# Expression of HuD (A Paraneoplastic Encephalomyelitis Antigen) mRNA in Lung Cancer

HuD, one of the Hu antigens (HuD and HuC), was recognized in the sera of small cell lung cancer (SCLC) patients with antibody-associated paraneoplastic encephalomyelitis/peripheral sensory neuropathy (PEM/PSN). Three forms of HuD mRNA, 197, 156, 110 nucleotides are made by alternative splicing at 868-909 residues and an additional 3'-splice site. To determine the diagnostic value of the HuD expression for small cell lung cancer, we examined 4 SCLC cell lines, 9 surgically resected SCLCs, and 12 surgically resected non-SCLCs using the reverse transcriptase-polymerase chain reaction with the HuD-specific primer pairs that spanned the putative alternative 3'-splicing site and direct DNA sequencing. None of the patients were associated with PEM/PSN. A single RNA transcript (156 nucleotides) among three forms (110, 156, 197 nucleotides) of the HuD gene was an alternatively spliced at 868-909 residues in SCLC cell lines. Expression of the HuD gene was stronger in three classic cell lines, but not in a variant cell line. Two of 9 SCLCs (22%) and 3 of 12 non-SCLCs (25%) expressed only the major RNA transcript (156 nucleotides) of the HuD gene, which was alternatively spliced in the same fashion as the cell line. These results revealed that no aberrant alternative splicing occurred in SCLC not associated with PEM/PSN and the expression of HuD gene was not specific for a particular histologic subtype of human lung cancer.

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Key Words : *HuD gene, Small cell lung cancer, Non-small cell lung cancer, RT-PCR, Direct DNA sequencing*

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## INTRODUCTION

Paraneoplastic encephalomyelitis sensory neuropathy (PEM/PSN) is one of the paraneoplastic neurological syndromes associated with small cell lung cancer (SCLC) and is characterized by dementia, sensory loss, and other neurological disabilities (1~6). Patients with PEM/PSN associated with SCLC harbor a characteristic antibody called anti-Hu in serum and CSF. The anti-Hu antibody, however, can be detected at low titer in the serum of about 15% of patients with SCLC not associated with paraneoplastic syndrome (7). A higher titer appears to be consistently associated with neurologic dysfunction, but the role of the antibody in the pathogenesis of the paraneoplastic syndrome has not been established. Until now less than 100 cases showing higher titer of serum anti-Hu antibody associated with PEM/PSN have been reported (1). Among them about 70% of patients are associated with SCLC (1, 2). The anti-Hu antibody reacts with a set of protein antigens (molecular weights 35~40 kd) expressed in neurons and SCLC cells (7). By immunohistology and Western blotting, previous studies (3, 7, 8) demonstrated the specific expression of the Hu antigen in all SCLCs, whether or not the tumor came from a patient who made the antibody.

HuD, one of the Hu antigens (HuD and HuC), recognized by the sera of patients with antibody-associated paraneoplastic encephalomyelitis, has been isolated by screening a cerebellar expression library (9). Reverse transcriptase-polymerase chain reaction(RT-PCR) assay revealed 3 forms of HuD mRNA, 197, 156, 110 nucleotides caused by alternative splicing at 868-909 residues and an additional 3'-splice site (9). The HuD mRNA is uniquely expressed in brain tissue. The major form of HuD mRNA is 156 nucleotides in human and monkey brains. The HuD protein shows a remarkable homology to *Drosophila* proteins, Elav and sex-lethal and is likely to be a neuron-specific RNA binding protein

that plays a role in neuronal development (10). However, whether the unique spliced transcript of HuD gene for SCLC is present is still not known.

Recently, RT-PCR has been used for detection of low levels of mRNA expression of certain genes using a small amount of RNA (11). SCLC is frequently detected in metastatic lesions and diagnosed by bronchoscopic biopsy without surgical resection. Thus, detection of specific transcripts of tumor antigenes for SCLC will provide supportive evidence for a differential diagnosis of SCLC, even with a small lung biopsy tissue. To address this issue and to determine the diagnostic value of the HuD expression for SCLC, we undertook a study by RT-PCR and direct DNA sequencing in SCLC cell lines and SCLC and non-SCLC tissues.

