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Ewing Sarcoma: influence of *TP53* Arg72Pro and *MDM2* T309G SNPs

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Abstract The Ewing Sarcoma is an important tumor of bone and soft tissue. The SNPs Arg72Pro of *TP53* and T309G of *MDM2* have been associated with many cancer types and have been differently distributed among populations worldwide. Based on a case–control design, this study aimed to assess the role of these SNPs in 24 Ewing Sarcoma patients, compared to 91 control individuals. DNA samples were extracted from blood and genotyped for both SNPs by PCR–RFLP and confirmed by DNA sequencing. The results showed an association between the G allele of the T309G and Ewing Sarcoma ($P = 0.02$). Comparing to the TT carriers, the risk of G allele carriers was 3.35 (95 % CI = 1.22–9.21) with $P = 0.02$. At the genotypic level, an association of the TT genotype with the control group ($P = 0.03$) was found. Comparing to the TT genotype, the risk of TG and GG was 2.97 (95 % CI = 1.03–8.58) with $P = 0.04$ and 5.00 (95 % CI = 1.23–20.34) with $P = 0.02$, respectively. No associations regarding the Arg72Pro SNP were found. Considering that the T309G has been associated with several types of cancer, including sarcomas, our results indicate that this SNP may also be important to Ewing Sarcoma predisposition.

Keywords Arg72Pro · T309G · TP53 · MDM2 · Ewing Sarcoma

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Introduction

The Ewing Sarcoma is part of the aggressive Ewing Sarcoma Family Tumors (ESFT). These tumors of bone and soft tissue occur mainly in children and young adults, being the second most common tumor of this type in such groups. The ESFT have a biological characteristic of a fusion of the *EWS* gene on chromosome 22 with one of the members of the *ETS* gene family of transcription factors. The fusion with *FLI1* gene on chromosome 11 is one of the most common genetic events in this regard, leading to the protein EWS/FLI that is important for oncogenesis [1–4].

The Ewing Sarcoma is rare. Its incidence in individuals up to 39 years old in northern England between 1981 and 2002 was 2.14 per million persons. On US, the annual incidence between 1973 and 2004 was 2.93 per million persons. Moreover, another study from US states that the incidence of Ewing Sarcoma is higher among Caucasians [5–7].

The p53 protein, encoded by tumor suppressor *TP53* gene, acts principally as a transcription factor of many genes involved in control of processes related to tumor growth, including cell cycle regulation, DNA preservation, apoptosis, angiogenesis inhibition and cellular senescence. The loss of p53 function by mutations in *TP53* or in genes of proteins that interact with p53 protein favors cellular proliferation and tumor initiation and progression. This gene is commonly mutated in tumors [8, 9]. The International Agency for Research on Cancer (IARC) registered 27,580 somatic mutations, 597 germline mutations and 2,314 mutant forms of this protein. Mutations in *TP53* that modified its transcriptional activity are present in approximately 50 % of all cancers [10, 11]. One of the most known polymorphisms of *TP53* is the single nucleotide polymorphism (SNP) at codon 72, located at the exon 4 of this gene. This SNP is a non-conservative change of the wild type variants Arginine (CGC) and Proline (CCC) (Arg72Pro–dbSNP ID: rs1042522), that results in different biological functions of p53. Previous studies indicate that the Pro variant is related to a stronger transcriptional activation while the Arg variant is better at inducing apoptosis. This SNP have been associated with cancer susceptibility [9, 12–14]. Cancer types like bladder [15], gastric [16] and thyroid [17] have been associated with Proline amino acid while breast [18] and colon and rectum [19] have been associated with Arginine amino acid. Moreover, a study found an association between the heterozygous genotype of Arg72Pro and cervical cancer risk [20]. The Arg72Pro SNP contributions to susceptibility to some cancers have been also published on a recent meta-analysis that confirms the associations of this SNP with some cancers. The study highlights the small number of studies and participants as a limiting aspect for the results and also suggests the roles of ethnicity and histological type of cancer in Arg72Pro association with cancer risk [21].

