Tumour necrosis factor-α –308G/A polymorphism is associated with insulin secretory defects in Bangladeshi prediabetic/diabetic subjects

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Abstract

Objectives: Tumour necrosis factor (TNF)-α, an adipocytokine, is closely linked to impaired glucose tolerance (IGT) and insulin resistance (IR) in type 2 diabetes (T2D) subjects. The relationship between the polymorphisms in the TNF-α gene and IR in Bangladeshi prediabetics and T2D subjects has not yet been fully identified. This study aims to reveal the association between TNF-α gene polymorphism and IR in hyperglycaemic patients of Bangladeshi origin.

Methods: In our study, 106 IGT, 100 T2D, and 109 healthy subjects of Bangladeshi origin were recruited to identify the impact of TNF-α gene polymorphism at position –308 with a G>A transition using PCR and subsequent restriction fragment length polymorphism (RFLP).

Results: The –308G>A TNF-α genotype frequency distribution within the control, IGT, and T2D groups showed a significant association ($\chi^2 = 21.077; P = 0.001$), although allele frequency distribution within the groups showed a
statistically non-significant difference ($\chi^2 = 1.696; P = 0.091$). β-cell functional deficiency (HOMA-B%) was observed to be significantly ($P = 0.034$) lower in subjects with a variant genotype. In addition, our results indicate that the study subjects’ body mass index (BMI) and residence status were positively correlated ($P \leq 0.05$) with $-308G>A$ TNF-$\alpha$ gene polymorphism.

**Conclusions:** Therefore, it can be concluded that $-308G>A$ TNF-$\alpha$ gene polymorphism may have a causative relationship with lower insulin secretory capacity and higher BMI in Bangladeshi IGT and T2D populations, while the urban population’s lifestyle might be associated with this polymorphism.

**Keywords:** -308G>A; Diabetes mellitus; Glucose intolerance; Insulin resistance; Polymorphism; Tumour necrosis factor-alpha

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**Introduction**

Type 2 diabetes (T2D) is an accredited metabolic and multifactorial disorder that is defined by a high blood glucose level due to defects in insulin functionality or a deficiency in insulin secretion.1,2 People with prediabetes have a greater chance of progressing to T2D.3 Prediabetes can be defined as a blood glucose level that is intermediate between normoglycaemia and the diabetes threshold. This can be ascertained either through impaired fasting glucose (IFG) or impaired glucose tolerance (IGT).4 Obesity, inactivity, and family history are the key factors that increase the possibility of developing T2D and have been found to be highly associated with complications related to this disease.5 Apart from the role of environmental determinants, numerous genetic factors influence disease susceptibility.6 Several studies have reported the existence of a close relationship between T2D and the different genes that encode molecules with diverse functions.7

Tumour necrosis factor (TNF)-$\alpha$, a cytokine that originated predominantly from macrophages and adipocytes, can promote insulin resistance (IR) and ultimately assist the progression of T2D.8 The TNF-$\alpha$ gene in humans is mapped at the 6p21.3 position of the chromosome encoding a polypeptide consisting of 157 amino acids.9 The interrelation between the TNF-$\alpha$ gene’s single nucleotide polymorphisms (SNPs) and T2D has been extensively examined in different populations. Kubaszek et al. reported that polymorphism in the promoter region of the TNF-$\alpha$ gene predicts the progression of T2DM from IGT.10–12 Among other SNPs, TNF-$\alpha$ promoter SNPs such as $-308G/A$ and $-238G/A$ are well-characterised and have been found to be correlated with IR and T2D in different studied populations.13,14 Indeed, $-308$ polymorphism has been shown to enhance transcriptional activity, affecting its gene expression with a rare A-allele over the G-allele, signifying the functionality of this SNP.15 The TNF-$\alpha$ gene with this unusual A-allele is supposed to be linked to body fat and hence obesity, as well as IR, contributing to the evolvement of T2D.10,16 Although TNF-$\alpha$ SNP and its association with T2D have been widely studied, the results are inconsistent. This is probably due to the variation from population to population, which is not yet fully understood.13,17,18 Previously, we reported that serum TNF-$\alpha$ is positively associated with Bangladeshi prediabetic populations and is also associated with insulin secretory defects.18 Therefore, this study aims to explore the impacts of TNF-$\alpha$ $-308G>A$ promoter SNP on IR and identify its association with prediabetes and T2D in the Bangladeshi population.

