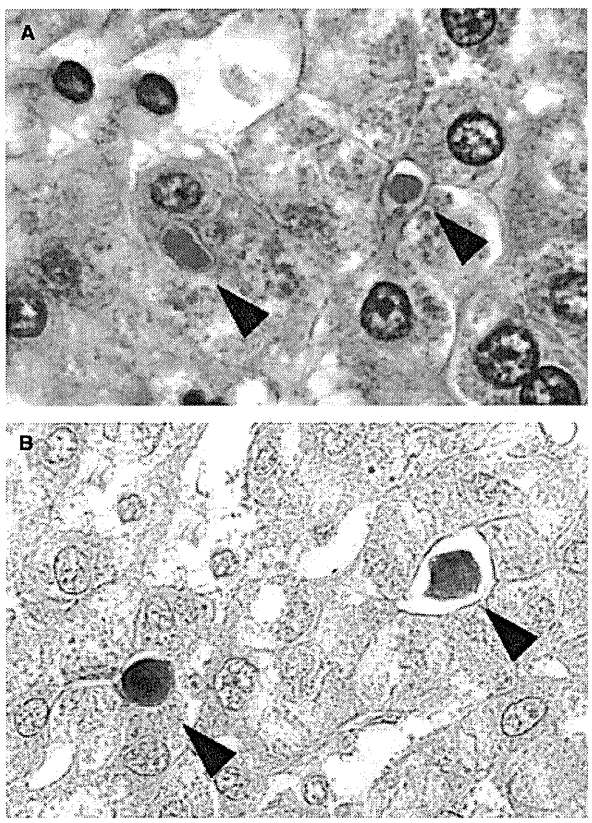

Figure 3. Bile production in hepatocellular carcinomas. Bile production (triangles) as determined using haematoxylin and eosin (**A**) and Hall's bile staining (**B**).

overexpression of glutamine synthetase has been reported to correlate well with the presence of *CTNNB1* mutations, and is regarded as an indicator of HCCs with *CTNNB1* mutations.[14,15,20] In agreement with these previous reports, *CTNNB1*-mutated HCCs showed significantly elevated levels of *GLUL* expression. At the same time, we found that *AMACR* overexpression is also highly correlated with the presence of *CTNNB1* mutations. The expression levels of *GLUL* and *AMACR* were also reflected by the expression of their protein products, which can be detected by immunohistochemistry. Diffuse and strong staining for AMACR and glutamine synthetase was observed almost exclusively in tumours with *CTNNB1* mutations. Conversely, none of the tumours negative for these proteins had *CTNNB1* mutations in the current series. We suggest that overexpression of AMACR might be a novel and excellent histological indicator of HCCs with *CTNNB1* mutations.

Bile production is occasionally observed in HCCs; notably, cholestasis has recently been suggested to be a feature of HCCs with *CTNNB1* mutations.[20] Audard *et al.*[20] noted a close correlation between strong glutamine synthetase expression and the presence of cholestasis, and suggested that cholestasis might be a marker of HCCs with *CTNNB1* mutations. In agreement with this hypothesis, tumour cholestasis was frequently associated with *CTNNB1* mutations and also with high AMACR expression in the present analysis. The biological significance of AMACR overexpression in cancers has been discussed in relation to its role in the β-oxidation of branched-chain fatty acids.[1] However, in addition to fatty acid metabolism, AMACR is also involved in bile acid synthesis in the liver, and is required for the conversion of C27 bile acids to C24 bile acids. C27 bile acids are more hydrophobic than their C24 products, and are more toxic to cells.[22–24] Considering the association between cholestasis and AMACR expression, AMACR might play a role in the processing of bile acid intermediates to avoid cell injury caused by the accumulation of C27 bile acids in *CTNNB1*-mutated HCCs.

In addition to its expression in HCCs, AMACR was also expressed in normal liver, predominantly in pericentral hepatocytes. Of note, recent studies have suggested that Wnt/β-catenin signalling plays a crucial role in the regulation of pericentral gene expression.[25] Wnt/β-catenin signalling is physiologically active in pericentral hepatocytes, and many of the previously identified β-catenin-regulated genes are localized to pericentral areas within the liver lobule.[25–28] Furthermore, Hailfinger *et al.*[26] demonstrated a similarity in the gene expression patterns of pericentral hepatocytes and *CTNNB1*-mutated HCCs, and suggested the common regulation of these genes by β-catenin-mediated signalling. Thus, the predominantly pericentral expression of AMACR implies that AMACR is also regulated by β-catenin-mediated signalling in normal liver. Overall, the present study suggests that AMACR is a target of β-catenin in the liver under both neoplastic and non-neoplastic conditions.

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Anti-HuC and -HuD autoantibodies are differential sero-diagnostic markers for small cell carcinoma from large cell neuroendocrine carcinoma of the lung

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Abstract. Aiming to identify novel sero-diagnostic markers for neuroendocrine carcinomas of the lung, the two-dimensional gel electrophoresis-immunoblot method was used to analyze tumor-associated autoantibodies in patients with small cell lung carcinoma (SCLC) and large cell neuroendocrine carcinoma (LCNEC). Several autoantigens were revealed and anti-HuC autoantibody was detected only in sera of SCLC patients. Since Hu family proteins including HuC are well-known causes of paraneoplastic encephalomyelitis/sensory neuronopathy (PEM/SN), the expression of HuC as well as HuD mRNAs and their proteins was studied in 11 lung cancer cell lines. The expression of HuC and HuD mRNAs and proteins was only detected in SCLC- and LCNEC-derived cells. To validate the existence of anti-HuC and -HuD auto-antibodies, we studied a large number of sera including those from lung cancer patients employing dot blot analysis. Anti-HuC and -HuD autoantibodies were detected only in SCLC cases with or without PEM/SN, and not in the sera of LCNEC patients. The mechanism leading to different anti-HuC and -HuD autoantibody production between SCLC and LCNEC is unclear; however, the results from the present and previous studies suggest that anti-HuC and -HuD auto-antibodies are novel differential sero-diagnostic markers for SCLC from LCNEC.

Introduction

Lung cancer is the leading cause of cancer-related death worldwide. Based on the presence or absence of cellular neuroendocrine differentiation, lung cancer can be grouped into non-neuroendocrine or neuroendocrine tumors. The former is roughly equal to the non-small cell lung cancer (NSCLC) largely comprising squamous cell carcinoma (SCC) and adenocarcinoma (AD). The latter ranges from low-grade typical carcinoid (TC), intermediate-grade atypical carcinoid (AC), to high-grade small cell lung carcinoma (SCLC) and large cell neuroendocrine carcinoma (LCNEC) (1,2), and the 5-year survival rates for TC, AC, LCNEC, and SCLC were 87, 56, 27, and 9%, respectively (2). Similar results were also reported by Garcia-Yuste *et al*, and the 5-year survival rates for TC, AC, LCENC, and SCLC were 96, 72, 21, and 14%, respectively (3). These results show that both SCLC and LCNEC are highly malignant and have a similar poor prognosis. Pro-gastrin-releasing peptide (pro-GRP) is a well-known sero-diagnostic marker for SCLC; however, its positive rate is lower in stage I and II (35-45%) than in stage III (55-70%) and IV (70-80%). At present, no specific sero-diagnostic markers for distinguishing SCLC from LCNEC have been reported.

Autoantibodies are antibodies detected in the sera of patients with various autoimmune diseases. They are also frequently observed in the sera of patients with various malignancies even in the early stages, and, thus, the possibilities for them to be used as potential tumor markers have been suggested (4-10). Hanash (11) has described that harnessing the immune response to identify novel cancer biomarkers is an attractive strategy, because the immune system performs biological amplification which is equivalent to a PCR reaction by generating a detectable signal, with antigenic tumor proteins as templates, beginning at an early stage during tumor development when the tumor may be otherwise undetectable. Many tumor-related autoantibodies have been reported in pulmonary carcinomas (12-15). Thus, an exhaustive search for novel tumor-specific autoantibodies, which may serve as early sero-diagnostic markers for cancers, has commenced.

In this study, we detected tumor-associated autoantibodies by immunoblotting based on two-dimensional gel electrophoresis

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(2-DE) from the sera of patients with pulmonary neuroendocrine carcinomas. Identified antigens were further assessed to confirm their specific expressions in neuroendocrine carcinomas by RT-PCR and immunoblotting. Finally, the usefulness of the autoantigens was validated using the sera of patients with various types of pulmonary carcinoma, along with non-cancerous and healthy controls.

Materials and methods

Cell lines. Six SCLC (N230, N231, H69, H82, Lu130, and N417), two LCNEC (LCN1 and LCN2) (16), two AD (A549 and LC-2/ad), and one SCC (RERF LC-AI) cell line were used in this study. All cell lines were grown in RPMI-1640 (Sigma, Steinheim, Germany) supplemented with 10% fetal bovine serum, 100 U/ml penicillin, and 100 μg/ml streptomycin (Gibco, Auckland, NZ). After being washed with phosphate-buffered saline without bivalent ions (PBS-), harvested cells were separated into two gropus, one was fixed in 10% formalin and embedded in paraffin, and the other was stored at -80°C until use for protein and total RNA extraction.

