

Evaluation of peptide-based approach for estimation of NSE and S-100 β towards the development of a cost-effective test for prognosis of AIS patients

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Here we compare the in-house ELISA technique for estimation of neuron-specific enolase (NSE) and S-100 β (glial-specific protein) peptides with a commercial kit in serum samples of acute ischaemic stroke (AIS) patients. Nineteen improved and four expired AIS patients were included for the study. NSE concentrations were significantly higher ($P < 0.05$) in expired as compared with improved AIS patients by both in-house ELISA and commercial kit method. Estimation of S-100 β by only in-house ELISA showed significantly high ($P < 0.05$) levels in expired AIS patients. Peptide-based estimation of NSE and S-100 β may be used for prognosis of AIS patients.

Keywords: Acute ischaemic stroke, glial-specific protein, neurological disease, neuron-specific enolase, prognosis.

EARLY diagnosis and prognosis of stroke is of utmost importance for clinicians in planning appropriate management strategies for acute ischaemic stroke (AIS) patients. In the current scenario, diagnosis and prognosis of AIS patients basically rely upon computed tomography (CT) scanning/magnetic resonance imaging (MRI), which are somewhat unsatisfactory. CT scan has limited sensitivity in detecting minor changes in the brain. MRI is known to be superior to CT scan, and overcomes all the limitations associated with CT¹. However, MRI is costly and cannot be performed repeatedly, and is unavailable in most hospital settings. There is a need for alternative methods which can be used for early diagnosis and prognosis of AIS patients.

During the last two decades, numerous biomarkers of the central nervous system (CNS), as well as non-CNS origins, have been evaluated in the body fluids of AIS patients for predicting any adverse neurological prognosis

in them². Some of these are neuron-specific enolase (NSE), S-100 β (glial-specific protein), myelin basic protein, creatine kinase isoenzyme, tau protein, polyamines and matrix metalloproteinase-9 (MMP-9) which are found to be promising markers in predicting the prognosis of AIS patients^{3,4}. Out of these, NSE and S-100 β are the most widely studied biomarkers for evaluating the prognosis of AIS patients⁵. Although NSE and S-100 β estimation is economical compared to CT and MRI, its use is limited in the developing countries because of unavailability and high import cost. Therefore, development of indigenous and more cost-effective experimental protocols are required for the estimation of NSE and S-100 β on a routine basis for prognosis of AIS patients.

In the present study, we compared our in-house cost-effective enzyme-linked immunosorbent assay (ELISA) developed using anti-peptide antibodies produced against selected peptides of NSE (i.e. NSE peptides 1 and 2) and S-100 β (i.e. S-100 β peptides 1 and 2) with a commercially available ELISA kit for the evaluation of predictive values of the test in serum samples of improved and expired AIS patients.

Forty AIS patients who were admitted to the Central India Institute of Medical Sciences (CIIMS), Nagpur, from December 2010 to May 2011 were included in the study. Diagnosis of AIS patients was based on the WHO definition of stroke, i.e. 'rapidly developing signs of focal (or global) disturbance of cerebral function lasting >24 h (unless interrupted by surgery or death), with no apparent non-vascular cause or history, neurological examination and CT'. All patients were admitted to the intensive care unit (ICU), where the ambient temperature was maintained between 20°C and 25°C. The protocol for this study was reviewed and approved by the Institutional Ethics Committee of CIIMS.

Patients with haemorrhagic stroke, transient ischaemic attack, brain malignancies, and those who had undergone brain surgery, severe systemic disease, dementia, psychiatric disease and active infection were excluded from the study. Patients who refused to participate and who took discharge against medical advice were also excluded from the study.

Detailed history was taken and CT scan was performed within 12 h of admission to exclude patients with stroke mimic, and severity of stroke was evaluated using National Institute of Health Stroke Scale (NIHSS). Based on the NIHSS score AIS patients were classified into four groups, i.e. score of 0 = no stroke, 1–6 = minor stroke, 7–18 = moderate stroke, 19–42 = severe stroke. The modified Rankin scale (mRS) was used for evaluation of prognosis of AIS patients by the clinician at the time of discharge.

