Comparative evaluation of risk factors, outcome and biomarker levels in young and old acute ischemic stroke patients

Anuja P Kawle[1], Amit R Nayak[1], Neha H Lande[1], Dinesh P Kabra[1], Nitin H Chandak[1], Shweta R Badar[2], Dhananjay V Raje[2], Girdhar M Taori[1], Hatim F Daginawala[1] and Rajpal S Kashyap[1]

[1]Biochemistry Research Centre, Central India Institute of Medical Sciences, Nagpur, Maharashtra, India; [2]MDS Bioanalytics Pvt. Ltd, 88/2, Sakar Enclave, Nagpur, Maharashtra, India

KEY WORDS

Neurological disorder
Prognosis
Immunoassay
ITIH4 protein
Neuron specific enolase

Corresponding Author:

Rajpal S. Kashyap
Tel : +91-712-2233381/2236441
E-mail : raj_ciims@rediffmail.com

ABSTRACT

Background: Stroke is the third leading cause of death and disability worldwide accounting for 400-800 strokes per 100,000 individuals each year.
Purpose: In the present study, we compared risk factors, clinical outcome, and prognostic biomarkers NSE, S-100 ßß and ITIH4 levels in young and old acute ischemic stroke (AIS) patients.
Methods: We compared the risk factors and clinical outcomes in young (n = 38) and old (n = 66) AIS patients admitted to tertiary health care centre in Central India. In addition, we also evaluated NSE, S100ββ & ITIH4 levels in admission and discharge samples of young and old AIS patients with different clinical outcome.
Results: Hypertension was a major risk factor in 45% of young and 80% of old AIS patients. Hospital outcome was less favorable in young AIS patients with higher dependent rates of 24% as compared to 12% in old AIS patients. Whereas long term outcome at 12 and 18 months after discharge was more favorable in young AIS patients with low dependency rates of 16% and 11% as compared to 41% and 24% in older AIS patients respectively. Similarly, serum NSE, S100ββ and ITIH4 levels showed a distinct pattern of expression at discharge time in AIS patients with improved and dependent outcome in both the age groups.
Conclusion: Young males with hypertension and smoking habits are at a high risk of AIS while old AIS patients are at a greater risk of worse long term outcome. Serum levels of NSE and S100ββ are independent predictors of outcome in AIS patients. Similarly, it also suggests that serum ITIH4 levels could be used as a potential biomarker for predicting the outcome in AIS patients.

doi : 10.5214/ans.0972.7531.220204

Introduction

Stroke is the third leading cause of death and disability worldwide accounting for 400-800 strokes per 100,000 individuals each year.[1] Although stroke is a disease associated with old age, recent studies suggest that rates of stroke in younger population (<50 year age) has been increasing.[2] Overall incidence rates of stroke in younger age were reported to range from 7 to 15 in 100,000 people/year.[3–7] Also a greater incidence of young stroke was reported in developing countries ranging from 45 to 70 in 100,000 people/year.[8–10] India contributes about 1.44–1.64 million acute stroke cases every year[11–12] out of which 15–30% was of younger age group.[13]

Although young stroke patients are reported to have better chances of survival than old patients, about half of them do not return to work and remain dependent throughout their life for routine activities.[14] Thus an early identification of AIS patients who are at high risk of poor outcome may be immensely helpful in planning better management strategies for them. Although the risk factors and outcome has been well described in number of worldwide studies including India, there is lack of current data based on them.[15,16] Hence, there is a dire need for research especially on population based studies in order to get recent updates on AIS.

Among the stroke subtype, acute ischemic stroke (AIS) accounts for 85% of total stroke cases. Similarly, it has also been reported that chances of better recovery is very high among AIS cases than hemorrhagic stroke (HS) cases. We aimed to compare the risk factors and clinical outcomes in young and old AIS patients admitted to tertiary health care centre in Central India. In addition, we also evaluated NSE, S100ββ & ITIH4 levels (estimated using 9 anti ITIH4 peptides) in admission and discharge samples of young and old AIS patients which could be used as potential biomarkers for prognosis of outcome.

Methods

The study was approved by the Institutional Ethics Committee of Central India Institute of Medical Sciences (CIIMS), Nagpur. 104 AIS patients who were admitted to intensive care unit (ICU) of CIIMS from November 2012 to January 2014 who were enrolled in the current study. Diagnosis of AIS was based on the WHO definition of stroke i.e. "rapidly developing signs of focal (or global) disturbance of cerebral function lasting >24 hrs (unless interrupted by surgery or death), with no apparent non vascular cause, history, neurological examination and computerized tomography".

A formal consent was taken from each of the participants who were included in the study and a predesigned questionnaire was recorded and stored for all AIS patients. A baseline of clinical characteristics such as age, gender, risk factors and behavioral factors, past history of stroke and duration of hospital stay were prepared. Smoking was defined as current use of >1 cigarette per day. Diabetes mellitus was defined as receiving oral hypoglycemic agents/insulin treatment. Hypertension was defined as receiving antihypertensive treatment.

