studies agree that invasive lobular carcinomas are more common than invasive ductal carcinomas among breast cancers that metastasize to the stomach [1-5]. The distinction of metastatic breast carcinomas from primary gastric carcinomas is clinically important because these lesions require different treatment strategies. Patients with metastatic breast carcinomas are usually treated with chemotherapy; on the other hand, if the lesion is a primary gastric carcinoma, further evaluation of the clinical stage is required before determining an appropriate therapeutic management. However, the histologic diagnosis of metastatic breast carcinomas is sometimes problematic because the typical histologic features of invasive lobular carcinomas, for example, linear, dissociated, and a single-file growth pattern, are similar to those of diffuse-type gastric carcinomas. In particular, invasive lobular carcinomas frequently show a signet ring morphology that can be easily confused with that of primary signet ring cell–type gastric carcinomas [2,5-8]. Furthermore, the stomach is the initial site of presentation of advanced breast cancers in some instances [1,5,9-11].

Immunohistochemistry using a panel of antibodies has been reported to be capable of differentiating metastatic breast carcinomas from primary gastric carcinomas [6,12,13]. Estrogen receptor (ER) α, progesterone receptor (PR), mammaglobin, and gross cystic disease fluid protein 15 (GCDFP-15) have been used as markers of breast carcinomas [6,8,12-20], whereas CDX2, MUC5AC, MUC6, and CK20 are highly specific to gastric carcinomas [6,13,15,21-27]. However, these antibodies have limitations in their sensitivities; thus, the use of multiple antibodies is often required.

Hepatocyte nuclear factor (HNF) 4A is a member of the nuclear receptor superfamily and is a critical developmental regulator of the visceral endoderm [28]. Recent studies have shown that HNF4A is expressed in an organ-specific manner in normal as well as neoplastic tissues [28-30]. Among nonneoplastic tissues, HNF4A is expressed in epithelial cells of the gastrointestinal tract, liver and pancreas, and the proximal tubules of the kidney but not in most other organs including mammary glands [28]. The expression of HNF4A in neoplastic lesions has not been extensively studied, but previous reports imply that tumors originating from HNF4A-positive organs generally retain HNF4A expression [28-30]. This suggests that HNF4A might be useful for determining the primary sites of metastatic tumors. Here, we tested the use of HNF4A, in addition to a previously tested panel of antibodies, for the diagnosis of metastatic breast carcinomas in the stomach.

2. Materials and methods

2.1. Study group

Endoscopic biopsy specimens of 21 metastatic breast carcinomas were included in the present study. All the cases were retrieved from the surgical pathology files of the National Cancer Center Hospital, Tokyo, Japan, between 1997 and 2010. Patients with metastatic breast carcinomas involving the stomach had been diagnosed in cases that (1) had a history of surgical treatment of primary breast cancer or concurrent gastric and breast tumors of identical histologic type and (2) were immunohistochemically consistent with metastatic breast carcinomas using 1 or more of the following antibodies: ERα, PR, GCDFP-15, mammaglobin, CK20, MUC5AC, MUC6, and CDX2. Paraffin-embedded specimens of the corresponding primary lesions were available in 12 cases of metastatic breast carcinomas, and these specimens were also subjected to immunohistochemical analysis for comparison with the metastatic lesions.

For histologic and immunohistochemical comparisons, endoscopic biopsy samples of primary gastric cancers were also examined. A consecutive series of 33 poorly differentiated and/or signet ring cell adenocarcinomas of the stomach were retrieved from our case files without taking age and sex into account. These patients were clinically confirmed not to have any breast tumors.

This study was approved by the Ethics Committee of the National Cancer Center, Tokyo, Japan.

2.2. Histologic evaluation

The histology of breast carcinomas involving the stomach was classified into poorly differentiated adenocarcinoma, signet ring cell carcinoma, or others. Both metastatic breast and primary gastric carcinomas were histologically examined for the presence of signet ring cell components. The cytologic features of signet ring cells, with either univacuolated or multivacuolated cytoplasms, were further classified as previously described [7]. Briefly, the univacuolated type is characterized by a single well-circumscribed intracytoplasmic lumen with/without a central eosinophilic inclusion. The multivacuolated type is characterized by foamy cytoplasm with an abundance of mucin-filled vesicles.

2.3. Immunohistochemistry

Deparaffinized 4-μm-thick sections from each paraffin block were exposed to 0.3% hydrogen peroxide for 15 minutes to block endogenous peroxidase activity and then washed in deionized water for 2 to 3 minutes. For heat-induced epitope retrieval, the sections were subjected to citrate buffer (pH 6.0) at 121°C for 10 minutes. The primary antibodies that were used are monoclonal and listed in Table 1. For staining, we used an automated stainer (Dako, Glostrup, Denmark) according to the vendor's protocol. ChemMate EnVision (Dako) methods were used for detection. Appropriate positive and negative controls were used for each antibody.

Table 1 Antibodies used in the present study

Antibody	Clone	Dilution	Source
HNF4A	H1415	1:100	Perseus Proteomics (Tokyo, Japan)
ERα	1D5	1:100	Dako, Glostrup, Denmark
PR	PgR636	1:400	Dako
Mammaglobin	304-1A5	1:200	Dako
GCDFP-15	D6	1:200	Signet laboratories, Inc (Dedham, MA)
CDX2	CDX2-88	1:100	Bio Genex (San Ramon, CA)
MUC5AC	CLH2	1:200	Novocastra Laboratories,
MUC6	CLH5	1:100	Ltd (Newcastle upon Tyne, UK)
CK20	KS20.8	1:50	Dako

The extent of positive staining was graded semiquantitatively as follows: − (negative), ± (1%-10% positive cells), + (11%-50% positive cells), and ++ (>51% positive cells). To determine the specificity and sensitivity of each antibody, cases with completely negative staining were regarded as negative, whereas cases with any positive staining were considered positive. Two observers (T. K. and S. S.) evaluated the immunohistochemical results. Discrepant cases were reviewed using a multiheaded microscope to achieve consensus.

2.4. Statistical analysis

The Fisher exact test was used to analyze each 2-by-2 table of discrete data. $P < .05$ was considered statistically significant.

3. Results

3.1. Clinicopathologic features

The clinicopathologic features of metastatic breast carcinomas are summarized in Table 2. All the patients with metastatic breast carcinomas to the stomach were women, with a mean age of 59.6 years (range, 43-77 years). The mean interval between the diagnosis of primary breast carcinomas and the detection of gastric metastases was 65.8 months (range, 0-270 months). In 4 cases (cases 1, 8, 9, and 19), gastric metastasis was pointed out concurrently with or before the detection of the primary lesions.

The endoscopic appearance of gastric lesions was linitis plastica-like in 8 cases (38%), multiple erosions in 6 cases

Table 2 Clinicopathologic features of metastatic breast carcinomas

Case no.	Age/sex	Primary histology	Interval[d] (mo)	Endoscopic finding	Histology	Signet ring cells
1	43/F	ILC[a]	0[e]	SMT with ulceration	POR > SIG	UNI
2	45/F	NA	76	SMT	SIG > POR	UNI > MULTI
3	46/F	IDC	45	Linitis plastica	POR	–
4	49/F	ILC	18	SMT with erosion	SIG = POR	UNI > MULTI
5	51/F	ILC	87	SMT	POR	–
6	52/F	ILC	62	Linitis plastica	SIG = POR	MULTI
7	53/F	ILC	54	Multiple erosions	POR > SIG	UNI > MULTI
8	54/F	ILC	0[e]	Multiple SMTs	SIG > POR	MULTI > UNI
9	54/F	ILC	0	Multiple erosions	SIG > POR	MULTI > UNI
10	55/F	NA	17	Multiple erosions	POR	–
11	55/F	ILC	7	Multiple SMTs	POR > SIG	MULTI > UNI
12	55/F	ILC	145	Linitis plastica	SIG > POR	UNI > MULTI
13	59/F	ILC	1	Linitis plastica	SIG = POR	UNI
14	62/F	ILC	107	Linitis plastica	SIG > POR	MULTI > UNI
15	70/F	IDC	270	SMT with erosion	POR	–
16	71/F	Mixed[b]	183	Multiple erosions	POR > SIG	MULTI > UNI
17	73/F	IDC	30	Linitis plastica	SIG > POR	MULTI
18	73/F	ILC	121	Multiple erosions	POR > SIG	MULTI
19	76/F	IDC	0	Multiple erosions	POR > SIG	MULTI
20	77/F	ILC + IDC[c]	109	Linitis plastica	SIG	UNI
21	77/F	ILC	39	Linitis plastica	SIG	MULTI

Abbreviations: F indicates female; ILC, invasive lobular carcinoma; IDC, invasive ductal carcinoma; SMT, submucosal tumor; POR, poorly differentiated; SIG, signet ring cell; UNI, univacuolated; MULTI, multivacuolated; NA, not available.

[a] Diagnosis was made by axillary lymph node biopsy.

[b] Mixed invasive ductal lobular carcinoma.

[c] Bilateral tumors, invasive lobular in the right breast, and invasive ductal in the left breast.

[d] Interval between diagnosis of primary breast carcinomas and the detection of gastric metastases.

[e] Gastric lesions were detected before the primary lesions.

(28%), a submucosal tumor with a central ulceration/erosion in 3 cases (14%), solitary submucosal tumor in 2 cases (10%), and multiple submucosal tumors in 2 cases (10%). The histologic subtypes of the primary lesions of the metastatic breast carcinoma cases were invasive lobular in 13 cases (62%), invasive ductal in 4 cases (19%), and mixed invasive ductal lobular in 1 case (5%). One case had bilateral tumors, with an invasive lobular carcinoma in the right breast and an invasive ductal carcinoma in the left breast (case 20). Detailed information on the histology of the primary lesions was not available in 2 cases (cases 2 and 10).