## MATERIAL AND METHODS

### Cell lines and tumor tissues

Four SCLC cell lines (N230, N231, Lu141, N471) were examined. Three (N230, N231, Lu141) were classic SCLC cell lines and one (N471) was a variant SCLC cell line (12, 13). All of these cell lines were established from tumors of patients without PEM/PSN.

Nine surgically resected SCLCs, and 12 surgically resected non-SCLCs were analyzed (Table 1). None of the patients with SCLC had the PEM/PSN syndrome and most tumors manifested as a solitary mass in the peripheral portion of the lung. Eight out of 9 patients were males. In one case, the tumor was associated with the syndrome of inappropriate secretion of antidiuretic hormone (SIADH). In 9 SCLCs, 6 of which were the mixed small cell/large cell type, two of which were small cell type, and one of which was the combined small cell type (14). Non-SCLCs comprised 3 squamous cell carcinomas, 3 adenocarcinomas, 4 large cell carcinomas, and 2 carcinoid tumors. Normal lung tissues from each patients were examined simultaneously as control tissue. All specimens of lung tumors analyzed were acetone-fixed paraffin-embedded tissues (15). Two non-SCLCs were examined along with fresh tissue in order to compare to the expression in acetone-fixed samples.

**Table 1.** Results of HuD expression in lung cancers

| Samples | HuD mRNA Expression[a] (major transcript) |
|---|---|
| SCLC Cell Lines | 3/4 (156 nucleotides) |
| Classic cell lines | 3/3 |
| N230, N231, Lu141 | |
| Variant cell lines | 0/1 |
| N417 | |
| SCLC Tissues | 2/9 (156 nucleotides) |
| Mixed small cell/large cell type | 1/6 |
| Small cell type | 0/2 |
| Combined small cell type | 1/1 |
| non-SCLC Tissues | 3/12 (156 nucleotides) |
| Squamous cell carcinoma | 1/3 |
| Adenocarcinoma | 1/3 |
| Large cell carcinoma | 1/4 |
| Carcinoid | 0/2 |

[a]: No. of cancers expressing HuD gene per total cancers examined ; SCLC : Small cell lung cancer

The brain and spinal cord tissues for a positive control were obtained by autopsy of a neurologically normal individual, snap frozen in isopentane chilled by liquid nitrogen, and kept frozen at -70℃.

### Extraction of total RNA

After deparaffinization with xylene, total RNA was isolated from two to three 50 μm-thick acetone-fixed paraffin-embedded sections, trimmed off tumorous areas, by using the acid-guanidium-phenol-chloroform method (16). After hematoxylin-eosin staining and confirmation of tumor tissue, five 15 μm-thick fresh samples from the spinal cord, brain and 2 non-SCLCs were used in the same way. Poly(A)+RNA was prepared from the 4 SCLC cell lines using the guanidium thiocyanate and oligodeoxy thymidylate cellulose method (17).

### Reverse transcriptase-polymerase chain reaction (RT-PCR)

Total RNA (0.2 μg~1.0 μg) from surgical samples and poly(A)+RNA(10 ng) from SCLC cell lines were incubated with RT in a solution containing the following : 2.5 mM of each dNTP, 10 U of RNase inhibitor, 5X reaction buffer (250 mM Tris-HCl, 375 mM KCl, 15 mM $MgCl_2$) and 5 nmol of random hexamer primer (Synthetic Genetics) (sample overlaid with 2 drops of mineral oil). RT reactions were terminated by incubation at 98℃ for 30 min and used directly by subsequent PCR amplification of specific cDNAs with a PCR reaction mixture (50 μl final volume : 1X PCR buffer (100 mM Tris-HCl, 500 mM KCl, 15 mM $MgCl_2$), 0.25 mM each dNTP, and 20 μM each 5'(748~764) and 3'(929~945) primers (CCAGGCCCTGCTGTCCC, AGGCTTCTCAT-TCCATC) for HuD which spanned the putative alternative 3'-splice site described previously (9). With these primers it was possible not only to determine the presence of HuD mRNA but also to determine the 3'-splice site (Fig. 1). After denaturation at 95℃ for 5 min, 2.5 μl *Taq* polymerase mixture (2.5 U *Taq* polymerase (Perkin Elmer Cetus, CA. USA), 1 U/ μl PCR

