

## Association of +10211T/G (rs17846866) Variant of Adiponectin Gene with Type 2 Diabetes Mellitus

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Various adiponectin gene (ADIPOQ) variants, located on chromosome 3q27 were associated with Type 2 diabetes mellitus (T2DM) in different ethnicity. In this study, it is aimed to find the association of +10211T/G (rs17846866) variant of ADIPOQ with T2DM and healthy controls in North Indians. In this study, 150 T2DM and 150 healthy control subjects aged between 25-75 years were recruited. Circulatory adiponectin levels were measured by commercially available ELISA kit. For genotype analysis, Polymerase chain reaction-restriction fragment length polymorphisms (PCR-RFLP) method was used. The genotypic analysis of rs17846866 variant of ADIPOQ has shown that there were no significant association of TT versus TG genotype ( $P=0.13$ ) as well as TT versus GG genotype ( $P=0.11$ ) with T2DM patients and healthy controls. However, the G allele frequency of the rs17846866 has shown significant association with T2DM (13.7%) as compared to healthy controls (7.7%,  $P=0.02$ ). In T2DM, circulatory adiponectin level was significantly lower in TT genotype than TG genotypes ( $P=0.01$ ). However, the circulatory adiponectin level was lower in GG genotype than TG genotype ( $P=0.49$ ), but not significant. The result showed that rs17846866 variant of ADIPOQ was associated with altered circulatory adiponectin levels. The TT genotype may be the major contributor to reduce the circulatory adiponectin levels in T2DM. However, the G allele may be increased the risk of T2DM in North Indians.

**Keywords:** ADIPOQ variant, rs17846866, Type 2 diabetes, Circulatory adiponectin.

Adiponectin was first identified in 1995, Scherer *et al* first characterized adiponectin as a novel 30-kDa secretory protein (Acrp30)<sup>1</sup>. Later in 1996, Nakano *et al* characterized adiponectin as mRNA transcripts of gelatin-binding protein of 28kDa size (GBP28)<sup>2</sup> and Hu *et al* described adiponectin as a protein found highly expressed in adipose tissue (AdipoQ)<sup>3</sup>. Arita *et al* isolated adipose most abundant gene transcript 1 (apM1) and named it adiponectin<sup>4</sup>. Further studies

demonstrated the multifactorial effects of adiponectin in adipocyte cells of adipose tissue and energy homeostasis which have a great impact on circulatory and storage sugar levels<sup>5,6</sup>. Lara-Castro *et al* identified that it is a highly expressed transcript in preadipocytes differentiating into adipocytes<sup>7</sup>.

The circulating adiponectin levels in plasma have a wide range (2 to 30 $\mu$ g/ml), and that contributes about 0.01% of plasma proteins in adult individuals. The plasma level of this protein

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varies in different ethnicity. Indo-Asian has lower plasma adiponectin levels than Caucasians (3.3 to 4.9 µg/ml) and even than the Japanese population (5 to 10 µg/ml). Genome-Wide Association Studies (GWAS) among Asian and European populations identified ADIPOQ locus as the major gene for alteration in the circulatory adiponectin levels<sup>8,9</sup>. ADIPOQ and its transcript has a versatile impact and linked multiple diseases such as diabetes<sup>10</sup>, metabolic syndrome<sup>11</sup>, obesity<sup>12</sup>, non-alcoholic fatty liver disease<sup>13</sup>, cardiovascular diseases<sup>14</sup>, neurodegenerative diseases<sup>15</sup>, and cancer<sup>16</sup>.

The ADIPOQ consists of 3 exons and 2 introns, contains about 16 kb of the genomic sequence, and located on chromosome 3q27<sup>17</sup>. We have included Intron 1 located +10211T/G (rs17846866) variant of ADIPOQ and associated with T2DM. Several studies reported that +10211T/G (rs17846866) variant of ADIPOQ contributed to T2DM<sup>18-20</sup> (Figure 1).

Indians are highly susceptible to diabetes compared to other Europeans, Americans, and Asians<sup>21</sup>. It is an urgent need to find the possible genetic link of diabetes and its pathophysiology in a different region of India to reduce the burden of diabetes and its complication. Therefore, in this study, it is aimed to find the association between rs17846866 variant of ADIPOQ and T2DM.

## METHODS

### Materials: Human blood samples

#### Subject selection

In this case-control study, 150 T2DM and 150 healthy controls were recruited aged between 25-75 years from outpatient diabetes clinic of Medical University. The study was carried out from May 2014 to October 2017. The study was approved by the ethical standards of the institutional committee. This study adhered to the principles of the 1964 Helsinki declaration and its later amendments or comparable ethical standards<sup>22</sup>. Written informed consent was taken from each subject recruited in the study.

