

## Lack of association between *MDM2* SNP309 and *TP53* Arg72Pro polymorphisms with clinical outcomes in myelodysplastic syndrome

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*MDM2*/p53 pathway plays an important role in the control of apoptotic and proliferation mechanisms, and alterations in this pathway have been described in myelodysplastic syndromes (MDS). We investigated the frequency of *MDM2* SNP309, *TP53* Arg72Pro polymorphisms in *de novo* MDS and the association of these polymorphisms with clinical characteristics. Our results showed that the frequencies of genotypes for *MDM2* SNP309 and *TP53* Arg72Pro did not differ between MDS and healthy controls and that these polymorphisms were not associated with clinical and laboratory parameters, disease progression and overall survival, suggesting that *MDM2* and *TP53* polymorphisms are not involved in risk for MDS, or in the clinical and laboratory characteristics of the disease.

*Key words:* myelodysplastic syndromes, *MDM2*, *TP53*, p53, polymorphism

Abnormalities in p53 and its negative regulator, Mouse double-minute (*MDM2*) play a major role in cancer [1]. The p53 is a transcription factor encoded by *TP53* gene that mediates cellular responses to DNA damage by regulating cell-cycle arrest, senescence and apoptosis [2]. *MDM2* is an ubiquitin E3 ligase that regulates the stability of p53 [2]. Single nucleotide polymorphisms (SNP) in *MDM2* and *TP53* have been identified and have been proposed to affect tumorigenicity [3]. The change of G to the T allele in the nucleotide 309 (SNP309, rs2279744) of the *MDM2* gene results in a higher affinity for Sp1, hence leading to enhanced *MDM2* transcription [3]. Higher *MDM2* levels can attenuate p53 response [4], which may lead to insufficient DNA repair, reduced apoptosis and tumor formation [5]. A SNP in the codon 72 of the *TP53* gene results in either a C or G nucleotide and leads to either a Proline (Pro) or Arginine (Arg) residue at codon 72, respectively (Arg72Pro, rs1042522) [1, 3, 6]. The Arg variant has been shown to be more potent in apoptosis induction [1, 6] and the Pro variant has been shown to be better for inducing cell-cycle arrest and in its ability to repair damage DNA [3].

Myelodysplastic syndromes (MDS) encompass a heterogeneous group of clonal hematopoietic stem cell disorders characterized by morphological dysplasia, impaired dif-

ferentiation and defective cellular functions, resulting in peripheral cytopenias and has a tendency to progress towards acute myeloid leukemia (AML) [7]. Genetic abnormalities in *TP53* have been reported in MDS and may be involved in disease progression [8, 9]. As far as we know, no study has yet investigated the role of *MDM2* SNP309 and *TP53* Arg72Pro in *de novo* MDS. Thus, the aim of the present study was to investigate the incidence of *MDM2* SNP309 and *TP53* Arg72Pro polymorphisms in MDS patients and to correlate the frequency of these polymorphisms with age, neutrophils and platelets at diagnosis, low risk *versus* high risk disease according to FAB, IPSS, cytogenetic risk, disease progression and overall survival.

### Patients and methods

**Subjects.** One hundred-three controls and seventy-seven patients with a diagnosis of *de novo* MDS were included in the study. The diagnosis of MDS was based on bone marrow morphology, including bone marrow aspirate and biopsy, cytogenetics and exclusion of systemic disease. Karyotypes were analyzed by conventional G-banding of bone marrow cells. Twenty metaphases were then analyzed and karyotyped

in accordance with ISCN (2009) criteria[10]. Patients' characteristics are described in Table 1. The control group consisted of healthy volunteers; mean age 37 (17-89). All healthy volunteers and patients provided informed written consent and the study was approved by the ethics committee of the University of Campinas.

