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Research Article

The cytokine gene polymorphisms (TNF-α, IL-10 And IFN-γ) and the role of inflammatory cytokines in diabetic neuropathy

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ABSTRACT

Background: One of the most frequently-occurring micro vascular complications is diabetic neuropathy (DN). Diabetic nephropathy (DN) affects approximately one third of people with type 1 or type 2 diabetes mellitus The objective of the study is an attempt to examine functional SNPs primarily at the position on gene of TNF-α (-308 G/A, rs 1800629), IL-10 (-1082 G/A, rs 1800896) and IFNγ (+874 A/T, rs 62559044) in order to establish their association with peripheral neuropathy patients with type 2 diabetes.
Methods: 150 cases presenting Diabetic neuropathy and 160 cases of age and sex matched healthy controls were included in the study. ARMS PCR was done for genotyping of TNF-α (-308), IL-10 (1082 G/A) and IFNγ (+874) polymorphism using allele specific primers for detection of single nucleotide polymorphisms. Analysis of the data was carried out using Epi Info 5 software. In addition, the gene frequencies were estimated and goodness of fit between the observed and expected phenotype frequencies was tested. Multifactor Dimensionality Reduction (MDR) analysis was performed to study case-control data and gene-gene interactions.
Results: The results revealed that the chi- square test for heterogeneity for IL-10 system was found to be significant (χ^2 = 16.2380; d.f = 2; p >0.001) between patients and controls, indicating a significant departure from the HWE. Thus, the test of association of both homogeneity and heterogeneity of IL-10 showed a significant difference, indicating an association of IL-10 with diabetic neuropathy.SNPs at position −308 promoter gene of TNF-α and IFNγ (+874) were not significantly associated with development of Diabetic Neuropathy.
Conclusion: This case-control study suggests that IL-10-1082G/G polymorphism is associated with the susceptibility to diabetic neuropathy in type 2 DM patients. IL-10 serves as an important bio marker in Indian population for their susceptibility to Diabetic Neuropathy as it may play a role in alteration of IL-10 production and the inflammatory responses.

Keywords: Diabetic Neuropathy, Tumor Necrosis Factor Alpha, Interleukin – 10, Interferon Gamma

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).[1] Untreated diabetes may cause severe health complications which can be largely divided into macro vascular and micro vascular complications. The macro vascular complications include cerebrovascular disease, coronary heart disease, and peripheral vascular disease. The micro vascular complications include diabetic retinopathy, diabetic neuropathy, and diabetic nephropathy.

Cytokines are key mediators which regulate immune response; and their expression by immune cells depends on several factors such as infection, inflammation, hormonal conditions and also relevant gene polymorphisms.[2] Recent studies have shown that inflammation, and more specifically pro-inflammatory cytokines, play a determinant role in the development of micro vascular diabetic complications. One of the most frequently occurring micro vascular complications is diabetic neuropathy (DN). Diabetic nephropathy (DN) affects approximately one third of people with type 1 or type 2 diabetes mellitus.[3] It is a multifactorial disease. It develops as a result of hyperglycemia-induced local metabolic, enzymatic and micro vascular changes. The disease gradually progresses and involves small and large sensory fibers.[4] 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. These results in significant disability and morbidity.[5] Complications of diabetic neuropathy include severe pain, loss of ambulation and increased risk of foot ulceration and amputation. Lifetime risk of foot amputation is 15% in patients with diabetic neuropathy.[6] 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[7] and angiotensin converting enzyme inhibitors (ACE) inhibit the progression of neuropathy irrespective of blood glucose level.[8] 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.[9]

In recent years, our knowledge of the pathophysiological processes that lead to diabetic nephropathy has notably improved on a genetic and molecular level.

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.[10] TNF-α is a multifunctional cytokine. It can directly inhibit phosphorylation of insulin receptor's substrate and reduce glucose uptake by peripheral tissues.[11] In human, the gene is located on chromosome # 6 [p21.3].[12] Several studies have shown that SNP at position -308 A/G were associated with various inflammatory conditions.[13] Interleukin [IL-10] is a potent inflammatory cytokine. The main function of IL-10 is terminating the inflammatory signal in inflammatory cells. It promotes B cell activation. The human IL-10 gene is located on chromosome # 1 [1q31-q32] and encodes for five exons. It has been shown to limit the cascade of proinflammatory cytokines activation[14] and to down regulate T cell-mediated immune responses.[15] INF-γ is aTh1 cytokine which supports the immune system to perform cytolysis of target cells and also was reported to be increased in diabetes mellitus.[16] IFN-γ gene intron-1 polymorphism was speculated to influence immune complex disease susceptibility which is characterized by an imbalance of various immunoregulatory systems.[17] The gene is located on chromosome # 12 [12q14].

