Associations of −308G/A polymorphism of \textit{tumor necrosis factor (TNF)} − \( \alpha \) gene and serum TNF-\( \alpha \) levels with measures of obesity, intra-abdominal and subcutaneous abdominal fat, subclinical inflammation and insulin resistance in Asian Indians in north India

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Abstract. Objectives: Obesity is associated with high levels proinflammatory cytokines like tumour necrosis factor alpha (TNF-\( \alpha \)), which may play an important role in the genesis of insulin resistance. We evaluated the relationship of −308G/A polymorphism of TNF-\( \alpha \) gene with obesity and insulin resistance in Asian Indians in north India.

Methods: This cross-sectional study included 151 apparently healthy individuals (79 males, 72 females) 18–50 yrs of age from New Delhi, India. Body composition by dual-energy x-ray absorptiometry (DEXA) and abdominal fat by magnetic resonance imaging (MRI) were measured. Biochemical measurements included OGTT, lipids, fasting insulin, hs-CRP and TNF-\( \alpha \) levels. We analysed −308G/A polymorphism of TNF-\( \alpha \) gene and studied its association with obesity and biochemical parameters.

Results: At comparable BMI, abdominal obesity was more prevalent in females (50%) as compared to males (20%). The wild genotype (GG) was present in 78.8%, GA in 17.9%, and AA in 3.3% subjects. Measures of body composition, abdominal fat distribution, lipids, insulin, hs-CRP and TNF-\( \alpha \) levels were not influenced by the presence of −308G/A polymorphism. Serum TNF-\( \alpha \) levels correlated significantly with fasting insulin in both genders.

Conclusion: TNF-\( \alpha \) levels correlate with fasting insulin but not with indicators of body composition in Asian Indians. The −308G/A polymorphism of TNF-\( \alpha \) gene is not associated with differences in the serum levels of TNF-\( \alpha \) in Asian Indians.

Keywords: TNF-\( \alpha \) gene polymorphism, obesity, abdominal fat, insulin resistance, Asian Indians

1. Introduction

Obesity is rapidly increasing in developing countries [1]. Obesity, in particular abdominal obesity, is associated with insulin resistance which may contribute to the development of hypertension, type 2 diabetes and dyslipidemia. Ethnicity influences the development of
abdominal obesity and insulin resistance significantly. In particular, Asian Indians are at a higher risk of development of insulin resistance and its complications at a lower degree of adiposity as compared to Caucasians [22,17]. This increased risk may be the result of a complex interplay of several factors including genetic, nutritional and environmental influences.

Adipose tissue secretes several adipocytokines that have pro-inflammatory properties and these may affect insulin sensitivity [24]. Tumor necrosis factor alpha (TNF-α), a pro-inflammatory cytokine produced by several cell types in response to various stimuli including infectious agents and other cytokines, is an extremely pleotropic cytokine and may influence the expression of a broad range of genes. Adipose tissue is a significant source of TNF-α production and its expression is elevated in adipose tissue and muscle tissues in obesity [10]. Elevated expression of TNF-α in turn has been shown to influence insulin sensitivity adversely by suppressing phosphorylation of insulin receptor and its substrates [10]. Other mechanisms such as quantitative regulation of glucose transporters by TNF-α may also have a role in the genesis of insulin resistance [13].

The gene for human TNF-α is located on chromosome 6p21.3 which codes for a 157-amino acid polypeptide processed from a 233-amino acid precursor [5]. Among the various variants, a G→A transition at position −308 in the promoter region of TNF-α gene has been most well characterized. This polymorphic variant has been shown to affect the promoter region of TNF-α gene with consequent increase in transcriptional activation of the TNF-α protein [4]. Studies which have evaluated the influence of −308G/A polymorphism of TNF-α gene on obesity and insulin sensitivity have reported conflicting results. A significant association of -308A variant with insulin resistance, higher body mass index (BMI) and higher leptin levels was reported by Fernandez-Real et al. [12]. In another study, subjects homozygous for A allele were observed to have higher fasting insulin levels, higher systolic blood pressure (SBP) and lower HDL-c levels as compared to those homozygous for G allele (7). On the other hand, several other studies have reported no correlation between −308G/A polymorphism and insulin resistance or indicators of obesity [26,11]. It is noteworthy that the previously mentioned studies involved white population.

