Infertility caused by an association between *Arg72Pro* polymorphism of the *p53* gene and *Glu298Asp* of the *eNOS* gene in patients with endometriosis

T.R. Santos[2], K.S.F. e Silva[1,3], R.C.P.C. Silva[3], K.K.V.O. Moura[1,2,3], L.A. Guillo[3], C.L. Ribeiro Júnior[1] and I.R. Costa[1,2]

[1] Núcleo de Pesquisas Replicon - Pontifícia Universidade Católica de Goiás, Goiânia, GO, Brasil
[2] Escola de Ciências Médicas, Farmacêuticas e Biomédicas - Pontifícia Universidade Católica de Goiás, Goiânia, GO, Brasil
[3] Instituto de Ciências Biológicas, Universidade Federal de Goiás, Goiânia, GO, Brasil

Corresponding author: K.S.F e Silva
E-mail: smallbinho@hotmail.com

Genet. Mol. Res. 17 (3): gmr18046
Received June 03, 2018
Accepted July 06, 2018
Published July 27, 2018
DOI http://dx.doi.org/10.4238/gmr18046

ABSTRACT. Endometriosis is characterized by ectopic endometrial tissue and affects millions of women worldwide. The disease leads to various symptoms such as chronic pelvic pain and infertility and does not yet have a well-defined etiology. The pathology is similar to cancer, since endometrial cells are highly proliferative, invade tissues and may be associated with tumor suppressor genes, including p53. Genetic polymorphisms are responsible for phenotypic variations in the population and the development of endometriosis is due to a combination of multiple genes and environmental factors. The Arg72Pro polymorphism of the p53 gene alters the amino acid 72 from arginine to proline and studies indicate that there is a considerable increase in the risk of cancer in patients with this polymorphism, because the protein becomes more susceptible to degradation. The eNOS gene participates in angiogenesis, a necessary factor for endometrial cells to survive outside the uterus and has increased

expression in patients with endometriosis during the menstrual cycle. The Glu298Asp eNOS polymorphism is due to a point mutation of glutamate to aspartate, which can generate changes in its enzymatic activity. Aspartate generates protein products with different susceptibility to cleavage, functionally affecting the protein. We detected the Arg72Pro polymorphism of p53 and the Glu298Asp polymorphism of eNOS and estimated their prevalence in fertile and infertile patients diagnosed with endometriosis. The techniques used were conventional PCR and ARMS-PCR. Patients with Pro or Asp polymorphic alleles were found to be more susceptible to the development of endometriosis, and an association of these two polymorphisms is directly linked to infertility. The target genes p53 and eNOS can be used as molecular markers for endometriosis diagnosis, guiding prognosis and treatment, and may contribute to a better understanding of the pathophysiology of endometriosis.

Key words: Endometriosis; polymorphism; *p53; eNOS; Arg72Pro; Glu298Asp*

INTRODUCTION

Endometriosis is characterized by ectopic endometrial tissue. It can affect ovaries, peritoneum, uterine ligaments, retrocervical region, rectovaginal septum, bladder, rectum, sigmoid, and other portions of the digestive tract (Bellelis *et al.*, 2010; Crosera *et al.*, 2010; Marqui, 2012). Prevalence is up to 20% in women of reproductive age and 30 to 50% in infertile women, representing one of the most common gynecological diseases, with rare cases before menarche. It is estimated that more than 70 million women in the world are affected by endometriosis (Bellelis *et al.*, 2010; Bellelis *et al.*, 2014; Bianco *et al.*, 2011; Nácul and Spritzer, 2010).

This disease is responsible for causing both physical and emotional problems in patients. Endometriosis reduces the patient's quality of life by causing chronic pelvic pain, infertility and reducing the capacity to perform common activities (Minson *et al.*, 2012). The causes for the development of endometriosis are not completely clear. The most accepted theory is described by Sampson (1927) regarding retrograde menstruation. During the menstrual period, endometrial tissue leaks through the fallopian tubes, causing the tissue to grow in the peritoneum, ovary and other organs (Sampson, 1927). Moreover, genetic, hormonal and immunological factors may contribute to the development of the disease (Bellelis et al., 2011; Nácul and Spritzer, 2010). Since it is an estrogen-dependent pathology, conditions that increase exposure to this hormone also increase the risk of onset of endometriosis (Bellelis *et al.*, 2010).