The objectives of the study is an attempt to examine functional SNPs primarily at the position on gene of TNF-α (-308 G/A, rs 1800629), IL-10 (-1082 G/A, rs 1800896) and IFNγ (+874 A/T, rs 62559044) in order to establish their association with peripheral neuropathy patients with type 2 diabetes.

METHODS

A total of 150 patients (85 males and 65 females) presenting diabetic neuropathy attending local Government King George General Hospital, Visakhapatnam, Andhra Pradesh were included in the study. The diagnosis of diabetic neuropathy was established by clinical analysis. 160 members of age and sex matched healthy individuals with no known history of any disease were taken as controls (85 males and 75 females). 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 DNA isolation. DNA was isolated by salting out method.[18] All the three cytokine gene polymorphisms were typed by using amplification refractory mutation system polymerase chain reaction [ARMS – PCR][19] was done for genotyping of TNF-α(-308 G/A), IL-10 (-1082 G/A) and IFNγ (+874 A/T) polymorphism using allele specific primers for detection of single nucleotide polymorphisms. The amplified products were separated on 2% agarose gels stained with ethidium bromide and visualized under a UV transilluminator.

Analysis of the data was carried out using Epi Info 5 software. In addition, the gene frequencies were estimated by using maximum likelihood methods[20] and goodness of fit between the observed and expected phenotype frequencies were tested.[21] 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 IL-10 gene polymorphisms with diabetic neuropathy. Pooled odds ratios and relative risk were calculated by the random-effects method.[22] For odds ratio, confidence interval was calculated. Increased risk was calculated using the formula: Increased 5Risk = (Relative Risk – 1.00) x 100. The significance level was 5%.

Multifactor Dimensionality Reduction (MDR) analysis was performed using MDR software (v. 3.0.2) to study

case-control data, gene-gene interactions, and gene-environment interactions.[23,24] Best models with possible combinations of the polymorphisms were considered based on 10-fold cross validation and maximum testing accuracy. Once MDR identifies the best combination of factors, the final step is to determine which multifactor levels (genotypes) are high risk and which are at low risk using the entire data set. This final evaluation is conducted with a threshold ratio that is determined by the ratio of the number of affected individuals divided by the number of unaffected individuals in the data.

RESULTS

Distribution of TNF-α, IL-10 and IFNγ polymorphisms in the study groups is presented in Table 1. The genotyping was done for the entire group of 150 patients with diabetic neuropathy and 160 controls. The genotypic distributions of TNF- α A/A, A/G and G/G genotypes in diabetic neuropathy patients were 17.3%, 56% and 26.7% respectively and in controls it was 17.5%, 53.7% and 28.7% respectively. The genotypic distributions of IFNγ A/A, A/T and T/T genotypes were 30%, 48.7% and 21.3% in diabetic neuropathy patients and in controls it was 21.9%, 53.1% and 25% respectively. According to Hardy-Weinberg equilibrium model, the frequencies of observed genotypes of both patients and controls did not deviate significantly from those expected (TNF-α: χ^2 = 2.5474; df = 1, p>0.10; IFNγ: χ^2 = 0.0594; df = 1, p>0.80). Observed genotype frequencies of IL-10 A/A, G/A and G/G phenotypes in patients was 18%, 23.3% and 58.7% and in controls, it was 8.1%, 43.1% and 48.7% respectively. Our results indicate that there is a significant difference in the genotype frequencies in the IL-10 gene at this position i.e. −1082 amongst the diabetic neuropathy patients (χ^2 = 29.3594; d.f = 1; p > 0.001) indicating a significant deviation from the Hardy-Weinberg equilibrium.

Table 1: Distribution of the TNF-α, IL-10 and IFNγ phenotypes in patients and controls.