The p53 protein interacts with the MDM2 protein. In its main pathway, this protein binds to p53 leading to its degradation via proteasome in a normal ("non-stressed") situation. In stressed cells, p53 is phosphorylated, which interrupts the interaction with MDM2 complex, which is degraded, resulting in activated p53 that will act regulation the transcription of several genes [22]. The *MDM2* gene contains two promoter regions where the first controls the MDM2 levels, and the second region, (which is the first intron), increases its expression following p53 response. In this second promoter region there is a SNP at nucleotide 309 characterized as a change of T to G (T309G–dbSNP ID: rs2279744) [23]. This SNP has been related to cancer susceptibility. The studies of Bond et al. [23, 24] states that the G allele of T309G increases the levels of MDM2 by enhancing the binding site of SP1 transcription factor of the MDM2 promoter. This results in p53 inhibition, thus attenuating its apoptotic and tumor suppression responses. This SNP has recently been associated with the susceptibility to osteosarcoma [25] and other cancers [26] [27] [28], and two recent meta-analyses reported that this SNP can be an important cancer risk marker by interacting with Arg72Pro SNP [29, 30]. Thus, considering the importance Arg72Pro and T309G in cancer, this study aimed to assess the association of these SNPs with Ewing Sarcoma.

Materials and methods

Study population

Both cases and controls are from Southern Brazil. The case group included 24 Ewing Sarcoma patients recruited between 1991 and 2004. The DNA was previously extracted from the blood following the protocol of Lahiri and Nurnberger [31]. Additional phenotypic and genotypic information about these patients are presented in Silva et al. [32].The control group was composed by 91 clinically healthy individuals sampled from the 1982 Birth Cohort Study of Pelotas. The individuals of this cohort have been followed since their birth, and its methodological aspects have been well reported previously [33, 34]. The DNA was extracted as previously described [35]. This study was approved by the Research Ethics Committee of each institution.

Phenotyping

The Ewing Sarcoma diagnosis for all patients was confirmed by image and clinic analyses made by oncologists of Child Cancer Institute of Porto Alegre Clinic Hospital.

Genotyping

Both SNPs (TP53 Arg72Pro and MDM2 T309G) were genotyped by PCR–RFLP using GoTaq® qPCR Master

Mix (Promega, USA) in a total volume of 12 µl. Both SNPs were genotyped using primers and restriction enzyme previously described [36, 37]. For the Arg72Pro SNP, the 199 bp amplicon was digested using the restriction enzyme *Bst*UI (New England Biolabs, MA) and later analyzed on 2.5 % agarose gel stained with GelRed™ (Biotium Inc., CA). For this SNP, the genotyping was performed as the following: Pro/Pro genotype resulted in one fragment of 199 bp; the Arg/Pro genotype had three fragments of 199, 113 and 86 bp and the Arg/Arg genotype had two fragments of 113 and 86 bp. For the T309G SNP, the 157 bp amplicon was incubated with the restriction enzyme *MspA*1I (New England Biolabs, MA) and analyzed on 2.5 % agarose gel stained with GelRed GelRed™ (Biotium Inc., CA). The three genotyping possibilities were: TT presented one fragment of 157 bp; TG had three fragments of 157, 109 and 48 bp and the GG genotype showed two fragments of 109 and 48 bp.

Sequencing

The genotypes of Arg72Pro SNP and T309G were confirmed by sequencing using the same primers used for genotyping. The amplicons were purified using the GFX PCR DNA and Gel Band Purification Kit (GE Healthcare, USA) and sequencing was carried out in a MegaBACE 1000 DNA sequencer (GE Healthcare, USA using the Dynamics ET-terminator technology. The sequences were analyzed with ContigExpress® of Vector NTI 10.0 (Invitrogen, USA).

Statistical analyses

For statistical analyses, the Fisher's exact test was performed to evaluate the association between the genotypes, alleles and combinations of the SNPs with the occurrence of Ewing Sarcoma. For allele analysis the following patterns were used: Arg carriers (Arg/Arg and Arg/Pro genotypes); Pro carriers (Arg/Pro and Pro/Pro genotypes); T carriers (TT and TG genotypes); G carries (TG and GG genotypes). The significance was defined as $P \leq 0.05$. Hardy–Weinberg equilibrium and linkage disequilibrium were also evaluated. Logistic regression was used to obtain the odds ratio (OR) and the 95 % confidence intervals (95 % CI) for genotypes, alleles and SNP combination analyses between cases and controls. For this analysis the genotypes Arg/Arg (for Arg72Pro SNP) and TT (for T309G) were considered as the reference categories. For SNP combinations, a variable containing the genotype information for both SNPs simultaneously was generated. The four logistic regression models were assessed for overdispersion using a Chi squared test. The data were analyzed using SPSS 16.0 and R 2.13.1.

