Tumor necrosis factor-alpha (−308G/A, +488G/A, −857C/T and -1031 T/C) gene polymorphisms and risk of ischemic stroke in north Indian population: A hospital based case–control study

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ABSTRACT

Background: Genetic factors may play a role in the susceptibility of Ischemic stroke (IS). Previous studies have shown that Tumour necrosis factor-α (TNF-α) gene polymorphisms were associated with the risk of IS in multiple ethnicities. The present case–control study tested the hypothesis that genetic polymorphisms of the TNF-α gene may affect the risk of IS in North Indian population. We investigated the association of four single nucleotide polymorphisms (−308G/A, +488G/A, −857C/T and -1031 T/C) within TNF-α gene promoter and their haplotypes with the risk of IS.

Methods: IS was classified using the Trial of Org 10,172 in Acute Stroke Treatment (TOAST) classification. Genotyping was performed for 250 IS patients and 250 age- and sex-matched IS free controls by using SNaPshot technique. Multivariate logistic regression was used to control the confounding effects of demographic and risk factor variables. Haplotype analyses were done by using PHASE software and Linkage disequilibrium (LD) analyses were done by using Haploview version 4.2 software.

Results: An independent association between TNF-α +488G/A (OR = 2.59; 95%CI 1.46 to 4.60; p = 0.001) and -857C/T (OR = 1.77; 95%CI 1.01 to 3.11; p < 0.04) and risk of IS was observed under dominant model. However, no significant association between -308G/A and -1031 T/C gene polymorphisms and risk of IS was observed. Haplotype analysis showed that A308-G488-C857-T1031 haplotypes were significantly associated with the increased risk of IS [OR = 1.66; 95%CI 1.02 to 2.71; p = 0.003]. Strong linkage disequilibrium (LD) was observed for +488G/A and -857C/T (D' = 0.41, r^2 = 0.004).

Conclusions: Two SNPs (+488G/A and -857C/T) of TNF-α gene and their haplotypes are significantly associated with the risk of IS in the population enrolled from North India. Our findings indicate that polymorphisms and haplotypes of TNF-α gene may be used as a genetic marker for identifying individuals at increased risk for developing IS.

1. Introduction

Ischemic stroke (IS) is a complex multifactorial disease which accounts for 80–85% of stroke and its pathophysiology is regulated by a combination of lifestyle, environmental and unclear genetic risk factors (Bevan and Markus, 2011). Recent data suggested that inflammatory processes are involved in the pathogenesis of IS. Several frequent polymorphisms have been identified in the Tumour necrosis factor-α (TNF-α) gene (Carr et al., 2002; Matarin et al., 2009; Hansson, 2005; Flex et al., 2004; Hollegaard and Bidwell, 2006). TNF-α is one of the main pro-inflammatory cytokines and plays a central role in initiating and regulating the inflammatory response (Zaremba, 2000).

Human TNF-α gene is located on chromosome 6p21.3 which consists of four small exons and encodes protein of 233 amino acid residue (Nedwin et al., 1985). TNF-α increases capillary permeability, activates endothelium, and causes a significant neutrophil adherence and accumulation in capillaries and small blood vessels. TNF-α also exacerbates ischemic brain injury and increases the infarct size by various mechanisms that include thrombus formation, release of endothelin 1 and nitric oxide (potent vaso-active agents), promotion of leukocyte adhesion, and infiltration in addition to blood-barrier breakdown and

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tissue swelling (Feuerstein et al., 1994, 1998; Barone et al., 1997; Liu et al., 1994; Maemura et al., 1992; Pinto et al., 2006; Tuttolomondo et al., 2014, 2015). TNF-α regulates the inflammatory response and activates blood coagulation and therefore is an important candidate gene for stroke (Bazzoni and Beutler, 1996). Genetic screening has revealed four polymorphic regions (−308G/A, +488G/A, −857C/T and -1031 T/C) in the promoter region of TNF-α gene. A number of studies have shown the association of − 308 G/A polymorphism with stroke. However, the results have not been consistent across population. The A allele, which has been associated with elevated TNF levels (Wilson et al., 1997), was found to be protective in Korean adults with IS (Um and Kim, 2004). On the other hand, it conferred an increased risk of IS in younger Italian patients (Rubattu et al., 2005). Patients with high TNF-α level might be at an increased risk of developing thrombotic complications because of the effect of this cytokine on the endothelium.

