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ORIGINAL ARTICLE

Evaluation of TP53 Pro72Arg and MDM2 SNP285–SNP309 polymorphisms in an Italian cohort of LFS suggestive patients lacking identifiable TP53 germline mutations

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Abstract Li-Fraumeni syndrome (LFS) is a rare genetic cancer predisposition disease, partly determined by the presence of a *TP53* germline mutation; lacking thereof, in presence of a typical LFS phenotype, defines a wide group of 'LFS Suggestive' patients. Alternative LFS susceptibility genes have been investigated without promising results, thus suggesting other genetic determinants involvement in cancer predisposition. Hence, this study explores the single and combined effects of cancer risk, age of onset and cancer type of three single nucleotide polymorphisms (SNPs)—*TP53* Pro72Arg, *MDM2* SNP285 and SNP309—already described as modifiers on *TP53* mutation carriers but not properly investigated in *LFS* Suggestive patients. This case–control study examines 34 Italian LFS Suggestive lacking of germline *TP53* mutations and 95 tumour-free subjects. A significant prevalence of homozygous *MDM2* SNP309 G in the *LFS* Suggestive group ($p < 0.0005$) confirms its contribute to cancer susceptibility, also highlighted in LFS *TP53* positive families. Conversely its anticipating role on tumour onset has not been confirmed, as in our results it was associated with the SNP309 T allele. A strong combined outcome with a 'dosage' effect has also been reported for *TP53* P72 and *MDM2* SNP309 G allele on cancer susceptibility ($p < 0.0005$). Whereas the *MDM2* SNP285 C allele neutralizing effect on *MDM2* SNP309 G variant is not evident in our population. Although it needs further evaluations, obtained results strengthen the role of *MDM2* SNP309 as a genetic factor in hereditary predisposition to cancer, so improving LFS Suggestive patients management.

Keywords Suggestive of Li-Fraumeni syndrome · *TP53* Pro72Arg · *MDM2* SNP285 · *MDM2* SNP309 · Rare disease · Osteosarcoma

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Introduction

TP53, also called "The Guardian of the Genome", encodes for a protein playing a major role in both regulation of cell growth and maintenance of biological processes [1]. In fact, TP53 protein is a transcription factor upregulating the expression of target genes involved in cell cycle arrest, DNA repair, apoptosis and senescence, in response to DNA damage [2]. Inactivation of TP53 has been proven to be a key event in the development of a large number of human cancers. In particular, germline mutations inactivating TP53 are related to Li-Fraumeni syndrome (LFS; MIM #151623), a rare autosomal dominant disorder, characterized by early onset of specific cancer types, as pre-menopausal breast cancer, soft tissue sarcoma (STS), osteosarcoma, brain

tumours, adrenocortical carcinoma (ACC) and a variety of other neoplasms [1, 3]. From a clinical point of view the first definition of the classic Li-Fraumeni Syndrome was established by Li et al. [4] with specific stringent clinical criteria then implemented by Chompret [5], reviewed by Tinat [6] and recently updated by the French LFS working group [7]. These rules, known as "Chompret criteria", define a patient as Suggestive of LFS if one of three distinct clinical situations is applied: (a) familial presentation [a proband with an LFS tumour (breast cancer, STS, osteosarcoma, Central Nervous System tumour, ACC, leukemia, bronchoalveolar lung cancer) under 46 years and one first or second degree relative with an LFS tumour under 56 years or with multiple tumours], or (b) multiple tumours (two of which belong to the LFS spectrum, the first developed before 46 years) or (c) rare cancers (ACC or choroid plexus carcinoma or rhabdomyosarcoma, irrespective of family history).

As outlined by Tinat et al. [6], the presence of a *TP53* mutation—detected in the 30 % of this group of patients—is a discriminating factor which classify a subject as LFS; those without a *TP53* alteration are otherwise defined as 'Suggestive of Li-Fraumeni syndrome' (LFS Suggestive). The genetic predisposition to cancer in all patients without *TP53* mutations implies the contribution of hereditary determinants; to this, alternative LFS susceptibility genes have been investigated, but none has been proven to play a pivotal role in the pathology [8]. Therefore, it has been proposed that additional genetic factors like single-nucleotide polymorphisms (SNPs) may contribute to alter the predisposition to cancer development [9], with also a potential role in the determination of cancer age of onset [10].

