

# Allelic Frequency of C509T Polymorphism of the Transforming Growth Factor Beta 1 Gene on the Alveolar Bone Disorders in Beta Thalassemia Major in Indonesia

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**Abstract: Objectives:** Beta thalassemia major is a disorder that is homozygous beta thalassemia and often called Cooley anemia. The hypothesis of this research is the allele T is the allele most frequently found in patients with beta thalassemia major. This study used two groups of patients groups and control groups. **Patients:** The sample used was 66 patients with beta thalassemia major and 15 control groups from Suku Sunda Indonesia. Research methods and results: examination of polymorphisms C509T Transforming Growth Factor Beta 1 (*TGFβ1*) gene using PCR-RFLP method to determine the genotype and allele. **Measurement and Result:** Allele frequency was calculated by using odds ratio and tested with Fisher exact test (OR = 95% CI (0.71 to 5.92)). Allele T in patients with beta thalassemia major there are 88 (66.7%) and in control groups 24 (80%). Allele C in patients with beta thalassemia major there are 44 (33.3%) and in control groups 6 (20%). There are more T alleles in patients and control groups so that there is no significant difference between beta thalassemia major and control groups. **Conclusion:** T allele is the allele most frequently found in patients with beta thalassemia major and the control groups and the highest risk factor for the occurrence of the jaw bone disorder beta thalassemia major.

**Key words:** Polymorphism, TGFβ1 gene, thalassemia, Indonesia.

## 1. Introduction

Thalassemia is a hereditary hemolytic anemia with various degrees of severity that is characterized by absent or decreased globin chain synthesis due to abnormal circumstances quantitative globin chains. A total of five cases of the disease was first discovered by Thomas Cooley and Lee in 1925 [1].

Thalassemia has been identified as having a high frequency in the subtropical region, its distribution extends from southern Europe to Southeast Asia. The main distribution area includes the border the Mediterranean Sea, most of Africa, the Middle East, Indian Sub-continent, and Southeast Asia, including Indonesia, with the incidence of 5-20%. Some areas of

Southeast Asia as much as 40% of the population has one or more thalassemia gene. In North America there are probably 750 to 1,000 patients with homozygous beta thalassemia and only 15 to 20 new cases diagnosed each year [1-2].

Currently in Indonesia is estimated there are approximately 5,000 patients with beta thalassemia major. A total of six to ten of every 100 people of Indonesia to bring this disease genes. According to the Minister of Health, an estimated one of 1,600 newborns in Indonesia suffered from thalassemia major and thalassemia minor with 200,000 people. Thalassemia center in RSCM Jakarta Indonesia every month to serve 1200 patients, whereas in the Thalassemia Clinic Dr. Hasan Sadikin found approximately 380 patients who need care [3].

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One of the characteristic facial Cooley thalassemia facies or facies of the rodent. Patient's face will look maxillary anterior teeth that looked protrusi/prominent. Clinically facies of Cooley cause problems for patients. Aesthetic disturbances and malocclusion are the main problems that arise. Protrusi maxillary teeth will result in clearly visible when the patient's mouth clenched. Relationships Angle Class II malocclusion is a clinical picture which is clearly visible. Skull bones will stretch (tower skull) with protrusion of the frontal and posterior, maxillary hypertrophy, upper lip retraction, prominent molar eminence, and anterior open bite. Bridge of the nose will shorten and the eyes will look into Mongoloid slant [1-4].

In accordance with the important role of *TGFβ1* gene in bone remodeling, the bone abnormalities in the pathogenesis of this gene has a role as an important stimulator of osteoblast formation causing kemotaksis movement, proliferation and differentiation in the osteoblast. However, in vitro is still being conducted research mainly on the effects of *TGFβ1* exogenous gene in the culture system of osteoblasts. In vivo secretion of *TGFβ1* causes the growth of the matrix and the stimulation of osteoblast mineralization, giving rise to the effect of inhibiting osteoclast differentiation and resorption of mature osteoclasts [5].