Only single study conducted in South Indian population by Munshi et al. (2011) reported that +488G/A polymorphism in TNF-α gene is an important risk factor for IS. Limited number of studies are available for the association between TNF-α (-857C/T and -1031 T/C) gene polymorphisms with the risk of stroke. As per our knowledge, no information is available from North Indian population on the association between these four SNPs with the risk of IS. Hence this study was undertaken to investigate the association of (−308G/A, +488G/A, −857C/T and -1031 T/C) polymorphisms in TNF-α gene with risk of IS.

2. Materials and methods

2.1. Subjects

The present case–control study was a hospital based study and was completed in one and a half years (October 2013 to April 2015). The study was conducted in the Department of Neurology, All India Institute of Medical Sciences (AIIMS), New Delhi in collaboration with Institute of Genomics and Integrative Biology (IGIB), New Delhi. A total of 250 patients were recruited in the study after radiologic confirmation of IS by computed tomography (CT) or magnetic resonance imaging (MRI) scans of the brain. All patients had clinical signs consistent with the World Health Organization (WHO) definition of stroke. A control group comprising of 250 age and sex matched individuals was recruited from volunteers and healthy persons accompanying the patients in the general outpatient department (OPD) and was assessed by questionnaire for verifying stroke free status (QVSFS) (Jones et al., 2001). Written informed consent was obtained from all the subjects before the collection of information and blood samples. Patients with a history of transient ischemic attack, fever, rheumatologic disease, autoimmune disease, any acute or chronic infection, CT/MRI proven hemorrhagic stroke, and a history of regular immunosuppressive or analgesic therapies were excluded. The study was approved by the Local Institutional Ethics Committee.

2.2. Clinical examination

A detailed history and clinical evaluation was carried out by neurologist. IS was categorized using the Trial of Org 10,172 in Acute Stroke Treatment (TOAST) classification (Meschia, 2002). The National Institutes of Health Stroke Scale (NIHSS), modified Rankin Scale (mRS) and Barthel Index (BI) scores were used for the determination of clinical severity and independency. At six months, disability and functional independence was assessed telephonically by mRS and BI.

2.3. Definition of variables

Definitions of variables were modified from the study (Feigin et al., 1998) and are as follows: Hypertension: Subjects will be considered to have hypertension if they either have the diagnosis of hypertension or treated for hypertension before the stroke or reference date. In addition,

if a control will have no recorded blood pressure before the reference date, but diastolic pressure of 90 mmHg or more or a systolic pressure of 140 mmHg or more on two or more occasions during the study evaluation, he or she will be considered to have hypertension. Diabetes: if a subject will have the diagnosis documented by a physician in the medical record or if fasting blood sugar level will be >126 mg/dl, Dyslipidemia: if they either will have the diagnosis of dyslipidemia or treated for dyslipidemia. Smoker: Person will be defined as regular smoker if a person smoking ≥1 cigarettes daily, Bidis, Cigar for proceeding > 3 months. Body Mass Index (BMI): BMI will be calculated by weight in kilograms divided by the square of height in meters. Family history of Stroke: A positive family history of stroke will be considered if a subject's first-degree relative (parent or sibling) had a stroke. Socioeconomic Status: It was classified into two classes based on four items, mainly two wheeler, refrigerator, computer or car. Low – not possessing any of the four, High: possessing either two- wheeler or refrigerator or computer or car. Occupational behaviour: It comprised of Sedentary or sitting occupations (mostly sitting e.g. shopkeeper, clerk, etc.), Moderate physical work (involves walking e.g. salesman, nurses, housework etc), Heavy physical work (carrying, lifting e.g. labourer, coolie etc.). Physical activity: Physical activity was defined if a person engaged in morning or evening walk/running/jogging/swimming/cycling at least half an hour in four days or more in a week (Kumar et al., 2014, 2015).

2.4. DNA isolation and genotyping

Single time one teaspoon (4 ml) venous blood samples were taken from IS patients and controls in a tube containing ethylene diamine tetra acetic acid (EDTA). Genomic DNA was isolated from whole blood through standard phenol-chloroform method. The primers were designed for the four selected SNPs using the Primer3 online tool, (http://bioinfo.ut.ee/primer3-0.4.0/). The TNF-α regions were amplified in T-100 thermal cycler (Bio-Rad) using the primer sequences and conditions for Polymerase Chain Reaction (PCR) are listed in Table 1. Genotyping was performed on 3130xl automated DNA sequencer (Applied Biosystems) using the SNaPshot method.