Blood samples were taken from AIS patients at the time of admission. Similarly, blood samples were also taken from a healthy individual as control sample ($n = 10$). Blood was allowed to clot and after centrifugation (100 g

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Table 1. Anti-neuron-specific enolase (NSE) peptide and anti-S-100 $\beta\beta$ (glial-specific protein) peptide antibodies produced against the selected antigenic sequence of NSE and S-100 $\beta\beta$

Antibody produced	Start position	Selected antigenic sequence of NSE and S-100 $\beta\beta$	End position
Anti-NSE peptide-1	18	PTVEVDLYTA-C	27
Anti-NSE peptide-2	58	LGKGV LKAVDH-C	68
Anti-S-100 $\beta\beta$ peptide-1	5	EKAMVALIDVFHQ-C	17
Anti-S-100 $\beta\beta$ peptide-2	49	C-KEQEVVDKVM	58

Table 2. Sample dilution and antipeptide antibody dilution used for the estimation of NSE and S-100 $\beta\beta$ in the serum sample of AIS patient

Type of marker	Anti-NSE peptide-1	Anti-NSE peptide-2	Anti-S-100 $\beta\beta$ peptide-1	Anti-S-100 $\beta\beta$ peptide-2
Sample dilution	1 : 200	1 : 200	1 : 500	1 : 200
Primary Ab dilution	1 : 5000	1 : 1000	1 : 10000	1 : 5000
Secondary Ab dilution	1 : 10000	1 : 10000	1 : 10000	1 : 10000

for 10 min) the serum was separated and stored at -20°C until further use.

Serum NSE levels were estimated using Can Ag NSE EIA kit (Sweden) according to instructions of the manufacturer. This test is based on a solid-phase, non-competitive immunoassay using two monoclonal antibodies (derived from mice) directed against two separate antigenic determinants of NSE molecule. The monoclonal antibodies (MAbs) used bind to the γ -subunit of the enzyme and thereby detect both $\gamma\gamma$ and $\alpha\gamma$ which are iso-enzymes of NSE. All the analyses were carried out in triplicate.

Quantitative determination of S-100 $\beta\beta$ in human serum was also performed using Can Ag S-100 $\beta\beta$ EIA (Sweden). This is a two-step enzyme immunometric assay (EIA) based on two monoclonal antibodies derived from mouse, specific for two different epitopes of S-100 $\beta\beta$. All the analyses were carried out in triplicate.

The antigenic peptides of NSE and S-100 $\beta\beta$ were determined based on the method of Kolaskar and Tongaonkar (<http://bio.dfci.harvard.edu/Tools/antigenic.html>) using on-line software (Molecular Immunology Foundation (MIF)-Bioinformatics software) and reference sequences of NSE (sequence ID: NP_001966.1) and S-100 $\beta\beta$ (sequence ID: NP_006263.1) available in the National Centre for Biotechnology Information (NCBI) reference sequence. These antigenic peptide sequences were then subjected to BLAST analysis of NCBI, to obtain the sequence similarities with other non-redundant protein database sequences. Based on the results of the BLAST analysis, four antigenic sequences (two of NSE and two of S-100) were finally selected, namely NSE peptide 1 (PTVEVDLYTA-C), NSE peptide 2 (LGKGV LKAVDH-C), S-100 $\beta\beta$ peptide 1 (EKAMVALIDVFHQ-C) and S-100 $\beta\beta$ peptide 2 (C-KEQEVVDKVM). These peptide

sequences were sent for synthesis followed by antibody production in GenicBio Limited, Shanghai, China. The purity of the synthesized peptides was checked using HPLC and their quantification was done using mass spectrometry. All the designed peptides were synthesized with purity $>90\%$. Specificity of the produced antibodies was checked by evaluating them in comparison with the pre-immune serum collected before immunizing the rabbits with the peptides. The produced antibodies were not cross-reacting with any other serum protein (data not given). The anti-peptides produced were named based on their peptide name (Table 1).

For the estimation of NSE and S-100 $\beta\beta$ using anti-peptide antibody, a microtiter ELISA well plate was coated with 100 μl serum samples and incubated for 45 min at 37°C . The plate was then washed once with wash buffer, i.e. 0.5% Tween-20 in phosphate buffered saline (PBST). Microtitre wells were then blocked by adding 200 μl of blocking buffer (0.5% BSA in PBST) and incubated at room temperature for 90 min. The wells were washed again three times with PBST. Then 100 μl (concentration of the antibody stock is 1 mg/ml, which is then further diluted to working concentration as mentioned in Table 2) of primary antibodies (anti-peptide antibody raised in rabbit) was added and incubated for 45 min. The wells were again washed three times with PBST. Then 100 μl anti-rabbit horseradish peroxidase (HRP) conjugated secondary antibody (dilution 1 : 10,000) was added and incubated at 37°C for 45 min. After incubation, the microtitre wells were washed four times with PBST and antibody reactivity was detected by adding 100 μl tetramethylbenzidine hydrogen peroxide (TMB/ H_2O_2) substrate. The reaction was then stopped 5 min after adding 100 μl of 2.5 N sulphuric acid (H_2SO_4) and intensity of the developed colour was measured at 450 nm in an ELISA reader.

