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Maternal SNPs in the p53 pathway: Risk factors for trisomy 21?

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Abstract. The p53 family and its regulatory pathway play an important role as regulators of developmental processes, limiting the propagation of aneuploid cells. Its dysfunction or imbalance can lead to pathological abnormalities in humans. The aim of this study was to evaluate the effect of maternal polymorphisms *TP53* c.215G>C (P72R), *TP73* 4 c.-30G>A and 14 c.-20C>T, *MDM2* c.14+309T>G (SNP309), *MDM4* c.753+572C>T and *USP7* c.2719-234G>A as risk factors for Down Syndrome (DS) birth. A case-control study was conducted with 263 mothers of DS children and 196 control mothers. The distribution of these genotypic variants was similar between case and control mothers. However, the combined alleles *TP53* C and *MDM2* G, and *TP53* C and *USP7* A increased the risk of having offspring with DS (OR = 1.84 and 1.77; 95% CI; $P < 0.007$ and 0.018, respectively). These results suggest that, although the individual polymorphisms were not associated with DS birth, the effect of the combined genotypes among *TP53*, *MDM2* and *USP7* genes indicates a possible role of *TP53* and its regulatory pathway as a risk factor for aneuploidy.

Keywords: Down syndrome, *TP53*, *TP73*, *MDM2*, *MDM4*, *USP7*

1. Introduction

Down Syndrome (DS), characterized by trisomy of chromosome 21, is the most common cause of mental retardation in humans [23], occurring in 1 in 700–800 births [30]. The meiotic nondisjunction is the main cause of free 21 trisomy, event responsible for the aneuploidy 21 in 95% of affected individuals [3]. In 95% of cases, the nondisjunction occurs during maternal meiosis [1], mainly in the first meiotic division [2,59]. It is well established that advanced maternal age is a risk factor for aneuploidy and is associated specifically with errors that occur during oogenesis [59].

Given the important role played by p53 family proteins as regulators of crucial developmental processes, their dysfunction or imbalance can lead to pathological abnormalities in humans. Genomic instability, aneuploidy and copy number polymorphisms that originate in the female germline and contribute to a number of developmental defects can be explored through investigations of the *TP53* gene family [26]. Encoded by *TP53* gene, the p53 protein has known importance in the prevention of tumors and genomic stability in somatic cells, acting as a transcription factor that regulates a large number of genes in response to cell damage, including activation of oncogenes and DNA damage [12,35,45]. When activated, p53 initiates cellular responses, such as cell cycle arrest, DNA repair, senescence and apoptosis [22,27]. The loss of p53 allows the accumulation of aneuploid cells as a result of chromosomal instability. Thus, p53 and its regulatory pathway play a critical role in limiting the propagation of aneuploidy and preserving the nature of diploid human cells [55].

The central control of the p53 regulatory pathway consists of three major genes and their products:

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MDM2 (*Mouse double minute p53 binding protein homolog 2*), *MDM4* (*Mouse double minute p53 binding protein homolog 4*) and *USP7* (*ubiquitin specific peptidase 7* (*herpes virus-associated*)), also known as *HAUSP* [10,29]. The main negative regulator of the *TP53* is the protein *MDM2*, which acts on the p53 as an E3 ubiquitin ligase, leading to degradation of p53 [9, 31]. *MDM2* is upregulated by *TP53*, where the increase in p53 levels leads to increased transcription of *MDM2*. Thus, the product degrades p53 by inhibiting their levels, resulting in a negative feedback loop. This process maintains the p53 protein at low level in the absence of stress signals, allowing normal cell proliferation [42, 47]. Participating in the same metabolic pathway, the *TP73* gene plays a crucial role in maintaining the rate of ovulation and acting on the spindle checkpoint, reducing aneuploidy in the offspring [57]. p73 plays an important role in maintaining genomic integrity as well, which is particularly important when p53 function is compromised [5].