Histologically, all the gastric involvements by metastatic breast carcinomas showed a poorly differentiated morphology. None of the cases showed gland formation. Signet ring cell components were identified in 17 cases (81%). In 11 cases, signet ring cell components were predominant or equal to the poorly differentiated components; and in the other 6 cases, the signet ring cell components were only minor. In 4 cases (19%), the entire biopsy specimen was composed of poorly differentiated components.

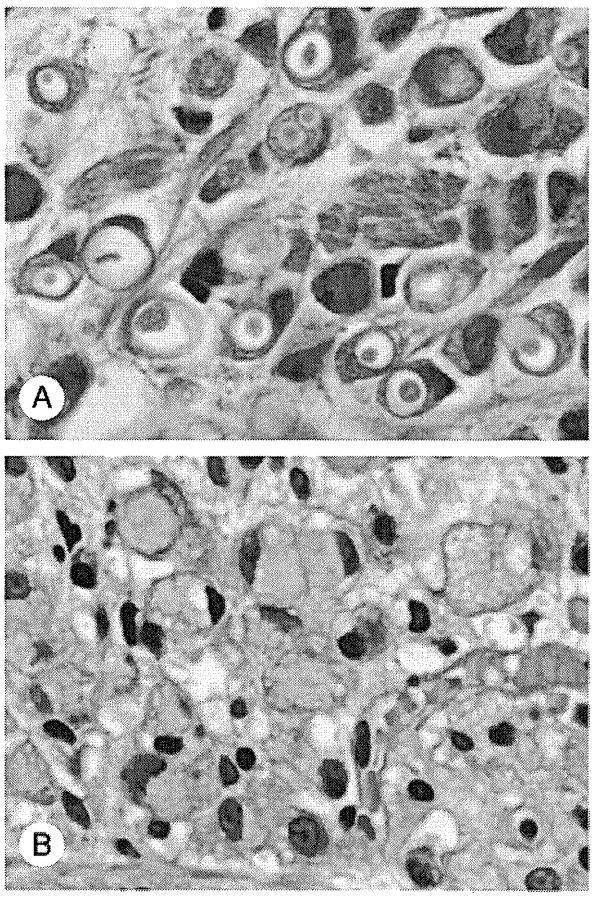

Fig. 1 Histologic features of gastric involvement by metastatic breast and primary gastric carcinomas of signet ring cell type. A, Metastatic breast carcinoma to the stomach. The tumor cells are mainly composed of univacuolated signet ring cells that are characterized by sharply demarcated intracytoplasmic lumina with central eosinophilic inclusions. B, Primary gastric adenocarcinoma. The signet ring cells are a multivacuolated type with predominantly foamy and abundant mucin-filled cytoplasm that pushes the nuclei against the cell membrane (hematoxylin and eosin, original magnification ×400).

Among the 17 cases with signet ring cells, all the signet ring cells were a univacuolated type in 3 cases (Fig. 1A), whereas all the signet ring cells were a multivacuolated type in 5 cases. The other cases had both univacuolated and multivacuolated signet ring cell components.

With regard to the primary gastric carcinoma cases used as a control, the male-female ratio of the patients was 19:14; and their mean patient age was 65.5 years (range, 42-85 years). Histologically, signet ring cells were present in 31 of the 33 cases. In all the patients, signet ring cells were predominantly or exclusively the multivacuolated type (Fig. 1B), whereas minor components of univacuolated signet ring cells were observed in 13 (39%) of the 31 cases with signet ring cells.

3.2. Immunohistochemistry

The results of the immunohistochemical analysis are summarized in Tables 3 and 4. In 21 gastric lesions of metastatic breast cancers as well as 12 primary sites of the breast cancers, HNF4A was consistently negative (Fig. 2A). In contrast, in the 33 primary gastric cancers, HNF4A was diffusely positive in 31 cases and focally positive in 2 cases (Fig. 3A). HNF4A was consistently expressed in nonneoplastic gastric epithelium but was negative in stromal cells in all the cases that were examined. For the discrimination of these 2 entities, the sensitivity and specificity of HNF4A were both 100%.

In gastric lesions of metastatic breast carcinomas, ERα, PR, mammaglobin, and GCDFP-15 were expressed in 16 (76%), 7 (33%), 11 (52%), and 13 (62%) cases, respectively (Fig. 2B-E). One case (case 15) was negative for all these antibodies. None of the primary gastric carcinomas expressed any of these 4 antibodies.

Primary lesions of metastatic breast carcinomas were also stained for the same panel of antibodies in 12 cases. ERα, PR, mammaglobin, and GCDFP-15 were positive in 10 (83%), 9 (75%), 6 (50%), and 4 (33%) cases, respectively. The immunophenotypes were entirely concordant between the primary and metastatic lesions in only 1 of the 12 cases, but the other cases showed some differences. In 3 cases, ERα was positive in the primary lesions but negative at metastatic sites. Similarly, the loss of PR expression at the metastatic sites was observed in 6 cases. Mammaglobin expression was lost in the metastatic lesions in 3 cases. On the other hand, mammaglobin expression was observed only in the metastatic lesions in 3 cases. In 4 cases, GCDFP-15 was negative in the primary lesions but positive in metastatic lesions of the stomach.

Table 3 Results of immunohistochemical staining

Antibody	Breast carcinoma								Primary gastric carcinoma			
	Metastasis to the stomach				Primary lesion							
	−	±	+	++	−	±	+	++	−	±	+	++
HNF4A	21	0	0	0	12	0	0	0	0	0	2	31
ERα	5	1	7	8	2	2	0	8	33	0	0	0
PR	14	1	3	3	3	4	3	2	33	0	0	0
Mammaglobin	10	3	3	5	6	3	3	0	33	0	0	0
GCDFP-15	8	1	4	8	8	2	2	0	33	0	0	0
CDX2	21	0	0	0	12	0	0	0	21	5	4	3
MUC5AC	20	0	1	0	12	0	0	0	5	1	9	18
MUC6	21	0	0	0	11	1	0	0	24	3	5	1
CK20	21	0	0	0	12	0	0	0	15	4	7	7

NOTE. −, negative; ±, 1% to 10% positive cells; +, 11% to 50% positive cells; and ++, more than 51% positive cells.

In primary gastric carcinomas, CDX2, MUC5AC, MUC6, and CK20 were expressed in 12 (36%), 28 (85%), 9 (27%), and 18 (55%) cases, respectively (Fig. 3B-E). These 4 markers were negative in all the metastatic and primary breast carcinomas except for 1 metastatic breast carcinoma (case 2) that stained positive for MUC5AC and 1 primary breast carcinoma (case 6) that stained positive for MUC6.

With regard to the reactivity of these antibodies in nonneoplastic gastric mucosa, CDX2 was expressed in metaplastic epithelium, MUC5AC was expressed in foveolar epithelium, MUC6 was expressed in pyloric glands and mucous neck cells of the fundic glands, and CK20 was expressed in metaplastic and foveolar epithelium. The expressions of ERα, PR, mammaglobin, and GCDFP-15 were completely absent in nonneoplastic gastric mucosa.

The sensitivity and specificity of each antibody for metastatic breast and primary gastric carcinomas are summarized in Table 4. ERα, PR, mammaglobin, and GCDFP-15 were 100% specific to metastatic breast carcinomas; but the sensitivities were variable, ranging from 33% to 76%. CDX2, MUC5AC, MUC6, and CK20 were highly specific to primary gastric carcinomas. Their sensitivities varied from 27% for MUC6 to 85% for MUC5AC.

4. Discussion

Gastric metastases of breast carcinomas can be clinically confused with primary gastric carcinomas, particularly linitis plastica, because of the similarity of their endoscopic and histologic findings. In agreement with previous studies, a linitis plastica–like gross appearance was the most common endoscopic finding for metastatic breast carcinoma [1,2,6]. However, metastatic breast carcinomas can also present with various gross appearances, including submucosal tumor–like, erosive, and ulcerated lesions [1,5,10]. In the present study, we confirmed these results and showed that metastatic tumors frequently occur as multiple lesions.

The histologic subtypes of the primary breast lesions were predominantly invasive lobular carcinomas, as previously reported [1-5]. All metastatic breast carcinomas showed a poorly differentiated morphology; and none of the cases exhibited gland formation. Signet ring cells were identified in most of the metastatic breast carcinomas, including some of the metastatic invasive ductal carcinomas.

Previous studies have suggested that metastatic breast and primary gastric carcinomas can potentially be differentiated based on morphology using hematoxylin and eosin–stained sections [7,26,31]. These previous studies suggested that the signet ring cells of lobular carcinoma are of a univacuolated type, which is characterized by a single, well-circumscribed intracytoplasmic lumina. On the other hand, signet ring cells of gastric carcinomas usually have multivacuolated cytoplasm with foamy and abundant mucin-filled vesicles.

In the present study, the cases in which all or most of the signet ring cells were univacuolated were limited to metastatic breast carcinomas. However, significant proportions of metastatic breast and primary gastric carcinomas contained both univacuolated and multivacuolated signet ring cells. Thus, a definitive diagnosis of metastatic breast carcinomas based solely on their histologic features may be difficult, whereas lesions predominantly composed of univacuolated signet ring cells are suggestive of metastatic breast carcinomas, rather than primary gastric carcinomas.

Immunohistochemically, all the primary gastric carcinomas but none of the metastatic breast carcinomas were positive for HNF4A. Thus, the use of this antibody alone allowed metastatic breast carcinomas to be discriminated from primary gastric carcinomas. The expression of HNF4A has not been extensively studied in tumors. Remarkably, however, all the primary gastric carcinomas that have been previously examined (total of 49 cases) were uniformly positive for HNF4A [28,29]. Moreover, the staining for HNF4A was mostly diffuse and strong, which is a major advantage for its use in the diagnosis of biopsy specimens, where only a limited amount of tissue is available.

