9568036 observed a statically significant difference in the RB patients compared to the control group, and the nonwild-type allele A increased the chance of susceptibility to developing RB by 2.92 times.

Conclusion: The rs9568036 SNP in the *RB1* gene may increase susceptibility to the development of RB in the affected patients. In spite of that, this polymorphism does not influence RB patient's invasion. Further investigation with a large enough sample size is recommended to validate this hypothesis.

Keywords: CDKN1A rs1801270, Genetics, Polymorphism, RB1 rs9568036, Retinoblastoma

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Introduction

Retinoblastoma (RB) (OMIM#180200) is the most frequent intraocular tumor in children, with 5000–8000 new pediatric cases every day worldwide. In 95% of cases, including both hereditary (40%) and sporadically (60%) types, it affects children under 5 years[1-3] by inactivating two alleles of the *RB1* gene in the 13q14 locus via translocations, point mutations, insertions, and deletions (INDELs).[4,5] In the hereditary type, these mutations are present in the germline, but they occur only in the tumor tissue in the sporadic type.[6,7]

Deletion of members of the RB family results in a compensatory increase in the 21 kD protein (p21) (cyclin-dependent kinase inhibitor),[8] which the *CDKN1A* gene encodes at 6p21.2. After activation of p53 in response to cellular DNA damage, the p21 protein is upregulated. p21 is an important protein that plays a vital role in cell cycle check by inhibiting the activity of cyclin complexes (E/cdk2 and A/cdk2).[9,10]

Polymorphisms are present in all individuals, and because they are kind of changes in the genomic sequence, they are always passed on to the next generation. In fact, the reason they are called polymorphisms is that although they are changes in everyone's genomes, they often do not cause a specific disease or phenotype. However, some of them can be associated with some traits or diseases such as RB. The

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most common type of polymorphism is single-nucleotide polymorphisms (SNPs). These changes can either protect people or make them susceptible to certain diseases. In RB, in addition to mutations in the *RB1* gene, polymorphisms have been reported in several genes that are associated with disease onset and invasiveness.[11,12]

p21 SNP converts serine to arginine in codon 31 (rs1801270 C > A), which is part of the zinc finger region with highly conserved,[13] it can lead to a lack of p21 accumulation during cellular DNA damage.[14] Thus, this *p21* SNP may affect the activity and expression of p21. As a result, it disrupts the activity of the p53 pathway and plays a role in susceptibility to different types of cancers, including RB.[15,16]

In the following, *RB1* rs9568036 and *P21* rs1801270 were selected for this experiment from the HapMap SNP database. For the first time, we studied these SNPs in the *RB1* and *P21* genes to investigate their association with RB cancer risk and invasiveness in an Iranian population.

Methods

Our study included 50 RB patient samples (36 bilateral and 14 unilateral) who were treated between April 2018 and August 2020. The 50 control samples were selected from normal individuals (25 females and 25 males) with similar socioeconomic status and geographical origins that had been referred to the hospital for examination of other problems (without eye involvement). Clinical data of the RB patients (gender, age-onset of disease, laterality, status of global, focality, and international classification of retinoblastoma (ICRB) classification was collected from their medical records.

Our study with the ethics code 1398.723 was approved by the Ethical Committee of the Iran University of Medical Sciences, and written informed consent was received from the caregivers of the patient and control groups.

Three ml of blood samples was collected in EDTA anticoagulant tubes from all the participants.

Genomic DNA was extracted from peripheral blood samples using extraction kit (BIORAN AccuPrepk, Germany). Then, the extracted DNA was preserved at −20°C for the next step of the study. A spectrophotometer (Thermo Scientific™ NanoDrop™ 2000/2000c, USA) was used to measure the amount, purity, and concentration of the extracted DNA.

The exon 2 of the *CDKN1A* gene and intron 17 of the *RB1* gene fragments were amplified using gene-specific oligonucleotide primers. The primer sequences and polymerase chain reaction (PCR) conditions are shown in Table 1.

