TGFB1 and *TGFBR2* gene polymorphisms and pathophysiology of severe preeclampsia

Anita Deborah Anwar*, Firman Fuad Wirakusumah, Sofie Rifayani Krisnadi, Tono Djuwantono, Yelliantty, and Leri Septiani

Department of Obstetrics and Gynecology, Faculty of Medicine, Universitas Padjadjaran–Dr. Hasan Sadikin General Hospital, Bandung, Indonesia
*All **correspondence** to: Anita D. Anwar, MD., PhD
Department of Obstetrics and Gynecology, Faculty of Medicine,
Universitas Padjadjaran–Dr. Hasan Sadikin General Hospital, Bandung, Jl. Pasteur No. 38, Bandung 40161, West Java, Indonesia
E-mail: anitadebbyanwar@gmail.com

Abstract

Transforming growth factor β1 (*TGFB1*) and transforming growth factor β type 2 receptor (*TGFBR2*) are key components of TGF-β signaling and play an important role in vascular remodeling in placentation. Several polymorphisms have been identified in *TGFB1* and *TGFBR2* genes, including those associated with the regulation of TGF-β1 levels and TGF binding to its receptor. The decrease of TGF-β1 levels and the disruption of TGF TGF-β1 signaling can cause abnormal placentation, hypoxia, and increased soluble endoglin (sEng), which can lead to preeclampsia.This study aims to determine the role of gene polymorphisms C-509T *TGFB1* and C1167T *TGFBR2*and their relation to TGF-β1 and sEng levels in cases of severe preeclampsia. Genotyping performed using restriction fragment length polymorphism (RFLP) and a direct sequencing method was used to study 26 cases of preeclampsia against 26 control matched normal pregnancies. The results showed that the TGF-β1 levels in severe preeclampsia were significantly lower compared to normal pregnancies, whereas the levels of sEng in severe preeclampsia were significantly higher compared to normal pregnancies. In addition, the genotypes -509CT/TT *TGFB1* and 1167CT/TT *TGFBR2* appeared in both groups. This analysis highlights the absence of a significant association between the genotype -509C/T *TGFB1* and TGF-β1 levels in both

groups. The results did not indicate any significant associations between the genotypes -509C/T *TGFB1* and 1167C/T *TGFBR2* and the levels of sEng in both groups. Based on these results, it can be concluded that the levels of TGF-β1 and sEng are associated with severe preeclampsia but that the gene polymorphisms C-509T *TGFB1* and C1167T *TGFBR2* are not directly related to severe preeclampsia.

Key Words: Severe preeclampsia, *transforming growth factor β*1, *transforming growth factor β* type 2 receptor, single nucleotide polymorphism, soluble endoglin

1. Introduction

Preeclampsia is a pregnancy-specific syndrome appearing after 20 weeks gestation in women who were previously normotensive and non proteinuric.Preeclampsia is categorized as severe if some of the following criteria are met: blood pressure higher than 160/110 mm Hg on at least two examinations within an interval of 6 hours; proteinuria higher than 5 g/day; oliguria (less than 400 mL/24 hours); neurological and ophthalmologic changes; nausea and vomiting; pulmonary edema and cyanosis; thrombocytopenia; hemolytic anemia; and restricted fetal growth.[1] The incidence of severe preeclampsia in Indonesia from 1980-2001 accounted for 6-8% of all pregnancies and a maternal mortality rate by of 9.8%. From

2006-2008, Dr. Hasan Sadikin Hospital, Bandung, Indonesia, encountered 524 cases (7.2%) of preeclampsia out of 7285 deliveries, with 22 cases of maternal mortality (0.3%) and 15 cases (0.21%) of perinatal death [2].

Pathogenesis of preeclampsia involves three stages: abnormal vascular remodeling of the placenta, placental ischemia, and endothelial cell dysfunction [3]. In previous studies, it has been hypothesized that the original cause of preeclampsia involves genetic factors, immunological factors, and other factors resulting in a failure of trophoblast proliferation and invasion. These factors lead to abnormal placental vascular remodeling and endothelial dysfunction causing the syndrome of preeclampsia.

