

# 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

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## **Authors' contributions**

*This work was carried out in collaboration among all authors. All authors read and approved the final manuscript.*

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## **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.

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**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 ( $27.41 \pm 16.7 \mu\text{g/ml}$ ) of T2DM patients was significantly lower than that of non-diabetic subjects ( $37.19 \pm 26.77 \mu\text{g/ml}$ ), ( $p =0.002$ ). 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- $\alpha$  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 palmitoyl 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 susceptibility 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 2241766 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 guanine (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)

5ml 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).

**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

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 94°C for 3 minutes, annealing at 94 °C 1minute, 57 °C 1 minute, 72 °C 1 minute for 34 cycles and final extension at 72 °C for 5 minutes and were hold at 4 °C. PCR cycle condition for SNP+276 were heat denaturation at 94 °C for 3 minutes, annealing at 94 °C 1minute, 60 °C 1 minute, 72 °C 1 minute for 35 cycles and final extension at 72 °C for 6 minutes and were hold at 4 °C.

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.

## 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. Heterozygote (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.

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)].

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.

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).

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).

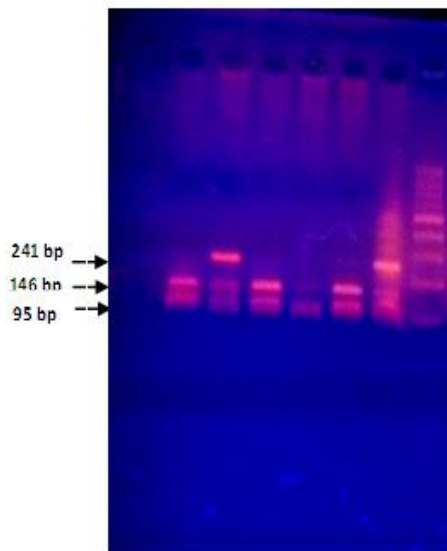


Fig. 1. PCR-RFLP of SNP +276

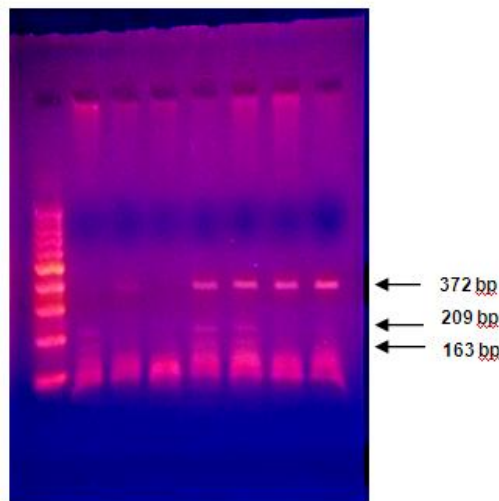


Fig. 2. PCR-RFLP of SNP +45

**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

$\chi^2=6.57, p=0.037$

**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

$\chi^2=9.48, p=0.008$

**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.

**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.

#### 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  $r^2 > 0.1$ ] were applied as a criterion for meaningful LD and [ $D' = 1$ ,  $r^2 = 1$ ] 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-γ 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.

## 5. 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.

## 6. 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.

## CONSENT

As per international standard or university standard, patients' written consent has been collected and preserved by the author(s).



## 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.

## COMPETING INTERESTS

Authors have declared that no competing interests exist.

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