Since 80.95 % (34 out of 42) of patients coming to the attention of the Department of Medical Genetics of Istituto Ortopedico Rizzoli (Bologna, Italy) with a clinical suspect of LFS do not carry a *TP53* mutation, we decided to investigate the effect of three SNPs already related to Li-Fraumeni syndrome: the Pro72Arg polymorphism located in the coding sequence of *TP53* [11], the *MDM2* SNP309 in the murine double minute 2 (*MDM2*) gene [11] and the *MDM2* SNP285 located in the *MDM2* promoter region, 24 bp upstream of the previous polymorphism [12]. The combined effect of these polymorphisms on cancer risk has been already evaluated in previous studies [13, 14] taking into account cohorts of patients and groups of LFS subjects with a *TP53* germline mutation but, to date, no proper attention has been dedicated to all patients without a causative *TP53* mutation.

The polymorphism at codon 72 in the *TP53* gene (NM_000546.5: c.215C>G, p.Pro72Arg, Chr17(GRCh37): g.7579472G>C, rs1042522) causes an amino acid replacement of Pro (CCC) with Arg (CGC) in the transactional domain of the protein, which seems to be responsible of a better capability in inducing apoptosis [15]; whether or not the different apoptotic potential of *TP53* Pro72Arg alters cancer risk is currently unclear and controversial [14, 15]. Meta-analyses studies on patients with various tumour types demonstrated that the Pro–Pro genotype is associated with an increased risk of carcinogenesis [16, 17]; moreover Khan et al. [17] found this effect related to the ethnicity. As a matter of fact, an increased risk of Pro–Pro genotype on cancer susceptibility had been evaluated in *TP53* mutation carriers by many studies, but its role in a *TP53* negative LFS case study has never been considered.

A meta-analysis study also reported how a large number of researches supported the existence of an associated effect between *TP53* Pro72Arg and another single nucleotide polymorphism (SNP309, NM_002392.3: c.14 + 309T>G, Chr12 (GRCh37): g.69202580T>G: rs2279744) in the *MDM2* promoter regarding an altered tumour susceptibility [16]. MDM2 is the primary negative regulator of TP53 function, which inhibits TP53-mediated transcriptional activity and targets TP53 towards proteasomal degradation [18]. A naturally occurring T to G sequence variation (SNP309) in the second promoter enhancer region of *MDM2* gene has been shown to increase the binding affinity of the trascriptional activator Sp1 resulting in higher levels of MDM2 protein, in the formation of transcriptionally inactive TP53–MDM2 complexes and in the alteration of the TP53 pathway [19]. Studies on humans and mice have shown how MDM2 over- or under-expression effectively influences TP53 function [20–25]. In particular, MDM2 and TP53 form an oscillating auto-regulatory feedback loop which is tightly controlled to allow the appropriate response to the stresses in order to suppress neoplasms development. When MDM2 activity is inappropriately heightened, TP53 activity is attenuated and consequently tumour susceptibility arises [26]. Moreover, results from clinical studies demonstrated that SNP309G allele was associated with an earlier onset of tumours in both hereditary Li-Fraumeni individuals [27] and in patients with sporadic tumour [28, 29] suggesting that this polymorphism may contribute to individual susceptibility to cancer also in LFS suggestive patients [19].

The third polymorphism—*MDM2* SNP285 (NM_002392.3:c.14 + 285G>C, Chr12(GRCh37): g.69202556G>C: rs117039649)—has recently been described within the *MDM2* promoter [12]. Population studies revealed that the SNP285C variant has occurred on the SNP309G allele and is present only in Caucasians, so defining only three possible haplotypes: 285G–309T, 285G–309G, 285C–309G [30, 32]. In vitro analyses showed that the 285C variation is able to antagonize the effect of the 309G variation through

a reduction of the Sp1 transcription factor binding strength to the *MDM2* promoter [30]; this effect was confirmed by Renaux-Petel et al. [12] who associated the *MDM2* 285G–309G haplotype with an earlier age of tumour onset in patients with Li-Fraumeni syndrome. Its role in a *TP53* negative population has never been investigated.