Sera. Sera from 80 pulmonary carcinoma patients (SCLC: 31, LCNEC: 7, AD: 21, and SCC: 21) and 21 non-neoplastic lung disease (interstitial pneumonia: 7, tuberculosis: 5, non-lung cancer diseases: 3, acute inflammation: 1, epithelioid granuloma: 1, cryptogenic organizing pneumonia: 1, nontuberculous mycobacteria: 1, aspergillosis: 1, inflammatory granuloma: i) patients treated at Kitasato University Hospital. Sera from 26 healthy volunteers were also used as a normal control. All sera were kept at -80°C until use. The SCLCs was further divided into 15 limited and 12 extensive disease cases. This study was approved by the Ethics Committee of Kitasato University School of Medicine. All patients were informed of the aim of the study and gave consent to use their samples.

2D-immunoblotting (IB). Sample preparation and the two-dimensional gel electrophoresis (2-DE) used in this study were described in our previous study (17).

Proteins extracted from the mixture of two LCNECs (LCN1 and LCN2) or the mixture of three SCLCs (N231, H69, and Lu130) were separated by 2-DE. Two pieces of gel were prepared for each sample, one was transferred to a polyvinylidene difluoride (PVDF) membrane (Millipore Corp,. Bedford, MA, USA) for immunoblotting and the other was visualized by coomassie brilliant blue R-350 (CBB) staining (PhastGel Blue R, GE Healthcare, Uppsala, Sweden).

Blotting membranes were blocked with 0.05% casein/TBS (0.01 mol/l Tris-HCl, pH 7.5, 150 mmol/l NaCl) for 30 min at room temperature (RT). Membranes prepared with LCNEC proteins were reacted with 100-times diluted pooled sera of five LCNEC patients and the membranes with prepared SCLC proteins were reacted with 100-times diluted pooled sera of five SCLC patients, respectively, for 1 h at RT. The dilution buffer was 0.0025% casein/TBS-T (TBS containing 0.1% Tween-20). Then, the membranes were incubated with 1,000-times diluted horseradish peroxidase (HRP)-conjugated rabbit anti-human IgG polyclonal antibody (Dako, Glostrup, Denmark) for 30 min at RT. Finally, signals were developed by stable DAB solution (Invitrogen, Carlsbad, CA, USA).

Protein identification. In brief, protein spots that reacted with patients, sera were excised from 2-DE gels and destained with 50% (v/v) acetonitrile (ACN)/50 mM NH_4HCO_3, dehydrated with 100% (v/v) ACN, and then dried under vacuum conditions. Tryptic digestion was performed for 24 h at 37°C in a minimum volume of digestion solution containing 0.5 ng/μl sequencing grade modified trypsin (Promega Corp., Madison, WI, USA) and 25 mM Tris-HCl buffer (pH 9.0). After incubation, digested protein fragments eluted in solutions were collected, and gels were washed once in 50% (v/v) ACN/5% trifluoroacetic acid (TFA) and collected in the same tube. Solutions containing digested protein fragments were measured by MALDI-TOF/TOF MS (autoflex-III; Bruker Daltonik GmbH, Bremen, Germany).

Fragment ion spectra from MS and MS/MS were submitted to MASCOT (http://www.matrixscience.com/) for a database search and the identification of corresponding proteins employing the following database: IPI human 20091026 (86379 sequences; 34740790 residues, http://www.ebi.ac.uk/IPI/IPIhuman.http://www.ebi.ac.uk/IPI/IPIhuman.html/).

RT-PCR. Total RNAs from the above-mentioned 11 lung cancer cell lines were extracted with Isogen (Nippon Gene, Tokyo, Japan) according to the manufacturer's instructions. Primers were designed with Oligo Primer Analysis Software, version 6.0 (Takara Bio Inc, Otsu, Japan) according to HuC and HuD mRNA sequences (18,19). HuC forward primer: 5'-TGCAAGTTGGTTCGGGACAAG-3' (582-602); reverse primer: 5'-GGCGGATGACTGGTAGAGG-3' (1031-1049). HuD forward primer: 5'-GTCTCTTCGGGAGCATTGGT-3' (415-434); reverse primer: 5'-CCTCTTATCAAAGCGGAT GAA-3' (753-773). PCR was performed with pretreatment at 94°C for 2 min and 30 cycles of denaturation at 94°C for 30 sec, annealing at 60°C for 30 sec, and extension at 72°C for 30 sec. Beta-2-microglobulin was used as an internal control. PCR products were electrophoresed on 3% agarose gel and stained with ethidium bromide.

1D-immunoblotting. Proteins were extracted from lung cancer cell lines with detergent lysis buffer (20) using an ultra-sonic homogenizer (UH-50; SMT Co., Tokyo, Japan). Each extracted protein (10 μg) was boiled and separated by 10% sodium dodecyl sulfate-polyacrylamide gel electrophoresis (SDS-PAGE) with a constant current at 15 mA. The immunoblotting methods were generally the same to those used for 2D-IB with some modifications. Transferred membranes were blocked with 0.5% casein/TBS for 30 min at RT, followed by reaction with 200-times diluted HuC- and HuD-positive serum as anti-Hu protein antibody with dilution buffer for 1 h at RT, because purchased anti-HuC antibody did not show specific reactivity. Then, the membranes were incubated with 1,000-times diluted HRP-conjugated rabbit anti-human IgG polyclonal antibody (Dako) for 30 min at RT. Finally, signals were developed using Immobilon Western reagent (Millipore Corp.).

Micro-dot blot array. Anti-HuC and -HuD autoantibodies in sera were detected employing the automatic dot blot system, and the micro-dot blot array with a 256-solid pin configuration (Kakengeneqs Co., Ltd., Chiba, Japan) was used. In brief, 1 μl

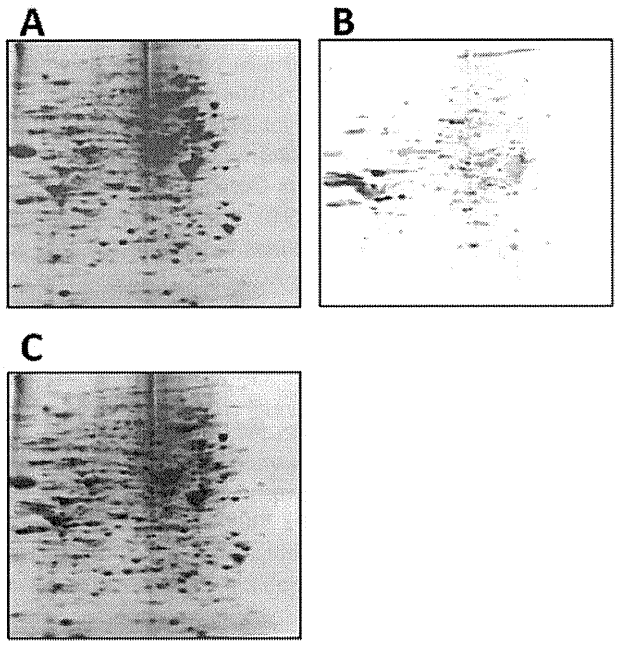


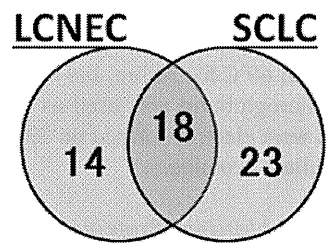
Figure 1. Detection of autoantibodies by 2D-immunoblotting. (A) Protein lysates from SCLC cell lines were separated by 2-DE and stained with CBB. (B) Immunoblot analysis was performed with mixed sera from patients with SCLC as primary antibodies, visualized with DAB solution. (C) A and B were merged.

each of proteins was spotted onto PVDF membranes, which were prepared by a wheat germ cell-free system (21). Then the membranes were blocked with 20% N101 (NOF Corp., Tokyo, Japan)/TBS for 1 h at RT. After being washed in TBS, the membranes were reacted with 100-times diluted sera with 1% N101/TBS for 30 min at RT. After TBS-T washing, the membranes were incubated with 1,000-times diluted HRP-conjugated rabbit anti-human IgG polyclonal antibody for 30 min at RT. Finally, signals were developed with Immobilon Western reagent. The data were analyzed using DotBlotChip-System software ver. 4.0 (Dynacom Co., Ltd., Chiba, Japan). Normalized signals are presented as the positive intensity minus background intensity around the spot. Statistical analysis was performed using the Mann-Whitney U test. The area under the curve (AUC) and best cut-off point were calculated employing receiver operating characteristic (ROC) analysis.