Research involving *TGFβ1* gene polymorphisms have been conducted in patients with atherosclerosis, cancer, asthma, and several other diseases, but no study conducted on patients with beta thalassemia major. The results of these studies still showed that there were differences in the role of each genotype on each ethnic group and disease. Two single-base polymorphism in the *C509T* is known through the examination in patients with atherosclerosis, bone disorders, and several kinds of cancer. Polymorphism *C509T* polymorphism position is one of the most frequently found, allegedly this position closest to the TATA Box that strongly influence the transcription process is going to happen [5-6].

## **2. Objective**

*C509T* polymorphism of *TGFβ1* gene may consist of allele C and T that have each have a role in the occurrence of jaw bone abnormalities in patients with beta thalassemia major. The hypothesis developed is allele T has a larger role against the occurrence of jaw bone abnormalities in patients with beta thalassemia major.

## **3. Patients, Materials and Methods**

The object of research is beta thalassemia major who were treated at Dr. Hasan Sadikin. When selecting subjects research conducted in January 2008 to July 2008. Materials research is a number of 2 ml of blood taken from patients with beta thalassemia major, and then used for examination of *TGFβ1* gene in Laboratory Medicine Research Unit, Universitas Padjadjaran Dr. Hasan Sadikin. Sampling was based on consecutive sampling and found 66 people beta thalassemia major.

In the preparation phase is carried out as follows:

(1) Submission of ethics proposals to the Commission for Health Research Ethics FK-Unpad/RSUP Dr. Hasan Sadikin.

(2) Information provided to parents about the research done, then parents signed the informed consent form.

(3) Screening of beta thalassemia major patients treated at the Thalassemia Clinic Dr. Hasan Sadikin in accordance with the status of existing patients and is a diagnosis that is determined by the consultant pediatrician hematology-oncology children.

Blood samples were taken from the vein cubiti 2 ml using 10 ml syringe. If venous cubiti not palpable, the blood was taken on the veins in the patient's head. Blood is inserted into a tube containing EDTA vacuette 2 ml for DNA isolation.

### *3.1 DNA Analysis*

Analysis of DNA consists of DNA isolation and PCR-RFLP. DNA was isolated from blood by using

the DNA Isolation Kit from Pharmacia, and then 200 ng of DNA used as template for PCR.

### 3.2 Polymerase Chain Reaction (PCR) and Restriction Fragment Length Polymorphism (RFLP)

RFLP method is a method polymorphism analysis using a specific enzyme. Primers used for *C509T* are:

Forward 5'CAGACTCTAGAGACTGCTAG3'  
Reverse 3'GCTACCAGAGAAAGAGGAC5'.

The tube containing the PCR mixture incorporated into the PCR machine (Corbette) with PCR conditions as follows: initial denaturation at 95°C for 4 minutes, followed to a temperature cycle consisting of:

- (1) Denaturation at 95°C for 1 minute.
- (2) Annealing at a temperature of 57°C for 1 minute.
- (3) extension at 72°C for 1 minute.

Stages are carried out and followed by 35 cycles final extension at 72°C for 10 minutes.

Examination RFLP for the *C509T* polymorphism of *TGFβ1* gene using the enzymes that have a regional Eco8II CCTANGG introduction. T allele would produce truncated fragments of 230 bp and 188 bp, while allele C is not interrupted by using the enzyme (418 bp) (Fig. 1).

Restriction enzyme mixture to the *C509T* polymorphism of *TGFβ1* gene (volume of 10 mL) was

1 mL 10X Buffer tango, 0.3 mL Eco8II enzyme concentration of 10 μ/mL, and 8.7 mL of PCR results. The whole mixture was centrifuged for 20 seconds with a speed of 13,000 rpm, and then incubated at 37°C for 24 hours. The result of electrophoresis performed incubation for 60 min with 100 V voltage fixed. The next stage is to electrophoresis on agarose gene.

## 4. Results

Results of PCR using primers TLR and TLF to obtain a base target gene polymorphism *C509T TGFβ1* are shown in Fig. 1. The result of PCR electrophoresis products is 418 bp DNA band.

PCR products were then digested with restriction enzymes Eco8II and the picture shows electrophoresis DNA band of 230 bp and 188 bp, the circumstances indicate the presence of 509T allele, whereas if not cut off the only visible PCR product of 418 bp. It indicates the 509C allele (normal) (Fig. 2).

