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RESEARCH ARTICLE

THE TNF- α SINGLE NUCLEOTIDE POLYMORPHISM AND THE ROLE OF INFLAMMATORY CYTOKINES IN DIABETIC NEUROPATHY

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ABSTRACT

Immunological and inflammatory reactions play a pivotal role in the initiation and perpetuation of Diabetic Neuropathy (DN). The present study is an attempt to estimate the levels of adenosine deaminase (ADA) and C-reactive protein (CRP) activity and for genotyping TNF alpha (-308) polymorphism in patients with Diabetic neuropathy.

Methods: 50 cases presenting Diabetic neuropathy and 50 cases of age and sex matched healthy controls were included in the study. Serum ADA activity was measured spectrophotometrically at 630 nm and serum C-reactive protein was detected using Avitex CRP kit, which is a rapid latex agglutination test and ARMS PCR was done for genotyping of TNF alpha (-308) polymorphism using allele specific primers.

Results: The mean ADA levels were 37.2 ± 5.0 in patients and 18.2 ± 5.6 in controls, significant at $p< 0.01$. CRP test was found to be positive in 20/20 cases of Diabetic Neuropathy and none of the controls. SNP at position −308 promoter gene of TNF-α was not significantly associated with development of Diabetic Neuropathy.

Interpretation & conclusions: The present study observed the importance of ADA as a serum marker in addition to CRP for better therapeutic management of DN. SNP at position −308 promoter gene of TNF-α was not significantly associated with development of Diabetic neuropathy. The odds ratio and relative risk estimates of AA phenotype showed an increased risk to have the disease when compared with the other phenotypes.

INTRODUCTION

Diabetes mellitus (DM) is one of the most widespread chronic diseases in the world. It can be caused by the genetically predisposed lack of insulin or by the body unresponsiveness to insulin resulting in elevated blood sugar levels. According to the International Diabetes Federation, diabetes affects more than 230 million people worldwide and is expected to affect 350 million by 2025. In 2003, the five countries with the largest number of people with diabetes were India (35.5 million), China (23.8 million), the United States (16 million), Russia (9.7 million) and Japan (6.7 million) (Christian Nordqvist, 2006). Untreated diabetes may cause severe health complications which can be largely divided into macrovascular and microvascular complications. The macrovascular complications include cerebrovascular disease, coronary heart disease, and peripheral vascular disease. The microvascular complications include diabetic retinopathy, diabetic neuropathy, and diabetic nephropathy. One of the most frequently-occurring microvascular complications is diabetic neuropathy (DN).

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Diabetic neuropathy is a decrease in nerve function typically affecting the lower limbs in people with diabetes. The peripheral nerves become damaged by persistently elevated blood sugar levels. This results in significant disability and morbidity (Braunwald *et al.,* 2001). Complications of diabetic neuropathy include severe pain, loss of ambulation and increased risk of foot ulceration and amputation. Life-time risk of foot amputation is 15% in patients with diabetic neuropathy (Feldman *et al.,* 1999). Different hypotheses have been proposed to explain the various modes of progression of diabetic neuropathy. It has been suggested that consumption of oral hypoglycemic agents such as glyburide (Quasthoff, 1998) and angiotensin converting enzyme inhibitors (ACE) inhibit the progression of neuropathy irrespective of blood glucose level (Martinez-Blasco *et al.,* 1998). Early diagnosis and treatment of diabetic neuropathy is important for preventing secondary complications and improving quality of life. Considering that diabetes affects an estimated 177 million people worldwide, more than 20 million people suffer from diabetic neuropathy with a remarkable range of clinical manifestations (WHO, 2002). Most human diseases linked to specific genetic polymorphisms are chronic in nature. Among the few known genetic polymorphisms that seem to affect the

