TNF-alpha –308G/A and –238G/A polymorphisms and its protein network associated with type 2 diabetes mellitus

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Abstract Several reports document the role of tumor necrosis factor alpha (TNF-\textalpha) and lipid metabolism in the context of acute inflammation as a causative factor in obesity-associated insulin resistance and as one of the causative parameter of type 2 diabetes mellitus (T2DM). Our aim was to investigate the association between –308G/A and –238G/A polymorphisms located in the promoter region of the TNF-\textalpha gene in T2DM in the Indian population with bioinformatics analysis of TNF-\textalpha protein networking with an aim to find new target sites for the treatment of T2DM. Demographics of 100 diabetes patients and 100 healthy volunteers were collected in a structured proforma and 3 ml blood samples were obtained from the study group, after approval of Institutional Ethics Committee of the hospital (IEC). The information on clinical parameters was obtained from medical records. Genomic DNA was extracted; PCR–RFLP was performed using TNF-\textalpha primers specific to detect the presence of SNPs. Various bioinformatics tools such as STRING software were used to determine its network with other associated genes. The PCR–RFLP studies showed that...
among the $-238G/A$ types the GG genotype was 87%, GA genotype was 12% and AA genotype was 1%. Almost a similar pattern of results was obtained with $TNF-\alpha$ $-308G/A$ polymorphism. The results obtained were evaluated statistically to determine the significance. By constructing $TNF-\alpha$ protein interaction network we could analyze ontology and hubness of the network to identify the networking of this gene which may influence the functioning of other genes in promoting T2DM. We could identify new targets in T2DM which may function in association with $TNF-\alpha$. Through hub analysis of $TNF-\alpha$ protein network we have identified three novel proteins RIPK1, BIRC2 and BIRC3 which may contribute to $TNF$-mediated T2DM pathogenesis. In conclusion, our study indicated that some of the genotypes of $TNF-\alpha$ $-308G/A$, $-238G/A$ were not significantly associated to type 2 diabetes mellitus, but $TNF-\alpha$ $-308G/A$ polymorphism was reported to be a potent risk factor for diabetes in higher age (>45) groups. Also, the novel hub proteins may serve as new targets against $TNF-\alpha$ T2DM pathogenesis.

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1. Introduction

Recent times have witnessed large-scale human genetic studies that have made dramatic progress in identifying type 2 diabetes susceptibility genes, increasing the list from three important genes ($PPARG$, $KCNJ11$, and $TCF7L2$) to about more than 20 other genes in the last 5 years. Diabetes, a chronic metabolic disorder, that affects $>170$ million people worldwide has become one of the leading causes of death and disability. It has been estimated that by 2030, the number of affected people would rise to about 366 million (Wild et al., 2004). Of the two types of diabetes, the majority (~90%) is affected by type 2 diabetes (T2DM), characterized by impaired insulin secretion from pancreatic beta cells and insulin resistance. As per the estimates reported by Wild et al. (2004) the three leading countries in terms of the number of individuals affected by T2DM are India (31.7 million in 2000; 79.4 million in 2030), China (20.8 million in 2000; 42.3 million in 2030) and the US (17.7 million in 2000; 30.3 million in 2030). These figures emphasize that T2DM has become an epidemic in the 21st century, with India in the lead for the largest number of diabetics in the world.

T2DM is most often associated with older age, obesity, family history of diabetes, previous history of gestational diabetes, physical inactivity and certain ethnicities. About 80% of people with T2DM are overweight. The long-term complications of diabetes affect almost every organ of the body. The disease often leads to retinopathy, nephropathy, heart and blood vessel disease, amputations and nerve damage. Uncontrolled diabetes can complicate pregnancy and cause birth defects in new-born babies (Singh, 2011).

Earlier T2DM was generally regarded as a disease of the middle-aged group or elderly, but recent evidences suggest that even children and adolescents aged less than 30 years are becoming caught up in the diabetes epidemic, attributed mainly to the rise in obesity in these groups. Numerous reports exist of T2DM in children in countries such as Japan, USA, India, Australia and UK (Bloomgarden, 2004). This scenario of decrease in the age for the onset of diabetes may be a cause for concern with grave consequences in terms of resources and healthcare, as future generations become afflicted at a younger age may find their productivity affected. Thus, it has become pertinent not only to devise preventive measures, but also to counteract this rising epidemic with new knowledge, or else find new druggable targets.