Results

Autoantigen identified by 2D-IB. The immunoreactivity of autoantibodies in sera was assessed by 2D-IB, and the representative positive protein spots on the membrane are shown in Fig. 1. Sixty-two and 63 positive spots were detected with sera from LCNEC and SCLC patients, respectively. In total, 32 proteins for LCNEC and 41 proteins for SCLC were identified as autoantigens. The identified proteins are summarized in Fig. 2. Twenty-three proteins including HuC

A



Classification	Spots	Antigens
LCNEC	62	32
SCLC	63	41

B

Symbol	Protein	MW	IPI	Molecular Function
ATP5A1	ATP synthase subunit alpha, mitochondrial	59714	IPI00440493	Transporter activity
DRG1	Developmentally regulated GTP-binding protein1	40517	IPI00031836	Unknown
EIF3I	Eukaryotic translation initiation factor 3 subunit I	36479	IPI00012795	Translation regulator activity
EIF4A1	Eukaryotic initiation factor 4A-I	46125	IPI00025491	Translation regulator activity
EIF4A2	Isoform 1 of eukaryotic initiation factor 4A-II	46373	IPI00328828	Translation regulator activity
EZR	Ezrin	69378	IPI00643975	Cytoskeletal anchoring activity
ELAVL3	Hu antigen C (HuC)	39547	IPI00031552	RNA binding
FKBP4	FK506-binding protein 4	51772	IPI00219005	Isomerase activity
HNRNPK	Isoform 1 of heterogeneous nuclear ribonucleoprotein K	50944	IPI00216049	Ribonucleoprotein
FSCN1	Fascin	54498	IPI00183187	Structural molecule activity
HNRPA3	Isoform 1 of heterogeneous nuclear ribonucleoprotein A3	39571	IPI00418373	RNA binding
HPRT1	Hypoxanthine-guanine phosphoribosyltransferase	24564	IPI00219492	Enzyme, Ribosyltransferase
HSP90AA1	Isoform 2 of heat shock protein HSP 90-alpha	98069	IPI00382478	Chaperone activity
HSPA1A	Heat shock 70 kDa protein 1	70008	IPI00304925	Chaperone activity
HSPA8	Isoform 1 of heat shock cognate 71 kDa protein	70854	IPI00003865	Heat shock protein activity
KRT8	Keratin, type II cytoskeletal 8	53671	IPI00554649	Structural molecule activity
HSPD1	60 kDa heat shock protein, mitochondrial	61019	IPI00784154	Heat shock protein activity
MDH2	Malate dehydrogenase, mitochondrial	35503	IPI00291009	Catalytic activity
PHB	Putative uncharacterized protein PHB	29723	IPI00793442	Receptor signaling complex scaffold activity
MSN	Putative uncharacterized protein MSN (Fragment)	97645	IPI00872814	Structural constituent of cytoskeleton
RPSAP15	RPSA 32 kDa protein (ribosomal protein SA)	33293	IPI00413109	Cell adhesion molecule activity
RUVBL2	RuvB-like 2	51135	IPI00009104	Transcription regulator activity
VTA1	Vacuolar protein sorting-associated protein VTA1(C6orf59)	33858	IPI00017193	Unknown

Figure 2. Identified autoantigens in LCNEC and SCLC patients. (A) Compared autoantigens identified in sera from patients with LCNEC or SCLC. Thirty-two and 41 autoantigens were identified from LCNEC and SCLC, respectively. The number of approved autoantigens identified both in LCNEC and SCLC was 18. (B) Twenty-three autoantigens including ELAVL3 (HuC) were identified only in SCLC patients.

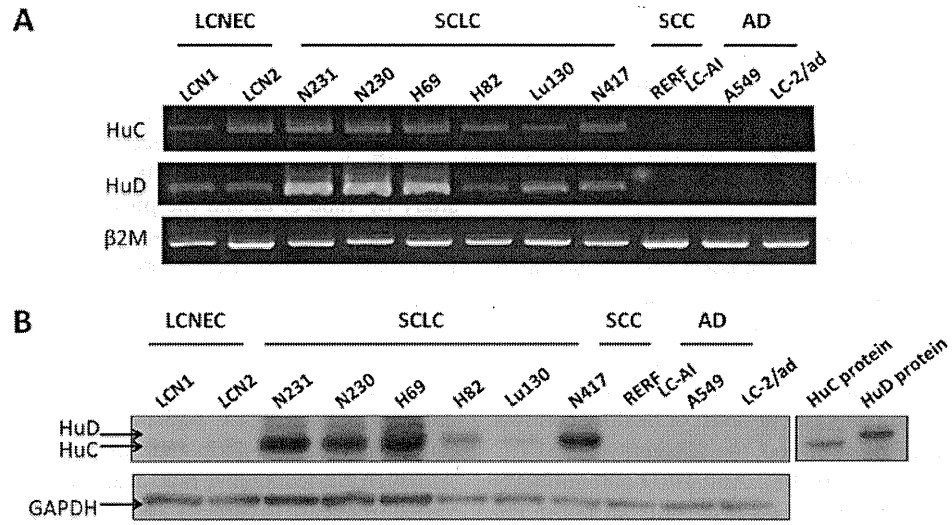


Figure 3. HuC and HuD expressions in lung cancer cell lines. (A) HuC and HuD mRNAs were detected by RT-PCR. Both mRNAs were expressed only in LCNEC and SCLC cell lines. The internal control was beta-2-microglobulin (β2M). (B) HuC protein levels were detected by immunoblot analysis. HuC and HuD recombinant proteins were used as a positive control and GAPDH was used as an internal control. HuC protein was also expressed only in the majority of SCLC and LCNEC cell lines, and HuD protein was expressed only in a part of SCLC cell lines.

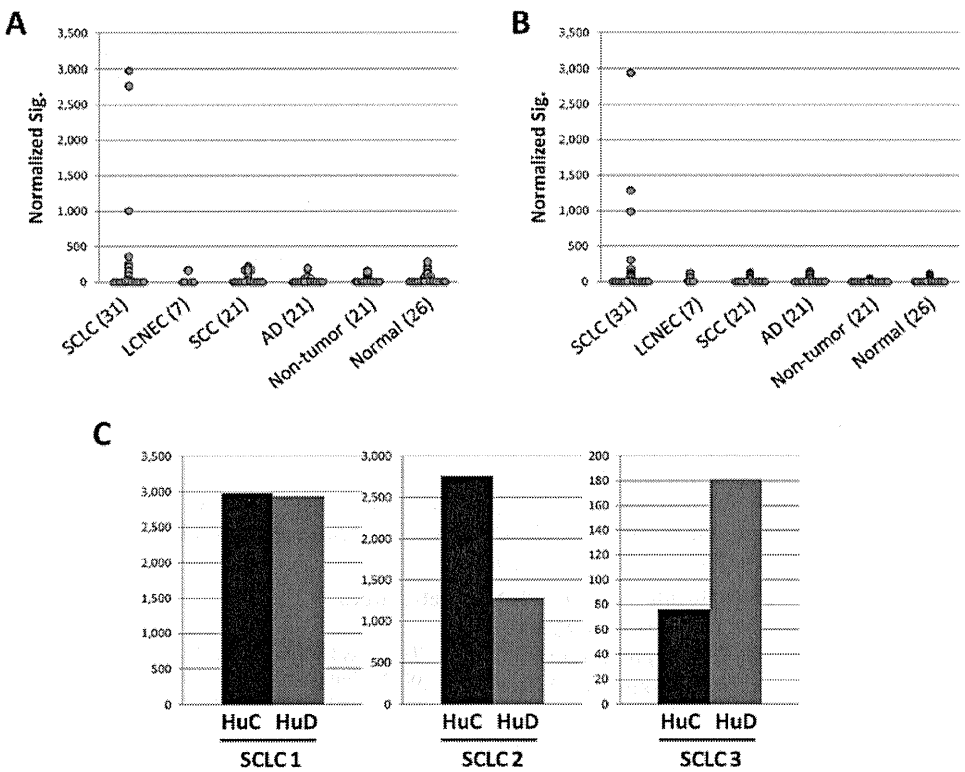


Figure 4. Anti-HuC and -HuD autoantibody levels in sera by dot blot analysis. Anti-HuC autoantibody was detected in 4/31 SCLC patients and not in the others (A). Anti-HuD autoantibody was also detected only in 6/31 SCLC patients (B). The quantitative ratio of anti-HuC and -HuD autoantibodies varied from case to case (C).

were detected only in SCLC, 14 only in LCNEC, and 18 were observed in both.

HuC and HuD expressions in lung cancer cell lines. To examine whether HuC and HuD are specifically expressed in neuroendocrine tumors of the lung, we performed RT-PCR using 11 lung cancer cell lines (Fig. 3A). The expressions of HuC and HuD mRNAs were detected in all neuroendocrine carcinoma-derived cell lines, but not in SCC or AD cell lines.

To confirm that HuC and HuD proteins were also specifically expressed in neuroendocrine tumors, we performed immunoblot analysis using the same 11 lung cancer cell lines (Fig. 3B).

Both proteins were also detected only in neuroendocrine carcinoma-derived cell lines. HuC protein was detected in 5 of 6 SCLC and the two LCNEC cell lines. HuD protein was also detected in 4 of 6 SCLC, but not in the two LCNEC cell lines. These results were generally in accordance with those of mRNA expression analyses by RT-PCR.

Validation of anti-HuC and -HuD autoantibodies. To confirm the utility of anti-HuC and -HuD autoantibodies as potential biomarkers in neuroendocrine carcinoma of the lung, we investigated their levels in patient and control sera by dot blot analysis. Anti-HuC autoantibody was detected in 4 of 31 SCLCs (Fig. 4A), but not in other lung cancer subtypes including LCNEC, non-neoplastic lesions, or healthy controls ($p=0.003$). At a cut-off point of 360, the sensitivity for SCLC was 12.9% (95% CI: 0.036-0.298). Anti-HuD autoantibody was also detected in 6 of 31 SCLCs (Fig. 4B), but not in the others ($p<0.001$). At a cut-off of 176, the sensitivity for SCLC was 19.6% (95% CI: 0.075-0.375). When compared with the others, both anti-HuC and -HuD autoantibodies showed 100% specificity for SCLC. The AUC-ROC levels were 0.577 and 0.602, respectively.

Anti-HuC and -HuD autoantibodies are detected in SCLC patients with and without PEM/SN. In this study, these autoantibodies were detected in patients without rather than with PEM/SN. Furthermore, the quantitative ratio of anti-HuC and -HuD autoantibodies varied from case to case (Fig. 4C).