2.5. Statistical analysis

The chi-square test was used to determine whether the allelic frequencies were in accordance with Hardy-Weinberg equilibrium (HWE). The conditional logistic regression analysis was used to estimate Odds Ratio (OR) and 95% confidence intervals (CIs) for the strength of association between TNF-α gene polymorphisms and risk of IS. Multivariate logistic regression was used to control the confounding effects of demographic and risk factor variables. Tests were considered significant at $p < 0.05$. Data was analyzed using the STATA, version 13.0 (StataCorp. 2013. Stata Statistical Software: Release 13. College Station, TX: StataCorp LP). The linkage disequilibrium (LD) analyses were performed using HaploView 4.2 software (Barrett et al., 2005) and haplotype analyses were done by PHASE software. The threshold value of the frequencies of the haplotypes included in the analysis was set to 2%.

3. Results

After screening 389 stroke cases, 250 IS cases were included in the study. For the control group, 321 people were screened and 250 age and sex matched controls were recruited for the study. The mean age of IS patients was 52.8 ± 12.5 years and control group was 50.9 ± 12.7 years and both groups consisted of 203 males and 47 females. The clinical characteristics of IS patients and controls are presented in Table 2. The risk factors examined such as history of hypertension (cases 58.4% vs controls 16.8%), diabetes (cases 31.6% vs controls 10.4%), smoking (cases 38.8% vs controls 26.8%), alcohol intake (cases 32.4% vs controls 22.4%) and dyslipidemia (cases 22.8% vs controls 5.6%) were found significantly more often in cases than in controls

Table 1
List of primer sequences and PCR conditions used for TNF- α gene polymorphisms.

SNPs	rsID	Primers	Annealing (°C)	Amplicon Size (bp)
−308G/A	rs1800629	F.P- 5-AGGCAATAGGTTTTGAGGGCCAT-3 R.P- 5-TCCTCCCTGCTCCGATTCCG-3 S.P- 5- CAATAGGTTTTGAGGGGCATG -3	55	107
+488G/A	rs1800610	F.P- 5-GCCAGACATCCTGTCTCTCC-3 R.P- 5-CAGAGGGAAGAGGTGAGTGC-3 S.P – 5- TGCATCCCCGTCTTTCTCCA -3	60	220
−857C/T	rs1799724	F.P- 5-GGCTCTGAGGAATGGGTTAC-3 R.P- 5-CCTCTACATGGCCCTGTCTAC-3 S.P- 5- GTATGGGGACCCCCCCTTAA -3	56.5	127
−1031 T/C	rs1799964	F.P- 5-TATGTGATGGACTCACCAGGT-3 R.P- 5-CCTCTACATGGCCCTGTCTT-3 S.P- 5- CAAAGGAGAAGCTGAGAAGA -3	63	264

Abbreviations: F.P-Forward primer; R.P-Reverse Primer; S.P-SNaPshot Primer; bp-base pair.

(p < 0.05). Out of 250 cases, 157 (62.8%) cases were recruited from the outpatient department (OPD) and 93 (37.2%) cases were recruited from the inpatient department (IPD). 240 cases (96.0%) completed full 6 month telephonic follow-up, 7 patients (2.8%) died, 2 patients (0.4%) had a recurrence of ischemic stroke and 10 (4.0%) were lost to follow-up. The mean and standard deviation (S.D) was 12.55 ± 13.42 for the NIHSS at admission, 3.06 ± 1.05 for mRS and 63.56 ± 16.69 for BI at discharge. After telephonic follow up at 6 months, the mean and S.D. was 1.30 ± 1.16 for mRS and 85.31 ± 15.26 for BI.

All genotype and allelic frequencies were in HWE in both IS patients and controls. Genetic analysis for TNF-α (−308G/A, +488G/A, −857C/T and -1031 T/C) gene polymorphisms were conducted for all 250 IS cases and 250 age-sex matched controls and are summarized in Table 3. Adjusted conditional logistic regression analysis showed an independent association of TNF-α +488G/A (OR 2.59; 95%CI-1.46 to 4.60; P = 0.001) and -857C/T (OR 1.77; 95%CI 1.01 to 3.11; P < 0.04) with the risk of IS under dominant model. However, no significant association was observed for -308G/A and -1031 T/C gene polymorphisms

with the risk of IS. After further analysis based on TOAST classification, we observed significant association between TNF-α -308G/A gene polymorphism and risk of IS under dominant (OR 4.57; 95%CI 1.39 to 15.0; P = 0.01) and allelic (OR 2.67; 95%CI 1.19 to 5.95; P = 0.01) models for others (Stroke due to determined + undetermined etiology) subtype of IS.

