Polymorphism in Glutatione S-Transferase P1 and Manganese-Superoxide Dismmutase Genes in Egyptian Women with Preeclampsia

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Abstract: *Susceptibility to preeclampsia is believed to have a genetic component. Several studies have reported associations between polymorphisms of oxidative stress- related genes and preeclampsia. The aim of the present study was to study the polymorphisms in anti-oxidant genes glutathione S-transferase P1 (GSTP1) and Mn-superoxide dismutase (Mn-SOD) in patients with of preeclampsia. Seventy four preeclampsia patients and fifty age-matched healthy pregnant female controls were genotyped for GSTP1 and Mn-SOD. DNA was extracted from peripheral blood of all patients and control women. DNA analysis was carried out by polymerase chain reaction (PCR), and then digestion of the PCR products by restriction enzymes (RFLP) for both genes was performed. As regards GSTP1, the carriers of Val (G) allele were significantly more frequent among preeclampsia patients when compared to the control group (45.95% Vs. 19%, X^2=10.40, OR=2.418, 95%CI (1.3698 - 4.269); p=0.0023). Preeclampsia patients had a lower frequency of GSTP1 Ile/Ile (AA) genotype (31.1% Vs. 68% in control; X^2=13.09, OR=0.457, 95%CI (0.241 - 0.866); p =0.0164). The frequency of GSTP1 Ile/Val (AG) and Val/Val (GG) genotypes was higher in preeclampsia patients than control (68.9%Vs. 32%; X^2= 12.83, OR=2.154, 95%CI (1.106 - 4.194); p =0.0241). However, non significant frequencies differences as regards Mn-SOD genotypes or alleles were found between preeclampsia patients and control. It could be concluded that, pregnant Egyptian women carrying the Val (G) allele of GSTP1 GSTP1 -105 Ile →Val (-313 A to G) polymorphism may be more susceptible to preeclampsia either in homozygous or heterozygous state.*

Keywords*: Preeclampsia, GSTP1, Mn-SOD, gene polymorphism.*

I. Introduction

Preeclampsia is a potentially life-threatening disease that occurs exclusively in pregnant women during late gestation (>20 weeks). The two hallmark symptoms are hypertension and proteinuria, complicating at least 5% of pregnancies and are usually resolved upon delivery of the placenta **(Lain and Roberts, 2002).** Although preeclampsia is a leading cause of maternal death and a major contributor to maternal and prenatal morbidity, its precise cause has not been completely elucidated **(Chamy et al., 2006).** Susceptibility to preeclampsia is believed to have a genetic component and several studies have reported associations between polymorphisms of oxidative stress and hypertension-related genes with preeclampsia. The mechanism of oxidative stress-associated hypertension has been continuously evolving with the popular theme of endothelial dysfunction. The etiology of oxidative stress in preeclamptic women has therefore been the focus of extensive research **(Zhang et al., 2008).** Low levels of reactive oxygen species (ROS) are crucial in maintaining normal cellular physiologic functions, such as proliferation, apoptosis, and cell cycle arrest; whereas increased levels of ROS induce oxidative stress and cause an imbalanced hemostatic microenvironment **(Aggarwal and Gehlot, 2009).**

The products of the glutathione S-transferase-P1 (GSTP1) and manganese superoxide dismutase (Mn-SOD; SOD-2) genes are involved in the regulation of ROS metabolic processes and some single nucleotide polymorphisms (SNPs) within these genes result in dysregulated protein translation and/ or altered function **(Xu et al., 2012).** The GSTP1, a member of the glutathione S-transferase family of phase 2 detoxification isozymes, is a candidate enzyme protecting epithelial cells from ROS-induced oxidative stress and detoxifying free radicals by catalyzing the nucleophilic addition of hydrophobic and electrophilic compounds to reduced glutathione **(Zusterzeel et al., 1999).** The GSTP1 gene, located at 11q13, has a single nucleotide polymorphic site at codon 105 (exon 5). This SNP (rs1695) is a substitution of adenosine-to-guanosine at nucleotide 313 (A-313-G) results in an isoleucine (Ile, ATC)-to-valine (Val, GTC) change at residue 105 (Ile-105-Val) **(Zusterzeel et al., 2000).** This amino acid substitution, in the GSTP-1 binding site, decreases its catalytic activity and causes less effective detoxification. The GSTP1 enzymes with Val-105 have altered heat stability and represented with specific activity of the Val-containing isoform **(Hurl et al., 2005).**

