

RESEARCH ARTICLE

The TP73 Gene Polymorphism (rs4648551, A>G) Is Associated with Diminished Ovarian Reserve

Laura Diniz Vagnini¹, Adriana Renzi¹, Gabriela Ravanelli Oliveira-Pelegrin¹, Maria do Carmo Tomitão Canas¹, Claudia Guilhermino Petersen², Ana Lucia Mauri², João Batista Alcantara Oliveira², Ricardo Luiz Razera Baruffi², Mario Cavagna², José Gonçalves Franco Junior^{2*}

1 Paulista Center for Diagnosis, Research and Training, Ribeirão Preto, São Paulo, Brazil, **2** Center for Human Reproduction Prof. Franco Jr, Ribeirão Preto, São Paulo, Brazil

* franco@crh.com.br



OPEN ACCESS

Citation: Vagnini LD, Renzi A, Oliveira-Pelegrin GR, Canas MdCT, Petersen CG, Mauri AL, et al. (2015) The TP73 Gene Polymorphism (rs4648551, A>G) Is Associated with Diminished Ovarian Reserve. PLoS ONE 10(3): e0120048. doi:10.1371/journal.pone.0120048

Academic Editor: Wan-Xi Yang, Zhejiang University, CHINA

Received: October 8, 2014

Accepted: February 3, 2015

Published: March 20, 2015

Copyright: © 2015 Vagnini et al. This is an open access article distributed under the terms of the [Creative Commons Attribution License](https://creativecommons.org/licenses/by/4.0/), which permits unrestricted use, distribution, and reproduction in any medium, provided the original author and source are credited.

Data Availability Statement: All relevant data are within the paper.

Funding: This research was supported by Merck Serono grant (GFI-2014-16). The funders had no role in study design, data collection and analysis, decision to publish, or preparation of the manuscript.

Competing Interests: All authors have contributed substantially to the manuscript and approved its submission, declaring no competing interests. Both Paulista Center for Diagnosis, Research and Training and Center for Human Reproduction Prof. Franco Jr

Abstract

It's known that the members of the TP53 family are involved in the regulation of female reproduction. Studies in mice showed that the TP73 gene (member of this family) plays a role in the size of follicular pool, ovulation rate and maintenance of genomic stability. In the present study we analyzed data from 605 patients with ≤ 37 years attending their first intracytoplasmic sperm injection (ICSI). The association between the TP73 polymorphism (rs4648551, A>G) and the following parameters related to ovarian reserve, like age, antral follicular count (AFC), anti-Mullerian hormone levels (AMH) and ovarian response prediction index (ORPI) was evaluated. Our results showed an association of the AA genotype with diminished ovarian reserve (AMH < 1 , AFC ≤ 9). Women presenting the AA genotype had a 2.0-fold increased risk for having AMH < 1 and AFC ≤ 9 (OR 2.0, 95% CI 1.23-3.31, P = 0.005). Patients presenting AA genotype had the lowest levels of AMH (P = 0.02), the lowest number of antral follicles (P = 0.01) and the lowest ORPI (P = 0.007). Analyzing the alleles, we can see an enrichment of the A allele in the group of diminished ovarian reserve (OR 1.4, 95%CI 1.02-1.83, P = 0.04). To the best of our knowledge, the present study is the first to analyze this polymorphism in humans for assessing the numbers of ovarian follicles and AMH levels and, therefore, the ovarian reserve. Our findings can contribute to the use of this polymorphism as a potential marker of diminished ovarian reserve.

Introduction

Current studies show that about 17% of couples are unable to conceive and their infertility could stem from different causes, genetic factors representing a significant component [1–3]. The members of the p53 family (p63, p73 and p53), besides their roles in cell cycle regulation, transactivation and apoptosis, are involved in female reproduction and blastocyst implantation [4]. The p53, p63 and p73 are powerful transcription factors, and small variations in their

are clinics and research centers. All authors are researchers in these companies and declare no real or perceived competing interests. This does not alter the authors' adherence to PLOS ONE policies on sharing data and materials.

activation status can cause modifications on the expression pattern of genes that they transcribe, modifying the fate of the cell [5].