PCR was conducted in a 30 µl mixture containing 10 pM of each primer, 200 ng of genomic DNA, 25 µl of 2× Master Mix containing Taq DNA polymerase, MgCl2, dNTPs, and reaction buffers. PCR products were conducted in 1% agarose gel electrophoresis with constant V90 voltage and alternating current for 60 min with GelRed and then observed under ultraviolet light.

Then, 25 µl of each PCR product with forward primer for direct sequencing of *P21 SNP* and *RB1 SNP* (exon 2 and intron 17, respectively) was sent to South Korea. All samples were sent for Sanger sequencing on an ABI 3730 sequencer (Bioneer, South Korea), and the results were analyzed using Chromas and Bio Edit software.

Electropherograms were used to find the polymorphisms in sense directions.

Statistical analysis

Molecular data were analyzed using logistic regression ($P < 0.05$ was assumed to be statistically significant). Odds ratios (ORs) were checked with a 95% confidence interval (CI) using SPSS version 22 software (IBM Corp. Armonk, NY, USA). The Hardy–Weinberg equilibrium (HWE) was measured by the Guo and Thompson method.[17]

Results

Fifty RB patients (mean of 22.5 ± 17 months) and the control group of 50 individuals without RB (mean of 20.5 ± 17 months), with age onset of disease of 0–60 months, were included in the study. Both the groups had an almost equal ratio of both sexes. According to the ICRB, 15 eyes

Table 1: Primer sequences of polymorphisms and polymerase chain reaction conditions for target amplification

Polymorphism	Sequence (5'-3')	Product length (bp)	PCR conditions
RB1 rs9568036	F[a]: GGGTCTAAGGGAGGGATAGC R[b]: GCCTTCCTGAGTTGTAGCTCTC	510	ID[c]: 95°C/3 min (1 cycle) D[d]: 94°C/1 min (40 cycles) A[e]: 57°C/30 s (40 cycles) E[f]: 72°C/30 min (40 cycles) FE[g]: 72°C/5 min (1 cycle)
p21 rs1801270	F: AGGGCCTTCCTTGTATCTCTG R: TCTGAGAATCCTGGTCCCT	526	ID: 95°C/3 min (1 cycle) D: 94°C/1 min (40 cycles) A: 56°C/30 s (40 cycles) E: 72°C/30 min (40 cycles) FE: 72°C/5 min (1 cycle)

[a]Forward primer, [b]Reverse primer, [c]Initial denaturation, [d]Denaturation, [e]Annealing, [f]Extension, [g]Final extension. PCR: Polymerase chain reaction, RB: Retinoblastoma

from 50 patients in the RB group were enucleated, including 9 eyes (60%) in Group D and 6 eyes (40%) in Group E.

Genotype distribution and the frequencies of allelic variants of *RB1* and *CDKN1A* genes in patients and controls are presented in Table 2. Figure 1a-f demonstrates the sequencing results using Chromas software.

The polymorphisms studied in this experiment were observed in the HWE in the control groups. There was a significant difference between the minor allele frequency (MAF) of the two selected SNPs between RB patients and the control group (all $P > 0.05$).

In RB patients, the frequency of nonwild-type allele A for rs9568036 was significantly higher than in the control group (OR = 2.92, 95% Confidence interval [CI]: [0.35–4.3], $P = 0.014$). Conversely, there was no significant difference in RB patients compared to the control groups for the frequency of nonwild allele A in rs1801270 (OR = 1.21, 95% CI: [0.37–3.8], $P = 0.766$).