(748)ccaggccctgctctcccAGCTCTACCAGTCCCCTAACCGGCGCTACCCAGGTCCACTTCACCACCA
GGCTCAGAGGTTCAGGCTGGACAATTTGCTTAATATGGCCTATGGCGTAAAGA**G/ACTGATGT**
**GTGGACCAGTCCCCCCTTCTGCTTGTTCCCCCAG/G**TTCTCCCCAATTACCATTgatggaatgag
aagcct (945)

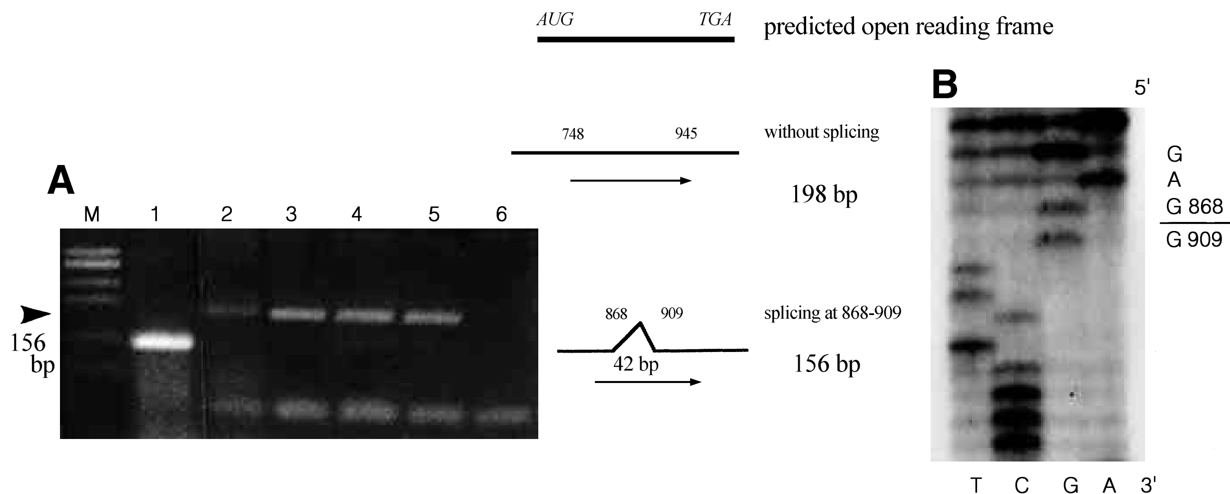


**Fig. 1.** A sequence of an amplified segment of the open reading frame of HuD gene was printed in the top. Small letters are primers and bold letters are spliced segments. **A:** Detection of a single mRNA transcript compatible with 156 nucleotides (arrow) from the spinal cord as a positive control (lane 2) and three classic SCLC cell lines [ lane 3 (N230), lane 4 (N231), and lane 5 (Lu141)]. A variant cell line [ lane 6 (N471)] showed no expression of HuD gene. RT-PCR was performed on each of these samples, using primers which spanned the putative alternative 3'-splice site of HuD gene. A product of exon 7 of p53 gene (110bp) (lane1) from the normal lung tissue was examined for marker of the shortest spliced transcript of HuD gene. Marker(M) was loaded with *Hae*III-digested X174 DNA. **B:** Direct DNA sequencing of cDNA products representing a single band in RT-PCR ; deletion of 42 nucleotides produced by the alternative splicing at 868-909 residues was confirmed.

enhancer (Stratagene, CA, USA), and 1U Bind-aid amplification enhancer for decreasing background (United State Biomedical Corporation, USA)) were added. PCR analyses were performed in an automated DNA clonal cycler (Perkin Elmer Cetus) with the following temperature profile according to the previous report (9) : 35 cycles of 1 min at 94℃, 30 sec at 47℃, and 1 min at 72℃. A low annealing temperature is suitable for PCR reaction of HuD gene because of the short length of primers (less than 20 base pairs) and the high proportion of purines in one primer. One-fifth (10 $\mu$l) of the PCR product was electrophoresed in 3% agarose gel (Nuseive, FMC, USA), the PCR fragment was stained with ethidium bromide and analyzed with ultraviolet light at 320 nm. In order to check the integrity of total viable RNA, the same samples were assayed with primers (1,556~1,575 and 3,658~3,677 in genomic sequence) specific for $\beta_2$-microglobulin (the size of the amplified band : 261 bp). Exon 7 of p53 (110 bp) from the normal lung tissue was examined for marker of the shortest spliced transcript of HuD gene. RT-PCR for p53 gene was performed with primers of exon 7 (GTTGGCTCTGACTGTACCAC, ATCACACTGGAAGACTCCAG) and at 55℃ of annealing temperature using the same protocol. Negative controls without added RNA or normal lung tissue were used for each sets of RT-PCR reactions.