There is lack of data as to how the TNF-α gene polymorphism influences insulin resistance and obesity in Asian Indians. In a recent study involving Asian Indians diagnosed to have acute myocardial infarction in South Africa, the GG genotype was more frequent in those having metabolic syndrome based on NCEP ATP III criteria [19]. However, no association of TNF-α gene polymorphism was observed with individual components of the metabolic syndrome. In a recent study involving obese Asian Indian subjects, the frequency of −308A allele of TNF-α gene was observed to be higher in individuals with obstructive sleep apnea as compared to those without obstructive sleep apnea [6]. In the current study we evaluated the association of −308G/A polymorphism of TNF-α gene with insulin resistance and indicators of adiposity in urban Asian Indians in north India.

2. Methods

2.1. Subjects and measurements

This cross-sectional study included 151 apparently healthy subjects (79 males and 72 females) aged 18 to 50 years. These individuals responded to a local advertisement and volunteered to take part in the study. It included some members of staff of the institute and also relatives of patients who were seeking treatment at the hospital. Subjects included in the study were asymptomatic for any acute or chronic disease state and were not taking any medications at the time of inclusion in the study. Subjects with any body deformity and metallic implants were excluded. The study was approved by the institutional ethics committee. All the subjects gave written informed consent before participating in the study. Evaluation included recording of a detailed demographic and clinical history, followed by a detailed physical examination. Blood pressure was measured over the right arm in sitting position using a standard mercury sphygmomanometer by a single physician after the subject had rested for five minutes. Anthropometric measurements (height, weight, body mass index [BMI], waist circumference [WC], and skinfold thickness at triceps [TR] and subscapular [SS] regions) were obtained according to the methods described earlier [29].

2.2. Total body fat and abdominal fat

Subjects underwent whole body DEXA scan (Lunar Prodigy Advanced Whole Body DEXA system, GE Medical Systems, USA) for determination of regional and global measurement of whole body fat (%BF) and other measures of body composition like fat free mass,
lean body mass and bone mineral content as mentioned earlier [15]. Abdominal fat area and its distribution was measured by single slice magnetic resonance imaging (1.5 Tesla, SIGNA High Definition MR, GE Medical Systems, USA) at lumbar vertebrae L2–3 level as indicated earlier [15].

2.3. Biochemical measurements

Venous blood samples were obtained after an overnight fast of at least 10 hours. Subsequently, the subjects underwent 75 grams oral glucose tolerance test as per the standard criteria. Estimation of fasting (FBG) and 2-hr post glucose load (2hrFG) blood glucose, total cholesterol (TC), serum triglycerides (TG) and high-density lipoprotein cholesterol (HDL-c) was performed using commercially available reagent kits as mentioned elsewhere [20]. Serum levels of low-density lipoprotein cholesterol (LDL-c) levels were calculated using Freidewald’s equation [30].

2.4. Serum insulin and hs-CRP assays

Fasting serum insulin levels were measured by radio immunoassay (RIA) using commercially available radioimmunoassay kits (IMMUNOTECH, Prague, Czech Republic) as mentioned earlier [20]. Homeostasis model assessment (HOMA) was estimated by the following equation: Insulin (μU/ml) x Blood glucose (mmol/l)/22.5. The inter- and intra-assay variation was 2.5 and 3.7%, respectively. Serum levels of high-sensitivity C-reactive protein (hs-CRP) were measured using ELISA based kit (Biocheck, Inc. CA, USA) as mentioned earlier [21]. The inter- and intra-assay variation determined using duplicate samples was less than 5%.

2.5. Serum TNF-α levels

Blood samples for estimating serum levels of TNF-α were collected in the morning between 8 am and 9 am in all subjects. Serum TNF-α levels in all subjects were measured by ELISA based kits (eBiosciences, San Diego, CA) as mentioned earlier [6]. The sensitivity range of the kit was from 4 to 500 pg/ml. The intra-assay variation was 2.0–2.5% and inter-assay variation was 3–3.5%.

2.6. TNF-α promoter polymorphism (−308G/A)

The procedure used to determine the gene polymorphism of TNF-α gene was similar to that mentioned earlier [6]. Briefly, venous blood (about 10 ml) was drawn into EDTA containing tubes for DNA isolation. Polymerase chain reaction-restriction fragment length polymorphism (PCR–RFLP) was used to determine the variants of TNF-α gene promoter region (−308G/A). The upstream primer sequence was ‘AGGCAATAG-GTTTTGAAGGCCAT3’ and downstream primer sequence was ‘TCCCTGCTCCGATTCCG3’. The reaction was carried out in a final volume of 25 μl containing 0.25 mM of each dNTP (Bangalore Genei, India), 0.1 mmol of each primer and 1.25 U of Taq DNA polymerase (Bangalore Genei, India). DNA was amplified during 35 cycles with 1 min denaturation at 94°C, 1 min annealing at 60°C and 1 min extension at 72°C. Initially, 1 cycle of denaturation at 94°C, 1 min annealing at 60°C and 1 min extension at 72°C was performed. Finally, a cycle of 1 min denaturation at 94°C, 1 min annealing at 60°C and 5 min extension at 72°C was included. The PCR product was digested with 0.5 μl of Nco I restriction enzyme (New England Biolabs). After restriction digestion, three bands of different sizes were obtained. 107 bp fragment corresponding to variant allele (Restriction site absent) and a set of 87 bp and 20 bp corresponding to wild type allele (Restriction site present). Band pattern was checked by using 10% native polyacrylamide gel electrophoresis (PAGE) followed by silver staining.

