Original Research Article

Single Nucleotide Polymorphisms At +45 T>G And At +276 G>T Of The Adiponectin Gene And Plasma Adiponectin Level In Myanmar Type 2 Diabetic Patients

ABSTRACT

Introduction: Type 2 *Diabetes Mellitus* (T2DM) is one of the most common metabolic diseases and poses a substantial burden on health care systems globally. There is compelling data that genetic susceptibility to T2DM is polygenic. Genome-wide association studies have identified almost 50 loci associated with T2DM risk. Adiponectin gene polymorphism may be a causal role in the pathogenesis of insulin resistance and T2DM.
Aims: The aim of the study was to investigate the association between single nucleotide polymorphisms (SNP) at rs 2241766 (SNP+45 T>G) and at rs 1501299 (SNP+276 G>T) of adiponectin gene and plasma adiponectin level in Myanmar Type 2 Diabetic patients.
Study design: It was a cross-sectional analytical study.
Methodology: 100 Type 2 Diabetic patients and 104 non-diabetic subjects were included. Genotype frequencies were determined by PCR-RFLP method and plasma adiponectin level was measured by ELISA method.
Results: Carrier groups (TG and GG genotypes of SNP+45) were more likely to develop T2DM risk than non-carrier groups (TT) [OR =1.8 (95% CI = 0.89-3.63, p = 0.09) and OR = 3.51 (95% CI = 1.07-11.54, p =0.04)] respectively. Carrier groups (GT and TT genotypes of SNP+276) were more likely to develop T2DM risk than non-carrier groups (GG) [OR =1.98 (95% CI =1.10-3.55, p = 0.02) and OR = 4.07 (95% CI =1.34-12.3, p = 0.01)] respectively. Therefore, G allele of SNP+45 was found to statistically increase T2DM risk than T allele and T allele of SNP+276 was found to statistically increase T2DM risk than G allele. Mean plasma adiponectin level (27.41±16.7µg/ml) of T2DM patients was significantly lower than that of non-diabetic subjects (37.19±26.77 µg/ml), (p =0.002). Mean plasma adiponectin levels of carrier groups (TG and GG of SNP+45) were significantly lower than that of non-carrier groups (TT). Mean plasma adiponectin levels of carrier groups (GT and TT of SNP+276) were significantly lower than that of non-carrier groups (GG).
Conclusion: SNP+45 T>G and +276 G>T of adiponectin gene are significantly associated with low plasma adiponectin level and type 2 *diabetes mellitus* in this study groups of Myanmar population.

Keywords: Adiponectin gene, SNP, plasma adiponectin, type 2 diabetes

1. INTRODUCTION

Diabetes Mellitus is a multi-factorial, polygenic metabolic disorder which can affect nearly every organ system in the body. The prevalence of T2DM is increasing worldwide and in 2003, the number of people with diabetes in South East Asia was 39.3 millions and this will increase upto 81.6 millions in 2025.The risk of developing T2DM is determined by both genetic and environment factors. Insulin resistance is considered to be the core factor in the pathogenesis of T2DM. Genetic and epidemiological studies strongly suggest that insulin resistance is, at least in part, genetically determined [1]. The prevalence of T2DM is increasing worldwide and in 2003, the number of people with diabetes in South East Asia was 39.3 millions and this will increase to 81.6 millions in 2025. In 2003, the overall prevalence of diabetes mellitus in Yangon division was 11.9% (urban 13.9 % and rural 7.3%) [2].

Adiponectin is one of the most abundant proteins which is derived from adipose tissue and is encoded by adiponectin gene, located on chromosome 3q27. It plays an important role in regulating energy homeostasis, glucose and lipid metabolism and anti-inflammatory responses in the vascular system. It is also likely to modulate insulin sensitivity and to play a role in both human and animal models of insulin resistance. Insulin resistance is a fundamental element in the etiology of type 2 diabetes mellitus (T2DM) and is quite often associated with obesity [3].

The two main actions of adiponectin are insulin sensitizing action and anti-atherosclerotic action. Adiponectin acts through two types of receptors (AdipoR-1 and AdipoR-2). AdipoR-1 is most abundantly expressed in skeletal muscle, whereas AdipoR-2 is in liver. Adiponectin decreases tissue TG contents and up-regulates insulin signaling via activating PPAR-α activity. It also reduces TG contents in muscle by activating AMPK kinase. In skeletal muscle, adiponectin binds AdipoR-1 and stimulates phosphorylation of acetyl-coenzyme A carboxylase (ACC) leads to inhibition of ACC activity and a consequent reduction in the malonyl CoA content, thereby depressing carnithine palmatoyl transferase-1 (CPT-1) activity and increasing fatty acid oxidation. These changes lead to decreased tissue TG content which contributes to improve insulin signal transduction.

In the liver, adiponectin binds AdipoR-2 and inhibits gluconeogenesis by AMPK dependent phosphorylation. It decreases the expression of the key enzymes involved in gluconeogenesis such as phosphoenol pyruvate carboxykinase (PEPCK) and glucose 6 phosphatase, thereby decreasing hepatic glucose production [4].

Fifty two candidate genes in a variety of biochemical, regulatory and signal transduction pathways have involved in contribution to T2DM. Adiponectin is one such gene [5]. Genome-wide scans in humans have mapped a suspectibility locus for T2DM and metabolic syndrome to chromosome 3q27, where the gene encoding adiponectin is also located. It spans 17 kilo base (kb) and consists of 3 exons and 2 introns [6].

Genetic variations in the adiponectin gene can affect plasma adiponectin concentration and it is estimated that a 30-70% variation in normal circulating adiponectin level can be attributed to genetic factors. Hara et al [3], 2002 also stated that the serum concentrations of adiponectin are heritable, thus making it a strong candidate gene for T2DM, obesity and coronary artery disease (CAD). A total of 42 single nucleotide polymorphisms (SNPs) in adiponectin gene and its regulatory region with a minor allele frequency of >1.5% have been identified.

The association between adiponectin gene polymorphisms and plasma adiponectin level in T2DM had been proved by various studies in Asian populations as well as in Western populations. The T>G polymorphism of SNP+45 in exon 2 and the G>T polymorphism of SNP+276 in intron 2 of adiponectin gene have been found to be related to type 2 diabetes in Japanese subjects [3], in Iranian obese individuals [7] and in non-diabetic Greek women [8].Therefore, the adiponectin gene polymorphisms were found to have genetic effects on diabetes, obesity and insulin resistance but its effects also influenced by different genetic backgrounds and environmental factors in different ethnic populations.