The lifetime risk for type-2 diabetes is 38% if one parent has diabetes and 60% if both the parents are affected (Stumvoll et al., 2005). Multiple susceptibility genes have been reported in pathogenesis of type-2 diabetes and associated complications such as neuropathy, nephropathy and retinopathy. Current evidence suggests that oxidative stress may be one of the underlying pathological conditions associated with the development of pre-diabetic and diabetic conditions and may also be responsible for the complications of diabetes (Evans et al., 2002). About 70–80% of all T2DM patients are reported as obese, the key insights into understanding type 2 diabetes may be obtained by exploring the mechanisms through which obesity can bring about insulin resistance in the vital tissues. It is clear that the molecular etiology of insulin resistance is multifactorial where numerous independent mechanisms contribute to the final phenotype (Martyn et al., 2008). Several such molecular targets involved in inhibiting insulin action have been characterized in recent years (Moller, 1993; Katz et al., 1995; Hotamisligil, 1999; Arora, 2012).

Since $TNF-\alpha$ is a multifunctional, pro-inflammatory cytokine which plays an important role in several autoimmune diseases such as rheumatoid arthritis, pernicious anemia, diabetes mellitus, etc we hypothesized that $TNF-\alpha$ may be a mediator of obesity-linked insulin resistance in type 2 diabetes. This is also supported by studies in human obesity that have shown an increased adipose production in cases where $TNF-\alpha$ expression was high. Ethnic differences have also been observed to contribute to risk of development of T2DM. These reports support the need to investigate the role of $TNF-\alpha$ in influencing/or promoting diabetes.

1.1. Tumor necrosis factor alpha ($TNF-\alpha$) gene

The $TNF-\alpha$ gene is located within the HLA III region in chromosome 6p21 (Wilson et al., 1997; Nishimura et al., 2003) (Fig. 1). This gene was first isolated by Carswell et al. (1975) in a study exploring tumor necrosis in sarcoma Meth A. It is an important pro-inflammatory adipocytokine (Fig. 2, (Dushay and Abrahamson, 2005)) that is involved in regulating cellular processes such as differentiation, proliferation and cell death, as well as in inflammation and immune responses. It has also been implicated in pathogenesis of many human diseases (Qidwai and Khan, 2011; Sikka et al., 2014). $TNF$ is believed to be one of the main pro-inflammatory cytokines involved in the destruction of pancreatic $\beta$-cells.
moter region of the examined single nucleotide polymorphisms (SNPs) in the probability to inflammatory related diseases. Some studies have TNF-α predisposed to diabetes (Vaux and Flavell, 2000).

Genetic variations in the promoter region may regulate TNF-α production, transcription and may influence susceptibility to inflammatory related diseases. Some studies have examined single nucleotide polymorphisms (SNPs) in the promoter region of the TNF gene, such as −238G/A, −308G/A, −857C/T, and −1031T/C in humans (Feng et al., 2009a). TNF-α −308G/A single nucleotide polymorphism in the promoter region of TNF was found to increase the expression of this pro-inflammatory cytokine in culture cells and was positively associated with risk for T1DM (Feng et al., 2009b; Nishimura et al., 2003). TNF-α is reported to impair insulin signal pathways (Peraldi et al., 1996) thus playing a central role in the development of T2DM. Although a few studies have focused on the association between TNF-α −308G/A polymorphism and T2DM, their results remain unclear, necessitating the need for further investigations (Feng et al., 2009a; Sharma et al., 2014). Hence, the aim of our study was to investigate the association of two key TNF-α promoter polymorphisms, −308G/A and −238G/A, as a risk factor in T2DM patients (Guzmán-Flores et al., 2011). Further, we analyzed the networking of this gene using STRING software to find its association with other genes in influencing the disease progression. Our investigation also aims to provide new targets which could be useful in drug discovery.

2. Materials and methods

2.1. Ethics statement

The study protocol was approved by the Ethics Committee of the Institute (Bhagwan Mahavir Medical Research Centre, Hyderabad). This is a hospital based case-control study; eminent doctors helped us identify diabetic patients who matched our selection criteria. Non-diabetic volunteers were age and sex matched and formed the control group. We obtained written informed consent from all study participants for the use of their blood samples for the investigation.