Discussion

In this study, 55 autoantigens in total were identified employing 2D-immunoblotting. Our results confirmed the utility of this approach to identify tumor-associated antigens including HuC recognized by autoantibodies in sera from patients with lung cancer (12,13).

Hu proteins are a family consisting of four RNA-binding proteins, three of which are normally expressed in the nervous system (22). All four members have three RNA-interacting domains known as RRM (RNA recognition motif) (23). PEM/SN, which occurs in less than 1% of SCLC patients, is related to high titers of autoantibodies for neuronal Hu proteins (24-26). The mechanism by which the immune system identifies Hu proteins from tumor cells as foreign proteins and generates anti-Hu autoantibodies is still unknown. A few studies have focused on the genetic causes of ectopic neuronal Hu gene (ELAV) expression in neuroendocrine tumors and their roles in the onset and progression of such tumors (27,28). Dalmaou *et al* (29) and Graus *et al* (30) reported that approximately 16% of SCLC patients without PEM/SN have detectable levels of anti-Hu autoantibodies in their sera. In this study, both SCLC patients with and without PEM/SN were detected in 12.9% for anti-HuC and 19.4% for -HuD autoantibodies in their sera. This positive rate was almost the same as those reported previously. In a mouse model study, Kazarian *et al* found that anti-Hu reactivity appeared to arise prior to chemical evidence of cancer in these mice, suggesting the possibility of using anti-Hu for the early detection of SCLC (31). The present results on anti-HuC and -HuD autoantibodies supported this possibility, because these autoantibodies were found from limited to extensive diseases. Although the follow-up period was short and only a few controls were used, Tsou *et al* reported that SCLC patients with high levels of anti-Hu reactivity survived for longer than those with low levels ($p=0.08$) (32). In agreement with the study by Tsou *et al* and the present study, Verschuuren *et al* also reported that anti-Hu-positive SCLC cases survived longer, and Dalmau *et al* reported that anti-Hu-positive SCLC patients have relatively limited disease (29,33). Larger scale studies are need to classify the reason for the favorable prognosis of SCLC patients with anti-Hu autoantibodies.

In this study, anti-HuC and -HuD autoantibodies were detected only in SCLC patients, and not in those with other lung cancers, non-neoplastic disease, or healthy controls. Although the quantitative ratio of anti-HuC and -HuD antibodies varied from case to case, 23.3% of SCLC patients were positive for either anti-HuC or anti-HuD or both antibodies. The positive rate may rise using a more sensitive methodology. In spite of this, we detected HuC and HuD mRNAs and proteins in LCNECs, and failed to detect anti-HuC and -HuD autoantibodies in the sera of LCNEC patients, who share many biological features with SCLC patients. Although only a small number of cases were analyzed in a previous study, somatic mutations of the HuD gene were detected in a part of SCLC, TC, and AC cases of different neuroendocrine lung tumors including LCNEC (34). Thus, genetic mutations of HuC and HuD may contribute to the production of autoantibodies.

The present results suggest that anti-Hu autoantibodies are differently expressed between SCLC and LCNEC, and they may be used as novel sero-diagnostic and differential markers for these two tumor types.

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Original Research

LUNG CANCER

Prognostic Significance of Nestin Expression in Resected Non-small Cell Lung Cancer

Shinichiro Ryuge, MD; Yuichi Sato, PhD; Guo Qin Wang, MD; Toshihide Matsumoto, MS; Shi Xu Jiang, MD; Ken Katono, MD; Hayato Inoue, MD; Yukitoshi Satoh, MD; and Noriyuki Masuda, MD, FCCP

Background: **Nestin is a class 6 intermediate filament protein expressed in stem/progenitor cells during CNS development. Nestin expression has been detected in many kinds of tumors and was reported in a recent small-scale study in non-small cell lung cancer (NSCLC). We investigated the relationships between nestin expression and clinicopathologic parameters and determined its prognostic significance concerning survival in patients with resected NSCLC.**

Methods: **Nestin expression in tumor cells was studied immunohistochemically in 171 consecutive patients with NSCLC, and associations with clinicopathologic parameters were evaluated. Kaplan-Meier survival analysis and Cox proportional hazards models were used to estimate the effect of nestin expression on survival.**

Results: **Nestin expression was observed in tumor cell samples in 27 of the 171 patients with NSCLC (15.8%). Nestin had only cytoplasmic expression. Clinicopathologically, nestin expression was significantly associated with squamous cell carcinoma ($P = .001$), poorer differentiation ($P = .007$), lymph node metastasis ($P = .008$), intratumoral vascular invasion ($P = .003$), intratumoral lymphatic invasion ($P = .008$), pleural invasion ($P = .039$), and poorer prognosis ($P < .001$). Multivariable analysis confirmed that nestin expression increased the hazard of death after adjusting for other clinicopathologic factors (hazard ratio, 2.75; 95% CI, 1.39-5.46).**

Conclusions: **The present study suggests that nestin expression is a prognostic indicator of poorer survival probability for patients with resected NSCLC and may be used as a potential marker for select patients who should receive adjuvant chemotherapy.** ***CHEST 2011; 139(4):862–869***

Abbreviations: AD = adenocarcinoma; Hh = hedgehog; HR = hazard ratio; NSCLC = non-small cell lung cancer; p-TNM = pathologic TNM; SCC = squamous cell carcinoma

Primary lung cancer is the leading cause of cancer mortality worldwide.[1] Although surgical resection is the optimal treatment of early-stage, non-small cell lung cancer (NSCLC), 5-year survival rates for surgically resectable NSCLC are still unsatisfactory and range from 19% for stage IIIA to 63% for stage IA.[2] Recurrence causing mortality occurs most commonly in distant extrathoracic regions. Recently, adjuvant cisplatin-based chemotherapy has been recommended to improve survival for patients with NSCLC with completely resected stage II and stage IIIA cancers.[3,4] Although adjuvant chemotherapy shows some improvement in 5-year overall survival ranges, from 4% to 15%, it is also associated with serious adverse side effects.[5,6] Moreover, the benefit of platinum-based adjuvant chemotherapy for patients with stage IB cancer has not been established. Therefore, the identification of predictive and/or prognostic markers is important to stratify patients with resected NSCLCs and to select high-risk patients who should receive aggressive adjuvant chemotherapy.

Nestin is a class 6 intermediate filament protein that is specifically expressed in stem/progenitor cells of the developing CNS.[7] Although little is known about the biologic function of nestin, recent studies have indicated that nestin may play an important role in the distribution and organization of critical cellular factors involved in regulating cell proliferation, survival, and differentiation.[8,9] Although nestin is expressed in the dividing cells of the CNS and myogenic tissues during the early stages of development, its expression becomes rapidly downregulated and is replaced by

tissue-specific intermediate filaments upon differentiation.[7,10] However, nestin could be re-expressed in adult tissues under pathologic conditions, such as in the formation of the glial scar after injury to the CNS, regeneration of injured skeletal muscle tissue, and CNS tumors.[11-13] Moreover, recent studies have shown that nestin is also expressed in epithelial tumors, such as pancreatic cancer,[14] prostate cancer,[15] and breast cancer.[16] Kawamoto et al[14] reported that nestin expression in tumor cells might contribute to nerve and stromal invasion in pancreatic cancer. In prostate cancer, nestin is identified as a critical component of a novel pathway for metastasis.[15] In breast cancer, nestin is a selective marker for basal-like and triple-negative ($ER^-/PR^-/HER2^-$) breast cancer, and its expression is associated with tumor aggressiveness.[16] Nestin is localized in the cytoplasm of tumor cells[13-16] and Leydig cells[17] in these studies.

Nestin has been detected in small cell lung cancer cell lines,[15,18] and one recent research study has revealed nestin expression in the nuclei of tumor cells in NSCLC.[19] To our knowledge, no report has been published concerning the relationships between nestin expression and clinicopathologic features and patients' prognoses in a large number of NSCLC cases. Therefore, the objectives of this study were (1) to immunohistochemically examine nestin expression in tumor cells with samples of 171 patients with NSCLC, (2) to evaluate the relationships between nestin expression in tumor cells and the clinicopathologic parameters of NSCLC, and (3) to estimate the prognostic impact of nestin on survival in patients with resected NSCLC.

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Materials and Methods

Patients and Tissue Specimens

A total of 171 consecutive patients with NSCLC who underwent complete resection from January 2002 to September 2004 at the Kitasato University Hospital were included in this retrospective cohort study. Patients were excluded if they received preoperative chemotherapy and/or radiotherapy. Ten percent formalin-fixed and paraffin-embedded samples were collected from all the patients, and 3-μm-thick sections were stained with hematoxylin and eosin. The histologic diagnosis was based on the criteria of the World Health Organization/International Association for the Study of Lung Cancer classification of lung and pleural tumors.[20] Each case was reassigned for TNM classification and pathologic stage on the basis of the new International Association for the Study of Lung Cancer staging system.[21] The following clinical and pathologic parameters were reviewed retrospectively and analyzed for each case: age at surgical resection, gender, smoking habits, histologic type, tumor differentiation, pathologic TNM (p-TNM) stage, nodal status, intratumoral vascular invasion, intratumoral lymphatic invasion, pleural invasion, received adjuvant chemotherapy, viability status, and survival time after surgery. Viability status was determined based on whether NSCLC-related death occurred, and survival time was defined as the duration from the date of surgery to the date of death or the end of the follow-up. We treated all other causes of death and lost to follow-up as censored cases. The study was approved by the ethics committee of Kitasato University School of Medicine. Appropriate informed consent was obtained from all patients.