System Cytokine	Phenotype	Diabetic Neuropathy (Patients)		Controls	
		Obs.	Exp.	Obs.	Exp.
TNF-α (-308 G/A)	A/A	26	30.38	28	30.98
	A/G	84	74.25	86	78.85
	G/G	40	45.37	46	50.18
	Total	150	150.00	160	160.00
		χ^2= 2.5474 (0.20>P>0.10)		χ^2= 1.2831 (0.30>P>0.20)	
IL-10 (-1082 G/A)	G/G	27	13.05	13	13.92
	G/A	35	62.39	69	66.55
	A/A	88	74.56	78	79.53
	Total	150	150.00	160	160.00
		χ^2= 29.3594 (P>0.001)		χ^2= 0.1804 (0.70>P>0.50)	
IFNγ+874		45	44.56	35	37.64
	A/A	73	74.39	85	79.93
	A/T	32	31.05	40	42.43
	T/T	150	150.00	160	160.00
	Total	χ^2= 0.0594 (0.90>P>0.80)		χ^2= 0.6460 (0.50>P>0.30)	

The allelic distributions of TNF-α, IL-10 and IFNγ polymorphisms are given in Table 2. The frequency of the A and G alleles in diabetic neuropathy patients were 45% and 55% and in controls it was 44% and 56% for TNF-α. For IL-10, the frequency of G and T alleles were 70% and 29% in patients. Same frequencies were observed in controls also. The frequency of A and T alleles in patients were 54% and 45% and in controls were 48% and 51% for IFNγ. The results revealed that the chi- square test for heterogeneity for IL-10 system was found to be significant (χ^2 = 16.2380; d.f = 2; p >0.001) between patients and controls, indicating a significant departure from the HWE. Thus, the test of association of both homogeneity and heterogeneity of IL-10 showed a significant difference, indicating an association of IL-10 with diabetic neuropathy.

Association between different combinatory forms of alleles was estimated. Test of association of TNF-α, IL-10 and IFNγ 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 3. In TNF-α, the heterozygous (GA) individuals are equally likely to get the disease (RR = 1.04), with an overall odds ratio of 1.10 (95% CI: 0.68, 1.76; p = 0.6907). Individuals with the other two groups (AA and GG) were at a reduced risk of diabetic neuropathy. In IL-10, the Risk estimates show a significant association of

GG and AA phenotypes with diabetic neuropathy individuals (RR = 2.22 & 1.20 respectively), with an overall odds ratio of 2.48 (95% CI: 1.17, 5.33, p = 0.0095) and 1.49 (95% CI: 0.93, 2.40, p = 0.0802) respectively. The result shows an increased risk of 100% and 49% more, indicating that individuals with GG phenotypes are 2 times more likely to get the disease when compared with the other phenotypes of the IL-10.

In IFNγ the Risk estimates show a significant association of AA phenotypes with diabetic neuropathy individuals (RR = 1.37), with an overall odds ratio of 1.53 (95% CI: 0.89, 2.64, p = 0.1022). The result shows an increased risk of 30% more, indicating that individuals with AA phenotype are 1.3 times more likely to get the disease when compared with the other phenotypes of the IFNγ.

Table 2: Allele frequencies in diabetic neuropathy patients and controls.

System (Allele)	Diabetic Neuropathy	Controls	Intergroup Heterogeneity	d.f
TNF-α A	0.4500 ± 0.0287	0.4400 ± 0.0277	0.1932	
G	0.5500 ± 0.0287	0.5600 ± 0.0277	(0.95>p>0.90)	2
IL-10 G	0.2950 ± 0.0263	0.2950 ± 0.0255	16.2380	
A	0.7050 ± 0.0263	0.7050 ± 0.0255	(p>0.001)	2
IFN γ A	0.5450 ± 0.0287	0.4850 ± 0.0279	2.7305	
T	0.4550 ± 0.0287	0.5150 ± 0.0279	(0.30>P>0.20)	2

Table 3: Test of Association, Relative Risk, Odds Ratio and 95% Confidence Interval Estimates of TNF α, IL-10 and IFN γ Phenotypes in DN patients and Control Group.