2.2. Selection of study group

Clinicians identified 100 diabetes patients and we identified 100 healthy volunteers as controls which formed our study group (200 subjects). Three millilitre blood samples were obtained from the study group. Demographic details were collected in a structured pro forma. The patient case details were obtained from medical records and documented in the excel sheet. The clinical profile consisted of age, initial diagnosis, social habits, lifestyle, and medications used.

2.3. Blood sample collection

Three milliliters of venous blood was collected in an EDTA containing vacutainer tube (Vacuette®) from the study group (100 + 100) of adult patients with T2DM and non-diabetic controls with informed consent.

2.4. Genomic DNA isolation

Genomic DNA was isolated from blood samples using the protocol standardized in our laboratory (Jamil et al., 2014). It is a rapid non-enzymatic method for salting out cellular proteins with saturated solution and precipitation by dehydration using the standard Phenol–Chloroform method. This was followed by lysing RBC using RBC lysis buffer and removal of protein content by precipitation. The precipitated DNA was suspended in 70% ethanol to remove salts. Finally the DNA was suspended in Tris buffer and stored at −20°C until used. The presence of genomic DNA was detected by running the isolated samples on 1% agarose gel, electrophoresed at 60 V. A visual quantification of DNA was done using a 100 bp DNA marker. The DNA was then considered for PCR–RFLP procedure.

2.5. Polymerase chain reaction–restricted fragment length polymorphism (PCR–RFLP)

After checking for the purity of DNA, PCR was carried out in 20 μl reactions using standard procedures (Jamil et al., 2014). The genomic region encompassing the −308G/A polymorphism was amplified using the following primers: forward 5’-AGGCAATAGGGTTTGGAGGCCCCAT-3’ and reverse 5’-TCCTCCGGCTCCGAGTCCG-3’. The 107 bp PCR product was digested with NeoI restriction enzyme, according to the manufacturer’s instructions for RFLP.

To identify the −238G/A polymorphism, we used the following primers: forward 5’-AGAAGACCCCGTTGCTCCGAGGCCCCAT-3’ and reverse 5’-ATCTGGAGGAAGGCGG TAGTG-3’. The PCR product was digested with MspI restriction enzyme, according to the manufacturer’s instructions for RFLP.
2.5.1. RFLP
After restricted digestion the samples were used for RFLP analysis by standard protocol and the genotypes of various bps were obtained. These were categorized into homo, hetero and wild type genotypes and statistically analyzed to determine the \( p \) values.

2.6. Statistical analysis
All the above experiments were repeated in triplicates. The distribution of the genotype frequencies of TNF-\( \alpha \) promoter polymorphisms for patients and control subjects were compared using the chi-squared test; Odds ratio (OR) with respective confidence interval (95% CI) for disease susceptibility was also calculated. The observed frequency of polymorphism between patients and controls were tested for Hardy–Weinberg equilibrium (HWE) using the \( v^2 \) method. The clinical parameters such as age, sex, BMI, and random sugar levels were correlated and calculated for patients with different genotypes. Values were expressed as percentage, mean, and standard deviation. Fisher’s two tailed test and Pearson’s correlation were used to evaluate statistical significance and calculate \( p \) value of the parameters tested, all the statistical analysis was done using SPSS statistical software (18.0, Chicago Illinois). A \( p \)-value of < 0.05 was considered significant.

2.7. Bioinformatics analysis

2.7.1. Construction of TNF-\( \alpha \) protein–protein interaction network, analysis of gene ontology and network hub analysis
To understand the networking of TNF-\( \alpha \), we used the STRING v 9.05 database (Search Tool for the Retrieval of Interacting Genes/Proteins http://string-db.org/ (Szklarczyk et al., 2011)) to retrieve interactions pertaining to this gene. We analyzed interactions pertaining only to \textit{Homo sapiens} and restricted the network to 50 additional interactors. We further filtered the network to retain only those interactions with confidence score > 0.9 which signifies more than 80% probability that these interactions may be replicated in KEGG (Kyoto Encyclopedia of Genes and Genomes) database. Additionally, we grouped the interactors into five clusters based on shared similarity/relatedness, using k-means algorithm (MacQueen, 1967) provided by STRING database. The gene ontology enrichment of the network interactors was performed using KOBAS 2.0 server (KEGG Orthology Based Annotation System, http://kobas.cbi.pku.edu.cn/, (Xie et al., 2011)). We also analyzed the hubness of the network using cytoHubba plugin (Lin et al., 2008) in Cytoscape visualization software (Smoot et al., 2011). For hub analysis we chose degree and betweenness ranking methods to identify proteins that may be biologically significant, due to their high interconnectivity, and hence concomitantly may play a role in the disease.