At rs 2241766 (+45T>G) of adiponectin gene, it can have 3 forms of genotype distribution (TT, TG, GG) when the base thymine (T) changes to guanine (G). TG heterozygous form at rs 2441766 has 3 times increased risk of T2DM and GG homozygous has 3.8 times increased risk of T2DM [9]. At rs 1501299 (+276 G>T) of adiponectin gene, it can have 3 forms of genotype distribution (GG, GT, TT) when the base guinine (G) changes to thymine (T).

Moreover, hypoadiponectinemia is found to be strongly linked to obesity, insulin resistance and T2DM and may be used to predict the overall risk of developing insulin resistance and T2DM. The findings of Snehalatha et al in 2003 [10] and Aleidi et al in 2015 [11] also proved that plasma adiponectin is an independent predictor of T2DM in Asian Indian population and in Jordanian population respectively.

Many studies found the association between the genetic variation and serum adiponectin level in different study groups. This study will find out the genetic variation of adiponectin in Myanmar study groups and also try to verify the link between the two adiponectin gene polymorphisms (+276 G>T and SNP +45 T>G) and its gene product, adiponectin protein in relation to T2DM patients.

2. METHODOLOGY

2.1 Type of study

It is a cross-sectional, analytical study.

2.2 Study Population

Total 204 numbers (T2DM=100 and non-diabetic subjects=104) were included. T2DM patients were recruited from out-patient department and diabetes clinic of North Okkalapa General and Teaching Hospital. Non-diabetic subjects were selected by simple random sampling from population of Quarter B, North Okkalapa Township, Yangon, Myanmar. From those subjects, who had fasting plasma glucose level less than 6.1 mmol/l or less than 110 mg/dl were regarded as non-diabetic subjects according to WHO, 2006 criteria [12].

2.3 Study Procedure

Materials used for the procedure

For biochemical parameters determination

1. Adiponectin ELISA Kit (Shanghai Taoyu International Company)
2. ELISA plate reader
3. Spectrophotometer

For genotyping

1. Thermocycler (Astec 700)
2. Microcentrifuge
3. Water bath
4. Taq polymerase enzyme, dNTP mix, PCR buffer (Vivantis)
5. Specific primer sets for SNP +45 and +276 (AIT Biotech Pte Ltd)
6. Restriction enzymes: SmaI for SNP +45 and BsmI for SNP +276 (New England Biolabs, NEB**)**

5 ml of venous blood were taken from both subjects for the determination of plasma adiponectin and for genotyping. Determination of plasma adiponectin level was done by ELISA method (Shanghai Taoyu company).

DNA extraction was carried out by salting out method [13]. PCR master-mix consisted of total final volume 50 µl which included genomic DNA sample-2µl, Taq polymerase-0.5 µl, dNTP-2.5 µl, PCR buffer-10 µl, MgCL2-3 µl, forward and reverse primers-8 µl each and distilled water-16 µl. Purity of DNA was checked by agarose gel electrophoresis. PCR cycle condition for SNP+45 were heat denaturation at 94ºC for 3 minutes, annealing at 94 ºC 1minute, 57 ºC 1 minute, 72 ºC 1 minute for 34 cycles and final extension at 72 ºC for 5 minutes and were hold at 4 ºC. PCR cycle condition for SNP+276 were heat denaturation at 94 ºC for 3 minutes, annealing at 94 ºC 1minute, 60 ºC 1 minute, 72 ºC 1 minute for 35 cycles and final extension at 72 ºC for 6 minutes and were hold at 4 ºC.

Specific DNA fragments consisting SNP +45 and +276 was amplified from genomic DNA by specific primer sets PCR products are identified in 2% agarose gel and seen at 372 bp for SNP+45 and 241 bp for SNP+276 respectively. The PCR products of SNP +45 were digested by SmaI and SNP +276 by BsmI (New England Biolab, NEB). The digested products were separated by 2% agarose gel to analyze for RFLP. Master-mix for enzyme digestion (SmaI) consisted of total final volume 25 µl which included PCR product-5 µl, 20000 unit/ml enzyme -0.3µl, 10X buffer-2.5 µl and distilled water-17.2 µl. Master-mix for enzyme digestion (BsmI) consisted of total final volume 25 µl which included PCR product-5 µl, 10000 unit/ml enzyme -0.3µl, 10X buffer-2.5 µl and distilled water-17.2 µl.

Chart 1 . Primers for SNP+45 [16]

Primer	Sequence 5'–3'
rs2241766	Forward primers GCA GCT CCT AGA AGT AGA CTC TGC TG
rs2241766	Reverse primers GCA GGT CTG TGA TGA AAG AGG CC

Chart 2 .Primers for SNP+276 [7]

Primer	Sequence 5'–3'
rs1501299	Forward primers CCT GGT GAG AAG GGT GAG AA
rs1501299	Reverse primers AGA TGC AGC AAA GCC AAA GT

2.4 Statistical analysis

Plasma adiponectin level was expressed as mean and standard deviation. Genotype and allele frequencies were expressed as percentage. Mean plasma adiponectin levels were compared across genotypes using ANOVA and Tukey HSD test and between T2DM and non-diabetic subjects by Student's t-test. Hardy-Weinberg equilibrium (HWE) and the association between disease status and the genetic variants were tested by Pearson's Chi square test. Odds ratio, 95% confidence intervals and all statistical tests were carried out using SPSS software version v.16.0.

3. RESULTS

A total of 204 participants [100 patients with T2DM and 104 non-diabetic subjects] who fulfilled the inclusion criteria were accounted for analysis in this study. The mean age of T2DM subjects was 50.24 ± 9.87 years old.

In SNP+45, wild (TT) genotype can be seen as single band at 372 bp. Heterozygone (TG) genotype was seen as three bands at 163 bp, 209 bp, 372 bp and mutant (GG) genotype was seen as two bands at 163 bp and 209 bp. In SNP+276, wild (GG) genotype was seen as two bands at 95 bp and 146 bp. Heterozygous (GT) genotype was seen as three bands at 95 bp, 146 bp, 241 bp and mutant (TT) genotype was seen as single band at 241 bp.