In this study, we show that $-308G>A$ TNF-$\alpha$ gene polymorphism may have a causative relationship with lower insulin secretory capacity and higher BMI in Bangladeshi IGT and T2D populations, while the urban population’s lifestyle might be associated with this polymorphism.

**Materials and Methods**

**Study subjects**

This research was carried out in the laboratory of the Department of Physiology and Molecular Biology, Bangladesh University of Health Science (BUHS), Dhaka. A total of 106 IGT and 100 T2D subjects of Bangladeshi origin were recruited, along with 109 age-matched healthy controls. World Health Organization (WHO) guidelines (1999) were followed to identify IGT and T2D subjects.19 The study participants submitted signed informed consent letters. Data were collected using a pretested standard questionnaire. Patients who were newly diagnosed as either IGT or T2D were included, and patients suffering from any chronic illness, gestational diabetes, or mental disorder were excluded from our study. The power of the study was not calculated, but we calculated the sample size to derive the appropriate proportion of the population needed for this research, using the following statistical formula:

$$\text{Sample Size (SS)} = Z^2 \times (p \times (1-p)) / \frac{C^2}{2}$$

where $Z = Z$ value (e.g. 1.96 for a 95% confidence level), $p =$ percentage picking a choice, expressed as a decimal (0.5 used for the sample size needed), and $C =$ confidence interval, expressed as a decimal (e.g. 0.04 = ±0.4).

**Assessment of demographic and anthropometric parameters**

Demographic information was collected by interviewing the subjects. Anthropometric parameters such as weight, height, and waist and hip circumference were measured for each of the subjects using standard methods, according to the WHO guidelines.6

**Blood sample collection**

Blood was drawn by venipuncture under the overnight fasting condition (10–12 hours) and after 2 hours of glucose administration (75 g dissolved in 250 ml water). After 30 minutes, samples were then centrifuged at 3000 rpm for 10 minutes to produce serum. The serum was preserved in the freezer.
Electrophoresis (2% agarose gel) following ethidium bromide staining.  

**Biochemical analysis**

The glucose–oxidase method was used to measure the serum glucose level. Serum total cholesterol (TC), triglyceride (TG), and HDL-C were measured using cholesterol oxidase assay, glycerophosphate oxidase assay, and cholesterol oxidase assay, respectively. To calculate serum LDL-C, Friedewald’s equation was used. Serum creatinine and serum SGPT were measured with the alkaline picro method and the UV-spectrophotometric method using commercial kits (Randox Laboratories, Ltd., UK), respectively. Fasting serum insulin levels were determined using the ELISA method (Linco Research Inc., USA). We employed homeostatic model assessment (HOMA) to measure β-cell functional deficiency (HOMA B%), insulin sensitivity (HOMA S%), and insulin resistance (HOMA IR) based on fasting serum glucose and fasting serum insulin level. HOMA IR and HOMA B% were obtained using the following formulas:  

(a) HOMA-IR = Glucose x Insulin/22.5  
(b) HOMA-B % = 20 x Insulin/Glucose-3.5, if the glucose in molar units (mmol/L)  

**Genetic analysis of TNF-α genotypes**

**Extraction of DNA and PCR analysis**

We used the Commercial GenElute DNA extraction kit (QIAGEN, USA), and then performed PCR using specific primers for TNF-α gene amplification [5'-AGGCAATAGGTGGTAGGGCCAT 3'- (sense); 5'-TCTCCTCCGTCCGCATTCCG-3' (antisense)] [31]. The master mix included 4 μL of genomic DNA (10–50 ng/μL), 10 pmol of each of the primers, 200 μmol/L dNTPs, 0.35 units of HotStar Taq polymerase (QIAGEN, USA), 1 μL of PCR buffer, and 3.23 μL of nuclease-free water in a final volume of 10 μL. PCR was carried out in a thermal cycler (Biometra, Germany) according to the following steps: i) initial denaturation (95 °C for 15 minutes), ii) denaturation (95°C for 30 seconds and 34 cycles), iii) annealing (58°C for 45 seconds), iv) elongation (72°C for 45 seconds), and v) final elongation (72°C for 10 minutes). Amplicons were identified via agarose gel electrophoresis (2% agarose gel) following ethidium bromide staining.  