3. Results

3.1. Demographic results
The demographics of the subjects included in this study were collected using a questionnaire. The details included their age, sex, start of illness, period of illness, any treatment and family history. A total of 100 patients were randomly included in this study, out of which 56% were males and 44% were females. Care was taken to recruit equal number of healthy volunteers of similar age and sex. We utilized the help of attending clinicians in the selection of the study group. Incidentally, there were more number of males in the study group.

3.1.1. Age group
In our study, the age group of patients varied from 20–90 years, of which 8% were in the age group of 20–30 years, 22% in 31–40 years, 39% in 41–50 years, 23% in 51–60 years and 8% in 61–90 years (Fig. 3). Based on our data, T2DM was more prevalent in the age group of \( \geq 41–50 \) years; this may be referred to as the susceptible group.

3.2. Random blood sugar level, body mass index and physical activity
We found that 78% of the patients showed a high body mass index and 74% of the patients had a high random blood sugar level. Among these, only 30% of the patients had the habit of regularly exercising. Based on our data, patients with the highest random blood sugar level were mostly obese or over weight and based on our demographic data might be attributed to their poor diet control or lack of exercise (Fig. 4).

3.3. Genotyping
DNA extracted from blood samples by standard procedure (salting out method (Jamil et al., 2014)) was checked for quality and quantity on 1% agarose gel. After checking for the purity of DNA, PCR was carried out in 20 \( \mu l \) reactions as previously described. The genomic region encompassing the \( -308G/A \) polymorphism was amplified using the specific primers and the 107 bp fragment produced was checked on a 2% agarose gel. Amplification of \( -238G/A \) promoter polymorphism with specific primers yielded a 152 bp PCR product, which was also visualized on a 2% agarose gel (figure not shown). A sample gel electrophoresis image is presented in Fig. 5 depicting results from \( -308G/A \) polymorphism analysis.
A 100 bp DNA ladder (Life Technologies) was simultaneously run on the gels to confirm the PCR product (bps) sizes. Photographs of gels were scanned and analyzed.
3.3.1. Restriction fragment length polymorphism (RFLP)

Following enzymatic digestion of the PCR product using NcoI restriction enzyme and incubation at 37°C for 12 h, the TNF-α (−308G/A) PCR-RFLP products were visualized on a 2% agarose gel. Three types of bands were observed: a complete NcoI cut representing homozygous TNF-α (−308G/G), resulting in two fragments of 87 and 20 bp; a partial cut representing heterozygous TNF-α (−308G/A), resulting in three fragments of 107, 87 and 20 bp; and an uncut 107 bp fragment representing homozygous TNF-α (−308A/A) (Fig. 6).

The MspI digested −238G/A PCR products were of two types: a complete MspI cut which yielded a 152 bp fragment and two fragments of 133 and 19 bp for the wild-type allele (−238G/G) and a partial cut representing heterozygous TNF-α (−238G/A), resulting in three fragments of 152, 133 and 28 bp.

Of the 100 diseased individuals genotyped for −308G/A, the GG genotype was found in 88%, GA genotype in 10% and AA genotype in 2% of the patients. In the control group the results were not much different, since we observed GG genotype in 87%, GA in 12% and AA in 1% of the healthy individuals (Table 1). The distribution of −238G/A polymorphism in cases were as follows: 85% GG, 12% GA, and 1% AA, whereas in controls it was 87% GG, 13% GA and 2% AA (Table 1). Since a lot of work has been reported on haplotypes, we did not include the data here.

