Molecular Vision 2017; 23:20-25 <http://www.molvis.org/molvis/v23/20>
Received 5 September 2016 | Accepted 26 January 2017 | Published 28 January 2017

Study of polymorphisms in the *TP53* and *RB1* genes in children with retinoblastoma in northern Mexico

Edwin J. Anaya-Pava,[1] Jesús Nares-Cisneros,[2] Rubén I. Cárdenas-Hernández,[3] Yolanda Jaramillo-Rodríguez,[4] Graciela Zambrano-Galván[5]

[1]Retina Deparment, Instituto de la Visión, Hospital La Carlota, Montemorelos, Nuevo León, México; [2]Department of Pediatric Gastroenterology, High Specialty Medical Unit No. 71, Mexican Institute of Social Security, Torreón, Coahuila, México; [3]Departament of Ophthalmology, High Specialty Medical Unit No. 71, Mexican Institute of Social Security, Torreón, Coahuila, México; [4]Department of General Pathology and Health Research Division, High Specialty Medical Unit No. 71, Mexican Institute of Social Security Torreón, Coahuila, México; [5]Laboratory of Molecular Stomatology, Faculty of Dentistry, Juarez University of Durango State, Durango, Durango, México

Purpose: To determine the frequency and association of polymorphisms in the *TP53* and *RB1* genes with clinical characteristics in a group of children with retinoblastoma (RB) in northern Mexico.
Methods: A prospective, longitudinal, and analytical study of 11 patients diagnosed with RB was conducted. Endpoint PCR and high-resolution real-time PCR were performed. Chi-square and Student *t* tests were used to evaluate associations between variables. Allelic frequencies, as well as genotypic and Hardy–Weinberg equilibriums, were evaluated using Guo and Thompson's method.
Results: We found a statistically significant difference between the polymorphism RB1-GG/rs9568036 and tumor chemoresistance ($p<0.05$). The allelic variants RB1-AA and AG/rs9568036 were determined to be associated with tumor chemosensitivity ($p<0.05$). A statistically significant relation between the polymorphism RB1-GG/rs9568036 and males ($p = 0.0386$), rate ratio (RR) = 2.0 (95% confidence interval [CI] = 0.76–5.32), as well as between the allelic variants RB1-AA and AG/rs9568036 and females ($p = 0.0027$), RR = 8.0 (95% CI = 1.28–50.04), was observed. We also observed a statistically significant association between the rs1042522 polymorphism in the *TP53* gene and unilateral presentation of the disease.
Conclusions: The rs9568036 polymorphism in the *RB1* gene and the allelic variants can be associated with type of response to medical therapy and associated with male sex, while the allelic variant rs1042522 polymorphism in the *TP53* gene is associated with the unilateral presentation of the disease in a group of Mexican children with RB.

Retinoblastoma (RB) is a primary intraocular tumor that is most commonly malignant in childhood; this condition arises from primitive neural retinal cells destined to become photoreceptors and corresponds to 11% of all cancers in the first year of life [1,2]. The incidence of RB worldwide is estimated at between 5,000 and 8,000 new cases per year, and although RB can occur at any age, this tumor occurs most often in preschool children (95% of cases diagnosed before 5 years of age) [3-5]. The primary genetic event associated with RB is the inactivation of both alleles of the *RB1* gene (Gene ID: 5925, OMIM 614041) in the 13q14 locus by translocations, deletions, insertions, and point mutations [6,7].

Genetically, RB presents as hereditary (40%) or non-hereditary (60%). In the inherited form, a constitutional mutated allele is always transmitted via germs; meanwhile, in the non-hereditary form, both alleles are inactivated by somatic mutations [8,9].

Correspondence to: Edwin J Anaya-Pava, Camino al Vapor 209, CP 67512, Montemorelos, Nuevo León, México; Phone: +52 (826) 263-31-88; FAX: +52 (826) 263-34-04; email: ejanayap@hotmail.com

The *TP53* gene (Gene ID: 7157, OMIM 191170) is located at 17p13.1 and provides instructions for making the p53 tumor protein (p53); this protein acts as a tumor suppressor and binds directly to DNA. When p53 is damaged, other genes are activated to repair the damage. If the DNA cannot be repaired, this protein prevents the cell from dividing and sends signals for apoptosis; thus, the protein has been nicknamed the "guardian of the genome" [10,11].