Placental vascular remodeling initiated by the proliferation and invasion of trophoblast into the spiral arteries in the endovascular and perivascular progestation is influenced by the cytokine TGF-β1 expressed by trophoblast cells and is a combination of mediators between proinflammatory and anti-inflammatory activity [4]. TGF-β1 is found in the placenta and decidua from the first trimester until the end of pregnancy. TGF-β has three isoforms (TGF-β1, -β2, and -β3) and has a role in tissue regeneration, cell differentiation, embryonic growth, and immune system regulation. Several studies have shown that TGF-β1 is involved in proliferation and invasion of trophoblast in the pathogenesis of preeclampsia. During placental development, endoglin expressed by endothelial and syncytiotrophoblast is further up-regulated because it plays an important role in the process of proliferation, migration, and trophoblast invasion [5]. Endoglin binds TGF-β1 and TGF-β3 with high affinity [4].

Induced biological effects of TGF-β1 include target gene regulation and subsequent cell growth, differentiation, and apoptosis [6]. TGF-β binds to specific receptors on the cell surface, namely TGF-β type 1 receptor (R-1), TGF-β type 2 receptor (R-2), betaglycan, and endoglin [5]. The biological effects of TGF-β1 are determined by the precision of the signal between the TGF-β1 with its receptor. The accuracy of the signals of TGF-β1 is determined

by the amount and structure of the protein TGF-β1. There is disruption of the TGF-β1 signaling if there are changes in protein levels or damage to the TGF-β1. Changes in the level of TGF-β1 affect the transcription of genes and underscore the biological effects that follow. Damage to the structure of the TGF-β1 protein will interfere with the receptor ligand binding. The TGF-β1 gene is located on chromosome 19q13.1 [5], and is expressed by various cell types, including eosinophils, monocytes, macrophages, T helper cells, and airway epithelial cells [5]. The TGF-β1 gene has polymorphisms in various locations.Three polymorphisms are located in the promoter region at -988 (C/A), -800 (G/A), and -509 (C/T); and two polymorphisms are located at codon 10 (Leu10Pro) and 25 (Arg25Pro) of exon 1. Polymorphisms in the promoter region will alter the regulation and levels of protein expression as well as whether the merger of introns and exons fails will trigger changes in mRNA splicing [9].

In addition to the amount and structure of the protein TGF-β1, the expression and structure of the TGF-β type 1 receptor in the cell membrane also determines the signaling of TGF-β1. Changes in the expression and structure of the TGF-β1 receptor cause interference with the receptor ligand binding. TGF-β1 receptor is a complex consisting of TGF-β types 1 receptor, 2, and 3. Among the three types of TGF-β1 receptors, the TGF-β1 type 2 receptor (TβR-2) is a receptor protein that binds directly to TGF-β1. Because TβR-2 has a higher affinity than the other types, it is considered instrumental in the process of TGF-β1 signaling. Changes or disturbances in TβR-2 can affect and even inhibit the mechanism of TGF-β1 signaling, although forming a ligand in normal conditions. Because TβR-2 underwent a conformational change (changes in protein structure), the ligand TGF-β1 cannot bind to it, blocking the transcription of genes that play a role in cell proliferation and preventing the process of angiogenesis from occurring.

TGFBR2 gene is a gene encoding TβR-2, and changes in the *TGFBR2* gene would affect the state of TβR-2 protein. Lucke *et al*. [7], found that mutations in the *TGFBR2* gene affect the presence of TβR-2 in breast cancer. Oshima *et al*. [8], found the presence of deletions of genes coding for TβR-2 in mice, resulting in early embryonic death caused by failure of yolk-sac vascularization. One form of the mutations found by Lucke *et al*. [7], is a point mutation or *single nucleotide polymorphism* (SNP). Mutations are more common in TβR-2 kinase domain and result in the loss of TβR-2 expression on the cell surface. Loeys *et al.* [9], found that the point mutation in the gene *TGFBR2* is associated with changes in blood vessel wall structure. Decreased levels of TGF-β1 and a disruption in the TGF-β1 signaling can cause abnormal placentation and hypoxia as well as increased soluble endoglin (sEng).Increased sEng can lead to the inactivation of TGF-β1 signaling. Soluble endoglin is a fraction endoglin in endothelial cells and induces proteolytic processes at the cell membrane due to hypoxia. Several studies have revealed that the sEng from the placenta is increased in serum of preeclampsia in accordance with the severity of the disease and decreased after delivery [10-11]. Accordingly, this study aims to determine the role of gene polymorphisms TGF-β1, *TGFBR2*, and sEng in relation to the expressed cases of severe preeclampsia.