3.4. Analysis of TNF-α protein interaction network, gene ontology and hubness: bioinformatics approach

On analysis of TNF-α network, we found that the fifty-one network proteins were interconnected via 264 interactions. Through analysis of KEGG pathway ontology of the network clusters (Fig. 7), we found that the network was significantly enriched in several essential pathways. We observed that cluster 1 comprised of nine proteins mainly involved in TNF signaling pathway, NFκB signaling pathway and Toll-like receptor pathway. The fourteen cluster 2 interactors were found to be enriched in NFκB signaling pathway and apoptosis signaling pathway. Our analysis of the twelve proteins of cluster 3, which included TNF-α, showed a significant enrichment in TNF signaling pathway. We found that cluster 4 was comprised of eight proteins, mainly interleukins and cell adhesion proteins. Cluster 5 comprised six proteins significantly enriched in the JAK–STAT signaling pathway.

On analysis of hubness of the network, we found that six proteins were common to the degree and betweenness methods and were among the top ten hub proteins in the network. These included the proteins TNFRSF1A, BIRC2, BIRC3, TRAF2, RIPK1, and TRAF6. Of these TNFRSF1A, TRAF2 and TRAF6 have been reported earlier in diabetes. Their hubness further validates their biological and hence disease relevance. Further, we have identified three novel hub proteins—RIPK1, BIRC2 and BIRC3 that are involved in numerous interactions in the protein network. RIPK1 (cluster 1, Fig. 7) functions in regulation of TNF-α production on inhibition of caspases. BIRC2 and BIRC3 (cluster 2; Fig. 7) function in suppression of apoptosis via binding to TNF-α associated receptors, TRAF1 and TRAF2, and they are also involved in immune response modulation and other cell growth and development processes. Thus, these three genes may serve as...
| Polymorphism   | Genotype | T2DM n (%) | Control n (%) | Odds ratio | 95% CI     | p-value |
|---------------|----------|------------|---------------|------------|------------|---------|
|              | Wild type | GG 88 (88%) | 87 (87%) | 1.09 | 0.47–2.53 | 0.83     |
|              | Heterozygote | GA 10 (10%) | 12 (12%) | 0.81 | 0.33–1.98 | 0.65     |
|              | Homozygote | AA 2 (2%) | 1 (1%) | 0.49 | 0.04–5.54 | 0.56     |

**Figure 7**  Clustered TNF-α protein interaction network, retrieved from STRING database. TNF-α has been highlighted in red box while the novel hub proteins BIRC2, BIRC3 and RIPK1 have been highlighted using blue ovals.
novel targets in type 2 diabetes mellitus patients with altered/dysregulated TNF-α.

4. Discussion

We have studied two bi-allelic polymorphisms previously identified in the TNF-α promoter region, which have been considered to play an important pathogenic role in T2DM, insulin resistance and obesity (Guzmann-Flores et al., 2013; Saxena et al., 2013; Sikka et al., 2014; Wilson et al., 1992). The −308G/A and −238G/A polymorphisms have been associated with insulin resistance, obesity and T2DM in different ethnic groups (Guzmán-Flores et al., 2013; Saxena et al., 2013; Sikka et al., 2014; Wilson et al., 1992). In this study, interestingly, we did not find any significant correlation between these polymorphisms and T2DM subjects of South Indian origin. Cytokines are important mediators of immunity and there is now convincing evidence that cytokines also play an important role in the pathogenesis of autoimmunity. TNF-α is pivotal in host defense against infections and has a major role in autoimmune diseases as well. It is also a crucial cytokine for granuloma formation. The level of TNF-α varies from individual to individual and is genetically determined. There are many biallelic single nucleotide polymorphisms (SNPs) in and around the TNF-α gene. The G/A polymorphism, which we studied, is located upstream of the gene at −308 and is known to influence TNF-α levels. It has been reported that as compared to the TNF-α −308 G-allele, A-allele has a higher transcriptional activity. TNF-α −308 promoter gene polymorphism has been reported to be associated with several autoimmune disorders and metabolic syndrome pathogenesis (Hajeer and Hutchinson, 2000; Sookoian et al., 2005).