Aim of this study is to evaluate, the single and combined role/effect of the three mentioned SNPs as cancer susceptibility genetic determinants in an Italian cohort of LFS Suggestive patients without a *TP53* mutation, with the ultimate purpose to identify potential risk factors which could help genetic counselling to better estimate the tumour risk and, consequently, to improve the clinical management of these patients.

Materials and methods

Patients

A Caucasian population of 34 Italian unrelated subjects, suggestive for Li-Fraumeni syndrome which came to the attention of the Department of Medical Genetics of Istituto Ortopedico Rizzoli for oncologic genetic counselling from 2004 to 2015, was recruited for this study. All patients have been screened for *TP53* germline mutations—considering both point mutations and big indel—at the Laboratory of Molecular Genetics of Istituto Ortopedico Rizzoli. This dataset is composed of 10 males and 24 females with a mean age of 39.90 (from 12 to 77 years); as control cluster 95 Italian healthy subjects composed by 42 males and 53 females with a mean age of 44.42 (15–70 years) and defined by the absence of any previous malignant manifestations were enrolled.

According to the updated Chompret criteria [5], the 34 LFS Suggestive patients fitted in one of the three described above:

- 28 out of 34 had a typical familial presentation with one first or second degree relative with an LFS tumour under 56 years; to be more detailed, these patients developed a first tumour as follow: 43 % osteosarcoma, 25 % breast cancer, 11 % limphoma, 3.5 % adenocarcinoma, 3.5 % epithelial cancer, 3.5 % gastric cancer, 3.5 % thyroid cancer, 3.5 % melanoma, 3.5 % nephroblastoma
- 5 out of 34 developed at least two tumours belonging to LFS spectrum (osteosarcoma, STS, lymphoma, breast cancer) before 46 years;
- 1 out of 34 patients developed a rare cancer, the alveolar rhabdomyosarcoma, with a negative family history.

Considering all 34 oncological patients, the mean age of onset for the first tumour is 21.85 (ranging from 1 to 46 years); in particular, males have a mean age of onset of 19.3 years while for female it is 23.2 with no statistical gender-difference ($p = 0.47$, Mann–Whitney test).

Of note, being an orthopaedic institute, the most of considered patient came to our attention for muscoloskeletal problems; to this, observing the LFS tumour spectrum, osteosarcoma has an higher prevalence in our cancer patients population (44.11 %, 15 out of 34 cancer patients) compared to what described in literature [7].

Personal, clinical, genetic and genealogical data including gender, tumour type, age of onset, number of primary cancers and family history were collected for all enrolled subjects and their family members on a dedicated digital platform, named GePhCARD.

The general protocol of this study (n. 21651 "POLIMORFISMI") was approved by the Ethical Committee of Istituto Ortopedico Rizzoli; informed consent was achieved from all enrolled patients.

Mutation screening and SNP genotyping

Genomic DNA of all subjects was extracted from peripheral blood according to standard procedures.

Sanger Sequencing was used to evaluate *TP53* Pro72Arg, *MDM2* SNP285 and SNP309 genotype in our patients group and healthy control cluster as subsequently described; of note, due to the unavailability of enough genomic DNA, 2 out of 34 and 9 out of 34 patients have not been evaluated for—respectively—*MDM2* SNP309 and *MDM2* SNP285 genotype.