2.7. Definitions

Obesity was defined as BMI of ≥ 25 kg/m² [3]. Abdominal obesity was defined as WC ≥ 90 cm and ≥ 80 cm in males and females, respectively [3]. High intra-abdominal fat area at L2–3 level was defined as > 135.3 cm² in males and > 75.7 cm² in females [2]. Dyslipidemia was defined according to NCEP, ATP III guidelines [23] as: hypercholesterolemia > 200 mg/dL, hypertriglyceridemia ≥ 150 mg/dL and low HDL-c as < 40 mg/dL in males and < 50 mg/dL in females. Impaired fasting glucose (IFG) was defined as FBG ≥ 100 and < 126 mg/dL and impaired glucose tolerance (IGT) was defined 2hrPG ≥ 140 and < 200 mg/dL [9]. Fasting hyperinsulinemia was defined as a value > 20 μU/l which corresponded to the 75th percentile of the distribution of insulin values in the study population [20]. The metabolic syndrome was defined according to the modified NCEP, ATP III criteria [28] criteria.

2.8. Statistical analysis

The data were managed on and Excel spreadsheet. Continuous variables were assessed for normalcy and
were compared between both genders using student’s ‘t’ test. Variables that were not normally distributed were compared using the Mann-Whitney rank-sum test among the males and females. Categorical variables were compared between the two genders using the Chi-square test. The relationships of TNF-α levels with body composition and biochemical measure including fasting insulin were evaluated using Spearman’s correlation method. The difference in the values of anthropometric and biochemical measures in subjects with and without the −308G/A polymorphism was assessed by using student’s ‘t’ test or Mann-Whitney rank-sum test, wherever applicable.

3. Results

3.1. Clinical and body composition characteristics (Table 1)

Females were older than males ($p = 0.03$). Obesity was present in 30.0% (24.0% males and 36.6% females, $p = 0.09$) and abdominal obesity in 36% (22.8% males and 50.7% females, $p < 0.001$) subjects. High abdominal fat was present in 51.6% (48.9% males and 54.5% females) subjects. Hypertension (blood pressure $\geq 140/90$) was present in 23.2% (25.3% males and 20.8% females, $p = NS$) subjects. The mean blood pressure values, BMI and WC were comparable among both genders. Females had higher skinfold thickness and %BF as compared to males. Among the abdominal fat compartments, TAF area was comparable among both genders, SCF area was higher in females and IAF area was higher in males.

3.2. Biochemical profile (Table 1)

Mean fasting and 2-hr post glucose load glucose values were higher in females than males. IFG was present in 29.1% (22.8% males and 36.1% females, $p = 0.07$). Serum levels of TC, TG and LDL-c were comparable among both genders, whereas those of HDL-c were higher in females. Among the dyslipidemias, hypercholesterolemia was present in 17.2% (20.2% males and 13.9% females, $p = NS$), hypertriglyceridemia in 36.4% (38% males and 34.7% females, $p = NS$) and low HDL-c in 30.4% (3.8% males and 59.7% females, $p < 0.001$) subjects.

Fasting serum insulin levels were comparable among both genders. Hyperinsulinemia was present in 37.8% (40.6% males and 24.8% females, $p = NS$) subjects. Serum levels of TNF-α and hs-CRP were comparable among both the genders. Metabolic syndrome as defined by the modified NCEP criteria was present.
in 32.4% subjects, with higher prevalence in females (44.4%) as compared to males (21.5%, \( p = 0.003 \)). The prevalence of hyperinsulinemia was higher in subjects with metabolic syndrome as compared to those without metabolic syndrome (55.5% vs. 23.4%, \( p = 0.009 \)).