System	Phenotype combinations	Control (n)	Diabetic Neuropathy					
			(n)	RR	OR	95% CI	χ^2 values	p-value
TNF α	AA vs GG + GA	28	26	0.99	0.99	0.53-1.85	0.00	0.9691
	GA vs GG + AA	86	84	1.04	1.10	0.68-1.76	0.16	0.6907
	GG vs GA + AA	46	40	0.93	0.90	0.53-1.53	0.17	0.6822
IL-10	GG vs GA + AA	13	27	2.22	2.48	1.17-5.33	6.72	0.0095
	GA vs GG + AA	69	35	0.54	0.40	0.24-0.67	13.60	0.0002
	AA vs GG + GA	78	88	1.20	1.49	0.93-2.40	3.06	0.0802
IFN γ	AA vs TT + TA	35	45	1.37	1.53	0.89-2.64	2.67	0.1022
	TA vs TT + AA	85	73	0.92	0.84	0.52-1.34	0.62	0.4326
	TT vs TA + AA	40	32	0.85	0.81	0.46-1.43	0.58	0.4448

Table 4: Results of MDR analysis on genetic factors.

No. of loci	Polymorphism Model	Testing Accuracy	CVC	Prediction Error (%)
1	IL-10	0.5990	10/10	40.10*
2	IL-10, IFN-γ	0.5612	7/10	43.88
3	IL-10, IFN-γ, TNF-α	0.5946	10/10	40.54*

*P≤0.01 based on 1000 permutations.

MDR software was used to analyze the interaction of the 3 factors that may affect the diabetic neuropathy, and the results were detailed in Tables 4 and 5. We found that the cross-validation (CV) consistency of the three-factor model (IL-10*IFN-γ*TNF-α) was maximal (10/10), and the accuracy of the test samples was the highest (0.5946). Permutation testing was used to perform a hypothesis test and evaluate its statistical significance. Thus, the three-factor interaction model was the best model, which shows that there was an interaction between the three SNP's (p≤0.01). Table 5 summarizes the three locus genotype combinations associated with high risk and with low risk, along with the corresponding distribution of cases and of controls, for each multilocus genotype combination. The cell is labeled as either high risk if the case–control ratio reaches or exceeds a predetermined threshold (for example, ≥1) and low risk if it does not reach this threshold (Fig 1). Figure 2 illustrates the MDR

interaction information analysis of the three polymorphisms, represented in the form of a dendrogram. The interaction information analysis revealed a strong synergism between the three SNPs markers TNF-α, IL-10 and IFNγ contributing to Diabetic Neuropathy.

Table 5: Distribution of high-risk and low-risk genotypes in the best three locus model.

Pattern	Multilocus–genotype combinations			No. of cases (DN)	No. of controls	Association with DN
	TNF- α	IL – 10	IFN – γ			
1	A/A	A/A	T/T	5	2	High-risk
2	A/A	A/A	A/A	7	5	High-risk
3	A/A	G/G	T/T	2	0	High-risk
4	A/A	G/G	A/A	1	0	High-risk
5	A/A	G/G	A/T	4	1	High-risk
6	A/G	A/A	A/A	14	8	High-risk
7	A/G	A/A	A/T	26	18	High-risk
8	A/G	G/G	T/T	2	2	High-risk
9	A/G	G/G	A/T	10	3	High-risk
10	A/G	A/G	A/A	8	5	High-risk
11	G/G	A/A	T/T	6	6	High-risk
12	G/G	A/A	A/A	8	6	High-risk
13	G/G	G/G	A/A	3	0	High-risk
14	G/G	G/G	A/T	4	3	High-risk
15	A/A	A/A	A/T	5	7	Low-risk
16	A/A	A/G	T/T	0	4	Low-risk
17	A/A	A/G	A/A	1	3	Low-risk
18	A/A	A/G	A/T	1	6	Low-risk
19	A/G	A/A	T/T	10	16	Low-risk
20	A/G	G/G	A/A	0	2	Low-risk
21	A/G	A/G	T/T	5	6	Low-risk
22	A/G	A/G	A/T	9	26	Low-risk
23	G/G	A/A	A/T	7	10	Low-risk
24	G/G	G/G	T/T	1	2	Low-risk
25	G/G	A/G	T/T	1	2	Low-risk
26	G/G	A/G	A/A	3	6	Low-risk
27	G/G	A/G	A/T	7	11	Low-risk