Table 4 Summary of immunohistochemical results

Antibody	Metastatic breast carcinoma	Primary gastric carcinoma	*P*	Sensitivity	Specificity
Markers for breast carcinoma					
ERα	16/21 (76%)	0/33 (0%)	9.6×10^{-10}	76%	100%
PR	7/21 (33%)	0/33 (0%)	6.6×10^{-4}	33%	100%
Mammaglobin	11/21 (52%)	0/33 (0%)	3.7×10^{-6}	52%	100%
GCDFP-15	13/21 (62%)	0/33 (0%)	1.8×10^{-7}	62%	100%
Markers for gastric carcinoma					
HNF4A	0/21 (0%)	33/33 (100%)	1.9×10^{-15}	100%	100%
CDX2	0/21 (0%)	12/33 (36%)	1.6×10^{-3}	36%	100%
MUC5AC	1/21 (5%)	28/33 (85%)	3.0×10^{-9}	85%	95%
MUC6	0/21 (0%)	9/33 (27%)	2.0×10^{-2}	27%	100%
CK20	0/21 (0%)	18/33 (55%)	6.0×10^{-5}	55%	100%

NOTE. *P* values indicate the significance of the difference between gastric involvement by metastatic breast cancer and primary gastric carcinoma.

Our study also confirmed that previously reported markers could differentiate metastatic breast carcinomas and primary gastric carcinomas with a high specificity. ERα, PR, mammaglobin, and GCDFP-15 were specific markers of breast carcinomas. Although several older studies have reported that up to 28% of gastric carcinomas expressed ER [32,33], these findings were based upon studies using a particular antibody against ER, clone H222, which is no longer used in standard practice. Similarly, 1 study reported that mammaglobin was expressed in 13% of gastric carcinomas that had metastasized to the lymph nodes; however, they used a polyclonal mammaglobin antibody, which is not widely used [18]. Overall, these previous reports and the present study indicate that these 4 markers can specifically detect metastatic breast carcinomas when appropriate antibodies are used. On the other hand, their sensitivities for the identification of metastatic breast carcinomas were variable. ERα was the most sensitive marker for breast carcinomas, followed by GCDFP-15, mammaglobin, and PR.

CK20, MUC5AC, MUC6, and CDX2 were confirmed to be useful for identifying primary gastric carcinomas. In our study, 1 case of metastatic breast carcinoma expressed MUC5AC. Indeed, O'Connell et al [6] also reported a similar

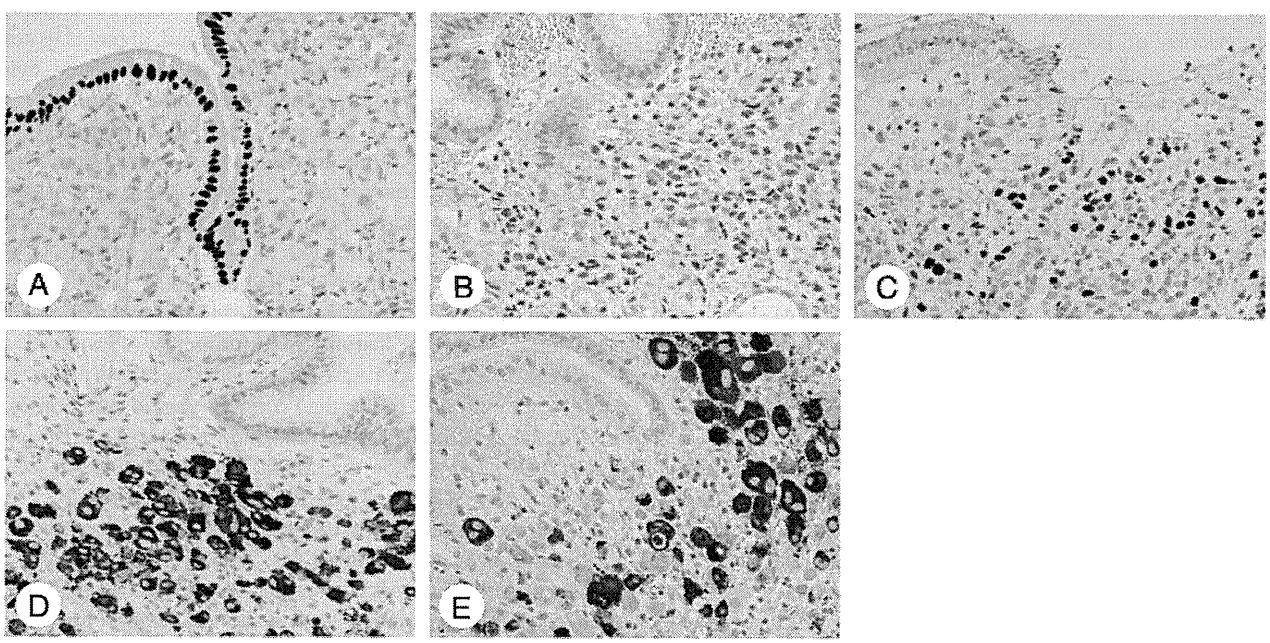

Fig. 2 Immunohistochemical staining of metastatic breast carcinoma to the stomach. HNF4A is expressed in nonneoplastic gastric epithelium but is negative in metastatic breast carcinoma cells infiltrating the lamina propria (A). A metastatic breast carcinoma to the stomach shows the nuclear expression of ERα (B) and PR (C) and the cytoplasmic expression of mammaglobin (D) and GCDFP-15 (E) (original magnification ×200).

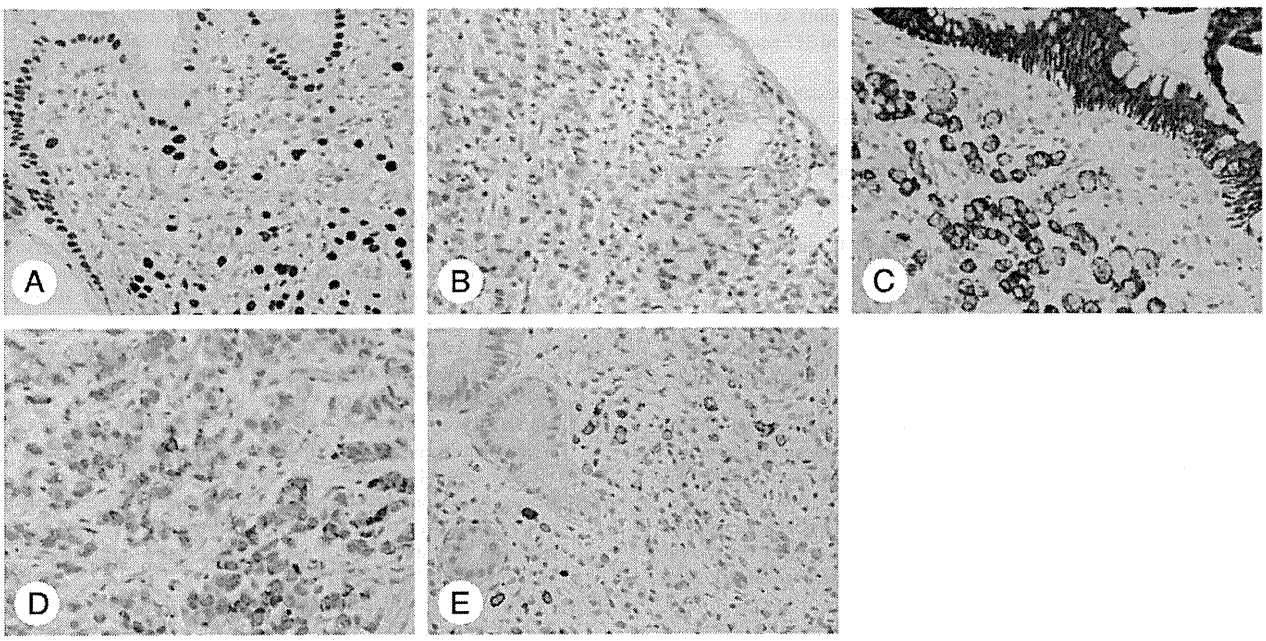

Fig. 3 Immunohistochemical staining of primary gastric carcinoma. Primary gastric carcinoma showing the nuclear expression of HNF4A (A) and CDX2 (B) and the cytoplasmic expression of MUC5AC (C), MUC6 (D), and CK20 (E). The nonneoplastic gastric epithelium is strongly positive for HNF4A (A) and MUC5AC (C) (original magnification ×200).

result: 1 of the 19 breast carcinomas that had metastasized to the gastrointestinal tract expressed MUC5AC in their study. In addition, studies on primary breast carcinomas have shown that minor subsets of breast carcinomas express CK20, MUC5AC, or MUC6 [15,26,27,34,35]. Thus, although our study showed that these gastric carcinoma markers are quite specific, the expression of CK20, MUC5AC, or MUC6 may not completely exclude the possibility of a metastatic breast carcinoma. The expression of CDX2 has never been reported in breast carcinomas, but its sensitivity for the identification of gastric carcinomas was relatively low in the present study.

In summary, breast carcinomas that metastasize to the stomach consistently show a poorly differentiated or signet ring cell morphology, regardless of the histology of the primary lesion. A predominance of univacuolated signet ring cells favors a diagnosis of metastatic breast carcinomas, but it may be difficult to conclusively differentiate these lesions from primary gastric carcinomas based solely on morphology. The use of immunohistochemistry is effective for the diagnosis of gastric metastases of breast carcinomas. ERα, PR, mammaglobin, and GCDFP-15 are specific markers for breast carcinomas, whereas HNF4A, CK20, MUC5AC, MUC6, and CDX2 are useful for identifying primary gastric carcinomas. Remarkably, HNF4A was able to distinguish all primary gastric carcinomas from metastatic breast carcinomas. We suggest that HNF4A may be a highly useful marker for excluding metastatic breast carcinomas in the diagnosis of gastric biopsy specimens.