PCR was performed on a Veriti 96 well thermal cycler by Applied Biosystem in a 30 µl volume containing 50 ng of genomic DNA, 25 mM MgCl2, 10 mM of dNTP Mix, 10uM of each primer, 5X Colorless GoTaq Flexi Buffer and GoTaq G2 Hot Start Polymerase by Promega. PCR conditions included a denaturation step of 2 min at 95 °C followed by 35 cycles of 94 °C for 35 s, annealing for 35 s at 60 °C or 62 °C according to melting conditions and 35 s at 72 °C and a final extension at 72 °C for 5 min. Primers for exon 4 of *TP53* and *MDM2* intron 1, as well as PCR Tm conditions are shown in Table 1. Direct sequencing using the BigDye Terminator v3.1 cycle sequencing kit (Applied Biosystems, Foster City, CA) according to the manufacturer's instructions on a AB3130XL Automated DNA Sequencer and Sequence Analysis 5.3.1 software (Applied Biosystems, Foster City, CA) was performed for all 129 subjects. All analyses were performed in duplicate.

Sequences evaluation had been performed using as reference sequences NM_000546.4 for TP53 and NM_002392.3 for MDM2.

Table 1 PCR amplification conditions for exon 4 of *TP53* and *MDM2* intron 1

Primer name	Forward	Reverse	Amplicon size bp	PCR (Tm, °C)
TP53 Exon 4	ACCTGGTCCTCTGACTGCTC	GAGGAATCCCAAAGTTCCAA	407	62
*MDM2*309 Intron 1	CGGGAGTTCAGGGTAAAGGT	AGCAAGTCGGTGCTTACCTG	471	62
*MDM2 SNP*285/309 Intron 1	AGGGCGGGATTTCGGACG	AGCAAGTCGGTGCTTACCTG	414	60

Statistical analysis

All continuous data were expressed as mean while categorical variables were expressed as frequency and percentages. The ANOVA test was performed to assess the differences between groups of continuous, normally distributed and homoscedastic data; the Mann–Whitney test was used otherwise. The Kruskal–Wallis test followed by the Mann–Whitney test with the Bonferroni correction as well Jonckheere–Terpstra test for multiple comparison were used otherwise. Fisher Chi square test was performed to investigate the relationships between dichotomous variables. Pearson Chi square test, evaluated by Exact Methods for small samples, were performed to investigate the relationships between grouping variables. The Kendall Tau correlation analysis was used to test the relationship between ordinal variables. All non parametric analyses were performed applying Monte–Carlo Method for small sample size. The Generalized Linear Model (GLM) with negative binomial distribution and Log-link function was used as multivariate analysis to perform the correction for gender and type of cancer on the relationships involving dichotomic variables. The Generalized Linear Model (GLM) with Gamma distribution and Log-link function was used as multivariate analysis to perform the correction for gender on the relationships involving continuous variables. The same analysis stratified by tumour type and/or ag of onset had not been performed due to the small sample size.

All statistical analysis were performed using SPSS v.19.0 (IBM Corp., Armonk, NY, USA). For all tests $p < 0.05$ was considered significant.

Results

Molecular evaluations for each polymorphism performed on cases and controls gave the results summarized in Table 2 and subsequently detailed.

In particular, according to sequencing results for *TP53* Pro72Arg polymorphism, the genotype is estimated as follows: Arg/Arg genotype was observed in 18 out of 34 LFS Suggestive patients (53 %) and in 57 out of 95 (60 %) unaffected subjects. The heterozygous Pro/Arg state was found in 13 out of 34 patients (38.2 %) and in 32 out of 95 (33.7 %) controls. The Pro/Pro homozygous state was detected in 3 out of 34 (8.8 %) LFS Suggestive patients and in 6 out of 95 (6.3 %) controls. The genotype frequencies of *TP53* Pro72Arg revealed no significant difference between case group and controls (Table 2, Pearson's χ^2, $p = 0.77$); no gender-specific differences have been observed (Wald χ^2, $p = 0.104$). Of note, the genotype frequencies of Pro72Arg SNP in the control group was in line with what reported for Caucasian population in specific catalogues of human genetic variation (1000 Genomes, http://browser.1000genomes.org/index.html and dbSNP, http://www.ncbi.nlm.nih.gov/SNP/). As this SNP has been shown by Bougeard et al. [27] to influence the age of first tumour onset in LFS patients with a germline *TP53* mutation, we decided to investigate this effect on our *TP53* negative case study population. To this, in our population the average age of first tumour onset is 21.86 years. Grouping these patients for the *TP53* Pro72Arg genotype—as reported in Table 3—the mean ages were respectively 14.67 years for the Pro/Pro group, 19.89 years for the Pro/

