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# Influence of *MDM2* and *MDM4* on Development and Survival in Hereditary Retinoblastoma

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***Background.*** Retinoblastoma (RB) accounts for 3% of all childhood malignancies, with different incidences around the world. This malignancy results from loss-of-function of both *RB1* alleles although other genes, like *MDM2* and *MDM4*, have been proposed to be involved in tumor development. ***Procedure.*** We genotyped rs2279744T>G and rs937283A>G in *MDM2*, and rs4252668T>C and rs116197192G>A in *MDM4*, in 104 unrelated RB patients and 104 controls. Sixty-month survival Kaplan–Meier curves and $\chi^2$-tests were performed for estimating the putative effect of *MDM2* and *MDM4* alleles on disease progression and survival of RB patients. ***Results.*** *MDM2* rs2279744G was significantly more frequent in controls, indicating an apparently protective effect on RB development. However, survival of patients who carried a constitutional *RB1* mutation was significantly lower with rs2279744TG or GG than with rs2279744TT. Presence of rs2279744G and a constitutional *RB1* mutation was sixfold more frequent in the 0–12 month age group than other age groups at onset of symptoms ($P = 0.0401$). *MDM4* rs4252668C was present at a significantly higher frequency in controls while the frequency of *MDM4* rs116197192G was significantly higher in RB patients, suggesting that this allele might increase the risk of developing RB. ***Conclusion.*** Our results indicate that *MDM2* and *MDM4* polymorphisms may influence development and/or survival in RB. Pediatr Blood Cancer 2012;59:39–43.


**Key words:** *MDM2* and *MDM4* polymorphisms; retinoblastoma; *RB1* and *TP53* tumor suppressor genes

## INTRODUCTION

Retinoblastoma (RB) accounts for 3% of all childhood malignancies, with considerable variation in incidence around the world. In children between 0 and 14 years from US and Europe, RB incidence has been reported to be 4 per million, while higher incidence rates have been observed in developing countries like Brazil, where age-adjusted incidence rates for children 0–4 years varied from 11 to 27 per million, with a higher incidence in poorer regions of the country [1].

Both hereditary and sporadic forms of this malignancy are associated with loss-of-function of both *RB1* alleles. In the hereditary form, one *RB1* mutation is constitutional and the other is somatic; carriers of constitutional mutations show variable penetrance and expressivity, a reason why the identification of potential genetic modifiers of the RB phenotype is relevant. Recently, the *MDM2* and *MDM4* oncogenes have been proposed as genetic modifiers of this condition [2,3].

*MDM2* is located in 12q14.3-q15 and is involved, by regulating *TP53* function in cell cycle control, apoptosis, senescence, and DNA repair [4,5]. It also regulates *RB1* function in two ways: (1) blocking ligation of the retinoblastoma protein (pRB) to the E2F–DNA complex and inhibiting growth suppression by pRB and (2) degrading pRB in a proteasomal-dependent, ubiquitin-independent process, through a 20S proteasomal vector [6–8].

Although rarely mutated, *MDM2* is highly polymorphic and at least 152 single nucleotide polymorphisms (SNPs) and 18 polymorphic insertions have been observed [9]. Recently, researchers identified one SNP (rs2279744T>G) in the intronic *MDM2* promoter region which creates a site with greater affinity for Sp1 transcription factor and increases *MDM2* mRNA levels [10], a reason why its likely association with different tumor types has been investigated [11–14]. The association of the rs2279744G allele and RB development in patients carrying a constitutional *RB1* mutation has been recently demonstrated [2]. Another SNP (rs937283A>G), located at position −628 in exon 1 of *MDM2*, has been associated with a two- to three-fold increased risk of lung cancer when present with the rs2279744G allele [9].