Haplotype analysis showed that A308-G488-C857-T1031 haplotypes were significantly associated with the increased risk of IS [OR 1.66; 95%CI 1.02 to 2.71; P = 0.003] (Table 4). Strong linkage disequilibrium (D' = 0.41, r^2 = 0.004) was detected between two SNPs (+488G/A and -857C/T) in the TNF-α gene (Fig. 1).

4. Discussion

The present study was the first study from North India which revealed that TNF-α (+488G/A and -857C/T) gene polymorphisms and their haplotypes were significantly associated with increased risk of IS. Case–control genetic association studies are being used for

Table 2
Demographic and risk factor variables for ischemic stroke (IS) patients and control subjects.

Characteristics	Controls (N = 250) n (%)	Ischemic stroke (N = 250) n (%)	Crude OR [95% CI], p value	*Adjusted OR [95% CI], p value
Age in years (Mean + S.D)	52.83 ± 12.59	50.97 ± 12.70	Matched	
Male/Female, n	203/47	203/47		
LVD	-	107 (42.8)		
SVD	-	83 (33.2)		
CE	-	26 (10.4)		
ODE	-	22 (8.8)		
UDE	-	12 (4.8)		
NIHSS at admission (Mean + S.D)	-	12.55 ± 13.42		
BI score at discharge (Mean + S.D)	-	63.56 ± 16.69		
BI score at 6 months (Mean + S.D)	-	85.51 ± 15.26		
mRS score at discharge (Mean + S.D)	-	3.06 ± 1.05		
mRS score at 6 months (Mean + S.D)	-	1.3 ± 1.16		
Hypertension	42 (16.8)	146 (58.4)	8.4 [4.8 to 14.6],<0.0001	6.2 [3.2 to 12], <0.0001
Diabetes	26 (10.4)	79 (31.6)	3.5 [2.1 to 5.7], <0.0001	2.1 [1.1 to 4.2],0.02
Dyslipidemia	14(5.6)	57 (22.8)	5.2 [2.6 to10.4], <0.0001	2.4 [1.0 to 5.7],0.04
Smoking	67 (26.8)	97 (38.8)	1.7 [1.1 to 2.5],0.005	1.1 [0.6to1.9],0.69
Alcohol	56 (22.4)	81 (32.4)	1.8 [1.1 to 2.8],0.008	1.8 [0.9 to 3.5],0.05
Myocardial Infarction	4 (1.6)	17 (6.8)	5.3 [1.5 to 18.3],0.008	1.8 [0.4 to 7.1],0.36
Migraine with Aura	8 (3.2)	10 (4)	1.2 [0.4 to 3.1],0.63	1.6 [0.4 to 5.8],0.40
Migraine without Aura	4 (1.6)	5 (2)	1.2 [0.3 to 4.6],0.33	1.3 [0.1 to 10.9],0.78
Low Socioeconomic Status	14 (5.6)	66 (26.4)	5.0 [2.7 to 9.0], <0.0001	7.5 [3.0 to18.2],<0.0001
High BMI	89 (35.6)	77 (30.8)	0.7 [0.4 to 1.0],0.11	0.8 [0.4 to1.4],0.50
Sedentary Life Style	106 (42.4)	127 (50.8)	1.4 [1.0 to 2.1],0.04	1.1 [0.6 to1.9],0.6
Physical activity	136 (54.4)	107 (42.8)	0.6 [0.4 to 0.8], 0.009	0.7 [0.4 to 1.3],0.36
Family history of stroke	9 (3.6)	32 (12.8)	3.5 [1.6 to 7.4],0.001	6.8 [2.2 to 20.9],0.001
Family history of diabetes	28 (11.2)	48 (19.2)	1.8 [1.1 to 3.1],0.015	3.1 [1.4 to 6.9],0.004
Family history of hypertension	34 (13.6)	61 (24.4)	2.0 [1.2 to 3.1],0.003	1.8 [0.9 to 3.4],0.06
Family history of heart Attack	14 (5.6)	19 (7.6)	1.4 [0.6 to 3.1],0.33	1.7[0.5 to 5.9],0.38