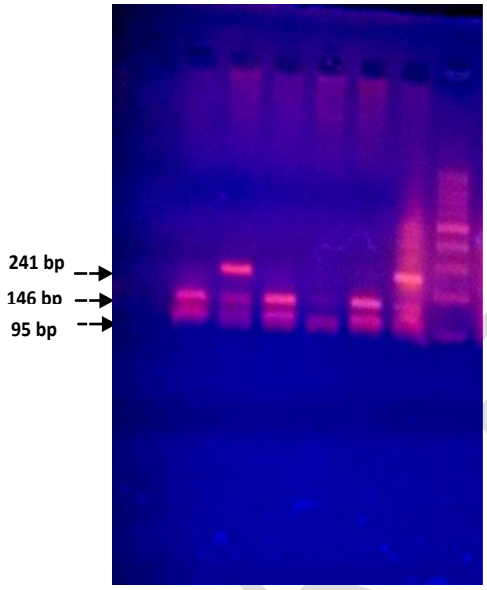

Figure (1) PCR-RFLP of SNP +276

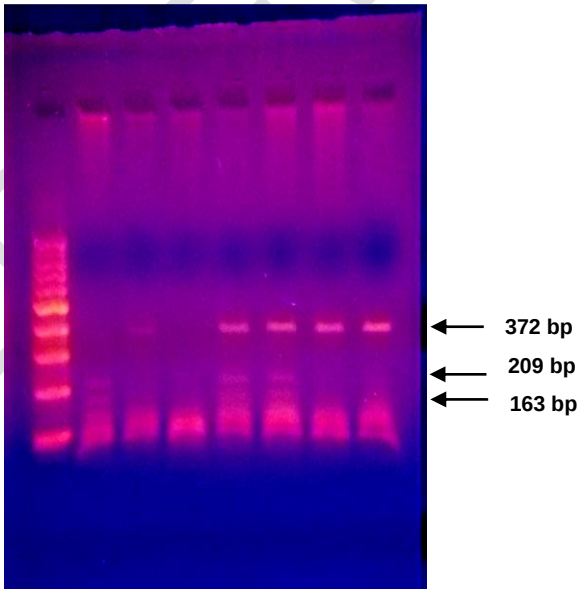

Figure (2) PCR-RFLP of SNP +45

Table 1. Association of TG and GG genotypes of SNP +45 and T2DM

Genotype	T2DM	Non-diabetic	Odds ratio	95%CI	*p*-value
TT (n=148)	65 (65%)	83 (80%)	-	-	-
TG (n=41)	24 (24%)	17 (16%)	1.80	0.89-3.63	0.09
GG (n=15)	11 (11%)	4 (4%)	3.51	1.07-11.54	0.04

X2=6.57, p=0.037

The subjects with heterozygous (TG) genotype were more likely to develop T2DM than those with wild (TT) genotype [Odds ratio = 1.8 (95% CI = 0.89-3.63)].The subjects with mutant (GG) genotype were more likely to develop T2DM than those with wild (TT) genotype [Odds ratio = 3.51 (95% CI =1.07-11.54)].

Table 2. Association of allele frequencies of SNP +45 and T2DM patients

Subjects	Allele		Odds ratio	95%CI	*p*-value
	T	G			
T2DM	154	46	2.14	1.25-3.64	0.005
Non-diabetic subject	179	25			

G allele of SNP+45 was found to be associated with the risk of developing T2DM [Odds ratio= 2.14 (95% CI= 1.25-3.64)].

Table 3. Association of GT and TT genotypes of SNP +276 and T2DM

Genotype	T2DM	Non-diabetic	Odds ratio	95%CI	*p*-value
GG (n=100)	39 (39%)	61(59%)	1.0	-	-
GT (n=86)	48 (48%)	38(36%)	1.98	1.10-3.55	0.02
TT (n=18)	13 (13%)	5 (5%)	4.07	1.34-12.30	0.01

X^2=9.48, p=0.008

The subjects with heterozygous (GT) genotype were more likely to develop T2DM risk than those with wild (GG) genotype with an odds ratio of 1.98 (95% CI = 1.10-3.55) and it was statistically significant.

The subjects with mutant (TT) genotype were more likely to develop T2DM risk than those with wild (GG) genotype with an odds ratio of 4.07 (95% CI =1.34-12.30) and it was statistically significant.

Table 4. Association of allele frequencies of SNP +276 and T2DM patients

Subjects	Allele		Odds ratio	95%CI	*p*-value
	G	T			
T2DM	126	74	1.91	1.24-2.94	0.003
Non-diabetic subject	156	48			

T allele of SNP+276 was found to be associated with the risk of developing T2DM [Odds ratio = 1.91 (95% CI =1.24-2.94)].

Table-5. Comparison of plasma adiponectin levels with different genotypes of SNP +45 in T2DM patients

Parameter	Genotypes			*p*	Comparison between different genotypes	*p*
	TT (*n*=65)	TG (*n*=24)	GG (*n*=11)			
Plasma adiponectin (ug/ml)	33.32 ± 17.98	24.28 ± 14.56	21.18 ± 11.37	0.02	TT vs TG TT vs GG TG vs GG	0.02 0.007 0.5

Results were shown in Mean ± SD.

A one-way between subjects ANOVA was conducted to compare the plasma adiponectin levels with different genotypes (TT, TG and GG). Post hoc comparisons using the Tukey HSD test indicated that mean adiponectin level of TT (33.32 ± 17.98) was significantly different than both mean adiponectin level of TG (24.28 ± 14.56) and GG (21.18 ± 11.37).

Table-6. Comparison of plasma adiponectin levels of with different genotypes of SNP+276 in T2DM patients

Parameter	Genotypes			*p*	Comparison between different genotypes	*p*
	GG (*n*=39)	GT (*n*=48)	TT (*n*=13)			
Plasma adiponectin (ug/ml)	31.64 ± 18.95	25.92 ± 15.02	19.77 ± 11.85	0.05	GG vs GT GG vs TT GT vs TT	0.13 0.01 0.13

Results were shown in Mean ± SD.

A one-way between subjects ANOVA was conducted to compare the plasma adiponectin levels with different genotypes (GG, GT and TT). Post hoc comparisons using the Tukey HSD test indicated that the mean adiponectin level of GG (31.64 ± 18.95) was significantly different than the mean adiponectin level of TT (19.77 ± 11.85).

4. DISCUSSION

Type 2 Diabetes Mellitus is one of the most common metabolic diseases and poses a substantial burden on health care systems globally. There is compelling data that genetic susceptibility to T2DM is polygenic. Genome-wide association studies have identified almost 50 loci associated with T2DM risk. Adiponectin gene polymorphism may be a causal role in the pathogenesis of insulin resistance and T2DM [14].