To prove that the allele frequencies follow Hardy-Weinberg equilibrium theory used Chi Square to the formula  $\chi^2 = \sum (O_i - E_i)^2 / E_i$  with value  $O_i$  = observed value of genotype I and  $E_i$  = expected value (expectation) genotype i.

Table 1 shows the group of patients with beta thalassemia major appear three types of genotypes, namely CC, CT, and TT. The number of CT genotype

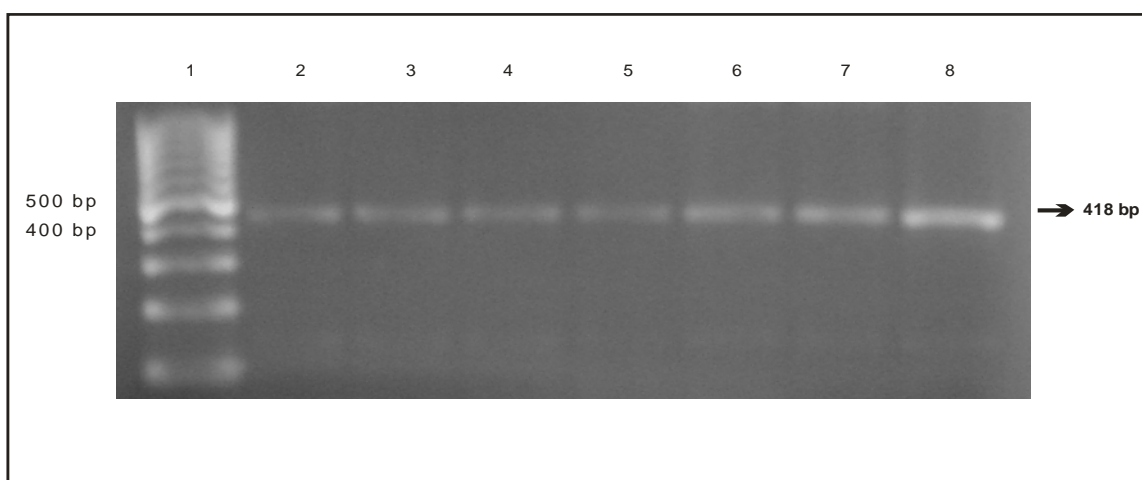
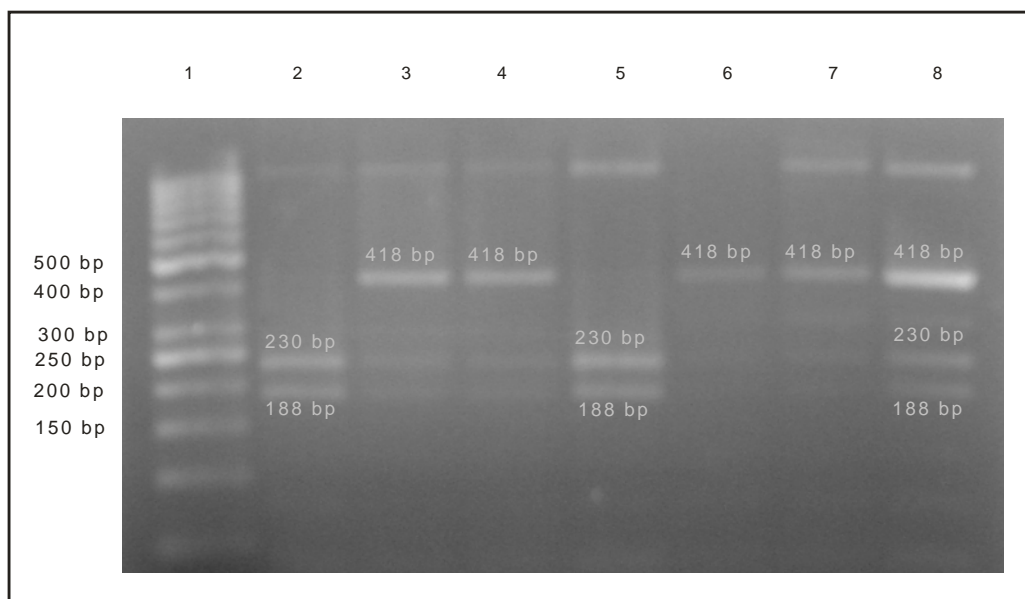


Fig. 1 Electrophoresis of Polymorphism PCR Products *C509T TGFβ1* gene (Lane 1: Alert DNAΦX174 Hae III; Lane 2 to lane 8 of 418 pb).