Conditional Logistic Regression Analysis.
*Adjusted variables include Hypertension, Diabetes, Dyslipidemia, Smoking, Family History of stroke, Alcohol, Sedentary Life Style and Low socioeconomic status.
Abbreviations: BMI- Body Mass Index; OR- Odds Ratio; CI- Confidence Interval; SD- Standard Deviation;LVD-Large Vessel Disease;SVD-Small Vessel disease;CE-Cardioembolic;ODE-Other determined etiology; UDE-Undetermined etiology.

Table 3

Genotype and allelic frequencies of TNF-α (−308G/A, +488G/A, −857C/T and -1031 T/C) gene polymorphisms in IS patients and controls.

Polymorphisms			LVD N = 107	SVD N = 83	CE N = 26	Others N = 34	IS N = 250	Controls N = 250
G308A	Genotype	GG, n (%)	93 (86.9)	75 (90.3)	23 (88.5)	27 (79.4)	218 (86.8)	225 (90)
		GA, n (%)	14 (13)	7 (8.4)	3 (11.5)	5 (14.7)	29 (11.6)	23 (9.2)
		AA, n (%)	0	1 (1.2)	0	2 (5.8)	3 (1.2)	2 (0.8)
	Allele	G, n (%)	200 (93.4)	157 (94.5)	49 (94.2)	59 (86.7)	465 (93)	473 (94.6)
		A, n(%)	14 (6.6)	9 (5.5)	3 (5.8)	9 (13.2)	35 (7)	27 (5.4)
	Dominant GA + GG vs. AA	Adjusted OR (95% CI), P value	1.40 (0.59–3.35), 0.43	1.04 (0.37–2.88), 0.93	1.59 (0.35–7.29), 0.54	**4.57 (1.39–15.0), 0.01**	1.42 (0.73–2.76), 0.3	
		Unadjusted OR (95% CI), P value	1.35 (0.67–2.72), 0.39	0.96 (0.41–2.21), 0.92	1.17 (0.32–4.18), 0.80	2.33 (0.92–5.90), 0.07	1.36 (0.75–2.47), 0.29	
	Recessive AA vs. GA + GG	Adjusted OR (95% CI), P value	NE	4.94 (0.41–58.79), 0.20	NE	NE	4.15 (0.63–27.30), 0.13	
		Unadjusted OR (95% CI), P value	NE	1.51 (0.13–16.89), 0.73	NE	**1.75 (1.05–56.93), 0.04**	1.49 (0.25–8.97), 0.65	
	Allelic A vs. G	OR (95% CI), P value	1.22 (0.62–2.38), 0.54	1.00(0.46–2.18), 0.56	1.07 (0.31–3.66), 0.55	**2.67(1.19–5.95), 0.01**	1.31 (0.78–2.21) ,0.29	
Polymorphisms			LVD N = 107	SVD N = 83	CE N = 26	Others N = 34	IS N = 250	Controls N = 250
G488A	Genotype	GG, n (%)	68 (63.5)	59 (71)	18 (69.2)	17 (50)	162 (64.8)	192 (76.8)
		GA, n (%)	36 (33.6)	22 (26.5)	8 (30.8)	16 (47)	82 (32.8)	55 (22)
		AA, n (%)	3 (2.8)	2 (2.4)	0	1 (3)	6 (2.4)	3 (1.2)
	Allele	G (%)	172 (80.4)	140 (84.3)	44 (84.6)	50 (73.5)	406 (81.2)	439 (87.7)
		A (%)	42 (19.6)	26 (15.7)	8 (15.4)	18 (26.4)	94 (18.8)	61 (12.2)
	Dominant (GG + GA vs. AA)	Adjusted OR (95% CI), P value	**2.23 (1.20–4.14), 0.01**	**2.33 (1.17–4.65), 0.01**	1.89 (0.65–5.50), 0.23	**4.88 (1.90–12.52), 0.01**	**2.59 (1.46–4.60), 0.001**	
		Unadjusted OR (95% CI), P value	**1.89 (1.16–3.10), 0.01**	1.34 (0.77–2.35), 0.29	1.47 (0.60–3.55), 0.39	**3.31 (1.58–6.89), 0.001**	**1.78 (1.20–2.66), 0.004**	
	Recessive (GG vs. AA +GA)	Adjusted OR (95% CI), P value	2.12 (0.22–19.93), 0.50	1.61 (0.14–18.29), 0.70	NE	0.58 (0.03–10.91), 0.72	1.75 (0.29–10.33), 0.53	