The Mn-SOD, SOD2, is one of the major superoxide scavengers in mitochondria, and it catalyzes accumulated superoxide radicals into hydrogen peroxide (H_2O_2). The Mn-SOD gene contains five exons and spans almost 20 kb located at 6p25. The 47 cytosine-to-thymine (C-47-T) transition at codon 16 in the Mn-SOD gene (rs4880) creates a sense mutation of alanine (Ala, GCT)-to-valine (Val, GTT) (Ala-16-Val) in the 24-amino acid signal sequence of Mn-SOD protein, which affects protein folding and localization **(Cai et al., 2004; Wang et al., 2009).** This SNP has been identified in exon 2 of the human Mn-SOD gene. Molecularly, the Val variant is predicted to form a β-sheet structure on the protein, while the Ala variant results in an α-helical conformation. MnSOD is synthesized in the cytoplasm and transported into the mitochondria via an N-terminal mitochondrial targeting sequence (MTS) **(Zelko et al., 2002).** The Ala variant is more efficiently imported into the mitochondria than the Val variant. Miscoding of Ala to Val will result in arresting of Mn-SOD in the inner mitochondrial membrane but not in the mitochondrial matrix, therefore reducing its functional activities **(Sutton et al., 2005).** The inefficient Mn-SOD protein in the mitochondria may leave the cell vulnerable to oxidative damage without its full defense against superoxide radicals, which leads to protein oxidation and DNA mutations **(Bag and Bag, 2008).** Several studies have revealed that this Mn-SOD polymorphism is associated with cardiovascular and metabolic diseases in the general population **(Tian et al., 2011, Kariz et al., 2012).**

Results of studies seeking associations of the Mn-SOD C-47-T and GSTP1 A-313-G polymorphisms with preeclampsia have not always been consistent among different population analyses. Thus, our objective was to clarify a possible association between these two SNPs and the risk for developing preeclampsia among Egyptian women.

II.   Subject And Methods

Patient selection:

During the period from July 2011 to November 2012, a case- control study was conducted in Obstetric and Gynecology and Medical Biochemistry Departments, Mansoura Faculty of Medicine. Seventy four primigravida with preeclampsia with singleton pregnancy, were recruited from Obstetrics Unit of Obstetrics and Gynecology Department. The control group included 50 age-matched healthy pregnant women with singleton pregnancy free from any diseases. All participants underwent history taking, physical examination for measurement of blood pressure, body weight, obstetric examination and obstetric ultrasonography. Basic laboratory tests as complete blood count, serum creatinine, ALT, AST, and repeated urine analysis for proteinuria were done. Informed consent was taken from each participant. The study was approved by The Ethical Committee of Mansoura Faculty of Medicine, Egypt.

Preeclampsia was diagnosed when hypertension was accompanied with proteinuria (>300 mg/dl/24h) Hypertension was defined when systolic blood pressure ≥140 mmHg and/or diastolic blood pressure ≥90 mmHg measured on two consecutive occasions at least 6 hour apart.

Pregnant women with chronic hypertension, superimposed preeclampsia, patients with eclampsia were excluded. Also, patients with gestational diabetes mellitus, associated diabetes mellitus, and chronic renal disease, and twin pregnancies were excluded.

Biochemical investigation:

Venous blood samples were taken from all participants. Three ml were delivered to centrifuge tubes containing K_2EDTA (stored as EDTA anti-coagulated blood sample at -30ºC for DNA extraction). Another 5 ml blood sample was allowed to clot for 15 minutes and centrifuged at 7000 rpm for 10 minutes for serum separation to determine: serum creatinine, ALT and AST. Samples were centrifuged at 4000 xg for 20 min at 4ºC to avoid the contamination of the serum with platelets. They were then stored at –20ºC until used.

DNA extraction:

Genomic DNA was extracted from EDTA–anticoagulated peripheral blood leucocytes using QIA amp DNA Blood Mini Kit supplied by Qiagen GmbH (Cat, No.51104, Hiden, Germany) **(Schur et al., 2001).** The average DNA concentration (0.161±0.002μg/μl) was determined from absorbance at 260 nm (Jenway, Genova Model, UK). All samples had a 260/280 nm absorbance ratio between 1.4 and 1.63. The integrity of the DNA was checked by electrophoresis on 2 % agarose gel stained with ethidium bromide.

