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Application of Serum ELAVL4 (HuD) Antigen Assay for Small Cell Lung Cancer Diagnosis

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Abstract. *Background/Aim: The ELAVL4 (HuD) is a neuron-specific RNA-binding protein expressed in 100% of small cell lung cancer (SCLC) cells and over 50% of neuroblastoma cells. The aim of this study was to investigate the serum HuD concentration in SCLC patients and the possibility of its utilization as a biomarker of small cell lung cancer. Materials and Methods: Our study included 47 SCLC cases and 29 normal controls. Indirect competitive inhibition ELISA method was established to detect HuD antigen of serum samples. To design the ELISA system, purified antigen and real positive and negative serum samples were used, and checkerboard titration was performed. The value of current serum biomarkers (Pro-GRP, NSE, CYFRA21-1 and CEA) was obtained from a clinical laboratory. Results: The HuD antigen concentration in the SCLCgroup was significantly higher than that in the normal group ($p<0.01$). The cut-off value, specificity and sensitivity were 60 ng/ml, 89.7% and 74.5%, respectively. The best linear range was 9.75~600 ng/ml. The sensitivity of the HuD-ELISA assay was much better than the current biomarkers-CEA, NSE and pro-GRP. Also,it was equal to or better than the combined use of two or three indicators. Conclusion: The high titres of HuD in SCLC patients and preferable consistency suggested that HuD may serve as a potential diagnostic criterium for SCLC and may serve as a marker of disease progression.*

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Lung cancer is the leading cause of cancer mortality with high incidence in both men and women worldwide (1). Small cell lung cancer (SCLC) is a high-degree malignant neuroendocrine tumor, accounted for 10% to 15% in all diagnosis of lung cancer cases (2). It is very difficult to diagnose SCLC in early stages, because it has a tendency to metastasize in lymph nodes and distant organs (3, 4). The overall 5-year survival rate is less than 5% (5). The current clinical diagnosis of SCLC, includes CT, X-ray, tumor biomarkers and pathological section. The relevant tumor biomarkers are progastrin releasing peptide (ProGRP), neuron-specific enolase (NSE), cytokeratin-19 fragments (CYFRA21-1), and carcinoembryonic antigen (CEA). It was reported that their specificities were 84.1%, 82.5%, 83.6% and the sensitivities were 47.1%, 35.3%, 35.3%, respectively (6). However, the positive rate of early-stage disease is lower than that of advanced stages (stage I and II 35-45%, stage III of 55%-70%, stage IV 70%-80%). Hence, it is urgent to develop biomarkers for early diagnosis of SCLC.

ELAVL (Embryonic Lethal, Abnormal Vision, *Drosophila*-Like)/Hu protein is a neuron-specific RNA-binding protein, that contains four family members: HuR/A, HuB, HuC and HuD (7). Hu antigen D (HuD) protein is highly conserved and homologous to the *Drosophila* ELAV protein. It is a newly-discovered neuron marker and specific antigen that regulates neuron-specific gene expression at the transcriptional level, participating in neuronal development, survival, plasticity as well as the disease process (8). HuD protein was first recognized in the paraneoplastic syndrome associated with small cell lung cancer. In recent years there has been increasing evidence that the erroneous regulation and mutations of *HuD* are involved in the development of neuroendocrine and neurological disorders (9). Certain studies have found that is overexpressed in the SCLC (10). Some scholars have determined the presence of HuD mRNA expression in SCLC cells using RT-PCR methods and obtained positive data (11). However, the false-positive

identification of HuD in peripheral blood by the comparison test has hindered its utilization as an SCLC marker.

In the present study, we purified high-quality human HuD protein as a coating antigen and established a competitive inhibition ELISA method to detect the concentration of HuD in 76 clinical serum samples. The results showed that the HuD antigen concentration in the SCLC group was significantly higher than that in the normal group. Its specificity and sensitivity were superior to the current clinical biomarkers, ProGRP, NSE and CEA. Taken together, the results of this study provided a new prospect for the diagnosis of SCLC.

