The Potential effect of G915C polymorphism in regulating TGF-β1 transport into Endoplasmic Reticulum for cytokine production

Hani Susianti[1]*, Atma Gunawan[2], Jayarani Fatimah Putri[3], Basuki B Purnomo[4] Kusworini Handono[1] & Handono Kalim[2]

[1]Departement of Clinical Pathology, Faculty of Medicine, Brawijaya University, Malang, Indonesia; [2]Departement of Internal Medicine, Faculty of Medicine, Brawijaya University, Malang, Indonesia; [3]Departement of Biology, Faculty of Sciences, Brawijaya University, Malang, Indonesia; [4]Department of Urology, Faculty of Medicine, Brawijaya University, Malang, Indonesia; Hani Susianti – Email: hanisusianti.fk@ub.ac.id; *Corresponding author

Abstract:
The TGF-β1 cytokine concentration is known to be higher in nephritis with implied Lupus Nephritis severity. The production of TGF-β1 cytokine is associated with G915C polymorphism. Therefore, it is of interest to study G915C polymorphism. The G915C polymorphism changes codon 25 which encodes arginine into proline in the signal peptide of TGF-β1. The amino acid substitution affects signal peptide properties that may inhibit the transport of TGF-β1 into the endoplasmic reticulum and eventually decline the cytokine production. Hence, the effect of G915C polymorphism on the properties of the signal peptide, the ability of TGF-β1 transport into the endoplasmic reticulum and the concentrations of urinary TGF-β1 in Lupus Nephritis patients was studied. The arginine substitution into proline decreased the polarity of the signal peptide for TGF-β1. The increased hydrophobicity with increased binding energy of the signal peptide for TGF-β1 to Signal Recognition Particle (SRP) and translocon is shown. This implies decreased protein complex stability in potentially blocking the transport of TGF-β1 into the endoplasmic reticulum. This transport retention possibly hampers the synthesis and maturation of TGF-β1 leading to decreased cytokine production.

Background:
The etiology of lupus disease is not yet clearly entirely understood. A number of study support that genetic factor plays a role in disease manifestation **[1, 2]**. Hence, studies focus on the affiliation of genetic polymorphism to lupus susceptibility and severity **[1]**. Several genetic predispositions to lupus have been investigated, one of which is a cytokine polymorphism **[3]**. The TGF-β1 cytokine concentration is likely higher in nephritis, which nephritis is one of the mortality causes in Lupus patients **[4]**. Furthermore, the increasing of TGF-β1 production may be projected to the severity of Lupus Nephritis **[4, 5]**. Another study showed that G915C polymorphism of TGFB1 gene is related to the progression of liver fibrosis in patients with chronic Hepatitis C **[6]**. Therefore, the G915C polymorphism is possibly related to renal fibrosis and it is a dominant feature in renal failure.

The G915C polymorphism changes codon 25 which encodes arginine into proline of the signal peptide of TGF-β1. The change from arginine into proline alters the signal peptide properties that may inhibit transport of TGF-β1 into the endoplasmic reticulum **[7, 8]** and eventually decline the cytokine production. Consequently, Individuals with homozygote genotype (arginine/arginine) have more concentrations of serum TGF-β1 than those with heterozygote genotype (arginine/proline) **[7, 9]**. We further analyze the effect of G915C polymorphism on the properties of the signal peptide, the ability of TGF-β1 transport into the endoplasmic reticulum and the concentrations of urinary TGF-β1 in Lupus Nephritis patients at Saiful Anwar Hospital, Malang.

Methodology:
Sample
The blood samples for analysis of G915C polymorphism and serum creatinine were taken from 45 patients with Lupus

Nephritis and 45 subjects as controls. The urine samples for measuring TGF-β1 and protein were collected from those subjects. The Lupus Nephritis was determined by ACR (American College of Rheumatology) 1997 criteria and renal biopsy was conducted on all patients as the gold standard of Lupus Nephritis diagnosis.

TGFB1 measurement
Midstream morning urine specimens were collected in a sterile container, then immediately centrifuged and stored at -80ºC refrigerator until analysis. TGF-β1 was measured by sandwich enzyme-linked immunosorbent assay (ELISA) using human kit TGF-β1 (Novatein Bioscience®, USA; detection limit <1 pg/ml). TGF-β1 assays was performed strictly according to the manufacturer's instruction.