Results

We genotyped the *TP53* Arg72Pro and *MDM2* T309G SNPs in 24 Ewing Sarcoma patients and 91 control individuals. Of the 24 patients diagnosed with Ewing's sarcoma, 14 (58.33 %) were female and 10 (41.67 %) were male. All 24 patients had white skin color. The age of the patients ranged from 1 to 21 years old. The sarcoma was present in several locations, including bone and soft tissues. The main sites affected were hips, pelvis, femur, tibia, humerus, elbow, chest and jaw. The diagnoses were performed using X-rays, exploratory surgeries, biopsies and lumbar puncture. With respect to treatment, patients underwent chemotherapy and radiotherapy sessions. Some patients also underwent surgery. After the different types of treatment, most patients showed complete disease remission.

The genotype distributions between cases and controls were in Hardy–Weinberg equilibrium ($P < 0.05$) and linkage disequilibrium was not significant ($P = 0.19$). Moreover, none of the logistic regression models showed overdispersion ($P > 0.4$). The Table 1 shows the genotypic and allelic frequency distributions of both SNPs among cases and controls and also the OR (95 % CI) of genotypes and alleles. Regarding the Arg72Pro SNP of *TP53*, no statistical differences were observed between groups (Ewing Sarcoma patients and controls) regarding genotypes and alleles ($P > 0.05$). Regarding the analyses of the T309G SNP of *MDM2*, the genotype TT was associated to the control group ($P = 0.03$) and the G allele was associated with the Ewing Sarcoma patients ($P = 0.02$). The TG and GG genotypes showed a significant increase in cancer risk in comparison to TT genotype ($P = 0.04$ and 0.02, respectively). Moreover, the OR of G allele carriers was significant in comparison to the TT carriers ($P = 0.02$). Regarding the combined genotypes analysis (combining both SNPs), a $P = 0.02$ showed that the genotypic combination Arg/Pro + TT (23.1 %) were related to the control group while the genotypes Arg/Arg + TG (33.3 %) were associated with Ewing Sarcoma.

Discussion

Ewing Sarcoma is a rare disease with a frequency of 2–3 per million in Caucasian populations in Western countries [38]. Given this information and the fact that the state of Rio Grande do Sul has a population of 10.5 million people [39], it is expected a frequency of 21–35 affected individuals. We recruited 24 patients for this study. Therefore, even though we used a modest sample size, it is in accordance with the number of cases of Ewing Sarcoma expected to occur in Rio Grande do Sul.

Table 1 Genotypic and allelic frequencies of Arg72Pro and 309T>G in Ewing Sarcoma patients and controls

	Ewing Sarcoma n (%)	Controls n (%)	*P*	OR (95 % CI)	*P*
p53 Arg72Pro SNP					
Genotypes					
Arg/Arg	14 (58.3)	40 (44.0)	0.42	1[a]	0.44
Arg/Pro	8 (33.3)	43 (47.3)		0.53 (0.20–1.40)	0.20
Pro/Pro	2 (8.3)	8 (8.8)		0.71 (0.13–3.77)	0.69
Allele					
Arg carriers	22 (91.7)	83 (91.2)	1.00		
Pro carriers	10 (41.7)	51 (56.0)	0.25	0.56 (0.22–1.39)[b]	0.21
MDM2 309T>G					
Genotypes					
TT	6 (25.0)	48 (52.7)	0.03	1[a]	0.05
TG	13 (54.2)	35 (38.5)		2.97 (1.03–8.58)	0.04
GG	5 (20.8)	8 (8.8)		5.00 (1.23–20.34)	0.02
Allele					
T carriers	19 (79.2)	83 (91.2)	0.14		
G carriers	18 (75.0)	43 (47.3)	0.02	3.35 (1.22–9.21)[c]	0.02
Total n (%)	24 (100)	91 (100)			

[a] Reference category

[b] The Arg/Arg carriers were considered as reference category versus the Pro allele carriers (Arg/Pro and Pro/Pro)

[c] The TT carriers were considered as reference category versus the G allele carriers (TG and GG)

This study evaluated the roles of *TP53* Arg72Pro and *MDM2* T309G SNPs in Ewing Sarcoma. Regarding the Arg72Pro SNP, no associations were found either on genotypic and allelic analysis. For the T309G SNP, the genotypic analysis showed a $P = 0.03$, associating the TT genotype to the control group. Compared to the TT genotype, the genotypes TG and GG increased cancer risk: 2.97 (1.03–8.58) and 5.00 (1.23–20.34), respectively. At the allelic level, the G allele was associated with Ewing Sarcoma ($P = 0.02$) and with an increased risk of Ewing Sarcoma, since the risk of the G allele carriers was 3.35 (1.22–9.21), compared to TT carriers. Previously, the studies of Bond et al. [23, 24] elucidated the molecular mechanisms that could explain our associations. The authors affirmed that the G allele amplify the MDM2 levels, inhibiting p53 response. Furthermore, the authors affirm that in some GG genotype individuals, the apoptosis and cell cycle arrest functions are low permitting that cells containing mutations could multiply allowing cancer at younger ages [23, 24].