		Unadjusted OR (95% CI), P value	2.37 (0.47–11.96), 0.29	2.03 (0.33–12.380, 0.44	NE	2.49 (0.25–24.69), 0.43	2.00 (0.50–7.99), 0.32	
	Allelic G vs. A	OR (95% CI), P value	1.75 (1.14–2.70), 0.009	1.33 (0.81–2.19), 0.25	1.30 (0.58–2.91), 0.50	2.59 (1.41–4.72), <0.001	1.66 (1.17–2.36), 0.003	
Polymorphisms			LVD N = 107	SVD N = 83	CE N = 26	Others N = 34	IS N = 250	Controls N = 250
C857T	Genotype	CC, n (%)	71 (66.3)	59 (71)	18 (69.2)	22 (64.7)	170 (68)	172 (68.8)
		CT, n (%)	29 (27.1)	21 (25.3)	7 (26.9)	10 (29.4)	67 (26.8)	68 (27.2)
		TT, n (%)	7 (6.5)	3 (3.6)	1 (3.8)	2 (5.8)	13 (5.2)	10 (4)
	Allele	C (%)	171 (79.9)	139 (83.7)	43 (82.6)	54 (79.4)	407 (81.4)	412 (82.4)
		T (%)	43 (20.1)	27 (16.3)	9 (17.4)	14 (20.5)	93 (18.6)	88 (17.6)
	Dominant CC + CT vs. TT	Adjusted OR (95% CI), P value	1.57 90.86–2.86), 0.13	1.74 (90.89–3.36), 0.10	1.51 (0.53–4.29), 0.43	1.52 (0.61–3.82), 0.36	**1.77 (1.01–3.11), 0.04**	
		Unadjusted OR (95% CI), P value	1.11 (0.69–1.81), 0.65	0.89 (0.52–1.54), 0.69	0.98 (0.40–2.35), 0.19	1.20 (0.56–2.55), 0.63	1.03 (0.71–1.51), 0.84	
	Recessive TT vs. CT + CC	Adjusted OR (95% CI), P value	2.32 (0.69–7.73), 0.17	1.17 (0.25–5.28), 0.83	0.76 (0.07–8.01), 0.82	3.14 (0.53–18.47), 0.20	1.70 (0.62–4.60), 0.29	
		Unadjusted OR (95% CI), P value	1.68 (0.62–4.53), 0.30	0.90 (0.24–3.35), 0.87	0.96 (0.11–7.81), 0.11	1.50 (0.31–7.15), 0.61	1.33 (0.56–3.16), 0.51	
	Allelic T vs. C	OR (95% CI), P value	1.17 (0.78–1.76), 0.43	0.90 (0.56–1.45), 0.68	0.97 (0.46–2.08), 0.56	1.21 (0.64–2.28), 0.54	1.06 (0.77–1.47), 0.68	
Polymorphisms			LVD N = 107	SVD N = 83	CE N = 26	Others N = 34	IS N = 250	Controls N = 250
T1031C	Genotype	TT, n (%)	64 (59.8)	39 (46.9)	13 (50)	18 (52.9)	134 (53.6)	121 (48.4)
		TC, n (%)	34 (31.7)	26 (31.3)	12 (46.1)	13 (38.2)	35 (38)	115 (46)
		CC, n (%)	9 (8.4)	8 (9.6)	1 (3.8)	3 (8.8)	21 (8.4)	14 (5.6)
	Allele	T (%)	162 (75.7)	114 (68.6)	38 (73)	49 (72)	363 (72.6)	357 (71.4)
		C (%)	52 (24.2)	52 (31.3)	14 (27)	19 (28)	137 (27.4)	143 (28.6)
	Dominant (TT + TC vs. CC)	Adjusted OR (95% CI), P value	0.54 (0.31–0.96), 0.03	1.09 (0.60–1.99), 0.76	0.96 (0.36–2.54), 0.94	0.83 (0.35–1.97), 0.67	0.54 (0.31–0.91), 0.02	
		Unadjusted OR (95% CI), P value	0.63 (0.39–0.99), 0.04	1.05 (0.64–1.74), 0.82	0.93 (0.41–2.10), 0.87	0.83 (0.40–1.70), 0.62	0.81 (0.57–1.15), 0.25	
	Recessive CC vs. TC + TT	Adjusted OR (95% CI), P value	1.94 (0.69–5.45), 0.20	2.46 (0.84–7.16), 0.09	0.66 (0.05–8.53), 0.75	2.31 (0.48–11.10), 0.29	1.60 (0.70–3.64), 0.26	
		Unadjusted OR (95% CI), P value	1.54 (0.64–3.69), 0.32	1.79 (0.72–4.45), 0.20	0.67 (0.08–5.34), 0.70	1.63 (0.44–5.99) 0.46	1.50 (0.76–2.94), 0.24	
	Allelic C vs. T	OR (95% CI), P value	0.80 (0.55–1.15), 0.23	1.13 (0.77–1.60), 0.56	0.91 (0.48–1.74), 0.79	0.96 (0.55–1.70), 0.92	0.94 (0.71–1.24), 0.67	