Neurological deficit was assessed using the National Institute of Health Stroke Scale (NIHSS) score at the time of admission. AIS patients were classified into three groups based on NIHSS score; these were minor stroke (NIHSS score 1–6), moderate stroke (NIHSS score 7–18), and severe stroke (NIHSS score 19–42). Similarly, Modified Rankin Scale (mRS) was used for evaluation of outcome in AIS patients at discharge time and at 12 months and 18 months after discharge. Out of 104 patients, 12 months follow-up was obtained for 60 AIS patients and 18 months follow-up was obtained for 56 AIS patients. Based on m-RS score AIS patients were classified into two outcome groups: improved group (n = 87; m-RS score 0–2) and dependent/expired group (n = 17; m-RS score 3–6).

AIS patients were then classified into two groups on the basis of their age. These are young AIS patients group and old AIS patient groups. Young AIS group included patients with ≤50 year's age. Likewise, old AIS group included patients who were >50 years of age.

Treatment

All AIS patients received antiplatelet agents (aspirin 150 mg and clopadrigel 75 mg) once a day. Out of 104 AIS patients, 10 were thrombolyzed using intravenous recombinant tissue plasminogen activator, while 3 patients were treated with decompressive hemicraniectomy and duroplasty for malignant middle cerebral artery syndrome.

Exclusion criteria

Stroke patients with hemorrhagic stroke, brain malignancies transient ischemic attack patients who underwent brain operation patients who presented with severe systemic disease, dementia, psychiatric disease, active infection and patients who took discharge against medical advice were excluded from the study.

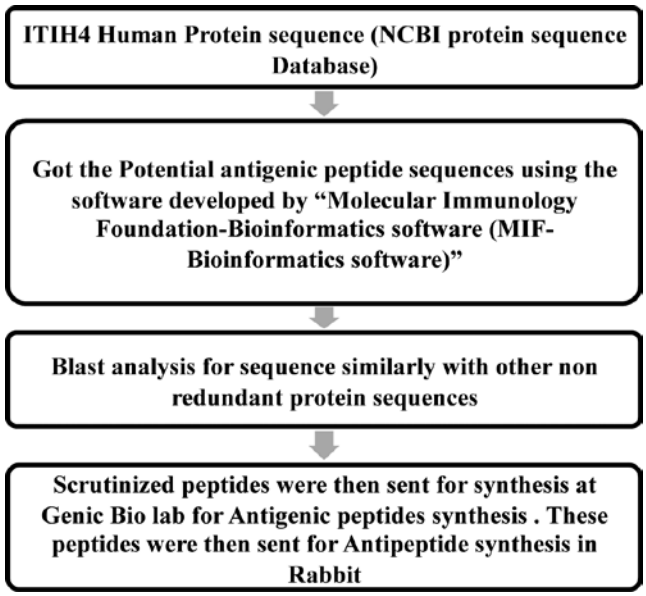
Samples

For evaluation of stroke, biomarkers NSE, S100ββ and ITIH4 peptide levels, were analysed from blood of AIS patients performed at admission and discharge/expiry time. Blood was allowed to clot and after centrifugation (100 × g for 10 min), serum was separated and stored at −20°C use.

Designing and synthesis of peptides and anti-peptide of ITIH4 protein

The reference sequences of ITIH4 were obtained through NCBI reference sequence databases. The antigenic peptides of ITIH4 were determined on the basis of Kolaskar and Tongaonkar (1990) method by using online software titled "Molecular Immunology Foundation-Bioinformatics software (MIF-Bioinformatics software)".[17] These antigenic peptide sequences were then subjected to multiple sequence alignment using NCBI BLAST to obtain the sequence similarities with other non redundant protein database sequences. Based on the results of the blast analysis, nine potential sequences of ITIH4 were selected (*as shown in supplementary file 1 & 2*). These peptide sequences were then sent for antibody production (Antipeptide 1 to 9) in GenicBio lab, Shanghai, China. Then, the produced antipeptides were evaluated individually for detection of ITIH4 in the serum samples of AIS patients. The antipeptide antibodies, giving the most specific reactivity, were reported.

Supplementary file 1: Steps for synthesis of antigenic peptides of ITIH4 and its antibody.



Supplementary file 2: List of Antipeptide antibody generated against the selected peptides of ITIH4.

Antibody	Sequence
Anti ITIH-4 Peptide -1	LLLKVRPQQLVKH-C
Anti ITIH-4 Peptide -2	REALIKILDD-C
Anti ITIH-4 Peptide -3	PEGSVSLIILLT-C
Anti ITIH-4 Peptide -4	RYSLFCLGFGFDVSY-C
Anti ITIH-4 Peptide -5	C-GPDVLTATVSGK
Anti ITIH-4 Peptide -6	C-LNLSLAYSFV
Anti ITIH-4 Peptide -7	C-TFFKYYLQGAKIPKPEA
Anti ITIH-4 Peptide -8	C-LLLSDPDKVT
Anti ITIH-4 Peptide -9	C-LGQFYQEVLWG

ITIH4 estimation using anti-ITIH4 peptide antibody

ITIH4 was estimated using Indirect Enzyme-Linked Immunosorbant Assays (ELISA). In brief, microtiter ELISA wells were coated with 100 µl (1:400) of serum samples taken from stroke patients and blocked with 200 µl of 2.5% BSA in phosphate buffer saline (PBS) for 2hrs. After washing with PBS, the polyclonal antibody (i.e. Anti peptide antibody) against ITIH4 was added, and plates were incubated at 37°C for 45 min. The wells were then washed, followed by addition of the secondary antibody (goat anti rabbit immunoglobulin horseradish peroxidase; IgG-HRP) and incubated for 45 min at 37°C. After another wash, antibody reactivity was detected via the addition of 100 µl *Tetramethylbenzidine* hydrogen peroxide

(TMB/H2O2) substrate solution to the wells, which were then incubated at room temperature for about 5 min. The reaction was stopped with 100 µl of 2.5 N H2SO4, and the absorbance of each well was read at 450 nm.

