



Identification of TNF- α -308G/A (rs1800629) polymorphism in Bangladeshi population related to type-2 diabetes

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ABSTRACT

This study was designed to inspect the association between tumor necrosis factor (TNF)- α -308G/A (rs1800629) polymorphism with diabetes mellitus type-2 (DMT2) in Bangladeshi population. Besides, the role of TNF- α -308G/A in early proliferation of DMT2 has been investigated. The allelic frequency of TNF- α -308G/A and their association with DMT2 was studied using high resolution melting (HRM) analysis and confirmed using sequencing. A few demographic risk factors associated with DMT2 were also investigated routinely. The significance of these risk factors was analyzed statistically. We have analyzed 657 individuals who were distributed into two groups: 330 non-diabetic controls and 327 DMT2 individuals. HRM analysis shows that 11 individuals bare G/A and 2 bare A/A genotype in DMT2 patients. Within non-diabetic individual, we found only one with G/A genotype. The frequency of TNF- α -308G>A are within the Hardy-Weinberg equilibrium (0.00482) at 95% confidence level. TNF- α -308G>A frequency in two age group based on first time diagnosed, we found association with early proliferation of DMT2 with a *p*-value of 0.008965 in Fisher's test at 95% confidence level. Our result suggested that the single nucleotide polymorphisms TNF- α -308G>A is closely associated with DMT2 patients in Bangladeshi population. Besides, presence of TNF- α -308G>A polymorphism increases the risk of early proliferation of DMT2.

1. INTRODUCTION

Genotype screening in human disease frequently results in the identification of sequence variations whose direct connection with occurrence of disease is often remains unclear. Extensive research work has elucidated now that many of these seemingly harmless changes are actually part of etiology of several diseases. DMT2 is a recognized metabolic disorder that has turned to a major health issue throughout the world. DMT2 is a complex polygenic disorder [1] illustrated by impaired insulin resistance, insulin secretion, and deregulation of lipid and protein metabolism with environmental and multiple genetic susceptibility [2,3].

Tumor necrosis factor (TNF) is a proinflammatory cytokine, which is responsible for the inflammation process. In addition, it reasons in upregulation of cell adhesion molecule expression, leukocyte recruitment, apoptosis induction, and monocyte chemo-attraction. It also is responsible for the amplification of the immune response through stimulation of the expression of various transcription factors, growth factors, and other inflammatory mediators [4]. However, several studies have demonstrated that elevated levels of TNF- α production in both rodents and human subjects as a causative factor for insulin resistance and the pathogenesis of DMT2 [1,5-7]. This observation was further complemented with administration of exogenous TNF- α to animals to induce insulin resistance, whereas neutralization of TNF- α can improve insulin sensitivity [8-10]. This implies the association of TNF- α expression level with DMT2. For this reason, etiological studies of single nucleotide polymorphisms (SNPs) in promoter region of TNF- α have drawn significant attention as potential risk factor for DMT2 because it regulates the expression of the protein. The 308G>A

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(rs1800629) is one such mutation that is located at position 308 in the promoter region of the TNF- α gene and involves a substitution of adenine (A) for guanine (G). The 308G>A polymorphism was previously shown to be associated with the progression of impaired glucose tolerance in case of type-2 diabetes in Finns [11] and Asians [12,13]. Although there are some conflicts of findings within distinct ethnic groups [14–16] but confirmed using meta-analysis for Asian population [13]. However, based on the literature review, research regarding allelic frequency of 308G>A polymorphism has not conducted on Bangladeshi population, which represents a significant ethnic group within Asia in terms of population size. Besides, correlation between age and 308G>A polymorphism has not been studied yet.

2. MATERIALS AND METHODS

2.1. Sample collection, biochemical test, and genomic DNA isolation

This study was performed in accordance with guidelines approved by the Institutional Animal, Medical Ethics, Biosafety and Biosecurity Committee (IAMEBBC) for Experimentations on Animal, Human, Microbes and Living Natural Sources (Memo number: 58/320/IAMEBBC/IBSc), Institute of Biological Sciences (IBSc), University of Rajshahi, Rajshahi, Bangladesh. A total of 657 individuals were accumulated in this study that was distributed into two groups: 330 non-diabetic controls and 327 DMT2 individuals. Peripheral blood samples were collected from clinically diagnosed DMT2 patients and non-diabetic individuals. Non-diabetic individuals served as control. Written informed consent from all the participating individuals was taken. Samples were randomly collected in EDTA.K₃ vacuomed tubes from different parts of the country. A little part of the blood was used for genomic DNA isolation and the rest was used for selected blood parameter testing, i.e., HbA1c, triglyceride (Tg), and cholesterol. Genomic DNA was isolated from blood using Wizard Genomic DNA isolation Kit (Promega, USA) according to the manufacturer's instructions. DNA concentration was measured by using UV spectrophotometer as well as agarose gel visualization. A further salt removal stage was performed using Sephacryl S-400 (GE Healthcare, USA) gel filtration.