risk and progression of infection, single-nucleotide polymorphisms in the cytokine cascade stand out (Kovar and Florian, 2007). Tumor Necrosis Factor- Alpha (TNF-α) plays a major role in the immune system.. A study in type 1 diabetes found increased levels of TNFα in subjects with diabetic neuropathy (Ronald Goldberg, 2009). Adenosine deaminase (ADA) is related to lymphocytic proliferation and differentiation. As a marker of cell mediated immunity, its activity is found to be elevated in the diseases in which there is a cell – mediated immune response (Galanti *et al.,* 1981). C-reactive protein (CRP), an acute phase protein is synthesized by hepatocytes in response to proinflammatory cytokines in particular IL-6. It was recently reported that CRP was independently associated with development of diabetic neuropathy (Ronald Goldberg, 2009). Therefore the present study is an attempt to estimate the levels of adenosine deaminase (ADA) a marker for cell mediated immunity and C-reactive protein (CRP) a marker for inflammation ; and to examine functional SNPs primarily at the position on gene of tumor necrosis alpha (TNFα) −308G/A in order to establish their association with peripheral neuropathy in type 2 diabetes.

MATERIALS AND METHODS

A total of 50 patients presenting diabetic neuropathy attending local Government King George General Hospital, Visakhapatnam were included in the study. The diagnosis of diabetic neuropathy was established by clinical analysis. Equal number of age and sex matched healthy individuals with no known history of any disease were taken as controls. All the subjects were examined clinically and information pertaining to age, sex, habits and health status were recorded. The patient's ages were ranged between 30 and 80 years. Blood samples were collected in sterile vials containing 15% EDTA as an anticoagulant from both controls and patients for the estimation of serum ADA and C-reactive protein and for DNA isolation.

The serum was assayed immediately after collection of samples for ADA activity at 37 ˚C by a spectrophotometric method using adenosine as the substrate. This method is based on the Bertholet reaction, the formation of colored indophenol complexes from ammonia liberated from adenosine and quantified by spectrophotometrically at 630 nm (Guisti, 1974). The activity of ADA is expressed in units per liter. For the detection of CRP in serum, Avitex -CRP kit was used which is a rapid latex agglutination test. The latex suspension is mixed with serum containing elevated CRP levels, clear agglutination was observed within minutes. Avitex –CRP has detection limit of 6 mg per liter of CRP in the patient's serum. The test is considered as positive when the CRP serum concentration is above 6mg/litre and negative when it is at 6 mg per liter and below. DNA was isolated by salting out method as described by Lahari *et al*. (1992). ARMS PCR was done for genotyping of TNF alpha (-308) polymorphism using allele specific primers for detection of single nucleotide polymorphisms. The amplified product of 183bp was viewed on 2% agarose gels stained with ethidium bromide. The data of the study subjected to statistical analysis is expressed as mean ± SD. Analysis of the data was carried out using Epi Info 5 software. In addition, the gene frequencies were estimated by using maximum likelihood methods of Balakrishnan (1988) and goodness of fit between the observed and expected phenotype frequencies were tested according to Taylor & Prior (1938). Genotype frequencies were checked for deviation from Hardy–Weinberg equilibrium and were not significantly different from those predicted. Odds ratios and 95% confidence interval (95% CI) were calculated to assess the strength of the relationship between the TNF-α gene polymorphisms with diabetic neuropathy. Pooled odds ratios and relative risk were calculated by the random-effects method of DerSimonian and Laird (1986). For odds ratio, confidence interval was calculated. Increased risk was calculated using the formula: Increased Risk = (Relative Risk – 1.00) x 100. The significance level was 5%.