The pathology is similar to that of cancer since endometrial cells require neovascularization to establish, grow and invade tissues. It is a highly mobile and proliferative disease (Marqui, 2012; Yang *et al.*, 2015). The loss of sensitivity of the endometrium to progesterone during the menstrual cycle is another recognized causative factor of endometriosis. As progesterone levels decrease in the secretory phase, the cytokines, proinflammatory chemokines and matrix metalloproteinase (MMPs) levels increase, in order

to prepare the endometrium tissue for the intense inflammatory process caused by menstruation (Bellelis *et al*., 2014).

Non-invasive laboratory methods often do not allow an accurate diagnosis, making it necessary to perform surgery to obtain histological material and confirm the disease. Pelvis videolaparoscopy is the most used technique for confirming endometriosis diagnosis (Bellelis *et al*., 2011; Santos *et al.*, 2010). Therapeutic treatment stimulates a hypoestrogenic environment that breaks the estrogen cycle and reduces the endometrial implant rate. The standard procedure for endometriosis treatment is laparoscopic surgery, which removes all endometrial tissue through electrocauterization or laser destruction of endometriotic implants (Crosera *et al.*, 2010; Pereira *et al.*, 2010). Other treatment possibilities include a levonorgestrel intrauterine device (LNG-IUS), a second-generation hormonal contraceptive. Whether inserted during surgery or after, it is effective for five years. In addition there are options such as intramuscular injections administered every three months and the use of combined oral contraceptives (Sanghera *et al.*, 2016).

Genomic alterations may be important factors in the development of endometriosis. Genetic polymorphisms have been defined as variations in the DNA sequence responsible for phenotypic variations in populations with a frequency greater than 1% in the general population (Santos *et al.*, 2010). A combination of multiple genes and the environment determine the genetic influence in endometriosis. The risks of this disease in women who have affected relatives are greater compared to the general population. Multiple genes have been associated with endometriosis and among them are the cytochrome P450 (CYP) family, tumor suppressor genes, estrogen regulatory genes, proinflammatory cytokines, vascular function regulators and pro-angiogenic genes (Nastasi-Catanese *et al.*, 2013).

P53 is a tumor suppressor gene, located on chromosome 17p13 and related to cell proliferation and progression of several types of tumors. The protein coded by *p53* induces apoptosis or blocks the cell cycle in response to DNA damage, making it possible to destroy or repair cells before DNA restarts replication (Gallegos-Arreola *et al.*, 2012; Ribeiro Júnior *et al.*, 2009; Zhou *et al*., 2012). There are several single nucleotide polymorphisms (SNPs) within the *p53* gene, and the polymorphism of codon 72 (Arg72Pro, rs1042522 G>C) in exon 4 is the most common. This polymorphism changes the amino acid residue 72 from arginine to proline and both alleles show oncogenic properties. The allele *Arg72* induces apoptosis more rapidly and suppresses transformation more efficiently than Pro72, which is more susceptible to protein degradation, increasing the risk of cancer (Gallegos-Arreola *et al.*, 2012).

Nitric oxide (NO) is a multifunctional biomolecule synthesized from L-arginine by three isoenzymes of Nitric Oxide Synthase (NOS): neuronal nitric oxide synthase (*nNOS*), induced nitric oxide synthase (*iNOS*) and endothelial nitric oxide synthase (*eNOS*) (Seckin *et al.*, 2016; Zhao *et al.*, 2016). Nitric oxide is responsible for the regulation of vasodilation, exerting its effect by regulating the migration of endothelial cells, and it takes part in several physiological processes including angiogenesis, thrombosis, coagulation and fibrinolysis (Zhao et al. 2016). The *eNOS* is a gene located on chromosome 7 (7q35-q36), with 26 exons and 25 introns and encodes a protein of 1,203 amino acids (Zhao et al., 2016); *eNOS* is an important pro-angiogenic factor, associated with the development of endometriosis since angiogenesis is needed for ectopic endometrial tissue to survive outside the uterus (Kim *et al.*, 2009).