Among the SNPs of adiponectin gene, an intronic SNP+276 G>T at rs-1501299 an exonic SNP+45 T>G at rs-2241766 were considered the important known genetic risk factors for the development of insulin resistance and T2DM. Since adiponectin regulates both glucose and lipid metabolisms, derangement of these metabolism due to reduced adiponectin will lead to insulin resistance and T2DM. The aim of the present study was to provide the supportive evidence for the involvement of adiponectin gene and its effect on plasma adiponectin level in Myanmar T2DM patients.

In the present study, the genotype distribution of SNP+45, (TG & GG) genotypes were more likely to develop T2DM than TT as shown in table-1. The association is not significantly high when there is only one G allele but when both alleles are G, the risk for developing T2DM become double and the association of SNP+45 and T2DM also become statistically significant. These results were consistently found in several studies conducted by Hara et al (2002) [3] in the Japanese population, Li et al (2007) [6] in Han Chinese population, Gable et al (2007) [15] in European subjects, Khodeer et al (2011) [16] in Egyptian patients and Biswas et al (2011)[17] in the South Indian population. The association between genotype distribution and the risk of T2DM in these studies reported the range between the odd ratios of 1.5 to 4.9. This variation may be due to the contribution of other factors such as diet pattern, race, lifestyle, environment which also influence on development of T2DM.

On studying the allele frequency of SNP+45, G allele was found to statistically increase T2DM risk than T allele as shown in table-2.The higher frequency of G allele in SNP+45 and the risk of developing T2DM were consistent with other studies in different populations such as in German [18], in Japanese [3] and in female nurses at Boston [19]. The recent

meta-analysis study (among 44 studies of Asians and Caucasians) conducted by Fan et al (2015) [20] found out that the AdipoQ gene +45 T>G polymorphism was significantly associated with the risk of T2DM in many Asian populations while it was not found to be associated with the risk of T2DM in Caucasians. Thus, G allele of SNP+45 might be a susceptible allele for T2DM and this polymorphism might be a predisposing factor to T2DM in Myanmar diabetic patients.

It was proved that the genotype distribution of SNP +276 conformed to the HWE in both T2DM ($\chi 2$ = 0.41, df = 1, p = 0.522) and non-diabetic group ($\chi 2$ = 0.67, df = 1, p = 0.413). The subjects with (GT and TT) genotypes were significantly greater risk of developing T2DM than those with (GG) genotypes as shown in table-3. These results were comparable with the findings of Yang and his groups [21] in Taiwan diabetic patients in 2007 which demonstrated that the risk of T2DM was more common in the subjects with GT and TT genotypes than GG of SNP+276.

On reviewing the allele frequency of SNP +276, T allele was found to statistically increase type 2 diabetes risk than G allele as shown in table-4.This finding was consistently found in the studies proved by the authors in Taiwanese subjects [22] and in Saudi Arabia population [23]. Therefore, the current study supported that T allele of SNP+276 might be a susceptible allele for T2DM and this polymorphism might be a predisposing factor to T2DM in Myanmar population.

The correlation of genetic variation at two loci (SNP+45 and +276) in the genome within a given population was assessed by the pattern of linkage disequilibrium (LD). Kruglyak [24] at 1999 stated that [D' > 0.33 and r2 > 0.1] were applied as a criterion for meaningful LD and [D' =1, r2 =1] can be regarded as perfect LD. So, it could be concluded that there is a significance of LD between the two loci and SNP+45 T>G was in linkage disequilibrium with SNP+276 G>T (D' = 0.47, p =0.00001). These result was in accordance with the report conducted by Berthier et al [25] in 2005 which stated that SNP+45 T>G was in linkage disequilibrium with SNP+276 G>T (D'=0.64, p =0.002) regarding as useful LD between the two SNPs.

The mean plasma adiponectin level (27.41+16.7µg/ml) of T2DM patients was significantly lower than that (37.19+26.77 µg/ml) of non-diabetic subjects (p=0.002). In SNP+45, mean plasma adiponectin levels of (TG&GG) genotypes were lower than that of TT. The reduction was statistically different between TT vs TG group and TT vs GG group (table-5). In SNP +276, mean plasma adiponectin levels of GT and TT genotypes were also lower than that of GG. The reduction was statistically different between GG vs TT group (table-6).

The findings of significantly reduced plasma adiponectin in T2DM were consistent with different populations in meta-analysis including 13 prospective studies of many races (eg. Whites, Asian Indians, African and native Americans) performed by Li et al [26] in 2009. The result of many different studies among different population pointed out that SNP+45 T>G and +276 G>T appeared to influence on plasma adiponectin level and that in turn influence on development of T2DM. In Myanmar, there was no similar study on the association between this SNPs and plasma adiponectin and development of T2DM previously. Although the study population was not big enough to represent the whole Myanmar population, the result of current study would be an important finding for Myanmar people and that would be an important data to be analyzed as part of the data for different races to support the above finding.

The mechanism by which the adiponectin gene polymorphism affects on its plasma adiponectin concentration may be due to alteration in mRNA level. In 2011, the recent report by Toy and groups [27] demonstrated that despite being a coding synonymous SNP+45, located in exon-2 of adiponectin gene which does not change the amino acid sequence, the G allele was associated with more than 80% reduction in mRNA expression in the subcutaneous adipose tissue. This observation was validated in an independent sample of omental adipose tissue where more than 50% reduction in adiponectin gene expression efficiency is associated with G allele of SNP+45. It is also speculated that SNP+45 T>G may alter RNA splicing or stability, suggesting an allele-specific differential expression of adiponectin. Since SNP +45 is in linkage disequilibrium with SNP +276 (intron 2), that destabilizes the pre-mRNA, results in reduced mRNA levels and finally leads to pathophysiological effects.

The mechanism by which decreased plasma adiponectin level due to SNP+276 was still conflicting. Because SNP +276 is placed in intron 2 of the adiponectin gene which is away from the consensus splice site and does not have a known function. Thus, it might be a marker of some other variants that affecting adiponectin gene expression. Menzaghi et al [28] in 2002 also shown that the SNP +276 G>T is in almost complete linkage disequilibrium with several polymorphisms placed in the 3' untranslated regions (3'UTR). 3' UTR is a region which is playing a pivotal role in the control of gene expression by binding proteins that regulate mRNA processing, translation (or) degradation.

As a therapeutic strategy for T2DM, the up-regulation of plasma adiponectin and adiponectin receptors (or) the development of adiponectin receptors agonists can be used. Insulin sensitizer, a PPAR-Ɣ agonist (hypoglycemic agent-thiazolidinediones, TZD) has been shown to increase plasma adiponectin level in mice and humans. So, the new interesting strategies are those to up-regulate the adiponectin receptors and those to stimulate the adiponectin receptors using small molecule agonists.