Table 2 Distribution of allele frequencies of *TP53* Pro72Arg polymorphism, *MDM2* SNP309 and SNP285 in the two groups

	TP53 Pro72Arg in 34 LFS suggestive patients n (%)			*MDM2* SNP309 in 32 LFS suggestive patients n (%)			*MDM2* SNP285 in 25 LFS suggestive patients n (%)		
	Pro/Pro	Pro/Arg	Arg/Arg	TT	TG	GG	GG	GC	CC
LFS-suggestive	3 (8.8 %)	13 (38.2 %)	18 (53 %)	7 (21.9 %)	17 (53.1 %)	8 (25 %)	21 (84 %)	4 (16 %)	0 (0 %)
Controls	6 (6.3 %)	32 (33.7 %)	57 (60 %)	77 (81.1 %)	14 (14.7 %)	4 (4.2 %)	93 (97.9 %)	2 (2.1 %)	0 (0 %)
p value	0.77			<0.0005			0.017		

p value calculated with Pearson's χ^2

Arg group and 24.31 for the Arg/Arg genotype. Even if not statistically significant (Pearson's χ^2, $p = 0.48$) probably due to the small sample size, an anticipated manifestation of the first tumour emerges in presence of the Pro allele with a potential dosage effect. Being our Institute a reference centre for musculo-skeletal diseases, we decided to evaluate also the *TP53* Pro72Arg genotype contribution on cancer type comparing two patients groups: those who developed a sarcoma as first tumour (14 osteosarcoma plus 2 STS) versus "other neoplasms" cases (18 patients). Although not significant (Pearson's χ^2 $p = 0.11$), we observed the absence of Pro/Pro genotype in the "other neoplasms" group.

Considering *MDM2* SNP309, TT variant was observed in 7 out of 32 (21.9 %) LFS Suggestive subjects and in 77 out of 95 controls (81.1 %). The heterozygous TG state was found in 17 out of 32 (53.1 %) of patients group and in 14 out of 95 (14.7 %) healthy subjects. GG genotype was found in 8 out of 32 (25 %) LFS Suggestive patients and in 4 out of 95 (4.2 %) controls. These data show an evident prevalence of the GG genotype in the affected patients (Pearson's χ^2, $p < 0.0005$, Table 2) confirming its already described contribution to cancer susceptibility in LFS *TP53* negative patients described by Rujis et al. [19]; no gender-related differences have been observed. Of note, the *MDM2* SNP309 genotype frequencies in our healthy group diverges from the European ones reported in 1000 Genomes and dbSNP databases; it is however important to consider that our Italian dataset represents a subgroup of the European population and different studies on the SNP309 identified variable frequencies depending on the considered geographical area [28]. Considering the influence of SNP309 on the age of onset we did not detect any significant difference (Pearson's χ^2, $p = 0.49$, Table 3); nevertheless we observed an anticipation in cancer onset in presence of the T variant; stratifying patients for the presence of the SNP309G risk allele we obtained that the GT/GG group showed a mean age of first tumour onset of 21.6 versus 15.67 years of the TT group (Pearson's χ^2, $p = 0.29$, Table 3). Considering the tumour type, statistical analyses did not detect any difference related to SNP309 genotype (Pearson's χ^2, $p = 0.419$).