**Fig. 2** Results Digestion with Enzymes Eco8II (Lane I. Ladder (ladder) DNA; Lanes 3, 4, 6, 7. Uncut (CC); Lane 2 and 5 truncated (TT); Lane 8 genotype CT).

in this study is the sum of allele T and allele C so that the total amount of two times each allele. Genotype C509T polymorphism *TGFBI* gene is homozygous TT genotype, and there is no allele C. Likewise, contrary to the CC genotype did not have allele T. The result of homozygous genotype frequencies will be multiplied by two. This occurs both in patient groups and control groups. The result of calculation shows the proportion of genotype CC = 1 (1.53%), CT = 42 (63.63%), and TT = 23 (34.84%). C allele frequency in a population of 0.333, while the T allele frequency of 0.667. This shows the T allele appears more often so it is considered dominant as compared with C allele is recessive.

Next in Table 2 is a computation of equilibrium test for patient group. From these calculations obtained by  $\chi^2 = 12.32$  which is greater than  $\chi^2$  table ( $\alpha = 5\%$ ). Thus, concluded the patient does not follow the Hardy-Weinberg law. That is, the frequency of allele

C509T polymorphism *TGFBI* gene in beta thalassemia major group not in accordance with Hardy-Weinberg theory.

Table 3, shown in the control group only appear 2, i.e., CT and TT genotypes, with the proportion of CT = 6 (40%) and TT = 9 (60%). C allele frequency in populations of 0.2, while the T allele frequency of 0.8. This shows the T allele appears more often so it is considered dominant as compared with C allele is recessive.

Table 4 is a fit test of Hardy-Weinberg model for the control group. The test results obtained by  $\chi^2 = 0.938$  which is smaller than  $\chi^2$  table ( $\alpha = 5\%$ ). Thus, allele frequencies of C509T polymorphism of *TGFBI* gene control group in accordance with Hardy-Weinberg theory. From the equilibrium calculations above, the patient thalassemia already been affected, whereas in the normal group did not experience interference.

**Table 1** C and T allele frequency in the C509T polymorphism of *TGFBI* Gene in Patients Group.

	Genotype			Total
	CC	CT	TT	
Number of Individuals	1 (1.53)	42 (63.63)	23 (34.84)	66
The number of T alleles	0	42	46	88
The number of alleles C	2	42	0	44
Total Number of alleles	2	84	46	132

Description: The frequency of allele C in the population =  $44/132 = 0.333$ ; T allele frequency in the population =  $88/132 = 0.667$ .

**Table 2 Model Fit Testing Hardy-Weinberg Gene Polymorphism C509T TGFBI Group of Patients.**

	Genotype			Total
	TT	CT	CC	
Observation	23	42	1	66
Expektasi ( E <sub>i</sub> )	nq <sup>2</sup> 66(0.667) <sup>2</sup> 29.363	n(2×p×q) 66(2×0.333×0.667) 29.319	np <sup>2</sup> 66(0.333) <sup>2</sup> 7.319	66
(O <sub>i</sub> - E <sub>i</sub> ) <sup>2</sup> / E <sub>i</sub>	1.379	5.485	5.456	12.32

Description:  $\chi^2 = \sum (O_i - E_i)^2 / E_i = 12.32$ ;  $\chi^2$  tables for  $\alpha = 5\%$  is 3.84 (df = 1).

**Table 3 The frequency of alleles T and C at the C509T polymorphism of TGFBI gene in the control group.**

	Genotype			Total
	CC	CT	TT	
Number of Individuals	0	6 (40)	9 (60)	15
The number of T alleles	0	6	18	24
The number of alleles C	0	6	0	6
Total Number of alleles	0	12	18	30

Description: The frequency of allele C in the population = 6/30 = 0.2; T allele frequency in the population = 24/30 = 0.8.