*MDM4*, located on 1q32, regulates p53 stability in an *MDM2* dependent process because p53 degradation occurs through stabilization of MDM2 by MDM4 [15–17]. MDM4 protein also regulates pRB levels because pRB ubiquitination mediated by MDM2 is inhibited by MDM4, due to competition between MDM2 and MDM4 for ligation to the C-terminal domain of pRB [8]. *MDM4* polymorphisms and mutations have been frequently studied due to their impact on *TP53* and *RB1* regulation and in association to risk of some cancer types, as is the case of rs4252668 or rs116197192 (c.458A>G) with breast cancer [18].

In view of the regulatory activity of MDM2 and MDM4 on *TP53* and *RB1*, we investigated the influence of rs2279744T>G

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and rs937283A>G in *MDM2*, and rs4252668T>C and rs116197192A>G in *MDM4* in tumor development and survival in RB patients.

## METHODS

We studied 104 RB patients seen at the genetic counseling clinic for RB of our institution between years 2001 and 2010. This group represents all patients treated for RB in this period. Control samples comprised 104 unrelated, healthy adults. All individuals signed an informed consent. This study followed the guidelines of the Helsinki declaration following approval by the local Ethics Committee.

Gender, ethnicity, and socio-economic status (SES) of patients were defined based on medical records. Our hospital is a reference center for pediatric cancer treatment primarily attending patients with low income. All patients included in this study were from the state of Rio de Janeiro. Controls of similar SES were recruited in a private clinic for paternity diagnosis located in the same region. The ethnicity of controls (Caucasian, Mulattos, or Black) was defined by physician who collected blood samples.

Genomic DNA was isolated from peripheral blood lymphocytes following a previously described protocol [19]. *MDM2* and *MDM4* polymorphisms genotyping was carried out by PCR/RFLP analysis as previously described [9,18,20]. Sequencing of randomly selected, amplified products was carried out for confirming genotypic data with an ABI PRISM 3100 automated platform (Applied Biosystems, Foster City, CA).

Analyses of *RB1* variants of 101 RB patients herein studied were previously carried out by sequencing, and were partially published [21–23]. Constitutional *RB1* mutations considered to be pathogenic due to presumptive loss-of-function comprised nonsense mutations, frameshift, splicing sites, and selected missense mutations, as well as 13q14 deletions (Table I). Constitutional *RB1* mutations were present in 56% of RB patients (52% unilateral and 48% bilateral). Some patients were only carriers of *RB1* polymorphisms (30%) and others did not carry pathogenic mutations or polymorphisms in the coding regions or intronic flanking regions (14%).

Molecular data were analyzed by Fisher's exact and $\chi^2$-tests. Odds ratios (OR) were estimated to test whether non-wild-type alleles were associated to RB risk or protection. Clinical and molecular data were used to estimate cumulative, 60-month survival rates by the non-parametric method of Kaplan–Meier in 101 RB patients. Comparisons of survival curves were tested using the log-rank test with the free software R. Survival time was estimated from the date of the first appointment to the hospital, which corresponds to the beginning of treatment, until hospital last visit. Results were considered statistically significant at $P < 0.05$ level.

## RESULTS

The phenotypic and demographic characteristics of patients and controls are listed in Table II. Gender differences between controls and patients were not statistically significant ($P = 0.88$). Similar results was observed with ethnicity ($P = 0.69$).

*MDM2* polymorphisms (rs2279744 and rs937283) were found to be in Hardy–Weinberg equilibrium (HWE) in both samples. Conversely, a significant difference ($P < 0.02$) was found between observed and expected frequencies of *MDM4* rs116197192, resulting from an excess of AG heterozygotes in the patients. In the case of *MDM4* rs4252668, all patients were homozygous for the wild-type (TT) genotype.

There was a significant difference in allele frequency between patients and controls for *MDM2* rs2279744G ($P < 0.05$; OR = 0.60; 95%CI = 0.40–0.91) and *MDM4* rs4252668C ($P = 0.024$; OR = 0.00; 95%CI = 0.00–0.76). *MDM4* rs116197192G showed a higher frequency in patients than controls ($P < 0.001$; OR = 5.41; 95%CI = 2.49–11.71) while no significant differences between samples were observed for *MDM2* rs937283C ($P = 0.18$; see Table III).