Inclusion for T2DM subjects was done as per World Health Organization criteria (WHO)<sup>23,24</sup>, whereas for healthy controls subjects who were unrelated and having no T2DM were included. Subjects with cardiovascular diseases, chronic liver disease, chronic kidney disease, any other

infectious disease such as tuberculosis, chronic obstructive pulmonary disease, thyroid disorder, pregnant women were excluded for both (T2DM and healthy control) groups. Detailed medical history was taken from each subject.

The formula used for the sample size calculation:

$$n = \left( \frac{r+1}{1} \right) \frac{\overline{P(1-P)} (Z_{\beta} + Z_{\alpha/2})^2}{(P_1 - P_2)^2}$$

Where n = required sample size.; r = ratio of controls to cases.

P = average proportion exposed;  $Z_{\alpha}$  = the desired power (0.84 for 80%);  $Z_{\alpha}$  = level of statistical significance (1.96); P1 = proportion of cases exposed (P1=0.35); P2 = proportion of control exposed (P2=0.20); n= 139H<sup>25</sup>150 per arm; Total sample size H<sup>25</sup>300. Power of study is 80%.

#### Laboratory investigations

Total 5ml venous blood was withdrawn from each subject for genotyping analysis and biochemical investigation like FBS, PPBS, Lipid profile and Serum creatinine using commercially available ortho-clinical diagnostics kits (Johnson & Johnson) using Vitros-250 system chemistry autoanalyzer. HbA1c was measured by using whole blood EDTA samples on Bio-Rad D10 high-performance liquid chromatography (HPLC) analyzer (Bio-Rad, Hemel Hempstead, UK)<sup>25</sup>. Circulatory adiponectin levels were evaluated in serum using commercially available ELISA kit (USCN, Life Science Inc. Wuhan).

#### DNA extraction and Genotyping

DNA was extracted from human whole blood EDTA samples by using HiPurA<sup>TM</sup> SPP Blood DNA Isolation Kit (Cat. No. MB541, HIMEDIA, India). Spectrophotometric analysis (Systronics-2205) and agarose gel electrophoresis using the Gel Doc system (Bio-Rad, Gel Doc XR<sup>+</sup>, Universal Hood II) revealed the concentration and the purity of the genomic DNA<sup>26</sup>. PCR-RFLP method was used for genotyping<sup>27</sup>. Primers were checked by using InSilico PCR online software. The rs17846866 (+10211T>G) variant of ADIPOQ was amplified using the forward primer, 5'-GCTAAGTATTACAGATTTTCAGGGCAG-3' and the reverse primer, 5'-CAGCAACAGCATCCTGAGC-3'<sup>18</sup>. The PCR product size was detected 228bp. After that,

the PCR products were digested with 10U of *HinfI* enzyme (New England Biolabs, UK), at 37°C for overnight. The restriction enzyme digested fragments of PCR products were observed on a 2.5% agarose gel. The wild type TT homozygote was detected as 101bp and 89bp fragments show *HinfI* enzyme restriction site absent. The mutant

GG homozygote was detected as 134bp and 89bp fragments show *HinfI* enzyme restriction site present. The TG heterozygous contained the three fragments of 134bp, 101bp and 89bp (Figure 2).

**Statistical Analysis**

The IBM SPSS software version 20.0 (Armonk, NY, USA) was used to analyze the data.