**Table 4 Model Fit Testing Hardy-Weinberg Gene Polymorphism C509T TGFBI Control Group.**

	Genotype			Total
	CC	CT	TT	
Observation	0	6	9	15
Expektasi ( E <sub>i</sub> )	np <sup>2</sup> 15(0.20) <sup>2</sup> 0.6	n(2×p×q) 15(2×0.2×0.8) 4.8	nq <sup>2</sup> 15(0.80) <sup>2</sup> 9.6	15
(O <sub>i</sub> - E <sub>i</sub> ) <sup>2</sup> / E <sub>i</sub>	0.60	0.30	0.038	0.938

Description:  $\chi^2 = \sum (O_i - E_i)^2 / E_i = 0.938$ ;  $\chi^2$  tables for  $\alpha = 5\%$  is 3.84 (df = 1).

#### 4.1 Analysis of Allele Frequency on Gene Polymorphism of TGFBI

Allele frequency of examination results are known then the genotype frequency of inspection sought. The result of statistical calculation on the C-509T polymorphism genotype of TGFBI gene showed the TT genotype frequency of 35%, CC 1% and CT 64% in patients with beta thalassemia major and TT by 40% and CT by 60% in controls. On examination there were no CC genotype in controls and in patients with beta thalassemia major there is only 1 case (1%) so there is no significant difference (Table 5).

Odds ratio testing against genotype CT and TT carried out to see greater opportunities between the two genotypes on the occurrence of the jaw bone disorder

beta thalassemia major. Therefore, the CC genotype obtained only one person and in the control group did not exist then the CC genotype was not included in the calculation.

Table 6 shows the number of individuals who have the allele C and T at the C509T polymorphism of TGFBI gene. Testing Odds ratio was also performed on both alleles. The purpose of testing is to find bigger role alleles between allele C and T in the jaw bone disorder beta thalassemia major group and controls. Allele T in patients with beta thalassemia major there for 88 (66.7%) and in control amounted to 24 (80%). Allele C in patients with beta thalassemia major there for 44 (33.3%) and in control of 6 (20%). T allele is more widely available in groups of patients and controls so there is no significant difference between beta thalassemia major and controls.

**Table 5 Genotype frequencies of C509T polymorphism of *TGFBI* Genes in Patients with Beta Thalassemia Major Compared to Control.**

Genotype	Patient n = 66 (%)	Control n = 15 (%)	$\chi^2$	Value p	Odds ratio	95% CI
CT	42 (64)	9 (60)				
TT	23 (35)	6 (40)	1.682	0.046	2.63	(0.73-9.64)
CC	1 (1)	-				

Note: \*) = based on exact-Fisher test.

**Table 6 Risk Factor Analysis of allele C and T C509T polymorphism *TGFBI* Gene in Patients with Beta Thalassemia Major and Control.**

Alele	Patient (%)	Control (%)	$\chi^2$	Value p	Odds ratio	95% CI
T	88 (66.7)	24 (80)	2.04	0.13	2	(0.71-5.92)
C	44 (33.3)	6 (20)				

Note: \*) = based on exact-Fisher test

## 5. Discussion

*TGFβ1* gene located on 19q13.1 locus has a major role in the regulation of biological processes such as cell proliferation, cell survival, cell differentiation, cell migration, and extracellular matrix production. Growth and development of intramembranous bones so desperately need extracellular matrix *TGFβ1* gene have a very important role in this process [5-7]. In accordance with the important role of *TGFβ1* gene in bone remodeling, the bone abnormalities in the pathogenesis of this gene has a role as an important stimulator of osteoblast formation causing kemotaksis movement, proliferation and differentiation in the osteoblast. However, in studies in vitro are still being debated about the effects of *TGFβ1* exogenous gene in osteoblast culture system. In the in vivo secretion of *TGFβ1* causes the growth of the matrix and the stimulation of osteoblast mineralization, giving rise to the effect of inhibiting differentiation of osteoclasts and osteoclast resorption that have been mature [5-8].

Tables 1 and 3 shows the distribution of genotype frequencies of C509T polymorphism of *TGFβ1* gene in patients and control groups. It appears that in both groups the T allele is the greatest number. This shows that the T allele is more dominant when compared with C allele. T allele is a mutant allele, meaning that at the time of the amputation by using restriction

enzyme was not recognized so it does not cut allele, while allele C could be cut to produce fragments of 230 bp and 188 bp.