### Direct sequencing of DNA by lamda-exonuclease digestion of double-stranded PCR products

Bands detected by RT-PCR were eluted from the gel and amplified using phosphated and non-phosphated 5'(751~767) and 3'(926~942) primers under the same profile of PCR. After confirming the PCR product(10 $\mu$l) by 3% agarose electrophoresis, the final PCR fragments were extracted with phenol-chloroform. The sequences primed with the phosphated primers were digested with lamda-exonuclease. The remaining single-strand products were precipitated with ethanol and subjected to sequencing using a 7-deaza-dGTP sequenase version 2.0 kit (USB, USA) with 5'-end labeled non-phosphated primers, whose sequences were the same as those of the phosphated primers in the previous step (18, 19). All cDNA products from SCLC cell lines and tissue samples were sequenced.

## RESULTS

### HuD expression in SCLC cell lines

Results of RT-PCR assay for HuD expression in SCLC cell lines are illustrated in Fig. 1 and Table 1. Three classic SCLC cell lines strongly expressed a single product of mRNA compatible with 156 nucelotides, which was expressed in the spinal cord as a positive control. In contrast, HuD expression was undetectable in one variant cell line (N471). To verify this product, direct DNA sequencing was performed seperately. A deletion of 42 nucleotides (from residues 868~909), which was previously predicted as an alternative 3'-splice site (9) was detected (Table 1 and Fig. 1). According to these results, an alternatively spliced form (156 nucleotides) at 868-909 residues was the dominant transcript found in SCLC cell lines.

### HuD expression in lung tumor tissues

We examined solitary SCLCs resected surgically and not associated with PEM/PSN (Table 1 and Fig. 2A). Histologically, 8 of them were pure small cell carcinomas and stained positively for anti-neural cell adhesion molecule antibody (N-CAM) such as Lu 243 (data not shown) (20). One case showed an adenocarcinoma component, but positive for N-CAM. Two of 9 SCLCs (22%) showed expression of 156 nucleotides transcript of HuD mRNA which was weaker than in SCLC cell lines (Fig. 2A). Histologically, one case was combined small cell type and the other was mixed small cell/large cell type.

Three of the 12 non-SCLCs (25%) expressed 156 nucleotides transcript of HuD mRNA (Table 1 and Fig. 2B); three of which were squamous cell carcinoma, adenocarcinoma and large cell carcinoma, respectively. Comparing to SCLC cell lines, the expression of HuD gene in these cases was weak. Neither of the carcinoid tumors expressed the HuD gene. Fresh samples from the two patients with non-SCLC showed stronger signals than the acetone-fixed tissues (Fig. 2C).

By Direct DNA sequencing of cDNA products from the SCLC tissues and non-SCLC tissues, we detected the same alternative splicing at 868-909 residues as that of SCLC cell lines (data not shown).