NSE measurement

Serum NSE levels were estimated with Can Ag NSE EIA kit (Sweden) as per the instructions of manufacturer. Test is based on solid phase, non-competitive immunoassay based on two monoclonal antibodies (derived from mice) directed against two separate antigenic determinants of NSE molecule. The monoclonal antibodies (MAb) used binds to γ subunit of enzyme and thereby detects both γγ and αγ enzymes of NSE. In brief, the required number of microplate strips were transferred to a strip frame. Each strip washed once with wash solution. 25 µL of NSE Calibrators (CAL A, B, C, D, and E) and patient specimens (unknowns-Uk) were added into the strip wells. The plate was incubated for 1 hour (±10 min) at room temperature (20–25°C) with constant shaking of the plate using a microplate shaker. After incubation, the content was aspirated and washed 6 times with wash buffer. About 100 µL of TMB HRP-Substrate was added to each well. It was incubated for 30 min (±5 min) at room temperature with constant shaking. After incubation add 100 µL of stop solution was added mixed and the absorbance was read at 405 nm in a microplate spectrophotometer within 15 min after addition of stop solution.

S-100ββ measurement

Quantitative determination of S-100 ββ in human serum was performed as per the instructions indicated in the manual (Can Ag S-100 EIA Sweden). In brief, the required number of microplate strips were transferred to a strip frame. Each strip was washed with wash solution. About 50 µL of S100BB calibrators and sample were washed into the strip wells. About 100 µL Biotin Anti-S100BB was added to each well and incubated for 2 hours (±10 min) at room temperature (20–25°C) with constant shaking using a microplate shaker. After incubation, the contents were aspirated and washed 3 times using wash buffer. About 100 µL of Tracer solution was added to each well. The frame was incubated for 1 hour (±5 min) at room temperature with constant shaking. After incubation the content was washed 6 times with wash buffer. About 100 µL of TMB HRP-Substrate was added to each well. It was incubated for 30 min (±5 min) at room temperature with constant shaking. 100 µL of Stop Solution was added and mixed and read at 405 nm in a microplate spectrophotometer within 15 min after addition of Stop Solution.

Statistical analysis

The categorical data on different baseline variables was expressed in terms of number and percentage and its association with study groups was determined using Chi-square test. The data on continuous variables was summarized in the terms of box plot and statistical significance of difference in the variable means between groups was determined using *t*-test. Outcome was defined as improved and patients expired or dependent after discharge. Considering death or dependency as outcome, the association of old age with outcome was analyzed using multivariate logistic regression after adjusting for potential confounders like age, sex, alcohol, thrombolysis, admission duration and hypertension and diabetes. All the analysis was performed using R-programming language (version: 3.0.0) and statistical significance was tested at 5% level.

Results

Young AIS patients account for 36% of total 104 AIS cases. Baseline characteristics of young (n = 38) and old (n = 66) AIS patients are given in Table 1. Results shows that 70% (73/104)

Table 1: Clinical characteristics of young and old AIS patients

Sr. No	Characteristics	Total No (%)	Young stroke (n = 38)	Old stroke (n = 66)	P value
1	**Sex**				
	Male (n = 73)	73 (70%)	30 (79%)	43 (65%)	0.207
	Female (n = 31)	31 (30%)	8 (21%)	23 (35%)	
2	**Associated risk factor**				
	Hypertension	70 (67%)	17 (45%)	53 (80%)	**0.0006***
	Diabetes	29 (28%)	5 (13%)	24 (36%)	**0.021***
	Ischemic heart diseases	5 (5%)	1 (3%)	4 (6%)	0.7557
	Cardiac disease	3 (3%)	2 (5%)	1 (2%)	0.3262
3	**Behavioral Factors**				
	Smoking	8 (8%)	6 (16%)	2 (3%)	0.4126
	Alcohol	11 (11%)	5 (13%)	6 (9%)	0.7502
4	**Past History of Stroke**	6 (6%)	1 (3%)	5 (8%)	0.5454
5	**Treatment**				
	a Thrombolysis	10 (10%)	5 (13%)	5 (8%)	0.5589
	b Decompressive surgery	3 (3%)	2 (5%)	1 (2%)	0.6232
6	**Disability Score on admission**				
	Mild (0–6)	23 (22%)	7 (18%)	16 (24%)	
	Moderate (7–15)	44 (42%)	17 (46%)	27 (41%)	0.5899
	Severe (16–58)	14 (13%)	7 (18%)	7 (11%)	

*P value <0.05 significant.

of AIS cases were predominately males. Hypertension was a major risk factor in 45% (17/38) for young and 80% (53/66) for old AIS patients. Diabetes was more frequently (P<0.05) associated with 36% (34/66) old AIS patients, while smoking was found to be a contributing risk factor for 16% (6/38) of young AIS patients. The ratio of AIS patients with ischemic heart disease (IHD), cardiac diseases, history of stroke and behavior associated risk factors (such as alcohol consumption) were similar in both the age groups. Severity of AIS was found to be 18% (7/38) mild, 46% (17/38) moderate and 18% (7/38) severe in younger age group as compared to 24% (16/66) mild, 41% (27/66) moderate and 11% (7/66) severe in older AIS age group as shown in Table 1.