Restriction fragment length polymorphism  

Genotyping of TNF-α –308G>A polymorphism was determined via the RFLP method using the Neo I restriction enzyme. Five μL of PCR products were taken in a separate tube and digested with the Neo I restriction enzyme at 37 °C for 4 hours (New England Biolabs, USA) in a water bath. Next, we performed agarose gel electrophoresis using 3.5% gel to resolve the fragments and then verified using UV spectrometry. In this experiment, we used ethidium bromide for staining.  

**Statistical analysis**

The Statistical Package for Social Science (SPSS), Windows version 17 (SPSS Inc., Chicago, Illinois, USA) was used to analyse the data. An unpaired Student’s t test was performed to assess the comparison between the two groups. ANOVA-Bonferroni analysis was performed to explore the relationship with the anthropological and biochemical characteristics. In appropriate situations, we performed a Chi-square ($\chi^2$) test. Statistically significant values were regarded as having a P value of <0.05.

**Results**

**Anthropological and biochemical parameters**

Among the anthropometric and biochemical parameters, IGT and T2D subjects’ BMI, WHR, SBP, FSG, PPG, TG, cholesterol, LDL-C, fasting insulin, and HOMA IR were observed to have higher values than in the control group ($P = 0.001$), whereas HOMA %B and HOMA %S were found to be significantly reduced in the IGT and T2D groups ($P = 0.001$) (Table 1, Figure 1).

**Allele and genotype frequency of the TNF-α gene –308 G>A**

The genotype frequency distribution within the groups showed a statistically significant association in the $\chi^2$-test ($\chi^2 = 21.077; P = 0.001$). The allele frequency distribution within the groups showed a non-significant association ($\chi^2 = 1.696; P = 0.091$) (Table 2).

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**Figure 1:** Representative electrophoretic patterns of TNF-α gene –308G>A polymorphism analysis by Neo I restriction enzyme digestion. Fragments produced were as follows: Homozygous wild genotype 87 bp and 20 bp; heterozygous (Ht) variant genotype 20 bp, 87bp, and 107 bp; and homozygous (Hz) variant genotype 107 bp. 20 bp is too small and cannot be resolved in 3.5% Agarose gel.
As clinical parameters, we considered age, BMI, WHR, blood pressure, FSG, PPG, HOMA %B, HOMA %S, and HOMA IR (Table 3). Among them, the study subjects’ BMI and HOMA %B had a significant association with the variant genotype (\(P = 0.037\) and \(P = 0.034\), respectively).

### Clinical parameters in regard to the \(-308G>A\) TNF-\(\alpha\) gene

As clinical parameters, we considered age, BMI, WHR, blood pressure, FSG, PPG, HOMA %B, HOMA %S, and HOMA IR (Table 3). Among them, the study subjects’ BMI and HOMA %B had a significant association with the variant genotype (\(P = 0.037\) and \(P = 0.034\), respectively).

### Frequency of the \(-308 G>A\) TNF-\(\alpha\) genotype in regard to residence

In regard to residential status, we divided our study population into a rural group and an urban group. The frequencies of the wild and variant genotype for both groups are shown in Table 4. A significant association was found among the groups in the \(\chi^2\)-test (\(\chi^2 = 14.78\) and...
Since T2DM and IGR were found to be higher in the urban population, TNF-\(\alpha\)-308G/A polymorphism was also found to be higher in this group. This suggests a direct correlation between hyperglycaemia and TNF-\(\alpha\)-308G/A polymorphism in the urban population.