## DISCUSSION

In this study, for the RT-PCR assay, we used the same

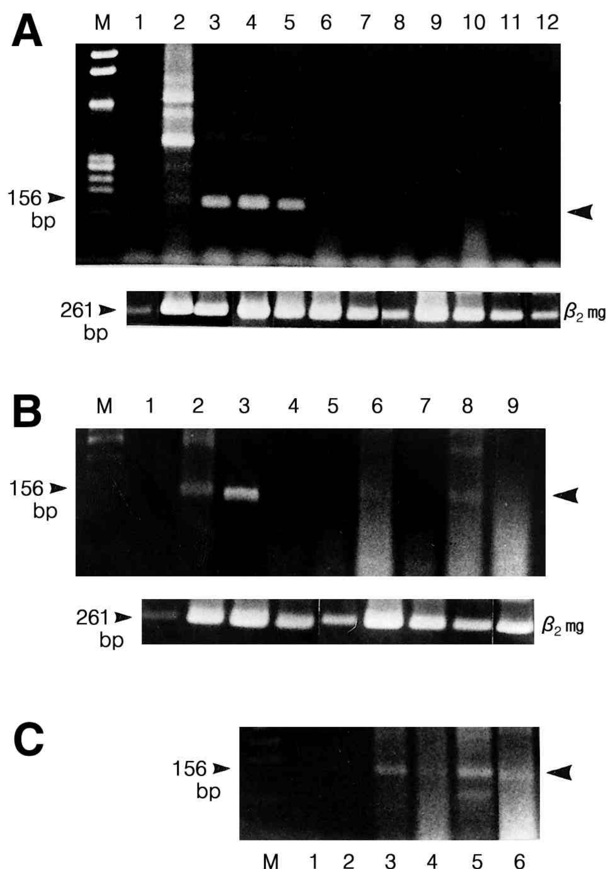


**Fig. 2. A:** HuD expression in SCLC tissues. Expression of 156 nucleotides transcript of HuD mRNA was detected in two cases (lane 11 and 12). One case (lane 11) was mixed small cell/large cell type and the other (lane 12) was the combined small cell type. The expression is weaker than in the spinal cord as a positive control (lane 2) and SCLC cell lines (lane 3-5). Five cases (lane 6-10) revealed no expression. Negative control reacts for RT-PCR were performed with RNA extracted from normal lung tissue (lane 1). In the bottom panel the same cDNA reaction products were assayed with primers specific to $\beta_2$-microglobulin (261 nucleotides) as a control for RNA content. **B:** Results of HuD expression in non-SCLC tissues. Expression of HuD gene was detected in a squamous cell carcinoma (lane 6) and an adenocarcinoma (lane 8). The expression was weaker than in the spinal cord as a positive conrtol (lane 2) and SCLC cell line (lane 3). Carcinoid tumor(lane 4) showed no expression of HuD gene. Negative control reacts for RT-PCR were performed with RNA extracted from normal lung tissue of each case. The same cDNA reaction products were assayed with primers specific to $\beta_2$- microglobulin as a control for RNA content. Lane 1: normal lung; lane 2: spinal cord; lane 3: SCLC cell line; lane 4 and 5: carcinoid tumor and normal lung counterpart, lane 6 and 7: squamous cell carcinoma and normal lung counterpart; lane 8 and 9: adenocarcinoma and normal lung counterpart. **C:** Comparison of HuD expression with fresh and acetone-fixed tissues. Expression of HuD gene is stronger in fresh samples than in acetone-fixed tissues. Lane 1: negative control without RNA; lane 2: normal lung; lane 3 and 4: fresh and aceton-fixed squamous cell carcinoma (the same case in lane 6 of Fig. 2B); lane 5 and 6: fresh and aceton-fixed adenocarcinoma (the same case in lane 8 of Fig. 2B)

HuD specific primers which spanned the putative alternative 3'-splice site as described by Szabo et al. (9). They reported detection in the human brain of 3 forms of HuD mRNA, 197, 156, 110 nucleotides caused by alternative splicing at 868-909 residues and an additional 3'-splice site. The major form of HuD mRNA is 156 nucleotides in human and monkey brains. Szabo et al. (9), however, suggested in their preliminary study that a minor product of 110 nucleotides may be the main product of SCLC. If this unique pattern of alternative splicing is present, we thought that it will be one of the diagnostic markers of SCLC. Then we examined SCLC cell lines and tissues. However, the single minor transcript (110 nucleotides) could not detected, but major transcript (156 nucleotides) was detected in both SCLC cell line and tissue as well as human brain and spinal cord. As a result, no aberrant alternative splicing occurred in SCLCs not associated with PEM/PSN. Whether RNA splicing is the same in tumors without PEM/PSN or in tumors with PEM/PSN warrants further study. In the preliminary study, we examined different sites of normal brain tissues in which the major transcript of the HuD gene was also 156 nucleotides (data not shown). However, we could not find 2 minor transcripts of HuD mRNA, 197 and 110 bp in the brain. The possibility of lower sensitivity of ethidium bromide staining than $P^{32}$-radiolabelling could be considered.