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NKX2.2 is a Useful Immunohistochemical Marker for Ewing Sarcoma

Akihiko Yoshida, MD,† Shigeki Sekine, MD, PhD,‡ Koji Tsuta, MD, PhD,* Masashi Fukayama, MD, PhD,† Koh Furuta, MD, PhD,* and Hitoshi Tsuda, MD, PhD**

Abstract: Ewing sarcoma is a high-grade round cell sarcoma that affects bones and soft tissues in children and young adults. Its diagnosis can be challenging, and the differential diagnoses include a wide variety of small round cell tumors. CD99 and FLI-1 are the currently accepted immunohistochemical markers for Ewing sarcoma, but their accuracy has been controversial. NKX2.2 is a homeodomain-containing transcription factor that plays a critical role in neuroendocrine/glial differentiation. The *NKX2.2* gene was recently identified as a target of EWS-FLI-1, the fusion protein specific to Ewing sarcoma, and was shown to be differentially upregulated in Ewing sarcoma on the basis of array-based gene expression analysis. However, the immunohistochemical diagnostic potential of this marker has not been tested. We immunostained representative sections of 30 genetically confirmed Ewing sarcomas and 130 non-Ewing small round cell tumors by using an antibody to NKX2.2. Nuclear staining in at least 5% of the cells was deemed positive. Twenty-eight (93%) of the 30 Ewing sarcomas were positive for NKX2.2. The staining was diffuse (> 50%) in all the positive cases and was moderate or strong in intensity for most cases (25 of 28). NKX2.2 was also positive in 14 non-Ewing tumors, including all the olfactory neuroblastomas and a minor subset of small cell carcinomas, synovial sarcomas, mesenchymal chondrosarcomas, and malignant melanomas. All the other non-Ewing tumors tested were negative for this marker. NKX2.2 is a valuable marker for Ewing sarcoma, with a sensitivity of 93% and a specificity of 89%, and aids in the differential diagnosis of small round cell tumors.

Key Words: Ewing sarcoma, NKX2.2, immunohistochemistry, diagnosis

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From the *Pathology and Clinical Laboratory Division, National Cancer Center Hospital; †Department of Pathology, The University of Tokyo; and ‡Division of Pathology, National Cancer Center Research Institute, Tokyo, Japan.

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Correspondence: Akihiko Yoshida, MD, Pathology and Clinical Laboratory Division, National Cancer Center Hospital, 5-1-1 Tsukiji, Chuo-ku, Tokyo 104-0045, Japan (e-mail: akyoshid@ncc.go.jp).

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Ewing sarcoma is a high-grade malignancy that typically affects bones and soft tissues of children and young adults.[6,31] Histologically, it is characterized by diffuse proliferation of uniform small round cells. Ewing sarcoma harbors specific recurrent chromosomal translocations, and in almost all cases these translocations create fusions of *EWS* to one of the *ETS* gene family members. The most frequent fusion partner of *EWS* is *FLI1* (90% to 95%), followed by *ERG* (5% to 10%) and other rare targets, such as *ETV1*, *E1AF*, and *FEV* (each < 1%).[31]

The diagnosis of Ewing sarcoma can be challenging, and a wide variety of small round cell tumors enter differential diagnosis. Difficulty exists not only because submitted materials may be less than ideal in quality and quantity but also because variable histologic patterns may be present in sarcoma tissue, such as primitive neuroectodermal tumor pattern, large cell pattern, spindle cells, adamantinoma-like nests, pseudoalveolar structure, angiomatoid growth, and prominent sclerosis.[11,31] Because patients with Ewing sarcoma are treated by specific chemotherapy regimens, accurate diagnosis is crucial. To this aim, several ancillary tests have been developed to supplement conventional morphologic assessment.

Molecular detection of specific fusion genes is accepted nowadays as the gold standard method for diagnosing Ewing sarcoma. However, this approach is costly, laborious, and only available at a limited number of large centers. Hence, immunohistochemistry has been an appealing substitute, and it also enables shorter turn-around time than do molecular assays. CD99 and FLI-1 are well-received immunohistochemical markers for Ewing sarcoma, but their accuracy has been a subject of debate. CD99 stains almost all Ewing sarcomas, but its reactivity is nonspecific, and a significant number of small round cell mimics are positively labeled.[31] The FLI-1 antibody has been tested over the past decade, but its sensitivities (63% to 100%) and specificities (60% to 97%) vary among studies,[7,9,11,12,14,15,21] likely reflecting the difference in the type of antibody used (monoclonal[9,14,21] versus polyclonal[7,10–12,14,15]) and the preset criteria for positivity (any positivity,[21] > 1%,[12] > 5%,[7,10,15] > 10%,[14] or > 30% [9]). The non-Ewing small round cell tumors that were reported to express FLI-1 include neuroblastomas,[12] olfactory neuroblastomas,[15] synovial sarcomas,[12,21] rhabdomyosarcomas,[15] desmoplastic small round cell tumors,[7,15] small cell carcinomas,[14,15] Merkel cell carcinomas,[15,21] malignant melanomas,[14,21] and lymphomas.[7,9,10,12,14,15] Taken

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together, the existing immunohistochemical markers, though helpful in many cases, seem to have met with limited success. Additional markers with a comparable or higher accuracy should be useful in solving the diagnostic dilemma, particularly when conventional ones yield equivocal results.

NKX2.2 is a member of the NK2 family of transcription factors, and it plays a critical role in development and differentiation in the central nervous system and gastrointestinal/pancreatic endocrine cells.[2,20,25,28] Recently, the *NKX2.2* gene has been identified as an important target of EWS-FLI-1, and the NKX2.2 protein was shown to be necessary for Ewing sarcoma oncogenesis,[23] likely through its repressive function.[19] *NKX2.2* was previously shown to be differentially upregulated in Ewing sarcomas when compared with neuroblastomas.[24] Similarly, Smith et al.,[23] by using previously published gene expression microarray data,[1] showed that *NKX2.2* was expressed in most (18 of 20) Ewing sarcoma clinical samples, whereas it was not expressed in virtually all (154 of 161) the other sarcomas tested. However, these molecular results have never been immunohistochemically extrapolated to the protein level for use by practicing pathologists. In this study, we investigated the diagnostic potential of a commercially available NKX2.2 monoclonal antibody to distinguish Ewing sarcoma from other small round cell tumors.

MATERIALS AND METHODS

Cases

After the approval of the institutional review board, 30 Ewing sarcomas (including those with primitive neuroectodermal tumor pattern) were retrieved from the pathology file of the National Cancer Center Hospital, Tokyo. The diagnosis of all the cases was confirmed by morphologic examination, conventional immunohistochemistry, and positive *EWS* rearrangement by fluorescence in situ hybridization. A total of 130 small round cell tumors that were thought to mimic Ewing sarcoma were selected on the basis of the literature and our own experience, and they were similarly retrieved from the archive. These comprised 15 neuroblastomas, 3 olfactory neuroblastomas, 9 poorly differentiated synovial sarcomas, 8 round cell liposarcomas, 15 alveolar rhabdomyosarcomas, 2 (round cell rich) embryonal rhabdomyosarcomas, 7 desmoplastic small round cell tumors, 3 soft tissue myoepitheliomas (small round cell pattern),[8] 1 cellular extraskeletal myxoid chondrosarcoma,[13] 3 solitary fibrous tumors (small round cell pattern),[17] 9 mesenchymal chondrosarcomas, 2 small cell osteosarcomas, 20 small cell carcinomas (15 pulmonary and 5 esophageal), 9 Merkel cell carcinomas, 3 medulloblastomas, 6 malignant melanomas (small round cell type), 11 lymphomas (6 lymphoblastic and 5 other types), and 4 plasma cell tumors. All the hematoxylin and eosin-stained slides were reviewed along with the relevant immunostained preparations and molecular data, and the diagnoses were confirmed.

Immunohistochemistry

Four-micrometer-thick sections from the representative block of each tumor were routinely deparaffinized. The sections were exposed to 3% hydrogen peroxide for 15 minutes to block endogenous peroxidase activity and then washed in deionized water for 2 or 3 minutes. The preparations were autoclaved in citrate buffer for antigen retrieval. The primary antibody used was NKX2.2 (clone 74.5A5, dilution 1:250; Developmental Studies Hybridoma Bank, Iowa City, IA). The slides were incubated for 1 hour at room temperature with the primary antibody and subsequently labeled with the Envision+/HRP system (Dako, Glostrup, Denmark). Diaminobenzidine was used as the chromogen and hematoxylin as the counterstain. Appropriate positive (pancreatic islet) and negative (skeletal muscle) controls were also prepared. The nuclear stains were considered positive. The intensity of staining was graded as follows: negative; weak; moderate; and strong. The extent of staining was classified as negative (0% to < 5%), focal (5% to 50%), and diffuse (> 50% to 100%). For the Ewing sarcoma cases, the CD99 and FLI-1 stainings performed at the original diagnosis were also reviewed and the results evaluated. The primary antibodies used at our laboratory are CD99 (O13, 1:50; Signet, Dedham, MA) and FLI-1 (monoclonal G146-222, 1:500; BD Biosciences, Franklin Lakes, NJ), and antigen retrieval by autoclaving is used for both markers.

Evaluation of Normal Tissues

In addition to the tumor samples, we carefully searched for any potential presence of NKX2.2-positive cells in the background normal tissues included in the tumor slides studied. These tissues comprised soft tissues (n = 33, including fat, skeletal muscle, tendon, fascia, vasculature, and small peripheral nerve), lung (n = 20), skin (n = 13), bone (n = 8), lymph node (n = 7), sinonasal tract (n = 6), esophagus (n = 5), bone marrow (n = 4), kidney (n = 3), salivary gland (n = 2), large peripheral nerve (n = 2), and ganglion (n = 1). We also stained a previously constructed tissue microarray (TMA) containing duplicate 2.0 mm cores from the following normal tissues: cerebrum, cerebellum, pituitary, salivary gland, thyroid, breast, lung, liver, esophagus, stomach, small bowel, large bowel, pancreas, gallbladder, spleen, kidney, endometrium, endocervix, prostate, testis, urinary bladder, ovary, fallopian tube, and skin. Whole sections of adrenal, parathyroid, and a variety of tissues from the central nervous system were further retrieved and stained for NKX2.2.