Single nucleotide polymorphisms (SNPs) in genes of the p53 regulatory pathway have been targeted for study in research relating to human reproduction [16, 17,29]. A common polymorphism in *TP53* c.215G>C (P72R, rs1042522) [11], a substitution at codon 72 that makes the induction of apoptosis less efficient [15,54]. The *MDM2* gene has an important functional polymorphism c.14+309T>G (SNP309, rs2279744), the result of a thymine to guanine change in its promoter region [7], increasing *MDM2* expression and attenuating the p53 function [7,16,29]. A substitution in intron 9 of *MDM4* gene c.753+572C>T (rs1563828) is correlated with human reproduction, as well as c.2719-234G>A change in intron 25 of *USP7* gene (rs1529916) [29]. In *TP73*, two closely linked polymorphisms in position 4 c.-30G>A and 14 c.-20C>T (rs2273953, rs1801173) are located before the initiating codon in exon 2. This region can form a clamp-shaped structure with the potential to interfere in gene expression [28].

Thus, we hypothesized that polymorphisms related to *TP53* and *TP73* genes and genes in their regulatory pathway – *MDM2*, *MDM4* and *USP7* – may be closely associated with human reproduction, where its fine regulation is extremely important in maintaining genomic stability of germline cells avoiding aberrations in its genome, as aneuploidies. This study investigated the influence of the *TP53* gene family and their regulators as risk factors for aneuploidy of chromosome 21. We analyzed the role of *TP53* c.215G>C polymorphism (rs1042522), *TP73* c.-30G>A (rs2273953) and c.-20C>T (rs1801173), *MDM2* c.14+309T>G (rs2279744), *MDM4* c.753+572C>T (rs1563828) and *USP7* c.2719-234G>A (rs1529916) as maternal risk factors for DS birth in a case-control study.

2. Materials and methods

2.1. Subjects

All cases were identified through Medical Genetic Service of Hospital de Clínicas de Porto Alegre (HCPA) and local support groups of DS (APAEs). The control group consisted of women with healthy children who were randomly selected to participate in this study during the blood collection for routine laboratorial analyzes in the HCPA. The case-control study was conducted with 263 case mothers and 196 control mothers. Further details on the selection and sample characteristics can be found in our previously published work [8].

This study was approved by the Ethics Committee of HCPA. All mothers who participated in the study signed an informed consent form. We collected 5 mL of peripheral blood in EDTA tubes for genetic analysis.

2.2. Analysis of polymorphisms

DNA was extracted from blood samples as described by Lahiri and Nurnberger [32]. The SNPs of five genes of the p53 signaling pathway were genotyped, including *TP53*, *TP73*, *MDM2*, *MDM4* and *USP7*. Genotypes were determined by using the following allelic discrimination Taqman probes (Applied Biosystems): C_2403545_10 (*TP53* gene), C_9493064_10 (*MDM4* gene) and C_9688119_1 (*USP7* gene). Since these two loci are closely linked, the genotype determination of polymorphisms c.-30G>A (rs2273953) and c.-20C>T (rs1801173) of *TP73* gene was performed with the probe C_16180357_10 determining polymorphism c.-30G>A (rs2273953), a method previously used by Hamajima and colleagues (2002) [21] and Scacchi and colleagues (2009) [51]. To determine the genotype of *MDM2* gene c.14+309T>G, probes were used which were labeled with FAM-TCCCGCGCCGCAG and VIC-CTCCCGCGCCGAAG fluorescence and primers forward 5'-CGGGAGTTCAGGGTAAAGGT-3' and reverse 5'-ACAGGCACCTGCGATCATC-3'. The real-time PCR reactions were performed in 96 well plates in each reaction containing: 10 ng of genomic DNA, 2x MasterMix Genotyping TaqMan (Applied Biosystems), probes specific for each polymorphism (40x) and enough water to reach 8 μL. The re-

actions were conducted in the StepOnePlus^{TM} PCR Real-Time System, with an initial cycle of 10 minutes at 95°C, followed by 45 cycles at 95°C for 15 s and 63°C for 1 minute. The reactions for c.14+309T>G *MDM2* gene were also conducted in 96 well plates in each reaction containing: 10ng of genomic DNA, 2x MasterMix Genotyping TaqMan (Applied Biosystems), 1 μM of each primer and probe, and sufficient water to reach 25 μL. This reaction was also conducted in StepOnePlus^{TM} PCR Real-Time Systems, with the initial cycle of 2 min at 50°C for 10 minutes and heating at 95°C, followed by 45 cycles at 95°C for 15 s and 60°C for 1 minute. The reaction products were analyzed on StepOne V2.2.2 Software.

