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ORIGINAL ARTICLE

p53 signaling pathway polymorphisms, cancer risk and tumor phenotype in *TP53* R337H mutation carriers

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Abstract Li-Fraumeni and Li-Fraumeni-like syndrome (LFS/LFL) are clinically heterogeneous cancer predisposition syndromes characterized by diagnosis of early-onset and often multiple cancers with variable tumor patterns and incomplete penetrance. To date, the genetic modifiers described in LFS/LFL have been shown to map to either *TP53* or its main negative regulator, *MDM2*. Additionally, all studies were focused on families with different *TP53* germline mutations. Hence, in this study we explored the effect of the most studied polymorphisms of p53 pathway genes on clinical manifestations of individuals carrying the founder *TP53* mutation R337H (n = 136) and controls (n = 186). Cancer-affected carriers had been diagnosed either with adrenocortical carcinoma (ACC, n = 29) or breast cancer (BC, n = 43). Allelic discrimation using TaqMan assay was used for genotyping *MDM2* SNP 309 (rs2279744) as well as *MDM4* (rs1563828) and *USP7* (rs1529916) polymorphisms. We found significantly higher *MDM2* SNP 309 GG genotype and G allele frequencies in the LFS cohort than in controls. Furthermore, median age at first diagnosis was earlier in *MDM2* SNP309 GG carriers when compared to other genotypes for both cancers (ACC: age 1 vs. 2 years; BC: age 35 vs. 43 years, respectively), although not statistically different. The allelic and genotypic frequencies for all SNPs did not differ between cancer affected and unaffected carriers, neither between patients with ACC or BC. In conclusion, our results suggest that MDM2 SNP 309 may contribute to the LFL phenotype and also to an earlier age at diagnosis of ACC and BC cancer in carriers of the R337H founder mutation.

Keywords P53 · R337H · Polymorphism · Li-Fraumeni syndrome

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Introduction

Li-Fraumeni Syndrome (LFS) and its variant, Li-Fraumeni-like Syndrome (LFL), are clinically heterogeneous cancer predisposition syndromes characterized by an autosomal dominant inheritance pattern, diagnosis of early-onset cancers and multiple primary tumors. The core tumors of LFS/LFL are bone and soft-tissue sarcomas, central nervous system tumors, breast cancer (BC) and adrenocortical carcinoma (ACC) [1–3]. Currently, germline mutations in the *TP53* gene are the only known genetic alterations underlying LFS/LFL [4].

In Southern and Southeastern regions of Brazil, a specific mutation in the *TP53* gene, R337H (c.1010G > A, p.ArgR337His), has been reported to occur at a high frequency both at population level and among individuals with clinical criteria for LFS/LFL [5–7]. Carriers display a wide range of cancers, within and beyond the spectrum of core tumors of the syndrome, but tumor penetrance appears to be lower than that observed in carriers of DNA-binding domain (DBD) mutations [5, 7–9]. The Arginine residue at codon 337 is a critical part of an alpha-helix motif involved in the protein oligomerization. Functional data have shown that the replacement of arginine by histidine disrupts the tetramer form in a pH-dependent manner, making the domain unable to oligomerize in conditions of slightly elevated pH [10]. Some authors have suggested that this peculiarity could explain, at least in part, the reduced penetrance observed in R337H carriers [11].

The p53 protein acts as a transcriptional factor that, in response to stress, regulates the expression of an array of different genes involved in growth arrest, DNA repair, apoptosis, metabolism and senescence [12]. Variations in p53 (multiple protein isoforms and/or mutant proteins) and in their partners are thought to underlie the wide range of clinical manifestations observed in the syndrome both within and between families. For instance, single nucleotide polymorphisms (SNPs) in the *TP53* and *MDM2* genes, a negative regulator of p53, have been associated to earlier age at cancer diagnosis in carriers of *TP53* germline mutations [13, 14].