RESULTS

50 cases presenting Diabetic neuropathy and 50 cases of age and sex matched healthy controls were included in the present study. The sex and mean age of the study group is represented in Table 1. The diabetic neuropathy patients had a mean age of 47.2 ± 13.92 years and included 51% males and 49% females, whereas the controls had a mean age of 46.2 ± 14.42 years with 48% males and 52% females. The ADA and CRP levels estimated in diabetic neuropathy patients and controls are presented in table 2. The mean ± SD of ADA levels of serum in diabetic neuropathy patients are 37.2±5.0 nmoles/ml and in controls are 18.2±5.6 nmoles/ml. The ADA levels in patients are significantly higher when compared to that of controls. All the diabetic neuropathy patients were positive for CRP, while all the controls were negative for the test. The CRP levels in patients were significantly higher when compared to the controls. Table 3 represents the TNF – α promoter polymorphisms (G-308A). Genotype frequency obtained from TNF-α gene analysis in patients with diabetic peripheral neuropathy revealed that the majority of them were G/G homozygotes (70%) followed by G/A heterozygotes (26%) and the least frequent are A/A homozygotes (4%). The control group had genotypic frequency of 80% homozygotes(GG) followed by diminished frequencies of 18% G/A and 2% A/A. The allelic frequency in patient group was 83% of G and 17% of A, where as the control group showed 89% of G allele and 11% of A allele. Genotype frequencies were in Hardy-Weinberg equilibrium. As only two subjects had A-308A genotype of the TNF-α gene, they were combined with subjects who had the G-308A genotype. Our results indicate that there is no significant ($p < 0.05$) difference in the genotype as well as allele frequencies of SNP in the TNF α promoter gene at this position i.e. −308 amongst the DN and control groups. Comparison between wild-type genotype and combination of hetero and mutant-type (GG vs. GA+AA) of SNP in the promoter region of TNF α in both study groups also showed non significant difference. The inter group heterogeneity was found to be (χ^2: 1.3936; d.f. =2; $0.50 > p > 0.30$), a non significant value observed between diabetic neuropathy patients and controls.

Test of association of TNF α phenotypes with the disease condition compared to the control group, the odds ratio and relative risks for each genotype versus the other two are shown in Table 4. AA homozygote were at increased risk of diabetic neuropathy, with an overall odds ratio of 2.04 (95 percent Confidence Interval: 0.10, 122.87; p = 0.5577) by the method of Dersimonian and Laird. Heterozygote (GA) were also at an increased risk of diabetic neuropathy, with an overall odds

Table 1: Sex and mean age of study group

	Sex Males/Females	Age Mean±SD
Patients	51/49	47.2 ±13.92
Controls	48/52	46.2 ±14.42

Table 2: CRP levels and ADA levels in diabetic neuropathy and controls

	C-Reactive Protein (CRP)		Adenosine Deaminase (ADA)	
	Diabetic Neuropathy	Controls	Diabetic Neuropathy	**Controls**
Mean ±SD	3.59 ± 1.03	0.98 ± 0.32	37.2 ± 5.00	18.2 ± 5.60

Table 3: Distribution of phenotypes and allele frequencies in diabetic neuropathy and controls

System	Phenotype	Diabetic Neuropathy		Controls	
		Observed	Expected	Observed	Expected
TNF	G/G	35	34.44	40	39.61
	G/A	13	14.11	9	9.79
	A/A	2	1.44	1	0.60
	Total	50	50.00	50	50.00
		$\chi^2 = 0.3141$ (0.90 > þ > 0.80)		$\chi^2 = 0.3341$ (0.90 > þ > 0.80)	
	Allele				
TNF	G	0.8300 ± 0.0376		0.8900 ± 0.0376	
	A	0.1700 ± 0.0376		0.1100 ± 0.0376	

Table 4: Test of Association, Relative Risk, Odds Ratio and 95% Confidence Interval Estimates of TNF- α phenotypes in disease and control groups

TNF α Phenotype combinations	Control (n)	Diabetic Neuropathy (n)	RR	OR	95% CI	χ^2 values
AA vs GG + GA	40	35	2.00	2.04	0.10 – 122.87	0.3400
GA vs GG + AA	9	13	1.44	1.60	0.55 – 4.76	0.9300
GG vs GA + AA	1	2	0.88	0.58	0.21 – 1.60	1.3300

ratio of 1.60 (95% CI: 0.55, 4.76; p = 0.3342). The odds ratio of diabetic neuropathy for homozygote (GG) were at reduced risk, with an overall odds ratio of 0.58 (95% CI: 0.21, 1.60; p = 0.2482). In the present study, Risk estimates show a significant association of AA and GA phenotypes with diabetic neuropathy individuals (RR = 2.00 & 1.44) respectively. The result shows an increased risk of 100% and 50% more, indicating that individuals with AA phenotype are two times more likely to get the disease when compared with the other phenotypes of the TNF α.