2.3. Statistical analysis

Statistical analysis were performed using SPSS software, version 14.0. The chi-square was used to test the Hardy-Weinberg equilibrium, to compare allelic and genotypic frequencies, to compare the ethnicity, and the frequency of spontaneous abortions between groups. The gene-gene additive effect was also analyzed by chi-square test and logistic regression models were used to control the effect of maternal age at the time of conception. For maternal age, a dichotomous variable was used ($<$ 35 or $\geqslant$ 35 years) due to high prevalence of children with DS in women aged over 35 years, and t test was performed to compare the mean maternal age. The ORs were used to quantify the association between each polymorphism and the risk of having a DS child. Using Epi-Info software version 6.0, we estimated to be able to detect an OR of 2.5 with a sample size of 150 cases and 150 controls with an assumed power of 80% and confidence level of 95%. The Bonferroni's correction was applied for five tests in logistic regression analisys ($\alpha_{Bonf} = 0.01$).

3. Results

A total of 263 case mothers and 196 control mothers were studied. As expected, the mean maternal age was higher in case mothers (34.75 years $\pm$ 8.05 vs. 28.26 $\pm$ 5.99, $P = 0.000002$) as well as the prevalence of mothers over 35 years of age in the case group (57% vs. 20%, OR = 5.31, 95% CI = 3.45–8.17, $P <$ 0.000001) (6 missing values in control group). The case group contained 237 (90.1%) mothers classified as Euro-descendants, 17 (6.5%) as African-descent and 9 (3.4%) classified as other ethnicity. In the control group, 175 (89.7%) mothers were classified as Euro-descendants, 10 (5.15%) as African-descent and 10 (5.15%) as other ethnicity (1 missing value). The ethnic groups did not differ significantly between groups ($P = 0.569$). A higher frequency of spontaneous abortions was observed in the case group (21.7% vs. 9.7%, $P < 0.001$).

Table 1 shows the distribution of genotypes and allele frequencies of the studied polymorphisms between case and control groups, as well as in other european and euro-descendant populations. The allelic and genotypic frequencies of polymorphisms were in Hardy-Weinberg equilibrium and did not differ between case and control groups when analyzed separately, even when controlled for maternal age, ethnicity and spontaneous abortion. Our observed allele frequencies are in accordance to those described for euro-descendants.

The gene-gene additive effect was analyzed by a combination of *TP53* risk allele with the risk alleles of other genes of its pathway (*TP53* C and *MDM2* G; *TP53* C and *MDM4* T; *TP53* C and *USP7* A; *TP53* C and *TP73* A/T). As shown in Table 2, the risk of having a child with DS in women with risk alleles for *TP53* + *MDM2* and *TP53* + *USP7* is 1.84 and 1.77 times higher, respectively (95% CI and $P < 0.007$ and 0.018), when adjusted for maternal age. We additionally tested the interaction of *TP53*, *MDM2* and *USP7* risk alleles together. The risk of having a DS child with this genotype is 2.04 higher (95% IC and $P < 0.020$) when controlled for maternal age. Interestingly, women under the age of 35 years at the time of conception showed a higher frequency of *TP53* C allele associated with *USP7* A allele (OR = 1.99; 95% CI = 1.08–3.66; $P <$ 0.026). When applying the Bonferroni's correction for multiple comparisons, the *TP53*/*MDM2* additive effect maintained its statistical significance both in the unadjusted and in adjusted for maternal age model. However, the gene-gene additive effect including *USP7* kept its significance only in the unadjusted analysis.

4. Discussion

Recent evidence has shown the important role played by p53 family as regulators of crucial processes related to human reproduction [14,26]. Our working hypothesis was based on two main pieces of evidence: 1) The loss of p53 function or genes that regulate this metabolic pathway may be related to the accumulation of aneuploid cells, increasing the risk of DS birth in women with these polymorphisms [55]. This same loss