Although significant progresses have been made in our understanding of the molecular biology of p53 and clinical/epidemiologic features of LFS/LFL, knowledge on risk modifiers and its effect on phenotype are still incomplete. Thus, in the present study we aimed to investigate whether selected SNPs in *MDM2, MDM4* and *USP7* were associated with cancer risk, age at first diagnosis and specific tumor types in carriers of the germline *TP53* mutation R337H.

Materials and methods

Subjects

For this study a total of 136 subjects were recruited from families attending Cancer Risk Evaluation clinics in the Hospital de Clínicas de Porto Alegre (Porto Alegre, Brazil), Hospital do Câncer A.C. Camargo (São Paulo, Brazil) and Hospital do Câncer de Barretos (Barretos, São Paulo). The LFL group included cancer-unaffected R337H mutation carriers (n = 60); R337H mutation carriers with a previous diagnosis of ACC (n = 29), BC (n = 43) or other tumors (n = 4). In addition, a control group consisted of cancer-unaffected individuals with no family history of cancer in first or second degree and without the R337H mutation (n = 186). Controls were recruited from a community based BC prevention program in Southern Brazil [15]. The institutional ethics committees of participating institutions approved the study and all participants provided written informed consent before recruitment.

Polymorphism analyses

Genomic DNA was extracted from white blood cells or non-tumoral tissue using commercial kits (Illustra Blood genomicPrep Mini Spin Kit, GE Healthcare and DNA FFPE Kit, FFPE Qiagen). Mutation testing was previously performed using Sanger sequencing of the entire coding region (exons 2–11) of *TP53* according to standard protocols (http://p53.iarc.fr/download/tp53_directsequencing_iarc.pdf).

TaqMan allelic discrimination analyses were performed according to Applied Biosystems standard protocols (Applied Biosystems, Carlsbad, USA). The analyzed SNPs were as follows: *MDM4* rs1563828 (C_9493064_10), *USP7* rs1529916 (C_9688119_1), and *MDM2* rs2279744 for which a custom-made TaqMan assay was made, using forward primer 5′-CGGGAGTTCAGGGTAAAGGT-3′, reverse primer 5′-ACAGGCACCTGCGATCATC-3′, VIC probe 5′-CTCCCGCGCCGAAG-3′ and FAM probe 5′-TCCCGCGCCGCAG-3′ (Applied Biosystems). PCR cycling reactions were performed on an ABI StepOne System (Applied Biosystems) and consisted of initial denaturation at 95 °C for 15 min, 40 cycles with denaturation 95 °C for 15 s, and then annealing and extension at 60 °C for 1 min.

Statistical analyses

Descriptive statistics was used to determine allelic and genotypic frequencies. Differences in the genotype distribution and Hardy–Weinberg equilibrium were assessed by Chi square analysis. Comparison of the age at first cancer diagnosis according to polymorphism status was assessed by the non-parametric Kruskal–Wallis and Mann–Whitney tests. A p value of <0.05 was considered statistically significant. SPSS V.18.0 (SPSS Inc., Chicago, IL) was used for data handling and for all analyses.

Results

Clinical characteristics of *TP53* R337H carriers and non-carriers enrolled in the study are shown in Table 1. Of the 136 carriers, 60 (44%) were cancer-unaffected, 29 (21%) had a previous diagnosis of ACC, 43 (32%) of BC and 4

Table 1 Clinical features of *TP53* R337H carriers and controls

	R337H mutation carriers*			Controls
	ACC	BC	Cancer-unaffected	
Number of patients	29	43	60	186
Age at siagnosis/recruitment, median (IQR)	2.5 (1–6.7)	42 (36–50)	33 (IQR 24–45)	52 (IQR 47–57)

ACC adrenocortical carcinoma, *BC* breast cancer

*Four patients developed other tumors

(3%) developed other tumors. As expected, the median age of ACC diagnosis was earlier [2.5 years; InterQuartile Range (IQR) 1.0–6.7] than that found in BC cases (42 years; IQR 36–50). In cancer-unaffected and controls, the median age at recruitment was 33 (IQR 24–45) and 52 (IQR 47–57) years, respectively.