Comparison of outcomes in young and old AIS patients are given in Table 2. Results show that 24% (9/38) of young AIS patients had ≥15 days hospital stay as compared to only 12% (8/66) of old AIS patients (p>0.05). Similarly, ratio of AIS patients with dependent hospital outcome was insignificantly (p>0.05) high in 24% (9/38) young AIS patients as compared to 12% (8/66) in old AIS patients. In contrast, long term outcome at 12 months and 18 month shows higher ratio of dependency outcome in old AIS patients (i.e. 41%; 17/41 and 24%; 9/38) as compared to young AIS patients (i.e. 16%; 3/19 and 11%; 2/18) respectively.

Table 3 depicts Cox multivariate logistic-regression analysis for predicting the long term outcome among young and old AIS patients. After adjusting for potential confounders (i.e. hypertension, diabetes, smoking, alcoholism, disability score, and admission time) we found that old AIS patients had 2.243 fold (95% CI.0.483–10.404) higher risk of dependency outcome at 12 months as compared to young AIS patients which further increased to 3.144 folds (95% CI.0.525–18.833) at 18 month after discharge.

Analysis of results of NSE, S100ββ and ITIH4 estimation in the serum samples of AIS patients with improved and dependent outcomes in both the age groups are given in Figure 1 & 2. We found an increased ITIH4 levels detected by antipeptide 1, 6 and 7 (P<0.05) at the time of discharge as compared to its admission level in young AIS patients with improved outcome. In contrast, its level decreased at discharge (vs. admission level) among young AIS patients with dependent/expired outcome. Similar results were seen in ITIH4 levels detected by antipeptide 6 in old AIS patients (as shown in Figure 1). Other antipeptides (i.e. antipeptide 2, 3, 4, 5, 8 and 9) did not show any differential pattern in young and old AIS patients with improved and dependent/expired outcome (*Data not shown*).

Results of NSE and S100ββ showed that their levels either remained stable or decreased at the time of discharge (vs. admission) in both young and old AIS patients who improved. While, its level further increased at discharge time in AIS patients with dependent/expired outcome (as shown in Figure 2).

Table 2: Comparison of outcomes in young and old AIS patients

Sr. No	Characteristics	Total No. (%)	Young stroke (n = 38)	Old stroke (n = 66)	P value
1	**Duration of Hospital Stay** *≤15 days* *>15 days*	 87 (84%) 17 (16%)	 29 (76%) 9 (24%)	 58 (88%) 8 (12%)	0.2076
2	**Hospital Outcome** *Dependent/Expired*	 17 (16%)	 9 (24%)	 8 (12%)	0.2076
3	**Long Term Outcome**				
a	**12 months*** *Dependent/Expired*	 20 (33%)	*n = 19* 3 (16%)	*n = 41* 17 (41%)	0.1053
b	**18 months©** *Dependent/Expired*	 16 (29%)	*n = 18* 2 (11%)	*n = 38* 9 (24%)	0.4349

*Out of 60 cases, © Out of 56 cases.

Table 3: Unadjusted and adjusted odds ratios of long term outcomes in young and old ischemic stroke patients

Sr. No	Outcomes	Unadjusted OR (95%CI)	Adjusted OR (95%CI)
1	**Outcome on discharge** *Dependent/Expired (n = 17)*	 0.44 (0.15–1.31)	 0.054 (0.007–0.41)
2	**Long term outcomes**		
a	**At 12 months** *Dependent/Expired (n = 20)*	 3.58 (0.98–18.11)	 2.243 (0.483–10.404)
b	**At 18 months** *Dependent/Expired (n = 16)*	 4.32 (1.00–33.11)	 3.144 (0.525–18.833)

OR indicates odds ratio; CI, confidence interval.