2. Methods

2.1. Research subjects

Pregnant women who came to the Hasan Sadikin hospital at a gestational age of 28-42 weeks and a diagnosis of severe preeclampsia (according to the protocol of the Division of Fetomaternal Department of Obstetrics and Gynecology Dr. Hasan Sadikin General Hospital –Faculty of Medicine, Universitas Padjadjaran, Bandung, Indonesia in 2008) served as the case group. Statistical calculations were obtained from a sample size of 26 people for both the case and control groups.

2.2 Clinical examination

The physical examination included the general condition, awareness, weight, height, and heart-lung examination. The examination also included systolic and diastolic blood pressure after the patient rested for 15 min on her left side. Measurement of blood pressure was obtained using a mercury-column sphygmomanometer and a Littman stethoscope. The blood pressure measurement obtained was the average of the two examinations performed at 5 min intervals.

2.3. Examination of levels of TGF-β1

The quantitative sandwich enzyme immunoassay technique was used to examine TGF-β1. Monoclonal antibodies specific to TGF-β1 were superimposed onto microplate standards and samples and then were pipetted into the well. The presence of TGF-β1 was determined using antibody immobilization. After washing the compounds that were not bound, enzyme-linked monoclonal antibody specific to TGF-β1 was added to the well. During washing to remove compounds that were not bound to the *antibody-enzyme* reagent, substrate solution was added to the well and coloring appeared in accordance with the proportion of TGF-β1 bound at an early stage. Once the development reached a peak, the color intensity was measured.

2.4. Measurement of soluble endoglin levels

Overall, 3.5 mL of venous blood was taken from the cubital vein using *vacutainer*/ pyrogen-free tubes. sEng levels were determined in duplicate by an ELISA technique using a Human soluble endoglin *immunoassay* kit (Quantikine) in accordance with the manufacturer's instructions (R & D Systems Inc., Minneapolis, MN).

2.5. Polymorphism detection method

Examination of the polymorphisms consisted of several stages, including DNA isolation, PCR sequencing, and RFLP. DNA isolation was performed using the Wizard ®*genomic* DNA

kit DNA *purification* (Promega). The sample used was 2 mL of whole blood mixed with anticoagulant *ethylenediaminetetraacetic acid* (EDTA).

2.6. Polymerase chain reaction (PCR)

The *TGFB1* fragment was isolated using *forward* primer 5 'CCC TCC GCC TCC AGG ATT TG 3' and *reverse* 5 'GGT CAC CAG AAG AGG AGA AC 3'. The *TGFBR2* fragment was isolated using *forward* primer 5 'CCT CCA TGA CAG TCA CAC TC 3' and *reverse* 5 'TAA AGG TCT GGA AGC AGC ACT 3' based on research by Lucke [10]. The PCR kit used was Go Green Master Mix-Taq Promega. The PCR conditions were as follows: Pre-heating was performed at 95°C for 3 min; denaturation at 95°C for 30 sec; annealing at 55°C for 1 min; extension at 72°C for 1 min; and extra-extension at 72°C for 10 min. The *TGFBR2* fragment was 218 base pair (bp), and the *TGFB1* fragment was 808 bp.

2.7. Restriction Fragment Length Polymorphism

PCR product *(TGFB1* gene fragments) were analyzed using RFLP method. Cutting is done with the restriction enzyme Bsu36I. Then incubated at 37°C for 20 h, then performed electrophoresis on 2% agarose gel.