**SNP genotyping.** Genomic DNA samples were obtained from peripheral blood samples using phenol-chloroform extraction. Identification of MDM2 and TP53 polymorphisms was performed using the polymerase chain reaction and restriction fragment length polymorphism methods as previously described [3, 11]. In brief, PCR was performed in a 50  $\mu$ L reaction volume consisting of 100 ng genomic DNA, 5  $\mu$ L reaction buffer 10X, 2  $\mu$ L MgCl<sub>2</sub> 50 mM, 2.5 unit of Taq polymerase and 200 nM of each forward and reverse primers. Primers sequences and annealing temperatures are described in Table 2. For RFLP analyses, MDM2 and TP53 PCR products were MspAII (New England Biolabs, Hitchin, UK) or BstUI (MBI Fermentas, St. Leon-Rot, Germany) digested, according to the manufacturer's protocol, and visualized on 3 or 1% agarose gel, respectively. The normal genotype for MDM2 was verified by a 157 bp fragment, the heterozygous genotype was verified by 157, 146 and 11 bp fragments, whereas homozygous polymorphic genotype demonstrated 146 and 11 bp fragments (Figure 1A-B). For TP53, the polymorphic genotype was verified by 839 bp fragment, the heterozygous genotype was verified by 839, 572 and 267 bp fragments, whereas homozygous normal genotype was demonstrated by 572 and 267 bp fragments (Figure 1C-D).

**Statistical analysis.** Statistical analyses were performed using R Foundation for Statistical Computing, version 2.10.1 (ISBN 3-900051-07-0). The Hardy-Weinberg equilibrium of MDM2 SNP309 and TP53 Arg72Pro was tested by Fisher's exact test. Fisher's exact test or Chi-square ( $X^2$ ) test were employed to determine the significance of differences in genotypes between patients and controls or in between patient groups. Comparison of median age at diagnosis between patients and different genotypes was carried out using Kruskal-Wallis test. The correlation of genotypes and neutrophils, platelets and bone marrow blast at diagnosis was analyzed by the Wilcoxon rank sum test or Kruskal-Wallis test. Cox regression was applied to evaluate the possible association between the genotypes with disease progression and overall survival. All analyses were performed for MDM2 SNP309, TP53 Arg72Pro and for both polymorphisms in combination. The frequency of G allele for MDM2 and Pro allele for TP53 were analyzed. A  $P$ -value < 0.05 was considered statistically significant.

## Results

To evaluate whether MDM2 and TP53 polymorphisms were associated with a predisposition to *de novo* MDS, we tested these polymorphisms in 77 patients with MDS and in 103 healthy volunteers (control). The control population was in Hardy-Weinberg equilibrium for MDM2 SNP309 ( $\chi^2=2.59$ ,  $P=0.27$ ) and TP53 Arg72Pro ( $\chi^2=0.677$ ,  $P=0.72$ ) polymorphisms. To determine whether there were significant differences in the distribution of MDM2 and TP53 polymorphisms, we used the T/T genotype for MDM2 SNP309 and the Arg/Arg genotype for TP53 Arg72Pro as reference for analyses and no differences were found (Table 3).

In our study, no association was found between MDM2 SNP309 and TP53 Arg72Pro and clinical parameters. Frequencies of MDM2 SNP309 and TP53 Arg72Pro distributed in female and male patient groups were not statistically different ( $P=0.156$ ,  $P=1.000$ ; respectively and  $P=0.516$  for MDM2 and TP53 interaction), indicating that both polymorphisms may not affect MDS susceptibility in a gender-specific manner. We also found no meaningful difference in initial platelet and neutrophil counts and

**Table 1. Patients characteristics.**

	Number of individuals
MDS Patients	77
Age (years), Median (range): 63, (21-89)	
Follow-up (month), Median (range): 36, (2-166)	
Number of patients that progressed	16
Time to progression (month), Median (range):10 (2-92)	
FAB	
RA/RARS	49/12
RAEB/RAEBt	11/3
CMMML	2
IPSS	
Low risk/ Intermediate-1	31/30
Intermediate-2/high risk	7/4
Not available	5
Cytogenetics	
Low risk	60
Intermediate/high risk	6/7
Not available	4

Abbreviations. FAB: French-American-British; RA: Refractory Anemia; RARS: Refractory Anemia with Ringed Sideroblasts; RAEB: Refractory Anemia with Excess of Blasts; RAEBt: Refractory Anemia with Excess of Blasts in transformation; CMMML: Chronic Myelomonocytic Leukemia; IPSS: International Prognostic Scoring System.