DNA Isolation and Sequencing
DNA was isolated from 200 µl vein blood sample by using Dneasy Blood and Tissue Kit (Qiagen). The TGFB1 gene was amplified by PCR machine with primer: 5'CTAGGTTATTTCCGTGGG (Forward) and 5'CCTTGGCGTAGTAGTCG (Reverse), 100 ng genomic DNA template, 0.5 unit Taq DNA polymerase and 2.5 µM dNTPs. The PCR conditions was denaturation at 95 ˚C for 5 minutes, followed by denaturation at 95 ˚C for 30 seconds, annealing at 50˚ C for 30 seconds, and elongation at 72˚ C for 30 seconds. It was repeated 30 cycles, and the final extension was conducted for 10 minutes at 72 ˚C. The PCR result was sequenced (Bioneer, Korea). The G915C polymorphism was analyzed following the sequencing of DNA from the subject by using BLAST-NCBI tool. The DNA sequences then were translated into amino acid sequence by Bio Edit Software for further analysis (molecule properties and docking).

Protein properties, molecular docking and molecular dynamic analysis
The structure of the signal peptide of TGF-β1 was built by using Phyre2 web server. Whereas Signal Recognition Particle (SRP) 54 M domain (1QB2) and translocon protein structure (Sec61_2wwb) were retrieved from PDB (http://www. rscb.org) followed by validating and checking using PROCHECK. The modelled-structure of the signal peptide of TGF-β1 was analyzed for hydrophobicity and polarity by Prot Param tool and ProtScale tool (www.expasy.org). The interaction among the signal peptide of TGF-β1 with SRP and translocon was examined by using **Escher NG molecular docking** from VEGAZZ. The binding stability among them was simulated by molecular dynamics simulation (YASARA, Amber 03). All visuals of protein structure were developed using the PyMol molecular graphic system.

Results:
The G915C polymorphism analysis found that none of the subject had CC genotype, and only one subject had GC genotype each in both Lupus Nephritis patients and control groups. So the majority of the population studied had the GG genotype. The subject with GC genotype tend to had lower levels of TGF-β1 than the GG genotype in both Lupus Nephritis patients and control groups. The Lupus Nephritis patients had higher levels of TGF- β1 and urinary protein, as well as serum creatinine compared with the control group. Increasing of   serum creatinine and urinary protein

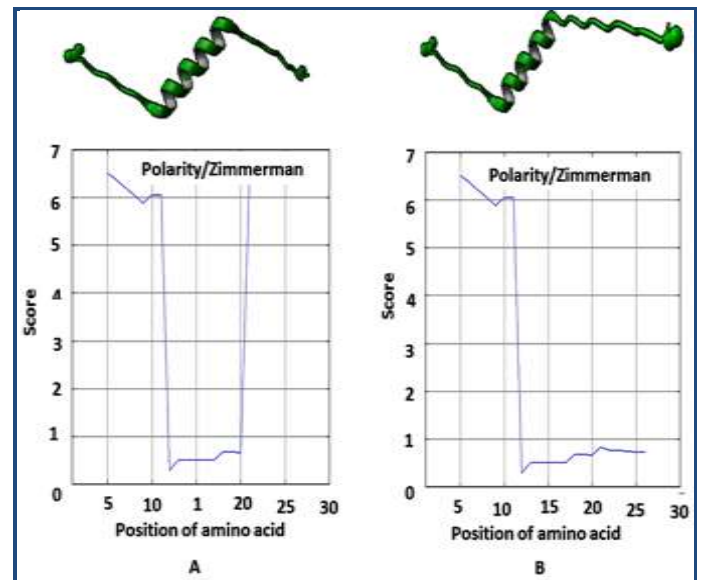concentration on patients showed the severity of Lupus Nephritis **Table 1 (see supplementary material).**

Figure 1: Substitution of arginine (R-25) **A)** into proline (P-25); **B)** did not affect the secondary structure (Green). However, this decreased the C-terminal polarity of the signal peptide for TGF-β1 (Graph below).