Genetic polymorphisms are variants of genome mutations that appear in certain individuals. These polymorphisms are transmitted to offspring and acquire a certain frequency in the population after many generations. The most common polymorphisms are single base changes, known as single nucleotide polymorphisms (SNPs) [12,13]. The aim of this study was to determine the frequency and association between the SNP rs9568036 (the *RB1* gene) and the SNP rs1042522 (TP53) and clinical characteristics in a group of Mexican children with RB.

Table 1. Polymorphisms and sequences of *RB1* and *TP53*.

Polymorphism	Sequence (5'-3')	Amplicon size
RB1 (rs9568036)	F: AAGGGAGGGATAGCATTAGGA R: GAATGTGCAGGTTTGTTACATAGG	105 bp
TP53 (rs1042522)	F: CATCACACCCTCAGCATCTC R: GCCTGGTCAGGAGCTTATTT	97 bp

METHODS

A total of 11 pediatric patients under 10 years of age with a diagnosis of RB confirmed by histopathological studies who presented at our hospital between March 2015 and August 2015 for close follow-up exams after completion of chemotherapy were recruited for polymorphism detection (men 63.6%, mean diagnosis age=16 months, range 3-37; woman 36.4%, mean diagnosis age=20 months, range 4-42). The mean duration of follow-up after diagnosis was 30 months (range 4-87). We excluded patients who had received blood transfusions in the previous 6 months or those who had received an organ donation. The study was approval by the Local Research Ethics Committee of High Specialty Medicine Unit, Specialty Hospital (UMAE) No. 71 of Mexican Institute of Social Security (IMSS) with code registered R2015–501–70 20, and informed consent was obtained from the responsible caregiver for all cases. The study was conducted in accordance with the Declaration of Helsinki and in compliance with the laws and regulations of the Mexican General Law of Health in Research for Health. Fifteen microliters of venous blood of patients with RB were obtained, placed in 100 µl of cell lysis solution, and immediately stored at 2 °C.

Polymorphism analysis was performed by amplification of the polymorphic site with conventional PCR using GoTaq Master Mix (Promega Inc., Madison, WI) to obtain similar amounts of amplification products from each sample. The 15 µl reaction contained 7.5 µl GoTaq Master Mix, 5.7 µl water, 0.3 µl the each primer (AAG GGA GGG ATA GCA TTA GGA (Sense), GAA TGT GCA GGT TTG TTA CAT AGG (AntiSense) for RB1(rs9568036) and CAT CAC ACC CTC AGC ATC TC (Sense), GCC TGG TCA GGA GCT TAT TT (AntiSense) for TP53 (rs1042522)) and 1.2 µl blood lysate. High-resolution melt (HRM) analysis, which is based on the characterization of the PCR products according the dissociation behavior of the DNA strand, was performed because this method is sensitive even to a simple change of base. A melting temperature (Tm) of 55 °C to 95 °C was used to cover all melting points according to the exchange rate base. A second amplification was performed using real-time PCR. The conditions of the conventional PCR amplification were: initial denaturation at 94 °C for 10 min, followed by an amplification of 55 cycles of 95 °C / 45 s; 54 °C / 45 s; 72 °C / 45 s. The reaction mixture for each sample (15 µl) was prepared according to the manual accompanying the HRM Type-it reagent (Qiagen Inc., Germantown, MD), and contained 7.5 µl, 5.9 µl water, 0.3 µl sense primer, 0.3 µl antisense primer, and 1 µl PCR product (Table 1). The conditions of a second amplification using real-time PCR were the same that conventional PCR, only that they used 20 cycles for both polymorphisms; for the analysis of the melt curves a RotorGene thermocycler (Quiagen Inc. Germantown, Maryland) was used with HRM channel. DNA melting curve analysis between 55 °C and 90 °C for both genes was used as an internal control to prevent false-negative or false-positive results (Figure 1).

The SNPs rs9568036 (RB1) and rs1042522 (TP53) selected for the study were taken from the HapMap SNP database and were genotyped by iSelect HD Bead-Chip (Illumina, San Diego, CA). Statistical analysis included measures of central tendency and dispersion (p values less than 0.05 were considered significant), and the chi-square and Student *t* tests were used to determine the association between variables using SPSS version 19.0 software (SPSS Inc., Chicago, IL). Genotype and allele frequencies, as well as Hardy–Weinberg equilibrium, were evaluated using Guo and Thompson's method [14].