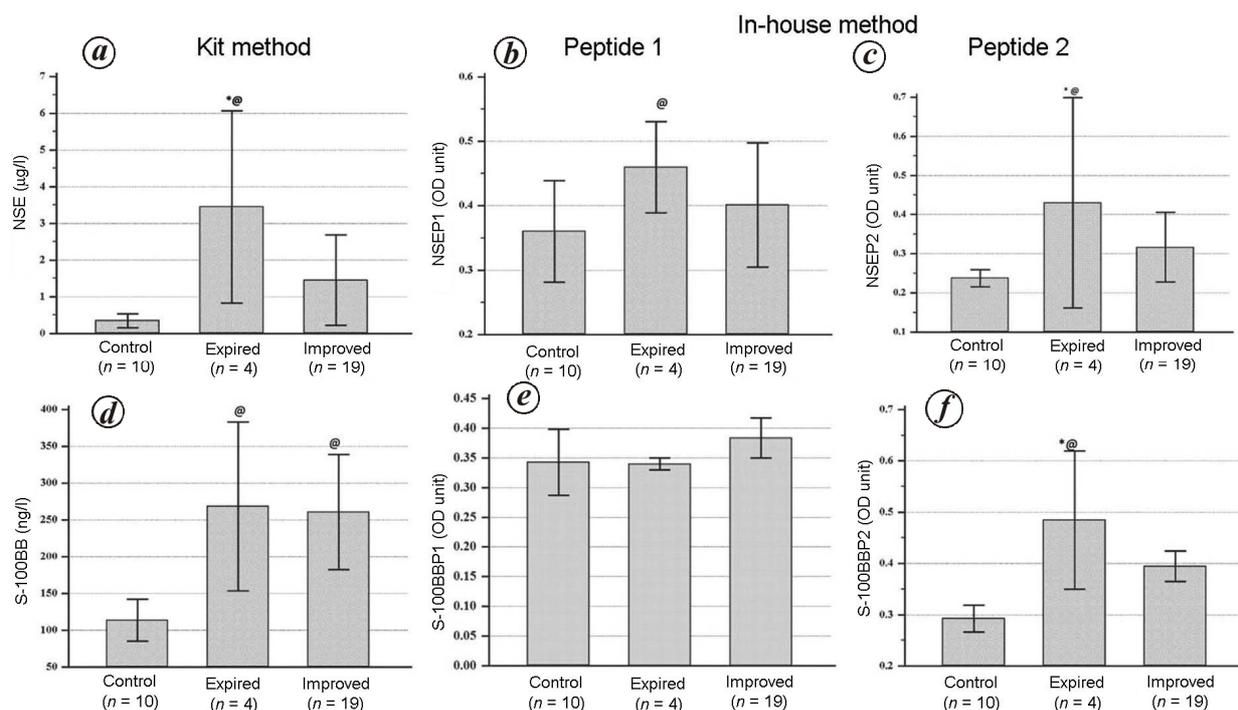


Figure 1. Estimation of serum level of neuron-specific enolase (NSE) and glial-specific protein (S-100ββ) in control individuals and AIS patient. **P* < 0.05 versus improved AIS patients; @*P* < 0.05 versus control.