Materials and Methods

Materials. Enzymes for recombinant DNA technology such as Taq polymerase, T4 DNA ligase, *Bam*HI and *Xho*I were purchased from New England Biolabs (NEB, Hitchin, Herts, UK). Polymerase chain reaction (PCR) amplification kit (including PCR buffer and dNTP mix) was also obtained from NEB. Plasmid mini kit I and PCR product recovery kit were purchased from Omega Bio-Tek (Norcross, GA, USA).

Patients. We collected serum samples from 47 SCLC patients who had undergone surgery at the Beijing Chest Hospital, Capital Medical University. Normal serum samples were from 29 healthy volunteers. The ages (mean±SD) of the SCLC Patients were 61.5±8.4 years. None of the patients had received chemotherapy or radiotherapy before surgery. Diagnosis was confirmed by conventional pathological morphology. Value of CEA, NSE and pro-GRP were tested by ELISA. Supernatants from serum samples were collected immediately after centrifugation (3,000 ×g for 15 min) and stored at −80˚C until required.

All patients provided written informed consent. All studies and procedures involving human serum samples were approved by the Beijing Chest Hospital Institutional Review Board. Patient samples were used in accordance with The Declaration of Helsinki.

Primer design and PCR amplification. The primers were designed according to the nucleotide sequence of ELAV4. In order to facilitate subsequent cloning, *Bam*HI and *Xho*I restriction endonuclease sites were attached to the 5'-termini of the upstream and downstream primers, respectively: forward: 5'-CGGGATCC GAGCCTCAGGTGTCAAATG-3'; reverse: 5'-CCGCTCGAG GTCAGGACTTGTGGGCTTTGTTG-3'. The polymerase chain reaction was carried out using the human genomic DNA of HuD as a template. The PCR mixture was subjected to 35 cycles of denaturation (30 s, 95˚C), annealing (30 s, 57˚C), and extension (60 s, 72˚C) using a DNA Thermal Cycler (Eppendorf). The PCR product was separated on a 1% agarose gel, purified using a PCR product recovery kit, and digested with *Bam*HI and *Xho*I overnight.

Plasmid constructs, protein induced expression and purification. The PCR product was cloned into the *Bam*HI and *Xho*I restriction-enzyme sites of the prokaryotic expression vector pET-28a (+). The recombinant plasmid was transformed into strain DH5α of *Escherichia coli*. Protein expression was induced by isopropyl β-D-1-thiogalactopyranoside (IPTG) (0.1 mM final concentration) when the OD_{600} of the lysogeny broth (LB) culture medium reached approximately 0.8. The cultures were allowed to grow at 37˚C overnight. Bacterial cells were harvested by centrifugation at 5000 rpm for 15 min. Then, the cell pellets were resuspended in ice cold lysis buffer (MCAC-0, pH 7.9, 1mM phenylmethane sulfonyl fluoride (PMSF)) and homogenized by sonication on ice. The cell debris were removed completely by centrifugation of the lysate at 20,000 ×g for 45 min at 4˚C. All subsequent purification steps were performed at 4˚C. The supernatant was added to a self-packaged Nickel-affinity column pre-equilibrated with lysis buffer. The contaminant protein was removed with MCAC-0 buffer and the target HuD protein was eluted with gradient concentration of 0-200 mM imidazole. The concentration of purified HuD protein was detected by BCA (Bicinchoninic Acid) method.

MALDI-TOF-TOF mass-spectrometric analysis. The purified protein was analyzed by SDS-PAGE and by MALDI-TOF-TOF mass spectrometry. 0.5 ml of digested sample was spotted onto an AnchorChip target (Bruker Daltonics, MA, USA). The sample droplet was dried at ambient temperature and coated with 0.5ml matrix solution. The mass spectrometer was calibrated externally using the monoisotopic (M+H) + ions of peptide-calibration standards (Bruker Daltonics, MA, USA). Mass data acquisitions were piloted using flexControl v.3.3 (Bruker Daltonics, MA, USA). The positive MALDI-TOF and MS/MS LIFT spectra of selected ions were collected and peak lists were generated using the SNAP peak-detection algorithm, with a signal-to-noise threshold higher than 6 and with top-hat baseline subtraction in flexAnalysis v.3.3 (Bruker Daltonics, MA, USA). The MS spectra and MS/MS spectra were combined in Biotools (Bruker Daltonics, MA, USA) and searched for Humanistic in the NCBI nonredundant database using the Mascot search engine (Matrix Science; London, England).