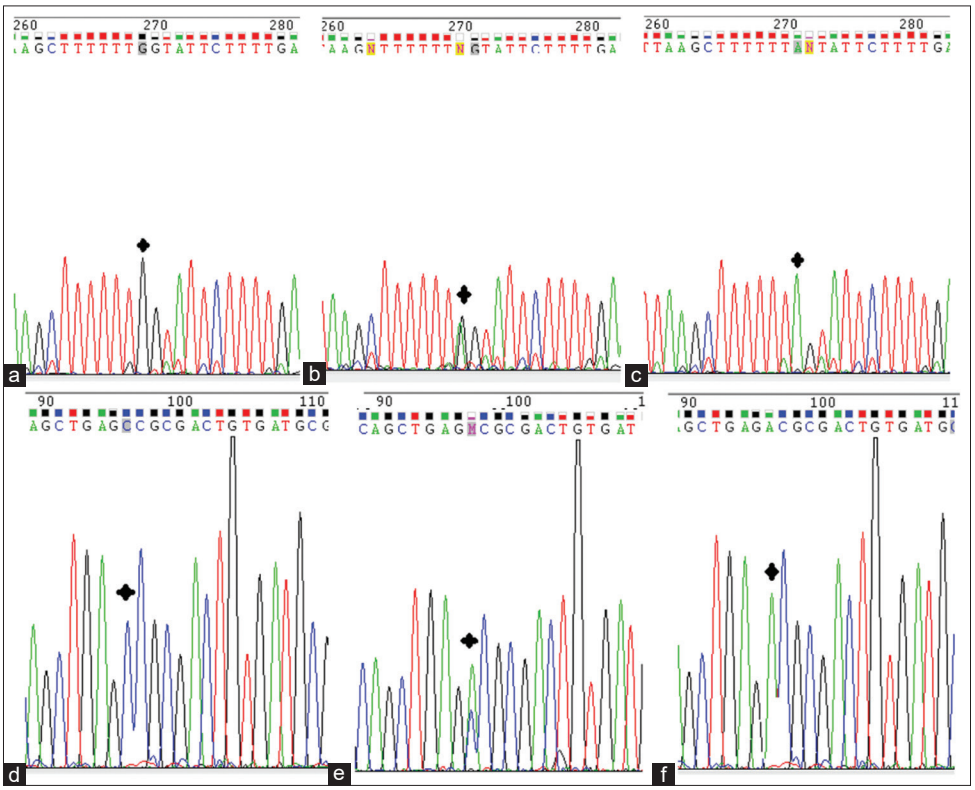

Figure 1: Sequence chromatogram of some individuals showing single nucleotide polymorphism (SNP) sites. The SNP sites are indicated by symbol*. (a) Homozygous wild-type for *RB1* rs9568036 (G > A); (b) Heterozygous nonwild-type for *RB1* rs9568036 (G > A); (c) homozygous nonwild-type for *RB1* rs9568036 (G > A); (d) Homozygous wild-type for *p21* rs1801270 (C > A); (e) Heterozygous nonwild-type or *p21* rs1801270 (C > A); (f) Homozygous nonwild-type for *p21* rs1801270 (C > A)

Table 2: Genotypes distribution and allele frequencies for *p21* rs1801270 and *RB1* rs9568036 polymorphisms

Polymorphisms	Group (*n*)	Homozygous for nonwild-type (%)	Heterozygous (%)	Homozygous for wild-type allele (%)	Allele	*P*
p21 rs1801270 (C>A)	Case (50)	AA	CA	CC	A	0.766
		1 (2)	5 (10)	44 (88)	0.07	1.21 (0.37-3.8)
	Control (50)	2 (4)	7 (14)	41 (82)	0.1	
RB1 rs9568036 (G>A)	Case (50)	AA	GA	GG	A	**0.014**
		16 (32)	22 (44)	12 (24)	0.5	2.92 (1.24-6.8)
	Control (50)	1 (2)	25 (50)	24 (48)	0.3	
Compound heterozygous	Case (50)	Yes	No			0.75
		6	44			1.22 (0.35-4.3)
	Control (50)	5	45			

Statistically significant data are shown in bold. RB: Retinoblastoma

Similarly, there was no statistically significant difference between the frequency of the combination of polymorphisms *RB1* rs9568036 and *p21* rs1801270 in the RB patients and the control group (OR = 1.22, 95% CI: [0.35–4.3], $P = 0.75$).

After establishing the above information, we examined the association of significant polymorphism (*RB1* rs9568036) and *p21* rs1801270 with RB invasion in Iranian patients using logistic regression analysis. The results are shown in Table 3 and Supplementary Table 1.