2.8. Sequencing

The PCR product (*TGFBR2* gene fragments) was sequenced using the services of Macrogen Inc. (Korea). The selected type of sequencing was direct sequencing. Most service checks were made online through the website http://www.macrogen.com. The results of sequencing were sent via e-mail to investigators. The resulting data were processed using the BioEdit and the software can be accessed online through the website http://www.ebi.ac.uk.

2.9. Data Analysis

The statistical analyses used in this study were Chi squared analysis (used to compare two groups of subjects and the frequency of characteristics of the genotype and *TGFBR2* genes polymorphism) and the Mann-Whitney U test (used to compare differences in plasma

protein levels in two groups). Data analysis was performed using SPSS for Windows version

13.0 at a 95% confidence interval (p ≤ 0.05).

3. Results

Subjects with preeclampsia (n=26) were identified after receiving a clinical diagnosis of

severe preeclampsia and presenting with hypertension 160/100 mmHg. Control subjects

(n=26) were normotensive pregnant women. The main clinical parameters listed in Table 1

include gestational age, body mass index, age, and parity. Between September 2008 and May

2009, a continuous series of 26 preeclamptic patients was studied.

Table 1. Characteristics of research subjects, both in terms of gestational age, BMI, maternal age, and parity in patients with severe preeclampsia and controls

Variable	Group		p-value [*)]
	Preeclampsia (n = 26)	Controls (n = 26)	
Gestational age (weeks)			0,124
28-32	9 (34,6%)	15 (57,7%)	
33-37	17 (65,4%)	11 (42,3%)	
Mean	33,3	32,7	
Range	31-37	30-36	
BMI (kg / m ^{2})			0,494
Mean	23,4	23,1	
Range	20-26	21-26	
Age (years)			0,427
<20	3	1	
20-34	23	24	
≥ 35	0	1	
Mean	26	28.9	
Range	19-34	19-37	
Parity			0,561
0-1	15	13	
2 -3	11	13	
≥ 4	0	0	

[*)]Significant: p<0,005

Based on the characteristics of the subjects, both in terms of gestational age, BMI,

maternal age, and parity (Table 1), no significant differences between preeclamptic and

normal pregnancies were found. Therefore, the characteristics of both groups can therefore be

considered homogeneous and the results of subsequent examination worthy of comparison. The results of this study show the frequency distribution of genotypes-509C/T gene *TGFB1* for severe preeclampsia and normal pregnancy (Figure 1).
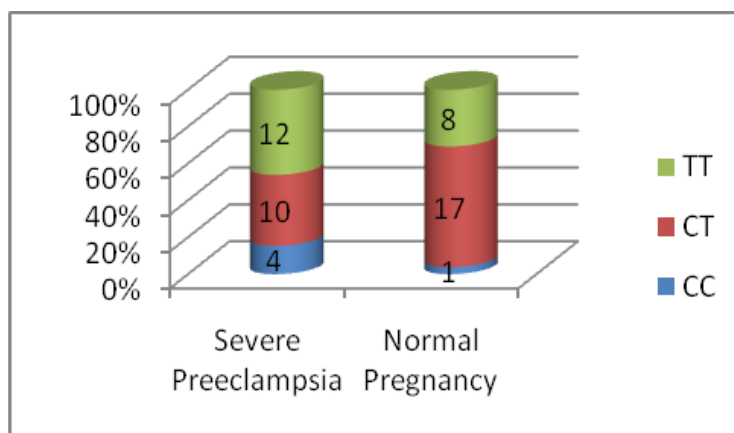

Figure 1. Proportions genotypes -509C/T *TGFB1* gene in patients with preeclampsia and normal pregnancy.