RESULTS

The frequencies of genotypes and alleles obtained for each of the examined SNPs are presented in Table 2. There was a statistically significant difference ($p<0.05$) between the frequency of the genotypes and the expected genotypes in the population according to the Hardy–Weinberg equilibrium (Table 3). There was a statistically significant difference between the RB1-GG/rs9568036 polymorphism and tumor chemoresistance, as well as between the RB1-AA and AG/rs9568036 polymorphisms and tumor chemosensitivity (Table 4).

There was no statistically significant association ($p = 0.6576$) between the RB1/rs9568036 polymorphism and any allelic variants with unilateral or bilateral disease

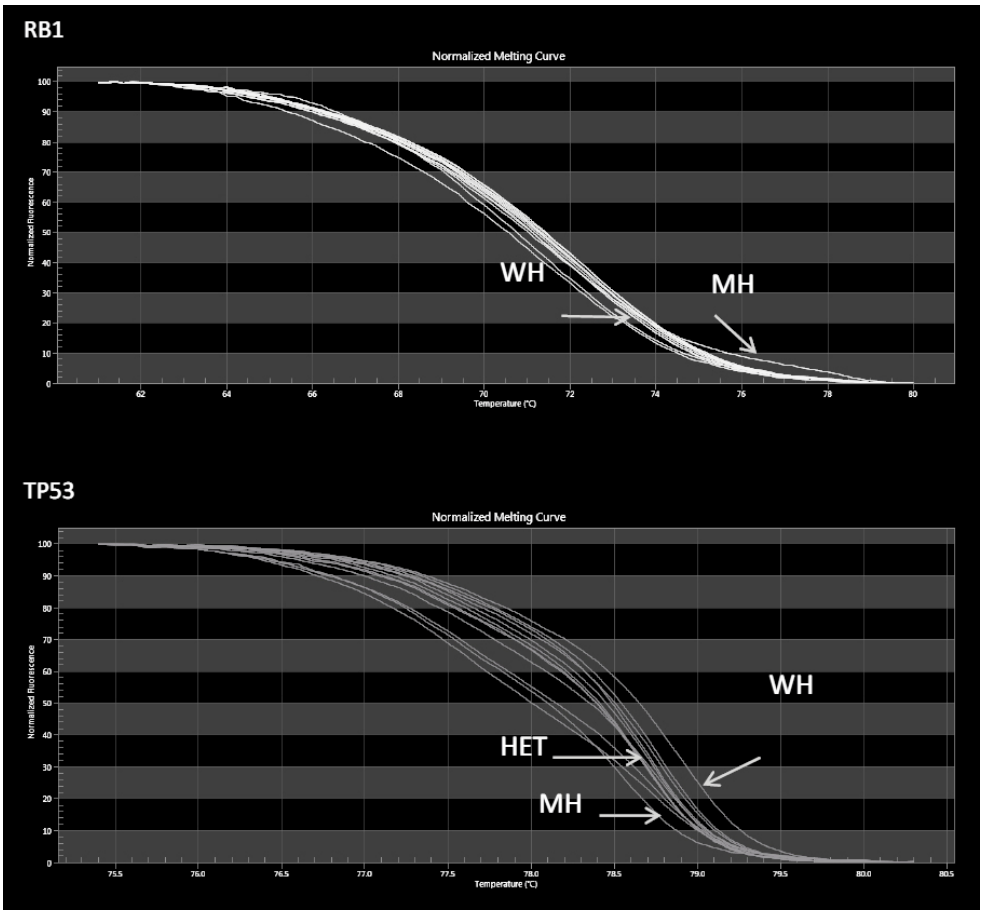

Figure 1. DNA melting curve analysis for the *RB1* and *TP53* genes. The change in fluorescence with increasing temperature was measured. As the temperature increased, the strands of the amplicon separate to form single-stranded DNA, causing the fluorescent intercalating dye to dissociate from the DNA and stop fluorescing. HET, heterozygote; WH, wild homozygous; MH, mutated homozygote.