RESULTS

The results are summarized in Table 1. Of the 30 Ewing sarcomas, 28 were positive for NKX2.2 (Fig. 1). The staining was diffuse in all the positive cases, and the intensity was weak in 3, moderate in 9, and strong in 16 cases. Two cases were negative for NKX2.2, and they occurred in the viscera of adult patients; both showed classic histomorphology and immunoprofile. In contrast,

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TABLE 1. NKX2.2 Comparative Immunohistochemistry in Small Round Cell Tumors

Tumor Type	Positivity	Extent	Intensity
Ewing sarcoma	28/30 (93%)	F0, D28	W3, M9, S16
Non-Ewing small round cell tumor	14/130 (11%)	F8, D6	W7, M4, S3
Neuroblastoma	0/15 (0%)	—	—
Olfactory neuroblastoma	3/3 (100%)	F0, D3	W2, M0, S1
Poorly differentiated synovial sarcoma	1/9 (11%)	F1, D0	W1, M0, S0
Round cell liposarcoma	0/8 (0%)	—	—
Rhabdomyosarcoma	0/17 (0%)	—	—
Desmoplastic small round cell tumor	0/7 (0%)	—	—
Soft tissue myoepithelioma	0/3 (0%)	—	—
Extraskeletal myxoid chondrosarcoma	0/1 (0%)	—	—
Solitary fibrous tumor (round cell rich)	0/3 (0%)	—	—
Mesenchymal chondrosarcoma	3/9 (33%)	F2, D1	W2, M1, S0
Small cell osteosarcoma	0/2 (0%)	—	—
Small cell carcinoma	5/20 (25%)	F4, D1	W1, M2, S2
Merkel cell carcinoma	0/9 (0%)	—	—
Medulloblastoma	0/3 (0%)	—	—
Malignant melanoma	2/6 (33%)	F1, D1	W1, M1, S0
Lymphoma	0/11 (0%)	—	—
Plasma cell tumor	0/4 (0%)	—	—

D indicates diffuse (> 50%); F, focal (5%-50%); M, moderate; S, strong; W, weak.

NKX2.2 was negative in most (89%) non-Ewing small round cell tumors (Fig. 2). Only 14 of the 130 non-Ewing tumors tested were immunoreactive (Fig. 3), and they comprised olfactory neuroblastomas (3 of 3), small cell carcinomas (5 of 20), synovial sarcoma (1 of 9), mesenchymal chondrosarcomas (3 of 9), and malignant melanomas (2 of 6). All the 3 olfactory neuroblastomas and 1 case each of small cell carcinoma, mesenchymal chondrosarcoma, and malignant melanoma showed diffuse staining, whereas the rest exhibited focal (5% to 30%) labeling. Staining of moderate or strong intensity was observed in 1 olfactory neuroblastoma, 4 small cell carcinomas, 1 mesenchymal chondrosarcoma, and 1 malignant melanoma. Evaluation of the conventional markers at the original diagnosis showed that all the 30 Ewing sarcomas were positive for CD99. FLI-1 staining results were available for 26 cases, and of these 24 were immunoreactive. Both NKX2.2-negative cases were positive for FLI-1, and both FLI-1–negative cases were positive for NKX2.2. In the normal tissues evaluated on whole slides or TMA, NKX2.2 was expressed strongly in the pancreatic islet and isolated endocrine cells in the stomach and the intestine and weakly and focally expressed in the oligodendrocytes of the central nervous system. The other tissues were negative for this marker.

DISCUSSION

NKX2.2 was expressed in 93% of the 30 genetically confirmed Ewing sarcomas. The reactivity was always diffuse and with moderate or strong intensity in most cases, rendering the staining results readily interpretable even in small biopsy specimens. In comparison, the immunostaining results were negative for most (89%) of the 130 other small round cell tumors that were morphologically similar to Ewing sarcoma. These results strongly suggest that NKX2.2 plays a valuable adjunctive role in the diagnosis of Ewing sarcoma. This study has successfully translated the published array-based gene expression data of Ewing sarcoma[23] into diagnostic immunostaining, analogous to the approach taken for the development of DOG1,[32] TLE1,[26] and MUC4.[5]

Two Ewing sarcomas (6.7%) were negative for NKX2.2. Both tumors showed classic morphologic features and immunoprofiles and were not otherwise different from the NKX2.2-positive examples. A previous study also showed the lack of *NKX2.2* gene expression in 2 of 18 Ewing sarcomas,[23] and the underlying mechanism for these rare negative events is unclear. It is conceivable that these cases harbor variant fusions that do not target *NKX2.2*, unlike common EWS-FLI-1. Indeed, 1 study[4] using mouse progenitor cells addressed that different Ewing-associated fusion proteins induce transcriptome changes with only limited similarity. However, a recent study[16] showed an increase in *NKX2.2* level in vitro by transfecting human mesenchymal progenitor cells with a variant fusion *EWS-ERG*. Furthermore, both NKX2.2-negative cases were diffusely positive for FLI-1, which may indicate the presence of *EWS-FLI-1* translocation in these samples.[7]

Non-Ewing small round cell tumors positive for NKX2.2 were limited to several entities, and over half of the positive cases exhibited only focal staining. Olfactory neuroblastoma is a site-specific tumor and should not be a diagnostic consideration in most instances. However, Ewing sarcoma rarely affects the sinonasal tract, and diffuse NKX2.2 staining in olfactory neuroblastoma may then be problematic. A characteristic lobular growth pattern, a conspicuous neuropil-rich background, and an S100-positive sustentacular cell population are morphologic clues. In contrast to Ewing sarcoma, olfactory neuroblastomas are typically negative for CD99 and positive

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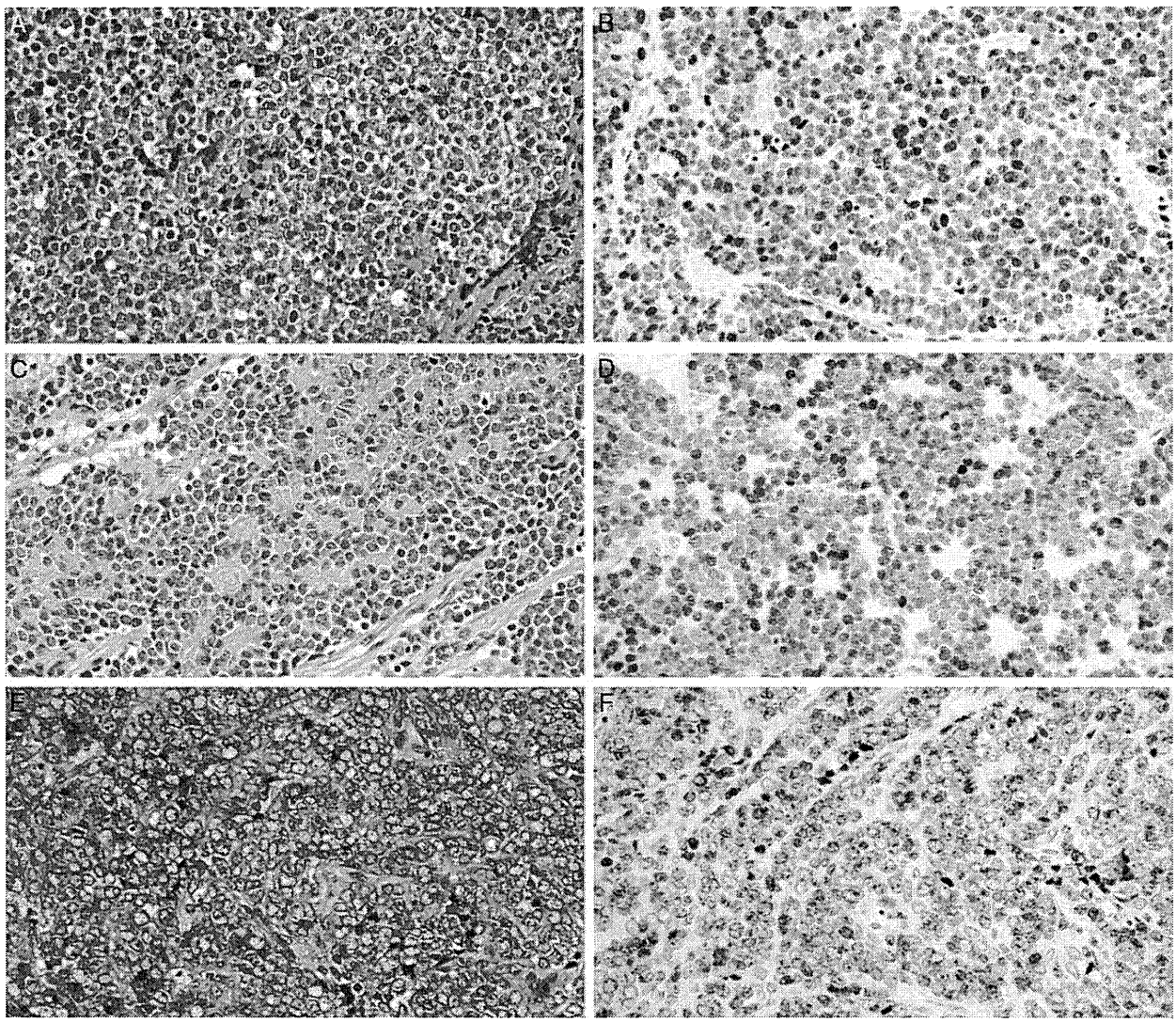

FIGURE 1. Most (93%) of the 30 Ewing sarcomas, irrespective of morphologic pattern, were immunoreactive to NKX2.2 in a diffuse manner: (A) classic morphology (H&E); (C) primitive neuroectodermal tumor pattern (H&E); (E) large cell pattern (H&E). B, D, and F, Positive NKX2.2 immunohistochemical staining for the cases depicted in (A), (C), and (E), respectively.

for calretinin.[31,33] Small cell carcinoma is clinically differentiated from Ewing sarcoma by old age of the patient and location of the tumor. Dense chromatin, spindling, nuclear pleomorphism, and molding are important findings that suggest small cell carcinoma. NKX2.2 expression in small cell carcinomas was focal in most cases, in contrast to diffuse reactivity in Ewing sarcoma.