3.3. Correlation of TNF-\( \alpha \) levels with anthropometric and biochemical parameters

The correlation of serum TNF-\( \alpha \) levels with anthropometric and biochemical parameters is mentioned in Table 2. Serum levels of TNF-\( \alpha \) did not correlate with any of the anthropometric and biochemical parameters except fasting insulin, with which a positive correlation was observed in both males and females. Serum levels of hs-CRP correlated positively with BMI, diastolic blood pressure (DBP), TR, SS, %BF, % truncal fat and 2-hr glucose value in both genders, the correlations being stronger in women. Serum levels of hs-CRP correlated with WC, TAF, IAF, SCF and TG in women only. Fasting insulin levels correlated positively with BMI and SS in both genders, with DBP, TR and TC in males and with WC, % truncal fat, TAF, SCF, 2-hr glucose value and TG in females.

3.4. TNF-\( \alpha \) gene polymorphism

The observed allelic frequency of \( G \) allele was 0.88 & that of \( A \) allele was 0.12. About 78.8% subjects were homozygous for GG (males 84.8%, females 72.2%), 17.9% were heterozygous for GA (males 11.4%, females 25%) and 3.3% were homozygous for AA (males 3.8%, females 2.8%) genotypes. The genotype frequencies followed Hardy Weinberg Equilibrium (chi value = 2.80, \( p = 0.09 \)). Due to small number of subjects who were homozygous for \( A/A \) genotype, for analysis the heterozygous and homozygous subjects were combined together. Comparison of the genotypes of TNF-\( \alpha \) gene and their association with clinical, anthropometric and biochemical parameters is mentioned in Table 3. The proportion of males with \( GG \) genotype was higher as compared to females (\( p = 0.05 \)). The various anthropometric and biochemical parameters, including serum TNF-\( \alpha \) levels did not differ among subjects with and without \( A \) allele. The prevalence of obesity, abdominal obesity, hypertension, IGT, hypercholesterolemia, hypertriglyceridemia and low HDL-c was comparable in subjects with and without at least one \( A \) allele. However, the difference in the prevalence of high IAF between subjects with and without \( A \) allele showed a trend towards significance (46.5% vs. 70.0%, respectively, \( p = 0.06 \)).

4. Discussion

This is the first study to evaluate the influence of \(-308G/A\) polymorphism of the TNF-\( \alpha \) gene on insulin
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Comparison of the clinical, anthropometric and biochemical parameters among genotypes of TNF-α gene

| Parameter                        | GG genotype (n = 119) | At least one A allele (n = 32) | P value |
|----------------------------------|-----------------------|-------------------------------|---------|
| Males (%)                        | 56.3                  | 37.5                          | 0.05    |
| Age (yrs)                        | 32.6 ± 9.8            | 29.9 ± 8.5                    | NS      |
| Systolic blood pressure (mm Hg)  | 123.8 ± 14.7          | 124.4 ± 10.4                  | NS      |
| Diastolic blood pressure (mm Hg) | 79.3 ± 9.5            | 79.4 ± 7.2                    | NS      |
| Body mass index (kg/m²)          | 23.1 ± 4.0            | 22.6 ± 4.1                    | NS      |
| Waist circumference (cm)         | 81.6 ± 11.8           | 79.7 ± 10.8                   | NS      |
| Triceps skinfold (mm)            | 18.9 ± 8.8            | 19.0 ± 9.7                    | NS      |
| Subscapular skinfold (mm)        | 24.4 ± 11.0           | 21.3 ± 10.3                   | NS      |
| % Body fat                       | 29.9 ± 11.5           | 30.4 ± 11.0                   | NS      |
| % Truncal fat                    | 32.6 ± 12.2           | 32.9 ± 11.8                   | NS      |
| Total abdominal fat (cm²)        | 239.8 ± 136.6         | 263.5 ± 140.8                 | NS      |
| Intra-abdominal fat (cm²)        | 103.5 ± 75.3          | 115.2 ± 63.6                  | NS      |
| Subcutaneous abdominal fat (cm²) | 138.9 ± 96.6          | 155.2 ± 94.0                  | NS      |
| Fasting blood glucose (mg/dL)    | 91.5 ± 12.9           | 93.7 ± 10.6                   | NS      |
| 2Hr Blood glucose (mg/dL)        | 116.6 ± 26.1          | 113.8 ± 21.5                  | NS      |
| Total cholestrol (mg/dL)         | 175.5 ± 32.3          | 173.4 ± 27.9                  | NS      |
| Triglycerides (mg/dL)            | 145.1 ± 60.9          | 139.9 ± 47.2                  | NS      |
| High-density lipoprotein cholesterol (mg/dL) | 47.0 ± 3.9 | 47.4 ± 4.2                  | NS      |
| TNF-α (pg/mL)                    | 57.5 ± 27.2           | 50.7 ± 26.8                   | NS      |
| hs-CRP (mg/L)                    | 3.40 ± 4.0            | 3.5 ± 4.5                     | NS      |
| Insulin (µU/mL)                  | 19.7 ± 6.1a           | 18.2 ± 5.3b                   | NS      |

Notes: a: n = 80; b: n = 20.
Values are mentioned in mean ± SD.