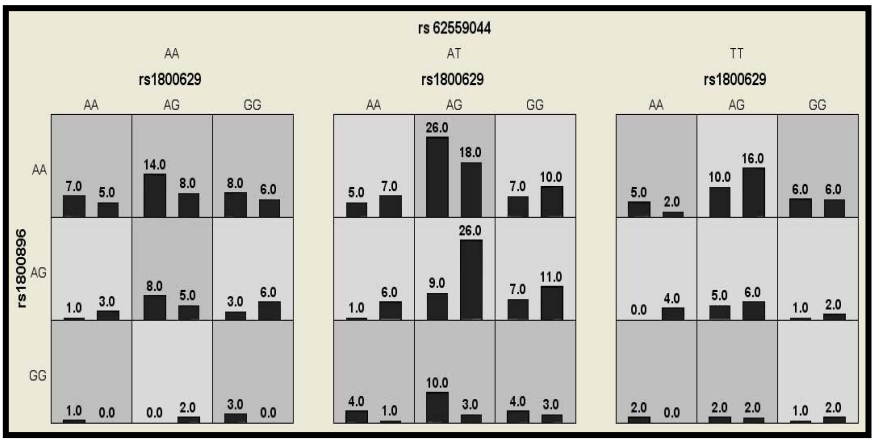

Figure 1: An MDR Analysis of the Three-factors (IL-10*IFN- γ*TNF-α) - Interaction Model of Diabetic Neuropathy.

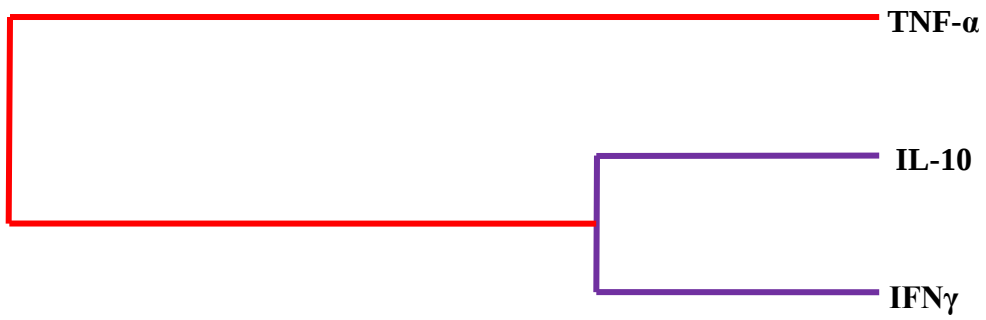

Figure 2: A Tree Diagram of the Interactions among three Factors (IL-10*IFN- γ*TNF-α) for Diabetic Neuropathy, as Analyzed by MDR.

In the cell in the figure, the left bands represent the disease case, and the right bands represent the control case. High-risk combinations are depicted as darkly shaded cells, low-risk combinations as lightly shaded cells.

DISCUSSION

The genetic basis of multifactoral diseases like diabetes probably consists of several predisposing risk factors that can interact with environmental factors to produce the disease phenotype. Diabetic neuropathy is one of the common complications of diabetes mellitus with high morbidity and impairment of quality of life reported an overall 28% of prevalence of peripheral neuropathy.[25] In diabetic patients the impaired insulin signaling and selective destruction of insulin producing beta cells in which cytokines play an important role. Certain pro-inflammatory cytokines are capable of interfering with insulin sensitive glucose uptake and can induce insulin resistance.[26] Higher levels of pro-inflammatory cytokines correlate with the incidence of neuropathy.[27] Spinal proinflammatory cytokines such as TNF-α, IFNγ and IL-10 are powerful pain-enhancing signals that contribute to neuropathic pain.[28,29] The present study focused on gene polymorphisms of pro and anti-inflammatory cytokines that may be responsible for nerve damage in diabetic neuropathy. The present study focused on three common functional SNPs primarily at the positions on genes of tumor necrosis alpha TNF-α 308G/A, interferon gamma (IFNγ) +874A/T and interleukin (IL-10) -1082G/A in order to establish their association with peripheral neuropathy in type 2 diabetes.