Reports on patient cohorts of mean ages 57 years (Hamann et al., 1995) and 39 years (Altshuler et al., 2000) have stated a lack of association of −308G/A polymorphism with type 2 diabetes. This could be due to the disparities in their study designs and variations in the genetic background and may also be due to environmental exposures of the T2DM patients enrolled in these studies. This may also indicate the involvement of other genes in T2DM pathogenesis in younger patients. The discrepancies in studies investigating association of TNF-α promoter polymorphisms with insulin sensitivity may also be ascribed to the differences in age of the patients in these studies (Fernández-Real et al., 1997; Rasmussen et al., 2000).

TNF-α −308G/A single nucleotide polymorphism (SNP) in the promoter region of TNF was found to increase the expression of this pro-inflammatory cytokine in culture cells and was positively associated with risk for T1DM (Wilson et al., 1997; Feng et al., 2009b). Additionally, TNF-α −308 and −238 promoter gene polymorphisms have been reported to be associated with several autoimmune disorders including Systemic Lupus Erythematosus, Rheumatoid Arthritis and infections such as tuberculosis (Qidwai and Khan, 2011). Inflammation with high levels of pro-inflammation cytokines such as IL-1, IL-6 and TNF-α is a key feature of T2DM. These observations suggest that TNF-α plays a role in the pathogenicity of T2DM and obesity (Feng et al., 2011).

We also found some reports documenting the significance of the SNPs in −308G/A and −238G/A in the promoter of the gene encoding TNF-α in the risk of diabetes/insulin resistance development in subjects aged 40–60 years (Elsaid et al., 2012; Fontaine-Bisson et al., 2007) and 85 years (Heijmans et al., 2002). Also, homozygosity for A-allele conferred a more than fourfold increased risk of diabetes. Hence, we took up bioinformatic studies to test our hypothesis of how TNF-α could influence the disease condition. The TNF gene is located in the HLA region characterized by strong linkage disequilibrium. Some studies have reported the lack of association of TNF-α (14 alleles) and the TNFc (2 alleles) short tandem repeats with the risk of diabetes despite their strong linkage disequilibrium with the −308G/A promoter polymorphism (Heijmans et al., 2002). Although this makes it difficult to categorically establish that the −308G/A polymorphism alone is the functional variation in association with diabetes, nevertheless this information formed an attractive candidate for investigation.

Results obtained through our in silico studies clearly identified three novel targets in T2DM-BIRC2, BIRC3 and RIPK1, through computational analysis of TNF-α protein network. Their hubness in a network enriched in proteins deregulated in diseases suggests that they may also play a role in diabetes mellitus, via their associations, contributing to pathogenesis mechanisms such as lowered insulin production, development of insulin resistance or diabetic nephropathy. Our network analysis also showed the interconnectivity between signaling pathways such as TNF receptor, NFκB, Toll-like receptor, apoptosis, etc.; this information could be essential in understanding disease mechanisms, especially in complex diseases such as diabetes, wherein multiple pathways are co-operatively engaged in pathogenesis of the disease. Further, our analysis of the hub proteins in the network has highlighted proteins that may act as crucial nodes, in synergism with TNF-α, in attributing insulin resistance to T2DM patients. Thus, the association of several proteins with TNF-α, observed in the protein interaction network, suggests a key role for these interactions in normal biological processes mediated by this protein and its signaling network. These proteins may be of significance in diabetes, wherein they may assist TNF-α in deregulation of pathways necessary to maintain insulin production, sensitivity and regulation of glucose levels and also in deregulation of pathways that lead to pathogenesis of diabetes induced/associated complications such as nephropathies and end stage renal disease. In conclusion, our study predicts the possible pathways that may contribute to TNF-α-mediated T2DM pathogenesis.

5. Conclusion

Despite the rapid progress in identifying diabetes related genes, what the precise causal variants are and how these variants increase susceptibility to type 2 diabetes mellitus (T2DM) remain unknown. However, we have identified new targets for T2DM using various Bioinformatics tools, as the promoter region polymorphisms did not show strong association with T2DM.

Authors’ contributions

kJ made the study design, analysis and interpretation of data and drafting the manuscript, AJ performed bioinformatics analysis, JA helped in the genetic analysis, SJ and SKY contributed to selection of patients, participated in the acquisition...
of patient consent, helping with the laboratory investigations. All authors participated in the discussions revised the manuscript and approved the final version.

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