

SHORT REPORT

Pattern of epitopic reactivity of the anti-Hu antibody on HuD with and without paraneoplastic syndrome

Nobuyuki Sodeyama, Kazuyuki Ishida, Kurt A Jaeckle, Lixin Zhang, Arata Azuma, Masahito Yamada, Hidehiro Mizusawa, Yoshiaki Wada

Abstract

Previous study has shown that the anti-Hu antibody titre of serum samples from patients with paraneoplastic encephalomyelitis/paraneoplastic sensory neuronopathy (PEM/PSN) was significantly higher than that from patients with small cell lung cancer without neurological disturbances (non-PEM/PSN). The aims of this study were (1) to identify the fine epitopes on HuD recognised by the anti-Hu antibody, (2) to determine if the pattern of epitopic reactivity differed between antibodies from patients with and without PEM/PSN, and (3) to determine if the pattern of epitopic reactivity correlated with the clinical features. Recombinant full length HuD and nine deletion fragments were constructed and immunoreacted by western blot analysis with 14 anti-Hu serum samples from eight patients with PEM/PSN and six without PEM/PSN. All anti-Hu serum samples reacted with the deletion fragments containing amino acids (aa) 90-101 or aa 171-206. Some anti-Hu samples reacted with the deletion fragments containing aa 223-234, aa 235-252, or aa 354-373. There was no difference in the pattern of epitopic reactivity between patients with and without PEM/PSN. There was no correlation between the pattern of epitopic reactivity and the clinical features. The anti-Hu antibody titre from patients with PEM/PSN was significantly higher than from patients without PEM/PSN, but there was overlap of their titre concentrations. In conclusion, aa 90-101 and aa 171-206 are the major epitopes with which all anti-Hu serum samples react, and aa 223-234, aa 235-252, and aa 354-373 are the minor epitopes with which only some anti-Hu serum samples react. The analyses suggested that the pattern of epitopic reactivity of the anti-Hu antibody on HuD was not a critical factor for the development or clinical features of PEM/PSN.

(J Neurol Neurosurg Psychiatry 1999;66:97-99)

Keywords: paraneoplastic syndrome; anti-Hu antibody; HuD; epitope; small cell lung cancer

Paraneoplastic encephalomyelitis/paraneoplastic sensory neuronopathy (PEM/PSN) is a rare neurological disorder which is commonly associated with small cell lung cancer.¹ Although there is a definite correlation between PEM/PSN and the presence of a characteristic antibody called "the anti-Hu antibody", the role of this antibody in the development of the disease is still unknown. Dalmau *et al* showed that the anti-Hu antibody was also detected in the serum and CSF from 16% of patients with small cell lung cancer without neurological disturbances (non-PEM/PSN).² They reported that the anti-Hu antibody titre from patients with PEM/PSN was significantly higher than patients without PEM/PSN,² suggesting that the presence of the high titre anti-Hu antibody was an important factor in the development of the disease. However, the injection of the anti-Hu antibody into experimental animals has failed to reproduce PEM/PSN.³ Mice immunised with recombinant HuD produced the high-titre anti-Hu antibody, but did not develop clinical symptoms of PEM/PSN.⁴ These findings indicated that the presence of the high-titre anti-Hu antibody was not the critical factor required for the development of PEM/PSN. Therefore, further examination for other possible differences in the anti-Hu antibodies between patients with and those without PEM/PSN might provide important keys to the understanding of the pathogenesis of PEM/PSN.

Three aims of this study were (1) to identify the fine epitopes on HuD recognised by the anti-Hu antibody, (2) to determine if the pattern of epitopic reactivity differed between antibodies from patients with and without PEM/PSN, and (3) to determine if the pattern of epitopic reactivity correlated with the clinical features of neurological disturbances. We have constructed recombinant full length HuD and nine deletion fragments. The immunoreactivities of these expression products with anti-Hu serum samples from patients with PEM/PSN and without PEM/PSN were investigated by western blot analysis.