Table 2. Genotype and allele frequencies of the analyzed SNPs.

SNP	Genotype	Frequency	p value	Allele	Frequency
rs9568036/RB1	Wild Homozygous AA	0.182	<0.001	A	0.9091
	Heterozygote AG	0		G	0.0909
	Mutated Homozygote GG	0.818			
rs1042522/TP53	Wild Homozygous CC	0.818	<0.001	C	0.875
	Heterozygote CG	0.91		G	0.125
	Mutated Homozygote GG	0.91			

Table 3. Distribution of genotypes for *RB1* and *TP53* genes, and Hardy–Weinberg equilibrium (HWE).

Alleles	AA	Aa	aa	p value
RB1 observed	10	0	1	<0.001
RB1 expected by HWE	9.090909	1.818182	1.818182	
TP53 observed	9	1	1	0.02
TP53 expected by HWE	9.1875	2.625	0.1875	

Table 4. Correlation between genotype - phenotype allelic variants of rs9568036 in the *RB1* gene and rs1042522 in the *TP53* gene polymorphisms.

Characteristic	Polymorphism RB1-GG/ rs9568036	Polymorphism RB1-AA and AG/ rs9568036	p value RB1	Polymorphism TP53-CC/ rs1042522	Polymorphism TP53-CG and GG/rs1042522	p value TP53
Leukocoria n=11/11	15* (range 1-42)	10* (range 8-12)	0.194	14.2* (range 1-42)	13.5* (range 8-19)	0.4645
Strabismus n=5/11	8* (range 3-14)	8*	0.3746	7.6* (range 3-14)	8.5* (range 8-9)	0.4121
Ocular hypertension n=5/11	20.75* (range 13-37)	15*	0.2315	21.3* (range 13-37)	17* (range 15-19)	0.3202
Diagnosis age	18* (range 3-42)	18*	0.4664	17.3* (range 3-42)	19* (range 18-20)	0.3726
Chemotherapy sessions	7.22	4	0.0431	6.7	8	0.244
Metastasis	0	1	N	0	1	NA

*months

presentation. There was a statistically significant relation between unilateral presentation of the disease and the polymorphism TP53-CC/rs1042522 (p = 0.0386), rate ratio (RR) = 2.0 (95% CI = 0.75–5.32), but the allelic variants TP53-CG and GG/rs1042522 had no relation with the laterality of the disease (p = 0.4611). There was a statistically significant association between the polymorphism RB1-GG/rs9568036 and men (p = 0.0386), RR = 2.0 (95% CI = 0.76–5.32), as well as between the RB1-AA and AG/rs9568036 polymorphisms and women (p = 0.0027), RR = 8.0 (95% CI = 1.28–50.04).

DISCUSSION

The *RB1* gene plays an important role in the negative control of cell cycling and tumor progression by translating the Rb protein and inhibiting cell division. In the hypophosphorylated state, the Rb protein is active and prevents the attachment of an E2 factor (E2F) transcription factor (related to DNA replication for division) [15]. Being hyperphosphorylated, the Rb protein receives kinases' phosphate groups and is inactivated, allowing cells to move from the G1 to the S phase and proceed with division. The absence of Rb protein activity, as a result of mutations that inactivate or deregulate its phosphorylated state, causes continuous cell division and leads to a large number of human cancers and metastasis, including cellular response to mitotic inhibition–induced cell death during chemotherapy [16,17].

To have equal concentrations of DNA, the first amplification was performed with end-point PCR, after which the PCR-HRM technique was performed to analyze the polymorphisms. The PCR-HRM technique used to analyze the polymorphisms in this study has been used in different clinical applications and successful investigations even in the analysis of SNPs in different types of cancer [18]. Each double-stranded DNA fragment presents a characteristic fusion pattern, which is determined by the content, length, and position of the CGs in the DNA sequence, thus allowing the detection of minimal alterations in the sequences, making this method faster, simpler, and less expensive compared with other methods used for amplification and quantification of DNA sequences [19,20].