In the group of subjects with severe preeclampsia, the TT genotype occurs with the highest frequency, whereas the CT genotype occurs with highest frequency in the normal pregnancy group. The results showed the frequency distribution of genotype 1167C/T *TGFBR2* gene for severe preeclampsia and normal pregnancy (Figure 2). The CT genotypes had the highest frequency of occurrence in both groups of subjects.
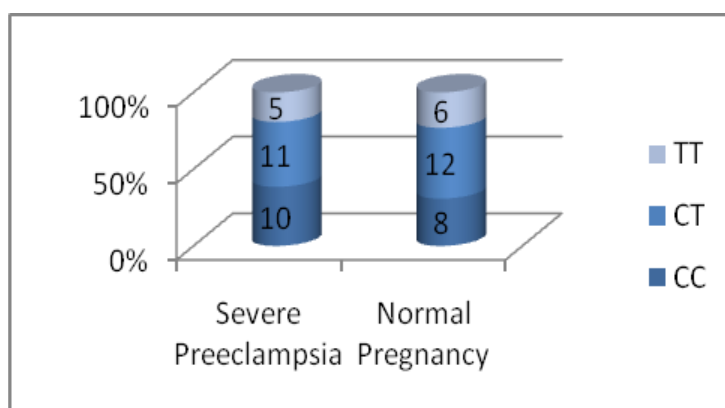

Figure 2. The proportion of genotype 1167C / T *TGFBR2* gene in patients with preeclampsia and normal pregnancy.

These results showed different levels of TGF-β1 in the severe preeclampsia group and the normal pregnancy control group (Table 2). The average TGF-β1 levels were lower in the subjects with preeclampsia compared to the normal pregnancy group. The statistical calculations indicate that the TGF-β1 levels differ significantly between the severe preeclampsia group and the normal pregnancy group at $p<0.005$. The results also indicate differences in sEng levels between the severe preeclampsia group and the normal pregnancy group. The average value of sEng in the severe preeclampsia group was higher than in the normal pregnancy group. Statistical calculations indicate a significant difference in the sEng levels between the severe preeclampsia group and the normal pregnancy group ($p<0.001$).

Table 2. Comparison of levels of *TGFB1* and levels of sEng in severe preeclampsia and normal pregnancy

Variable	Group		p-value [*)]
	Preeclampsia (n = 26)	Controls (n = 26)	
Levels of TGFB1			0,034
Mean (SD)	23.734,7 (11.329,4)	29.060,9 (9.534,3)	
Range	9.094 – 66.132,2	7.335,8 – 45.786	
Levels of sEng			<0,001
Mean (SD)	12,87 (3,24)	9,69 (3,19)	
Range	3,27 – 16,28	4,94 – 15,75	

[*)]Significant: $p<0,005$

The results (Table 3) showed levels of TGF-β1 in the severe preeclampsia and normal pregnancy groups according to the TT, CT, and CC genotypes. In the preeclampsia group, the mean levels of TGF-β1 were highest in the CT genotype, whereas the lowest levels were in the TT genotype. In the normal pregnancy group, the average levels of TGF-β1 were highest in the CT genotype, whereas the lowest levels were in the CC genotype.

Table 3. Comparative levels of TGFB1 according to genotype-509C/ T TGFB1 gene in severe preeclampsia and normal pregnancy

Group	-509 Genotype			p-value [*)]
	TT	**CT**	**CC**	
Preeclampsia				0.306
Average	23.314,51	25.173,38	24.766,17	
Range	10.000 – 66.132,2	10.915,8 – 34.362,1	15.957,6 – 29.950,8	
Normal pregnancy				0.712
Average	27.862,5	29.902,1	24.348,9	
Range	15.917,1 – 44.029.5	7.335,8 – 45.786	24.348,9 – 24.348,9	

[*)]Significant: p<0,005

4. Discussion

The results of this study showed that the frequency distribution of genotypes (-509 C/T) in the *TGFB1* gene differs between normal pregnancy and severe preeclampsia. In the severe preeclampsia group, the frequency of genotype -509 C/T *TGFB1* gene is dominated by the TT genotype. In the normal pregnancy group, however, the CT genotype was dominant. Single nucleotide polymorphism (SNP) at position -509 in this region promoted TGF-β1 and led to the substitution of the T/C. T allele, which is associated with changes in TGF-β1 concentrations in plasma. The difference in the C allele is more prevalent in the TT genotype compared to the CT genotype. Polymorphism in the promoter region alters the regulation and levels of protein expression, and if the merger of introns and exons fails, then changes in mRNA *splicing* occur.