NKX2.2 positivity in the minority of synovial sarcomas, mesenchymal chondrosarcomas, and malignant melanomas was unexpected, because these tumors are not believed to show neuroendocrine/glial differentiation, in which this protein plays an essential role. Poorly differentiated synovial sarcoma is often difficult to separate from Ewing sarcoma histologically. Nuclear spindling and TLE1 immunoreactivity favor synovial sarcomas.[26] The only NKX2.2-positive synovial sarcoma in our series exhibited weak focal staining in contrast to diffuse labeling of Ewing sarcoma. Identifying the cartilage component is critical to the diagnosis of mesenchymal chondrosarcoma, but matrix formation may be limited and obscured by uniform round cells in a given material. Diffuse positivity for CD99 is frequent in this sarcoma and can be a further source of confusion.[31] Coarse granular chromatin and prominent vascularity that may assume hemangiopericytoma-like features are characteristic of mesenchymal chondrosarcoma. FLI-1 and Sox9 immunostaining may be helpful in difficult cases.[9,30] The recently discovered specific *HEY1-NCOA2* fusion transcript[27] has not yet been applied to routine diagnosis. Malignant melanoma is notorious for its morphologic diversity, and a small round cell pattern may be rarely encountered.[18] Clinical history and conventional histologic hallmarks (eg, melanin

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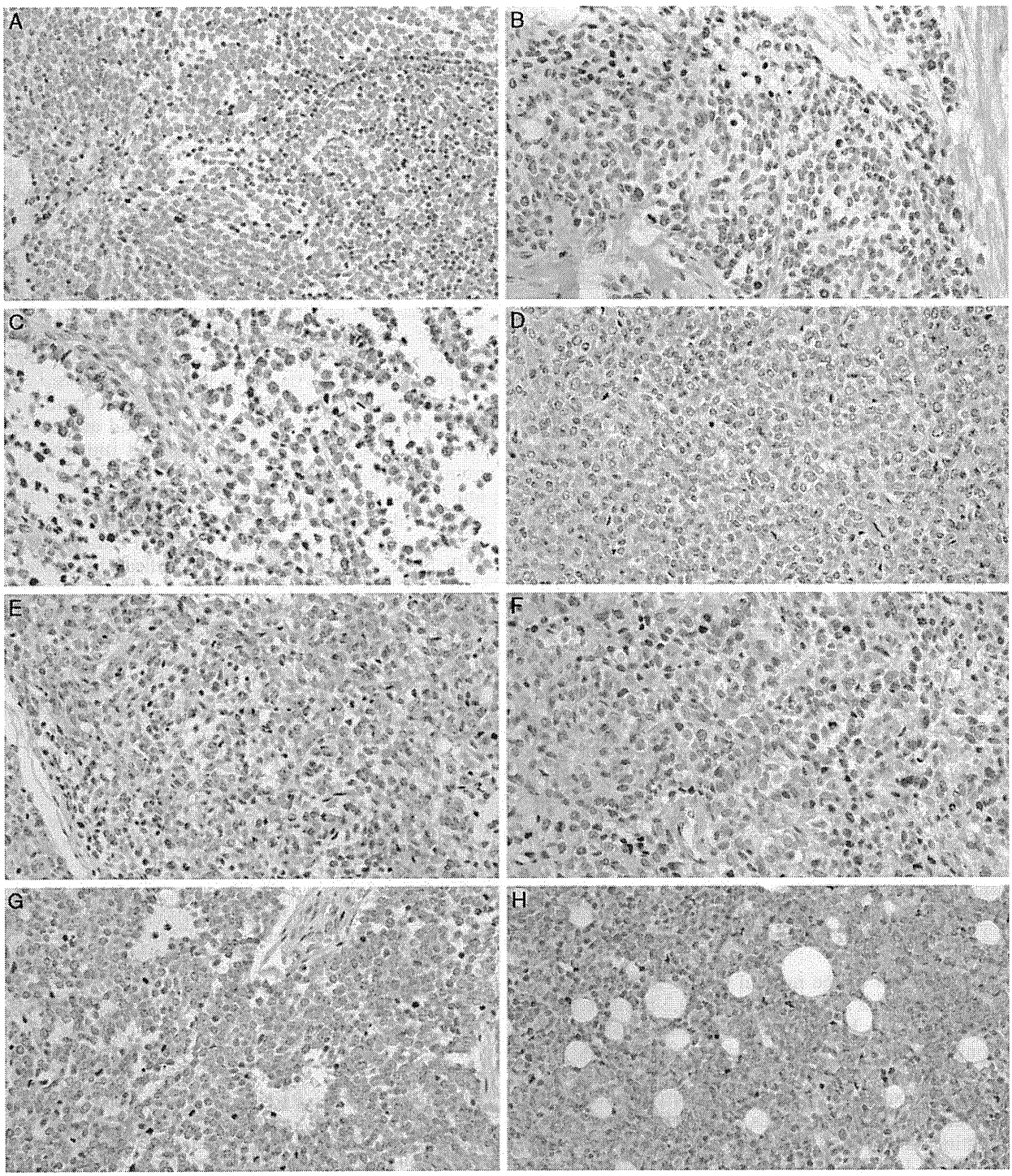

FIGURE 2. Most (89%) of the 130 non-Ewing small round cell tumors lacked NKX2.2 reactivity: (A), neuroblastoma; (B) desmoplastic small round cell tumor; (C) alveolar rhabdomyosarcoma; (D) poorly differentiated synovial sarcoma; (E) round cell liposarcoma; (F) small cell osteosarcoma; (G) Merkel cell carcinoma; and (H) lymphoblastic lymphoma.

pigment and intranuclear pseudoinclusion) are important. Ewing sarcomas are rarely positive for the S100 protein,[31] but they do not express more specific melanoma markers (eg, HMB45 and Melan A).

Although this study did not aim to make a formal comparison, NKX2.2 seems to have a virtually comparable sensitivity and specificity with FLI-1, judging from the published data.[7,9,10,12,14,15,21] In addition, NKX2.2

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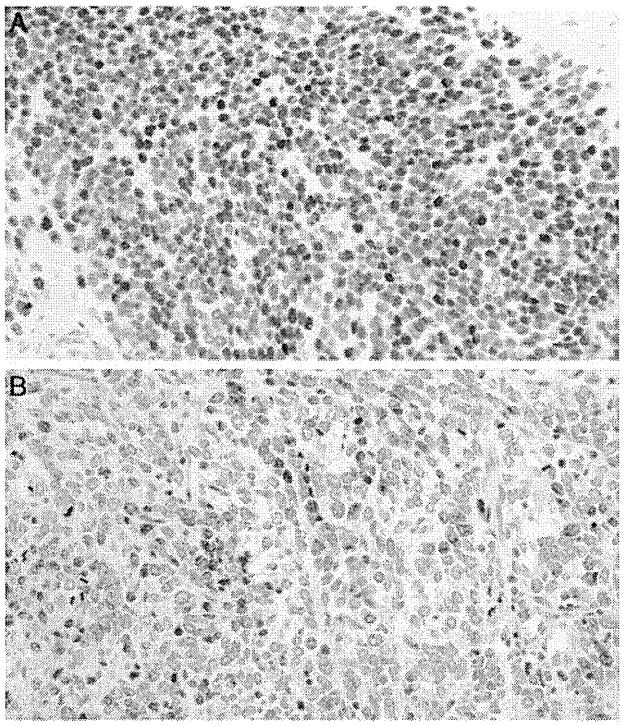

FIGURE 3. A small number of non-Ewing tumors were positive for NKX2.2. A, Olfactory neuroblastoma; (B) small cell carcinoma.

appears to have complementary diagnostic value to the conventional markers. Thus, both FLI-1–negative Ewing sarcomas were positive for NKX2.2, and both NKX2.2-negative tumors were positive for FLI-1. Many tumor entities that may be positive for FLI-1 on the basis of the literature lacked reactivity to NKX2.2, and mesenchymal chondrosarcoma, which may label for NKX2.2, was reportedly negative for FLI-1.[9] Similarly, olfactory neuroblastoma, which reacts with NKX2.2, is negative for CD99.[31,33] These data suggest that the usefulness of NKX2.2 is enhanced by combining with the conventional markers.

Examination of the normal tissues included in the tumor slides showed no intrinsic NKX2.2-positive native cells in soft tissues, bones, and lungs, the 3 tissues most commonly affected by Ewing sarcoma. Our TMA analysis also revealed that NKX2.2 expression was strictly regulated and exclusively observed in the pancreatic islet, gastrointestinal endocrine cells, and oligodendrocytes. The pristine background of preparation is in contrast to CD99 and FLI-1, which variably stain hematolymphoid and endothelial cells[14,15] and should facilitate staining interpretation. In addition, the clean background may also allow potential application to detection of lesional cells that are unapparent on routine examination. The latter approach may be particularly useful in identifying metastasis in the bone marrow or residual tumor cells after chemotherapy. According to Cheung et al,[3] *NKX2.2* gene expression was identified in 2 of 35 histologically negative bone marrow samples of patients with Ewing sarcoma, and the positive expression, when corroborated with the status of other markers, portended worse prognosis and suggested submicroscopic metastatic disease.