Studies in mice have showed that the p73 gene plays a role in female infertility. The p73 gene contains two promoters that drive the expression of two major groups of p73 isoforms with opposing cellular actions: pro-apoptotic and anti-apoptotic [6, 7] and both are required for normal embryogenesis [8]. Mice lacking either one of the groups of isoforms are infertile, but the root of the infertility differ [9]. Mice that are deficient for both p73 groups of isoforms have sensory defects that prevent them from mating normally [8]. On the other hand, female mice presenting the deletion of exons 2 and 3 of the gene mate normally, but have oocytes with reduced developmental competence, having an increased frequency of spindle defects in ovulated oocytes, reduced follicular pool size and poor oocyte quality [9], being similar to the set of embryonic defects observed in patients undergoing IVF, particularly older patients [10]. The expression of Tp73 in mice oocytes also declines with natural aging [9], suggesting that it is involved in maintaining genomic stability in oocytes. Steuerwald and collaborators [11] have also seen this down regulation of p73 transcripts in oocyte of older women patients. Another study with women submitted to in vitro fertilization (IVF) treatment showed an association of one single nucleotide polymorphism–SNP (rs4648551, A>G) on the TP73 gene with infertility, exhibiting a clear enrichment of the G allele in older infertile women (>35 years) [12]. Others studies have related the use of SNPs as possible markers to infertility problems [13–15].

Given the importance of this gene in fertility and the possible use of the polymorphisms as a marker to help treatment, we examined the association between one polymorphism on the TP73 gene (rs4648551, A>G) and the following parameters related with ovarian reserve: age, antral follicles count (AFC), anti-Müllerian hormone (AMH) levels and the ovarian response prediction index (ORPI = (AMH x AFC)/patient age) [16, 17]. The use of this polymorphism as a marker can be of interest as a potential prognostic factor in cases of human female infertility and could contribute to the improvement in the diagnosis of subfertility and may facilitate personalized strategies in the infertility therapy.

Materials and Methods

Patients

Our study included blood samples of 605 Brazilian women attending their first intracytoplasmic sperm injection (ICSI) cycle between February 2012 and June 2014. The criteria for inclusion were the following: age \leq 37 years, body mass index (BMI) between 20–30 kg/m², regular menstrual cycles, both ovaries present, no history of ovarian surgery, no severe endometriosis and no evidence for endocrine disorders or ovarian cysts.

In addition, for comparison of population genotype distribution, a total of 83 volunteer that had at least one healthy child with any infertility treatment were included. The study was authorized by the local ethical committee (the *Comitê de Ética em Pesquisa do Centro de Referência da Saúde da Mulher*; project reference: 045/11) and was conducted according to the principles expressed in the Declaration of Helsinki. A written informed consent was obtained from all recruited subjects.

DNA extraction and Genotyping

Genomic DNA for all subjects was extracted from the sample of blood for each patient using the QIAamp DNA blood mini kit (Qiagen) following the manufacturer's instructions.

Genotyping was performed in duplicate by real time polymerase chain reaction (PCR) amplification using a TaqMan SNP genotyping assay (C_26892242_10) according to Applied Biosystems standard protocols. Each 10 μ l reaction composed of 1 μ l of genomic DNA (100ng/ μ l),

5 μ l of UMM (TaqMan Universal Master Mix), 0.5 μ l probe (rs4648551), and 3.5 μ l of DNase-free water was performed according to the following amplification protocol: denaturation at 95°C for 10 min, followed by 40 cycles of 92°C for 15s and 60°C for 1min in StepOnePlus Real-Time PCR. Products were analyzed by TaqMan Genotyper Software v1.3 (ABI). Some samples were subjected to sequencing to validate the genotyping results.

AMH measurement

AMH was measured using an enzymatically amplified 2-site immunoassay kit (AMH Gen II ELISA, Beckman Coulter Inc.) according to the manufacturer's manual. The lowest detection limit of this assay is 0.01 ng/ml. To minimize the chances of bias in the assay, all sera were processed in duplicate during the same day, using the same measurement kits, and by the same operator. Low and high level controls were included in each assay.

Antral follicles count

All women had a transvaginal ultrasonographic evaluation performed during the early follicular phase of a previous cycle before the scheduled treatment. A single experienced sonographer, who was blinded to the results of any hormonal assays and the patient's age, performed the evaluation using a conventional 2-dimensional transvaginal ultrasound at 7 MHz (Medison Digital Color MT; Medison Co. Ltd., Seoul, Korea). The total number of 2–9 mm antral follicles in both ovaries was taken in account.