Department of Neurology, Tokyo Medical and Dental University, Tokyo, Japan
N Sodeyama
K Ishida
M Yamada
H Mizusawa
Y Wada

Department of Neuro-Oncology, Texas Medical Center, Houston, TX, USA
K A Jaeckle
L Zhang

Fourth Department of Internal Medicine, Nippon Medical School, Tokyo, Japan
A Azuma

Correspondence to:
Dr N Sodeyama,
Department of Neurology,
Tokyo Medical and Dental
University, Yushima 1-5-45,
Bunkyo-ku, Tokyo 113,
Japan. Telephone 0081 3
5803 5234; fax 0081 3 5803
5234; email
n-sodeyama.nuro@
med.tmd.ac.jp

Received 20 January 1998
and in final form
17 June 1998
Accepted 29 June 1998

The neurological conditions and anti-Hu antibody titre of PEM/PSN and non-PEM/PSN patients

Patient number	Type of cancer	Neurological symptoms	Anti-Hu antibody titre
PEM/PSN:			
1	SCLC	BE, En, My, SMN	102400
2	None known	BE, En, My, SMN, hearing loss	102400
3	None known	AN, BE, My, SMN	102400
4	Prostate	BE, LES, SCD	102400
5	Undifferentiated mediastinum	SMN, hearing loss	25600
6	SCLC	SN	25600
7	SCLC	BE, En, SCD	102400
8	SCLC	AN, BE, SMN	819200
Non-PEM/PSN:			
9	SCLC	No neurological disturbance	6400
10	SCLC	No neurological disturbance	6400
11	SCLC	No neurological disturbance	6400
12	SCLC	No neurological disturbance	51200
13	SCLC	No neurological disturbance	12800
14	SCLC	No neurological disturbance	102400

PEM/PSN=paraneoplastic encephalomyelitis/paraneoplastic sensory neuropathy; non-PEM/PSN=small cell lung cancer without neurological disturbances; SCLC=small cell lung cancer; BE=brainstem encephalitis; En=encephalitis; My=myelitis; SMN=sensorimotor neuropathy; AN=autonomic neuropathy; LES=Lambert-Eaton syndrome; SCD=subacute cerebellar degeneration; SN=sensory neuropathy. The titre was defined as the greatest dilution of each serum that stained the band of full length GST-HuD fusion protein on western blot. The medians (25 percentile, 75 percentile) of the anti-Hu antibody titre are 102400 (64000, 102400) in PEM/PSN patients and 9600 (6400, 51200) in non-PEM/PSN patients respectively.

Materials and methods

SERUM SAMPLES

Fourteen anti-Hu serum samples were obtained from eight patients with PEM/PSN (patient 1-8) and six non-PEM/PSN patients (patients 9-14). Normal control serum samples were obtained from 10 normal healthy persons. Neurological conditions of the patients are as shown in the table.

Isolation of full length HuD cDNA

Total RNA was extracted from normal human frontal lobe and was reverse transcribed.⁵ This reaction product was used as a template for polymerase chain reaction (PCR) amplification to produce full length HuD cDNA. The PCR was performed with the following temperature profile: 30 seconds at 94°C, 30 seconds at 57°C, 3 minutes at 72°C (35 cycles), and then 15 minutes at 72°C.

Construction of vectors for expression of full length GST-HuD fusion protein and its deletion fragments

The deletion constructs of HuD cDNA were produced by using restriction enzymes to cleave HuD cDNA, or by PCR with specific primers. HuD cDNA and its deletion constructs were separated by agarose gel, cut out from the gel, and then subcloned in frame into the plasmid expression vector which contained glutathione S-transferase (GST) (GST-HuD constructs). When the insert was digested with restriction enzymes, it was subcloned into the plasmid which had been predigested with the