Competitive-inhibition enzyme linked immune-absorbent assay (ELISA). To optimize the reaction conditions of indirect competitive ELISA, such as determination of the optimal coating concentration of HuD and the best working concentration of monoclonal antibody. The HuD protein was fold diluted in phosphate-buffered saline (0.05 mol/l PBS, pH 9.6) to yield working solutions (20 ng/ml), anti-HuD was proportionally diluted, and HRP labeled goat anti-rabbit IgG was diluted 1:2,500. Square titration was performed for ELISA detection, according to OD_{450} value equal to 1.0 was chosen as the ideal working concentration. The 96-well plates were coated with 20 ng/ml of HuD at 4˚C overnight. After washing the plate three times with 300 μl of PBST (0.05% Tween 20), the plate was blocked with PBS/3% non-fat milk at 37˚C for 1 h. Then different concentrations of HuD standard solution (marking standard curve) or the serum samples were mixed with the monoclonal antibody in each well. After incubation at 37˚C for 1 h, the wells were washed with PBST (phosphate buffered saline with Tween) 3 times and then horseradish peroxidase-conjugated goat anti-rabbit secondary antibody (1:2,500) was added and reacted for 1h at room temperature. After washing 3 times with PBST, 100 μl of 3,3',5,5'-tetramethylbenzidine (Tiangen, Beijing, China) was added and incubated at 37˚C for 15 min. The reaction was stopped with Stop Solution (2M H_2SO_4). All samples were examined in triplicate. The OD_{450} values were measured and the inhibition curves were plotted.

Standard curve and ROC curve analysis. Purified HuD protein as standard sample was serially diluted 2-fold in PBS (from 100 ng/ml) to yield an eight-point standard curve. The highest concentration of HuD was 100 ng/ml, followed by 50, 12.5, 6.25,

3.125, 1.56, 0.78 and 0.39 ng/ml respectively. The standard samples were mixed with equal anti-HuD mAb. The logarithmic values of the dilution were calculated and plotted as on the horizontal axis. The values of B/B_0 were estimated and plotted as the vertical axis. The optical density at 450 nm of each fold dilution were regarded as B, and the optical density of the standards as B0. The absorbance was read at 450 nm for all test samples. All samples were tested in triplicate. The HuD concentration for test samples (all patients and normal controls) were extrapolated from a standard curve through Curve Expert 1.4. The concentration of HuD in serum was plotted for ROC curve.

Statistical analysis. Statistical analyses were carried out with SPSS 16.0. The cut-off values and the area under the curve were defined by ROC curve. Sensitivity and specificity values were obtained in SCLC patients. To compare the concentration of HuD between all patients and normal control groups independent sample T test was used. A *p*-value less than 0.05 was considered statistically significant. In addition, Pearson Chi-Square was used to compare the relationship between HuD level and existing biomarkers, including CEA, NSE and pro-GRP. A *p*-value less than 0.01 was considered statistically significant.

Results

Forty-seven SCLC patients were included (Male:Female ratio of 31:16). The ages (mean±SD) of the SCLC patients were 61.5±8.4 years. Thirty-seven patients were smokers and 70% of the cohort were males. None of the patients had received chemotherapy or radiotherapy before surgery. Table I shows the characteristics of the studied cohort.

Cloning, expression and purification of HuD. A 1136-bp DNA fragment encoding HuD was obtained by PCR amplification and cloned into the prokaryotic expression vector pET-28a (+). The recombinant plasmid was transformed into *E. coli* strain BL21 (DE3). Identification by PCR, plasmid digestion (Figure 1A) and DNA sequencing confirmed that the recombinant plasmid contained the encoding sequence of human HuD that was identical to its sequence deposited in GenBank.

The recombinant HuD was expressed in a soluble form in *E. coli*. The proteins were eluted with MCAC buffer containing 0.2 mM imidazole. After a series of purification steps, the protein was judged to be >90% pure by SDS-PAGE stained with Coomassie Brilliant Blue (Figure 1B) and the result was confirmed by western blotting. The molecular weight of the purified protein was 42 kDa, as determined by MALDI-TOF-TOF MS and the peptide mass fingerprinting (PMF) analysis of trypsin-digested target protein confirmed that the purified protein was huan HuD (Figure 1C). The concentration of the purified protein was adjusted to 0.5 mg/ml.