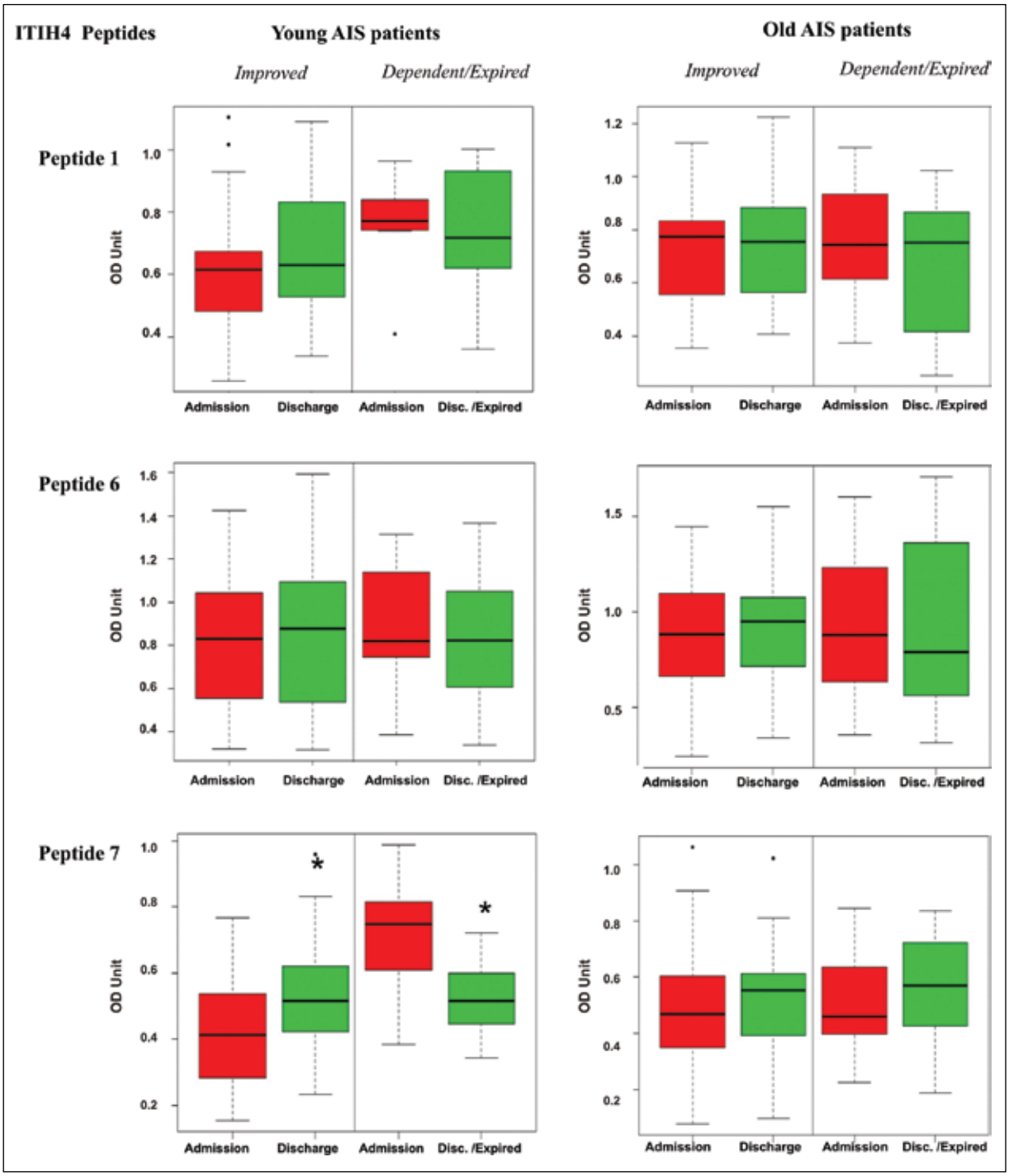


Fig. 1: Levels of ITIH4 in young (n = 38) and Old (n = 66) AIS patients with improved and dependent/Expired outcome.

Discussion

In the present study, we compared the risk factors and clinical outcome in young and old AIS patients. We found that young stroke account for 36% of total 104 AIS cases. Hypertension was found to be a major risk factor in both young and old AIS patients. Similarly, males were found to be at a higher risk of stroke than females in both the age groups. Diabetes was more frequently observed in old AIS patients, while smoking was observed in young AIS patients. Hospital outcome was poor in young AIS cases. Long term outcome at 12 and at 18 months, however, was better in young AIS patients.

In the earlier Indian studies, incidence of young stroke was reported to range from 15%–30% of total stroke cases.[18–21] However, in the current study, we observed 36% of young AIS cases. This suggests that incidence of young stroke was slightly higher in Central India population. Earlier it was also reported