Genotyping of GSTP1 -105 Ile □ Val (-313 A to G) SNP (rs1695) and Mn-SOD -16 Ala □ Val (-47 C to T) SNP (rs4880):

Conventional method of PCR amplification was used for genotyping of GSTP1 -105 Ile □ Val (-313 A to G) SNP (rs1695) in exon 5 of GSTP1 gene by the method described by **Li et al. (2010).** A 440 bp exon 5 region containing the polymorphism site was amplified by using the following primers sequences: a 20-mer GSTP1- forward primer 5′- ACG CAC A̲TC CTC TTC CCC TC -3′ and a 20-mer reverse primer 5′- TAC TTG GCT GGT TGA TGT CC -3′ (Fermentas Life Science, Ontario, Canada) in which the underlined nucleotide

represents the deliberated primer mismatch designed to introduce an artificial Bsm AI restriction site when the G allele is present at position -313. Genotyping of Mn-SOD -16 Ala → Val (-47 C to T) SNP (rs4880) was done by the method described by **Manica-Cattani et al. (2012).** A 110 bp exon 2 region containing the polymorphism site was amplified by using the following primers sequences: a 22-mer forward primer 5′- ACC AGC AGG CAG CTG GCG CCG G-3′ and a 20-mer reverse primer 5′-GCG TTG ATG TGA GGT TCC AG-3′ (Fermentas Life Science, Ontario, Canada) in which the underlined nucleotide represents the deliberated primer mismatch designed to introduce an artificial Hae III restriction site when the C allele is present at position -47.

PCR was carried out in 50 microliters final reaction volume using Ready Mix (RED. Taq-PCR Reaction Mix) (purchased from Sigma Aldrich, Saint Louis, USA).The following mixture was prepared for each sample: 25 µl RED-Taq PCR reaction Mix (1X), 2µl (20 pmol) of forward primer, 2µl (20 pmol) of reverse primer, 2µl (200 ng) of genomic DNA and 19 µl of deionized water. This mix was put in a thin wall PCR microcentrifuge tube and gently centrifuged to collect all components to the bottom of the tube. Thermal cycling was carried out using Techne TC-312 thermal cycler (Model FTC312D, Barlworld Scientific Ltd, UK). The following program was used: initial denaturation at 94 ℃ for 5 min followed by 35 cycles of denaturation at 94 ℃ for 30 sec, annealing at 57 ℃ (GSTP-1) or 60 ℃ (Mn-SOD) for 30 sec, and an extension step at 72 ℃ for 30 sec, followed by a final linear extension step at 72 ℃ for 5 min. Amplification products of 440 bp for GSTP-1 and 110 bp for Mn-SOD were obtained when electrophoresed in a 2.5% (wt/vol) agarose gel stained with ethidium bromide, and bands were visualized under ultraviolet (UV) via Light UV Transilluminator (Model TUV-20, OWI Scientific,Inc.800 242-5560).

The PCR products of GSTP-1 (440 bp) were digested using Bsm AI enzyme (Fermentas Life Science, Conventional restriction enzymes, Catalog number ER0031), 12µl of each PCR product was digested overnight with 3 µl (30 U) of the enzyme at 37 °C. The enzyme recognition site is 5'-GTCTC (N)1-3' and 3'-CAGAG(N)5-5'. Homozygous wild genotype (Ile/Ile; AA) was presented as a single band at 440 bp when electrophoresed in a 2.5% (wt/vol) agarose gel against DNA molecular marker. Two bands at 228 bp and 212 bp identified Val/Val (GG) homozygous genotype, and three bands at 440, 228 and 212 bp indicated heterozygous Ile/Val (AG) genotype **(Figure 1).**

To determine the Val (T) allele of Mn-SOD at position -47, PCR products of 110 bp were digested by Hae III enzyme (Fermentas Life Science, Conventional restriction enzymes, Catalog number ER0151), 12µl of each PCR product was digested overnight with 3 µl (30 U) of the enzyme at 37 °C. The enzyme recognition site is 5'-GGCC-3' and 3'-CCGG-5'. A single band (110 bp) indicated the Val/Val (TT) genotype, while two bands at 85 bp and 25 bp identified the wild Ala/Ala (CC) genotype. The presence of all the three bands (110 bp, 85 bp and 25 bp) indicated the Ala/Val (CT) genotype **(Figure 2).**

Statistical analysis:

Statistical package of social science (SPSS) version 16 was used for statistical analysis. The qualitative data were presented in the form of number and percentage. Chi-square test was used as a test of significance for qualitative data. Odds ratio and 95% confidence intervals were calculated for determination of disease association and expected risk for the disease. The quantitative data were expressed as mean and standard deviation. Significance was considered at p value less than 0.05.