Detection of serum HuD by indirect competitive ELISA. The optimal coating concentration of HuD protein was 20 ng/ml, and monoclonal antibody dilution degrees of 1:2,000. The best secondary antibody was diluted to 1:2,500. Data are shown in Table II. The standard curve was drawn with software Curve Expert 1.4. Under the concentration range of 9.75~600 ng/ml, the curve showed a good linear relationship. The correlation index was high (R^2=–0.969), indicating a significant correlation (Figure 2A). Indirect competitive ELISA method was used to detect HuD antigen in clinical serum samples. The concentration of HuD was calculated by the absorbance value of OD_{450}. The concentration of HuD in serum was analyzed for ROC curve. The cut-off value and area under the curve were 60 ng/ml and 0.897, respectively (Figure2B). The detection results of SCLC samples by ELISA showed that there were 35 specimens whose HuD concentrations were above 60 ng/ml (Figure 2C). Based on the data above, we concluded that 60 ng/ml was recognized as the positive cut-off value of HuD, and thus the resultant positive predictive value of the method was 85.4%. Moreover, compared with SCLC patients and normal controls, HuD antigen concentration of the SCLC group was significantly higher than that of the normal group (p<0.01) (Figure 2D). The specificity and sensitivity of HuD was 89.7% and 74.5% (Table III).

Relationship between serum HuD level and existing biomarkers. The current diagnostic serum markers of SCLC include CEA, NSE and pro-GRP. We evaluated the sensitivity of CEA, NSE and pro-GRP, which was 29.8%, 59.6% and 63.8%, respectively (Table IV). In order to examine the relationship between HuD and the other biomarkers, we analyzed the concordance of HuD and the other three indexes. As shown in Table V, the concordance of pro-GRP and NSE were much better than CEA (65.7%, 62.8% and 22.9%). Moreover, combined use of two indexes improved the sensitivity remarkably, and reached to 83% the three indexes were combined (Table VI). The sensitivity of HuD-ELISA assay was 76.6%, which was equal to or better than the combined use of two or three indices.

Discussion

HuD, a neuron-enriched RNA-binding protein (RBP) expressed early in embryonic neurogenesis, is one of the first markers of neuronal phenotype. It is widely expressed in the mature nervous system and involved in its development and maintenance (12). HuD can regulate the development of neurons in multiple stages, including the occurrence of neurons, differentiation, neurite outgrowth, neuronal dendritic maturation and neuronal circuitry development (13-15). In addition, HuD can play an important role in nerve injury and a variety of neuropathies, such as neuroendocrine tumorigenesis (16-18). HuD has been shown to be expressed in 100% of tissue samples of neuroblastoma and small-cell lung cancer (19). Few studies have focused on the genetic