There was no statistically significant association between *RB1* rs9568036 polymorphism and gender ($P = 0.730$), laterality of the disease (unilateral or bilateral) ($P = 0.638$), age onset of disease (<24 and >24) ($P = 0.164$), enucleating or preserved globe ($P = 0.125$), or in terms of focality (unifocal or multifocal) involvement ($P = 0.979$). Similarly, we found no association between *p21* rs1801270 and the risk of RB invasion [Supplementary Table 1].

Finally, we analyzed the frequencies of the *RB1* rs9568036 in the nonwild-type allele A based on ICRB classification. The results were as follows: 3 A (6%), 12 B (24%), 9 C (18%), 6 D (12%), and 8 E (16%). Furthermore, in the wild-type allele G, the frequencies of this SNP were 3 A (6%), 2 B (4%), 2 C (4%), 3 D (6%), and 2 E (4%). Similarly, the nonwild-type allele A in *p21* rs1801270 was 2 A (4%), 1 B (2%), and 3 C (6%), and the wild-type allele C was 18 A (36%), 13 B (26%), 9 C (18%), 2 D (4%), and 2 E (4%).

Discussion

RB is a malignant eye cancer that affects children under 5 years old.[1] In addition to translocations, point mutations, and INDLs, genetic polymorphisms can play a role in the development of this type of cancer as well.[4,5]

To the best of our knowledge, this is the first study that has evaluated the SNPs rs9568036 and rs1801270 in p53 and RB signaling pathways in the Iranian. Our results for *RB* rs9568036 revealed that the frequency of the nonwild-type A allele in patients was higher than in the controls. Compatible to our study, Anaya-Pava *et al*. also showed that the frequency of the nonwild-type A allele in patients with RB was higher than control.[18] We found that allele A in the nonwild-type increases the chance of developing RB by 2.92 fold. However, our results did not display any association between RB rs9568036 and RB invasion. Published studies on polymorphism *RB1* rs9568036 have demonstrated that it was not only associated with better tumor response to chemotherapy[18] but also can result in increased survival rates of nonsmall lung cancer cells patients.[19]

Although for *p21* rs1801270, our finding did not reveal any statistically significant difference in frequency of the nonwild-type A allele in patients compared to the control group, Carvalho *et al*. reported that the frequency of the nonwild-type A allele in patients was greater than in controls.[16] The frequency of the A allele (4.5) was relatively high in RB patients in Chen *et al*.'s report, compared to our study (0.07).[20]

Table 3: The association between the *RB1* rs9568036 with sex, laterality, diagnosis age, status of global and focality

RB1 rs9568036	Gender		*P*	Laterality		*P*	Diagnosis age		*P*	Status of global		*P*	Focality		*P*
	Male	Female	OR (95% CI)	Unilateral	Bilateral	OR (95% CI)	<24	>24	OR (95% CI)	Enucleating	Survival	OR (95% CI)	Unifocal	Multifocal	OR (95% CI)
GG (12)	7	5	0.730	4	8	0.638	11	1	0.164	4	8	0.125	7	5	0.979
GA + AA (38)	20	18	1.26 (0.33-4.6)	10	28	1.40 (0.34-5.6)	26	11	4.65 (0.53-40.5)	5	33	3.3 (0.71-15.16)	22	16	1.01 (0.27-3.8)

OR: Odds ratio, CI: Confidence interval

The frequency assessment indicates the MAFs of rs1801270 among ethnic groups displays a wide range of difference from 0.04 in Swedish Caucasians to 0.5 in Chinese. Relatively higher prevalence was observed in African and Indians than in Swedish Caucasians.[21] Similar to our study, a meta-analysis of case–control studies by Cao *et al*. indicated no statistically significant association between *p21* rs1801270 polymorphism and susceptibility to RB.[22]

In contrast to several studies reporting that *p21* rs1801270 SNP is associated with the increased risk of various types of cancer,[23-26] in our study, the difference was not statistically significant for the chance of developing RB in the presence of the nonwild-type allele A of *p21* rs1801270. In addition, Carvalho *et al*. found an increased RB risk in the CA genotype of Brazilian carriers.[16]

Despite a 38% decrease in p21 expression due to SNP rs1801270,[26] in our study, no statistically significant association was found between *p21* rs1801270 and RB invasion.