The −308G/A polymorphism in the promoter region of TNF-α gene has been associated with higher concentrations of TNF-α in vitro [4]. In a recent study involving Asian Indians, −308 GA genotype was found to be associated with higher production of TNF-α as compared to GG genotype [25]. However, in the present study, we did not find any difference in TNF-α levels in subjects with GG genotype and GA or AA genotype.

Association of −308G/A polymorphism with obesity has been reported in several studies involving White population [7,12]. Sookoian et al. [27] performed a meta-analysis of 31 studies involving about 3500 subjects on the association between −308G/A polymorphism of TNF-α gene and components of metabolic syndrome. The results of the meta-analysis indicated that subjects carrying A allele were at 23% higher risk of developing obesity, had higher systolic blood pressure and insulin levels as compared to subjects not carrying A allele. It is noteworthy that the majority of the studies considered in the meta-analysis included Caucasians and very few included Korean, Chinese Japanese, Arabian and Brazilian subjects. None of the study included subjects of Asian Indian origin. The authors further concluded that power of the meta-analysis was lower for Asian populations due to lesser number of studies and also the lower frequency of A allele in Asian populations compared to Caucasians.

The data on Asian Indians is very limited in this regard. The −308G/A polymorphism of TNF-α gene was not associated with any of the components of the metabolic syndrome in young Asian Indians with myocardial infarction in South Africa [19]. Several studies have reported negative associations between TNF-α gene polymorphism and insulin resistance and obesity in ethnic groups other than Asian Indians [11,26]. In a study involving obese Korean women, this polymorphism was not associated BMI and percentage body fat. However, significantly lower WHR was observed in subjects with GA or AA genotypes [14].

The influence of this polymorphism on abdominal fat distribution has been less investigated. In a study involving healthy and diabetic Japanese subjects, the...
−308G/A polymorphism was observed in only three out of 142 healthy subjects and three out of 132 patients. The allelic frequencies did not differ among the two groups. No significant effect of this polymorphism was observed on insulin resistance and abdominal fat distribution [18]. In our study though the IAF area was comparable in subjects with and without A allele, the prevalence of high IAF area was more in subjects carrying A allele as compared to those not carrying it \((p = 0.06)\), suggesting some role in the abdominal fat distribution in our population.

Increased production of TNF-α in the presence of −308G/A polymorphism may lead to increase in number of stromal-vascular cells which may transform to preadipocytes or may serve as infrastructure to support adipose tissue growth [16]. Insulin also enhances differentiation and lipid accumulation in adipocytes and interaction between TNF-α and insulin signaling pathways may play an important role in the differentiation of adipocytes.

Serum TNF-α levels did not correlate with any of the anthropometric parameter, abdominal fat compartment or biochemical parameter. The only significant correlation of TNF-α levels was observed with fasting insulin. In another study conducted on Asian Indian subjects residing in New Zealand, serum interleukin-6 levels correlated positively with percentage body fat, beta-cell function and inversely with appendicular skeletal muscle mass and insulin sensitivity [8]. However, TNF-α levels did not correlate with any of the variables examined.

Our study has some limitations. The relatively small sample size of the present study is a major limitation and it may not be adequate to draw firm conclusions. The sample size of the study was influenced to a great extent by the limited availability of resources during the present study to perform whole body DEXA scan and MRI of abdomen for determination of whole body fat and abdominal fat, respectively. The influence of factors like nutritional intake and physical activity that may have impact on body composition and insulin sensitivity could not be assessed due to lack of data in all subjects. We used fasting insulin instead of hyperinsulinemic euglycemic glucose clamp studies to define insulin resistance due to logistic and feasibility problems. However, fasting insulin has been shown to correlate fairly well with the gold standard of hyperinsulinemia euglycemic clamp for use in population based studies. The strength of our study is that it is the first study to evaluate the association of TNF-α gene polymorphism with insulin resistance and measures of adiposity including detailed abdominal fat distribution in Asian Indians in India.

In conclusion, −308G/A polymorphism of TNF-α is not associated with serum levels of TNF-α, insulin resistance and indicators of obesity in Asian Indians in India. Subjects with A allele did exhibit a trend towards higher prevalence of elevated IAF area. Serum TNF-α levels do not play a major role in generalized or abdominal adiposity, but may play a role in insulin resistance in Asian Indians.

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