The most widely studied and functionally relevant polymorphism associated with *eNOS* is G894T-Glu298Asp (rs1799983) in codon 298 of exon 7, which corresponds to a variable number tandem repeat (VPNR). This type of *eNOS* polymorphism promotes a point mutation from guanine into thymine leading to a change in the expected enzymatic activity promoted by the *eNOS* protein (Zhao *et al.*, 2016). Another polymorphism, T786C (rs2070744), changes thymine into cytosine at position 786 of the 5'-flanking region of the gene. T786C interferes with the activity of the promoter, damaging the endothelial synthesis of nitric oxide (Seckin *et al.*, 2016). Carriers of G894T alleles tend to exhibit decreased *eNOS* enzyme activity compared to GG homozygotes. The T allele generates protein products with a different susceptibility to cleavage, suggesting that this polymorphism has a functional effect on the *eNOS* protein (Kim *et al.*, 2009).

Here, we analyzed the *Arg72Pro* polymorphisms of the *p53* gene and the *Glu298Asp* polymorphism of the *eNOS* gene in order to estimate their prevalence in fertile versus infertile women diagnosed with endometriosis.

MATERIAL AND METHODS

We collected peripheral blood DNA samples from 35 patients with endometriosis in order to analyze polymorphisms of p53 and eNOS. The study included women with symptoms of endometriosis, such as progressive dysmenorrhea, dyspareunia, chronic pelvic pain and a confirmatory endometriosis diagnosis through laparoscopy. The patients were divided into two groups, the first with 18 women who were fertile and the second with 17 infertile women. The project was analyzed and approved by the Research Ethics Committee of the Pontifical Catholic University of Goiás (document number 0126.0.168.00008). The patients answered a questionnaire about social habits and signed the Informed Consent Form.

DNA from the blood samples was obtained with the Kaswi® extraction kit (Genomic DNA Purification Kit) and quantified with a NanoVue™ Plus (GE, Cambridge, UK) spectrophotometer. Only samples with a concentration greater than 5 ng/µl and with a purity of 1.8 to 2.0 were considered relevant. The DNA samples were submitted to ARMS-PCR and conventional PCR in order to check for the presence of eNOS and p53 polymorphisms, respectively. Table 1 presents the sequence of primers used for the detection of eNOS and p53 polymorphisms. The PCR reactions were performed according to the protocol proposed by Sambrook, with a final volume of 25 µl (Sambrook et al., 2001).

Table 1: Primer sequence for p53 and eNOS.

Gene	*Primer*	Sequence	Molecular size
p53	*Primer* p53-PRO (Ribeiro Júnior et al., 2009)	5' - GCC AGA GGC TGC TCC CCC – 3' (F) 5' - CGT GCA AGT CAC AGA CTT – 3' (R)	177 bp
	Primer p53- ARG (Ribeiro Júnior et al., 2009)	5' - TCC CCC TTG CCG TCC CAA – 3' (F) 5' - CTG GTG CAG GGG CCA CGC – 3' (R)	141 bp
eNOS	G894T (Adapted from Tajehmiri et al., 2014)	5'- AGG CCC AGC AAG GAT GTA GT 3' (FC) 5' - TGAAGGAAGAGTTCTGGTGGC 3' (R) 5' - TGAAGGAAGAGTTCTGGTGGA 3' (R)	 181 bp 171 bp

The thermocycling conditions for amplification of primers for the p53Pro polymorphism started with an initial denaturation for 5 minutes at 94°C followed by 35 cycles of denaturation for 1 minute at 94°C, annealing for 1 minute at 57°C, polymerization for 1 minute at 72°C and extension for 7 minutes at 72°C. For the p53Arg polymorphism amplification, there was an initial denaturation for 5 minutes at 94°C, followed by 35 cycles of denaturation for 1 minute at 94 °C, annealing for 1 minute at 59°C, polymerization for 1 minute at 72°C and extension for 7 minutes at 72°C.

Amplification of primers for the Glu298Asp polymorphism was performed according to the protocol established by Kim et al., (2009), with initial denaturation for 5 minutes at 94°C followed by 30 cycles of denaturation for 1 minute at 94°C, annealing for 1 minute at 58°C, polymerization for 1 minute at 72°C and extension for 10 minutes at 72°C. The PCR products were submitted to a 2% agarose gel electrophoresis in an electric field of 100 V/cm, stained with ethidium bromide (5mg/mL) and visualized with the photodocumentator BIORAD (Bio-Rad, Hercules, USA).