Table 1
Allelic and genotypic frequencies of SNPs in the *TP53* pathway

Gene	Genotype/Allele	Case n (%)	Control n (%)	P^*	Expected allele frequency**	
					1000 Genomes [52]	HapMap [53]
TP53	GG	116 (44.1)	99 (50.5)	0.322		
(rs1042522)	GC	123 (46.8)	78 (39.8)			
	CC	24 (9.1)	19 (9.7)			
	G	355 (67.5)	276 (70.4)	0.386	78.9	76.7
MDM2	TT	104 (39.5)	82 (41.9)	0.442		
(rs2279744)	TG	123 (46.8)	81 (41.3)			
	GG	36 (13.7)	33 (16.8)			
	T	331 (62.9)	245 (62.5)	0.949	64.0	NA
MDM4	CC	88 (33.5)	70 (35.7)	0.832		
(rs1563828)	CT	124 (47.1)	87 (44.4)			
	TT	51 (19.4)	39 (19.9)			
	C	300 (57.0)	227 (57.9)	0.843	67.9	65.0
USP7	GG	125 (47.5)	101 (51.5)	0.282		
(rs1529916)	GA	122 (46.4)	78 (39.9)			
	AA	16 (6.1)	17 (8.6)			
	G	372 (70.7)	280 (71.4)	0.873	67.3	68.9
TP73	GG/CC	161 (61.2)	109 (55.6)	0.381		
(rs2273953 and rs1801173)	GA/CT	87 (33.1)	71 (36.2)			
	AA/TT	15 (5.7)	16 (8.2)			
	G/C	409 (77.8)	289 (73.7)	0.181	79.9	NA

*Chi-square; **Data from european and euro-descendants populations; NA = not available.

Table 2
Risk allele combinations in the *TP53* pathway

Alleles†	Case n (%)	Control n (%)	P^*	OR (IC 95%)	P^{**}	OR (IC 95%)**
*TP53*C + *MDM2*G	94 (35.7)	46 (23.5)	0.006	1.81 (1.17–2.81)	0.007	1.84 (1.18–2.89)
*TP53*C + *MDM4*T	96 (36.5)	65 (33.2)	0.521	1.16 (0.77–1.74)	0.536	1.14 (0.75–1.74)
*TP53*C + *USP7*A	80 (30.4)	37 (18.9)	0.007	1.88 (1.18–3.00)	0.018	1.77 (1.10–2.85)
*TP53*C + *TP73*A/T	58 (22.0)	43 (21.9)	0.933	1.01 (0.63–1.61)	0.760	1.08 (0.66–1.75)
TP53 C + *MDM2*G + *USP7*A	51 (19.4)	19 (9.7)	0.006	2.24 (1.24–4.10)	0.020	2.04 (1.12–3.71)

†Includes the presence of the allele in homozygosis or heterozygosis. *Chi-square (Yates correction); ** P and OR adjusted for maternal age by logistic regression.

of function could also decrease the action of apoptotic mechanisms that would eliminate aneuploid embryos in women with wild alleles [56]. Even if the inactivation of p53 is not the primary cause of aneuploidy, its dysfunction strongly facilitates a tolerance to this chromosomal instability [5] and, 2) Loss of p73 function due to polymorphisms in its encoding gene may interfere in the control of the meiotic spindle during oogenesis, increasing the risk of aneuploidy in the offspring [56,57]. The expression of *TP73* naturally decreases with age, therefore the loss of *TP73* function may contribute to the increase of aneuploidy produced by old oocytes [26,56,57]. In our sample about 43% of women were younger than 35 years at the time of the trisomic fetus conception. Thus, it becomes evident that mutations affecting the function of *TP73* may be related to increased frequency of aneuploid pregnancies in young women. Supporting this hypothesis, a recent study showed that mice deficient in p73 presented spindle abnormalities, aneuploidy and little competence in fetal development [17].

The present study showed that polymorphisms in *TP53*, *TP73*, *MDM2*, *MDM4* and *USP7* genes do not represent a risk factor in the process of aneuploidy when analyzed separately, even when controlled for advanced maternal age. The distributions of allelic and genotypic frequencies found in this study are consistent with that expected for populations with European ancestry. Despite the ethnic admixture present in the Brazilian population, Southern Brazil has strong European ancestry, and the majority of our cases and controls were classified as Euro-descendants. This was confirmed by the similarity of allele frequencies observed in our sample compared to 1000 Genomes [52] and HapMap [53] databases for europeans and european-descendants.