**Discussion**

TNF-\(\alpha\), an important adipokine, can inhibit insulin activity by impairing insulin signalling pathways and exert a potent effect in the early development of T2D through the destruction of pancreatic beta cells.\(^{14,23}\) Polymorphism in the TNF-\(\alpha\) gene has a startling impact on susceptibility to T2D.\(^{14}\) The effect of the \(-308G>A\) TNF-\(\alpha\) SNP on obesity, IR, and T2D is inconsistent among different populations.\(^{23,17}\) Several studies have reported a probable correlation between the \(-308G>A\) variant of the TNF-\(\alpha\) gene and susceptibility to T2D in subjects from diverse populations.\(^{23,17}\) Some studies have also reported the opposite result through a negative association with IR and obesity.\(^{23,21}\) However, ethnic differences may be responsible for the variation in these results, since TNF-\(\alpha\) \(-308G>A\) polymorphism can vary among different ethnic populations. In addition, an inappropriate study design, an insignificant sample size, or related environmental factors may have played a role in obtaining such results.\(^{12}\)

In this study, multiple biochemical tools related to T2D and IGT were considered. Among them, the study subjects’ BMI, WHR, SBP, lipid profiles (TG, cholesterol, and LDL), and glucose and insulin levels showed a statistically significant difference within the control, IGT, and T2D groups (\(P \leq 0.05\)). These are common phenomena in prediabetic and diabetic subjects, as documented in other studies.\(^{21,24}\) HOMA is a widely used tool for assessing IR, insulin sensitivity, and the insulin secretory capacity of pancreatic \(\beta\)-cells. Our study subjects’ HOMA %S, HOMA%B, and HOMA IR were also found to exhibit a significant difference among the groups (\(P \leq 0.05\)). These findings are concordant with Song et al.’s study involving an exclusively female population; they showed that HOMA indices are associated with diabetes risk.\(^ {23}\)

Very limited evidence is available to prove the effect of TNF-\(\alpha\) gene promoter SNP on T2D progression in subjects with prediabetes. In our study, although allele frequency distribution between the groups showed a non-significant difference (\(P = 0.091\)), the genotype frequencies of the \(-308G>A\) variant of the TNF-\(\alpha\) gene among the control, IGT, and T2D subjects showed significant differences (\(P < 0.001\)). This finding could be an indication of the link between \(-308G>A\) TNF-\(\alpha\) polymorphism and prediabetes, which may influence the progression of T2D in the Bangladeshi population. Our findings are consistent with those of Liu et al., who observed a close link between \(-308G>A\) TNF-\(\alpha\) gene polymorphism and an increased chance of developing T2D in the Chinese population.\(^ {20}\) Our results also support a meta-analysis of the Asian population; however, they contradict Feng et al.’s meta-analysis, which found no significant association.\(^ {27,12}\) Although it appears that \(-308G>A\) TNF-\(\alpha\) polymorphism is only limitedly associated with Bangladeshi IGT and T2D subjects, further studies with a large dataset are required to validate the current results.

In our study, the wild and variant TNF-\(\alpha\) genotypes were compared with different variables. Our results indicate that the \(-308G>A\) genotype of TNF-\(\alpha\) is significantly linked to BMI (\(P = 0.037\)). Therefore, it can be suggested that BMI may be related to \(-308G>A\) TNF-\(\alpha\) polymorphism, probably acting as a predictor of obesity and ultimately a risk indicator in the development of T2D. Um et al. studied obese Korean women and reported that \(-308G>A\) TNF-\(\alpha\) polymorphism is unrelated to BMI, but Dalziel et al.’s study of a white population supports our findings, as \(-308G>A\) polymorphism was observed to have an association with obesity.\(^ {28,17}\) Another study showed that subjects with an A allele over the G allele were at a greater risk (23%) of developing obesity.\(^ {13}\) However, Um et al. indicated a significant association between this polymorphism and WHR based on lower WHR in subjects with GA or AA genotypes. However, their result is not consistent with our observations. Our data showed that \(-308G>A\) TNF-\(\alpha\) polymorphism has no significant effects on WHR.\(^ {28}\) Furthermore, our study indicated no significant role of \(-308G>A\) TNF-\(\alpha\) polymorphism with respect to age, which contradicts the findings of Heijmans et al., who reported that \(-308G>A\) TNF-\(\alpha\) polymorphism is significantly related to T2D in old age.\(^ {27}\) Ethnic variations within different countries could be the prime reason for such contradictions. For this reason, we were interested in performing this experiment on Bangladeshi subjects, as no studies have yet been done to confirm the effects of this polymorphism under the prediabetic condition and in the conversion of diabetes mellitus within this population.