The genotypic and allele frequencies of *MDM2, MDM4* and *USP7* SNPs among R337H carriers (with and without cancer) and controls are presented in Table 2. First, we compared the distribution of genotypes and alleles between all R337H mutation carriers, regardless of personal history of cancer, and non-carriers (control group). The genotypic and allelic distribution between groups did not differ significantly for *MDM4* and *USP7* SNPs. In contrast, *MDM2* SNP309 (T > G) was significantly associated to presence of the mutation for both, allelic ($p = 0.014$) and genotypic ($p = 0.042$) frequencies.

Second, we analyzed the overall impact of each SNP on cancer risk in the group of R337H mutation carriers, irrespective of the cancer type. No statistically significant differences in the allelic and genotypic frequencies of each SNP were found between R337H carriers with and without cancer, indicating similar frequencies among these groups (Table 2). Since the mean age at recruitment of cancer-unaffected R337H mutation carriers was earlier than that found in the BC group, meaning that some of these subjects may still develop BC, when we investigated the role of the SNPs on cancer risk we considered two different scenarios: (1) cancer unaffected *versus* all cancer-affected; and (2) cancer unaffected *versus* ACC group. Similar allelic and genotypic frequencies were observed in these comparisons.

Moreover, median age at first diagnosis was earlier in GG carriers (*MDM2* SNP309) when compared to others genotypes for both cancers (age of 23 vs. 37 years), and

Table 2 Distribution of *MDM2, MDM4* and *USP7* polymorphisms among *TP53* R337H mutation carriers (affected or not by cancer) and controls

Polymorphisms	*TP53* R337H carriers (cases)		Controls	p cancer versus no cancer	p cases versus controls
	Cancer-unaffected, N (%)	Cancer-affected, N (%)			
MDM2					
TT	18 (30.0)	25 (34.7)	79 (42.5)	0.600	0.042*
TG	26 (43.3)	33 (45.8)	83 (44.6)		
GG	16 (26.7)	14 (19.4)	24 (12.9)		
TG + GG	42 (70.0)	47 (65.3)	107 (57.5)	0.697	0.074
G	0.483	0.427	0.352		0.014*
MDM4					
TT	12 (20.0)	16 (21.3)	25 (13.5)	0.799	0.115
TC	28 (46.7)	38 (50.7)	87 (47.0)		
CC	20 (33.3)	21 (28.0)	73 (35.5)		
TC + TT	40 (66.7)	54 (72.0)	112 (60.5)	0.630	0.119
T	0.433	0.466	0.370		0.217
HAUSP					
AA	5 (8.3)	5 (6.6)	13 (7)	0.792	0.983
AG	26 (43.3)	30 (39.5)	75 (40.5)		
GG	29 (48.4)	41 (53.9)	97 (52.4)		
GA + AA	31 (51.7)	35 (46.1)	88 (47.6)	0.633	0.954
A	0.300	0.263	0.273		0.648

*Comparison of the *MDM2* rs2279744 genotypic and allelic frequencies between cases and controls (Chi square test)

also separately (ACC: age of 1 vs. 2 years; BC: age of 35 vs. 43 years, respectively), although with no significant difference. For *MDM4* and *USP7* gene SNPs, we did not find any pattern regarding to age at first diagnosis (Table 3).

Finally, we investigated the distribution of genotypes/alleles of the three SNPs according to tumor type among cancer-affected R337H mutation carriers. Although there were no differences on genotypic and allelic frequencies between ACC- and BC-affected carriers (Table 4) for any of the polymorphisms investigated, the *USP7* rs1529916 AG genotype was found in about 52% of ACC cases in comparison to 30% of the BC cases. Hardy–Weinberg equilibrium was achieved for all polymorphisms in both cases and control group.