The utility of NKX2.2 immunohistochemistry has been previously explored in a few other diagnostic settings. Wang et al[28,29] found this marker to be expressed in almost all (24 of 26) well-differentiated endocrine tumors of the gastrointestinal system, whereas all the 16 bronchopulmonary typical carcinoids tested lacked expression. These researchers thus suggested NKX2.2 as a marker of the site of origin for well-differentiated neuroendocrine neoplasms. Rousseau et al,[22] in contrast, applied NKX2.2 to glioma diagnosis. This protein was expressed in $\geq 25\%$ of cells in 76% of oligodendrogliomas but in only 19% of astrocytomas and was proposed as a marker for the oligodendroglial line of differentiation in diffuse gliomas.

In conclusion, NKX2.2 is a valuable immunohistochemical marker for Ewing sarcoma and aids in the differential diagnosis of small round cell tumors. A small number of non-Ewing tumors can be positive for NKX2.2 and should be separated from Ewing sarcoma on the basis of clinical findings, morphologic features, and conventional markers. The non-Ewing tumor entities that react with NKX2.2 do not seem to significantly overlap with those stained by CD99 and FLI-1, and a panel approach may be beneficial to enhance diagnostic accuracy.

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Prognostic impact of nestin expression in resected large cell neuroendocrine carcinoma of the lung

Shinichiro Ryuge[a], Yuichi Sato[b,*], Shi Xu Jiang[d], Guoqin Wang[c], Toshihide Matsumoto[d], Ken Katono[a], Hayato Inoue[f], Akira Iyoda[e], Yukitoshi Satoh[e], Hirokuni Yoshimura[e], Noriyuki Masuda[a]

[a] Department of Respiratory Medicine, School of Medicine, Kanagawa, Japan
[b] Department of Molecular Diagnostics, School of Allied Health Sciences, Kanagawa, Japan
[c] Kitasato Clinical Research Center, School of Medicine, Kanagawa, Japan
[d] Department of Pathology, School of Medicine, Kanagawa, Japan
[e] Department of Thoracic and Cardiovascular Surgery, School of Medicine, Kanagawa, Japan
[f] Kitasato University; Department of Thoracic Surgery, National Hospital Organization Sagamihara National Hospital, Kanagawa, Japan

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ABSTRACT

Background: Large cell neuroendocrine carcinoma (LCNEC) of the lung is categorized as a high-grade neuroendocrine carcinoma with an aggressive clinical behavior. Nestin is a class VI intermediate filament protein expressed in stem/progenitor cells during central nervous system development. Recently, we reported that nestin expression is a prognostic indicator of a poorer survival probability in patients with resected NSCLC. In the present study, we aimed to determine its prognostic significance concerning survival in patients with resected LCNEC.

Materials and methods: Nestin expression in tumor cells was immunohistochemically studied in 30 patients with resected LCNEC, and its associations with clinicopathologic parameters including the Ki-67 labeling index (LI) and TTF-1 expression 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 8 of the 30 (26.7%) LCNECs. Clinicopathologically, although no significant association between nestin expression and age, gender, smoking habits, p-TNM stage, tumor size, nodal status, or TTF-1 expression was observed, nestin expression was significantly associated with a high Ki-67 LI ($P=0.012$). On survival analysis, nestin expression was significantly associated with a poorer prognosis in patients with LCNEC ($P=0.016$). The Cox proportional regression model confirmed that the crude hazard ratio (95%CI) of nestin expression was 3.40 (1.18–9.77).

Conclusions: The present study suggests that nestin expression seems to be a prognostic indicator of a poorer survival probability in patients with resected LCNEC, although its prognostic significance still requires confirmation with larger patient populations.

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1. Introduction

Large cell neuroendcrine carcinoma (LCNEC) of the lung was first proposed by Travis et al. [1] in 1991 as one of the four categories of pulmonary NE tumors. In the 1999 World Health Organization (WHO) classification, LCNEC is a high-grade NE carcinoma as a variant of large cell carcinoma [2]. The incidence of LCNEC is low, and appears to be between 2.1% and 3.5% [3]. Although LCNEC is categorized as a variant of large cell carcinoma, it is considered to be aggressive clinically, and its clinical outcome is reported to be poorer than that of stage-comparable conventional non-small cell lung carcinoma (NSCLC) [4,5]. However, no prognostic difference was noted between LCNEC and small cell lung cancer (SCLC) [6], and the overall 5-year survival for surgically resected LCNEC ranged from 13% to 57% [4,7]. Several studies have reported the efficacy of adjuvant chemotherapy for patients with resected LCNEC [8–11], the identification of predictive and/or prognostic markers is important to stratify patients with resected LCNEC and to select high-risk patients who should receive aggressive adjuvant chemotherapy.

Nestin is a class VI intermediate filament protein that is specifically expressed in stem/progenitor cells of the developing central

Abbreviations: CNS, central nervous system; Hh, hedgehog; LCNEC, large cell neuroendocrine carcinoma; NSCLC, non-small cell lung cancer; p-TNM, pathologic TNM; SCLC, small cell lung cancer; WHO, World Health Organization.

* Corresponding author at: Department of Molecular Diagnostics, School of Allied Health Sciences, Kitasato University, 1-15-1 Kitasato, Minami-ku, Sagamihara, Kanagawa 252-0373, Japan. Tel.: +81 42 778 8013; fax: +81 42 778 9854.
E-mail address: yuichi@med.kitasato-u.ac.jp (Y. Sato).

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nervous system (CNS), and is a large protein (>1600 amino acids), structurally similar to other intermediate filaments with a highly conserved α-helical core domain of 300–330 amino acids flanked by amino- and carboxy-terminal domains [12]. Nestin contains a short N-terminus and an unusually long C-terminus, which interacts with other cellular components and microtubules, such as vimentin and desmin, to form heterodimers and mixed polymers [13,14]. Although little is known about the biological function of nestin, some studies 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 [15,16]. Moreover, recent studies have shown that nestin is also expressed in epithelial tumors, such as pancreatic cancer [17], prostate cancer [18], and breast cancer [19], and its expression is associated with tumor aggressiveness. Recently, we reported that nestin is expressed in a subset of NSCLC and its expression is related to clinicopathological factors, and nestin expression is a prognostic indicator of poor survival among patients with resected NSCLC [20]. In this study, cytoplasmic nestin expression was observed in 3 of 5 LCNECs. To our knowledge, no report has been published concerning the relationships between nestin expression and clinicopathological features including the Ki-67 labeling index (LI) as a marker of the proliferative activity of the tumor and TTF-1 expression that is expressed in 49% of LCNECs [21] and patients' prognoses in LCNEC. Therefore, the aims of the present study were: (1) to immunohistochemically examine nestin expression in tumor cells of LCNECs, (2) to evaluate the relationships between nestin expression in tumor cells and the clinicopathological parameters including the Ki-67 LI and TTF-1 expression, and (3) to estimate the prognostic impact of nestin on survival of patients with surgically resected LCNEC.

2. Materials and methods

2.1. Patients and tissue specimens

Of 903 patients who underwent surgery for primary lung cancer from 1999 to 2009 at Kitasato University Hospital, 32 (3.5%) consecutive patients were histologically diagnosed with primary LCNEC according to the criteria of the WHO classification [22] by experienced pathologists in the Department of Pathology, Kitasato University Hospital. Two of the 32 cases were excluded from this study because they received preoperative chemotherapy or radiotherapy, and the remaining 30 cases were enrolled in this retrospective cohort study. Of the 30 patients with LCNEC, 4 were diagnosed with combined LCNEC, with a component of adenocarcinoma in 3 and squamous cell carcinoma in 1 case. The postoperative pathologic TNM (p-TNM) staging was determined according to the 7th edition of TNM classification [23]. The following clinical and pathologic parameters were retrospectively reviewed and analyzed for each case: age at surgical resection, gender, smoking habits, p-TNM stage, tumor size, nodal status, surgical procedure, postoperative adjuvant chemotherapy, viability status, and survival time after surgery. The viability status was determined based on whether or not lung cancer-related death occurred, and the survival time was defined as the duration from the date of surgery to that of death or the end of follow-up. We treated death by all other causes and those 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.

2.2. 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 antinestin antibody has been described previously [24]. For Ki-67 and TTF-1 immunohistochemical staining, antigen retrieval was carried out by autoclaving in 0.01 M citrate buffer (pH 6.0) with 0.1% Tween 20 at 121 °C for 10 min. After blocking with 2% normal swine serum/Tris-bufer saline (0.01 M Tris–HCl pH 7.5, 150 mM NaCl) for 10 min, the sections were reacted with 100-times-diluted anti-Ki-67 monoclonal antibody (MIB-1, DAKO, Glostrup, Denmark) and 200-times-diluted anti-TTF-1 antibody (DAKO, Glostrup, Denmark) for 2 h at room temperature. 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 subsequently visualized with Stable DAB solution (Invitrogen; Carlsbad, California) and counterstained with Mayer's hematoxylin. Negative controls were prepared by substituting phosphate-buffered saline for the primary antibodies.

2.3. Evaluation of immunohistochemical staining

For nestin, cytoplasmic immunostaining in tumor cells was considered to be positive. Nestin-positive non-neoplastic cells, such as immature fibroblasts, were carefully excluded. The stainability of peritumoral vascular endothelial cells was used as an internal positive control. The 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. Tumors with a staining score of 2 or 3 were judged as positive. As, in most nestin-positive cases, usually more than 5% of the tumor cells are stained, tissues consisting of more than 5% positive tumor cells were considered significant.

For Ki-67, the expression of Ki-67 was assessed based on the labeling index determined by counting 400 tumor cells randomly selected in a high-power field. The median value of positive tumor cells was 45% in the current series; therefore, we defined tumors with ≥45% of Ki-67 LI were classified as high Ki-67.