The SCLC cell lines are subgrouped into 2 major classes mainly on the basis of their biochemical properties (12, 13). Classic SCLC cell lines express elevated levels of biomarkers (aromatic L-amino acid decarboxylase [AADC], neuron-specific enolase, creatin kinase, brain isoenzyme) and grow slowly, while variant cell lines fail to express AADC activity and grow quickly. In this study, we examined 3 classic cell lines which expressed the alternatively spliced form of HuD genes but not in a variant cell line. This finding implies that the HuD gene expression is one of the markers for classic SCLC cell line.

The classification of SCLC in tissues is contradictory. In this study, we used the revised International Association for the Study of Lung cancer (14) by which SCLCs are divided into 3 subtypes, small cell, mixed small cell/large cell and combined small cell type. The SCLCs examined in this study were pure small cell carcinoma, either small cell or mixed small cell/large cell type, except for one combined type. Two of 9 cases, one combined and one mixed small cell/large cell type, expressed the HuD gene. In another report (1), 15% of patients with SCLC not associated with PEM/PSN could be detected with low titer of the serum anti-HuD antibody. Our study also revealed that 22% of HuD expression in SCLCs not associated with PEM/PSN could be detected by RT-PCR.

The pathogenesis of PEM/PSN in SCLC is still not well known. One theory is that coexpression of MHC class I and Hu antigen by the tumor may result in a profound immune reaction. However, other factors may be involved in the pathogenesis of this immune disorder (2). SCLCs with the HuD antigen showed coexpression of other neuronal antigen (2, 20). Thus, SCLCs have been thought to be neuroectodermal in origin. This study also demontrated that 22% of SCLCs not associated with PEM/PSN expressed the HuD gene as well as other neuronal antigens.

In the previous study (7) all small cell carcinomas expressed the Hu antigen but less than 4% of non-SCLCs expressed it. The antigen was expressed by tumors other than lung cancer, including neuroblastoma, Merkel cell tumor, Ewing's sarcoma, melanoma, chondromyxosarcoma, small cell carcinoma of the stomach, and undifferentiated carcinoma of the prostate. In this study, 25% of non-SCLCs expresssed low level of the HuD gene. These tumors revealed no small cell carcinoma components with microscopic examination. Fresh tissues from the same patients revealed stronger expression of the HuD gene than the AMeX tissue. Clinical implication of low expression of HuD gene in non-SCLCs not associated with PEM/PSN is still unexplainable.

In a previous study (21) and our preliminary study, it was suggested that acetone fixation may not be better for RNA preservation than methanol fixation or the use of fresh-frozen tissue (data not shown). In this study, nevertheless, we successfully extracted 3.0 μg/cm (from normal lung tissue) to 9.7 μg (from tumor tissue) of viable total RNA from 2~3 sheets of 50 μm-thick tissue section. Compared to fresh tissue of the same samples, however, the RT-PCR product signal is slightly weak in acetone-fixed tissues. Therefore, this study indicates the possible diagnostic usefulness of RT-PCR assay using RNA extracted from paraffin-embedded tissues for other genetic studies.

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## REFERENCES

1. Dalmau J, Furneaux HM, Rosenblum MK, Graus F, and

Posner JB. *Detection of anti-Hu antibody in specific regions of the nervous system and tumor from patients with paraneoplastic encephalomyelitis/sensory neuropathy. Neurology 1991 ; 41 : 1757-64.*