Table I. *Clinical information of 41 cases of small cell lung cancer.*

Sample number	Gender	Age	Smoking history	Drinking history	Chronic Illness	CYFR A21-1	CEA	NSE	GRP	SCC	Pathologic diagnosis
c182	Female	57	No	No	Yes	N	↑	N	↑	N	SCLC
c584	Female	57	No	No	Yes	N	N	N	N	N	SCLC
c1114	Female	57	No	No	Yes	↑	N	N	↑	N	SCLC
c297	Male	63	Yes	yes	Yes	N	N	N	N	N	SCLC
c366	Male	61	Yes	No	No	N	↑	N	↑	N	SCLC
c384	Male	58	No	No	Yes	↑	N	N	N	N	SCLC
c1282	Male	57	Yes	Yes	No	N	↑	N	↑	N	SCLC
c442	Female	67	No	No	Yes	↑	↑	↑	↑	N	SCLC
c488	Male	58	Yes	No	Yes	N	N	↑	↑	N	SCLC
c1526	Male	58	No	No	Yes	N	N	N	↑	N	SCLC
c591	Male	51	Yes	No	Yes	N	↑	↑	↑	N	SCLC
c596	Female	74	No	Yes	Yes	N	↑	N	↑	N	SCLC
c608	Male	68	Yes	Yes	Yes	N	N	↑	N	N	SCLC
c714	Male	62	Yes	No	No	N	N	N	N	N	SCLC
c800	Female	73	Yes	Yes	Yes	N	N	↑	↑	N	SCLC
c965	Male	52	Yes	Yes	No	N	N	↑	N	N	SCLC
c1177	Female	60	Yes	No	Yes	N	N	↑	↑	N	SCLC
c1329	Male	62	Yes	Yes	No	N	N	↑	↑	N	SCLC
c1364	Male	42	Yes	Yes	No	N	↑	N	↑	N	SCLC
c29	Male	75	Yes	Yes	No	N	N	↑	↑	N	SCLC
c43	Male	65	No	No	No	N	N	N	↑	N	SCLC
c137	Male	54	Yes	No	Yes	↑	↑	↑	↑	N	SCLC
c153	Male	74	Yes	Yes	Yes	N	↑	N	↑	N	SCLC
c156	Female	78	No	No	Yes	↑	N	↑	↑	N	SCLC
c174	Female	57	No	No	Yes	N	N	↑	↑	N	SCLC
c181	Male	61	Yes	Yes	Yes	↑	↑	↑	↑	N	SCLC
c184	Male	55	Yes	Yes	No	↑	N	N	↑	N	SCLC
c187	Male	51	No	No	No	N	N	↑	N	N	SCLC
c203	Male	73	Yes	No	Yes	N	N	↑	N	N	SCLC
c227	Male	62	Yes	Yes	No	N	↑	N	N	N	SCLC
c231	Male	62	Yes	Yes	Yes	N	N	N	N	N	SCLC
c236	Male	60	Yes	No	No	N	↑	↑	N	N	SCLC
c244	Female	58	No	No	No	N	N	N	N	N	SCLC
c276	Female	56	Yes	No	Yes	↑	N	↑	N	↑	SCLC
c279	Male	79	Yes	Yes	Yes	↑	N	N	N	N	SCLC
c299	Male	68	Yes	Yes	Yes	N	N	↑	↑	N	SCLC
c302	Male	74	Yes	Yes	Yes	N	N	↑	N	N	SCLC
c327	Female	62	No	No	No	N	N	↑	↑	N	SCLC
c328	Female	54	No	No	No	N	N	↑	↑	N	SCLC
c333	Male	63	Yes	No	No	N	N	↑	↑	N	SCLC
c340	Female	65	Yes	No	Yes	N	N	↑	↑	N	SCLC
c357	Male	54	No	No	Yes	N	N	N	N	N	SCLC
c376	Female	61	Yes	No	Yes	N	N	↑	↑	N	SCLC
c390	Male	64	No	No	No	N	N	↑	N	N	SCLC
c468	Male	57	Yes	No	No	↑	N	↑	↑	N	SCLC
c477	Male	45	Yes	Yes	No	↑	↑	↑	↑	N	SCLC
c480	Male	60	No	No	No	↑	↑	↑	↑	N	SCLC

SCLC: Small cell lung cancer; CEA: carcino-embryonic antigen; NSE: neuron-specific enolase; pro-GRP: pro-gastrin-releasing Peptide; SCC: SccAg; N: Normal value; ↑: Higher than normal.

causes of the ectopic nELAV gene expression and their roles in the onset and progression of neuroendocrine lung tumor types, especially in SCLCs (20). Neuroendocrine tumor tissue samples and SCLC cell lines were subjected to *HuD* gene sequencing and LOH analysis. It was found that there were 13 heterozygous mutations in the *HuD* gene coding sequence in different lung tumor specimens, but with unknown function (9, 21).