In order to investigate the interaction of the *MDM2* SNP309 and the *TP53* Pro72Arg polymorphisms in our *TP53* negative population, the combined effect of both genotypes on cancer risk was analyzed stratifying both affected and control groups for the presence of the "higher risk alleles" *MDM2* SNP309G and *TP53* Pro72 variant. A strong significant difference with a $p < 0.0005$ in cancer susceptibility was noticed (Pearson's χ^2, Table 4), in fact only 5 out of 49 subjects (10.2 %) who did not present risk alleles at both loci (Arg/Arg + T/T) belonged to the LFS Suggestive group; considering the presence of risk alleles at both loci (Pro/Pro or Pro/Arg + T/G or G/G), 13 out of 18 subjects (72.2 %) are LFS Suggestive patients, whereas 14 out of 60 (23.3 %) of those who present a risk allele in just one locus (Arg/Arg + T/G or G/G and Pro/Pro or Pro/Arg + T/T) developed LFS-spectrum tumours. Noteworthy, a statistically significant ordinal correlation was observed between the "dosage" of risk alleles—like *MDM2* SNP309G and *TP53* Pro72—and the susceptibility to cancer development (Kendall Tau B test, $p < 0.0005$), suggesting a cumulative effect.

The combined effect of *MDM2* SNP309 and *TP53* Pro72Arg related to the age of first tumour onset has also been evaluated; to this, we stratified our group considering the presence of the "anticipative alleles"—*MDM2* SNP309T and *TP53* Pro72—previously observed but we did not detect any significant difference; patients who carry "anticipative alleles" on both loci showed a mean age of

Table 3 Mean age of first tumor onset in cancer patients according to *MDM2* SNP309 and *TP53* Pro72Arg genotypes

Genotype	No. of patients	Mean age of first tumour onset (years)	*p* value
MDM2 SNP309			
T/T	7	15.67	0.49
T/G	17	22.93	
G/G	8	18.50	
T/T	7	15.67	0.295
T/G + G/G	25	21.6	
TP53 Pro72Arg			
Pro/Pro	3	14.67	0.48
Pro/Arg	13	19.89	
Arg/Arg	18	24.31	
Arg/Arg	18	24.31	0.283
Pro/Pro + Pro/Arg	16	18.58	

p value calculated with Pearson's χ^2

Table 4 Combined *MDM2* SNP309 and *TP53* Pro72Arg risk genotypes of LFS suggestive patients and controls

Combination of risk genotypes	No. of LFS suggestive patients (%)	No. of controls (%)	*p* value
T/T + Arg/Arg	5 (10.2 %)	44 (89.8 %)	0.0005
(T/G or G/G + Arg/Arg) or (T/T + Pro/Pro or Arg/Pro)	14 (23.3 %)	46 (76.7 %)	
T/G or G/G + Pro/Pro or Arg/Pro	13 (72.2 %)	5 (27.8 %)	

p value calculated with Pearson's χ^2 and Kendall Tau b ordinal correlation

tumour onset of 16.89 years against a mean age of 19.50 for patients without any "anticipative allele" (Jonckheere–Terpstra test, $p = 0.762$). In addition, considering the tumour type no joined effect for these SNPs emerged.

As described in Table 2, taking into account the 25 patients analyzed for *MDM2* SNP285, GG genotype was observed in 21 out of 25 (84 %) individuals of LFS Suggestive group and in 93 out of 95 (97.9 %) controls. The heterozygous GC statewas found in 4 out of 25 (16 %) LFS Suggestive patients and in 2 out of 95 (2.1 %) healthy subjects. No CC genotype was observed in either patients and controls. Of note, genotype frequencies for SNP285 in our control group are similar to the ones reported for the European population (1000 Genomes, dbSNP). Despite the limited number of subjects considered and the rarity of SNP285C allele in the population, results described above show a statistically significant prevalence of this allele in the LFS Suggestive patients (Pearson's χ^2, $p = 0.017$, Table 2). Confirming previous results [12, 36], we detected the SNP285C variant only in subjects harbouring the SNP309GG or GT genotyping thus indicating its presence on the SNP309G allele. Then, considering the combined role of *MDM2* SNP285 and SNP309, the observed frequencies of the three possible genotype combinations—*MDM2* 285G-309T, 285G-309G and 285C-309G—in LFS suggestive patients are 24 % (6 out of 25), 60 % (15 out of 25) and 16 % (4 out of 25) respectively, different from those described in a previous study on *TP53* mutation carriers [12]. Their corresponding frequencies in the control population are 81.1 % (77 out of 95), 16.8 % (16 out of 95) and 2.1 % (2 out of 95).To evaluate the neutralizing effect of SNP285C on SNP309G variation [30] we removed patients carrying the SNP309TT genotype and considered the two possible genotype combinations 285G-309G and 285C-309G; according to our results which showed the presence of the *MDM2* 285G-309G haplotype in 15 patients and in 16 controls (48.4 and 51.6 % respectively) versus the *MDM2* 285C-309G haplotype detected in 4 patients and 2 controls (66.7 and 33.3 % respectively), we did not attest any significant antagonizing effect of SNP285C allele on the SNP309G variant on the cancer risk (Pearson's χ^2, $p = 0.67$).