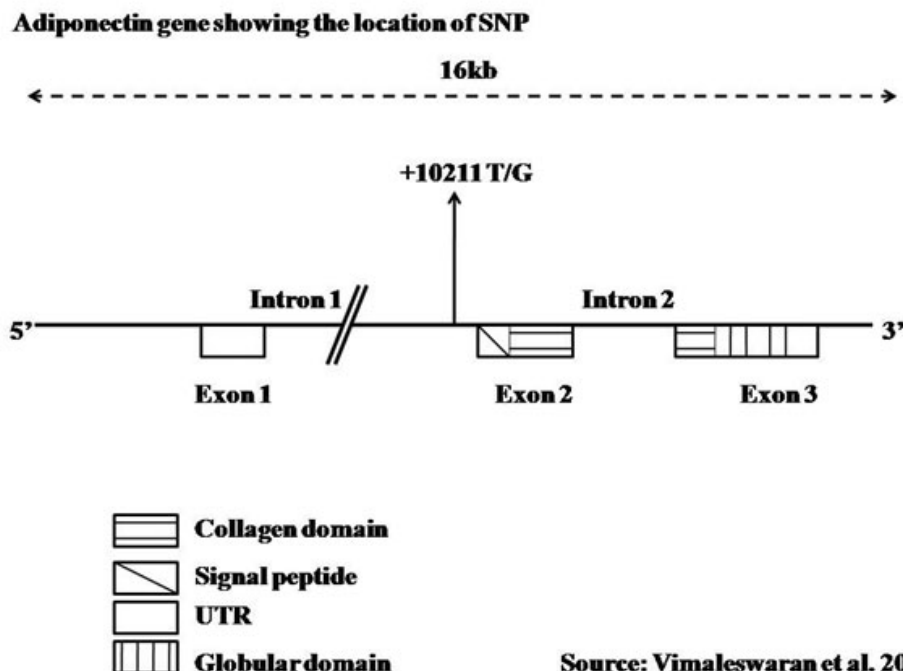


Fig. 1. The location of +10211T/G (rs17846866) variant of ADIPOQ identified in this study<sup>18</sup>

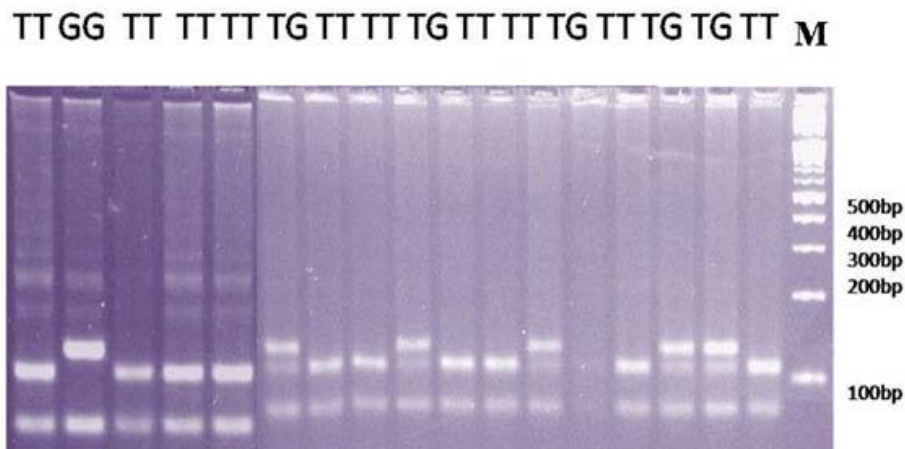


Fig. 2. Genotyping result for +10211T/G (rs17846866) variant of ADIPOQ. M: Marker, TT genotype: 101bp/89bp, TG genotype: 134/101/89bp, and GG genotype: 134/89bp

All the data were compared between the two groups by using the unpaired t-test. Values were given as a percentage (%) and mean  $\pm$  SD (Standard Deviation). Odds ratio (OR) and 95% confidence interval (CI) were used to present allelic and genotypic frequencies and that were analyzed using  $\chi^2$  test/Fischer's exact test. For all data, P-value  $<0.05$  was considered as statistically significant.

## RESULTS

In this study, 150 T2DM subjects with a mean age of (48.31 $\pm$ 10.88 years) and disease duration (4.79 $\pm$ 3.77 years), and 150 healthy control subjects with a mean age of (48.03 $\pm$ 11.83 years) were recruited. For the anthropometric and biochemical profile of T2DM and healthy control subjects referred to Khan *et al*<sup>17</sup>.

The genotype and allele frequencies of the rs17846866 first intronic region variant of ADIPOQ in T2DM and healthy controls are shown in (Table 1). The frequencies of the TT, TG and GG genotypes of rs17846866 were 76%, 20.7%, 3.3% in T2DM and 85.3%, 14%, 0.7% in healthy controls, respectively. The allele frequencies of the T and G were 86.3%, 13.7% in T2DM and 92.3%, 7.7% in healthy controls, respectively. There were

no significant association of homozygous TT and heterozygous TG genotype (OR: 0.60; CI: 0.33-1.11; P=0.13) as well as homozygous TT and GG genotype with T2DM and healthy controls (OR: 0.18; CI: 0.02-1.54; P=0.11). Although, T and G allele frequencies of the rs17846866 had a significant association with T2DM as compared to healthy controls (OR: 0.52; CI: 0.31-0.89; P=0.02).