The frequency of *MDM2* rs2279744G was not significantly different ($P = 0.07$) between 33 patients with unilateral RB, who did not carry a constitutional *RB1* mutation, and controls.

Patients with RB with presumed *RB1* pathogenic mutations were stratified in four age categories according to the onset of symptoms (0–12 months, 13–36 months, 37–48 months, and 48–60 months) and the frequency of each *MDM2* and *MDM4* allele was analyzed. In order to exclude a likely bias introduced by familial presentation of hereditary RB in the analysis, the median age of onset was estimated only in children without family history. This analysis showed that the simultaneous presence of *MDM2* rs2279744G and a constitutional *RB1* mutation was sixfold more frequent in the 0–12 months age group when compared to other age groups ($P = 0.04$). The median age of onset of symptoms was 5 months for patients who carried both a constitutional *RB1* mutation and the *MDM2* rs2279744G allele, versus 10 months for patients with only an *RB1* mutation ($P = 0.04$). Conversely, the onset of symptoms did not vary significantly with respect to any other polymorphism. Comparisons of survival between groups with different age of onset of symptoms, regardless of *RB1*, *MDM2*, and *MDM4* genotypes, did not show statistically significant differences ($P = 0.49$).

Comparison of survival estimates of patients with *MDM2* and *MDM4* polymorphisms and constitutional *RB1* mutations showed that only *MDM2* rs2279744 was significantly associated with RB survival. We observed a lower survival (64%) associated with the simultaneous presence of a constitutional *RB1* mutation and rs2279744TG or GG genotypes respective to similar patients

**TABLE I. Constitutional RB1 Mutations in 101 Retinoblastoma Patients**

| Mutation type | Pathogenic mutation | Number of patients (%) |
|---|---|---|
| Nonsense | R320X, R445X, R455X, R556X, R552X, R579X, R661X, Q702X, R787X, K844X | 16 (16) |
| Splice site | c.1-4, c.940-2, c.1128-1, c.1333-1, c.1422-2 | 18 (18) |
| Missense | G310E, K447E, K447Q, D856N, | 15 (15) |
| Frameshift | c.1_137del, c.772_776del, c.940_1049del, c.1050_1127del, c.1696_1814del | 5 (5) |
| 13q14 deletion | | 2 (2) |
| Total | | 56 (56) |

**TABLE II. Phenotypic and Demographic Characterization of Patient and Control Samples**

| | Characteristic | Cases (%) | Controls (%) |
|---|---|---|---|
| Gender | Male | 48 | 50 |
| | Female | 52 | 50 |
| Ethnicity | Caucasian | 59 | 53 |
| | Mullato | 27 | 31 |
| | Black | 14 | 16 |
| Family history | Present | 12 | |
| | Absent | 88 | |
| Laterality | Unilateral | 62 | |
| | Bilateral | 38 | |
| Onset of symptoms (months) | 0–12 | 47 | |
| | 13–36 | 38 | |
| | 37–48 | 10 | |
| | 49–60 | 5 | |

with rs2279744TT (96%; $P = 0.01$). Likewise, the survival of carriers of constitutional *RB1* mutations and rs2279744TG (68%) was significantly higher than similar patients with rs2279744GG (62%; $P < 0.05$; Fig. 1). Survival of children with rs2279744TT, TG, or GG genotypes, who did not carry *RB1* mutations, did not differ significantly ($P = 0.69$).