Abbreviations: LVD- large vessel stroke; SVD-small vessel stroke; CE-cardioembolic stroke; others includes- stroke due to undetermined aetiology + other determined aetiology; IS-Ischemic Stroke; NE- Not Estimable; OR- Odds Ratio; CI- Confidence Interval.

Table 4
Frequencies and association of Tumor Necrosis Factor Alpha (−308G/A, +488G/A, −857C/T and -1031 T/C) haplotypes in IS patients and controls.

| Haplotypes	IS cases n (%)	Controls n (%)	Odds Ratio (95% CI)	P value
G308-G488-C857-T1031	248 (49.6)	253 (50.6)	Reference	
G308-G488-C857-C1031	87 (17.2)	112 (22.4)	0.56 (0.56–1.10)	0.16
G308-G488-T857-T1031	31 (6.2)	26 (5.2)	1.21 (0.70–2.10)	0.69
G308-G488-T857-C1031	31 (6.2)	26 (5.2)	1.21 (0.70–2.10)	0.69
G308-A488-C857-T1031	13 (2.6)	23 (4.6)	0.57 (0.28–1.16)	0.12
G308-A488-C857-C1031	6 (1.2)	3 (0.6)	2.04 (0.50–8.24)	0.31
G308-A488-T857-T1031	**49 (9.8)**	**30 (6)**	**1.66 (1.02–2.71)**	**0.03**
A308-G488-C857-T1031	35 (7)	27 (5.4)	1.32 (0.77–2.25)	0.30
Total	**500**	**500**		

studying the genetic basis of complex multifactorial diseases. The TNF-α gene represents a strong candidate gene for the pathogenesis of stroke. In fact, TNF is known to play several pro-inflammatory and pro-coagulant effects on endothelium and, therefore, to expose vascular segments to local inflammation, thrombosis and hemorrhage (Terry et al., 1999; Mark et al., 2001; Hallenbeck, 2002). Its contributory role to stroke initiation and progression has been the topic of recent investigations. In this regard, the experimental evidence has clearly documented a critical role of TNF-stimulation on the increased sensitivity to induction of brain ischemia and hemorrhage (Sirén et al., 2001; Pinto et al., 2006; Tuttolomondo et al., 2014, 2015). The previous evidence in the favour of a relationship of TNF-α gene with occurrence of IS in humans has been reported in different populations as documented by the previous meta-analyses published by (Pereira et al., 2007) and (Gu et al., 2013) suggest that TNF-α -308G/A polymorphism might be a protective factor for IS in adult Asian population.

Recently several Genome Wide Association Studies (GWAS) for stroke have been reported, (Kubo et al., 2007; Ikram et al., 2009; Matarín et al., 2007, 2009; Yamada et al., 2009; Gretarsdottir et al., 2008) but most of these study's populations were of European origin and they did not detect the association of -308G/A gene polymorphism

in TNF-α gene with risk of stroke. Another study published by Cui et al. (2012) showed a significant association between −308G/A polymorphism and risk of stroke (OR 1.34; 95% CI 1.02 to 1.77) and did not show any association for -857C/T and -1031 T/C gene polymorphism with IS risk. TNF-α +488G/A was found to be an important risk factor for ischemic stroke in South Indian population (Munshi et al., 2011). Our present study suggests a significant association between +488G/A and -857C/T gene polymorphisms in TNF-α gene but shows no significant association between -308G/A and -1031 T/C gene polymorphisms with IS risk.