Statistical analysis

Statistical analysis was performed using StatsDirect version 2.7.9 software, and the Hardy-Weinberg equilibrium was applied using an online calculator, available on <http://ihg.gsf.de>. The differences in the frequencies of the SNP genotypes and/or alleles in the study and control groups were evaluated using Fisher's test. The odds ratio (OR) was calculated by logistic regression. All statistical tests were considered significant at a level of $P < 0.05$.

Results

A total of 605 patients and 83 control women were genotyped for the p73 rs4648551 A>G SNP. We found that the genotypes of the study group and control group were similar, having no significant difference in genotype and allelic frequency distributions of p73 rs4648551 A>G SNP ([Table 1](#)). All allelic and genotypic frequencies observed during this study are consistent with the Hardy-Weinberg equilibrium.

Analysis of the genotype distribution of the P73 (rs4648551, A>G) polymorphism in the study group, associated with the characters related to ovarian reserve investigated here, revealed a significant difference. Patients that presented the AA genotype had the lowest levels of AMH ($P = 0.02$), the lowest number of antral follicles ($P = 0.01$) and the lowest ORPI ($P = 0.007$). The genotype distribution and the means of AMH levels, AFC and ORPI are presented in [Table 2](#). There were no significant difference between the different genotypes and age.

Considering that there is an association of the investigated SNP with some of the characters analyzed, we decided to investigate the influence of the different genotypes with ovarian reserve. When patients with AA genotype were (isolated from patients with AG and GG genotypes, we observed a significant difference of this polymorphism associated with lower AMH levels ($P = 0.006$), AFC ($P = 0.003$) and ORPI ($P = 0.002$). When patients with the GG genotype were isolated, no significant difference was observed. [Table 3](#) summarizes our results.

Table 1. Genotypes and alleles frequencies distribution in the control and study groups.

P73 (rs4648551)	SNP Genotype	Control n (%)	Study group n (%)	P
Genotypes	AA	17 (20.5)	118 (19.5)	0.48
	GA	32 (38.5)	274 (45.3)	
	GG	34 (41.0)	213 (35.2)	
Alleles	A	66 (39.8)	510 (42.1)	0.56
	G	100 (60.2)	700 (57.9)	

doi:10.1371/journal.pone.0120048.t001

Based on the fact that AA genotype was associated with lower levels of AMH, AFC and ORPI, we divided the patients into two groups: diminished ovarian reserve (AMH <1ng/mL, AFC ≤9) and normal ovarian reserve (AMH ≥1ng/mL, AFC >9). The cut-off for diminished ovarian reserve of AMH <1ng/ml was used taken account Nardo, La Marca and colleagues [18, 19]. For AFC ≤9, we used the cut-off based on previous researches associating AMH and AFC [20]. Odds Ratio analysis was performed to verify an association of the AA genotype with decreased ovarian reserve. Women presenting the AA genotype had a 2.0-fold increased risk for having AMH <1 and AFC ≤9 (OR 2.0, 95% CI 1.23–3.31, P = 0.005). Analyzing the alleles, we detected an enrichment of the A allele in the group of diminished ovarian reserve (OR 1.4, 95%CI 1.02–1.83, P = 0.04). Results are shown in Table 4.

Table 2. Mean of age, AMH level, AFC and ORPI of SNP genotypes in the study group.

P73 (rs4648551)	AA	AG	GG	P
% (n/total)	19.5% (118/605)	45.3% (274/605)	35.2% (213/605)	
Age (years)	33.2±3.1 ^{a,b}	32.6±3.2 ^{a,c}	32.9±3.1 ^{b,c}	0.21
				0.07 ^a
				0.25 ^b
AMH level (ng/mL)				0.49 ^c
	1.7±1.4 ^{a,b}	2.1±2.1 ^{a,c}	2.4±2.8 ^{b,c}	0.02
				0.02 ^a
AFC				0.008 ^b
	12.7±7.4 ^{a,b}	14.9±8.4 ^{a,c}	16.0±11.1 ^{b,c}	0.49 ^c
				0.01
ORPI				0.004 ^a
	0.9±1.3 ^{a,b}	1.4±2.2 ^{a,c}	1.9±4.3 ^{b,c}	0.009 ^b
				0.94 ^c
			0.007	
			0.005 ^a	
			0.003 ^b	
			0.65 ^c	

The P value represents the comparison between genotypes:

^aAA versus AG;

^bAA versus GG;

^cAG versus GG.

doi:10.1371/journal.pone.0120048.t002

Table 3. Mean of age, AMH level, AFC and ORPI of associated SNP genotypes in the study group.