Immunohistochemical Staining for Nestin

Three-micrometer-thick sections were deparaffinized in xylene, rehydrated in a descending ethanol series, and then treated with 3% hydrogen peroxide for 10 min. After blocking with 0.5% casein for 10 min, the sections were reacted with 100-times-diluted antinestin polyclonal antibody (IBL; Takasaki, Japan) for 2 h at room temperature. The specificity of this antibody has been described previously.[22] After rinsing in tris-buffered saline (0.01 M Tris HCl pH 7.5, 150 mM NaCl) three times for 5 min each, the sections were reacted with Histofine Simple Stain MAX-PO (MULTI) (Nichirei; Tokyo, Japan) for 30 min at room temperature. The sections were visualized subsequently with stable DAB solution (Invitrogen; Carlsbad, California) and counterstained with Mayer hematoxylin. Negative controls were prepared by substituting phosphate-buffered saline for antinestin antibody.

Evaluation of Immunohistochemical Staining

For nestin, cytoplasmic immunostaining in tumor cells was considered to be positive. Nestin-positive nonneoplastic cells, such as immature fibroblasts, were excluded carefully. The stainability of peritumoral vascular endothelial cells was used as an internal positive control. Staining intensity was categorized into four groups by comparing the staining intensity of tumor cells with vascular endothelial cells: 0 = negative; 1 (weak) = weaker than endothelial cells; 2 (moderate) = the same as endothelial cells; 3 (strong) = stronger than endothelial cells. The tumors with a staining score of 2 or 3 were judged as positive. Because in most nestin-positive cases >5% of the tumor cells are usually recognized, the tissues consisting of >5% positive tumor cells were considered significant. Two investigators (S. R. and Y. Sato) separately evaluated all the specimens in a blinded manner. Variant cases were reviewed and discussed until a consensus was obtained for each of the specimens.

Statistical Analysis

Continuous variables were presented as median (range), whereas numeric variables were given as No. (%). The relationships between nestin expression and clinicopathologic parameters were assessed by Pearson χ^2 test or Fisher exact test, as appropriate. Cumulative survival of patients was estimated using the Kaplan-Meier method, and statistical significance of the difference of the survival rate between the nestin-positive and nestin-negative groups was tested using the log-rank test. For the Kaplan-Meier estimate of the survival curves, we truncated the data at a follow-up period of 5 years to avoid the number at risk being too small. Those with a survival time of > 5 years were reported to be 5 years, and events occurring after the end of the 5-year follow-up period were computed as censored data. Five-year cumulative survival probability was estimated using the life table method with the interval length set at 1 month. Mulivariable analysis was performed by employing the Cox proportional hazards regression model to examine the interaction between nestin expression and other clinicopathologic variables and to estimate the independent prognostic effect of nestin on survival by adjusting for confounding factors. Within the present study population, there were 53 lung cancer-related deaths, which allows a maximum of five variables to be included in a multivariable regression model. To avoid overfitting, all potential confounding factors of nestin expression were reduced to one single composite characteristic by applying a propensity score.[23] The conventional P value $\leq .05$ was used to determine the level of statistical significance. All reported P values are two sided. Analyses were performed independently at our clinical research center using SPSS software, version 17.0 (SPSS Inc; Chicago, Illinois).

Results

Patient Characteristics

The clinicopathologic characteristics of the patients are summarized in Table 1. A total of 107 men and 64 women were included, with ages ranging from 34 to 85 years (median, 64 years), of which 109 (63.7%) were smokers. There were 94 (55%) stage I (59 stage IA and 35 stage IB), 35 (20.5%) stage II (19 stage IIA and 16 stage IIB), and 42 (24.5%) stage III (36 stage IIIA and six stage IIIB) diseases, including 131 (76.6%) adenocarcinomas (ADs), 31 (18.1%) squamous cell carcinomas (SCCs), five (2.9%) large cell neuroendocrine carcinomas, three (1.8%) large cell carcinomas, and one (0.6%) adenosquamous carcinoma. Sixteen patients (9.4%) received adjuvant chemotherapy. The overall follow-up durations ranged from 3 to 91 months (median, 62 months). A total of 100 patients were alive at the end of the follow-up, 53 patients died of lung cancer, 11 patients died of other causes, and seven patients were lost to follow-up. In 11 other causes of death, the causes of death were idiopathic interstitial pneumonia (n = 4), pneumonia (n = 3), COPD (n = 1), cerebral infarction (n = 1), cholangiocellular carcinoma (n = 1), and gastric cancer (n = 1). None of these 11 patients died a surgery-related death. In seven lost-to-follow-up patients, all patients were lost to follow-up because of discontinued hospital attendance and inability to be contacted. The follow-up durations of these patients ranged from 12 to 52 months (median, 33 months).

Table 1—*Characteristics of the Patients*

Characteristics	Patients (N = 171)
Age	
Median age, y (range)	64 (34-85)
< 65 y	89 (52.0)
≥ 65 y	82 (48.0)
Gender	
Male	107 (62.6)
Female	64 (37.4)
Smoking habits	
NS	62 (36.3)
S	109 (63.7)
Histologic type	
AD	131 (76.6)
SCC	31 (18.1)
Others	9 (5.3)
Tumor differentiation	
Well/moderately	129 (79.1)
Poorly	34 (20.9)
p-TNM stage[a]	
Stage I	94 (55.0)
Stage II	35 (20.5)
Stage III	42 (24.5)
Receiving adjuvant chemotherapy	
Yes	16 (9.4)
No	155 (90.6)
Vital status	
Alive	100 (58.5)
Lung cancer-related death	53 (31.0)
Other causes of death	11 (6.4)
Unknown	7 (4.1)

Data are presented as No. (%) unless otherwise indicated. AD = adenocarcinoma; NS = never smoker; p-TNM = pathologic TNM; S = smoker; SCC = squamous cell carcinoma.

[a]Each case was reassigned for pathologic stage on the basis of the International Association for the Study of Lung Cancer Lung Cancer Staging Project (seventh edition).[21]

Nestin Expression in NSCLC

Cytoplasmic nestin expression in tumor cells was observed in 27 of the 171 NSCLC samples (15.8%) (Fig 1). They were further divided into 13 of 131 (9.9%) ADs, 11 of 31 (35.5%) SCCs, and three of five (60%) large cell neuroendocrine carcinomas, respectively. Nestin had only cytoplasmic expression. Nestin expression was also observed in the cytoplasm of vascular endothelial cells and fibroblasts in tumor stroma in each case. Nestin expression was not detected in nonneoplastic bronchial or alveolar epithelial cells. No expression was observed in the negative controls.

Relationships Between Nestin Expression and Clinicopathologic Characteristics

The relationships between nestin expression and clinicopathologic characteristics are summarized in

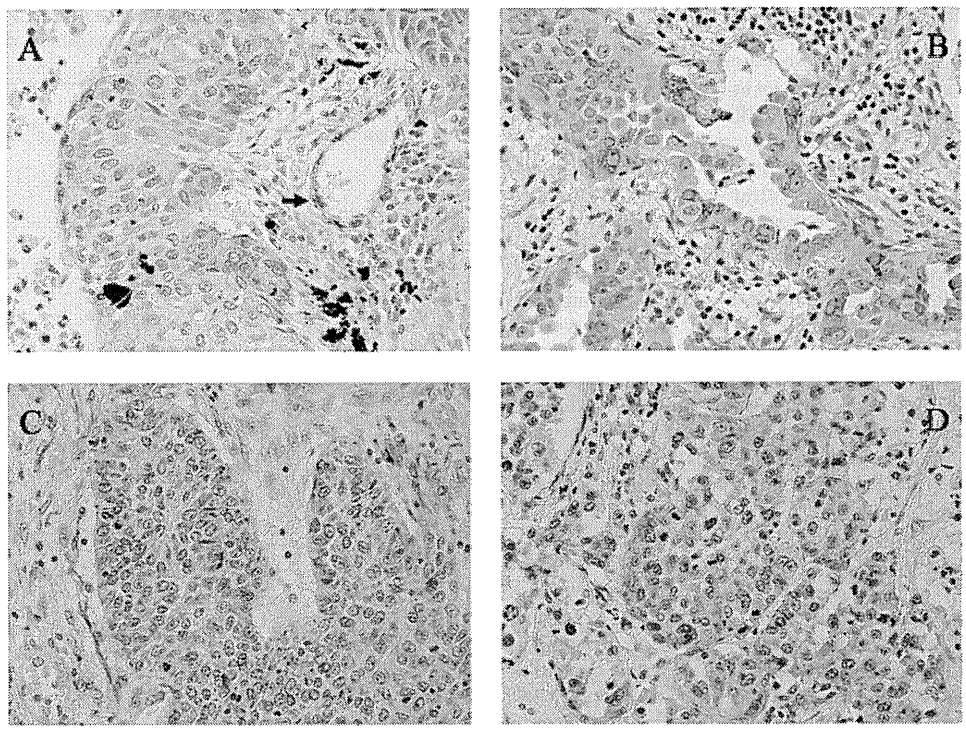

FIGURE 1. Immunohistochemical stain analysis of nestin expression in non-small cell lung cancer (NSCLC). Nestin was expressed in vascular endothelial cells (arrows). The stainability of peritumoral vascular endothelial cells was used as an internal control. A, Some tumor cells were observed in weak to moderate cytoplasmic staining (original magnification ×200). B, Adenocarcinoma (original magnification ×200). C, Squamous cell carcinoma (original magnification ×200). D, Large cell neuroendocrine carcinoma (original magnification ×200).

