

Tumour Necrosis Factor Alpha Serum Levels and its Gene Polymorphism 850 C/T (rs1799724) as Prognostic Markers of Traumatic Brain Injury Outcome

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ABSTRACT

Introduction: Road traffic accidents have been responsible for the high incidence of head injuries. In a study from India, it was reported that there were 150 Traumatic Brain Injury (TBI) cases per 1,00,000 population, out of which 20 succumbed to death. In the repair process of TBI many chemicals play a role, of which, Tumour Necrosis Factor alpha (TNF- α) is important as TNF- α was shown to possess both neurotoxic and neuroprotective activity.

Aim: To evaluate the serum levels of TNF- α and its functional polymorphism in TBI patients and assessing the outcome.

Materials and Methods: The prospective cohort observational study was conducted in the Department of Genetics and Molecular Medicine, Kamineni Hospitals, Hyderabad, Telangana, India, from June 2008 to May 2012. It included 126 patients of both sexes with severe and moderate head injuries based on Glasgow Coma Scale (GCS) scores. A 3 mL of plain/Ethylenediaminetetraacetic acid (EDTA) blood sample was drawn from individuals immediately after admission, which was used for serum TNF- α assessment and DNA isolation for TNF- α genotype analysis. The TNF- α levels were measured by Enzyme Linked Immunosorbent Assay (ELISA) using KRISHGEN Biosystems (Catalog No. KB 100-HTNF a) kit. Outcome assessment was done based on Glasgow Outcome Scale (GOS). Statistical analysis was accomplished by Open Epi version 3.01 software. Odd's Ratio (OR) were used to calculate risk of good/bad outcome with 95% confidence interval and results with a $p < 0.05$ were considered statistically significant.

Results: A total of 126 subjects of Indian origin were enrolled in this study. The mean age of the subjects was 36.7 ± 12.97 years. Out of the 126 patients, 46.8% (59) had multiple lesions. The patients with < 40 pg/mL (as per the kit specifications) TNF- α levels were 67.50% (85/126), while those with > 40 pg/L were 32.50% (41/126). The most common genotype found in the TBI patients was TT with a frequency of 60.3% (76/126), followed by CT with a frequency of 16.6% (21/126) and CC with a frequency of 23% (29/126). When genotype and TNF- α serum levels were studied together, it was seen that low TNF- α level (11-40 pg/mL) was associated with C allele and CC genotype, while those with TT genotype had high TNF- α level (> 40 pg/mL) when compared with other genotypes (CC, CT) and this was found to be statistically significant with an OR=3.4064, (95% CI= 1.8426 to 6.2975), $p=0.0001$. Higher percentage of death and disability was seen in patients with TT genotype while CC genotype showed good recovery at six months with OR=3.6441 (95% CI=1.911 to 6.946), $p=0.0001$. This suggests that T allele of TNF- α C850T polymorphism has a 3.6 fold higher risk of bad outcome in patients with TBI.

Conclusion: The C allele and CC genotype of rs1799724 polymorphism was associated with positive outcome at three to six months. Thus, evaluating TNF- α levels and evaluating the genotype of rs1799724 polymorphism at admission, can be taken as prognostic marker. It can also be used as a target for therapeutic intervention.

Keywords: Accidents, Glasgow coma scale, Genetic marker, Southern India, Trauma

INTRODUCTION

Road traffic accidents have been responsible for the high incidence of head injuries, which cause a great deal of morbidity and mortality. In a study from India, it was reported there were 150 Traumatic Brain Injury (TBI) cases per 1,00,000 population, out of which 20 succumbed to death [1]. This results in enormous loss of productivity and hence, there is a need for extensive multidimensional efforts to manage patients with head injuries [2,3]. In TBI there are several molecules involved in injury and repair of the brain tissue. These include growth factors, catecholamines, neurokinins, chemokines and cytokines like Interleukin 6 (IL6), Interferon (IFN) and TNF- α [4-8]. Post-traumatic inflammatory cascades have been documented both in clinical and experimental TBI. However, the exact role of these in both damage and recovery processes is not established [9,10].

It was observed that inspite of similar factors like age of patient, severity of injury, site of injury and the modality of treatment being the same, the outcomes of TBI are drastically variable, and hence,

prognostic markers are required to predict the outcome [1]. In various models of central nervous system disease and injury, TNF- α was shown to possess both neurotoxic and neuroprotective activity [11,12]. As the earlier study was on animal models [13] and no studies on TNF- α 850 polymorphism and its serum levels in Indian TBI patients were available, we decided to study the serum levels of TNF- α and its functional polymorphism in TBI patients and assessing the outcome.