Limitation of the study:

The limitation of this study was that the correlation between plasma adiponectin level and insulin resistance was not shown. Moreover, we could not show the molecular mechanism between these genetic polymorphisms and its protein, adiponectin.

5. CONCLUSION

The study demonstrated that G allele of SNP+45 and T allele of SNP+276 was associated with increased risk of T2DM and these gene polymorphism also effect on the plasma adiponectin level. Therefore, this study could provide evidence of the potential involvement of the adiponectin gene as a risk factor for T2DM in the Myanmar population. Moreover, the non-diabetic subjects who had risk alleles of SNP+276 of adiponectin gene and their family members should be done medical check-up regularly because they have potential to develop T2DM.

ETHICAL APPROVAL

The research was done according to international ethical guideline of CIOMS (Council for International Organization of Medical Science). Ethical approval was obtained from Ethical Review Committee of University of Medicine 2, Yangon, Myanmar.

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Determination of plasma adiponectin level

Sandwich Enzyme linked immunosorbent assay

(Shanghai Taoyu International Company

Principle

Two types of monoclonal antibodies against total human adiponectin are used in this assay. One type is used as primary antibody to capture the adiponectin in the sample or standard. The second antibody is labeled with horseradish peroxidase (HRP) enzyme and used as detection antibody.

The immunoplate is pre-coated with primary antibody specific for human adiponectin. When samples or standards are added into the pre-coated wells, adiponectin in the sample bind to the primary antibody and immobilized in the wells. When detection antibody is added to the wells, it bind to the adiponectin and trapped in the wells.

After that, unbound detection antibodies are removed by wash procedure, the substrate 3,3',5,5' tetramethylbenzidine (TMB) is added. Horseradish peroxidase enzyme converts TMB into coloured compound. The reaction is terminated by the addition of stop solution.

The intensity of colour is measured spectrophotometrically by the absorbance at 450 nm. The increase in absorbance is directly proportional to the amount of captured adiponectin in the unknown sample. The concentration of adiponectin in the sample can be calculated from a standard curve generated in the same assay with reference standards of known concentrations of adiponectin.

Reagents

1. Microtiter plate (96 wells) : coated with anti-human adiponectin monoclonal antibody
2. Wash solution : 20 ml
3. Sample diluent : 6 ml
4. Adiponectin standard : 0.5 ml
5. Standard diluent : 1.5 ml
6. Detection Antibody : monoclonal antibody against human adiponectin conjugated with HRP 6 ml
7. Substrate A : 6 ml
8. Substrate B : 6 ml
9. Stop Solution : 6 ml

Preparation of reagents

For plasma samples

The anti-coagulant (EDTA) containing tubes were used to collect the blood samples. After the condensation of 30 minutes, the supernatant plasma fluids were collected with the aliquot tubes and were frozen at temperature under-20° C.

Wash solution

Working wash solution was prepared by mixing 20 ml of 10x wash solution with 480 ml of distilled water to yield 500 ml of 1x wash solution. After preparation, working wash solution was stored at 2-8° C.

Sample dilution

The plasma sample (10 µl) was diluted with 40 µl of sample diluent and so the final dilution is five fold.

Standard Solution

Each adiponectin standard was prepared by serially diluting adiponectin standard solution with standard diluent.

2 ng/ml	5 Standard	150µl Original density Standard+150µl Standard diluent
1 ng/ml	4 Standard	150µl 5 Standard+150µl Standard diluent
0.5 ng/ml	3 Standard	150µl 4 Standard+150µl Standard diluent
0.25 ng/ml	2 Standard	150µl 3 Standard +150µl Standard diluent
0.125ng/ml	1 Standard	150µl 2 Standard +150µl Standard diluent

Procedure

1. All reagents and samples were brought to room temperature (18-25°C) before use.
2. The positions of standards and samples were marked on the plate map.
3. Standard solution (50 µl) and diluted samples (50 µl) were added to their assigned positions in the pre-coated microtiter plate.
4. After closing the plate with the plate membrane, it was incubated at 37°C for 30 minutes.
5. After incubation, all the liquids were discarded and washed 5 times with 300µl of working wash solution.
6. Then, 50 µl of HRP conjugate reagent was added to each well and incubated at 37°C for 30 minutes.

7. After incubation, all the liquids were aspirated and washed 5 times with 300µl of working wash solution. Residual wash buffer was removed by tapping the plate on the absorbent paper.
8. After washing, 50 µl of substrate A was added to each well followed by 50 µl of substrate B. The plate was incubated at 37℃ for 30 minutes.
9. After incubation, 50 µl of stop solution was added to each well. The absorbance of each well was read immediately using microplate reader set to 450 nm.
10. The standard curve was generated from the absorbance values of the known standards.
11. The concentration of each sample was determined by multiplying with 5 (dilution factor). Samples with concentration higher than that of the highest standard were further diluted and re-assayed. They were expressed in term of µg/ml after unit conversion. (multiply with 10^2)