P73(rs4648551)	AA	AG+GG	P	GG	AG+AA	P
% (n/total)	19.5% (118/605)	80.5% (487/605)		35.2% (213/605)	64.8% (392/605)	
Age (years)	33.2±3.1	32.7±3.2	0.10	32.9±3.1	32.8±3.2	0.96
AMH (ng/mL)	1.7±1.4	2.3±2.4	0.006	2.4±2.8	2.0±1.9	0.11
AFC	12.7±7.4	15.4±9.7	0.003	16.0±11.1	14.3±8.2	0.27
ORPI	0.9±1.3	1.6±3.3	0.002	1.9±4.3	1.2±2.0	0.13

doi:10.1371/journal.pone.0120048.t003

Discussion

The p53 family members are involved in many important processes, contributing to cell cycle regulation, transactivation and apoptosis in response to DNA damage [21]. Besides, the members of the p53 family have also been described as regulators of crucial process related to human reproduction [22, 23]. Observations have shown that the p73 plays a crucial role in maintaining the size of the follicular pool and ovulation rate, as well as acting at the spindle checkpoint [12], affecting the oocyte quality [9].

Recent studies have revealed that several genetic polymorphisms may play important roles in human reproduction [4, 13–15]. The present study investigated the association between the p73 rs4648551 A>G polymorphism and the ovarian reserve in Brazilian women undergoing IVF/ICSI treatment. Given that the p73 gene is associated with fertility, small variations in its activity can cause individual differences on the ovarian reserve and can present as potential targets of reproductive disorders. However, little is known about how this polymorphism acts in the fertility of women.

Our results showed a significant association of AMH levels, AFC and ORPI with the different genotypes of the p73 rs4648551 A>G polymorphism. Despite the fact that Feng and colleagues [12] have shown a relationship between this polymorphism and infertile patients with age ≥35 years, we did not observe this association. The lack of any relationship between this polymorphism and age could be explained in part because we did not include women older than 37 years in order to minimize the ageing effect on ovarian reserve.

Our findings showed an association of the AA genotype and lower mean levels of AMH, AFC and ORPI. Tomasini and colleagues [9] had already shown an association of this gene and follicular numbers in mice.

The division of the study group in two subgroups: diminished ovarian reserve (AMH<1, AFC≤9) and normal ovarian reserve (AMH≥1, AFC>9) showed more clearly the association

Table 4. Genotypes and alleles frequencies distribution in diminished and normal ovarian reserve groups.

	Diminished ovarian reserve	Normal ovarian reserve	P	OR	95%CI
Genotype	AMH<1+AFC≤9*	AMH≥1+AFC>9*			
AA	37.5% (33/88)	62.5% (55/88)	0.005	2.0	1.23–3.31
AG+GG	22.9% (88/385)	77.1% (297/385)			
Alleles					
A	29.1% (113/388)	70.9% (275/388)	0.04	1.4	1.02–1.83
G	23.1% (129/558)	76.9% (429/558)			

* In these groups, the population must necessarily have the two inclusion criteria (AMH and AFC).

doi:10.1371/journal.pone.0120048.t004

of the AA genotype with the first subgroup and an enrichment of the A allele in the same subgroup.

Data regarding treatment outcome (rFSH dosage, rLH dosage, number of retrieved oocytes and implantation, pregnancy and abortion rates) and TP73 gene polymorphism are under analysis for further publication. In conclusion, this study shows that the p73 rs4648551 A>G polymorphism can be involved in the ovarian reserve. Women with AA genotype have 2.0-fold increased risk for having a diminished ovarian reserve. To the best of our knowledge, the present study is the first to analyze this polymorphism in humans for assessing the numbers of ovarian follicles and AMH levels and, therefore, the ovarian reserve. Additional validation is needed to provide more information regarding the use of this polymorphism as a potential ovarian reserve marker.