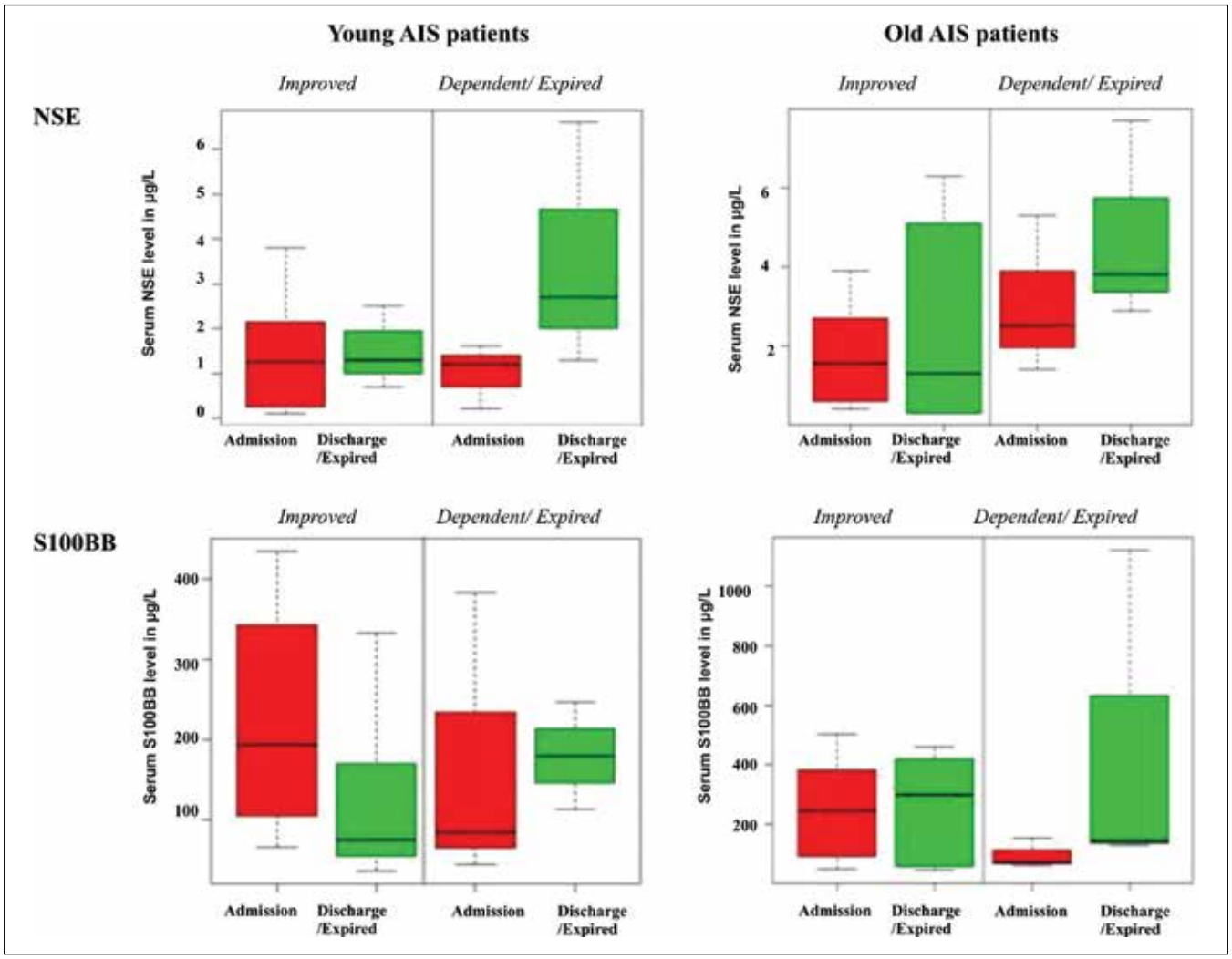


Fig. 2: Levels of NSE and S-100 (kit based assay) in young (n = 38) and Old (n = 66) AIS patients with improved and dependent/expired outcome.

that both short term and long term outcomes in young stroke patients were much better than old stroke patients (>50 yrs), with lower mortality and recurrence rate and better functional recovery.[22–24] However, we found slightly worse hospital outcome in young AIS patients as compared to the old AIS patients. The ratio of AIS patients with severe stroke was high in young age group in our study population. This could be the only possible explanation for the slightly worse hospital outcome in young AIS patients. We found good long term outcome among young AIS patients than old AIS patients. Old AIS patients showed poor outcome at 18 month follow-up. Poor outcome in old AIS patient was found to be associated with hypertension, diabetes, smoking, alcoholism, as suggested by multivariate analysis. Therefore, these factors should also be considered in order for better management of AIS patients after discharge.

Stroke was reported to be more common among men with 30%–45% higher incidence than in women.[25] Our results are also in agreement with the existing findings. In addition, we also found slightly higher incidence of stroke in men than women among young AIS patients (i.e. 58%).

Hypertension is one of the major risk factors for AIS, in both developing and developed countries.[26] A prospective study conducted by Indian Council of Medical Research has also reported hypertension, diabetes, tobacco use, and low hemoglobin concentration to be important risk factors for AIS.[27] Presence of diabetes, hypertension, heart disease, current smoking, and long-term heavy alcohol consumption are also identified as major risk factors for stroke in young adults.[28] We also found hypertension as a major risk factor for AIS in both the age groups. In contrast to earlier studies in which diabetes was reported to be a risk factor for stroke in all age groups, we observed diabetes more frequently among old AIS patient.[27,28] Similarly, smoking was found more recurrent in young AIS patients.

In addition, we also evaluated NSE, S100ββ & ITIH4 levels in admission and discharge samples of young and old AIS patients, as potential biomarkers for prognosis of outcome. We found that ITIH4 levels detected by antipeptide 1, 6 & 7 significantly increased in improved AIS patients at discharge. Its level decreased in those with dependent outcome. In our earlier studies, we have reported that serum level of ITIH4 protein is reduced in AIS patients as compared to normal individuals

and again returns to normal in improved AIS patients.[29,30] ITIH4, being a heavy chain-related protein inhibits the phagocytic activities of polymorphonuclear cells and acts as an anti-inflammatory protein.[31,32] Its serum levels also seem to correlate with the anti-inflammatory cytokine IL-10.[30] We observed that long term outcome was better in young AIS patients. Therefore, higher anti-inflammatory response as a result of high ITIH4 antipeptide 1, 6 and 7 levels among the young AIS patients possibly explains the superior outcome among young AIS patients than the older AIS patients.