Previous research has revealed the presence of this polymorphism (-509 C/T) in the *TGFB1* gene in several diseases. Research by Prasad *et al*. [7], on polymorphisms of the *TGFB1* gene promoter (-509 C/T) in patients with type-2 diabetes mellitus found the CC genotype (77%), CT genotype (21.2%), and TT genotype (1.8%). In patients with chronic

renal failure (CRF), the literature found the CC genotype (72%), CT genotype (24%), and TT genotype (4%). Sie *et al*. [12], in a study on gene promoter polymorphisms of TGF-β1 (-509 C / T) in patients who have a risk of myocardial infarction found CC genotype (48.16%), CT genotype (43.94%), and TT genotype (7,88%). Additionally, research on the polymorphism of the TGF-β1 promoter gene (-509 C / T) in patients who have a risk of stroke found the genotypes CC (45.59%), CT (45.21%), and TT (9.19%).

In the polymorphism C/T gene promoter at position -509 bp can alter the transcription factors and is associated with changes in TGF-β1 levels in the plasma.Although not yet fully explained, this is thought to be caused by the role of the T allele in the promoter region of TGF-β1. The results of this study show TGF-β1 levels in the preeclamptic subjects with respect to the genotypes TT, CT, and CC. The mean TGF-β1 levels were highest in the CT genotype (25173.38 pg/ml). The conclusion that polymorphism (-509C/T) *TGFB1* gene is associated with low levels of TGF-β1 is in accordance with Yamada *et al*. [13], who researched adult women and postmenopausal women, and Yokota *et al*. [14], who researched infarct myocardium.

The single nucleotide polymorphism (-509) in the gene TGF-β1 has been widely reported in diseases associated with cardiovascular illness and angiogenesis. Table 3 shows that the average TGF-β1 levels in the severe preeclampsia group (23734.7 pg/mL) are significantly lower than in the normal pregnancy group (29060.9 pg/mL) (p=0.034). This result is consistent with Farina *et al*. [15], who also found lower levels of TGF-β1 in preeclampsia subjects.

Yamada *et al*. [13], who researched adult women and postmenopausal women in Japan, showed that the TT or TC genotype groups had low TGF-β1 serum levels. In a study population in Europe, a relationship was found between the polymorphism -509 C/T of the *TGFB1* gene and chronic renal insufficiency [7]. Based on studies that have been conducted

in several countries examining the relationship between the polymorphism (-509) of the *TGFB1* gene and the risk of the occurrence of some diseases, it can be concluded that the polymorphism (-509) of the *TGFB1* gene is involved in the pathogenesis of several diseases. The incidence of disease is reportedly accompanied by a polymorphism (-509) of the *TGFB1* gene in people who are at risk for myocardial infarction, stroke, type-2 diabetes mellitus, chronic renal insufficiency, breast cancer, colorectal adenomas, and asthma [3-4].

The occurrence of abnormal vascular remodeling in the placenta in preeclamptic patients is based on the deregulation of placental angiogenetic factors that trigger ischemic and hypoxic placenta, as well as the release of anti-angiogenic factors such as sEng [16-19]. In the present study, we found a significant increase in sEng (p <0.001) in the severe preeclampsia group (12.87 ng / mL) compared to the normal pregnancy group (9.69 ng / mL). These results are consistent with the research by Daher *et al* [10], and Venkatesha *et al*. [11].

Some studies show *TGFBR2* SNPs in a number of genes associated with particular diseases, such as chronic renal disease, atherosclerosis, hypertension, Marfan syndrome, abdominal aortic aneurysm, breast cancer, carcinoma of the esophagus, and gastric cancer. At the *TGFBR2* gene, more than one SNP was found, including the SNP on the 1167 base (C/T) at codon 389. The *TGFBR2* gene polymorphism at codon 389 AAC/AAT is associated with hypertension and atherosclerosis. Yoshida *et al*. [20], found that a C1167T *TGFBR2* gene polymorphism was significantly associated with chronic kidney disease (CKD). C1167T is a unique form of SNP in that base changes that occur do not alter the amino acid produced (Asn389Asn) and therefore do not change the form of TβR-2. This phenomenon is known as a silent mutation. The silent mutation affects a protein by disrupting the translation process, resulting in decreased levels of these proteins.