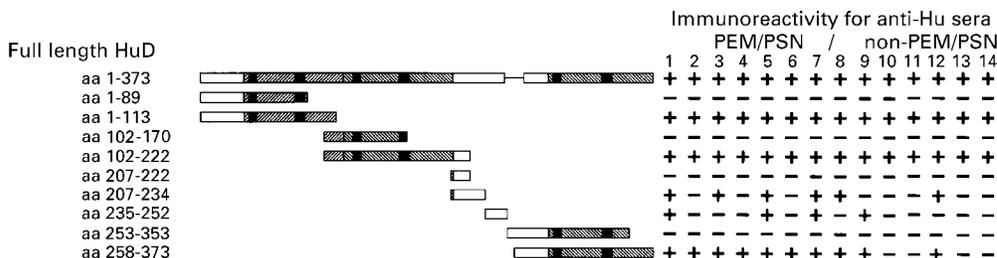
same restriction enzyme. When the insert was a PCR product, it was subcloned into the plasmid which was predigested by Sma I and then added with thimine at the 3' end.

Expression of full length GST-HuD fusion protein and its deletion fragments in *Escherichia coli*

The competent *Escherichia coli* cells were transformed with GST-HuD constructs. Transformed *E coli* cells were grown to an optical density of 1.0 at 600 nm and isopropyl-b-D-thiogalactopyranoside (Takara) was added. Cells were pelleted by centrifugation and were resuspended in phosphate buffered saline solution and followed by sonication. Glutathione sepharose 4B (Pharmacia) was added to the supernatant and the mixture was centrifuged. After glutathione, elution buffer (Pharmacia) was added and the mixture was centrifuged. The supernatant was used in western blot analysis. Recombinant proteins were obtained as fusion proteins with GST. These fusion proteins expressed by the above techniques are summarised in the figure.

Immunoblot of full length GST-HuD fusion protein and its deletion fragments

Fusion proteins were separated by 11% sodium dodecyl sulphate polyacrylamide gel electrophoresis⁶ and was electroblotted on to nitrocellulose membranes.⁷ After blocking, the membranes were immunolabelled with patients' serum samples, and followed by reaction with horse radish peroxidase labelled affinity isolated goat anti-human IgG (Sigma). Detec-



Schematic representation of full length HuD and its deletion fragments. The shaded boxes represent the RRM, and the closed boxes the RNP. The amino acid numbers contained in each deletion fragment are listed on the left.

Immunoreactivity of each deletion fragment for each serum sample is shown on the right. "+" or "-" means the presence or the absence of immunoreactivity for each deletion fragment respectively.

tion of the immunoreactive proteins was carried out with an enhanced chemiluminescence system (Amersham). We repeated the same examinations twice and confirmed the results.

Determination of the anti-Hu antibody titre

The anti-Hu antibody titre was determined by a semiquantitative western blot analysis. The titre was defined as the greatest dilution of each serum that stained the band of full length GST-HuD fusion protein on western blot.

Results

Western blot analysis showed that all 14 anti-Hu serum samples and anti-GST antibody reacted with full length GST-HuD fusion protein at 63 kDa. Ten serum samples from normal healthy persons did not react with full length GST-HuD fusion protein. The immunoreactivity of 14 anti-Hu serum samples with each deletion fragments was shown in the figure. The anti-GST antibody reacted with all deletion fragments at the presumed molecular weights. The results indicate that amino acids (aa) 90–101 and aa 171–206 are the major epitopes with which all anti-Hu sera react, and aa 223–234, aa 235–252, and aa 354–373 are the minor epitopes with which only some anti-Hu serum samples react. There was no difference in the pattern of epitopic reactivity between patients with PEM/PSN and patients without PEM/PSN. There was no correlation between the pattern of epitopes and the clinical features of neurological disturbances (table). The titre of each anti-Hu serum is also shown in the table. The anti-Hu antibody titre from patients with PEM/PSN was significantly higher than that of patients without PEM/PSN (Mann-Whitney *U* test, $p=0.02$). There was overlap of the titre of the anti-Hu antibody between the two groups of patients.