NSE and S-100ββ have been reported to be very promising biomarkers in predicting the outcome, extent of cerebral injury and neuronal damage in AIS patients.[33,34] During AIS, both NSE and S100ββ leak from the injured tissues and come into the blood after crossing the compromised blood brain barrier.[35] An elevated NSE levels in serum is reported after onset of stroke.[36] Increased NSE level in blood has also been reported to be an indicator of infarct volume and worse neurological outcome after ischemic stroke.[37] Similarly, increase in serum S100ββ protein levels have been demonstrated within 8h after onset of stroke.[38] Blood S100ββ levels were also reported to correlate with severity and functional outcome in stroke patients.[36] In the current study, we also compared NSE and S100ββ levels in improved and dependent groups, in both young and old AIS patients. We found that NSE and S100ββ levels either remained stable or decreased at the time of discharge as compared to its admission level in improved AIS patients. Its levels, however, further increased among AIS patient with dependent outcome. The above findings are consistent irrespective of age groups. This suggests that NSE and S100ββ are independent predictors of outcome in AIS patients.

Inspite of interesting findings, the study has a few limitations such as less number of sample size thereby curbing us to draw solid conclusions. Similarly, number of AIS patients for which the follow-up status was known is also limited. Therefore, similar studies with higher number of samples are required to confirm the findings.

Conclusion

Results of the current study lead to following conclusions. The incidence of young stroke was slightly higher in Central India as compared to earlier reports. Young males with hypertension and smoking habits are at a high risk of AIS. Old AIS patients are at a greater risk of worsening during long term outcome, as compared to young. Serum levels of NSE and S100ββ are independent predictors of outcome in AIS patients. Similarly, it also suggests that serum ITIH4 levels could be used as a potential biomarker for predicting the outcome in AIS patients.

Acknowledgment

All the authors would like to acknowledge Department of Biotechnology (DBT), India under the project number BT/PR14368/MED/30/525/210 for funding this project.

Authorship Contribution

Anuja P Kawle: Article drafting and data collection, **Amit R Nayak**: Conception & design and analysis and interpretation of data, **Neha H Lande**: Data collection, **Dinesh P Kabra**: Analysis & interpretation of data, **Nitin H Chandak**: Analysis & interpretation of data, **Shweta R Badar**: Statistical analysis, **Dhananjay V Raje**: Statistical analysis, **Girdhar M Taori**: Final approval of the article, **Hatim F Daginawala**: Final approval of the article, **Rajpal S Kashyap**: Conception & design, critical revision of the article and overall responsibility.

Abbreviations

Acute ischemic Stroke (AIS), Inter alpha trypsin inhibitor heavy chain 4 (ITIH4), Ischemic Heart Disease (IHD), Central Nervous System (CNS), Intensive Care Unit (ICU), Computerized Tomography (CT), National Institute of Health Stroke Scale (NIHSS), Modified Rankin Scale (mRS).

This article complies with International Committee of Medical Journal editor's uniform requirements for manuscript.

Conflict of Interest: None; Source of Funding: DBT

Received Date : 8 December 2014; Revised Date : 23 January 2015;
Accepted Date : 12 February 2015

References

1. Banerjee TK, Roy MK, Bhoi KK. Is stroke increasing in India—preventive measures that need to be implemented. J Indian Med Assoc. 2005; 103(3): 162, 164, 166.
2. Maaijwee NA, Rutten-Jacobs LC, Schaapsmeerders P, et al. Ischaemic stroke in young adults: risk factors and long-term consequences. Nat Rev Neurol. 2014; 10(6): 315–25.
3. Wolfe CD, Giroud M, Kolominsky-Rabas P, et al. Variations in stroke incidence and survival in 3 areas of Europe. European Registries of Stroke (EROS) Collaboration. Stroke. 2000; 31(9): 2074–9.
4. Vibo R, Kõrv J, Roose M. The Third Stroke Registry in Tartu, Estonia: decline of stroke incidence and 28-day case-fatality rate since 1991. Stroke. 2005; 36(12): 2544–8.
5. Jerntorp P, Berglund G. Stroke registry in Malmö, Sweden. Stroke. 1992 Mar; 23(3): 357–61.
6. Minelli C, Fen LF, Minelli DP. Stroke incidence, prognosis, 30-day, and 1-year case fatality rates in Matão, Brazil: a population-based prospective study. Stroke. 2007; 38(11): 2906–11.
7. Marini C, Totaro R, De Santis F, et al. Stroke in young adults in the community-based L'Aquila registry: incidence and prognosis. Stroke. 2001; 32(1): 52–6.
8. Radhakrishnan K, Ashok PP, Sridharan R et al. Stroke in the young: incidence and pattern in Benghazi, Libya. Acta Neurol Scand. 1986; 73(4): 434–8.
9. Morikawa Y, Nakagawa H, Naruse Y et al. Trends in stroke incidence and acute case fatality in a Japanese rural area: the Oyabe study. Stroke. 2000; 31(7): 1583–7.
10. Jacobs BS, Boden-Albala B, Lin IF, et al. Stroke in the young in the northern Manhattan stroke study. Stroke. 2002; 33(12): 2789–93.
11. World Health Organization. Preventing Chronic Diseases: A vital investment. Geneva, Switzerland. 2005.
12. Murthy J. Thrombolysis for stroke in India: Miles to go…… Neurology India 2007; 55(1): 3–5
13. Nayak SD, Nair M, Radhakrishnan K, et al. Ischaemic stroke in the young adult: clinical features, risk factors and outcome. Natl Med J India. 1997; 10(3): 107–12.
14. Varona JF, Bermejo F, Guerra JM, et al. Long-term prognosis of ischemic stroke in young adults. Study of 272 cases. J Neurol. 2004; 251(12): 1507–14.
15. Khalid Sher, Shahnaz Shah and Suneel Kumar. Etiologic Patterns of Ischaemic Stroke in Young Adults. Journal of the College of Physicians and Surgeons Pakistan. 2013; 23(7): 472–475.
16. Loes CA Rutten-Jacobs, Noortje AM Maaijwee, Renate M Arntz, et al. Risk factors and prognosis of young stroke. The FUTURE study: A prospective cohort study. Study rationale and protocol. BMC Neurology. 2011; 11: 109.
17. Kolaskar AS, Tongaonkar PC. A semi-empirical method for prediction of antigenic determinants on protein antigens. FEBS Lett. 1990; 276(1-2): 172–4.
18. Srinivasan K. Ischemic cerebrovascular disease in the young. Two common causes in India. Stroke. 1984 Jul-Aug; 15(4): 733–5.
19. Bansal BC, Prakash C, Jain AL, et al. Cerebrovascular disease in young individuals below the age of 40 years. Neurol India. 1973; 21(1): 11–8.