III.   Results

Clinical and demographic data of 74 patients and 50 control are summarized in **table (1).** The mean age of preeclampsia patients was 27.58±4.55 years with a mean age of gestation 29.47±2.58 weeks. Significant differences were found in the frequency of GSTP1 -105 Ile →Val (-313 A to G) SNP genotypes among preeclampsia patients and control as shown in **table (2).** Patients with preeclampsia had a lower frequency of GSTP1 -105 Ile/Ile (AA) genotype (31.1% Vs 68% in controls ; OR=0.457 with 95%CI (0.241 - 0.866); p =0.0164). The frequency of GSTP1 -105 Ile/Val (AG) genotypes was higher in preeclamptic patients than control (45.9% Vs 26%; OR= 1.767 with 95% CI (0.849 - 3.678); p=0.0251). GSTP1 -105 homozygous Val/Val (GG) genotype was found in 23% of preeclampsia patients and 6% of control carried that genotype. It was significantly more frequent in preeclampsia patients than in control (OR=3.829 with 95% CI (1.066 - 13.754); p =0.0396).

The frequency of GSTP1 -105 allele's distribution among the preeclampsia patients and control are shown in **table (2).** As regards carriers of Val (G) allele, it was significantly more frequent among preeclampsia patients with 2.418 folds higher risk of developing preeclampsia among patients group than controls (OR=2.418, 95% CI=1.3698 - 4.269, p=0.0023). On the other hand, the carriers of Ile (A) allele were significantly more frequent among control when compared to preeclampsia group (OR=0.667, 95% CI= 0.447 - 0.995; p=0.0474). **Table (3)** shows that the relative risk for preeclampsia was 4.712 folds higher in patients carrying Ile →Val (A to G) transition either in homozygote or heterozygote form than those carrying the dominant homozygote Ile/Ile (AA) form (OR=4.712, 95% CI=2.178-10.193, p=0.0001). Also, the relative risk for preeclampsia was higher in

patients carrying the Val (G) allele than those carrying the Ile (A) allele (OR=3.624, 95% CI= 1.999-6.570; p <0.0001).

As regards Mn-SOD -16 Ala → Val SNP (-47 C to T) genotypes, no significant differences were found in the genotype frequencies between patients and controls. Also, the frequency of Mn-SOD -16 Ala → Val allele's distribution among the preeclampsia patients and control shows non significant differences **(Table 4).** Neither of the Ala → Val transition genotypes nor alleles show any significant differences when compared to the dominant ones as regards preeclampsia relative risk **(Table 5).**

Table (1) Clinical and demographic data of patients and control:

Variable	Preeclampsia (n=74)	Control (n=50)	P value
Age (years)	27.58±4.55	25.78±4.21	0.052
Gravity	2.45±1.24	2.82±1.32	0.1
Parity	1.24±1.18	1.63±1.20	0.06
Gestational age (weeks)	29.47±2.58	29.33±2.54	0.6

Data are presented as mean ± SD

Table (2) Distribution of GSTP1 -105 Ile □ Val (-313 A to G) SNP genotypes and Ile and Val (A and G) alleles among patients and control:

GSTP1- 105 Ile □ Val genotypes	Preeclampsia (n=74) No (%)	Control (n=50) No (%)	X^2	OR (95%CI)	P value
Ile/Ile (AA)	23 (31.1%)	34 (68%)	13.09	0.457 (0.241 - 0.866)	**0.0164**
Ile/Val (AG)	34 (45.9%)	13 (26%)	5.01	1.767 (0.849 - 3.678)	**0.0251**
Val/Val (GG)	17 (23%)	3 (6%)	8.83	3.829 (1.066 -13.754)	**0.0396**
Ile/Val (AG) +Val/Val (GG)	51 (68.9%)	16 (32%)	12.83	2.154 (1.106 - 4.194)	**0.0241**
Ile (A)	80 (54.05%)	81 (81%)	5.01	0.667 (0.447 - 0.995)	**0.0474**
Val (G)	68 (45.95%)	19 (19%)	10.40	2.418 (1.3698 - 4.269)	**0.0023**

p is significant if < 0.05

Table (3): Association of GSTP1 -105 Ile □ Val (-313 A to G) SNP genotypes and alleles to the relative preeclampsia risk:

GSTP1 -105 Ile □ Val SNP	Preeclampsia (n=74) No (%)	Control (n=50) No (%)	X^2	OR (95% CI)	P value
Ile/Ile (AA)	23 (31.1%)	34 (68%)	16.3	4.712 (2.178-10.193)	**0.0001**
Ile/Val (AG) +Val/Val (GG)	51 (68.9%)	16 (32%)			
Ile (A)	80 (54.05%)	81 (81%)	19.02	3.624 (1.999-6.570)	**0.0001**
Val (G)	68 (45.95%)	19 (19%)			

P is significant if < 0.05
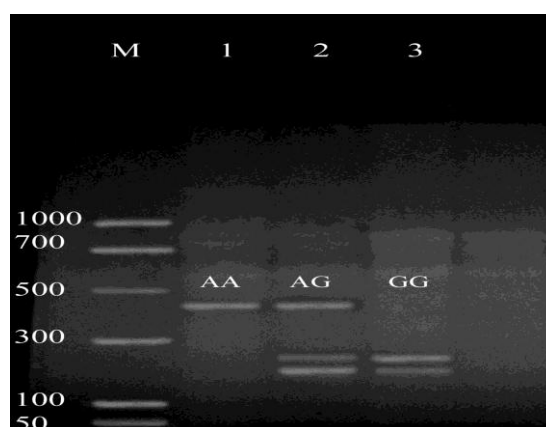
Figure 1: Agarose gel electrophoretic analysis of the GSTP1 -105 Ile □ Val (-313 A to G) (rs1695):

The genotypes of GSTP1 -105 Ile □ Val (-313 A to G) SNP (rs1695) after Bsm AI digestion analysis represented as follow: Ile/Ile (AA) genotype is presented by one single band at 440 bp. (lane 1) , Ile/Val (AG) genotype is presented by three bands at 440, 228 and 212 bp (lane 2) and.Val/Val (GG) genotype is presented by two bands at 228 bp and 212 bp (lane 3) Lane (M) represents the molecular marker (DNA molecular weight marker, purchased from Promega Technical Service, Catalog#G3161).

Table (4) Distribution of Mn-SOD -16 Ala □ Val (-47 C to T) SNP genotypes and Ala and Val alleles (C and T) among patients and control:

Mn-SOD -16 Val □ Ala genotypes	Preeclampsia (n=74) No (%)	Control (n=50) No (%)	X^2	OR (95%CI)	P value
Ala/Ala (CC)	53 (71.6%)	39 (78%)	0.24	0.918 (0.531 - 1.588)	0.7600
Ala / Val (CT)	18 (24.3%)	10 (20%)	0.21	1.216 (0.519 - 2.852)	0.6526
Val/Val (TT)	3 (4%)	1 (1%)	0.80	2.027 (0.205 - 20.046)	0.5456
Ala / Val (CT) + Val/Val (TT)	21 (28.4%)	11 (22%)	0.50	1.290 (0.572 - 2.908)	0.5394
Ala (C)	124 (83.78%)	88 (88%)	0.05	0.952 (0.656 - 1.382)	0.7963
Val (T)	24 (16.22%)	12 (12%)	0.32	1.351 (0.646 - 2.827)	0.4239

p is significant if < 0.05

Table (5): Association of Mn-SOD -16 Ala □ Val (-47 C to T) SNP genotypes and alleles to preeclampsia risk:

Mn-SOD -16 Val □ Ala SNP	Preeclampsia (n=74) No (%)	Control (n=50) No (%)	X^2	OR (95% CI)	P value
Ala/Ala (CC) Ala / Val (CT) + Val/Val (TT)	53 (71.6%) 21 (28.4%)	39 (78%) 11 (22%)	0.63	1.405 (0.607-3.250)	0.4269
Ala (C) Val (T)	124 (83.78%) 24 (16.22%)	88 (88%) 12 (12%)	0.85	1.420 (0.674-2.990)	0.3568

p is significant if < 0.05
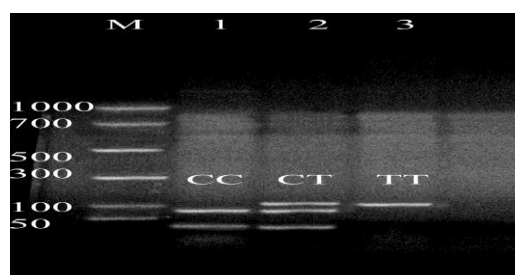
Figure 2: Agarose gel electrophoretic analysis of the Mn-SOD -16 Ala □ Val (-47 C to T) SNP (rs4880):