**Table 2. Primer sequences, restriction enzymes and annealing temperatures**

Gene	Polymorphisms	Primers Sequences	Restriction Enzyme	Annealing temperature
MDM2	SNP309	F: 5'-CGCGGGAGTTTCAGGGTAAAG-3' R: 5'-CTGAGTCAACCTGCCCACTG-3'	MspAII	63°C
TP53	Arg72Pro	F: 5'-TCAGACACTGGCATGGTGT-3' R:5'-AAGCCAAAGGGTGAAGAGGA -3'	BstUI	55°C

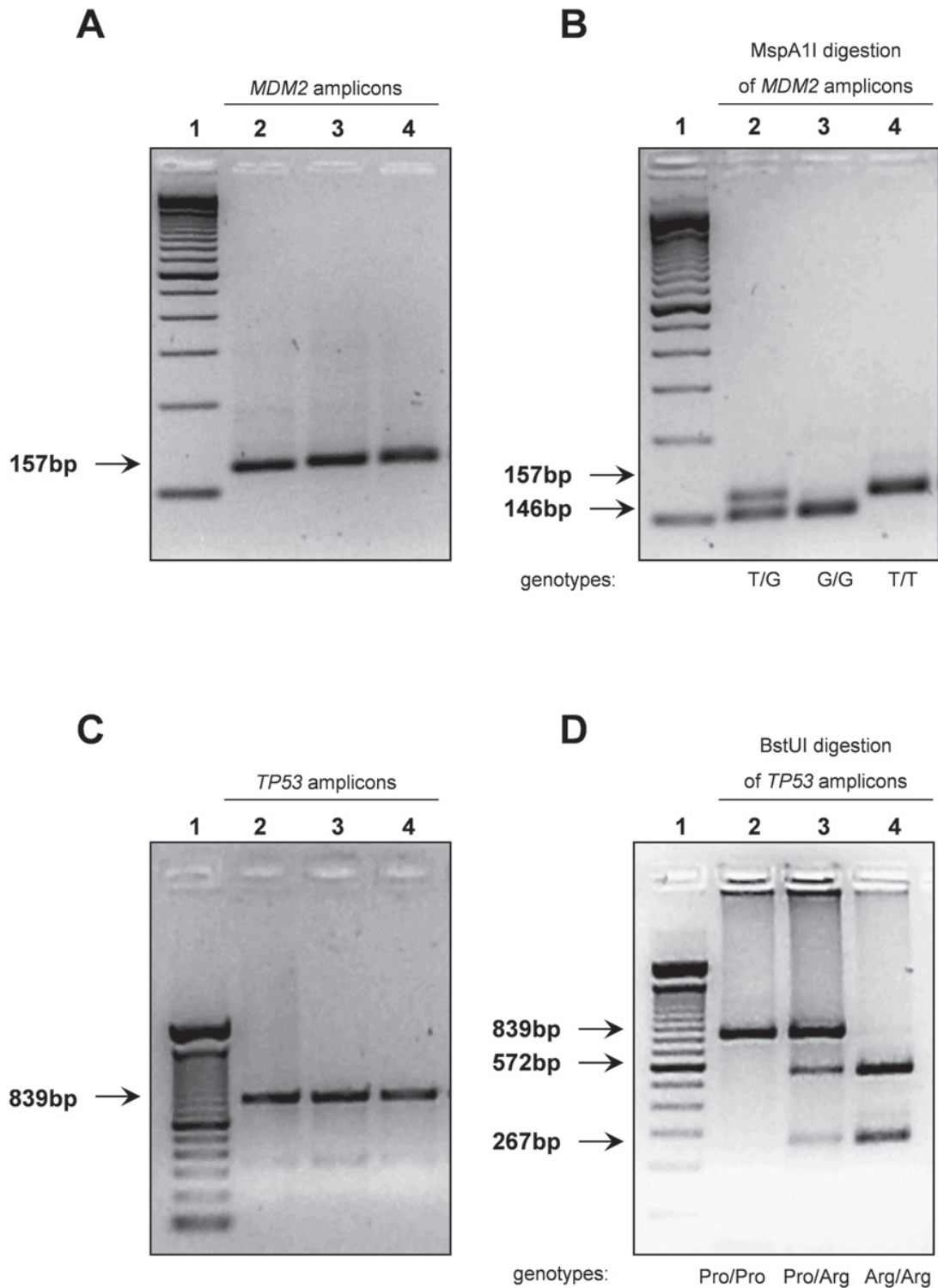


Figure 1. *MDM2* SNP309 and *TP53* Arg72Pro genotyping. (A) PCR amplification of *MDM2*: lane 1: Ladder 100bp fragments; lane 2 to 4: 157 bp amplicons obtained from genomic DNA of MDS patients. (B) MspA1I digestion of *MDM2* amplicons: lane 1: Ladder 100bp fragments, lane 2: digestion pattern observed in subjects with T/G genotype, lane 3: digestion pattern observed in subjects with G/G genotype and lane 4: digestion pattern observed in subjects with T/T genotype. (C) PCR amplification of *TP53*: lane 1: Ladder 100bp fragments; lane 2 to 4: 839 bp amplicons obtained from genomic DNA of MDS patients. (D) BstUI digestion of *TP53* amplicons: lane 1: Ladder 100bp fragments, lane 2: digestion pattern observed in subjects with Pro/Pro genotype, lane 3: digestion pattern observed in subjects with Pro/Arg genotype and lane 4: digestion pattern observed in subjects with Arg/Arg genotype. The fragments sizes are indicated in the figure.