Normal plasma adiponectin level

20-45 µg/ml

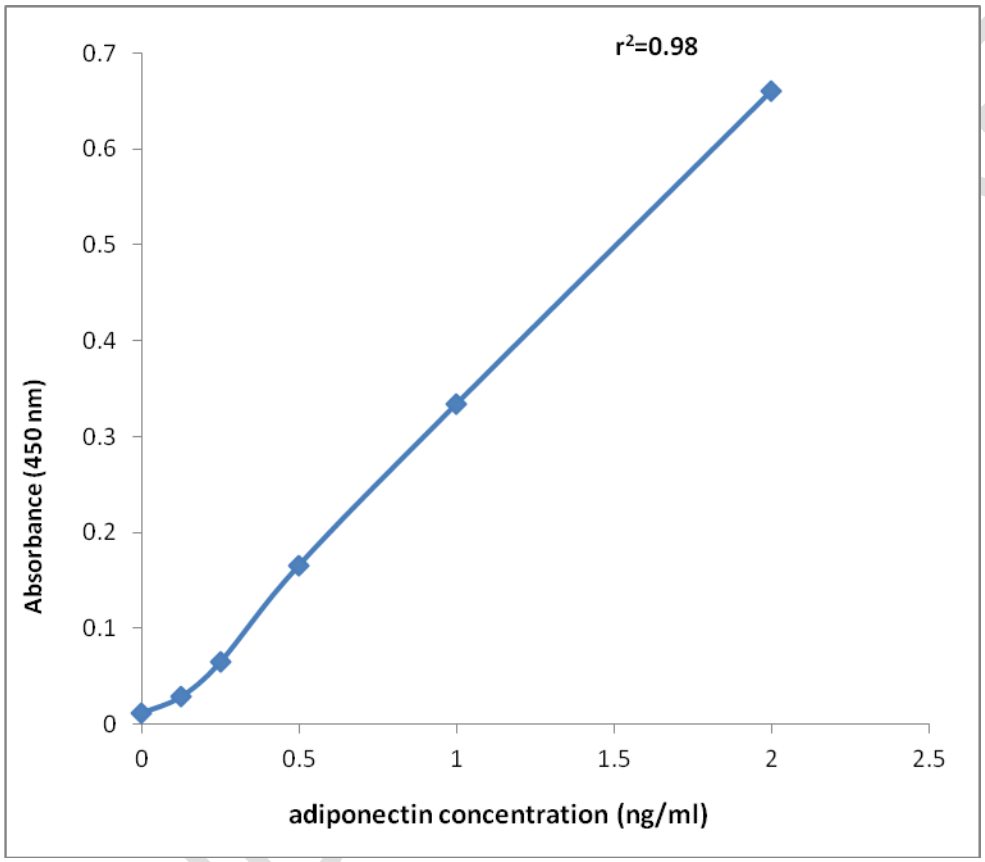

Figure-9: Standard curve for plasma adiponectin concentration

DNA extraction by salting out methods

(Miller et al, 1988)

Apparatus

1. Centrifuge and Micro-centrifuge
2. Water bath (65°C)
3. Vortex
4. Polypropylene tubes

Reagents

1. Red blood cell lysing (RLB) solution containing sterile distilled water 1L, NH_4CL 7.5g, Tris 1g
2. Nucleic lysis buffer containing 10mM Tri pH 8.2, 400mM NaCl, 2mM EDTA, pH 8.2 with HCl
3. Sodium Dodecyl Sulfate (SDS) solution (10%)
4. Proteinase K (2 mg/ml) in water
5. Sodium Chloride (5.3 M)
6. Cold 100% and 75% ethanol

Preparation of stock solution

1. Red cell lysis buffer (RLB) solution

Ammonium chloride (7.5 g) and Tris (1.0 g) were dissolved in a 1L of sterile distilled water. The pH was adjusted to 7.2 and it was kept as refrigerated form at
-4°C. Shelf life was approximately six months.

2. Tris-HCl (1M) (pH 8.0)

Trisma Base (60.5 g) was dissolved in 350 ml of deionized distilled water (ddw). Concentrated HCl was added until pH fell to 8.0 and volume was made up to 500 ml with ddw.

3. Sodium Chloride, NaCl (5M)

Sodium Chloride (146.1 g) was placed in a beaker and made up to the volume to 500 ml with ddw and the mixture was stirred well until it was completely dissolved.

4. Sodium Chloride, NaCl (5.3M)

Sodium Chloride (154.866 g) was placed in a beaker and made up to the
volume to 500 ml with ddw and was stirred well until it was completely dissolved.

5. Ethylene diamine tetrachloroacetic acid, EDTA (0.5M) (pH 8.0)

Disodium EDTA (93g) was added to 400 ml of ddw and was stirred until most of it dissolved. (0.5M) NaOH was added until pH raise to 8.0. The rest of the solid was dissolved completely. Volume was made up to 500 ml with ddw.

6. Nuclear lysis buffer

Tris (1M) =10 ml (pH 8.0), (5M) NaCl=0.8 ml and (0.5 M) EDTA =0.4 ml (pH8.0) were added to 90 ml of ddw to get 100 ml of nuclear lysis buffer with final concentrations of (10 mM) Tris (pH 8.2), (400 mM) NaCl and (2mM) EDTA. Then, the pH was adjusted with NaOH to 8.2.

7. Sodium dodecyl sulphate (SDS) (20%) solution

Sodium dodecyl sulphate **(**100g) was placed in a beaker and 350 ml of ddw was added. It was dissolved by stirring with magnetic stirrer at 68°C. The volume was made up to 500 ml with ddw and was stored at room temperature.

8. Sodium dodecyl sulphate (SDS) (10%) solution

Sodium dodecyl sulphate (20%) (5ml) was diluted with 5ml of ddw to get 10 ml of 10% SDS solution.

9. Ethanol (75%)

Ethanol 100% (37.5 ml) was diluted with ddw to get 50 ml of 75% ethanol.

10. Proteinase K (2mg/ml)

Proteinase K (100 mg) was dissolved in 50 ml of ddw and stored as frozen with 2 ml aliquots at -4° C.

11. Hydrochloric acid, HCl (1M)

Hydrochloric acid (41.3 ml) was dissolved in 458.7 ml of ddw to get 500ml of (1M) HCL.

12. Sodium hydroxide, NaOH (1M)

Sodium hydroxide (20g) was dissolved in 500 ml of ddw to get 500ml of (1M) NaOH.

Procedure for DNA extraction from plasma

1. Plasma (500 µl) was added to 1 ml of cold (0 to -5°C) RLB buffer. The mixture was vortexed for 30 seconds.
2. The mixture was centrifuged for 1 minute at 15000 rpm and poured off red supernatant. The rim of tube was blotted on a paper towel. A white to red pellet was remained in the bottom of the tube.
3. Red cell lysis buffer (1 ml) was added again and vortexed for 3-4 seconds to re-suspend pellet. Then, the mixture was centrifuged for 30 seconds at 15000 rpm. All fluid was drained off. The remaining pellet was white to pink and this step can be repeated as necessary.
4. Nuclear lysis buffer (200 µl) and 10% SDS (50 µl) were added to each pellet.
5. The pellet was broken up with pipette tip and was vortexed to get powdery, tiny flakes. Then, an additional 150 µl of nuclear lysis buffer was added and vortexed again.
6. After that, 100 µl of proteinase K (2mg/ml) was added and mixed. The mixture was incubated for 2 hours at 65°C.

7. After incubation, 175µl of 5.3M NaCl was added and centrifuged at 15000 rpm for 15 minutes in microfuge. And then, the supernatant was transferred to fresh tube.
8. Cold 100% ethanol (1ml) was added to supernatant. The mixture was inverted 6-10 times to precipitate DNA. It appeared as white to translucent stringy mass. Then it was centrifuged for10 minutes at top speed.
9. The supernatant was poured off carefully, not to lose pellet. The pellet was washed with 1 ml of cold 75% ethanol (break pellet by tapping) and was centrifuged again 1-2 minutes at 15000 rpm.
10. The ethanol was poured off and allowed to air dry with the cap open to evaporate the ethanol. The pellet was stored as dry in -20˚C.