Table 4 Distribution of *MDM2, MDM4* and *USP7* polymorphisms in *TP53* R337H carriers, according to tumor type

Polymorphisms	Adrenocortical carcinoma	Breast cancer	p
MDM2			
TT	7 (27.6)	15 (38.5)	0.408
TG	13 (44.8)	18 (46.2)	
GG	8 (27.6)	6 (15.4)	
TG + GG	21 (72.4)	24 (61.5)	0.498
MDM4			
TT	5 (17.2)	11 (26.2)	0.672
TC	15 (51.7)	19 (45.2)	
CC	9 (31)	12 (28.6)	
TC + TT	20 (69.0)	30 (71.4)	1.000
HAUSP			
AA	2 (6.9)	3 (7.0)	0.172
AG	15 (51.7)	13 (30.2)	
GG	12 (41.4)	27 (62.8)	
GA + AA	17 (58.6)	16 (37.2)	0.122

Discussion

Mdm2, Mdm4 and Usp7 are three critical p53 regulators. Mdm2 and Mdm4 degrade p53 through the binding and polyubiquitination of the protein, blocking its activity as transcriptional factor [16]. On the other hand, Usp7 play a role as a deubiquitinase, regulating the stability of p53 and the p53-binding protein Mdm2 [17–19]. Given the central role of these proteins on p53 signaling, in this study we investigated the impact of the most studied SNPs in the *MDM2, MDM4* and *USP7* genes on clinical manifestation (cancer risk, age at first diagnosis and tumor type) of *TP53* R337H mutation carriers.

To date, only a few genetic alterations have been shown to modify the LFS/LFL phenotype. Among these, a 16 bp duplication in the *TP53* gene, PIN3 (polymorphism intron 3), has been described as the germline variant with strongest modifier effect. In a study published by Marcel et al. the authors have found that cancer diagnosis occurred 19 years later in *TP53* germline mutation carriers with the 16 bp

Table 3 Distribution of mean age at first cancer diagnosis according to both polymorphism genotypes and cancer type

Polymorphisms	Adrenocortical carcinoma		p	Breast cancer		p	All cancer cases		p
	n (%)	Median (IQR)		n (%)	Median (IQR)		n (%)	Median (IQR)	
MDM2									
TT	8 (28.5)	2 (0.7–5.25)	0.415*	15 (38.5)	43 (37–48)	0.638*	23 (34.3)	37 (3–46)	0.481*
TG	13 (46.5)	3 (1.5–10.5)		18 (46.2)	42 (35–52)		31 (46.3)	32 (6–44)	
GG	7 (25.0)	1 (1.0–5.0)		6 (15.4)	35 (25–60)		13 (19.4)	23 (1–41)	
TG + GG	20 (72.4)	2.5 (1.5–9.2)	0.412**	24 (61.5)	41 (32–56)	0.452**	44 (65.3)	28 (3.2–42)	0.534*
MDM4									
TT	4 (14.3)	2.5 (1.2–38.2)	0.620*	11 (26.2)	44 (19–57)	0.891*	15 (21.4)	40 (22–50)	0.456*
TC	15 (53.6)	2 (1–6)		19 (45.2)	42 (37–50)		34 (48.5)	36 (2–43)	
CC	9 (32.1)	5 (0.8–11)		12 (28.6)	40 (36–54)		21 (30.0)	32 (5.5–42)	
TC + TT	19 (67.9)	2 (1–6)	0.487**	30 (71.4)	42 (36–50)	0.770**	49 (70.0)	36 (3–45.5)	0.667**
HAUSP									
AA	2 (7.1)	3–22	0.446*	3 (7.0)	36–39	0.821*	5 (7.0)	39 (19.5–42.5)	0.442*
AG	14 (50.0)	1.5 (1–10.2)		13 (30.2)	40 (36–54)		27 (38.0)	29 (1–42)	
GG	12 (42.9)	3 (1–5.7)		27 (62.8)	43 (32–47)		39 (55.0)	36 (6–46)	
GA + AA	16 (57.1)	2 (2.40.2)	0.888**	16 (37.2)	39.5 (36.2–37.7)	0.900**	32 (45.1)	36 (2–42)	0.293**

IQR interquartile range

*Kruskal–Wallis test

**Mann–Withney test

duplicated allele [13]. With regard to SNPs in *TP53*-related genes, although conflicting results, several publications have demonstrated the impact of *MDM2* SNP309 (T > G), a SNP located in the promoter region of *MDM2*, on earlier age of tumor onset in LFS patients carrying predominantly *TP53* DBD mutations [20–23].