**Table 3. Distribution of allele frequencies of *MDM2* and *TP53* polymorphisms or their interaction in MDS patients and controls.**

Genotypes		Controls (n=103), n <sup>o</sup> (%)	MDS cases (n=77), n <sup>o</sup> (%)	OR (95% CI)	P *
<i>MDM2</i> SNP309	<i>TP53</i> Arg72Pro				
T/T		55 (53.4)	40 (51.9)	1.00 ref.	
T/G		33 (32.0)	30 (39.0)	1.25 (0.66-2.37)	0.603
G/G		15 (14.6)	7 (9.1)	0.64 (0.24-1.71)	0.518
G allele		48 (46.6)	37 (48.1)	1.06 (0.59-1.91)	0.966
	Arg/Arg	46 (44.6)	35 (45.4)	1.00 ref.	
	Arg/Pro	42 (40.8)	36 (46.8)	1.12 (0.60-2.10)	0.831
	Pro/Pro	15 (14.6)	6 (7.8)	0.53 (0.19-1.49)	0.332
	Pro allele	57 (55.4)	42 (54.6)	0.96 (0.54-1.75)	0.916
T/T	Arg/Arg	19 (18.4)	19 (24.7)	1.00 ref.	
	Arg/Pro	28 (27.2)	18 (23.3)	0.64 (0.27-1.53)	0.379
	Pro/Pro	8 (7.8)	3 (3.9)	0.37 (0.09-1.63)	0.302
T/G	Arg/Arg	18 (17.5)	14 (18.2)	0.78 (0.30-2.00)	0.637
	Arg/Pro	11 (10.7)	13 (16.9)	1.18 (0.42-3.29)	0.798
	Pro/Pro	4 (3.9)	3 (3.9)	0.75 (0.15-3.82)	1.000
G/G	Arg/Arg	9 (8.7)	2 (2.6)	0.22 (0.04-1.17)	0.087
	Arg/Pro	3 (2.9)	5 (6.5)	1.67 (0.35-7.98)	0.702
	Pro/Pro	3 (2.9)	0 (0.0)	0.14 (0.01-2.96)	0.235