A previous study showed differences in the incidence of this polymorphism in the preeclampsia and control groups, which showed that the incidence of this polymorphism in

preeclamptic pregnancies smaller than in normal pregnancies. The appearance of polymorphisms in normal pregnancy patients indicates that not all individuals who have polymorphisms develop preeclampsia. Likewise, some individuals who have an otherwise normal genotype may still develop preeclampsia. These results show that TβR-2 expression is different for individual patients, despite having the same allele. This is a result of many factors that can influence the expression of TβR-2, including the condition of promoters, transcription factors, and the *splicing* process. Variations in TβR-2 gene expression are therefore very likely to occur with or without polymorphism.

Lucke *et al*. [7], conducted research on the C1167T *TGFBR2* gene polymorphism in breast cancer patients. Among some of the SNPs analyzed, the SNP on the 1167 base (C/T) was found to have no connection to breast cancer recurrence. Polymorphism was found in samples of both tumoral and normal DNA. The results suggest that the *TGFBR2* gene polymorphism C1167T at different bases not only occur in preeclamptic patients but also in the normal pregnancy control patients. Lucke *et al*. [7], does not address this phenomenon presumably due to the type of mutation in C1167T.

The type of C1167T mutation that occurs in the *TGFBR2* gene is a silent mutation that does not cause changes in the amino acid composition. Based on the principle of central dogma of molecular biology, this mutation has no effect on the shape and function of the TβR-2 protein, and the protein is considered to be normal despite the polymorphism. The TβR-2 protein has the same effect on individuals who have polymorphisms as it does on individuals who do not have polymorphisms – both groups function normally – which explains the presence of polymorphism in the preeclampsia group as well as the normal pregnancy group, indicating that the *TGFBR2* gene polymorphism is not directly associated with preeclampsia.

Different results were found in several other studies. Research by Yoshida [20] found that C1167T *TGFBR2* gene polymorphism is associated with CKD, which demonstrates the role of this polymorphism on the incidence of CKD, although its molecular mechanism has not yet been fully elucidated.

Study by Kudla *et al*. [21], states that a silent mutation can affect protein expression, including changing the expression levels of a protein. An increase in the stability of the mRNA adjacent to the ribosome binding site explains more than half of the variation of the protein levelsenes that do not efficiently produce mRNA molecules with close folds are inaccessible to the protein-making mechanism. Based on these folds and the mRNA level, translation initiation is predominantly related to maintaining and shaping the expression levels of individual genes in which codon bias can affect the efficiency of the global translational and cellular capabilities. Pearson *et al*. [22], also noted that proteins having at least one silent mutation will have a slightly different form than normal proteins.

TGF-β1 acts as an angiogenic factor that functions in trophoblast invasion and proliferation. Low levels of TGF-β1 cause impaired trophoblast invasion into spiral arteries, which can cause placental ischemia, thereby increasing levels of sEng. In addition, disruption of TGF-β1 signaling can also produce a similar effect, such as decreased levels of TGF-β1. Therefore, interference with the genotype *TGFB1* and *TGFBR2* genes can affect the sEng levels indirectly. The results of this study showed a difference in sEng levels based on genotype *TGFB1* and *TGFBR2* genes in the severe preeclampsia group. The difference, however, was not statistically significant. Similarly, the results showed no significant difference in sEng levels under the genotypes *TGFB1* and *TGFBR2* in the normal pregnancy group.

5. Conclusions

In summary, these results indicate a lack of influence and a lack of a meaningful relationship between the polymorphism-509C/T *TGFB1* and 1167C/T *TGFBR2* on sEng levels in subjects with severe preeclampsia.

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