The results were prepared in Excel 2016 and the statistical analysis was performed using the G Test with the software Bioestat version 5.3.

RESULTS

The endometriotic patient genotype with at least one Pro allele in the p53 gene and with the wild type eNOS was 2.7 times more frequent than the homozygotic Arg genotype. The patient genotype with at least one Asp allele in the eNOS gene and with the wild type p53 gene was 1.6 times more frequent than those with the homozygous or heterozygous Pro allele (Table 2). These results suggest that the Arg72Pro and/or Glu298Asp polymorphisms influence the development of endometriosis.

Table 2- Distribution of eNOS e p53 gene polymorphisms in the case group.

	Glu/Glu	Glu/Asp+Asp/Asp	
	n	n	p α
Arg/Arg	6	8	0.0453
Arg/Pro+Pro/Pro	16	5	

[a]*p*-value of the G-Test

Table 3 shows the genotype distribution according to the fertility of the patients. The distribution of the p53 and eNOS genotypes and their association with fertility shows that patients with endometriosis carrying the genotype Arg/Arg for the p53 gene had similar distributions in the fertile and infertile groups (pα=0.3834). However, the presence of at least one polymorphic allele for p53 (Arg/Pro + Pro/Pro) associated with a polymorphic allele of the eNOS gene (Glu/Asp + Asp/Asp) was found to be related to infertility (pα= 0.0167).

Table 3 – Distribution of *p53* and *eNOS* genotypes as a function of patient fertility.

	Glu/Glu	Glu/Asp+Asp/Asp		
	n	n	%	p α
Arg/Arg				
Infertile	1	3		0.3834
Fertile	5	5		
Arg/Pro+Pro/Pro				
Infertile	8	5		0.0167
Fertile	8	-		

[a]*p*-value of the G-Test

DISCUSSION

Advances in molecular biology have contributed significantly to the understanding of the functioning of our genes. Any genetic perturbation can lead to disease (Zatz, 2002). Currently, many molecular markers have been used to provide useful data for the study of genetic diseases, and according to Aguiar (2012) they are many molecular phenotypes derived from an expressed gene or a specific segment of DNA.

We found a significant difference between the Arg72Pro and Glu298Asp polymorphisms in our study group (p=0.0453). We found that women with endometriosis have the polymorphic p53 or eNOS gene more frequently than those without this disease. We showed that a single mutated allele in the gene is enough to increase the risk of endometriosis. Interestingly, all of the patients with a combination of p53 and eNOS polymorphisms (Arg/Pro + Pro/Pro and Glu/Asp + Asp/Asp) were infertile and for patients who presented only one polymorphic allele, the infertility rate was 50%. Our results indicate that combined p53 and eNOS polymorphisms is associated with a greater risk of infertility related to endometriosis.

Hsieh and Lin (2006) showed that the Pro/Pro and Arg/Pro genotypes are more frequent in patients with endometriosis, indicating that there is a relationship between the codon 72 polymorphism of the p53 gene and endometriosis. Chang et al. (2002) showed that women with the Pro/Pro or Arg/Pro genotypes were 3.9 times more likely to develop endometriosis than those with the Arg/Arg genotype.

Several studies have shown that p53 also relates to carcinogenic and carcinogenic-like diseases. Roh et al., (2004) and Kim et al. (2004) demonstrated that there is a significant association between p53 gene polymorphisms and the risk of cervical cancer in Korean women, while Zhou et al. (2012) demonstrated that the risk is increased in Indian women. Ribeiro Júnior et al. (2009) showed that patients who carry the Pro allele at codon 72 of the p53 gene (homo or heterozygous genotypes) had a higher frequency of infertility associated with pain and bleeding compared to patients who carry the Arg allele. Lattuada et al. (2004) suggested that the p53 polymorphism does not confer genetic susceptibility to endometriosis in the Italian population, but that there may be a relationship with the development of endometriosis to more severe forms of the disease.