If the samples were used to go for PCR reaction, this pellet was dissolved in 100 µl of sterile distilled water. The mixture was put in water bath for 15 minutes at 65˚C to dissolve the DNA sample. Then, the mixture was vortexed gently to resuspend.

Agarose Gel Electrophoresis of DNA

(Ausubel et al, 1998)

Principle

Electrophoresis is the process of separation of molecules according to their movement through an electrolyte subjected to an electric field. Agarose gel forms a porous support medium for better separation of molecules.

Due to the strong acidic nature of the phosphate groups in the backbone, DNA remains negatively charged at the pH used for most electrophoresis buffers. The charge density of nucleic acids is also constant. So that,the relative mobility of nucleic acids within agarose gels are determined by their size and structure.

Apparatus

1. Mupid submarine electrophoresis apparatus and power supply
2. Gel casting tray and combs
3. UV Transilluminator
4. Polaroid camera

Reagents

1. Agarose gel for nucleic acids (Nusieve, cat No. 50090 Cambrex Bio Science)
2. Autoclaved deionized distilled water (ddw)
3. Loading buffer containing bromophenol blue and xylene cyanol
4. Ethidium Bromide (10 mg/ml)
5. Trisma-Base
6. Boric acid

Preparation of reagents

1. Tris-Borate EDTA buffer (10x) (TBE buffer)

Tris base (18.15g) was mixed with 9 g of boric acid and 1.23g of EDTA.3a and was brought up to 300 ml with autoclaved ddw.

2. Tris-Borate EDTA buffer (1x)

Stock tris-Borate EDTA buffer (10x) (100 ml) was diluted with 900 ml of ddw to get 1x TBE solution with final volume 1000ml.

1. Ethidium bromide (10 mg/ ml)

Ethidium bromide powder (10 mg) was dissolved in 1ml of autoclave ddw.

2. Agarose gel (2%)

Tris-Borate EDTA buffer (1x) (100 ml) and a stir bar were added into a beaker and 2 g of agarose powder was slowly sprinkled into buffer while the solution was rapidly stirred on magnetic mixer, till the gel and TBE buffer were mixed thoroughly. Then, the beaker was covered with plastic wrap and a small hole was formed in the plastic wrap for ventilation. The beaker was heated in the microwave oven at (100℃) on medium power for 1 minute.

After the gel was completely dissolved, it was cooled on the mixer until the temperature fell to 50˚C – 60℃. At this point, 10 ul of ethidium bromide was added. The gel solution was poured into gel casting tray and the comb was placed, and was covered with brown paper and allowed to settle for 10- 30 minutes. Then, it was placed in 4℃ for about 2 hours and after that, the combs were removed. Finally, the gel blocks were removed from the casting tray and placed in TBE buffer and stored at 4℃.

Procedure for gel electrophoresis of DNA

First, the submarine electrophoresis unit was filled with 1 x TBE buffer and then 2% agarose gel block was placed on the support. The buffer solution was covered the gel. Then, DNA sample was mixed with loading dye and 1 x TBE to make 10 µl volume and was placed in the wells.

Then, the electrophoresis was run at 100 V for 30 minutes. After that, the DNA was visualized under ultraviolet light. Pure and unfragmented DNA was shown as clean single bands. Then, it was photographed by using Polaroid camera.

Polymerase Chain Reaction
(Ausubel, Brent, Kingstin et al, 1998)

Principle

Polymerase chain reaction (PCR) is a rapid and versatile in vitro method for amplifying defined target DNA sequences present within a source of DNA (e.g. total genomic DNA or a complex of cDNA population). Two oligonucleotide primers were designed which are specific for the known target sequence. When these primers were added to denatured template DNA, they bind specifically to complementary DNA sequences at the target site. In the presence of a suitably heat stable DNA polymerase and deoxynucleotide triphosphates, they initiated the synthesis of new DNA strands complementary to the individual DNA strands of the target DNA segment. The newly synthesized strands act as template strands for further DNA synthesis in subsequent cycles. After about 25 cycles of synthesis, the products of PCR includes about10^5 copies of specific target sequence, which can be easily visualized as a discrete band of specific size when submitted to agarose gel electrophoresis.

Reagents

1. Taq polymerase (Vivantis)
2. Deoxy nucleotide triphosphate mix, dNTP mix (10 mM of each dNTP)
3. PCR buffer (10x) which contain 500 mM KCL, 100 mM Tris - HCL, pH 9.1, 0.1% Triton
4. $MgCl_2$ (50 mM)
5. DNA sample in TE buffer
6. Forward and reverse primer 100 µM, stock solution
7. Autoclaved ddw

Primers for SNP +45 (Khodeer et al, 2011) (AIT Biotech Pte Ltd)

Primer	Sequence 5'–3'
rs2241766	Forward primers GCA GCT CCT AGA AGT AGA CTC TGC TG
rs2241766	Reverse primers GCA GGT CTG TGA TGA AAG AGG CC

Primer for SNP +276 (Mohammadzadeg and Zarghami, 2009)

Primer	Sequence 5'–3'
rs1501299	Forward primers CCT GGT GAG AAG GGT GAG AA
rs1501299	Reverse primers AGA TGC AGC AAA GCC AAA GT

Reference sequence for SNP +45 (exon-2) and SNP+276 (intron-2) (NCBI)

Instruments

1. Thermocycler (ASTEC PC-700)
2. Microcentrifuge (CAPSULEFUGE TOMY PMC -060)
3.

Procedure

The reagents were allowed to thaw completely and were mixed and placed on ice. The PCR reaction mixture was prepared as followed: For each sample, the PCR was performed on containing 10 x PCR buffer with $(NH4)_2SO_4$, 1.5 mmol of $MgCL_2$, 1.25 U of Taq polymerase, 250 µmol/L of each dNTP, 100 ng of each primer and 2 µl of genomic DNA in a final volume of 50 µl.

For working with multiple samples, a master mix consisting of $MgCL_2$, 10 x reaction buffer, dNTP mix, Taq polymerase and ddw were assembled. Appropriate multiples of the listed reaction components were combined, and appropriate volume was added. After adding the template DNA, the final volume was 50 µl and the reaction was started. Individual pipette tips were used for all combinations to prevent cross-contamination of the samples. The reaction tubes were placed in the heat block in thermocycler and proceeded with the individual PCR cycle conditions for the two SNPs of the adiponectin gene.