Regarding the *MDM2* SNP285 effect on the age of tumour onset, it was 18.85 years with the GG genotype and 23 years with the CG genotype (Pearson's χ^2, $p = 0.57$); even if not significant, we observed a delayed tumour onset in presence of the SNP285C allele. According to what described in previous study [30] we also considered the effect of *MDM2* SNP285C allele on the *MDM2* SNP309G variant related the tumour age of onset. Dichotomizing the patients carrying the *MDM2* 309G allele according to the presence of the *MDM2* 285C variant, we observed a higher but not significant (Pearson's χ^2, $p = 0.674$) mean age of tumour onset in patients with the 285C-309G haplotype (23 years) compared with those with 285G–309G (20.21 years); therefore the apparently neutralizing effect of 285C allele on the 'delaying' 309G variant is not evident in our population.

Discussion

Due to the high number of Li-Fraumeni patients without a *TP53* mutation—classified as Suggestive of Li-Fraumeni syndrome—our intent was to evaluate whether the presence of three SNPs involved in TP53 pathway could be related to cancer predisposition. To this, for the first time in a *TP53* negative Italian population of LFS Suggestive patients classified according to the revised Chompret criteria [7], we evaluated the effect of *TP53* codon 72 polymorphism, *MDM2* SNP309 and *MDM2* SNP285 already described as putative genetic determinants in tumour onset, though with debatable results [16, 17, 34]. In particular, *TP53* Pro72Arg has been controversially linked to cancer risk in different studies [14–17] but its role on *TP53*-negative patients has never been investigated. The *MDM2* SNP309 has been shown to be a modifier factor in *TP53* positive LFS patients, but only one previous study [19] focused the attention on a *TP53* negative combined Dutch-Finnish population. We also took into account the recently identified *MDM2* SNP285C which has been shown to antagonize the deleterious effect of the 309G variant in patients with a *TP53* germline mutation [12] but with controversial effect on sporadic cancer [33, 34].

In the LFS Suggestive group object of this study, the *TP53* Pro72Arg and *MDM2* SNP309 were analyzed in 34 and 32 patients respectively, while *MDM2* SNP285 in 25 patients; all three SNPs were genotyped in 95 tumour-free Italian controls. The identified dataset allowed us to evaluate both the "pure" and combined effect of these SNPs regardless the presence of a *TP53* mutation and to establish possible associations between their genotypes and cancer development, considering also their role on age of onset and tumour type.