Guanine to cytosine substitution on G915C changes codon number 25 of TGF-β1 signal peptide that changes arginine (R-25) to proline (P-25). Therefore, we implied that the peptide substitution will change 3D structure of the signal peptide of TGF-β1, which plays a role in the transport into the endoplasmic reticulum. Then, we further analyzed the structure of both types of signal peptides. The data showed that the secondary structure of the signal peptide of TGF-β1 arginine (R-25) variant and proline (P-25) variant at residue number 25 has similar secondary structure **(Figure 1)**. Hence, we implied that arginine substitution into proline manipulated peptide's properties. We further completed the polarity analysis, and the data indicated that the proline declined polarity of C-terminal of the signal peptide, so that the hydrophobic core of the signal peptide of TGF- β1 is increased **(Figure 1).** The polarity change is likely to cause differences in the binding pattern among the signal peptide of TGF-β1, Signal Recognition Particle (SRP) and translocon.

Further, we analyzed the interactions among the signal peptide of TGF-β1, SRP and translocon. The docking analysis result suggested that on the signal peptide of TGF-β1 R-25 variant and P-25 variant have different binding pattern when bond to SRP and translocon **(Figure 2)**. The binding stability between a complex of the signal peptide of TGF-β1 with SRP/translocon was analyzed by molecular dynamics simulation (YASARA). The results show that binding stability for both signal peptide variants was different. Protein complex stability of SRP/translocon with the TGF-β1 signal peptide variant R-25 was better than P-25, where RSMD scores for each was 4.03 Å and 6.01 Å respectively **(Figure 2).** The Arginine substitution into proline decreased the polarity of the signal peptide of TGF-β1 that change the binding pattern, and decreased the binding stability to SRP/translocon. This phenomenon assumed that the arginine substitution to proline might cause

retention for transportability of TGF-β1 to the endoplasmic reticulum. The transport retention might hinder TGF-β1 maturation that may reduce production of the cytokine.
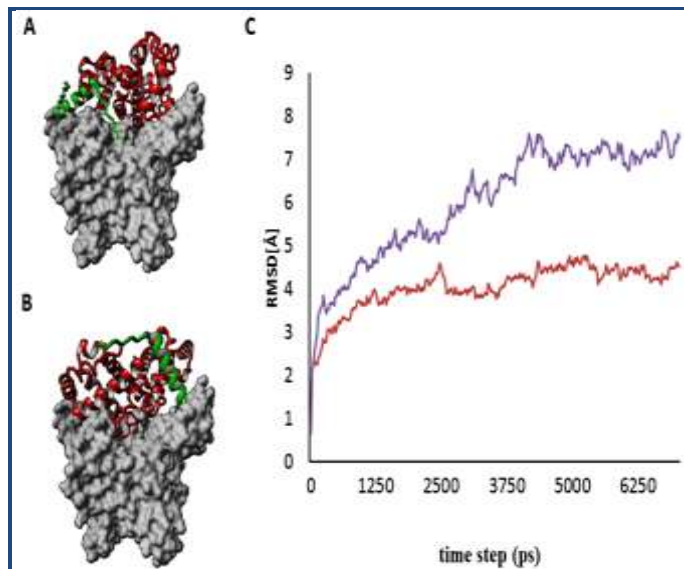

Figure 2: Substitution of arginine (R-25) into proline (P-25) changed binding pattern of the signal peptide of TGF-β1 with SRP (red) and translocon (grey), R-25 was bound closer to translocon: **A)** when compared with P-25; **B)** The substitution from arginine (red line) into proline (violet line) declined the binding stability of the signal peptide of TGF-β1 with SRP and translocon **C).**

Discussion:
The result indicated that GG genotype on SNP G915C of TGFB1 gene was more dominant (97.2%) in Malang population. This is different from the study by Wang et al. (2007) who stated that codon-25 was not polymorphic in East Asia population, and there was a polymorphism in Brazil and Iran ethnics **[3,10]**. The data showed that polymorphism dependent on region and ethnic type. Moreover, the urinary TGF-β1 concentration in genotype GG was higher than genotype GC for both in Lupus Nephritis and control groups. It suggested that Guanine substitution into Cytosine on G915C affect TGF-β1 production.