Table 2. Nestin expression was detected more frequently in SCC than in AD and other histologic subtypes ($P = .001$). Nestin expression was also related to poorer differentiation ($P = .007$), lymph node metastasis ($P = .008$), intratumoral vascular invasion ($P = .003$), intratumoral lymphatic invasion ($P = .008$), and pleural invasion ($P = .039$), whereas there was no significant association between nestin expression and age, gender, smoking habits, or p-TNM stage.

Kaplan-Meier Estimate of Survival for Patients With Nestin-Positive and Nestin-Negative Results

All the patients were included in the survival analysis. The overall follow-up periods ranged from 3 to 91 months (median, 62 months), and the mean survival time was 49.7 months, corresponding to a 5-year follow-up. Because a cumulative survival probability of 50% was not yet reached by the end of the 5-year follow-up, the overall median survival time was not determined. Five-year cumulative survival probability was 33% for the nestin-positive group and 77% for the nestin-negative group. The median survival time was 37.6 months for patients who had nestin-positive results; however, it was not available for patients who had nestin-negative results at the end of the 5-year follow-up period, indicating a significantly poorer rate of survival in the nestin-positive group compared with that in the nestin-negative group ($P < .001$) (Fig 2A). In further analyses, nestin expression was significantly associated with poorer survival for patients with stage II/III cancer ($P = .026$) (Fig 2B) and for patients with stage I cancer ($P < .001$) (Fig 2C). Five-year survival probability was 27% for patients who had nestin-positive results vs 57% for patients who had nestin-negative results with stage II/III cancer, and 42% vs 91%, respectively, in patients with stage I cancer.

Effect of Nestin Expression on Survival With Multivariable Analysis

A Cox proportional hazards model was applied to estimate the effect of nestin expression on survival. The crude hazard ratio (HR) of nestin-positive status compared with nestin-negative status was 4.19 (95% CI, 2.35-7.46; $P < .001$), which indicated that nestin-positive status increased the hazard of lung cancer-related death by four times that of nestin-negative status. With multivariable analysis, nestin expression, p-TNM stage, and adjuvant chemotherapy were significantly associated with survival. After controlling for

Table 2—*Relationship Between Nestin Expression and Clinicopathologic Parameters*

Clinicopathologic Parameters	Nestin Expression: Positive (n = 27)	Nestin Expression: Negative (n = 144)	Total	*P* Value
Age, y				.427
< 65	12 (13.6)	76 (86.4)	88	
≥ 65	15 (18.1)	68 (81.9)	83	
Gender				.178
Male	20 (18.7)	87 (81.3)	107	
Female	7 (10.9)	57 (89.1)	64	
Smoking habits				.098
NS	6 (9.7)	56 (90.3)	62	
S	21 (19.3)	88 (80.7)	109	
Histologic type				.001
AD	13 (9.9)	118 (90.1)	131	
SCC	11 (35.5)	20 (64.5)	31	
Others	3 (33.3)	6 (66.7)	9	
Tumor differentiation				.007
Well/moderately	14 (10.9)	115 (89.1)	129	
Poorly	10 (29.4)	24 (70.6)	34	
p-TNM stage[a]				.105
Stage I	11 (11.7)	83 (88.3)	94	
Stage II/III	16 (20.8)	61 (79.2)	77	
Nodal status				.008
N0	12 (10.5)	102 (89.5)	114	
N1/N2/N3	15 (26.3)	42 (73.7)	57	
Vascular invasion				.003
Yes	18 (22.5)	62 (77.5)	80	
No	5 (6.3)	75 (93.7)	81	
Lymphatic invasion				.008
Yes	14 (25.9)	40 (74.1)	54	
No	9 (9.5)	86 (90.5)	95	
Pleural invasion				.039
Yes	14 (23.7)	45 (76.3)	59	
No	13 (11.6)	99 (88.4)	112	
Adjuvant chemotherapy				.005
Yes	7 (43.8)	9 (56.2)	16	
No	20 (12.9)	135 (87.1)	155	

Data are presented as No. (%). See Table 1 legend for expansion of abbreviations.

[a]Each case was reassigned for pathologic stage on the basis of the International Association for the Study of Lung Cancer Lung Cancer Staging Project (seventh edition).[21]

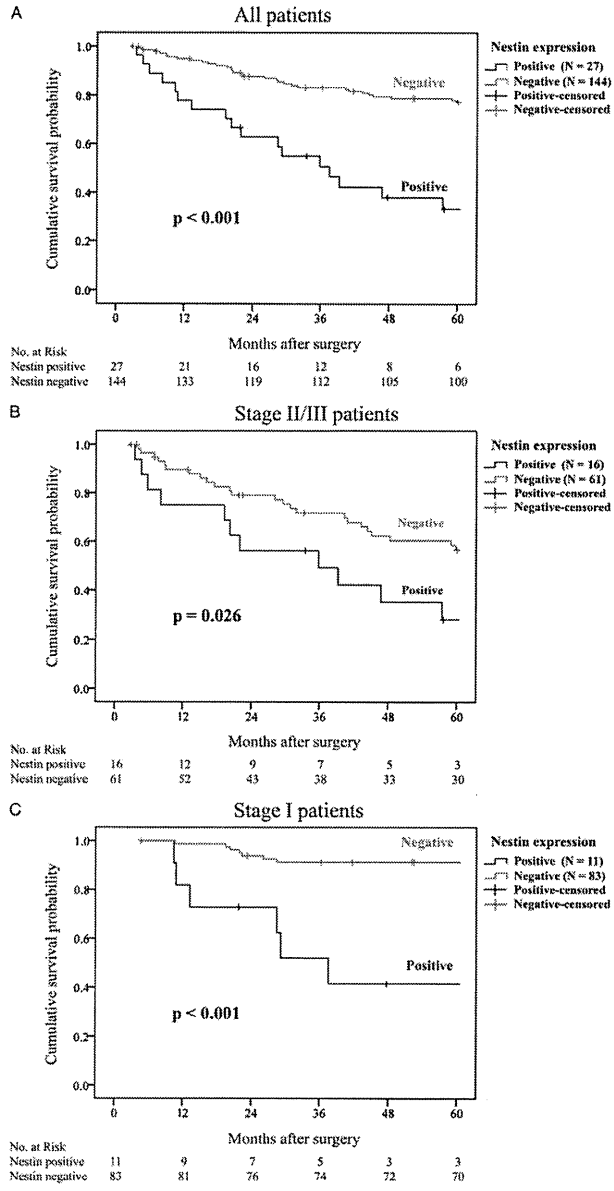

FIGURE 2. Cumulative survival of patients with NSCLC according to nestin expression estimated by the Kaplan-Meier method. Nestin expression was significantly associated with poorer survival in resected NSCLC. A, For all patients. B, For patients with stage II cancer and stage III cancer. C, For patients with stage I cancer. All other causes of death and lost to follow-up were treated as censored cases. See Figure 1 legend for expansion of abbreviation.

the effects of clinicopathologic factors, including age, gender, smoking habits, histologic type, p-TNM stage, and receiving adjuvant chemotherapy, the adjusted HR of nestin-positive status became 2.54 (95% CI, 1.30-4.94; $P = .006$) in comparison with nestin-negative status. We also performed an analysis by using a propensity score to adjust the effect of nestin expression by transforming all other confounding variables into a single estimator, and revealed that after the adjustment, the HR of nestin expression became 2.75 (95% CI, 1.39-5.46; $P = .004$) (Table 3). These findings suggest that nestin positive seems to be an independent and significant predictor of poorer survival.

Other factors, such as tumor differentiation, vascular invasion, lymphatic invasion, and pleural invasion, have been considered very important prognostic factors for survival. With respect to the effect of nestin on survival, these might be intermediate factors on the path of the nestin-survival relationship, such that nestin expression may first affect these factors, which in turn affect survival. Thus, adjusting for them may underestimate the effect of nestin. However, given the fact that the biologic relationships between nestin and these four factors have not yet been elucidated, in further analysis, tumor differentiation, vascular invasion, lymphatic invasion, and pleural invasion were controlled, in addition to those already evaluated, by

Table 3—*Univariable and Multivariable Analysis for the Effect of Nestin Expression on Survival*