Here, although not statistically significant, *MDM2* G/G genotype was found in higher frequency in ACC cases, usually a tumor of early-onset, than BC cases (27.6 vs. 15.4%, respectively). In the same way, age at diagnosis was lower in *MDM2* SNP309 GG carriers when compared to other genotypes. The fact that none statistically significant differences were observed between these comparisons might be explained by the lack of statistic power. In this context, it would be required to genotype at least 35 subjects in each genotype in order to identify a difference of 10 years in the age at diagnosis, taking into account the dispersal of ages observed in this study (with 80% power and 5% alpha). We can also hypothesize that *MDM2* SNP309 has a limited contribution when isolated assessed. In fact, Renaux-Petel et al. have only observed an effect of *MDM2* SNP309 GG genotype on age of tumor onset when haplotype analyses were performed. The authors showed that *MDM2* SNP309 along with *MDM2* SNP285 (MDM2 285–309 G-G) develop tumors 5 years earlier than patients harbouring other haplotypes [20]. In contrast, Wu et al. using a robust and reliable statistical method to evaluate cancer risk attributable to a measured hereditary susceptibility gene in family studies did not find a statistically significant interaction between *MDM2* SNP309 G allele and *TP53* mutation on cancer incidence [24].

Interestingly, we observed an enrichment of the *MDM2* SNP309 G allele in the R337H mutation carriers group (regardless of personal history of cancer) when compared to controls ($p = 0.014$), suggesting that this SNP may contribute to the LFL phenotype in families carrying the R337H germline mutation. Similar to our findings, Ruijs et al. showed higher percentage of SNP309 homozygotes (G/G) in *TP53*-negative LFS and LFS-related patients when compared to the general populations. The authors suggested that SNP309 G polymorphism act as an additional disease-causing factor [23].

We also did not observe a significant association between *MDM4* rs1563828 and *USP7* rs1529916 polymorphisms with cancer risk, age at first diagnosis and tumor type in R337H mutation carriers. Although we have found a higher frequency of *USP7* rs1529916 AG genotype in ACC cases, its potential role on cancer type in R337H mutations should be investigated in a larger series of patients and with other *TP53* germline mutation in order to confirm its potential role as a genetic modifier. If confirmed, these findings may have important clinical implications.

Our study has several limitations that must be considered in the interpretation of the results. Although the relatively small sample size, due to the rarity of the syndrome, this is one of the largest cohorts used in the context of genetic modifiers of LFS/LFL. In addition, we investigated just one SNP of each gene and the haplotype analyses have not been performed at this time. Finally, differently of *MDM2* SNP309, the mechanistic basis of a potential negative effect of *MDM4* and *USP7* on p53 signaling remains unknown. On the other hand, a positive aspect of our study is the homogeneity of the cases. Here, all patients were carriers of a particular germline mutation, the *TP53* R337H. Due the particularities found in the clinical presentation of DBD mutation and R337H carriers, we believe that different SNPs in genes of the p53 pathway may affect the p53 function in different ways. To our knowledge, this is the first study to investigate genetic modifiers in LFL patients carrying the same germline mutation.

The information about polymorphisms with modifier effect of the LFS/LFL phenotype may have important implications in the cancer risk assessment. Although several reports showing the impact of *MDM2* SP309 on clinical manifestations of LFS families carrying mostly *TP53* DBD mutations, our data indicate that this functional SNP may also play a role on age at first diagnosis in patients carrying the R337H germline mutation. The enrichment of G allele and GG genotype in LFL patients, regardless of personal cancer history, also suggest that *MDM2* SP309 may have an additive impact on LFL phenotype. Haplotype analyses as well as a larger number of patients are needed in order to confirm our findings.

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Compliance with ethical standards

Conflict of interest The authors have no conflict of interest to declare.

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