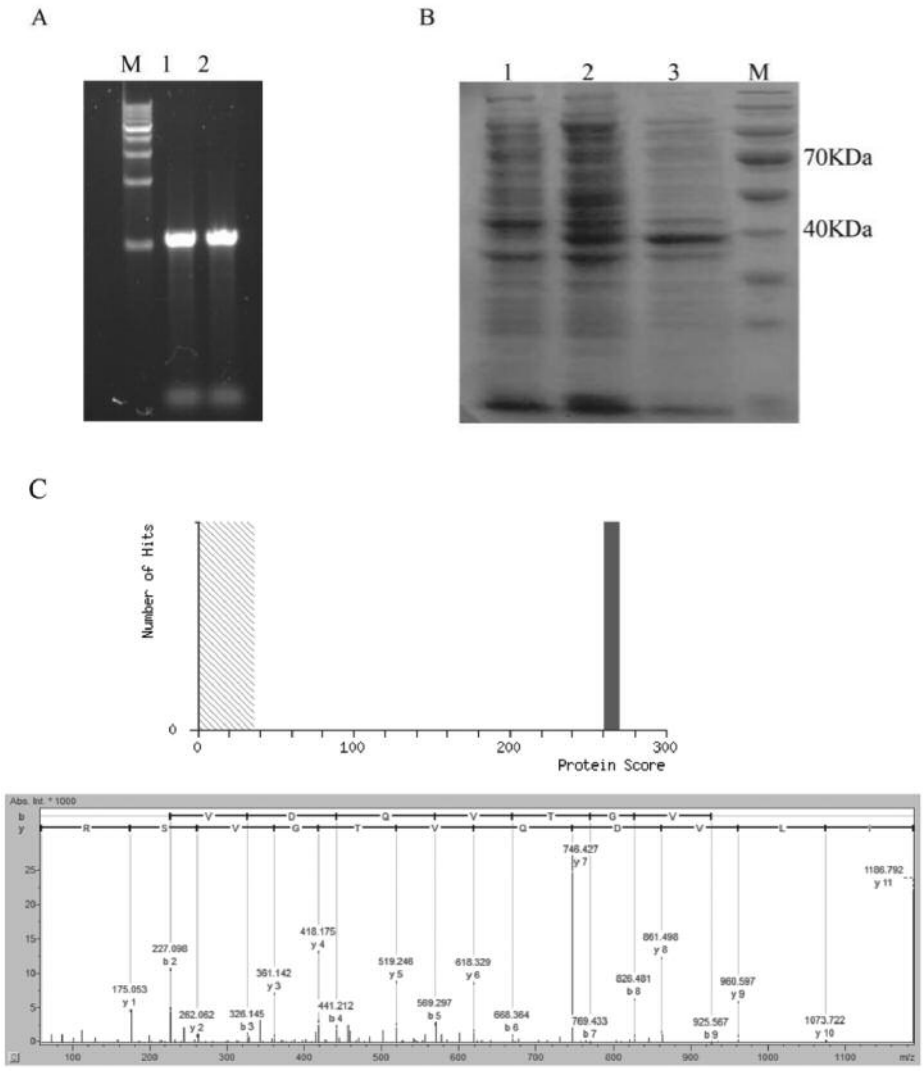

Figure 1. *Cloning, expression, and purification of HuD. (A) The bacteria containing the recombinant plasmid were identified by PCR. PCR products of HuD gene. M: DNA Marker 1 kb, 1 and 2: PCR product. (B) SDS-PAGE of the expression of HuD protein. Lane 1: recombinant E.coli BL21 (DE3) without induction; Lane2: recombinant E. coli BL21 (DE3) with induction; Lane 3: Supernatant of lysed E. coli BL21 (DE3) with induction; M: protein molecular weight marker (labelled in kDa). (C) MALDI-TOF-TOF MS spectrum of HuD peptide mass fingerprinting.*

SCLC is one of the most common malignant tumors, accounting for 15% of the newly diagnosed lung cancers. It is distinguished from non-small cell lung cancer by its rapid doubling time, high growth fraction and the early development as extensive metastases (22). One of the sequelae of SCLC is a paraneoplastic neurological autoimmune syndrome, including different types of paraneoplastic encephalomyelitis, which are caused by high titer of anti-Hu antibody (23-26). SCLC is initially sensitive to chemotherapy, but becomes resistant and easy to relapse after treatment (27). A credible biomarker will improve the prognosis of SCLC. Currently, the reliability of the diagnosis of SCLC has been improved by the use of biochemical markers such as CEA, NSE, CgA and Fas protein. Their combined applications have a higher sensitivity and specificity for the early diagnosis of SCLC, while there is no outstanding superiority for them alone (6).