MATERIALS AND METHODS

This prospective, cohort observational study was conducted in the Department of Genetics and Molecular Medicine, Kamineni Hospitals, LB Nagar, Hyderabad, Telangana, India, from June 2008-May 2012. Ethics Committee approval was obtained from the Institutional Ethics Committee of Kamineni Hospitals. (Registration Number: ECR/58/Inst/AP/2013). Since this was the prospective observational study, all the patients who visited during the study period and were appropriate based on the inclusion criteria were included in the study.

Inclusion criteria: All head injury patients of GCS between 3-13 without any other injuries like chest, abdominal injury or long bones fractures and co-morbidities.

Exclusion criteria: Patient's with polytrauma, hypertension, diabetes mellitus, sepsis, heart disease, with GCS>13 and those who presented more than 24 hours after injury were excluded. Patients who left against medical advice and those who did not come for follow-up were excluded from the study.

Study Procedure

The clinical parameters like GCS (defined by Teasdale and Jennet) [14] and other relevant details at admission, at discharge and GOS at first follow-up after one month and six months with radiological findings at admission and subsequent repeat Computed Tomography (CT) scan findings were recorded.

Biochemical test for serum TNF- α : A 3 mL of plain/EDTA blood sample was drawn from individuals immediately after admission, which was used for serum TNF- α assessment and DNA isolation for TNF- α genotype analysis. The serum samples were separated and stored frozen at -20°C until analysed. The TNF- α levels were measured by ELISA using KRISHGEN Biosystems (Catalog No. KB 100-HTNF a) kit.

For determination of TNF- α 100 microlitre of calibrators were used and optical density was read at 450 nm. Bichromatic measurements with a reference at 600-690 nm was used. A 100 microlitre/well of standards and samples were added to the plate. Six two-fold serial dilutions of the 500 pg/mL top standard were performed. Plates were sealed and incubated at room temperature for two hours with shaking. A 100 μ L of diluted Avidin modified Horseradish Peroxidase (Av-HRP) solution was added to each well plates were sealed and again incubated for 30 minutes. Plates were washed with buffer and then 100 μ L of freshly mixed TMB substrate solution was added and incubated in the dark for 15 minutes. Positive well turned blue. Reaction was stopped by adding 100 μ L of 2N H₂SO₄ to each well till positive well turn from blue to yellow. These plates were read at 450 nm within 30 minutes of stopping reaction. The antibodies in these tests were specific for human TNF- α , with no detectable cross reactivities to other cytokines present in serum samples. Normal value of TNF- α was taken as <40 pg/mL as per the serial dilutions done with the materials provided in kit.

Molecular tests: Genomic DNA was isolated from the blood according to the method routinely used in our laboratory. The DNA was kept at -20°C until analysis. Genotyping for the TNF- α 850 polymorphism (C/T) was carried out by a three step Polymerase Chain Reaction (PCR) [15] with annealing temp of 53°C, followed by restriction enzyme digestion with Hinc II for two hours at 37°C. Agarose gel electrophoresis followed by documentation under Ultraviolet (UV) Image gave bands of 120 bp in case of TT genotype, 140 bp and 120 bp in CT genotype, and an undigested 140 bp band in CC genotype.

Outcome assessment was done based on GOS, which gives the functional outcome of the patient. (five categories: GOS 1: dead, GOS 2: persistent vegetative state, GOS 3: severe disability, GOS 4: moderate disability and GOS 5: good recovery) [16].

STATISTICAL ANALYSIS

Statistical analysis was accomplished by Open Epi version 3.01 software online www.openepi.com. Mean age of the patients was expressed as mean \pm SD and other dependent variables like serum TNF- α levels are presented in percentiles whereas the independent variable like the genotype is represented with their allele frequencies. Odd's ratio's were used to calculate risk of good/bad outcome with 95% confidence interval and results with a p<0.05 were considered statistically significant.

RESULTS

A total of 126 subjects from southern India were enrolled in this study. The age range of the subjects was 18 to 63 years, with mean age of 36.7 \pm 12.97 years and patients in different age groups is presented [Table/Fig-1]. A 79% (99/126) of the patients were males and 21% (27/126) were females. A 44.44% (56/126) of cases had severe head injury (GCS <8) and 55.56% (70/126) patients had moderate head injury. These moderate head injury patients were subdivided into two groups. Group 1 with GCS 8-10 were 33.33% (42/126) and group 2 with GCS 11-13 were 22.22% (28/126).