Liu and colleagues studied some polymorphisms of the *RB1* gene and found that in response to platinum- and taxane-based chemotherapy, patients with the polymorphism rs4151510 GG had better survival rates for squamous cell lung cancer, while patients with the polymorphisms rs4151510, rs4151465, and rs9568036 had increased survival rates for non-small lung cancer cells [21]. In this study, we found that the polymorphism RB1/rs9568036 homozygous mutant GG was associated with twice the likelihood of RB in men refractory to conventional chemotherapy, while the SNP rs9568036 homozygous wild AA and heterozygous AG were associated with tumor sensitivity to chemotherapy.

Carvalho et al. found that in RB the polymorphisms rs1801270 C>A and rs1059234 C>T of the *CDKN1A* (Gene ID: 1026, OMIM 116899) gene were associated with an increased risk of RB (odds ratio [OR] = 2.5, 95% CI = 1.38–4.53), whereas patients with the CC genotype for both polymorphisms had a lower risk of RB (OR = 0.43, 95% CI = 0.25–0.74) [22]. In this study, we found that the polymorphism RB1/rs9568036 homozygous wild AA and heterozygous AG were associated with an increased likelihood (8X) of RB in women. Kadam-Pai and colleagues studied the Tsp509I polymorphism of the *RB1* gene in eight Asian populations and found an ethnic variation prevalent in Southeast Asia that confers increased susceptibility to RB [13]. In the present study of a northern Mexican population, we found

that the RB1/rs9568036 polymorphism and the allelic variants RB1-GG/rs9568036, RB1-AA, and AG/rs9568036 were associated with an increased risk for RB.

Furthermore, most of the somatic mutations in the *TP53* gene alter the individual amino acids in p53 leading to the production of an altered version of the protein that cannot bind DNA efficiently. This defective protein can accumulate in the nucleus of cells and prevent them from suffering apoptosis in response to DNA damage. Thus, the damaged cells continue to grow and divide an unregulated manner. In the rs1042522 polymorphism of the *TP53* gene, the substitution G>C exists in codon 72 and exon 4. Han and colleagues found that the GG genotype of the SNP rs1042522 of the *TP53* gene showed increased resistance to first-line chemotherapy compared with those with the genotypes CG or CC (60% versus 27%, p = 0.014) in a study of 148 patients with lung cancer with non-small cell carcinomas [23].

Shabazz and colleagues found a correlation between an increased risk of breast cancer and allelic variants of the rs1042522 polymorphism in women from Bangladesh (p = 0.0053, OR = 1.69) [24]. Meanwhile, Chen et al. reported in a Chinese population that this polymorphism is associated with local tumor invasion [25]. In this study, we found a statistically significant relation between the unilateral presentation of the disease and the same SNP.

Conclusion: The rs9568036 polymorphism in the *RB1* gene and its allelic variants can be associated with the type of response to medical therapy and associated with male sex, while the allelic variant rs1042522 polymorphism in the *TP53* gene is associated with the unilateral presentation of the disease in a group of Mexican children with RB.