In the present study, we established an indirect competitive ELISA method to detect the serum HuD antigen. This assay can distinguish SCLC patients from normal ones. The concentration of HuD antigen of the SCLC group was significantly higher than that of the normal group. The sensitivity and specificity of HuD antigen were 89.7% and 74.5%, respectively. The false

Table II. *Optimization of concentration for coating antigen and HuD mAb.*

HuD mAb Dilution fold (10^3)	Coating concentration (ng/ml)							
	Normol				SCLC			
	20	40	80	160	20	40	80	160
2	0.946	0.844	0.806	0.797	1.433	0.853	1.027	0.908
4	0.907	0.88	0.842	0.882	1.339	1.141	0.88	0.862

The optimal coating concentration of HuD protein was 20 ng/ml, and monoclonal antibody dilution radio 1:2000. The best secondary antibody was diluted to 1:2500.

Table III. *Analysis of HuD specificity and sensitivity.*

	SCLC	Normol
Positive	TP (n=35)	FP (n=3)
Negative	FN (n=12)	TN (n=26)
Total	47	29

Analyzing the specificity and sensitivity of HuD in SCLC group and Normol group. In SCLC group, true positive and false negative cases were 35 and 12. In normol group, false positive and true negative cases were 3 and 26. The specificity and sensitivity of HuD arrived at 89.7% and 74.5%.

Table IV. *The sensitivity of CEA, NSE and pro-GRP.*

Small lung cancer (n=47)		
Measurement methods	Positive specimens	Negative specimens
CEA	14	33
NSE	28	19
pro-GRP	30	17

The sensibility of CEA, NSE and pro-GRP arrived at 29.8%, 59.6% and 63.8%, respectively. CEA: Carcino-embryonic antigen; NSE: neuron-specific enolase; pro-GRP: pro-Gastrin-Releasing Peptide. The number represented positive or negative cases in small lung cancer.

Table V. *The concordance of HuD and current clinical biomarkers.*

	CEA		NSE		pro-GRP	
	Positive	Negative	Positive	Negative	Positive	Negative
HuD						
Positive	8	27	22	13	23	12
Negative	6	6	6	6	7	5

Analysis of the relationship between HuD and other clinical biomarkers. The concordance of HuD and CEA, NSE and pro-GRP were 22.9%, 62.8% and 65.7%, respectively. The number represented positive or negative cases in small lung cancer. CEA: Carcino-embryonic antigen; NSE: neuron-specific enolase; pro-GRP: pro-Gastrin-Releasing Peptide.

positive rate was 10%, which may have been because of cross reaction of antibody and autoantibodies. The false negative rate, which could cause a missed diagnosis, was 21.3%. The cause of false-negativity was unclear but may be due to the antibodies binding affinity of antigen coating the ELISA.

The comparison between the HuD-ELISA assay and the clinical diagnosis indexes showed that it had a better sensitivity than other clinical indexes. The combined use of two or three current diagnostic biomarkers remarkably improve specificity. The sensitivity of HuD (76.6%) was equal to NSE plus pro-GRP (78.7%) and better than CEA plus NSE (72.3%) and CEA plus pro-GRP (66%). The results indicated that the HuD-ELISA method was simple, fast, with high validity and non-invasive, which has a good prospect. However, HuD positivity may also appear in other diseases, such as neuroblastoma. Further investigation should focus on the differential diagnosis method.

In summary, we have developed a clinical detection method for HuD antigen as a potential diagnostic criterium for SCLC, which serves as a marker of disease progression.

Table VI. *Sensitivity of combined use of clinical diagnostic indexes.*

	NSE+ pro-GRP	CEA+ NSE	CEA+ pro-GRP	NSE+CEA+ pro-GRP
Cases	37	34	31	39
Sensitivity	78.70%	72.30%	66%	83%

The number represented positive or negative cases of clinical diagnostic biomarkers. CEA: Carcino-embryonic antigen; NSE: neuron specific enolase; pro-GRP: pro-Gastrin-Releasing Peptide.

Conflicts of Interest

There are no conflicts of interest.