Regarding predisposition to cancer development, no significant association with the *TP53* Pro72Arg genotypes within cases and controls emerged; we anyway noticed how the Pro/Pro genotype is more represented in the affected group whereas the Arg/Arg genotype is more prominent in the controls. This finding is in line with the more effective pro-apoptotic role of Arg variant compared to the Pro genotype described in literature [15, 31], also observed in a cohort study of carriers of *TP53* germline mutations [14] but without a strong association in previous studies on *TP53* negative cancer patient [17, 24]. These controversial results could be explained with a light effect of Pro genotype on cancer risk that if present in the single wild type gene copy of *TP53* mutation carriers, could have a heavier outcome. Considering *MDM2* SNP309, we detected a strong association between the presence of SNP309 G allele and the occurrence of cancer, in line with what reported by Ruijs et al. [19] and by Menin et al. [25] who also considered two *TP53* negative populations. From a molecular point of view, our results are justified by the lower percentage of cells undergoing apoptosis in individuals with a GG genotype [26] and by a more efficient surveillance system against DNA damage for TT carriers. Of note, no gender specific influence has been observed for both SNPs. Considering the combined effect of these SNPs, we observed a highly increased cancer risk in patients who carry both the *TP53* Pro72 variant and the *MDM2* 309G allele and a proportional decreasing in presence of one or none 'risk allele' at both loci. This result, which strengthens the deleterious effect observed for *MDM2* SNP309G, is in line with what demonstrated by Dumont et al. [15] through biochemical studies; in fact, considering Pro72 reduced capacity in inducing apoptosis and the TP53 basal level decrease caused by higher 309G induced MDM2 levels, SNP309G and Pro72 subjects have a less effective TP53-mediated tumour suppression, leading to an higher susceptibility in cancer development.

In contrast to the amount of studies on *TP53* Pro72Arg and *MDM2* SNP309, only a few have explored the effect of the *MDM2* SNP285, due to its recent identification. The SNP285C variant resides on the SNP309G allele and creates a distinct SNP285C/309G haplotype, typical of the Caucasian population [32]. In vitro analyses indicated that this SNP neutralizes the 309G variation effect, with reduced risk in different cancer types [33]; we did not detect this effect in our population.

Probably due to the limited number of LFS Suggestive patients, we did not detect any significant difference comparing *TP53* Pro72Arg, *MDM2* SNP309 and *MDM2* SNP285 genotypes with the age of tumour onset. It is however important to highlight that patients carrying the Pro/Pro genotype develop the first tumour 10 years earlier than those with the Arg–Arg genotype. Moreover, contrasting with what described for TP53 mutation carriers where *MDM2* SNP309 G appears to have anticipating effect [19, 27] and in line with what described by Ruijs et al. in a *TP53* negative LFS population, the presence of the SNP309 T allele seems to have an accelerating effect on first tumour development: in fact LFS Suggestive patients with SNP309 TT genotype develop the first tumour 6 years earlier than the others. No cumulative effect has been observed when combining the *MDM2* SNP309 and the *TP53* codon 72 polymorphism. Despite the small number of patients carrying the SNP285 C allele, we also evaluated the effect of the *MDM2* SNP285-SNP309 haplotype on age of tumour onset; this analyses revealed a not significant difference of 2.79 years comparing patients with the *MDM2* SNP285C-309G haplotype (23 years) versus the *MDM2* SNP285G-309G (20.21 years), in contrast with evidence reported in literature [12].

Being our Institute a reference center for musculoskeletal diseases we evaluated the *TP53* Pro72Arg, *MDM2* SNP309 and *MDM2* SNP285 contribution on the cancer type comparing patients who developed osteosarcoma and STS as first tumour with the other LFS spectrum neoplasms. No relevant observations emerged, apart from the presence of the Pro–Pro genotype only in 'sarcoma group'. It will be interesting to evaluate this result considering a wider dataset.

In conclusion, in order to justify a LFS phenotype in absence of a *TP53* mutation, potential causative genes or additional functional modifiers will probably have to be taken into consideration, including also the effect of other loci which have been shown to modulate the TP53-MDM2 pathway, such as miR-605 [35] or other novel SNPs. Despite this, the strong association between SNP309G and cancer predisposition in a LFS Suggestive *TP53* negative group lead us to hypothesize that it could heavily affect the LFS phenotype regardless the presence of a *TP53* germline alteration, so acting not only as a modifier but also as a potential disease causing factor. In addition, it would be interesting to further investigate in a wider Italian population, the role of the SNP309 T allele, as well as the combination with the *TP53* Pro72 variant as possible

'anticipative' elements. If confirmed, these data could be taken into account for patients' counseling, assuming that they may be considered cancer predisposing factors.

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Compliance with ethical standards

Conflict of interest The authors declare that they have no conflict of interest.

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