## DISCUSSION

We found a higher frequency of *MDM2* rs2279744TG and GG genotypes in controls than in patients, suggesting that, in individuals who were not carriers of *RB1* constitutional mutations, the G allele might be associated to a protective effect on tumor formation. Interestingly, the frequency of the rs2279744GG genotype varied from 14 to 24% between healthy Ashkenazi and Caucasians from Central Europe [10,12], similar to our findings (13.4%) in controls. In fact, no statistical difference was observed in the frequency of rs2279744G between 33 non-hereditary RB patients with unilateral RB and without a constitutional *RB1* mutation and controls. It must be considered, however, that our control sample was composed of adults, a fact that might have introduced a bias in our analysis in view that RB is a childhood malignancy. However, despite the possibility that a single SNP by itself might affect global survival, it is unlikely that this might substantially change allele frequencies between healthy children and adults, because one would have to expect a dominant effect of this polymorphism on survival.

The prevalence of *MDM2* rs2279744 genotypes in patients with different types of cancer has been shown to be variable, and their association with tumor development is controversial. Allele rs2279744G has been associated with a higher risk of hepatocellular carcinoma and bladder cancer [13,14], but without association with non-Hodgkin lymphoma and neuroblastoma [12,24]. In patients with Li-Fraumeni syndrome, a disease with a functionally affected the p53 pathway resulting from constitutional mutations, the rs2279744GG genotype was associated with a higher risk of cancer and accelerated tumor formation [25,26]. Recently, an association was observed between rs2279744G and RB development in patients with constitutional *RB1* mutations [2], a finding that was confirmed in this study. Interestingly, in the context of genetic predisposition to RB, a first mutational event at the *RB1* locus, the rs2279744G allele apparently enhances the subsequent effects of *RB1* haploinsufficiency, leading to earlier development of RB. This might explain the significant difference in the median onset of symptoms in patients with *RB1* mutations also carrying the TG or GG genotype in comparison to patients with *RB1* constitutional mutations and the TT genotype.

Only patients carrying a constitutional *RB1* mutation and the *MDM2* rs2279744TG or GG genotype were found to be associated with a lower survival rate, and not those with the TT genotype. Conversely, survival of children who did not carry constitutional

**TABLE III. Genotypes, Allelic Frequencies, Odds Ratio (OR), and Confidence Intervals (CI) for Polymorphisms rs2279744 and rs937283 in MDM2, and rs4252668 and rs116197192 in MDM4 in Patients and Controls**

| | Genotypes | | | Allele G | | | | |
|---|---|---|---|---|---|---|---|---|
| | N | | | Frequency | OR | 95% CI | $\chi^2$-test | *P*-value |
| MDM2—rs2279744 (T>G) | | | | | | | | |
| | TT | TG | GG | G | 0.60 | 0.40–0.91 | 5.72 | <0.05 |
| Patients | 53 | 44 | 7 | 0.28 | | | | |
| Controls | 37 | 53 | 14 | 0.40 | | | | |
| MDM2—rs937283 (A>G) | | | | | | | | |
| | AA | AG | GG | G | 1.31 | 0.88–1.95 | 1.75 | 0.186 |
| Patients | 37 | 51 | 16 | 0.40 | | | | |
| Controls | 45 | 48 | 11 | 0.33 | | | | |
| MDM4—rs116197192 (A>G) | | | | | | | | |
| | AA | AG | GG | G | 5.41 | 2.49–11.71 | 20.96 | <0.001 |
| Patients | 67 | 37 | 0 | 0.18 | | | | |
| Controls | 96 | 8 | 0 | 0.04 | | | | |
| MDM4—rs4252668 (T>C) | | | | | | | | |
| | TT | TC | CC | C | 0.00 | 0.00–0.76 | 5.06 | 0.024 |
| Patients | 104 | 0 | 0 | 0.00 | | | | |
| Controls | 99 | 5 | 0 | 0.02 | | | | |

Note: (N) number of genotype observed.