Acknowledgments

The authors wish to thank the nursing staff and laboratory technicians for their kind assistance during the study period.

Author Contributions

Conceived and designed the experiments: LDV AR GROP CGP JBAO JGFJ. Performed the experiments: LDV AR GROP MCTC CGP ALM JBAO RLRB MC JGFJ. Analyzed the data: LDV AR GROP JBAO JGFJ. Contributed reagents/materials/analysis tools: LDV AR GROP MCTC CGP ALM JBAO RLRB MC JGFJ. Wrote the paper: LDV AR GROP CGP JBAO JGFJ.

References

1. Bhattacharya S, Johnson N, Tijani HA, Hart R, Pandey S, Gibreel AF (2010) Female infertility. *Clin Evid* (Online) 2010.
2. Kohler HP, Rodgers JL, Miller WB, Skyttthe A, Christensen K (2006) Bio-social determinants of fertility. *Int J Androl* 29: 46–53. PMID: [16466523](#)
3. Kosova G, Abney M, Ober C (2010) Colloquium papers: Heritability of reproductive fitness traits in a human population. *Proc Natl Acad Sci U S A* 107 Suppl 1: 1772–1778. doi: [10.1073/pnas.0906196106](#) PMID: [19822755](#)
4. Kang HJ, Feng Z, Sun Y, Atwal G, Murphy ME, Rebbeck TR, et al. (2009) Single-nucleotide polymorphisms in the p53 pathway regulate fertility in humans. *Proc Natl Acad Sci U S A* 106: 9761–9766. doi: [10.1073/pnas.0904280106](#) PMID: [19470478](#)
5. Stehmeier P, Muller S (2009) Regulation of p53 family members by the ubiquitin-like SUMO system. *DNA Repair (Amst)* 8: 491–498. doi: [10.1016/j.dnarep.2009.01.002](#) PMID: [19213614](#)
6. Muller M, Schilling T, Sayan AE, Kairat A, Lorenz K, Schulze-Bergkamen H, et al. (2005) TAp73/Delta Np73 influences apoptotic response, chemosensitivity and prognosis in hepatocellular carcinoma. *Cell Death Differ* 12: 1564–1577. PMID: [16195739](#)
7. Wang J, Liu YX, Hande MP, Wong AC, Jin YJ, Yin Y (2007) TAp73 is a downstream target of p53 in controlling the cellular defense against stress. *J Biol Chem* 282: 29152–29162. PMID: [17693405](#)
8. Yang A, Walker N, Bronson R, Kaghad M, Oosterwegel M, Bonnin J, et al. (2000) p73-deficient mice have neurological, pheromonal and inflammatory defects but lack spontaneous tumours. *Nature* 404: 99–103. PMID: [10716451](#)
9. Tomasini R, Tsuchihara K, Wilhelm M, Fujitani M, Rufini A, Cheung CC, et al. (2008) TAp73 knockout shows genomic instability with infertility and tumor suppressor functions. *Genes Dev* 22: 2677–2691. doi: [10.1101/gad.1695308](#) PMID: [18805989](#)
10. Hardarson T, Hanson C, Sjogren A, Lundin K (2001) Human embryos with unevenly sized blastomeres have lower pregnancy and implantation rates: indications for aneuploidy and multinucleation. *Hum Reprod* 16: 313–318. PMID: [11157826](#)
11. Steuerwald NM, Bermudez MG, Wells D, Munne S, Cohen J (2007) Maternal age-related differential global expression profiles observed in human oocytes. *Reprod Biomed Online* 14: 700–708. PMID: [17579982](#)