Regarding the polymorphism of the eNOS gene, Wu et al. (2003) demonstrated that the levels of NO were significantly increased in the endometrial tissues of women with endometriosis. Kim et al. (2009) showed that the frequency of Glu/Asp and Asp/Asp genotypes was higher than that of the wild genotype in the group with endometriosis (22.4%) compared to the control group (13.3%). Their results are similar to what we found and indicate that there is a relationship between the eNOS gene and endometriosis. According to Najafi et al (2013), women with infertility showed a higher eNOS expression only in the uterine luminal epithelium compared to the control group. Moreover, studies reveal the increased expression of eNOS in the endometrium throughout the menstrual cycle in patients with endometriosis compared to women without endometriosis, and higher levels of NO in the ectopic endometrium compared to the eutopic endometrium (Kim et al., 2009; Seckin et al., 2016; Zhao et al., 2016).

Pena et al. (2000) demonstrated in his study that there is a great difficulty to obtain a genetically representative sample of the Brazilian population. There is a gradient relative to races, which varies greatly from the north to the south. Our study was carried out with

Caucasian Brazilians from four geographic regions; in most of them the patrilineage is European and the matrilineage is Amerindian or African. There are divergences regarding the ethnicities in Brazil, which is a highly mixed country with a pronounced genetic variation and prevalence studies may vary more than in other countries within this population. The existence of different types of genetic polymorphisms, classified according to their molecular nature and location in the genome, makes studies regarding influence in genetic diseases possible (Pena et al,.2000). Molecular diagnosis can avoid invasive examinations in order to identify pathologies and prenatal disease risk through genetic counseling.

CONCLUDING REMARKS

In conclusion, the Pro allele of p53 and the Asp allele of eNOS is directly linked with an increased risk of developing endometriosis in our study population. The association of both p53 and eNOS polymorphisms was found to be related to cases of infertility in endometriotic patients. Both genes can be candidates for molecular markers in order to diagnose endometriosis, guiding prognosis and a more efficient treatment in order to reduce time and costs, avoid infertility, controlling pain, and simplifying disease management. The investigation of genetic polymorphisms of p53 and eNOS, as well as other genes, may contribute to the clarification of endometriosis pathophysiology.

REFERENCES

Aguiar MS (2012). Marcadores Moleculares como ferramenta no Melhoramento Genético de Plantas. Sci. Agrar. Paran. 12: 1-5.

Bellelis P, Dias JA, Podgaec S, Gonzales M. et al. (2010). Aspectos epidemiológicos e clínicos da endometriose pélvica: uma série de casos. Rev. Assoc. Med. Bras. 56: 467–471.

Bellelis P, Podgaec S, Abrão MS (2011). Fatores ambientais e endometriose. Rev. Assoc. Med. Bras. 57: 456–461.

Bellelis P, Podgaec S, Abrão MS (2014). Fatores ambientais e endometriose: um ponto de vista. Rev. Bras. Ginecol. Obstet. 36: 433–435.

Bianco B, Christofolini DM, Brandes A, Lerner TG, et al. (2011). Analysis of codon 72 polymorphism of the TP53 gene in infertile women with and without endometriosis. Rev. Bras. Ginecol. Obstet. 33: 37–42.

Crosera AMLV, Vieira CHF, Samama M, Martinhago CD, et al. (2010). Tratamento da endometriose associada á infertilidade-revisáo da literatura. Femina. 38: 256-260.

Chang CC, Hsieh YY, Tsai FJ, Tsai CH, et al. (2002). The proline form of p53 codon 72 polymorphism is associated with endometriosis. Fertil. Steril. 77(1):43-5.

Gallegos-Arreola MP, Figuera-Villanueva LE, Puebla-Pérez AM, Montoya-Fuentes H, et al. (2012). Association of TP53 gene codon 72 polymorphism with endometriosis in Mexican women. Genet. Mol. Res. 11(2): 1401–1408.

Hsieh YY and Lin CS (2006). P53 codon 11, 72, and 248 gene polymorphisms in endometriosis. Int. J. Biol. Sci. 2(4): 188–193.

Kim H, Ku SY, Kim SH, Lee GH, et al. (2009). Endothelial nitric oxide synthase gene Glu298Asp polymorphism is associated with advanced stage endometriosis. Hum. Reprod. 24(10):2656-9.

Lattuada D, Viganò P, Somigliana E, Abbiati A, et al. (2004). Analysis of the codon 72 polymorphism of the TP53 gene in patients with endometriosis. Mol. Hum .Reprod. 10(9): 651-4.

Marqui ABT (2012). Polimorfismos genéticos e endometriose: a contribuição dos genes que regulam a função vascular e o remodelamento de tecidos. Rev. Assoc. Med. Bras. 58(5): 620–632.