2. Dalmau J, Graus F, Rosenblum MK, and Posner JB. *Anti-Hu-associated paraneoplastic encephalomyelitis/sensory neuropathy : a clinical study of 71 patients. Medicine 1992 ; 71 : 59-72.*
3. Dalmau J, Hurneaux HM, Gralla R, Kris M, and Posner JB. *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.*
4. Furneaux HF, Reich L, and Posner JB. *Autoantibody synthesis in the central nervous system of patients with paraneoplastic syndromes. Neurology 1990 ; 40 : 1085-91.*
5. Graus F, Cordon-Cardo C, and Posner JB. *Neuronal antinuclear antibody in sensory neuropathy from lung cancer. Neurology 1985 ; 35 : 538-43.*
6. Graus F, Elkon KB, Cordon-Cardo C, and Posner JB. *Sensory neuropathy and small cell lung cancer : Antinuclear antibody that also reacts with the tumor. Am J Med 1986 ; 80 : 45-52.*
7. Dalmau J, Furneaux HM, Cordon-Cardo C, and Posner JB. *The expression of the Hu (paraneoplastic encephalomyelitis/ sensory neuropathy) antigen in human normal and tumor tissues. Am J Pathol 1992 ; 141 : 881-6.*
8. Budde-Steffen C, Anderson NE, Rosenblum MK, and Posner JB. *Expression of antigen in small cell lung carcinoma lines detected by antibody from patients with paraneoplastic dorsal root ganglinopathy. Cancer Res 1988 ; 48 : 430-34.*
9. Szabo A, Dalmau J, Manley G, Rosenfeld M, Wong E, Henson J, Posner JB, Furneaux HM. *HuD, a paraneoplastic encephalomyelitis antigen contains RNA-binding domains and is homologous to Elav and sex-lethal. Cell 1991 ; 67 : 325-33.*
10. Robinow S, and White K. *The locus elav of Drosophila melanogaster is expressed in neurons at all developmental stages. Dev Biol 1988 ; 126 : 294-303.*
11. Deguchi T, Doi T, Ehara H, Ito S, Takahashi Y, Nishino Y, Fujihiro S, Kawamura T, Komeda H, Horie M, Kaji H, Shimokawa K, Tanaka T, and Kawada Y. *Detection of micro-metastatic prostate cancer cells in lymph nodes by reverse transcriptase-polymerase chain reaction. Cancer Res 1993 ; 53 : 5350-4.*
12. Carney DN, Gazdar AF, Bepler G, Guccion JG, Marangos PJ, Moody TW, Zweig MH, and Minna JD. *Establishment and identification of small cell lung cancer cell lines having classic and variant features. Cancer Res 1985 ; 45 : 2913-23.*
13. Matsumoto T, Terasaki T, Mukai K, Wada M, Okamoto A, Yikota J, Yamaguchi K, Kato K, Nagatsu T, Shimosato Y. *Relation between nucleolar size and growth characteristic in small cell lung cancer cell lines. Jpn J Cancer Res 1991 ; 82 : 820-8.*
14. Colby TV, Koss MN, Travis WD. *Tumors of the lower repiratory tract. Atlas of tumor pathology, third series, fascicle 13. Washington D.C., Armed Forces Institute of Pathology, 1995.*
15. Sato Y, Mukai K, Watanabe S, Goto M, Shimosato Y. *The AMeX method : a simple technique of tissue processing and paraffin embedding with improved preservation of antigens for immunostaining. Am J Pathol 1986 ; 125 : 431-5.*
16. Chomaczynski P, Sacchi N. *Single-step method of RNA isolation by acid guanidium thiocyanate-phenol-chloroform extraction. Anal Biochem 1987 ; 162 : 156-9.*
17. Sambrook J, Fritsh EF, Maniatis T. *Molecular cloning, A laboratory Manual 2. In : Fritsh EF, ed. NY : Cold Spring Harbor Laboratory, 1989 ; 726.*
18. Cho JH, Noguchi M, Ochiai A, Uchino S, Hirohashi S. *Analysis of regional differences of p53 mutation in advanced gastric carcinoma : relation to heterogeneous differentiation and invasiveness. Mod Pathol 1994 ; 17 : 205-11.*
19. Higuchi RG, Ochman H. *Production of single-strand DNA templates by exonuclease digestion following the polymerase reaction. Nucleic Acids Res 1989 ; 17 : 5865.*
20. Noguchi M, Hirohashi S, Shimosato Y. *Immunohistochemical detection of cluster 1 small cell lung cancer antigen and chromogranin A in lung carcinomas. Jpn J Cancer Res 1992 ; 22 : 10-6.*
21. Sato Y, Mukai K, Furuya S, Shimosato Y. *The AMeX method : a multipurpose tissue-processing and paraffin- embedding method. III Extraction and purification of RNA and application to slot-blot hybridization analysis. J Pathol 1991 ; 163 : 81-5.*