We also investigated the association between the double heterozygous polymorphisms (rs9568036 [G > A] and rs1801270 [C > A]) and susceptibility to RB and invasion. No association was found using logistic regression analysis on double heterozygotes between a group of patients and control children.

The first major limitation of the study is the selection of controls, which may be an important source of bias, and the second limitation is the small sample size that reduces the available statistical power, which makes it difficult to study low-frequency genotypes as well as any small effect size SNPs.

In summary, based on these results, we demonstrate that the minor alleles of the polymorphism *RB1* rs9568036 may play as risk factors for the development of RB in our samples. However, it does not seem to affect the invasion. Further functional studies on larger samples are needed for different ethnic groups to confirm that this polymorphism does indeed affect the development of RB.

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Conflicts of interest

There are no conflicts of interest.

References

1. de Aguirre Neto JC, Antoneli CB, Ribeiro KB, Castilho MS, Novaes PE, Chojniak MM, *et al*. Retinoblastoma in children older than 5 years of age. Pediatr Blood Cancer 2007;48:292-5.
2. Abramson DH. Retinoblastoma in the 20th century: Past success and future challenges the Weisenfeld lecture. Invest Ophthalmol Vis Sci 2005;46:2683-91.
3. Balmer A, Zografos L, Munier F. Diagnosis and current management of retinoblastoma. Oncogene 2006;25:5341-9.
4. Goodrich DW, Lee WH. Molecular characterization of the retinoblastoma susceptibility gene. Biochim Biophys Acta 1993;1155:43-61.
5. Leiderman YI, Kiss S, Mukai S. Molecular genetics of RB1-the retinoblastoma gene. Semin Ophthalmol 2007;22:247-54.
6. Dryja TP, Mukai S, Petersen R, Rapaport JM, Walton D, Yandell DW. Parental origin of mutations of the retinoblastoma gene. Nature 1989;339:556-8.
7. Kato MV, Ishizaki K, Shimizu T, Ejima Y, Tanooka H, Takayama J, *et al*. Parental origin of germ-line and somatic mutations in the retinoblastoma gene. Hum Genet 1994;94:31-8.
8. Conkrite K, Sundby M, Mukai S, Thomson JM, Mu D, Hammond SM, *et al*. miR-17 ~92 cooperates with RB pathway mutations to promote retinoblastoma. Genes Dev 2011;25:1734-45.
9. Harper JW, Adami GR, Wei N, Keyomarsi K, Elledge SJ. The p21 Cdk-interacting protein Cip1 is a potent inhibitor of G1 cyclin-dependent kinases. Cell 1993;75:805-16.
10. el-Deiry WS, Tokino T, Velculescu VE, Levy DB, Parsons R, Trent JM, *et al*. WAF1, a potential mediator of p53 tumor suppression. Cell 1993;75:817-25.
11. Porta M. The genome sequence is a jazz score. Int J Epidemiol 2003;32:29-31.
12. Kadam-Pai P, Su XY, Miranda JJ, Soemantri A, Saha N, Heng CK, *et al*. Ethnic variations of a retinoblastoma susceptibility gene (RB1) polymorphism in eight Asian populations. J Genet 2003;82:33-7.
13. Mousses S, Ozçelik H, Lee PD, Malkin D, Bull SB, Andrulis IL. Two variants of the CIP1/WAF1 gene occur together and are associated with human cancer. Hum Mol Genet 1995;4:1089-92.