Tzakas (2005) and Lau (2004) conducted a study on bone mineral density (BMD) were performed on women Caucasian tribes, Denmark, and Japan. In the Danish population polymorphism at position C509T resulted in disruption and factor binding of RNA polymerase other trinskripsi. Results Luedecking *et al.* (2000) in Ferrara (2002) found in patients with Alzheimer's that transcriptional activity at the -509 T allele variant is greater than the C allele. This activity led to greater concentration on TGF B1 allele T. In addition, it is evident also that the concentration of TGF B1 also differ in the amount two times more in the homozygous TT genotype compared with CC homozygotes. different situation was found in Japanese rates, genotype CC showed greater concentration when compared with TT genotype [9-11].

Seen in Table 5 that the CT alleles have odds ratios of 2.63 times compared with the allele TT/CC. This situation is evidence that mutant alleles have seen more risk on the C509T polymorphism of *TGFβ1* gene. C509T polymorphism of *TGFβ1* gene is a mutation found in the promoter region. This area is a region of DNA that facilitates gene transcription. In particular promoter is located in the *upstream* region

of the gene to be regulated on the same strand. Promoter consists of specific DNA sequences and response elements, which is where RNA polymerase and transcription factor proteins attach to and begin the process of transcription. Therefore, this region is an important element and will work actively with other regulators in the region of the gene transcription process [12].

Table 6 shows the proportion of alleles in patients and control groups. In the patient group, the proportion of T alleles larger than allele C. In the control group, the proportion of allele T allele was also higher than C. It shows that the T allele is dominant to the allele C. The proportion of allele T in patients greater than the proportion of allele C in the control group, while the proportion of allele C in the group of patients is less than the proportion of allele C in the control group. The statement above is in accordance with the results of research conducted by several researchers. The results Berndt (2007) of patients with *colorectal adenomas* showed the T allele had odds ratios of 1.10 for the severity of the disease. This is supported by the results of research by Silverman (2004), the T allele had odds ratio of 1.02. From both these studies showed that the T allele have a greater role on the severity of a disease. This shows the tendency of the T allele associated with the severity of the jaw bone. This situation is supported by the results of statistical analysis showed a significant difference in allele proportions between groups of patients with beta thalassemia major and controls (Table 2) [13-14].

The appearance of normal polymorphisms in patients indicate that not all individuals who experience abnormal polymorphisms jawbone. And vice versa, not all individuals bergenotip normal (nonpolimorfisme) will not experience the jaw bone disorders. The above shows that in each individual place of gene expression of different *TGF B1* despite having the same allele. This fact could be due to many factors that can affect gene expression conditions such

as *TGF B1* promoter, transcription factors, the process of *splicing*, etc., so that variations in gene expression of *TGF B1* each individual is likely to occur even without or with polymorphism [12].

*TGF B1* gene polymorphism does not work alone in causing abnormalities of the jaw bone. Genes and environmental factors as a multifactorial disorder that is the character of the jaw bone causes it to happen. The process of *remodeling* and angiogenesis as the pathogenesis of abnormalities of the jaw bone is a process that is influenced by various factors. Another factor that has influence in the pathogenesis of jaw bone defects are osteoprogenitor cells such as FGF, PDGF, IGF [11-14].

The results raise the possibility of *TGFβ1* gene mutations also affect the level of protein expression of *TGFβ1* so that the expression of *TGFβ1* protein on the cell surface becomes high. Overexpression resulted in increased osteoblastogenesis process [12-14].

Previous authors have much to explain about the overexpression of genes can be caused by mutations in polypeptide composition. Each gene is made up of DNA that carries the genetic code of the polypeptide spesik which will combine into a polypeptide chain and form a specific protein builder living organisms. DNA sequence located at a specific location on the chromosome that is composed with sequences that have a distinctive role. Each of these different roles can occur in the region of the promoter, transcription initials, followed by exons and introns are then the final code sequence transcription. Sequence part that does not inform the genetic code known as introns and the sequences that contain the genetic code known as exons.