Clinical characteristics of the T2DM according to rs17846866 genotypes of ADIPOQ are shown in (table 2). There was a significant impact of rs17846866 genotype on circulatory adiponectin level, TG, and VLDL (P=0.02, 0.04, and 0.04, respectively). FBS, PPBS, HbA1c, and SCr were not significantly associated with rs17846866 genotype. However, PPBS and HbA1c were found significantly higher in TT genotype as compared to GG genotype (P=0.02, P=0.009, respectively). Parameters of obesity and metabolic syndrome i.e. WC, WHR, BMI, HDL, and LDL did not show any significant association with rs17846866 genotype. Circulatory adiponectin level was significantly lower in TT genotype than TG genotype (P=0.01). However, the circulatory adiponectin level was lower in GG genotype than TG genotype (P=0.49), but not significant.

**Table 1.** The genotypes and allele distribution of rs17846866 variant of ADIPOQ in T2DM and healthy controls

rs17846866 Variant	T2DMN (%)	Healthy Controls N (%)	OR (95% CI)	P-value
Co-dominant				
TT	114 (76%)	128(85.3%)	1.00	
TG	31 (20.7%)	21 (14%)	0.60 (0.33-1.11)	0.13
GG	5 (3.3%)	1 (0.7%)	0.18 (0.02-1.54)	0.11
Dominant				
TT	114 (76%)	128(85.3%)	1.00	
TG+GG	36 (24%)	22 (14.7%)	0.54 (0.30-0.98)	0.06
Recessive				
TG+TT	145 (96.7%)	149 (99.3%)	1.00	
GG	5 (3.3%)	1 (0.7%)	0.19 (0.02-1.69)	0.21
Alleles				
T	259 (86.3%)	277 (92.3%)	1.00	
G	41(13.7%)	23 (7.7%)	0.52 (0.31-0.89)	0.02*

\*Significant considered as P<0.05.

Values are expressed as Number (N) and Percentage (%)

OR: Odd Ratio, CI: Confidence Interval

**Table 2.** Clinical characteristics of T2DM with reference to *rs17846866* genotypes of ADIPOQ

Parameters	Genotypes			TT vs. TG	P-value		
	TT (n=114)	TG (n=31)	GG (n=05)		TT vs. GG	TG vs. GG	P-value
FBS (mg/dl)	163.89±49.83	153.32±44.14	129.80±36.08	0.29	0.13	0.27	0.26
PPBS (mg/dl)	253.13±77.85	233.68±75.61	169.60±36.74	0.22	0.02*	0.07	0.21
HbA1c (%)	8.14±2.04	7.90±2.16	5.68±0.78	0.57	0.009*	0.03*	0.58
TC (mg/dl)	165.12±43.64	177.70±51.73	138.26±49.59	0.17	0.18	0.12	0.22
TG (mg/dl)	163.52±57.80	188.62±59.45	136.24±39.88	0.03*	0.29	0.07	0.04*
HDL (mg/dl)	39.00±11.36	39.11±11.47	40.72±20.58	0.96	0.75	0.79	0.96
LDL (mg/dl)	95.43±33.34	98.57±39.58	77.22±30.38	0.66	0.23	0.26	0.69
VLDL (mg/dl)	32.75±13.03	38.24±13.13	25.02±10.44	0.04*	0.19	0.04*	0.04*
SCr (mg/dl)	2.17±1.35	2.49±1.42	3.28±1.51	0.25	0.08	0.26	0.27
APN (ig/ml)	1.78±0.85	2.25±1.03	1.90±1.08	0.01*	0.76	0.49	0.02*
SBP (mmHg)	139.66±25.97	141.65±27.01	160.00±31.77	0.71	0.09	0.18	0.72
DBP (mmHg)	82.87±15.63	80.61±14.60	82.40±21.14	0.47	0.95	0.81	0.45
WC (cm)	97.94±6.81	97.45±5.56	100.60±4.84	0.71	0.39	0.24	0.68
WHR	0.98±0.07	0.97±0.06	1.01±0.05	0.47	0.35	0.17	0.43
BMI (kg/m <sup>2</sup> )	24.94±4.83	25.10±4.15	24.42±4.15	0.87	0.81	0.74	0.86

\*Significant considered as  $P < 0.05$ .

Values are expressed as Mean ± Standard Deviation

FBS: Fasting Blood Sugar, PPBS: Post-Prandial Blood Sugar, HbA1c: Glycated Haemoglobin, TC: Total Cholesterol, TG: Triglyceride, HDL: High-Density Lipoprotein, LDL: Low-Density Lipoprotein, VLDL: Very Low-Density Lipoprotein, SCr: Serum Creatinine, APN: Adiponectin, SBP: Systolic Blood Pressure, DBP: Diastolic Blood Pressure, WC: Waist Circumference, WHR: Waist to Hip Ratio, BMI: Body Mass Index