Age (in years)	N (%)
Mean \pm SD	36.7 \pm 12.97 years
18-25	37 (29.36%)
26-40	70 (55.56%)
>40	19 (15.08%)

[Table/Fig-1]: Distribution of patients in three different age groups.

The CT findings of all patients were recorded and the most common findings of CT scan were multiple lesions like Subdural Haematoma (SDH) with contusions, SDH along with Epidural Haematoma (EDH), EDH with fractures or multiple cerebral contusions. Of the 126 patients, 46.8% (59) had multiple lesions, 4.8% (6) had SDH, 9.5% (12) had EDH and 4.8% (6) had just contusions. About 31% (39) patients had diffuse brain injury and 3.2% (4) patients had other lesions like depressed fractures and pneumocephalus.

Serum TNF- α levels at admission were categorised into two groups, <40 pg/mL: Normal and >40 pg/mL: elevated. The patients with <40 pg/mL TNF- α levels were 67.50% (85/126), while those with >40 pg/mL were 32.50% (41/126) as seen in [Table/Fig-2]. It was observed that 63.41% of individuals with high levels of TNF- α had multiple lesions. None of the patients with EDH on CT scan had raised TNF- α levels [Table/Fig-3].

GCS	TNF- α levels	
	<40 pg/mL n=85	>40 pg/mL n=41
3-7	31 (36.4%)	29 (70.7%)
8-10	32 (37.6%)	8 (19.5%)
11-13	22 (26%)	4 (9.8%)

[Table/Fig-2]: Different TNF- α levels with GCS.

The values are presented as n (%)

CT scan findings	TNF- α levels		
	<40 pg/mL n=85	>40 pg/mL n=41	Total n=126
SDH	5 (5.88%)	1 (2.44%)	6 (4.8%)
EDH	12 (14.12%)	0	12 (9.5%)
Contusion	5 (5.88%)	1 (2.44%)	6 (4.8%)
Diffuse axonal injury	27 (31.76%)	12 (29.27%)	39 (30.1%)
Multiple lesions	33 (38.82%)	26 (63.41%)	59 (46.8%)
Others	3 (3.53%)	1 (2.44%)	4 (3.2%)

[Table/Fig-3]: TNF- α levels and CT scan findings of patients.

The values are presented as n (%)

TNF- α gene polymorphisms: TNF- α C850T polymorphism (rs1799724) was assessed in 126 individuals. The most common genotype found in the TBI patients was TT with a frequency of 60.3% (76/126), followed by CT with a frequency of 16.7% (21/126) and CC with a frequency of 23% (29/126).

When genotype and TNF- α serum levels were studied together, it was seen that low TNF- α level (11-40 pg/mL) was associated with C allele and CC genotype. While those with TT genotype had high TNF- α level (>40 pg/mL) when compared with other genotypes (CC, CT) and this was found to be statistically significant with an Odd's ratio of OR=3.4064, (95% CI=1.8426 to 6.2975), p=0.0001 [Table/Fig-4].

TNF- α levels genotype	<40 pg/mL	>40 pg/mL
CC (n=29)	24 (82.76%)	5 (17.24%)
CT (n=21)	17 (80.95%)	4 (19.05%)
TT (n=76)	44 (57.89%)	32 (42.11%)

[Table/Fig-4]: TNF- α levels and gene polymorphism (rs1799724).

Mortality was 30% in the first month following trauma and TNF- α serum levels did not appear to affect mortality. Subsequently based on GOS, patients were grouped as individuals with severe/moderate disability (GOS 2-3) taken as a bad outcome, and mild disability/good recovery were taken as good outcome (GOS 4-5). Post-trauma two patient's GOS improved in three months, while four cases improved by six months [Table/Fig-5].

Duration	Outcome		
	Death, n (%)	GOS 2-3, n (%)	GOS 4-5, n (%)
1 month, n=126	38 (30%)	25 (20%)	63 (50%)
3 months, n=88	-	23 (26.14%)	65 (73.86%)
6 months, n=88	-	19 (21.60%)	69 (78.40%)

[Table/Fig-5]: Distribution of patients and their outcome according to Glasgow Outcome Scale (GOS) at one month, three months and six months.

Patients with <40 pg/MI TNF levels showed a faster recovery while those with raised TNF- α levels had slow recovery.

Higher percentage of death and disability was seen in patients with TT genotype while CC genotype showed good recovery at six months with OR=3.6441 (95% CI=1.911 to 6.946), p=0.0001. This suggests that T allele of TNF- α C850T polymorphism has a 3.6 fold higher risk of bad outcome in patients with TBI [Table/Fig-6].