Taking into account that the p53 pathway studied depends on multiple protein interactions, we found that the combination of *TP53* and *MDM2* polymorphisms,

and possibly *TP53* and *USP7* contributes to this increased risk. The effect of *TP53* C allele associated with *USP7* A allele is probably maternal age independent as women under the age of 35 years at the time of conception showed a higher frequency of *TP53* C allele associated with *USP7* A allele. These results indicate a synergistic effect between genes that act in the same pathway in a multifactorial way. The allele P72 reduces the efficiency of p53 to induce apoptosis. Acting in the same signaling pathway, the G allele increases the expression of *MDM2*, degrading more p53 and negatively influencing the induction of apoptosis in these cells [16]. Some studies showed that a large amount of p53 protein is produced by the human placenta in abnormal pregnancies. It is suspected that p53 is an important factor in the pathogenesis of diseases through the induction of throphoblast apoptosis [19,24, 37,48]. Thus, the interaction of these polymorphisms could decrease the levels of the pro-apoptotic p53 protein, making it less functional in response to cell damage. As a consequence, the reaction would be attenuated by trophoblastic apoptosis and promote greater tolerance of aneuploidy.

Although these polymorphisms have never been investigated as possible risk factors for aneuploidy in humans, some studies showed an association between SNPs of p53 pathway and fertility, suggesting a specific role of p53 in the regulation of human reproduction. Pietrowski and colleagues (2005) [46] reported an association of the P72 allele and recurrent miscarriages. However, Fang and colleagues (2011) [16] did not find any difference in genotype and allele frequency of *TP53* P72 and R72 forms through a case-control study involving women with miscarriages. They also reported that women with *TP53* P72/P72 genotype and *MDM2* G/G have a significantly higher expression of the *MDM2* protein, which may attenuate the response of apoptosis after DNA damage. The genes *MDM2*, *MDM4* and *USP7*, which produce proteins that regulate p53 level, had their minor alleles enriched in women seeking clinics for *in vitro* fertilization for the same SNPs studied [29,36].

The R72 and P72 forms of *TP53* have different biological effects: R72 is more efficient in inducting apoptosis while P72 can promote a G1 arrest. This polymorphism seems to increase the chance of miscarriage in healthy women [46]. Thus, it is possible that genes that undergo selection in the *TP53* pathway may affect human reproduction [29]. The G allele of *MDM2* c.14+309T>G SNP is associated with a high risk of spontaneous abortion [16], which supports the combined effect between this *MDM2* polymorphism and R72P of p53 [7,58]. The *MDM4* gene, that is structurally homologous with the *MDM2* gene, is also involved in the regulation of p53. The *MDM4* protein indirectly affects p53, modulating its levels as well as *MDM2* activity [42], not only stimulating the ubiquitination of p53 mediated by *MDM2*, but also the self-ubiquitination of *MDM2* [39]. The transcriptional activation of *TP53* by *MDM4* can be inhibited regardless of *MDM2* [18], by binding to the *TP53* transactivation domain, that contributes to the total inhibition of p53 [41]. The T allele of this polymorphism is associated with fertility in women, suggesting that *MDM4* can regulate reproduction in a dependent and independent *TP53* pathways, and may also interact with *TP63* and *TP73* [29]. Another important regulator of the p53 signaling pathway, the *USP7* gene, acts in the stabilization of *MDM2*, *MDM4* and p53 by deubiquitinating these proteins [10,25,38]. Other studies also reported an association of *TP73* polymorphisms with increased risk for Alzheimer's disease, leukoplakia and several types of cancer [40,43,51].

There are no functional studies regarding the polymorphism c.2719-234G>A of the *USP7* gene. Kang and colleagues [29] found an association of the mutated A allele with infertility, showing the impact of this SNP on human fertility through the attenuation of the p53 pathway. As in this work, we also found a higher frequency of this allele in women younger than 35 years. The *MDM2*, *MDM4* and *USP7* proteins maintain the levels and activity of p53 that are critical to an appropriate transcriptional response signal after cell stress [29].

In humans, the p53 gene family appears to play other important roles in reproduction: *TP53* is involved in the regulation of the blastocyst and *TP73* regulates the integrity of germ line cells [17]. Some evidence supports the involvement of p53 as functional in germline cells. Studies in *C. elegans* showed similar functions of that analogous to the p53 protein: cep-1 (*C. elegans* p53-like-1). During the development of germ cells cep-1 ensures correct meiotic segregation [13]. In addition, the expression of CEP-1 at the end of pachytene, associated with the establishment of apoptotic competence, ensures that the germline cells with DNA damage or defects in meiotic recombination are eliminated before oogenesis. This process ensures that only healthy germ cells advance to the next generation [50]. Additionally, cks2 proteins, that are essential components of cyclins complexes, and are involved in cell cycle control, have their expression negatively controlled by p53 at

transcriptional and proteic level. This regulation contributes to control the transition from metaphase I to anaphase I in mammals' meiosis [49].