Abbreviations. OR: Odds ratio. \* *Chi-square* test

percentage of bone marrow blasts depending on *MDM2* SNP309 or *TP53* Arg72Pro polymorphisms (all  $P > 0.05$ ). Furthermore, *MDM2* and *TP53* polymorphisms and their interaction were not associated with the low-risk or high-

risk MDS according to FAB (RA and RARS *versus* RAEB and RAEBt), IPSS (Low-risk and Int-1 *versus* Int-2 and high-risk) and cytogenetic risk (low-risk *versus* intermediate and high-risk) (Table 4).

**Table 4. Effects of *MDM2* and *TP53* genotypes, or their interaction, in MDS, as stratified by disease risk groups**

Genotypes		FAB (n=77), n <sup>o</sup> (%)		IPSS (n=72), n <sup>o</sup> (%)		Karyotype category (n=73), n <sup>o</sup> (%)			
<i>MDM2</i> SNP309	<i>TP53</i> Arg72Pro	Low-risk	High-risk	Low and INT-1	INT-2 and high	good	Intermediate and poor		
<i>MDM2</i> or <i>TP53</i>		<i>P</i> *		<i>P</i> *		<i>P</i> *			<i>P</i> *
T/T		32 (41.5)	8 (10.4)	31 (43.1)	7 (9.7)	31 (42.5)	7 (9.6)		
T/G		25 (32.5)	5 (6.5)	25 (34.7)	2 (2.8)	25 (34.2)	4 (5.5)	0.691	0.475
G/G		5 (6.5)	2 (2.6)	5 (6.9)	2 (2.8)	4 (5.5)	2 (2.7)		
G allele		30 (39.0)	7 (9.1)	30 (41.6)	4 (5.6)	29 (39.7)	6 (8.2)	1.000	1.000
	Arg/Arg	28 (36.3)	7 (9.1)	28 (38.9)	5 (6.9)	26 (35.6)	7 (9.6)		
	Arg/Pro	29 (37.7)	7 (9.1)	28 (38.9)	5 (6.9)	30 (41.1)	5 (6.8)	1.000	0.717
	Pro/Pro	5 (6.5)	1 (1.3)	5 (7.0)	1 (1.4)	4 (5.5)	1 (1.4)		
	Pro allele	34 (44.2)	8 (10.4)	33 (45.9)	6 (8.3)	34 (46.6)	6 (8.2)	1.000	0.549
Interaction of <i>MDM2</i> and <i>TP53</i>		<i>P</i> †		<i>P</i> †		<i>P</i> †			<i>P</i> †
T/T	Arg/Arg	16 (20.7)	3 (3.9)	14 (19.4)	3 (4.2)	13 (17.8)	4 (5.5)	Ref.	Ref.
	Arg/Pro	13 (16.9)	5 (6.5)	14 (19.4)	4 (5.5)	15 (20.5)	3 (4.1)	1.000	0.691
	Pro/Pro	3 (3.9)	0 (0.0)	3 (4.2)	0 (0.0)	3 (4.1)	0 (0.0)	1.000	1.000
T/G	Arg/Arg	11 (14.3)	3 (3.9)	13 (18.1)	1 (1.4)	12 (16.4)	2 (2.7)	0.607	0.664
	Arg/Pro	12 (15.6)	1 (1.3)	11 (15.3)	0 (0.0)	12 (16.4)	1 (1.4)	0.258	0.355
	Pro/Pro	2 (2.6)	1 (1.3)	1 (1.4)	1 (1.4)	1 (1.4)	1 (1.4)	0.386	0.468
G/G	Arg/Arg	1 (1.3)	1 (1.3)	1 (1.4)	1 (1.4)	1 (1.4)	1 (1.4)	0.386	0.468
	Arg/Pro	4 (5.2)	1 (1.3)	4 (5.5)	1 (1.4)	3 (4.1)	1 (1.4)	1.000	1.000
	Pro/Pro	0 (0.0)	0 (0.0)	0 (0.0)	0 (0.0)	0 (0.0)	0 (0.0)		