DISCUSSION

Diabetic neuropathy is a common complication of diabetes mellitus with high morbidity and impairment of quality of life. Tesfye *et al.,* (1996) studied 3,250 diabetic patients and reported an overall prevalence of peripheral neuropathy in 28% of them. The incidence of neuropathy is significantly increased in older patients. Few studies have shown associations of neuropathy with age and duration of diabetes (Boulton *et al*., 2006). The results of the present study confirm previous reports. We found no difference in the diabetic neuropathy rate between the genders, which also has been confirmed by others (Janghorbani *et al.,* 2006). ADA has been considered as a marker for cell mediated immunity. It has strongly been suggested that serum ADA activity reflects monocyte/macrophage activity or turnover in different diseases (Ungerer *et al.,* 1992). The results of the present study in diabetic neuropathy patients indicated highly significant mean levels of ADA in patients compared to controls. Serum Adenosine deaminase activity was also found to be high in myocardial infarction (Jyothy *et al.,* 2003), suggesting the contribution of immunological and inflammatory process in the pathogenesis of coronary heart disease.

The prototypic marker of inflammation is C-reactive protein (CRP) a member of the pentraxin family. The production of CRP in the liver is triggered by various proinflammatory cytokines derived either from monocytes or macrophages. The proinflammatory response results in the increased secretion of interleukin –1 β and tumor necrosis factor - α which then results in the release of the messenger cytokine, interleukin - 6 which stimulates the liver to secrete CRP. It was thought as a bystander marker of inflammation, without playing a direct role in the inflammatory process. Recent studies suggest that CRP may also contribute directly to the proinflammatory state (Bharadways *et al.,* 1999). In the present study the levels of C-reactive protein were significantly high in the patients compared to controls. Similarly Christian Herder *et al.,* (2009) also observed high values of CRP indicative of active inflammation in diabetic neuropathy patients.

Inflammation is a well-known risk factor for the development of macro vascular disease. The present study indicates an association between a variety of inflammation

markers and the development of diabetic neuropathy. Tumor necrosis factor is a cytokine that is involved in systemic inflammation and is a member of a group of cytokines that stimulate the acute phase reaction (Locksley *et al.,* 2001). The promoter polymorphism in the TNF gene has been implicated in the regulation of TNF-α production and has been associated with a wide spectrum of inflammatory and infectious diseases, though results from different studies are not consistent. Majority of the studies till date reported lack of association between TNF-α (-308) polymorphism and diabetic retinopathy (Shabnam Montazeri *et al.,* 2010). Based on the statistical analysis of this study, we could detect no significant difference in the genotype and allele frequencies at this SNP between the control and diabetic neuropathy subjects. In contrast, a previous study has shown that −308 A allele in the promoter of the human TNF α gene was associated with higher risk for type 2 diabetes mellitus (Kubaszek *et al.,* 2003). However the correlation between SNP in TNF α gene promoter and type 2 diabetes is still controversial, because of discrepancies among different studies (Shiau *et al.,* 2003).

Furthermore the genotypic frequencies also vary in diverse populations. SNP database search for genotype frequency of TNF (rs1800629) showed a higher frequency of G/G genotype (95%), followed by G/A (6%) and negligible frequency of A/A in Asian population. The same trend was observed in our study. Whereas another study from South India population had observed a very high frequency of G/A heterozygotes (94%) in both patients and control groups (Venkata Karunakar *et al.,* 2009) Thus, it indicates that TNF-α 308 gene polymorphism may not be a precise marker for diabetic neuropathy in type 2 diabetic patients from South India. The TNFa gene may require further investigation on a functional basis, to elucidate the genetic role of the Th1/Th2 cytokine responses in the aetiopathogenesis of diabetic neuropathy.

Conclusions

The present study suggests the importance of measuring the biomarkers of inflammation assessed in the study not only to determine the severity of inflammation but also to evolve targeted treatment strategies for better management of the condition. The data indicate the elevated levels of serum adenosine deaminase and C- reactive protein in patients when compared with healthy individuals, indicating that subclinical inflammation is associated with diabetic neuropathy and neuropathic impairments. SNP at position −308 promoter gene of TNF-α was not significantly associated with development of diabetic neuropathy. However, the odds ratio and relative risk estimates of AA phenotype showed an increased risk to have the disease when compared with the other phenotypes. These results appear to encourage further investigation into the role of cytokines in the pathogenesis of diabetic neuropathy.

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