Despite the well established association between DS and advanced maternal age, the biochemical and molecular basis of nondisjunction are still not well understood. Altered patterns of recombination are known risks for nondisjunction [33,34]. More recently, Oliver and colleagues [44] have added support to the multifactorial etiology of nondisjunction in human meiosis. Their data suggested that pericentromeric chromatid exchanges during meiosis in females interact with maternal age-related risk factors, altering the susceptibility to nondisjunction. One year later, Gosh and colleagues [20] tested this hypotesis in an independent and ethnically different population in India and their results were consistent with those of Oliver and colleagues [44]. In our study we searched for different susceptibility factors that could predispose to aneuploidy, and we looked also for a possible interaction with advanced maternal age. However, a logistic regression considering maternal age failed to show an interaction between p53 pathway, maternal age and aneuploidy.

In this article we presented recent evidence linking the role of the *TP53* family and its regulators in the maintenance spindle stability during meiosis and embryo development. Allied to this evidence and because of the lack of studies investigating the relationship of these polymorphisms with the genesis of aneuploidy in humans, this work is the first to establish a relationship between polymorphisms in the *TP53* gene family and its regulatory pathway as a risk factor for aneuploidy of 21. Future studies in other populations should be conducted to confirm our findings, especially to clarify the factors independent of maternal age which may be involved in the development of aneuploid cells.

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Supplementary material

Table S1
Allelic and genotypic frequencies of SNPs in the *TP53* pathway in euro-descendants

Gene	Genotype/Allele	Case n (%)	Control n (%)	$P*$	Expected allele frequency**	
					1000genomes [52]	HapMap [53]
TP53	GG	106 (44.7)	92 (52.6)	0.164		
(rs1042522)	GC	113 (47.7)	67 (38.3)			
	CC	18 (7.6)	16 (9.1)			
	G	325 (68.6)	251 (71.7)	0.369	78.9	76.7
MDM2	TT	96 (40.5)	75 (42.9)	0.666		
(rs2279744)	TG	109 (46.0)	73 (41.7)			
	GG	32 (13.5)	27 (15.4)			
	T	301 (63.5)	223 (63.7)	0.991	64.0	NA
MDM4	CC	83 (35.0)	63 (36.0)	0.978		
(rs1563828)	CT	111 (46.8)	81 (42.3)			
	TT	43 (18.2)	31 (17.7)			
	C	277 (58.4)	207 (59.1)	0.895	67.9	65.0
USP7	GG	111 (46.8)	87 (49.7)	0.482		
(rs1529916)	GA	111 (46.8)	73 (41.7)			
	AA	15 (6.4)	15 (8.6)			
	G	333 (70.3)	247 (70.6)	0.982	67.3	68.9
TP73	GG/CC	149 (62.9)	98 (56.0)	0.269		
(rs2273953 and rs1801173)	GA/CT	76 (32.0)	63 (36.0)			
	AA/TT	12 (5.1)	14 (8.0)			
	G/C	374 (78.9)	259 (74.0)	0.117	79.9	NA

*Chi-square; **Data from european and euro-descendants populations. NA = not available.

Table S2
Risk allele combinations in the *TP53* pathway showing data for euro-descendants only

Alleles†	Case n (%)	Control n (%)	$P*$	OR (IC 95%)	$P**$	OR (IC 95%)**
*TP53*C + *MDM2*G	82 (34.6)	38 (21.7)	0.006	1.91 (1.19–3.06)	0.009	1.90 (1.17–3.06)
*TP53*C + *MDM4*T	82 (34.6)	55 (31.4)	0.569	1.15 (0.75–1.79)	0.551	1.14 (0.73–1.79)
*TP53*C + *USP7*A	72 (30.4)	32 (18.3)	0.007	1.95 (1.19–3.22)	0.016	1.85 (1.12–3.06)
*TP53*C + *TP73*A/T	50 (21.1)	34 (19.4)	0.770	1.11 (0.66–1.86)	0.502	1.19 (0.71–2.02)
TP53 C + *MDM2*G + *USP7*A	45 (19.0)	15 (8.6)	0.005	2.50 (1.29–4.88)	0.016	2.23 (1.16–4.60)

†Includes the presence of the allele in homozygosis or heterozygosis. *Chi-square (Yates correction). ** P and OR adjusted for maternal age by logistic regression.