PCR Master mix for SNP+45 (for one sample)

No	Reagents	Amount (µl)
1.	Genomic DNA sample	2
2.	Taq polymerase	0.5
3.	dNTP	2.5
4.	10 x PCR buffer	10
5.	$MgCL_2$	3
6.	Forward primer	8
7.	Reverse primer	8
8.	Distilled water	16
	Total final volume	50

PCR cycle condition

1. Heat denaturation at 94°C for 3 minutes
2. 94°C 1minute, 57°C 1 minute, 72°C 1 minute (34 cycles)
3. Final extension at 72°C for 5 minutes and were hold at 4°C

PCR Master mix for SNP+276 (for one sample)

No	Reagents	Amount (µl)
1.	Genomic DNA sample	2
2.	Taq polymerase	0.5
3.	dNTP	2.5
4.	10 x PCR buffer	10
5.	$MgCL_2$	3
6.	Forward primer	8
7.	Reverse primer	8
8.	Distilled water	16
	Total final volume	50

PCR cycle condition

1. Heat denaturation at 94°C for 3 minutes
2. 94°C 1minute, 60°C 1 minute, 72°C 1 minute (35 cycles)
3. Final extension at 72°C for 6 minutes and were hold at 4°C

Restriction enzyme analysis of PCR products

for detection of SNP +45 and SNP +276

Polymerase Chain Reaction – restriction fragment length polymorphism (PCR-RFLP) method (Xiao J et al, 2006)

Polymerase chain reaction products were digested with the corresponding restriction enzymes to analyze the different genotypes of SNP+45 (TT,TG, GG) and SNP+276 (GG,GT,TT).

For SNP +45

Restriction enzyme, *SmaI* (New England Biolabs, NEB)

Product source : An E.coli strain that carries the SmaI gene from *Serratia marcescens*

Features : CutSmart, Recombinant, Time saver

Recognition site:

5' CCCGGG 3'

3' GGGCCC 5'

Reagents

(1) Nuclease-free water
(2) Restriction enzyme buffer including bovine serum albumin (BSA)
(3) Restriction enzyme (fast digest *SmaI*)

Procedure for RFLP

1. Polymerase chain reaction products (372 bp) were digested using the restriction enzyme *SmaI*, which was stored at -20° C before used.
2. Polymerase chain reaction products and the reagents were put out to room temperature and mixed well by vortex and spinned down.
3. The master mix was made as shown in table. The reagents were mixed gently by pipetting and then kept on ice for 2 minutes.
4. Then, 20 µl of master mix was put into each tube and finally 5 µl of PCR product was added to the individual tube. They were mixed gently upto final volume of 25 µl.
5. The tubes were incubated at the optimum temperature of *SmaI* (25° C) for 15 minutes.
6. After incubation, the digested products were taken out and loaded onto a 2% agarose gel to analyze for the corresponding pattern of bands.
7. Then, DNA ladder was used to analyze the different genotypes.
8. The point mutation was replaced a T by a G at nucleotide position +45 relative to the ATG (methionine) start codon, creating a *SmaI* restriction site (CCC/GGG).
9. After agarose gel electrophoresis, the PCR product was visualized under ultraviolet light. Genotypes were determined by comparing the length of the restriction fragment.

Then, it was photographed by using Polaroid camera.

Master mix for enzyme digestion

No	Reagents	Amount (µl)
1.	20000 units/ml *SmaI*	0.3
2.	10X buffer	2.5
3.	Distilled water	17.2
4.	PCR product	5
	Total final volume	25

For SNP +276

Restriction enzyme, *BsmI* (New England Biolabs, NEB)

Product source : An E.coli strain that carries the BsmI gene from *Bacillus stearothermophilus*

Features : CutSmart, Recombinant, Time saver

Recognition site

5' GAATGCN 3'

3' CTTACGN 5'

Reagents

(1) Nuclease-free water
(2) Restriction enzyme buffer including BSA
(3) Restriction enzyme (fast digest *BsmI*)

Procedure for RFLP

1. Polymerase chain reaction products (241 bp) were digested using the restriction enzyme *BsmI*, which was stored at -20° C before used.
2. Polymerase chain reaction products and the reagents were put out to room temperature and mixed well by vortex and spinned down.
3. The master mix was made as shown in table. The reagents were mixed gently by pipetting and then kept on ice for 2 minutes.
4. Then, 20 µl of master mix was put into each tube and finally 5 µl of PCR product was added to the individual tube. They were mixed gently upto final volume of 25 µl.
5. The tubes were incubated at the optimum temperature of *BsmI* (65° C) for 15 minutes.
6. After incubation, the digested products were taken out and loaded onto a 2% agarose gel to analyze for the corresponding pattern of bands.
7. Then, DNA ladder was used to analyze the different genotypes.
8. The point mutation was replaced a G by a T at nucleotide position +276 relative to the ATG (methionine) start codon, removing a *BsmI* restriction site (GCN/CGN).
9. After agarose gel electrophoresis, the PCR product was visualized under ultraviolet light. Genotypes were determined by comparing the length of the restriction fragment.

Then, it was photographed by using Polaroid camera.

Master mix for enzyme digestion

No	Reagents	Amount (µl)
1.	10000 units/ml *BsmI*	0.3
2.	10X buffer	2.5
3.	Distilled water	17.2
4.	PCR product	5
	Total final volume	25

The Republic of Union of Myanmar
Ministry of Health and Sports
Department of Human Resource for Health
University of Medicine 2, Yangon

No. 677/709, Khaymarthi Road, North-Okkalapa Township, Yangon 11031
Tel: +95-1-9690127, +95-1-9690128, +95-1-9690129 Fax: +95-1-9690265

Letter No. 1/ERC (2012)
Dated: 24th December 2012

The Ethics Review Committee on Medical Research Involving Human Subjects, University of Medicine 2, Yangon, approves to conduct the following proposed research project.

SINGLE NUCLEOTIDE POLYMORPHISM AT +276G>T OF THE ADIPONECTIN GENE AND PLASMA ADIPONECTIN LEVEL IN TYPE 2 DIABETES MELLITUS

Investigator: Dr. Khin Thin Yu

Professor Dr. Tint Swe Latt
Chairperson
Ethical Review Committee
University of Medicine 2, Yangon

(This approval is valid for the period of one year from the date mentioned.)