2.2. High resolution melting (HRM) analysis and sequencing

A 110 bp sequence within TNF- α promoter region was targeted for HRM analysis. Primer sequences were as follows: TNF forward 5'-CCACAGACCTGGTCCCCA-3' and TNF reverse 5'-GGTCTTCTGGGCCACTGACT-3' (DNA Technology A/S, Denmark). PCR conditions were optimized as described previously [17] before HRM analysis and DNA concentration was again normalized based on qPCR Cq value. HRM was done using GoTaq qPCR master mix (2X) at 60°C for 40 cycles by using Eco™ qPCR system (Illumina, USA). However, each qPCR reaction mixture (10 μ l) for HRM analysis comprised of 5 μ l GoTaq® qPCR master mix (2X), 3 μ l nuclease free water, 0.5 μ l forward primer, 0.5 μ l reverse primer, and 1 μ l template DNA. qPCR was performed with the following cycling conditions: 95°C for 10 minutes, followed by 40 cycles of 95°C for 10 seconds, 60°C for 30 seconds, and 72°C for 15 seconds by using Eco™ qPCR system

(Illumina, USA). PCR reaction specificity was confirmed by melt curve analysis at 95°C for 15 seconds, 55°C for 15 seconds, 95°C for 15 seconds. HRM results were analyzed by Eco™ software (Illumina, USA). Identified genotypes were further confirmed using sequencing (1st BASE, Malaysia). Sequencing primers TNF sequence forward 5'-TGC CCC AGT GGG GTC TGT GA-3' and TNF sequence reverse 5'-AGC TTG TCA GGG GAT GTG GCG T-3' were targeted for 750 bp sequence from both directions.

2.3. Statistical analysis

Statistical analysis was carried out using the statistical program SPSS (version 19, IBM Corporation, USA). Frequencies of genotypic difference were compared using the χ^2 (Chi-square) test. One-way analysis of variance was utilized to compare the clinical features among groups. Fisher's *t*-test was performed to check the correlation between age and genotypes.

3. RESULTS

3.1. Demographic characteristics

The potential physiological or pathophysiological association of TNF- α in lipid metabolism and *in vivo* insulin sensitivity has been previously shown [18,19]. Therefore, in this study, besides the allelic distribution of 308G>A we also investigated the common obesity associated risk factors within the population. Our analysis shows that the means of body mass index (BMI), HbA1c, Tg, and cholesterol among DMT2 patients were higher than control group (Table 1). The mean BMI was 28.85 \pm 2.88 for control and 29.46 \pm 2.57 for DMT2 (Table 1). Mean of HbA1c of diabetic individuals (50.20 \pm 8.84) is higher than non-diabetic controls (37.36 \pm 2.61) (Table 1). The means of Tg and cholesterol between patients and non-diabetic control are also higher (Table 1). According to the data (Table 1), the significant difference for BMI (*p*-value = 0.0049), HbA1c (*p*-value = <0.0001), Tg (*p*-value = <0.0001), and cholesterol (*p*-value = <0.0001) was detected between DMT2 and non-diabetic control.

3.2. HRM-based genotyping analysis

PCR amplicon was melted using HRM and unmatched melting curve was detected with appropriate optical detection system. A characteristic melting curve was generated between 81.5°C and 86.7°C and processed by integrated HRM analysis software installed in the platform. Mutations were identified and confirmed by comparing the pattern of our HRM melt curve with a well-established melt curve pattern reported previously [20]. For each SNP, heterozygous genotype (G/A) resulted in an altered melting wider curve shape that was easily identified. Homozygous

Table 1: Demographic characteristics regarding the collected blood sample.

Parameters	DMT2	Non-diabetic control	<i>p</i> -value
BMI	29.46 \pm 2.57	28.85 \pm 2.88	0.0049 significant
HbA1c (mol/l)	50.20 \pm 8.84	37.36 \pm 2.61	<0.0001 significant
Tg (mg/dl)	156.02 \pm 27.93	145.45 \pm 19.70	<0.0001 significant
Cholesterol (mg/dl)	228.92 \pm 28.30	218.38 \pm 20.19	<0.0001 significant

allelic frequency differs in India between the Punjabi and Bengali Hindu population [37,38]. Bengali population of India and Bangladeshi population are anthropometrically very similar though separated through international borderline. However, our frequency distribution analysis showed association between 308G>A and DMT2. This observation is also similar to other observations within Asian population as well as Indian population [32–35]. Recent trend of meta-analysis for error correction of case-control study could have been good but unfortunately there is no other data of TNF- α -308G>A allelic frequency in case of Bangladeshi population.

DMT2 is a major cause of premature mortality in all over the world. In long term, this metabolic disorder exerts its effect through both micro-vascular and macro-vascular complications. For example, diabetic retinopathy within young adults is ultimately due to early proliferation of diabetes [39]. Similar observation has been also found for chronic kidney disease, risk of foot ulcers, and limb amputation [40–43]. However, over the past two decades, occurrence of DMT2 has changed from being a mild disorder of old age to one of the major causes of morbidity and mortality affecting young and middle-aged people, even at the adolescents [44–46]. Understanding the etiology, early proliferation of DMT2 has implication for better clinical management, as well as research. There are few epidemiological studies on young adults with DMT2 diagnosed before age 25 years have been performed since last decade [47–50]. This lacks the etiology concerned with the diabetes within young adults. Interestingly, correlating the allelic frequency with age in diabetic patients has not yet been done, particularly for TNF- α -308G>A. Young diabetic adults are often found to be obese. More often this relates with physical inactiveness and high calorie diet. However, TNF- α protein is profoundly present in adipose tissue and associated with lipid metabolism [18]. In case of TNF- α -308G/A polymorphism, it is well accepted that A allele increases the transcription of TNF- α while G allele is associated with low-level expression [51,52]. Therefore, we presume that TNF- α -308G/A polymorphism within Bangladeshi individual increases the obesity which indirectly helps to proliferate early diabetes. However, ethnic differences may attribute to this result, as well as some environmental factors may also affect the result [39,53,54].

Overall, our data showed that TNF- α -308G/A polymorphism is significantly involved with DMT2. Once we subdivided the DMT2 individual into two age groups, it was found that TNF- α -308G/A polymorphism causes early proliferation of DM type-2.

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AUTHOR'S CONTRIBUTION

Study design: AH; Molecular studies, data collection, and data analysis: DR, MSSU, BB, MMH, and MT; Manuscript preparation: AH and MMH.

CONFLICT OF INTEREST

The authors declared that they have no conflict of interest.

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