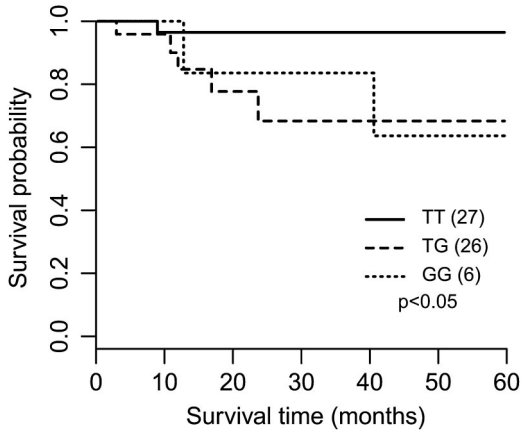


**Fig. 1.** Kaplan–Meier 60-month cumulative overall survival on the basis of rs2279744 TT, TG, or GG genotypes presence in RB patients with constitutional *RB1* mutation.

*RB1* mutations did not differ significantly regardless of rs2279744 genotypes. Our results suggest that the G allele might unfavorably affect survival in patients carrying *RB1* mutations, a finding supported by a significantly lower survival of carriers of rs2279744GG (62%) than children with the rs2279744TG genotype (68%). This provides the first evidence that rs2279744 genotypes might be associated with survival of RB patients, in agreement with the finding that rs2279744GG is associated with lower survival rate in renal [27], gastric [28], and esophageal carcinoma [29]. Conversely, one report showed that the TG genotype was associated with a better survival than the TT genotype in a case of esophageal carcinoma [30]. Furthermore, rs2279744GG and TG have been found to be associated to an earlier onset of symptoms in colorectal carcinoma [31], and in soft tissue sarcomas [32] although other studies did not confirm these associations [24,33–36]. These discrepancies might result from differences in ethnic composition, methodologies, or presence of mutations in different oncogenes and/or tumor suppressor genes.

The *MDM2* rs937283G allele was not significantly associated with RB, either by itself, in association with *RB1* mutations, or with *MDM2* rs2279744G. However, the simultaneous presence of *MDM2* rs937283G and rs2279744G was associated with a 2.3-fold higher risk of lung cancer [9].

The *MDM4* rs116197192G allele showed a significantly higher frequency in patients than in controls (18% vs. 4%), suggesting its association with an increased risk of RB development. The distribution of *MDM4* rs116197912 genotypes in patients, clearly departing from HWE due to excess of AG heterozygotes respective to GG homozygotes, indicates that rs116197912G might be a dominant allele associated to RB. This allele, of a polymorphic site in exon 7, results in a missense substitution (D153G) in a predicted casein kinase II (CK2) ligation site. CK1 and CK2 are two protein kinases that participate in a wide variety of cellular processes, including DNA repair and cell cycle control. Genotyping of this polymorphims in breast cancer patients did not reveal a preferential association with this neoplasia [18]. Further studies are necessary to confirm the association of this allele with cancer, in view of the paucity of data in the literature.

Analysis of *MDM4* genotypes at rs4252668 in controls showed departure from HWE. However, differences between expected and observed values were borderline, and probably due to the small size of our sample, and to the low frequency of *MDM4* rs4252668C in controls (2%).

The frequency of *MDM4* rs4252668C was significantly higher in controls than in patients. Analyses of larger samples might be revealing for understanding its relevance in cancer in view that rs4252668 is located within p53 binding domain of *MDM4*, probably affecting p53 regulation; partial data indicated that this allele was not associated with an increased risk of breast cancer [18].

Analyses of *MDM2* and *MDM4* polymorphisms allowed us to conclude that, despite *MDM2* rs2279744G being more frequent in controls than in RB patients, it was strongly associated with an earlier onset of symptoms and poorer survival rate in combination with *RB1* constitutional mutations. The *MDM4* rs4252668C allele was also more frequent in controls and was not associated to RB. On the other hand, the frequency of the *MDM4* rs116197192G allele was significantly higher in RB patients, indicating that this allele may be associated with an increased risk of RB in our population.

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