Minson F, Abrão MS, Júnior JS, Kraychete DC, et al. (2012). Importância da avaliação da qualidade de vida em pacientes com endometriose. Rev. Bras. Ginecol. Obstet. 34(1): 11–5.

Nácul AP and Spritzer PM. (2010). Aspectos atuais do diagnóstico e tratamento da endometriose. Rev. Bras. Ginecol. Obstet. 32(6): 298–307.

Najafi T, Novin MG, Khorram . (2012). Altered endometrial expression of endothelial nitric oxide synthase in women with unexplained recurrent miscarriage and infertility. Reprod. Biomed. Online. 25(4): 408–414.

Nastasi-Catanese, JA, Padilla-Gutiérrez JR, Valle Y, Ortega-Gutiérrez F, et al. (2013). Genetic contribution of CYP2C9, CYP2C19, and APOE variants in acenocoumarol response. Genet. Mol. Res. 10;12(4): 4413-21.

Pena SDJ, Carvalho-Silva DR, Alves-Silva J, Prado VF (2000). Retrato Molecular do Brasil. Ciência Hoje. 27(159):16–25.

Pereira NRGB, Leite MHVBB, Ribeiro RNT, Passarinho RM, et al. (2010). Laparoscopia na decisão da estratégia terapêutica para o casal infértil. Rev. Bras. Ginecol. Obstet. 32(9): 441–446.

Ribeiro Júnio RCL, Arruda JT, Silva CT, Moura KK (2009). Analysis of p53 codon 72 gene polymorphism in Brazilian patients with endometriosis. Genet Mol Res. 5;8(2): 494-9.

Roh JW, Kim JW, Park NH, Song YS, et al. (2004). p53 and p21 genetic polymorphisms and susceptibility to endometrial cancer. Gynecol. Oncol. 93: 499–505.

Sambrook J, Maccallum P, Russell D Molecular cloning: A laboratory manual. 3. ed. New York: Cold Springs Harbour Press, 2001.

Sampson JA (1927). Metastatic or Embolic Endometriosis, due to the Menstrual Dissemination of Endometrial Tissue into the Venous Circulation. Am. J. Pathol. 3(2): 93–110.

Sanghera S, Barton P, Bhattacharya S, Horne AW, et al. (2016). Pharmaceutical treatments to prevent recurrence of endometriosis following surgery : a model-based economic evaluation. BMJ Open. 6(4): 1–10.

Santos RP, Encinas F, Lasmar RB, Falagan-Lotsch P, et al. (2010). Polimorfismos nos genes MMP2, MMP13, CYP1A1, GSTM1 e EMX2 e endometriose. Femina. 39(6): 313–317.

Seckin Y, Yigit A, Yesilada E, Gulbay G, et al. (2016). Association of eNOS Gene Polymorphisms G894T and T-786C with Risk of Hepatorenal Syndrome. Gastroenterol. Res. Pract. 22:1-7.

Tajehmiri A, Mousamayali AR, Honari P (2014). Evaluation of eNOS G / T 894 polymorphism in Iranian patients affected by catatonic schizophrenia with type 2 diabetes. Adv. Biores. 6(1):102-106

Wu MY, Chao KH, Yang JH, Lee TH, et al. (2003). Nitric oxide synthesis is increased in the endometrial tissue of women with endometriosis. Hum. Reprod. 18(12): 2668–2671.

Yang CW, Chang CY, Lai MT, Chang HW, et al. (2015). Genetic variations of MUC17 are associated with endometriosis development and related infertility. BMC Med. Genet. 19;16:60.

Zatz M (2002). A biologia molecular contribuindo para a compreensão e a prevenção das doenças hereditárias. Cien. Saude Colet. 7(1): 85–99.

Zhao Y, Liao S, Lu R, Dang H et al. (2016). Endothelial nitric oxide synthase gene polymorphism is associated with Legg-Calvé-Perthes disease. Exp. Ther. Med. 11(5): 1913-1917.

Zhou X, Gu Y, Zhang SL (2012). Association between p53 codon 72 polymorphism and cervical cancer risk among Asians: a HuGE review and meta-analysis. Asian Pac. J. Cancer Prev. 13(10): 4909–4914.