Kubaszek et al. reported that lifestyle changes interact with TNF-\(\alpha\) \(-308G>A\) polymorphism.\(^ {10}\) Consistent with this, urban subjects participating in the present study showed a significantly (\(P = 0.001\)) higher frequency of variants in TNF-\(\alpha\) \(-308G>A\) polymorphism. This variation may be explained by lifestyle changes among urban people or by increased environmental pollution in urban areas. Thus, it can be suggested that the \(-308G>A\) TNF-\(\alpha\) SNP might be associated with lifestyle change or environmental pollution.

Our study demonstrated that the circulating insulin level did not differ in the subjects with the TNF-\(\alpha\) \(-308G>A\) wild versus variant genotypes and that there were also no differences for HOMA-IR and HOMA %S. Our findings are similar to those Liu et al. obtained within a Chinese

### Table 4: Frequency of the \(-308G>A\) TNF-\(\alpha\) genotype variant in regards to residence.

| Residence | Wild (n) | Variant (Ht & Hz) (n) | \(\chi^2/p-value\) |
|-----------|---------|----------------------|------------------|
| Rural     | 0.95 (152) | 0.05 (8) | 14.78/0.001 |
| Urban     | 0.81 (123) | 0.19 (29) | |

Data are represented as frequency (number). Ht: Heterozygous variant, Hz: Homozygous variant.
population. They did not observe any effects of the -308G>A TNF-α variant on fasting serum insulin level and HOMA-IR. In our study, differences in HOMA%B between the genotypes (wild GG and variant GA, AA) showed statistical significance (P = 0.034). A significant decrease in HOMA%B was observed in the study subjects with the variant genotype (Table 4), which indicates that β-cell function may be decreased in the presence of a variant genotype, leading to decreased glucose uptake, and may ultimately play role in the development of T2D. However, no data were reported to show such an association between HOMA%B and TNF-α variant genotypes.

Overall, our present experiment lays the foundation for determining the obvious effects of TNF-α gene polymorphism in Bangladeshi prediabetic and diabetic subjects and suggests that -308G>A SNP could be a potent susceptible target. The experiment is limited by a relatively small data size, which may limit some of our assumptions. Therefore, further research that includes gene and protein expression analyses with a large sample size will be beneficial to draw a certain conclusion by verifying the effects of TNF-α SNPs on Bangladeshi people’s disease (prediabetes or diabetes) susceptibility.

Conclusion

Based on our findings, we can assume that TNF-α–308G>A polymorphism may have a causative relationship with pancreatic β-cells’ lower insulin secretory capacity. Moreover, -308G>A variant genotypes of the TNF-α gene are significantly associated with a higher BMI, which might act as a predictor of obesity in prediabetic and T2D subjects. Finally, urban people seem to be largely affected by variant genotypes of the TNF-α–308G>A gene.

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Conflict of interest

The authors have no conflict of interest to declare.

Ethical approval

The work was carried out with ethical approval from the Institutional Animal, Medical Ethics, Biosafety and Biosecurity Committee (IAMEBBC), Institute of Biological Sciences, University of Rajshahi, Bangladesh (Resolution no 05 of the 2nd meeting held on 4th January, 2012). The certificate reference number is 21/320/IAMEBBC/IBSc (issued on 7th January, 2012).

Authors contributions

The authors have declared the following contributions: concepts and design by MMH, MOF, and ZH; experiments performed by SP, MD, and TS; data analysed by MMH, SP, MD, TS, MOF, and ZH; tables and figures prepared by MMH, SP, MD, and TS; manuscript written by MMH, SP, MD, MOF, and ZH. All authors have critically reviewed and approved the final draft and are responsible for the content and similarity index of the manuscript.

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