Discussion

In our study, there were two major epitopes with which all anti-Hu serum samples reacted and three minor epitopes with which only some anti-Hu serum samples reacted on HuD. A major epitope, aa 90–101, was in the first RNA recognition motif (RRM) outside the ribonucleoprotein consensus sequence (RNP), and another major epitope, aa 171–206 was in the second RRM, possibly including a portion of the RNP 1. One minor epitope, aa 354–373, was in the third RRM outside the RNP. These three epitopes were located between the carboxy terminal of the RNP 1 and the carboxy terminal of each RRM. Differing from these three epitopes, two minor epitopes, aa 223–234 and aa 235–252, were within the interval region between the RRM 2 and RRM 3.

Manley *et al* have already reported the epitope mapping of the anti-Hu antibody on HuD and showed that only deletion fragments of HuD which contained the first or second RRM were recognised by all the anti-Hu

antibodies.³ The presence of the major epitopes is also confirmed by our study. The novel finding in the current study is the discovery of three minor epitopes recognised by the anti-Hu antibody. This finding was considered to be relevant because all bands were detected at the presumed molecular weights and such reactivity was not seen with normal control serum samples under the conditions of western blot analysis we used.

There was no correlation between the pattern of epitopic reactivity and the development of PEM/PSN in patients positive for anti-Hu antibody, or the clinical neurological features. We could not identify a specific epitopic reactivity pattern which delineated patients likely to develop PEM/PSN. Our results suggest that the pattern of epitopic reactivity of the anti-Hu antibody on HuD was not a critical factor for the development or clinical features of PEM/PSN.

Dalmau *et al* showed that the anti-Hu antibody titre from patients with PEM/PSN was significantly higher than that from patients without.² Their results were also confirmed by our study. Overlap of the titre between those patients with and those without PEM/PSN was also shown by Graus *et al*.⁸ These findings might indicate that titre of the anti-Hu antibody is not a single critical factor for the development of PEM/PSN.

In conclusion, aa 90–101 and aa 171–206 of HuD are the major epitopes with which all anti-Hu serum samples react, and aa 223–234, aa 235–252, and aa 354–373 are the minor epitopes with which only some anti-Hu serum samples react. Our analyses suggested that the pattern of epitopic reactivity of the anti-Hu antibody on HuD was not associated with the development or clinical features of PEM/PSN.

We thank Drs Tomohiko Mizutani, Jun Takagiwa, and Hirotarō Miura for providing anti-Hu serum samples. This work was supported in part by Health Science Research Grants (Mechanism of Abnormal Deposition in Dementia Brain) of the Ministry of Health and Welfare of Japan to MY.

- 1 Graus F, Elkou KB, Cordon-Cardo C, *et al*. Sensory neuropathy and small cell lung cancer. Antineuronal antibody that also reacts with the tumor. *Am J Med* 1986;80:45–52.
- 2 Dalmau J, Furneaux HM, Gralla RJ, *et al*. Detection of the anti-Hu antibody in the serum of patients with small cell lung cancer—a quantitative Western blot analysis. *Ann Neurol* 1990;27:544–52.
- 3 Manley GT, Smitt PS, Dalmau J, *et al*. Hu antigens: reactivity with Hu antibodies, tumor expression, and major immunogenic sites. *Ann Neurol* 1995;38:102–10.
- 4 Sillevs Smitt PAE, Manley GT, Posner JB. Immunization with the paraneoplastic encephalomyelitis antigen HuD does not cause neurologic disease in mice. *Neurology* 1995; 45:1873–8.
- 5 Chomczynski P, Sacchi N. Single-step method of RNA isolation by acid guanidinium thiocyanate-phenol-chloroform extraction. *Anal Biochem* 1987;162:156.
- 6 Laemmli UK. Cleavage of structural proteins during the assembly of the head of bacteriophage T4. *Nature* 1970;227:68–5.
- 7 Towbin H, Staehelin T, Gordon J. Electrophoretic transfer of proteins from polyacrylamide gels to nitrocellulose sheets: procedure and some applications. *Proc Natl Acad Sci USA* 1979;76:4350–4.
- 8 Graus F, Dalmau J, Reñé R, *et al*. Anti-Hu antibodies in patients with small-cell lung cancer: association with complete response to therapy and improved survival. *J Clin Oncol* 1997;15:2866–72.