	Univariable Analysis			Multivariable Analysis								
				Model 1[a]			Model 2[b]			Model 3[c]		
Factors	HR	95% CI	*P* Value	HR	95% CI	*P* Value	HR	95% CI	*P* Value	HR	95% CI	*P* Value
Nestin expression												
Positive vs negative	4.18	2.34-7.46	< .001	2.54	1.30-4.94	.006	2.75	1.39-5.46	.004	2.50	1.11-5.63	.026
Age												
≥ 65 y vs < 65 y	1.07	0.62-1.84	.80	1.40	0.79-2.48	.24	n/d	n/d	n/d	n/d	n/d	n/d
Gender												
Male vs female	1.73	0.96-3.11	.06	1.04	0.43-2.48	.92	n/d	n/d	n/d	n/d	n/d	n/d
Smoking habits												
S vs NS	2.03	1.10-3.75	.02	1.43	0.59-3.46	.42	n/d	n/d	n/d	n/d	n/d	n/d
Histologic type												
Non-AD vs AD	2.82	1.60-4.96	< .001	1.35	0.68-2.68	.37	n/d	n/d	n/d	n/d	n/d	n/d
p-TNM stage[d]												
Stage II/III vs stage I	4.04	2.22-7.36	< .001	2.63	1.34-5.16	.005	n/d	n/d	n/d	n/d	n/d	n/d
Adjuvant chemotherapy												
No vs yes	4.08	2.13-7.83	< .001	2.17	1.02-4.59	.04	n/d	n/d	n/d	n/d	n/d	n/d
Tumor differentiation												
Poorly vs well/ moderately	2.83	1.53-5.21	.001	n/d	n/d	n/d	n/d	n/d	n/d	n/d	n/d	n/d
Vascular invasion												
Yes vs no	10.9	4.66-25.8	< .001	n/d	n/d	n/d	n/d	n/d	n/d	n/d	n/d	n/d
Lymphatic invasion												
Yes vs no	5.00	2.67-9.36	< .001	n/d	n/d	n/d	n/d	n/d	n/d	n/d	n/d	n/d
Pleural invasion												
Yes vs no	2.39	1.39-4.10	.002	n/d	n/d	n/d	n/d	n/d	n/d	n/d	n/d	n/d
Propensity score	n/d	n/d	n/d	n/d	n/d	n/d	0.13	0.03-0.53	.004	0.03	0.007-0.17	< .001

Analyses were performed using Cox proportional hazard regression. HR and 95% CI of the univariable analysis indicate the unadjusted effect of each of the clinicopathologic factors on survival. HR = hazard ratio; n/d = not done. See Table 1 legend for expansion of other abbreviations.

[a]Multivariable Model 1 indicates the adjusted effect of nestin by controlling age, gender, smoking habits, histologic type, p-TNM stage, and adjuvant chemotherapy.

[b]Multivariable Model 2 indicates the adjusted effect of nestin by applying propensity score which is a conditional probability of expressing nestin given by other clinicopathologic factors including age, gender, smoking habits, histologic type, p-TNM stage, and adjuvant chemotherapy.

[c]Multivariable Model 3 indicates the adjusted effect of nestin by applying propensity score additionally controlled for other variables including tumor differentiation, vascular invasion, lymphatic invasion, and pleural invasion.

[d]Each case was reassigned for pathologic stage on the basis of the International Association for the Study of Lung Cancer Lung Cancer Staging Project (seventh edition).[21]

applying a propensity score adjustment. The results revealed that after the adjustment, the HR of nestin changed to 2.50 (95% CI, 1.11-5.62; $P = .026$), which suggests that nestin expression may remain an independent risk factor for poorer survival after additionally controlling these four factors (Table 3).

Discussion

In the present study, we have demonstrated that nestin expression seems to be associated with poorer prognosis and is an independent prognostic factor for survival in patients with resected NSCLC. Although nestin was detected in both well/moderately and poorly differentiated tumors, its expression incidence was significantly higher in poorly differentiated ones ($P = .007$). Our results are in accordance with the previous finding that nestin expression is maintained in immature tissues and down-regulated during differentiation.[7,10] Moreover, in agreement with previous reports of pancreatic and prostate cancers,[14,15] our findings revealed that nestin expression was significantly associated with intratumoral vascular invasion, intratumoral lymphatic invasion, pleural invasion, and nodal status in NSCLC. Previous reports and the present study suggest that nestin expression may be important for the acquisition of migration and invasion capabilities of tumor cells, which subsequently results in poorer prognoses in patients with resected NSCLC.

A previous study reported that nestin was expressed in the mesenchymal stem cells of human fetal lungs.[24] However, in the present study, nestin expression was not observed in mature bronchial or alveolar epithelial cells in nonneoplastic peripheral lung tissues, but was detected only in tumor cells. Despite the fact

that only a small population of tumor cells was positive for nestin in most cases of the present study, nestin expression in these tumors may have imitated its early and transient expression pattern during fetal development. The small population of nestin-positive tumor cells in each tumor may thus represent those tumor cells with more immature natures, similar to the stem/progenitor cells of fetal development, and nestin-positive tumors may thus have more aggressive behavior resulting from higher abilities of tumor cell migration and invasion. This raises the question of how nestin-positive tumor cells could acquire and maintain the properties of stem/progenitor cells. A possible explanation could be the involvement of the hedgehog (Hh) signaling pathway. Beachy et al[25] suggested that Hh-dependent tumors may be derived at least partly from cancer progenitor cells. Several studies have reported that nestin expression is dependent on the activation of the Hh signaling pathway in Hh-dependent tumors,[26,27] including small cell lung cancer.[18] Although the involvement of the Hh signaling pathway in NSCLC has been controversial, Yuan et al[28] reported that a subset of NSCLC has also been found to be constitutively active for the Hh signaling pathway independent of the ligands by expressing high levels of GLI 1 protein. However, because the relationships between nestin expression and Hh signaling in NSCLC remain unclear, further studies are required to clarify this hypothesis.

Chen et al[19] reported that nestin was expressed in 45 of 52 NSCLC (86.5%), most of which were located in the nuclei of tumor cells. High nestin expression was significantly associated with poorer differentiation, AD, and N2 lymph node metastasis. Although there were some differences between our results and those of the study by Chen et al,[19] the most essential difference is the localization of nestin expression in tumor cells. In the present study, all 27 nestin-positive tumors showed in the cytoplasm of tumor cells. Given that nestin is an intermediate filament protein as one component of cytoskeleton, it is reasonable to deduce its cytoplasmic location in tumor cells. Our results are consistent with findings in other studies in which nestin was located in the cytoplasm of most tumor cells.[13-16,29] On the other hand, the patients' prognoses in our study and those in the study by Chen et al[19] were similar. This might be explained by assuming that cytoplasmic nestin-positive tumor cells were positive in our study and were included in the nestin-high-expression group in the study by Chen et al.[19]

Customized or individualized adjuvant chemotherapy may be indicated by identifying prognostic and predictive biomarkers such as p53[29] and ERCC1,[30] and prognostic biomarkers such as the lung metagene model.[31] Within our study population, we have demonstrated that nestin expression is a new independent prognostic marker for patients who have undergone resections for NSCLC. The results among patients with stage I cancer and stage II/III cancer were consistent, and nestin positivity decreased survival probability, especially among the stage I subgroup. In the 11 patients who were nestin-positive with stage I cancer, there were three patients with stage IA cancer and eight patients with stage IB cancer. Nestin expression could serve as a useful marker to stratify high-risk patients who should receive adjuvant chemotherapy, especially among patients with stage I cancer with resected NSCLC. However, further studies are warranted to determine whether nestin expression is a prognostic indicator to help select patients who might benefit from receiving adjuvant chemotherapy. In prostate cancer, nestin-positive tumor cells are detected not only in primary lesions but also in metastatic ones.[15] Thus, it seems likely that nestin-positive tumor cells are directly involved in micrometastasis. The aim of adjuvant chemotherapy in patients with resected NSCLC is to eradicate micrometastasis, reduce the risk of recurrence, and improve survival. Regarding drug resistance in cancer cells, it is suggested that cancer stem cells are resistant to chemotherapy through their quiescence, their capacity for DNA repair, and their adenosine triphosphate-binding cassette transporter expression.[32] Although we have not demonstrated the relationships between nestin expression and chemotherapy sensitivity, given that nestin-positive tumor cells possess features of cancer progenitor/stem cells, those nestin-positive tumor cells may be resistant to chemotherapy.

Conclusions

In conclusion, we have reported that nestin is expressed in a subset of NSCLC and its expression is related to clinicopathologic factors. We demonstrated that nestin expression is a prognostic indicator of poor survival among patients with resected NSCLC, although its prognostic significance still requires confirmation with larger patient populations.

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Dr Ryuge: contributed to the design, analysis, and interpretation of the data, and the writing and revision of the manuscript.
Dr Sato: contributed to the study concept and design and the writing and revision of the manuscript.
Dr Wang: contributed to the data analysis and the writing and revision of the manuscript.
Mr Matsumoto: contributed to the study concept and design.
Dr Jiang: contributed to the interpretation of the data and the writing and revision of the manuscript.
Dr Katono: contributed to the collection and interpretation of the data.
Dr Inoue: contributed to the study concept and design.

Dr Satoh: contributed to the collection and interpretation of the data.
Dr Masuda: contributed to the collection and interpretation of the data.
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The balance between the expressions of hASH1 and HES1 differs between large cell neuroendocrine carcinoma and small cell carcinoma of the lung

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ABSTRACT

To clarify the biological differences between small cell lung carcinoma (SCLC) and large cell neuroendocrine carcinoma (LCNEC), we investigated the expression of two bHLH type transcription factors, human achaete-scute homolog 1 (hASH1) and hairy/enhancer of split 1 (HES1), which positively and negatively regulate the neuroendocrine differentiation of respiratory epithelial cells, respectively. Eighty-eight formalin-fixed and paraffin-embedded pulmonary carcinomas (32 SCLC, 32 LCNEC, 14 adenocarcinomas, and 10 squamous cell carcinomas) and 14 SCLC and 1 LCNEC derived cell lines were used. hASH1 and HES1 mRNA were detected using a highly sensitive *in situ* hybridization method with digoxigenin-labeled cRNA probes and biotinylated tyramide. The staining results were scored from 0 to 12 by multiplying the staining intensity by the percentage of positive tumor cells. The mean staining score of hASH1 mRNA was significantly higher in SCLC than in LCNEC ($p < 0.01$); conversely, that of HES1 mRNA was lower in SCLC than in LCNEC ($p < 0.01$). These findings reveal that SCLC more strongly expresses the neuroendocrine phenotype, while LCNEC shows characteristics more similar to the ciliated epithelium phenotype, suggesting that the biological characteristics of these two tumors are different.