Genotypes	Bad outcome (Death+Disability with GOS 1-3) (n=57)	Good outcome (GOS 4-5) (n=69)
CC (n=29)	5 (17.24%)	24 (82.76%)
CT (n=21)	6 (28.57%)	15 (71.43%)
TT (n=76)	46 (60.53%)	30 (39.47%)

[Table/Fig-6]: Genotypes of TNF- α and the number of good and bad outcomes in terms of recovery after six months.

DISCUSSION

Brain injury is among the leading cause of mortality and disability. The common causes of head injury in adults are road traffic accidents, fall from height, assault, industrial accidents, fall of heavy objects and bullet or missile injuries and head injury due to road traffic accident has been most common [2,17,18]. The most common age group affected by head injury were young people between 20-40 years [19,20]. In the present study, 55.56% patients were in the 26-40 years of age group. In a study done by Gururaj G [2,18] it was shown that the age group of 15-44 years contributed for 60-70% of injuries.

There is conflicting evidence on the role of TNF- α showing its potential effect in both repair and of damage. Clinical and experimental studies on the stimulation of TNF- α production after TBI and its consequences have been a subject of interest since the 1990's, but still whether its effects are protective or toxic is a matter of debate. According to Fan L et al., serum TNF- α is elevated within first 24 hours of injury, while the TNF- α mRNA expression in the traumatized brain area was seen to be elevated one hour post injury [13]. In the present study, serum TNF- α levels at admission i.e., average time of 7.2 hours post trauma were evaluated. They were categorised into two groups with less than or greater than 40 pg/mL of TNF- α . A 70.7% (29) of patients with increased TNF- α levels had severe head injury based on the GCS scores. In the present study, TNF- α levels were found to be elevated in patients with diffuse axonal injury, but none of the patients with EDH had elevated TNF- α levels, suggesting that injury involving the cerebral parenchyma may lead to increased secretion of TNF- α because of injury to neuronal cells.

As EDH is not associated with parenchymal injury, TNF- α secretion is not increased in EDH. Patients with EDH are therefore known to have a good prognosis because of reduced parenchymal injury.

Low TNF- α levels at admission appeared to have a good recovery based on our data, while TBI patients with high TNF- α levels had bad outcome. Hence, TNF- α level post-TBI can be used as good prognostic marker for predicting head injury outcomes based on GOS, indicating survival with mild disability or complete recovery with independent life style by six months. Result of present study are similar to those of Graham EM et al., who showed that, preventing adverse effects of raised TNF- α after TBI could be mitigated with monoclonal TNF antibody therapy [21].

A TNF- α promoter polymorphism nucleotides upstream from the transcription initiation site is associated with elevated TNF- α levels (Wilson AG et al., Pujhari SK et al., Sharma S et al., but since this polymorphism was not identified in our population (Lakshmi KV et al.) [17] the present study assessed TNF- α -850 C/T polymorphism (rs1799724) [22-24]. The TNF- α homozygous TT genotype was associated with high TNF- α levels in serum and relatively poorer outcome at one month and also after six months indicating that initially elevated TNF- α is harmful during early stages of TBI and may contribute to the secondary brain injury responsible for bad prognosis associated with low or no recovery.

Outcome of TBI cannot be predicted only on the basis of clinical features or radiological findings, suggesting that genetic variability, may determine a person's response to head injury. Both brain and serum increased levels of TNF- α at early stage of brain injury have been associated with neuroinflammatory response and the magnitude and duration of this may influence clinical outcome. The TNF- α gene polymorphisms studies with ischaemic brain injury resulting in stroke have been conducted in India, but there are no studies associating it with TBI. This preliminary study from Southern India indicates that both serum TNF- α levels assessed within 24 hours of trauma and TNF- α rs1799724 polymorphism can be used as prognostic markers of TBI outcome. To the best of our knowledge for our study population this is the first study on TNF- α and its association with TBI which gives serves as a prognostic marker. This data is valuable for providing the framework and justification for further research aimed at describing the potential role and pathological mechanisms that TNF- α plays in secondary brain injury. It can also be used as a target for therapeutic intervention.

Limitation(s)

The number of samples included in the study were relatively less. Larger studies with different subjects including various ethnic groups are required for stronger evidence.

CONCLUSION(S)

This study demonstrates the relationship between TNF- α serum levels and its gene polymorphism with outcome of TBI, based on their GCS and GOS scores. Initial elevated serum TNF- α was associated with poor neurological recovery after three and six months. The C allele and especially CC genotype of rs1799724 polymorphism was associated with positive outcome at three to six months. Thus, evaluating TNF- α levels and evaluating the genotype of rs1799724 polymorphism at admission, can be taken as prognostic marker.

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