The G915C polymorphism is located in the signal peptide (15-30 residues in length) of TGF-β1 implying that it altered the properties of the signal peptide, necessary for protein transport to endoplasmic reticulum. It usually consists of three parts namely N-terminal (region n) followed by a region with hydrophobic amino acid (region h) consisting of 7-15 residues of hydrophobic amino acid and more polar C-terminal region **[11, 12]**. The signal peptide is involved in targeting of nascent proteins to their sites of translocation at the endoplasmic reticulum membrane, initiating a stable interaction between the ribosome and translocon, and providing a ligand for the opening of the translocation channel **[13]**. Guanine to cytosine substitution on G915C will cause change to codon number 25

of the TGF-β1 signal peptide that encodes arginine (R-25) to proline (P-25). The amino acid substitution decreased polarity of C-terminal of the TGF-β1 signal peptide , which was very important for the cleavage process of signal peptide by peptidase enzyme. This cleavage is substantial to continue the synthesis process of TGF-β1 protein. On the other hand, the presence of proline in the TGF-β1 signal peptide in bacteria is predicted to be the reason exporting protein efficiency to outside the cell was declined **[11,12,14].** Moreover, the presence of proline in the TGF-β1 signal peptide had changed its binding pattern to SRP and translocon. Furthermore, the Arginine substitution to proline decreased its binding stability with SRP and translocon. It could possibly start an interaction disorder with SRP on translocon, that triggering a retention for transporting TGF-β1 to the endoplasmic reticulum **[15].** Thus, data suggests G915C polymorphism is associated with the synthesis and maturation of TGF-β1 leading to decreased cytokine production.

Conclusion:
The G915C polymorphism is shown to affect the signal peptide of TGF-β1 involved in potential maturation and production of TGF-β1.

References:
[1] Liu C & Ahearn JM, *Best Pract Res Clin Rheumatol*. 2009 **23** : 507 [PMID:19591781]
[2] Mok CC, *J Biomed Biotechnol*. 2010 **2010:** 638413 [PMID: 20414362]
[3] Wang B *et al*. *Kobe J Med Sci*. 2007 **53**: 15 [PMID: 17579298]
[4] Guarnizo-Zuccardi P *et al*. *Tissue Antigens.* 2007 **70**: 376 [PMID: 17711410]
[5] Babel N *et al*. *Kidney Int*. 2004 **66**: 428 [PMID: 15200452]
[6] Wang H *et al*. *World J Gastroenterol.* 2005 **11**: 1929 [PMID: 15800982]
[7] Awad MR *et al*. *Transplantation*. 1998 **66**: 1014 [PMID: 9808485]
[8] Yu SK *et al*. *J Korean Med Sci*. 2010 **25**: 564 [PMID: 20357999]
[9] August P & Suthanthiran M, *Kidney int Suppl.* 2003 **64**: S99 [PMID: 14531781]
[10] Romani S *et al*. *Hepat Mon*. 2011 **11**: 901 [PMID: 22363353]
[11] Cheng Z, *Biosci Rep.* 2010 **30**: 201 [PMID: 20156192]
[12] Matoba S & Ogrydziak DM, *J Biol Chem*. 1998 **273**: 18841 [PMID: 9668059]
[13] Rutkowski DT *et al*. *Proc Natl Acad Sci U S A*. 2001 **98**: 7823 [PMID: 11416167]
[14] Hessa T *et al*. *Nature*. 2005 **433**: 377 [PMID: 15674282]
[15] Morris DR *et al*. *Plos one*. 2012 **7**: e37878 [PMID: 22662243]

BIOINFORMATION

Supplementary material:

Table 1: Serum creatinine, urinary protein and TGF-β1concentration, and genotype distribution of Lupus Nephritis patient and Control groups.

Parameters	Lupus Nephritis Patient Group		Control Group	
	Genotype GG	Genotype GC	Genotype GG	Genotype GC
Amount of subjects (%)	97.7	2.3	97.7	2.3
Age, years (mean±SD)	28 ±8	16	30 ± 5	34
Gender				
Female (n, %)	41(91.1)	1(2.3)	41(91.1)	1(2.3)
Male (n, %)	3(6.6)	0	3(6.6)	0
Urinary TGF-β1 (mean±SD) *	54.33±41.21[a]	27.19	23.67±9.86	12.91
Urinary Protein (mean±SD) **	762.69±708.12[a]	1007.20	57.30 ±57.36	62.60
Serum Creatinine (mean±SD) **	1.14 ± 0.92[a]	2.0	0.59± 0.12	0.62

Notes: * pg/ml; ** mg/dl; [a] $p < 0,05$ for the difference between GC genotype of lupus nephritis patients and control group