The genotypes of Mn-SOD -16 Ala → Val (-47 C to T) SNP (rs4880) after Hae III digestion analysis represented as follow: Ala/Ala (CC) wild genotype is presented by two bands at 85 bp and 25 bp (lane 1), Ala/Val (CT) genotype is presented by three bands at 110 bp, 85 bp and 25 bp (lane 2), and Val/Val (TT) mutant genotype is presented by one single band at 110 bp (lane 3). Lane (M) represents the molecular marker (DNA molecular weight marker, purchased from Promega Technical Service, Catalog#G3161).

IV. Discussion

Maternal susceptibility to preeclampsia is a multifactorial and complex process, involving the contribution of a series of essential gene variants **(Oudejans et al., 2007),** affecting hemodynamic and vascular parameters. The etiology and pathophysiology of preeclampsia are not fully understood, however oxidative stress has been strongly linked to the occurrence of that disease **(López-Jaramillo et al., 2001).** The oxidative stress pathways cause endothelial damage and induction of metabolic and coagulation aberrations. There is a suggested involvement of glutathione S-transferases, major detoxificating enzymes, in the pathophysiology of preeclampsia **(Pappa et al., 2011).** In the present study, we tried to assess a possible association between glutathione S- transferase P1 -105 Ile □ Val (-313 A to G) SNP (rs1695) as well as Mn-superoxide dismutase -16 Ala → Val (-47 C to T) SNP (rs4880) and patient risk for preeclampsia among Egyptian women.

The frequency of GSTP1-105 Ile/Val (AG) genotypes, Val/Val (GG) genotypes (both heterozygote and homozygote forms) and the frequency of carrying the Val (G) allele were significantly higher in preeclampsia patients than control group**.** The relative risk of preeclampsia is higher in the carriers of Ile/Val (AG) and Val/Val (GG) genotypes and the carriers of Val -105 (G) alleles than Ile-105 (A) allele**.** In accordance of the observed results of the current study, some studies reported the same associations as the Val-105 (G) allele was associated with an increased risk of developing preeclampsia in a Dutch population **(Zusterzeel et al., 2000)** and with severe hypertension in elderly pregnancy in a Japanese population **(Ohta et al., 2003).** It is of interest that homozygosity for the Val-105 polymorphism was associated with severe preeclampsia because of the production of a GSTP1 enzyme with reduced detoxifying capacity **(Zusterzeel et al., 2002).**

However, a study performed by **Canto et al. (2008)** on Maya-Mestizo women reported different associations in which the GSTP1-G (Val) allele conferred a reduced risk of preeclampsia under a dominant model, since the comparison of AG and GG vs. AA genotype frequencies was significantly different. Also, no association was found in the colored population of South Africa **(Gebhardt et al., 2004).** Moreover, **Pappa et al. (2011)** speculated that the Val-105 allele frequency was higher in normal pregnancies than in preeclampsia among Greek population. In fact, candidate gene studies suppose that single-gene effects may be variable across populations **(Zintzaras et al., 2006).**

For Mn-superoxide dismutase-16 Ala → Val (-47 C to T) polymorphism, it was found that no significant differences in the genotype frequencies and allelic distribution were detected among preeclampsia and control groups **(Table 4, 5).** These findings are in accordance with the result of **Zhang et al (2008)** who reported that the effects of the Ala-9Val are not fully understood and therefore it is difficult to hypothesize the effects of the variant on the risk of preeclampsia. Also, **Kim et al. (2005)** found that there was no significant association between Mn-SOD polymorphism and the risk for preeclampsia. Whereas, in contrary to our results, **Procopcius et al. (2012)** reported significant association between Val/Val (TT) and preeclampsia in Romanian population. No other studies were found for Mn-SOD polymorphism association to preeclampsia, so these conflicting findings warrants further investigation in other populations.

In summary, we found that glutathione S- transferase P1 -105 Ile □ Val (-313 A to G) SNP (rs1695) is associated with the susceptibility to preeclampsia in pregnant Egyptian women. Whereas, our data did show any significant difference between Mn-superoxide dismutase -16 Ala → Val (-47 C to T) SNP (rs4880) in the samples taken from normal and preeclamptic pregnancies.

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