The results of our present case–control study provide more convincing evidence of the association between TNF-α gene polymorphisms and risk of IS after adjusting the confounding variables including hypertension, alcohol, diabetes, dyslipidemia, family history of stroke, sedentary life style and low socioeconomic status. A high degree of LD was observed between the two SNPs (+488G/A and -857C/T) in our study. The study results published by (Banerjee et al., 2008; Munshi et al., 2011; Sultana et al., 2011; Tong et al., 2010) showed the protective role of TNF-α -308G/A gene polymorphism with the risk of IS. Our study results are in accordance with the study published by (Wawrzynek et al., 2014) showing non-significant association with the risk of IS in Caucasians living in Poland. Our findings suggest significant association of TNF-α -308G/A gene polymorphism with others subtype (Stroke due to determined + undetermined etiology) of IS. Tuttolomondo et al. (2012) showed no differences in the genotype and allelic distributions (Tuttolomondo et al., 2012). The most studied and interesting aspects of -308A/G polymorphism remain unexplained: there are many discrepancies between the results. However, the cause of this is not clear. Differences in the ethnicity of the studied population may be taken as one of the possibilities.

However, there were a few limitations in our study. Firstly, the study was conducted in a single hospital and the participants might not have been the representatives from other areas. Therefore, further large sample size and multicentric studies are needed to confirm our findings. Secondly, we did not evaluate the plasma level of TNF-α in IS patients and controls. Despite these limitations, our study provides strong evidence for the association between TNF-α (+488G/A and -857C/T) gene polymorphisms and risk of IS.
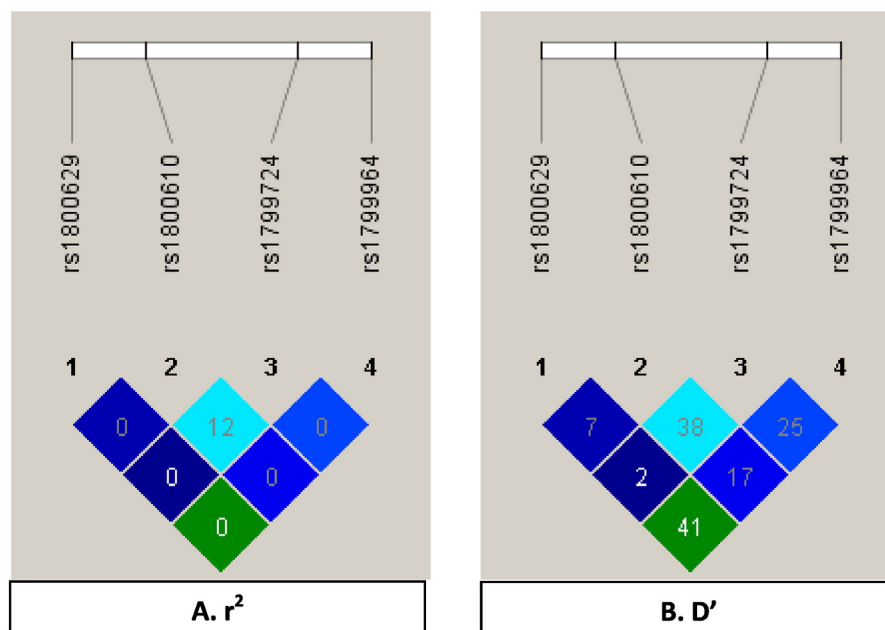
Fig. 1. LD plots of the four SNPs (−308G/A, +488G/A, −857C/T and -1031 T/C) of TNF-α gene in North Indian population. The values in the squares are the pair-wise calculations of r^2 (**A**) or D' (**B**). The squares with the "0" indicate $r^2 = 0$ (i.e., No LD between a pair of SNPs). The square with the "41" indicate $D' = 0.41$ (i.e., medium LD between a pair of SNPs).

5. Conclusion

Two SNPs ($+488$G/A and -857C/T) of TNF-α gene and their haplotypes are significantly associated with the risk of IS in the population enrolled from North India. Our findings indicate that polymorphisms and haplotypes of TNF-α gene may be used as a genetic marker for identifying individuals at increased risk for developing IS.

Conflict of Interest

The authors have declared that no competing interests exist.

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