Regarding the combined genotypes analysis, the Arg/Pro + TT combination was associated with the control group, while Arg/Arg + TG was associated with cancer. Atwal et al. [40] considered that the presence of the G allele, that has lower apoptotic function than the T allele, could compensate for the higher apoptotic activity of Arg.

Our finding suggests, for the first time, the importance of the G allele of T309G for Ewing Sarcoma. This result is corroborated by previously studies that evaluated the association between this SNP and other sarcoma types, including osteosarcoma (the most common bone tumor in children). The authors found that the frequency of GG genotype was higher in osteosarcoma cases than in controls. Moreover, they found that GG females had ~4 fold higher risk for osteosarcoma compared with TT females and ~2 fold increase comparing females carrying at least one G allele to males. An association of Pro/Pro of Arg72Pro SNP with risk of osteosarcoma was not found, but Arg/Pro was reported to reduce cancer risk compared with Arg/Arg. The study also showed that the Pro/Pro genotype increased death risk [25]. Recently, a meta-analysis including cancer types such as osteosarcoma, soft tissue sarcomas, Kaposi's sarcoma and Uterine leiomyosarcoma confirmed that the T309G—but not Arg72Pro—have been associated with risk of different sarcomas, with the G allele carriers where at increased risk of developing sarcomas [41]. All these studies, combined with our results, indicate the roles of these SNPs in susceptibility to important sarcomas.

The *MDM2* T309G SNP has also been described in association with diverse tumors. Recently, a meta-analysis showed that the GG genotype is associated with increased risk of endometrial cancer and may be a potential marker for this type of cancer. The authors still affirm that the GG genotype could increase in 65 % the risk for endometrial cancer [26]. Similarly, another study showed that the GG

genotype increased susceptibility to Acute Myeloid Leukemia among children [27]. A meta-analysis showed that the G allele may be a risk factor for hepatocellular carcinoma, but indicates that more studies—with larger samples and including different ethnicities—are needed. [28].

Recently, the importance of T309G in susceptibility to various tumor types was confirmed on a meta-analysis of Wan et al. [29]. The authors found that the GG and TG genotypes were associated with an increased cancer risk and proposed a relation of GG genotype of T309G and Pro/Pro genotype of Arg72Pro in increasing cancer risk. Another interesting aspect of this study is that the T309G frequency differed among populations. The meta-analysis indicated an association of GG genotype with tumor risk among the populations from Europe and Asia [29]. Another meta-analysis also associated the T309G SNP to increased risk of cancer, suggesting this SNP as a possible biomarker for cancer risk. The authors propose further studies to elucidate ethnic differences in cancer risk [30].

These ethnic differences in the frequencies of the T309G have also been discussed for diverse populations. Sucheston et al. [42] showed a variation of the frequencies of the T309G SNP in populations worldwide affirming that the G allele had elevated prevalence in populations of West Asian, Native Americans and East Asian in comparison with Sub Saharan Africa in which it was uncommon.

With respect to the limitations of our study, the cases ranged from 1 to 21 years old individuals from Southern Brazil, while the control group was composed of 23 years old individuals, all from Pelotas (a city also in Southern Brazil). This difference could introduce a selection bias if the association between the genotype and disease was modified by the place of residence. Because there is no evidence of such association, we believe that our study is not susceptible to selection bias.

In conclusion, we found an association of G allele of *MDM2* T309G SNP with Ewing Sarcoma patients and the TT genotype with the control group. Therefore, our results showed that this SNP might be important regarding Ewing Sarcoma predisposition. Thus, considering the reported importance of this SNP in risk of diverse types of sarcomas and cancer types, the suggestion of Wo et al. [30] to evaluate the ethnic differences in cancer risk and also regarding the differential distribution of the T309G among populations stated by the study of Sucheston et al. [42], it seems interesting to evaluate the value of this SNP in Ewing Sarcoma patients of populations with different characteristics in order to contribute to elucidate these relations.

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