1. Introduction

It is now widely recognized that neuroendocrine tumors of the lung range from low-grade typical carcinoid (TC) and intermediate-grade atypical carcinoid (AC) to high-grade small cell lung carcinoma (SCLC) and large cell neuroendocrine carcinoma (LCNEC) [1,2]. Generally, neuroendocrine tumors share some common morphological characteristics such as organoid structures, a palisading basal cell arrangement, and rosette formation. In 1991, Travis et al. introduced the term LCNEC to describe a distinct entity of high-grade neuroendocrine tumors with light microscopic characteristics that differ from those of high-grade SCLC [1]. Although LCNEC is considered to be a disease entity that is distinct from SCLC, these two types of lung cancer share a great number of features, such as similar gene expression profiles and clinical characteristics [2–4]. Pro-gastrin-releasing peptide (pro-GRP) is a specific serodiagnostic marker of SCLC [5]; however, its positivity ratio is low in the early stages of SCLC, and no specific markers have been developed for LCNEC. Many studies have reported that no significant differences in outcome between LCNEC and SCLC patients were observed [3,6,4], and it is difficult to differentiate LCNEC from SCLC, and no definitive discrimination points except for its morphological characteristics and the details of its biological behavior (including tumor aggressiveness and the degree of differentiation) have been established.

Transcriptional factors play an important role in the neuroendocrine differentiation of immature respiratory epithelial cells, and two basic-helix–loop–helix transcription factors, achaete-scute complex homolog 1 (ASCL1, termed hASH1 in humans, MASH1 in rodents) and hairy/enhancer of split 1 (HES1), regulate neuronal differentiation positively and negatively, respectively [7,8]. hASH1 plays a crucial role in neural commitment and differentiation [9] and is selectively expressed in normal fetal pulmonary neuroendocrine cells and lung cancers with neuroendocrine features [7,10].

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Disruption of the MASH1 gene affects neuroendocrine differentiation during fetal development, resulting in the loss of pulmonary neuroendocrine cells [11].

HES1, a key effector of the Notch signaling pathway, is expressed broadly in non-neuroendocrine cells in the airway epithelium [12]. HES1 directly represses hASH1 expression by binding to a class C site in the hASH1 promoter [13]. In the developing lung, Notch1 and HES1 are strongly expressed in non-neuroendocrine airway epithelial cells, whereas MASH1 is restricted to clustered pulmonary neuroendocrine cells [11,12]. In the mice deficient in HES1, the number of pulmonary neuroendocrine cells increased markedly, and MASH1 was overexpressed [12]. Conversely, MASH1 gene knockout disrupted neuroendocrine differentiation and resulted in the loss of pulmonary neuroendocrine cells [11]. Consistent with its well-established role in inhibiting commitment and differentiation in neuronal precursors, Notch signaling appears to play a critical role in restricting neuroendocrine cell development within the airway epithelium. In human tumors, it is reported that SCLC cells express hASH1 but lack HES1 expression. On the contrary, non-neuroendocrine carcinoma cells do not express hASH1, but show high HES1 expression [13]. However, no studies have been carried out to examine the expression levels and the balance between the expression levels of hASH1 and HES1 in LCNEC and SCLC. In this study, we compared the expression levels of hASH1 and HES1 mRNA in both tumors using a highly sensitive *in situ* hybridization (ISH) method employing biotinylated tyramide.

2. Materials and methods

2.1. Cell lines

Eleven classical (Lu24, Lu130, Lu134A, Lu135C, Lu139, Lu140, Lu143, Lu165, N230, N231, and H69) and three variant (H82, N417, and Lu135v) SCLC cell lines and a LCNEC cell line (LCN1) [14] were used in this study. The SCLC cells were donated by Dr. Hirohashi (National Cancer Center, Japan). Each cell line was grown in RPMI1640 (Sigma, Steinheim, Germany) supplemented with 10% fetal bovine serum (Biowest, Miami, FL, USA), 100 U/mL penicillin, and 100 μg/mL streptomycin (Gibco, Auckland, New Zealand). The harvested cells were washed with phosphate-buffered saline without bivalent ions and were fixed in 10% formalin and embedded in paraffin for *in situ* hybridization or were stored at −80 °C until their use in the reverse transcription-polymerase chain reaction (RT-PCR).

2.2. Tumor tissues

Eighty-eight formalin-fixed and paraffin-embedded pulmonary carcinomas, which were surgically resected at Kitasato University Hospital, were used in the present study. They were divided into 32 SCLC, 32 LCNEC, 14 adenocarcinomas (AD), and 10 squamous cell carcinomas (SCC). This study was approved by the Ethical Committee of Kitasato University School of Medicine. All patients were informed of the aim of the study and gave their consent for their samples to be used.

2.3. RT-PCR

Total RNA were extracted from the fifteen cell lines with Isogen (Nippon Gene, Tokyo, Japan) and reverse transcribed with the First Strand cDNA Synthesis kit (Roche Diagnostics, Mannheim, Germany) according to the manufacturer's instructions. The primers used for the PCR were designed with Oligo Primer Analysis Software, version 6.0 (Takara Bio, Otsu, Japan) according to defined hASH1 mRNA (accession number: NM_004316), HES1 mRNA (accession number: NM_005524), and β-2-microglobulin (β_2M) mRNA sequences (accession number: NM_004048) (Table 1). PCR amplification was performed using Taq DNA polymerase (Roche Diagnostics) and the PCR system 2700 (Applied Biosystems, Warrington, UK). The PCR consisted of denaturing at 94 °C for 5 min, followed by 30 cycles of 30 s at 94 °C, 30 s at 55 °C, and 1 min at 72 °C and a final extension step of 7 min at 72 °C.

Table 1
Primer sets used for the reverse transcription-polymerase chain reaction.

hASH1	
Sense primer	5′-TCTCCCCCAACTACTCCAAC-3′
Antisense primer	5′-CGCGTGTGCTGCTCCCTTCT-3′
HES1	
Sense primer	5′-GTCAACACGACACCGGATAAA-3′
Antisense primer	5′-GCGGGTCACCTCGTTCA-3′
β-2-Microglobulin	
Sense primer	5′-TTCTGGCCTGGAGGGCATCC-3′
Antisense primer	5′-ATCTTCAAACCTCCATGATG-3′

hASH1, human achaete-scute homolog 1; HES1, hairy/enhancer of split 1.

2.4. Preparation of the cRNA probes

For non-radioactive ISH, digoxigenin (DIG)-labeled cRNA probes were generated with T7 RNA polymerase promoter region (CTTAATACGACTCACTATAGGG)-tailed PCR, as described in our recent study [15]. The PCR conditions were the same as those used for the RT-PCR, and the core sequences of the primers used were the same as those of the primers used for the RT-PCR mentioned above without the T7 RNA polymerase promoter sequence. The PCR products were transcribed *in vitro* using T7 RNA polymerase and labeled with DIG-dUTP using a DIG *in vitro* transcription kit (Roche Diagnostics) to produce sense and antisense cRNA probes. The specificity of the PCR-generated templates was confirmed by direct sequencing with a cycle sequencing method (Big Dye Terminator cycle sequencing kit, Applied Biosystems).

2.5. Highly sensitive ISH

The ISH was carried out as described previously [15,16] with some modifications. In brief, deparaffinized 3-μm-thick tissue sections and cells were treated with 10 μg/mL proteinase K (Roche Diagnostics) for 20 min at 37 °C and 5 μg/mL proteinase K for 10 min at 37 °C, respectively. The sections were post-fixed in 4% paraformaldehyde and then treated with 0.2 N HCl and 0.25% acetic anhydride in 0.1 mol/L tri-ethanol amine (pH 8.0) for 10 min each. After treatment with 3% hydrogen peroxide for 60 min, the sections were dehydrated and air-dried. Fifty microliters of the hybridization mixture (Hybridization Solution I, Maxim Biotech, Inc., South San Francisco, CA) and 50 ng of the sense or antisense cRNA probe were loaded onto each section and hybridized for 16–18 h at 50 °C. After hybridization, the sections were washed in 50% formamide/2× standard sodium citrate (SSC) for 30 min at 55 °C and then treated with 10 μg/mL RNase A (Roche Diagnostics) for 30 min at 37 °C. The sections were then stringently washed with 2× SSC, 0.2× SSC, and 0.1× SSC for 20 min each at 55 °C. After being placed into 0.01 mol/L Tris–HCl (pH 7.5), 0.3 mol/L NaCl, and 0.1% Tween-20 three times for 5 min each, and then in 0.5% casein/0.01 mol/L Tris–HCl (pH 7.5) and 0.15 mol/L NaCl for 10 min, the sections were reacted with 400 times diluted horseradish peroxidase (HRP)-conjugated rabbit anti-DIG Fab′ fragmented polyclonal antibody (Dako, Glostrup, Denmark), 0.07 μmol/L biotinylated tyramide [17,18], and 500 times diluted HRP-conjugated streptavidin (Dako) for 15 min each. Finally, the sections were visualized with DAB solution (Liquid DAB Substrate Chromogen System) (Dako) and counterstained with Mayer's hematoxylin.