Table 3. Clinical characteristics of the studied AIS patients (*n* = 23)

Characteristics	Improved	Expired	<i>P</i> value
<i>n</i>	19	4	
Age (years)	51 ± 17	53 ± 13	0.8
Sex (M/F)	13 (68)/6 (32)	3 (75)/1 (25)	0.7
Admission within 24 h	14 (74)	3 (75)	0.5
Duration in the hospital (days)	13 ± 15	4 ± 3	0.2
Associated risk factors			
Hypertension	8 (42)	2 (50)	0.8
Diabetes mellitus	8 (42)	1 (25)	0.9
Cerebrovascular event	10 (53)	2 (50)	0.7
Ischemic heart disease	2 (11)	0	0.8
Past history of stroke	2 (11)	0	0.8
Smoking habit	2 (11)	1 (25)	0.9
Drinking habit	3 (16)	2 (50)	0.4
Other disorders	9 (47)	2 (50)	0.6
Thrombolysis	3 (16)	3 (75)	0.07
Decompression surgery	2 (11)	0	0.8
National Institute of Health Stroke Scale score at admission	12 ± 5	19 ± 10	0.04

Informed consents were taken from all enrolled participants and their kin for the study. As mentioned earlier, the study was approved by the Institutional Ethical Committee of CIIMS.

All the statistical analysis was performed using MedCalc (version 13.1). Test for proportion was used to compare baseline characteristics associated with the improved and expired AIS patients. Similarly, a *t*-test was used to compare the mean values of NSE and S-100ββ between

expired and improved AIS patients. The statistical level of significance was set at *P* < 0.05.

Out of 40 patients, 23 were finally included in the study which comprised of 19 improved and 4 expired AIS patients. The baseline and clinical characteristics of improved and expired AIS patients showed that there was no significant difference between them except for the severity score (NIHSS). Expired AIS patients showed significantly higher (*P* < 0.05) NIHSS score (18 ± 3) compared to improved AIS patients (12 ± 5) (Table 3).

Figure 1 shows the mean levels of NSE protein estimated by the kit method and peptide-based approach in serum samples of improved and expired AIS patients and control individuals. The mean levels of NSE concentration (kit method) were significantly higher in expired AIS patients compared to improved AIS patients and control samples (Figure 1 a). Similar results were observed when NSE was estimated by in-house ELISA using anti-NSE anti-peptides 1 and 2 (Figure 1 b and c).

Figure 1 d-f shows the mean levels of S-100ββ protein estimated by the kit method and peptide-based approach. The result shows that although the level of S-100ββ increases (*P* < 0.05) in the serum samples of AIS patients, there is no significant difference in the mean S-100ββ concentration (kit method) in expired and improved AIS patients (Figure 1 d). On the contrary, S-100ββ estimation using in-house ELISA with S-100ββ anti-peptide shows significantly high (*P* < 0.05) S-100ββ levels in expired compared to improved AIS patients when tested with S-100ββ peptide 2 (Figure 1 f).

During the past few years, use of anti-peptide antibodies has been widely reported in the diagnosis of various diseases. Pattnaik *et al.*⁷ have shown that anti-peptide antibodies produced against peptides of conserved regions of flaviviruses selectively recognized flaviviruses. Dambinova *et al.*⁷ evaluated the anti-*N*-methyl-D-aspartate receptor subunit NR2 (anti-NR2) peptide antibody for the detection of NR2 protein levels for diagnosis of AIS patients. Santana *et al.*⁸ reported monospecific antibody generated against synthetic peptides of interleukin-8 (IL-8) as a versatile tool for detecting IL-8 by different immune techniques such as ELISA, dot blot, Western blotting and immunocytofluorescence. Although peptide designing and antibody production is a costly affair, if the peptide and anti-peptide are prepared in bulk, it will reduce the cost of synthesis. Similarly, the amount of antibody produced will be sufficient to perform the test on a large number of samples without the problem of batch-to-batch variation. Further, the development of in-house protocol using peptide and anti-peptide further reduces the cost of the test, since there is no involvement of dealers, distributors or manufactures. Thus, adapting the peptide-based approach is more cost-effective than the market kit. Apart from this, use of synthetic peptides and anti-peptide antibodies instead of whole antigen avoids the possibilities of non-specific cross-reactivity and increases the possibility of specific reactivity. Therefore, the development of antipeptide antibody-based diagnostic or prognostic test could be a cost-effective approach.

In the present study, we compared the levels of NSE and S-100 β in serum samples of AIS patients using anti-peptide antibodies and a commercial kit for the development of cost-effective prognostic test. We found that the NSE concentration using the kit method is significantly higher in expired as patients compared with improved AIS patients at the time of admission. Similarly, anti-NSE-peptides 1 and 2 antibodies are able to differentiate between expired and improved AIS patients. There is no difference in S-100 β concentration between improved and expired AIS patients when estimated by the kit method. However, estimation of S-100 β concentration using anti-S-100 β peptides shows significantly higher S-100 β levels in expired AIS patients when tested with anti-S-100 β peptide 2 antibodies.