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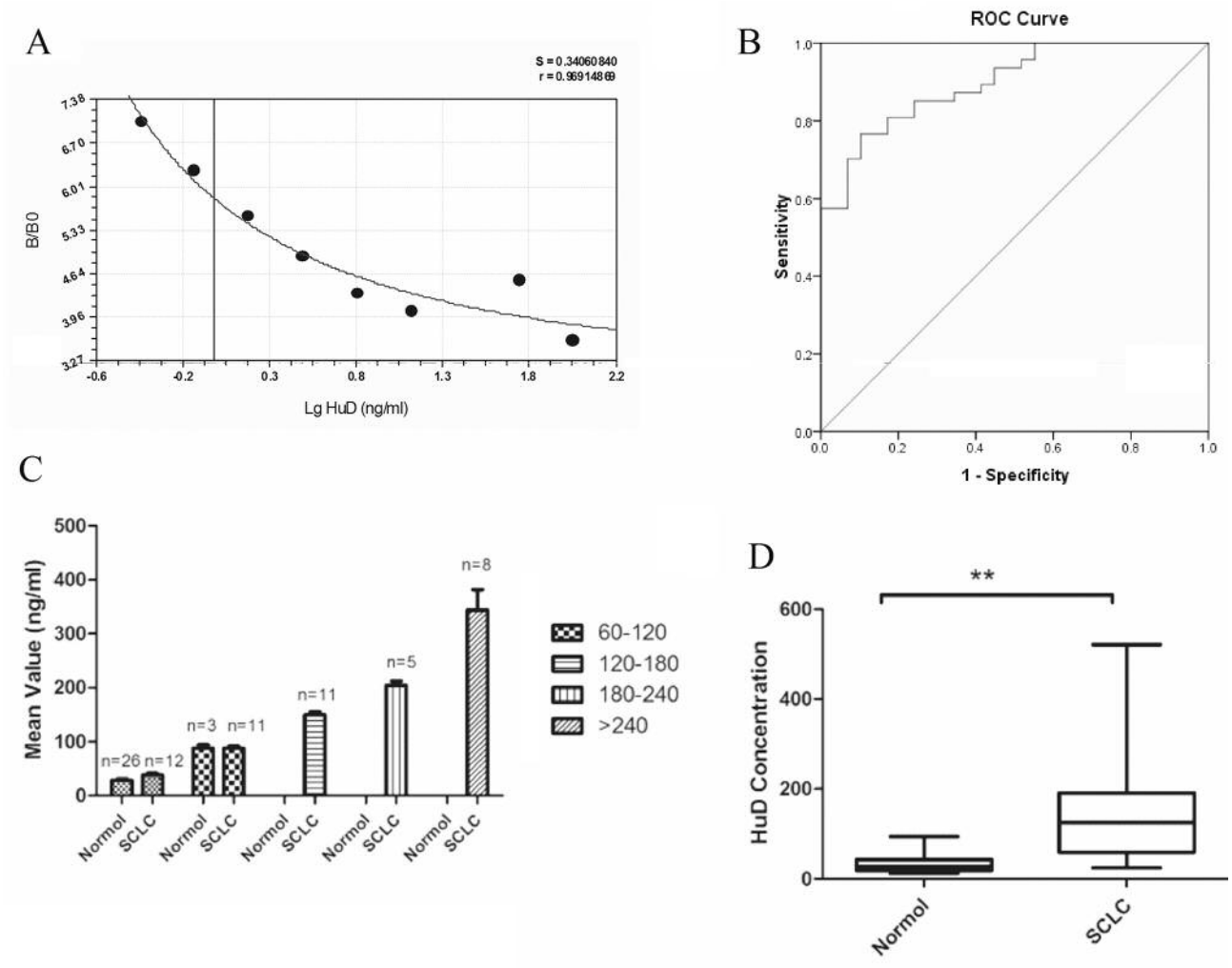

Figure 2. *Competitive-inhibition enzyme linked immunoabsorbent assay (ELISA). (A) Optimized standard inhibition curve of HuD mAb by icELISA. (B) Curve of ROC on HuD antigen concentration. (C) Concentration distribution of HuD antigen in SCLC and normal control. (C) Comparative HuD antigen concentration in Normol and SCLC (n=77, *p<0.05).*

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