## DISCUSSION

This study was performed to explain the role of genetic susceptibility to T2DM. Pathogenesis of T2DM is not much known. It is a multifactorial disease which develops by the interaction of genetic and environmental factors. Genetic factor includes multiple genes involved in development of T2DM. In the present study, we included *rs17846866* variant of ADIPOQ to assess the genetic risk factor for T2DM. ADIPOQ variants were studied in different diseases<sup>28-30</sup> as well as in T2DM<sup>31-33</sup>. We found a significant association of hypoadiponectinemia with T2DM. Similar results were reported in the literature in different ethnic group<sup>27, 33-34</sup>. Though, genetic studies were performed, limited and with inconsistent results. Alteration in circulatory levels of adiponectin was significantly associated with *rs17846866* variant of ADIPOQ in T2DM. The *rs17846866* variant dominant mode of inheritance showed nearly significant (TT vs. TG+GG,  $P=0.06$ ) association with T2DM. Vimalaswaran *et al.* explained that the dominant mode of inheritance because the G allele (TG genotype) found a significantly higher

risk than TT genotype while GG genotype did not have a higher risk than TG genotype. The G allele was found a significant association with T2DM in our results ( $P=0.02$ ) and T allele is more common in healthy controls. Vimalaswaran *et al* found that TG genotype of *rs17846866* had significantly higher risk for diabetes as compared to TT genotype and also associated with hypoadiponectinemia<sup>18</sup>. However, GG genotype has not associated with diabetes (OR: 0.18, CI: 0.02–1.54,  $P=0.11$ ). The lack of GG genotype association with diabetes may be due to low frequencies of that GG genotype in both the T2DM and NHC groups. Saxena *et al* reported that *rs17846866* variant of ADIPOQ increases the risk of T2DM<sup>19</sup>. Haplotype analysis and linkage disequilibrium study should be conducted between *rs17846866* and other variants of ADIPOQ, because GG genotype and G allele of other variants of this gene reported a significant association with hypoadiponectinemia and T2DM<sup>35, 36</sup>. We observed that genotypic variation of *rs17846866* variant of ADIPOQ had a significant association with hypoadiponectinemia and GG genotype have shown 1.5 folds higher serum creatinine as compared to TT genotype.

This indicated that the ADIPOQ variant may be contributing the risk for diabetic nephropathy in T2DM patients. The previous study was reported that ADIPOQ variants have a strong correlation with the progression of diabetic nephropathy in T2DM patients<sup>37</sup>. Nazir *et al* have also found a significant positive association of ADIPOQ with diabetic nephropathy<sup>38</sup>. In addition, we observed that there was a significant impact of rs17846866 variant on TG, VLDL, and circulatory adiponectin levels. Raised TG and VLDL indicated dyslipidemia in T2DM patients. Hypoadiponectinemia and dyslipidemia in T2DM patients may contribute to increasing the risk for CVD. Verges reviewed that abnormalities of lipoprotein metabolism and increased TG and VLDL are one of the major risk factors for CVD in T2DM patients<sup>39</sup>. However, several studies have shown a discrepancy in the association of hypoadiponectinemia and CVD. Elevated circulatory adiponectin levels were independently linked with lower 10-year CVD risk in adults<sup>40</sup>. Similarly, Cheung *et al* were reported that ADIPOQ gene variant and hypoadiponectinemia is the independent predictor of CHD<sup>41</sup>. However, Menzaghi *et al* reviewed that high serum adiponectin is simply a marker of insulin sensitivity and glucose homeostasis, not a marker of CVD risk<sup>42</sup>. Similarly, the ADIPOQ variant associated with adiponectin levels was not associated with cardiometabolic risk factors<sup>43</sup>.

We did not find any association of WC, WHR, BMI, HDL, and LDL; this indicating that the variant of ADIPOQ included in our study is not associated with obesity or metabolic syndrome. The majority of study supported that ADIPOQ variants are associated with body weight, BMI and WHR i.e. obesity<sup>44, 45</sup>. Reasons for this is that the variant of ADIPOQ included in our study not associated with obesity but other variants of ADIPOQ gene is responsible for this<sup>30, 46</sup>.

#### Limitations and recommendations

Our study sample size might be smaller and we have taken only one variant rs17846866 of ADIPOQ. This study should be duplicated in a larger sample size with including other variants of ADIPOQ. Haplotype analysis and linkage disequilibrium study should be conducted between rs17846866 and other variants of ADIPOQ.

## CONCLUSION

The result showed that rs17846866 variant of ADIPOQ was associated with altered circulatory adiponectin levels. The TT genotype may be the major contributor to reduce the circulatory adiponectin levels in T2DM. However, the G allele may be increased the risk of T2DM in North Indians.

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