Normally NSE is present only in negligible amounts in the peripheral blood. Elevated NSE levels were reported within 4 h after the onset of stroke, where it has a half-life of around 48 h (ref. 9). It has been reported to be an indicator of infarction volume and worse neurological outcome after ischaemic stroke¹⁰. Tiainen *et al.*¹¹ have reported that decrease in serum NSE levels after therapeutic hypothermia attenuates neuronal death in cardiac arrest patients. We observed higher NSE concentration among expired AIS patients. This confirms the current findings which suggest that serum NSE levels could be an indicator of severity and a bad prognosis.

Estimation of NSE using anti-peptides has also shown similar results.

Similar to NSE, serum S-100 β protein levels were known to increase 8 h after the onset of stroke^{12,13}, which then reached peak values at 24 h (ref. 14). Blood S-100 β levels were also reported to correlate with severity and functional outcome in stroke patients¹⁵. However, the difference is best described in the blood samples collected between 48 and 72 h of stroke^{15,16}. In our earlier study, we also reported higher levels of S-100 β in an expired patient at 72 h (ref. 17). We did not observe any difference in S-100 β levels between improved and expired AIS patients using the kit method, while the estimation of S-100 β by anti-S-100 β peptide-2 antibody showed a higher concentration in expired AIS patients even at the time of admission. S-100 β is an acidic calcium-binding protein found in glial and Schwann cells¹⁸. It has also been reported in other cell types¹⁹. Expression of NSE is reported only in neurons and cells of neuroendocrine origin²⁰. The higher pool of S-100 β proteins from all cell types could be possibly masking the alteration in S-100 β concentrations specifically occurring due to neuronal injury. The antibody produced against the specifically designed peptides and anti-peptides of S-100 β showed higher specificity compared to the kit method for estimation of S-100 β at the time of admission.

The results of the present study suggest that in-house ELISA developed using the selected peptides and anti-peptides of NSE and S-100 β can be used in a cost-effective manner for prediction of severity and mortality in AIS patients. The study also opens an avenue for the development of less expensive diagnostic protocols.

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Molecular analysis of genetic stability in *in vitro* regenerated plants of broccoli (*Brassica oleracea* L. var. *italica*)

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Genetic integrity of tissue culture-raised plantlets of broccoli cv. Solan green head from India was assessed using RAPD. First, highly efficient, reliable and high-frequency shoot regeneration was achieved in leaf (62.96%) and petiole (91.11%) explants on MS medium containing 4.5 mg/l BAP + 0.019 mg/l NAA, and 4.0 mg/l BAP and 0.5 mg/l NAA respectively. Maximum rooting ability (93.99%) with healthy and vigorous roots was observed on MS medium containing 0.20 mg/l NAA. The regenerated plantlets with well-developed shoot and root system were acclimatized successfully. For genetic stability studies, a total of 66 amplicons were amplified using 15 informative primers with a high degree of monomorphism (88.45%) across the mother plant and 20 randomly selected *in vitro* regenerated plantlets.

Keywords: Broccoli, genetic fidelity, leaf and petiole explants, plant regeneration, RAPD-PCR.

BROCCOLI is an important vegetable crop of the cabbage family Brassicaceae (formerly Cruciferae) with chromosome number $2n = 18$. It is nutritionally rich with medicinal property and classified as the *italica* cultivar group of the species *Brassica oleracea*. It is high in vitamins C and A, soluble fibre and contains the medicinally important anticancerous compound sulphoraphane with potential application in the pharmaceutical industry^{1,2}. Plant tissue culture is an important aspect of plant biotechnology because genetic manipulation is now necessary to harness its potential to overcome crop yield losses due to biotic and abiotic stresses. So establishment of a highly efficient, reliable and stable plant regeneration system without the risk of genetic instability is a major step in genetic improvement. Scaling-up of any micropropagation protocol is severely hindered due to incidence of somaclonal variations³. The occurrence of somaclonal variations is a potential drawback when the propagation of elite plant is intended⁴, where assessment of the tissue culture-raised variations using clonal fidelity is required to maintain the advantages of the desired elite genotypes such as superior growth, resistance to abiotic and biotic stresses and other horticultural and agronomically important

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