Abbreviations. FAB: French-American-British ; IPSS: International Prognostic Scoring System; INT-1: Intermediate-1; INT-2: Intermediate-2. \* *Fisher's exact* test; † *Chi-square* test.

*MDM2* SPN309 and *TP53* Arg72Pro were not associated with age of onset, disease progression and overall survival. The median ages of onset for *MDM2* genotypes were 65.5 years (27.5-88.9) for T/T, 61.1 years (21.1-86.7) for T/G and 61.0 years (25.1-71.1) for G/G. For *TP53* genotypes the median ages were 68.8 years (22.1-88.9) for Arg/Arg, 60.6 years (21.1-82.4) for Arg/Pro and 60.1 years (38.4-69.4) for Pro/Pro. Comparison of overall survival between patients according to *MDM2* and *TP53* polymorphisms revealed no significant difference ( $P>0.05$ ). The median survival based in *MDM2* genotypes were 34.4 months (range, 164.5-2.2 months) for T/T, 39.7 months (range, 166.0-7.3 months) for T/G and 30.9 months (range, 88.1-4.1 months) for G/G. Based on *TP53* genotypes the median survival were 38.9 months (range, 150.2-2.0 months) for Arg/Arg, 30.8 months (range, 166.0-2.2 months) for Arg/Pro and 46.9 months (range, 134.3-2.7 months) for Pro/Pro. Similarly, the interaction between *MDM2* and *TP53* polymorphisms did not affect overall survival ( $P>0.05$ ).

## Discussion

Several studies have investigated the association between *MDM2* and *TP53* polymorphisms with risk of different cancers, including AML [2, 3, 12], however, the results are inconclusive. The G allele of *MDM2* SNP309 was associated with increased risk of AML, but no association was found between *MDM2* or *TP53* polymorphisms and age of onset or other clinical manifestations [12]. In contrast, in another study, the G allele was associated with a reduced risk of leukemia, and again no association was observed between these polymorphisms with clinical parameters [3]. Ellis *et al* [2] described an interaction of *MDM2* SNP309 and *TP53* Arg72Pro polymorphisms with disease susceptibility and responses to genotoxic therapy in therapy-related MDS/AML.

Mutation of *TP53* plays an important role in the pathogenesis of MDS and studies involving the p53/MDM2 pathway have emerged, suggesting a relationship of p53 expression with risk and prognosis [13, 14]. However, *TP53* Arg72Pro has rarely been addressed in MDS and *MDM2* SNP309 has not been reported in this disease. Herein, we investigated the association of both polymorphisms and their interaction in *de novo* MDS for the first time. Our results showed no association of these polymorphisms with risk of MDS and clinical parameters, even when the polymorphisms were analyzed individually or combined. Interestingly, McGraw *et al* (Blood, ASH Annual Meeting Abstracts 2011;116:269) recently reported that *TP53* Arg72Pro homozygous Pro/Pro genotype occurs with significantly greater frequency in del5q MDS, compared to both non-del5q MDS and healthy controls, and no correlation was found with clinical parameters in MDS 5q and non-del5q. Regarding patients with non-del5q, our results are in agreement with McGraw *et al*, as no correlations were found in either studies. Interestingly our cohort included

only four patients with del5q, which showed a *TP53* Arg72Pro homozygous Arg/Arg genotype.

In summary, in our study, *MDM2* SNP309 and *TP53* Arg72Pro were not significantly associated with the risk for MDS and with clinical variables, including clinical parameters such as FAB, IPSS, cytogenetic risk, age of onset, disease progression and overall survival.

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