20. Chopra JS, Prabhakar S. Clinical features and risk factors in stroke in young. Acta Neurol Scand. 1979; 60(5): 289–300.
21. Radhakrishnan K, Ashok PP, Sridharan R, et al. Stroke in the young: incidence and pattern in Benghazi, Libya. Acta Neurol Scand. 1986; 73(4): 434–8.
22. Marshall J. The cause and prognosis of strokes in people under 50 years. J Neurol Sci. 1982; 53(3): 473-8.
23. Varona JF. Long-term prognosis of ischemic stroke in young adults. Stroke ResTreat. 2010 20; 2011: 879817.
24. Palmcrantz S, Widén Holmqvist L, Sommerfeld DK. Young individuals with stroke: a cross sectional study of long-term disability associated with self-rated global health. BMC Neurol. 2014 28; 14: 20.
25. Appelros P, Stegmayr B, Terént A. Sex differences in stroke epidemiology: a systematic review. Stroke. 2009; 40(4): 1082–90.
26. Kearney PM, Whelton M, Reynolds K, et al. Worldwide prevalence of hypertension: a systematic review. J Hypertens. 2004; 22(1): 11–9.
27. Dalal PM, Dalal KP, Rao SV, Parikh BR. Strokes in westcentral India: a prospective case-control study of "risk factors" (a problem of developing countries). In: Bartko D, et al. eds. Neurology in Europe. John Libbey and Co.Ltd., London, 1989; 16–20.
28. You RX, McNeil JJ, O'Malley HM, et al. Risk factors for stroke due to cerebral infarction in young adults. Stroke. 1997; 28(10):1913-8.
29. Kashyap RS, Nayak AR, Deshpande PS, Kabra D, Purohit HJ, Taori GM, Daginawala HF. Inter-alpha-trypsin inhibitor heavy chain 4 is a novel marker of acute ischemic stroke. Clin Chim Acta. 2009; 402 (1-2): 160–3.
30. Nayak AR, Kashyap RS, Kabra D, Purohit HJ, Taori GM, Daginawala HF. Time course of inflammatory cytokines in acute ischemic stroke patients and their relation to inter-alfa trypsin inhibitor heavy chain 4 and outcome. Ann Indian Acad Neurol. 2012; 15(3): 181–5.
31. Choi-Miura NH, Takahashi K, Yoda M, et al. The novel acute phase protein, IHRP, inhibits actin polymerization and phagocytosis of polymorphonuclear cells. Inflamm Res. 2000; 49: 305–10.
32. Choi-Miura NH. Quantitative measurement of the novel human plasma protein, IHRP, by sandwich ELISA. Biol Pharm Bull. 2001; 24: 214–7.
33. Hill MD, Jackowski G, Bayer N, et al. Biochemical markers in acute ischemic stroke. CMAJ. 2000; 162: 1139–40.
34. Wunderlich MT, Ebert AD, Kratz T, et al. Early neurobehavioral outcome after stroke is related to release of neurobiochemical markers of brain damage. Stroke.1999; 30:1190–5.
35. Stevens H, Jakobs C, de Jager AE, et al. Neurone-specific enolase and N-acetyl-aspartate as potential peripheral markers of ischaemic stroke. Eur J Clin Invest. 1999; 29(1): 6–11.
36. Nagdyman N, Grimmer I, Scholz T, et al. Predictive value of brain-specific proteins in serum for neurodevelopmental outcome after birth asphyxia. Pediatr Res. 2003; 54(2): 270–5.
37. Fassbender K, Schmidt R, Schreiner A, et al. Leakage of brain-originated proteins in peripheral blood: temporal profile and diagnostic value in early ischemic stroke. J Neurol Sci. 1997 1; 148(1): 101–5.
38. Foerch C, Otto B, Singer OC, et al. Serum S100BBB predicts a malignant course of infarction in patients with acute middle cerebral artery occlusion. Stroke. 2004; 35(9): 2160–4.