Inflammation is a well-known risk factor for the development of macro vascular disease. Excess TNF-α can result in harmful inflammatory responses.[30] Whereas too little can contribute to persistent infection.[31] In that regard, some polymorphisms in the TNF-α gene have been associated with susceptibility to certain autoimmune diseases,[32,33] infectious diseases,[34] certain cancers[35] and diabetes mellitus.[36] However, it has been shown to be non-significant in many other inflammatory diseases.[37-39] Our study also, could not find any association of TNF-α -308 gene polymorphism with diabetic peripheral neuropathy. Similarly, a study in South Indian population also could not find any association of TNF-α with diabetic peripheral neuropathy.[40] Very few reports are available on the association of TNF-α gene polymorphisms with risk of diabetic neuropathy. Interestingly, two studies from South Indian population, present study and other study[40] observed a higher frequency of G/A heterozygotes. To reach a more reliable and comprehensive conclusion, a further investigations on functional basis, to elucidate the genetic role of the cytokine responses in the pathogenesis of Diabetic neuropathy is required.

IL-10 gene is an anti-inflammatory cytokine that may regulate the complex network of reactions. The difference in anti-inflammatory profile is determined not only by the levels of IL-10 production with neurological deterioration and also by the functional polymorphisms of the gene. Several functional IL-10 gene polymorphisms have been described.[41] One of the polymorphism that is associated with low, medium or high production of IL-10 situates in the promoter region of the gene at position -1082.[42] Based on clinical evaluation of diabetic neuropathy, the 'high-producer' IL-10-1082 G/G genotype may be responsible for the down regulation of immune response leading to inflammation in diabetic neuropathy patients. The most prominent feature from the present study is the significant higher frequency of IL-10 (-1082) GG genotype with higher frequency of GG allele among diabetic neuropathic patients. These findings are in agreement with other study.[40] Studies conducted in France and Spain did not confirm any significant association of DM with different genotypes of IL-10 promoter polymorphisms in Caucasians.[43-45] Whereas few reports from Taiwanese,[46] Turkish[47] and Egyptian subjects[48] has shown association of IL-10-1082 homozygous GG with DM cases.

The SNP at position +874 A/T plays a fundamental role in the induction of IFNγ production.[49] In the first intron of the IFNγ gene, there is a CA repeat polymorphism that affects transcription. Moreover, an adenine (A) to thimine (T) transition at position þ874 (intron 1) has been associated with increased IFNγ expression.[50] The T allele of IFNγ+874A/T provides a binding site for the transcription factor NF-kB, which is able to regulate IFNγ expression.[50] It is possible that low IFNγ production will facilitate not as much of immune response against inflammation rendering

these individuals more susceptible to the disease as the downstream process would eventually leads to nerve damage.[40] Our observation shows profound increase in the distribution of IFNγ +874 homozygous A/A genotype which is significantly associated with diabetic neuropathy. The same trend was observed in another South Indian diabetic neuropathy cases.[40] Egyptian diabetic cases[50] and Greece diabetic populations[51] and a North Indian population with cervical cancer.[52]

The pathogenetic vision of diabetes mellitus has changed in the last several years, with inflammatory pathways playing major roles in the development and progression of diabetic complications. Many of the proposed mechanisms are interdependent and it is likely that more than one mechanism is involved in the development of the chronic complications of diabetes. Understanding the role of risk factors and molecular biomarkers may help in early diagnosis and risk prediction of the condition. In addition to that, there may be genetic influences in either protecting or making them susceptible to the development of complications. Effective treatment and/or prevention will therefore require an integrated approach combining multiple strategies to target the underlying inflammatory processes. Diabetic Neuropathy designed to target specific cytokines, chemokines, growth factors and even transcription factors is already well underway. Besides the assessment of pharmacological safety, bioavailability, and efficacy *in vivo*, more clinical studies will further support the potential of such strategies to be used in diabetes therapy. It is possible that in the coming years the hope of new therapeutic and preventive strategies based on inflammatory properties with beneficial actions on diabetic complications can be translated into real clinical treatment.

CONCLUSIONS

In conclusion, this case-control study suggests that IL-10-1082G/G polymorphism is associated with the susceptibility to diabetic neuropathy in type 2 DM patients. The results shows an increased risk, indicating that individuals with GG phenotypes of IL-10 and AA phenotypes of IFNγ are two times more likely to get the disease. Analysis of gene-gene interaction of the 3 factors that may affect the diabetic neuropathy shows that there was an interaction between the three SNP's. IL-10 serves as an important biomarker in Indian population for their susceptibility to Diabetic Neuropathy as it may play a role in alteration of IL-10 production and the inflammatory responses during the course of the disease. Repeated studies with large sample size are required for further validation of our findings and also to understand the association between cytokine gene polymorphisms and the development of Diabetic Neuropathy.

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