12. Feng Z, Zhang C, Kang HJ, Sun Y, Wang H, Naqvi A, et al. (2011) Regulation of female reproduction by p53 and its family members. *FASEB J* 25: 2245–2255. doi: [10.1096/fj.10-180166](https://doi.org/10.1096/fj.10-180166) PMID: [21402718](https://pubmed.ncbi.nlm.nih.gov/21402718/)
13. Boudjenah R, Molina-Gomes D, Torre A, Bergere M, Bailly M, Boitrelle F, et al. (2012) Genetic polymorphisms influence the ovarian response to rFSH stimulation in patients undergoing in vitro fertilization programs with ICSI. *PLoS One* 7: e38700. doi: [10.1371/journal.pone.0038700](https://doi.org/10.1371/journal.pone.0038700) PMID: [22701696](https://pubmed.ncbi.nlm.nih.gov/22701696/)
14. Simoni M, Casarini L (2014) Mechanisms in endocrinology: Genetics of FSH action: a 2014-and-beyond view. *Eur J Endocrinol* 170: R91–107. doi: [10.1530/EJE-13-0624](https://doi.org/10.1530/EJE-13-0624) PMID: [24288354](https://pubmed.ncbi.nlm.nih.gov/24288354/)
15. Altmae S, Hovatta O, Stavreus-Evers A, Salumets A (2011) Genetic predictors of controlled ovarian hyperstimulation: where do we stand today? *Hum Reprod Update* 17: 813–828. doi: [10.1093/humupd/dmr034](https://doi.org/10.1093/humupd/dmr034) PMID: [21862569](https://pubmed.ncbi.nlm.nih.gov/21862569/)
16. Oliveira JB, Baruffi RL, Petersen CG, Mauri AL, Nascimento AM, Vagnini L, et al. (2012) A new ovarian response prediction index (ORPI): implications for individualised controlled ovarian stimulation. *Reprod Biol Endocrinol* 10: 94. doi: [10.1186/1477-7827-10-94](https://doi.org/10.1186/1477-7827-10-94) PMID: [23171004](https://pubmed.ncbi.nlm.nih.gov/23171004/)
17. Oliveira JBA, Mauri AL, Petersen CG, Cavagna M, Baruffi RLR, Jr JGF (2013) Clinical decision in ovarian stimulation guided by the ovarian response prediction index (ORPI). *Fertility and Sterility* 100: S275.
18. La Marca A, Sighinolfi G, Radi D, Argento C, Baraldi E, Arsenio AC, et al. (2010) Anti-Mullerian hormone (AMH) as a predictive marker in assisted reproductive technology (ART). *Hum Reprod Update* 16: 113–130. doi: [10.1093/humupd/dmp036](https://doi.org/10.1093/humupd/dmp036) PMID: [19793843](https://pubmed.ncbi.nlm.nih.gov/19793843/)
19. Nardo LG, Gelbaya TA, Wilkinson H, Roberts SA, Yates A, Pemberton P, et al. (2009) Circulating basal anti-Mullerian hormone levels as predictor of ovarian response in women undergoing ovarian stimulation for in vitro fertilization. *Fertil Steril* 92: 1586–1593. doi: [10.1016/j.fertnstert.2008.08.127](https://doi.org/10.1016/j.fertnstert.2008.08.127) PMID: [18930213](https://pubmed.ncbi.nlm.nih.gov/18930213/)
20. Nelson SM (2013) Biomarkers of ovarian response: current and future applications. *Fertil Steril* 99: 963–969. doi: [10.1016/j.fertnstert.2012.11.051](https://doi.org/10.1016/j.fertnstert.2012.11.051) PMID: [23312225](https://pubmed.ncbi.nlm.nih.gov/23312225/)
21. Levine AJ, Tomasini R, McKeon FD, Mak TW, Melino G (2011) The p53 family: guardians of maternal reproduction. *Nat Rev Mol Cell Biol* 12: 259–265. doi: [10.1038/nrm3086](https://doi.org/10.1038/nrm3086) PMID: [21427767](https://pubmed.ncbi.nlm.nih.gov/21427767/)
22. Dotsch V, Bernassola F, Coutandin D, Candi E, Melino G (2010) p63 and p73, the ancestors of p53. *Cold Spring Harb Perspect Biol* 2: a004887. doi: [10.1101/cshperspect.a004887](https://doi.org/10.1101/cshperspect.a004887) PMID: [20484388](https://pubmed.ncbi.nlm.nih.gov/20484388/)
23. Hu W (2009) The role of p53 gene family in reproduction. *Cold Spring Harb Perspect Biol* 1: a001073. doi: [10.1101/cshperspect.a001073](https://doi.org/10.1101/cshperspect.a001073) PMID: [20457559](https://pubmed.ncbi.nlm.nih.gov/20457559/)