14. Johnson GG, Sherrington PD, Carter A, Lin K, Liloglou T, Field JK, *et al.* A novel type of p53 pathway dysfunction in chronic lymphocytic leukemia resulting from two interacting single nucleotide polymorphisms within the p21 gene. Cancer Res 2009;69:5210-7.
15. Liu F, Li B, Wei Y, Chen X, Ma Y, Yan L, *et al*. P21 codon 31 polymorphism associated with cancer among white people: Evidence from a meta-analysis involving 78,074 subjects. Mutagenesis 2011;26:513-21.
16. Carvalho IN, Reis AH, Cabello PH, Vargas FR. Polymorphisms of CDKN1A gene and risk of retinoblastoma. Carcinogenesis 2013;34:2774-7.
17. Guo SW, Thompson EA. Performing the exact test of Hardy-Weinberg proportion for multiple alleles. Biometrics 1992;48:361-72.
18. Anaya-Pava EJ, Nares-Cisneros J, Cárdenas-Hernández RI, Jaramillo-Rodríguez Y, Zambrano-Galván G. Study of polymorphisms in the TP53 and RB1 genes in children with retinoblastoma in northern Mexico. Mol Vis 2017;23:20-5.
19. Liu D, Xu W, Zhang ZW, Qian J, Zheng H, Zhang J, *et al.* RB1 polymorphism contributes to the efficacy of platinum-taxanes in advanced squamous cell lung cancer. Asian Pac J Cancer Prev 2015;16:775-81.
20. Chen R, Liu S, Ye H, Li J, Du Y, Chen L, *et al.* Association of p53 rs1042522, MDM2 rs2279744, and p21 rs1801270 polymorphisms with retinoblastoma risk and invasion in a Chinese population. Sci Rep 2015;5:13300.
21. Birgander R, Själander A, Saha N, Spitsyn V, Beckman L, Beckman G. The codon 31 polymorphism of the p53-inducible gene p21 shows distinct differences between major ethnic groups. Hum Hered 1996;46:148-54.
22. Cao Q, Wang Y, Song X, Yang W. Association between MDM2 rs2279744, MDM2 rs937283, and *p21* rs1801270 polymorphisms and retinoblastoma susceptibility. Medicine (Baltimore). 2018;97:e13547.
23. Ebid GT, Sedhom IA, El-Gammal MM, Moneer MM. MDM2 T309G has a synergistic effect with P21 ser31arg single nucleotide polymorphisms on the risk of acute myeloid leukemia. Asian Pac J Cancer Prev 2012;13:4315-20.
24. Valentin MD, Canalle R, Queiroz Rde P, Tone LG. Frequency of polymorphisms and protein expression of cyclin-dependent kinase inhibitor 1A (CDKN1A) in central nervous system tumors. Sao Paulo Med J 2009;127:288-94.
25. Akhter N, Dar SA, Haque S, Wahid M, Jawed A, Akhtar MS, *et al*. Crosstalk of Cyclin-dependent kinase inhibitor 1A (CDKN1A) gene polymorphism with p53 and CCND1 polymorphism in breast cancer. Eur Rev Med Pharmacol Sci 2021;25:4258-73.
26. Su L, Sai Y, Fan R, Thurston SW, Miller DP, Zhou W, *et al*. P53 (codon 72) and P21 (codon 31) polymorphisms alter *in vivo* mRNA expression of p21. Lung Cancer 2003;40:259-66.

Supplementary Table 1: The relation between the *p21* rs1801270 with sex, laterality, diagnosis age, status of global and focality

p21 rs1801270	Gender		*P* OR (95% CI)	Laterality		*P* OR (95% CI)	Age diagnosis		*P* OR (95% CI)	Status of global		*P* OR (95% CI)	Focality		*P* OR (95% CI)
	Male	Female		Unilateral	Bilateral		<24	>24		Enucleating	Survival		Unifocal	Multifocal	
CC (44)	25	19	0.292	14	30	0.458	31	13	0.894	13	31	0.324	21	23	0.135
CA + AA (6)	2	4	2.63 (0.43-15.9)	1	5	2.33 (0.24-21.8)	4	2	1.19 (0.19-7.3)	3	3	0.41 (0.07-2.3)	5	1	0.18 (0.02-1.6)

OR: Odds ratio, CI: Confidence interval