As a result of environmental influences such as evolution which lasts longer than the genetic code that was taken is not always stable, so there will be variations, known as genetic polymorphisms. Polymorphism genetic code can occur in all locations, one on chromosome 19q13.1 is composed of 7 exons and introns alternating stretches reached 2217 bp

sequence of this region which is the genetic information for gene *TGF B1*. [11-14]

*TGF B1* gene polymorphisms are known in the promoter region was C-800A, G-1639A, C509T, and C-1348T. polymorphism at position C-509 T has been found associated with several diseases. The prevalence of each gene polymorphism of *TGF B1* for each race and disease could be different and the causes of these differences is not clearly known [14].

Population based on specific phenotype to the disease have a different prevalence, as shown in the results of research conducted by Tzakas (2005) on *peak bone mass* in the normal group, Berndt (2007) against *colorectal adenomas*, Silverman (2004) of patients with asthma, Yim (2007) of patients with urinary tract infection and *reflux vesicorectal*, Crobu (2008) against *myocardial infarction*, Salam (2007) against asthma in children, and Tamizifar (2007) of *ulcerative colitis*. Results genotypes obtained also show similarities with the results of research on the *peak bone mass*, asthma, urinary tract infection and *vesicorectal reflux*, and *ulcerative colitis*. Genotypes obtained in patients with beta thalassemia major is the TT by 35%, CC 1% and CT 64% (Table 4.10). CT genotype is the genotype most obtained. This situation shows that there is polymorphism therefore has obtained more than one percent of the heterozygous mutant genotype (*wild type*) [11-14].

T allele contained in the individual polymorphisms can alter the structure of the different mRNA folds compared with the mRNA encoded from genes that have alleles C. The difference occurs in the form of folds of mRNA, which is caused by differences in base that has a different strength of interaction with other bases, so that the fold structure that forms would be different. Due to differences in the folds will cause an effect on mRNA binding to ribosomes and influential in the process of translating the mRNA code. Amino acid produced is not different, but the ribosome will translate the mRNA, especially the T

allele at codon 509 folds longer due form before the ribosomes continue translation stage [13].

The statement above is in accordance with the results obtained by Tamizifar (2007), namely the highest levels of *TGF B1* mRNA is located in the lamina propria cells. Lamina propria was the most close to the surface epithelium that *TGF B1* gene has an important role during the formation of epithelial restitution processes during phase change. In addition, the T allele have a greater ability to produce so it will happen TGB B1 overexpression on the experience polymorphism genotype. The possibility of structural changes that may occur in the form of changes that result in protein folds are not functional so that excessively expressed on the cell membrane. Condition overexpression also occurred in the C509T polymorphism of *TGF B1* gene that lead to increased osteoblastogenesis in bone process. The results are consistent with the Tamizifar (2007), namely the acquisition of the T allele have a greater risk factor than the C allele on the occurrence of ulcerative colitis [15].

Fit Analysis Hardy-Weinberg model used to estimate distribution of certain genotypes in the population. Table 2 shows Fit Testing Hardy-Weinberg model for groups of patients. It appears that in this group did not follow the Hardy-Weinberg Model Fit proved by the results of analysis of  $\chi^2 = 12.32$  which is greater than  $\chi^2$  table ( $\alpha = 5\%$ ). This can be caused by the genotype of beta thalassemia major group was an interruption. Hardy-Weinberg Law imbalance can be caused by several things, such as the selection process, migration, subpopulations, mutation, and genetic drift. In this study, used methods of SNP, which is one form of mutation, i.e., point mutations (*point mutation*). Then there is the possibility that beta thalassemia major cause of population do not meet the Hardy Weinberg equilibrium, is a mutation that appears in the population. Point mutations causing polymorphisms, so that it can be concluded that the



group of patients with beta thalassemia major experienced polymorphism.

Hardy-Weinberg law is applied to the control case, the examination of the link between the emergence of binary a disease and are often based on a comparison of SNP genotype distribution found in patients of the control.

Fit Testing The control group followed the Hardy-Weinberg model, as seen in Table 4. The result  $\chi^2 = 0.938$  is smaller than  $\chi^2$  table ( $\alpha = 5\%$ ). These results indicate that the genotypes of the normal group without any disturbance and the distribution is very uneven.

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