REFERENCES

1. Yun J, Li Y, Xu C-T, Pan B-R. Epidemiology and Rb1 gene of retinoblastoma. Int J Ophthalmol 2011; 4:103-9. [PMID: 22553621].
2. Butros LJ, Abramson DH, Dunkel IJ. Delayed diagnosis of retinoblastoma: analysis of degree, cause, and potential consequences. Pediatrics 2002; 109:E45-[PMID: 11875173].
3. de Aguirre Neto JC, Antoneli CB, Ribeiro KB, Castilho MS, Novaes PE, Chojniak MM, Arias V. Retinoblastoma in children older than 5 years of age. Pediatr Blood Cancer 2007; 48:292-5. [PMID: 16847922].
4. Abramson DH. Retinoblastoma in the 20th century: past success and future challenges the Weisenfeld lecture. Invest Ophthalmol Vis Sci 2005; 46:2683-91. [PMID: 16043839].
5. Balmer A, Zografos L, Munier F. Diagnosis and current management of retinoblastoma. Oncogene 2006; 25:5341-9. [PMID: 16936756].
6. Goodrich DW, Lee WH. Molecular characterization of the retinoblastoma susceptibility gene. Biochim Biophys Acta 1993; 1155:43-61. [PMID: 8504130].
7. Leiderman YI, Kiss S, Mukai S. Molecular genetics of RB1–the retinoblastoma gene. Semin Ophthalmol 2007; 22:247-54. [PMID: 18097988].
8. 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. [PMID: 2733786].
9. Kato MV, Ishizaki K, Shimizu T, Ejima Y, Tanooka H, Takayama J, Kaneko A, Toguchida J, Sasaki MS. Parental origin of germ-line and somatic mutations in the retinoblastoma gene. Hum Genet 1994; 94:31-8. [PMID: 8034292].
10. Godefroy N, Lemaire C, Mignotte B, Vayssière JL. p53 and Retinoblastoma protein (pRb): a complex network of interactions. Apoptosis 2006; 11:659-61. [PMID: 16554964].
11. Park J-H, Zhuang J, Li J, Hwang PM. p53 as guardian of the mitochondrial genome. FEBS Lett 2016; 590:924-34. [PMID: 26780878].
12. Porta M. The genome sequence is a jazz score. Int J Epidemiol 2003; 32:29-31. [PMID: 12690001].
13. Kadam-Pai P, Su XY, Miranda JJ, Soemantri A, Saha N, Heng CK, Lai PS. Ethnic variations of a retinoblastoma susceptibility gene (RB1) polymorphism in eight Asian populations. J Genet 2003; 82:33-7. [PMID: 14631100].
14. Guo SW, Thompson EA. Performing the exact test of Hardy-Weinberg proportion for multiple alleles. Biometrics 1992; 48:361-72. [PMID: 1637966].
15. Giacinti C, Giordano A. RB and cell cycle progression. Oncogene 2006; 25:5220-7. [PMID: 16936740].
16. Julian LM, Palander O, Seifried LA, Foster JE, Dick FA. Characterization of an E2F1-specific binding domain in pRB and its implications for apoptotic regulation. Oncogene 2008; 27:1572-9. [PMID: 17891180].
17. Zhao J, Zhang Z, Liao Y, Du W. Mutation of the retinoblastoma tumor suppressor gene sensitizes cancers to mitotic inhibitor induced cell death. Am J Cancer Res 2014; 4:42-52. [PMID: 24482737].
18. Montgomery JL, Sanford LN, Wittwer CT. High resolution DNA melting analysis in clinical research and diagnostics. Expert Rev Mol Diagn 2010; 10:219-40. [PMID: 20214540].
19. Wittwer CT. High-resolution DNA melting analysis: advancements and limitations. Hum Mutat 2009; 30:857-9. [PMID: 19479960].
20. Wang J, Pan X, Liang X. Assessment for Melting Temperature Measurement of Nucleic Acid by HRM. J Anal Methods 2016; 2016:5318935-[PMID: 27833775].
21. Liu D, Xu W, Zhang ZW, Qian J, Zheng H, Zhang J, Su B. RB1 polymorphism contributes to the efficacy of platinum-taxanes in advanced squamous cell lung cancer. Asian Pac J Cancer Prev 2015; 16:775-81. [PMID: 25684524].

22. Carvalho IN, Reis AH, Cabello PH, Vargas FR. Polymorphisms of CDKN1A gene and risk of retinoblastoma. Carcinogenesis 2013; 34:2774-7. [PMID: 24045412].

23. Han JY, Lee GK, Jang DH, Lee SY, Lee JS. Association of p53 codon 72 polymorphism and MDM2 SNP309 with clinical outcome of advanced nonsmall cell lung cancer. Cancer 2008; 113:799-807. [PMID: 18618574].

24. Shabnaz S, Ahmed MU, Islam MS, Islam MR, Al-Mamun MM, Islam MS, Hasnat A. Breast cancer risk in relation to TP53 codon 72 and CDH1 gene polymorphisms in the Bangladeshi women. Tumour Biol 2015; 2015:1-9. [PMID: 26666818].

25. Chen R, Liu S, Ye H, Li J, Du Y, Chen L, Liu X, Ding Y, Li Q, Mao Y, Ai S, Zhang P, Ma W, Yang H. Association of p53 rs1042522, MDM2 rs2279744, and p21 rs1801270 polymorphisms with retinoblastoma risk and invasion in a Chinese population. Sci Rep 2015; 5:13300-[PMID: 26289323].

Articles are provided courtesy of Emory University and the Zhongshan Ophthalmic Center, Sun Yat-sen University, P.R. China. The print version of this article was created on 28 January 2017. This reflects all typographical corrections and errata to the article through that date. Details of any changes may be found in the online version of the article.