For TTF-1, alveolar type II cells of the normal lung served as an internal positive control. The staining intensity was categorized into four groups by comparing the staining intensity of tumor cells with alveolar type II cells: 0 = negative; 1 (weak) = weaker than alveolar type II cells; 2 (moderate) = the same as alveolar type II cells; 3 (strong) = stronger than alveolar type II cells. Staining was considered positive when ≥10% of tumor cells showed intensity 2 or 3 [21]. Concerning combined LCNEC cases, nestin, Ki-67 and TTF-1 expression was evaluated in the LCNEC component.

Two investigators (R.S. and S.Y.) 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.

2.4. Statistical analysis

Continuous variables were presented as median (range), whereas numerical variables were given as no. (%). The relationships between nestin expression and clinicopathologic parameters were assessed employing Fisher's exact test. 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. Three- and 5-year cumulative survival probabilities were estimated using the life table method with the interval length set at 1 month. Cox proportional hazards models were used to

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Table 1
Characteristics of the patients.

Characteristics	Patients, N (%) (N = 30)
Age	
Median age, y (range)	67 (50–85)
Gender	
Male	27 (90.0)
Female	3 (10.0)
Smoking habits	
Smoker	28 (93.3)
Never smoker	2 (6.7)
Details of histologic diagnosis	
LCNEC, pure	26 (86.7)
LCNEC, combined	4 (13.3)
With adenocarcinoma	3 (10.0)
With squamous cell carcinoma	1 (3.3)
p-TNM stage*	
IA	4 (13.3)
IB	9 (30.0)
IIA	6 (20.0)
IIB	3 (10.0)
IIIA	6 (20.0)
IIIB	2 (6.7)
Surgical procedure	
Wedge resection	2 (6.7)
Lobectomy	25 (83.3)
Pneumonectomy	3 (10.0)
Adjuvant chemotherapy	
Yes	7 (23.3)
No	23 (76.7)
Vital status	
Alive	11 (36.7)
Lung cancer-related death	16 (53.3)
Other cause of death	1 (3.3) (cerebral infarction)
Unknown	2 (6.7)

Data are presented as no. (%) unless otherwise indicated. p-TNM = pathologic TNM.
* Each case was reassigned for pathologic stage on the basis of the 7th edition of TNM classification [23].

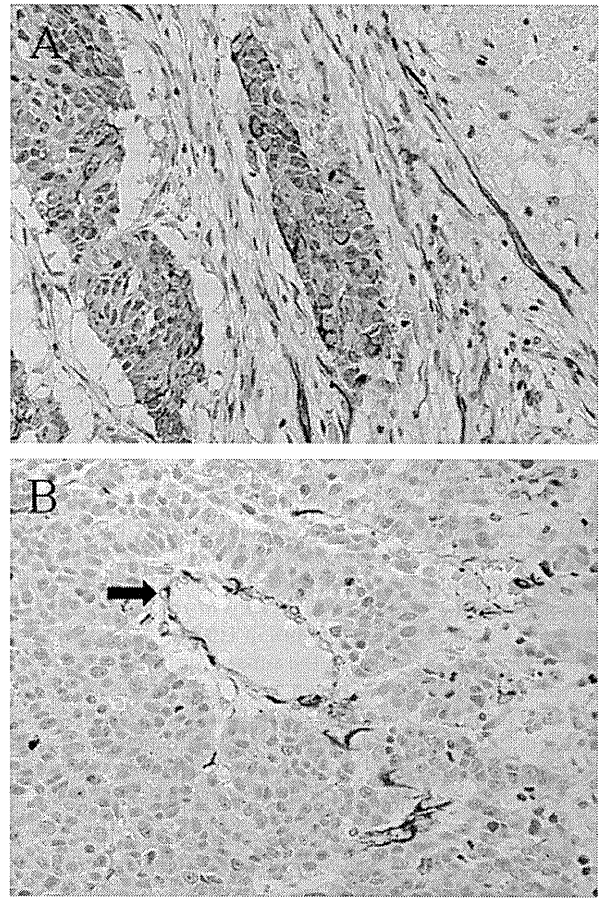

Fig. 1. Immunohistochemical staining of nestin in LCNEC. Nestin was expressed in the cytoplasm of vascular endothelial cells (arrows). The stainability of peritumoral vascular endothelial cells was used as an internal positive control. (A) Nestin was expressed in the cytoplasm of tumor cells (original magnification ×200). (B) The tumor cells were negative for nestin (original magnification ×200).

estimate the effect of nestin expression on survival. The conventional *P*-value of 0.05 or less 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 version 17.0 software (SPSS Inc., Chicago, Illinois).

3. Results

3.1. Patient characteristics

The clinicopathologic characteristics of the patients are summarized in Table 1. Pathological findings revealed that 29 patients underwent complete resection with negative surgical margins, 1 patient underwent incomplete resection with microscopic tumor invasion on the bronchial stump. Seven patients (23.3%) received adjuvant chemotherapy as follows: cisplatin plus etoposide ($n = 2$), cisplatin plus irinotecan ($n = 1$), cisplatin plus docetaxel ($n = 1$), and tegafur-uracil (UFT) ($n = 3$). The overall follow-up durations ranged from 2 to 124 months (median, 22 months). No patients died from surgery-related death. After 59 and 10 months follow-up, respectively, 2 patients were lost to follow up because of discontinued hospital attendance and inability to be contacted.

3.2. Nestin expression in LCNEC

Cytoplasmic nestin expression in tumor cells was observed in 8 of the 30 (26.7%) LCNECs (Fig. 1A). There is no difference between nestin-positive and -negative tumors in morphological features. Nestin expression was also observed in the cytoplasm of vascular endothelial cells and fibroblasts in the tumor stroma in each case. Nestin expression was not detected in non-neoplastic bronchial or alveolar epithelial cells. No positive staining was observed in the negative controls.

3.3. Relationship between nestin expression and clinicopathologic characteristics

The relationships between nestin expression and clinicopathologic characteristics summarized in Table 2. Nestin expression was significantly associated with a high Ki-67 LI ($P = 0.012$).

3.4. The effect of nestin expression on survival

All the patients were included in the survival analysis. The overall follow-up periods ranged from 2 to 124 months (median, 22 months), the median overall survival time was 28.4 months, and the 5-year cumulative survival probability was 47% (Fig. 2A). Of the 8 patients whose tumors were nestin-positive, there were 2 patients with stage IA, 3 with stage IB, 2 with stage IIB, and 1 with stage IIIB cancer. The follow-up duration of the nestin-positive group had not yet reached 5 years during the study, the 3-year cumulative survival probability was 25% for the nestin-positive group and 55% for the nestin-negative group. The median overall survival time was 8.1 months for patients with nestin-positive tumors and 81.4 months for those with nestin-negative tumors, and nestin expression was significantly associated with a poorer prognosis ($P = 0.016$, Fig. 2B). Cox proportional hazard regression was applied to

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Table 2
Relationships between nestin expression and clinicopathologic parameters.

Clinicopathologic parameters	Nestin expression		Total	*P*-value
	Positive (*N*=8)	Negative (*N*=22)		
Age, y				1.00
<65	3(25.0)	9(75.0)	12	
≥65	5(27.8)	13(72.2)	18	
Gender				0.166
Male	6(22.2)	21(77.8)	27	
Female	2(66.7)	1(33.3)	3	
Smoking habits				1.00
Smoker	8(28.6)	20(71.4)	28	
Never smoker	0(0)	2(100)	2	
p-TNM stage*				0.391
Stage I/II	7(31.8)	15(68.2)	22	
Stage III	1(12.5)	7(87.5)	8	
Tumor size				0.682
≤3 cm	2(20.0)	8(90.0)	10	
>3 cm	6(30.0)	14(70.0)	20	
Nodal status				0.199
N0	7(36.8)	12(63.2)	19	
N1/N2/N3	1(9.1)	10(90.9)	11	
Adjuvant chemotherapy	1.00			
Yes	2(28.6)	5(71.4)	7	
No	6(26.1)	17(73.9)	23	
Immunohistochemistry				
Ki-67 labeling index				0.012
Low (<45%)	1(6.3)	15(93.7)	16	
High (≥45%)	7(50.0)	7(50.0)	14	
TTF-1 expression				0.666
Negative	5(23.8)	16(76.2)	21	
Positive	3(33.3)	6(66.7)	9	

Data are presented as no. (%).
See Table 1 legend for expansion of abbreviations and note on asterisk.

Table 3
Univariable analysis for the effect of nestin expression on survival.

Factors	Univariable analysis		
	HR	95% CI	*P*-value
Nestin expression			
Positive vs negative	3.40	1.18–9.77	0.023
Age			
≥ 65 y vs < 65 y	0.73	0.27–1.96	0.54
Gender			
Male vs female	2.16	0.48–9.65	0.31
Smoking habits			
Smoker vs never smoker	1.36	0.17–10.5	0.76
p-TNM stage*			
Stage III vs stage I/II	2.47	0.87–7.02	0.08
Adjuvant chemotherapy			
No vs yes	5.42	0.71–41.3	0.10

CI = confidence interval; HR = hazard ratio.
See Table 1 for note on asterisk.

estimate the effect of nestin expression and other clinicopathologic variables on survival. Only nestin expression showed significance. The crude hazard ratio (HR) of nestin expression compared to nestin absence was 3.40 (95% CI, 1.19–9.77; $P=0.023$, Table 3), indicating that nestin expression increases the hazard of death by three times.

4. Discussion

The incidence (3.5%) and 5-year cumulative survival probability (47%) of LCNEC in the present study were similar to those of previous reports [3,10,25]. In the present study, we showed that nestin expression was related to a high Ki-67 LI and seemed to be associated with a poorer prognosis in patients with resected LCNEC. The present study indicated that nestin expression is associated with tumor cell proliferation activity in LCNECs. Moreover,

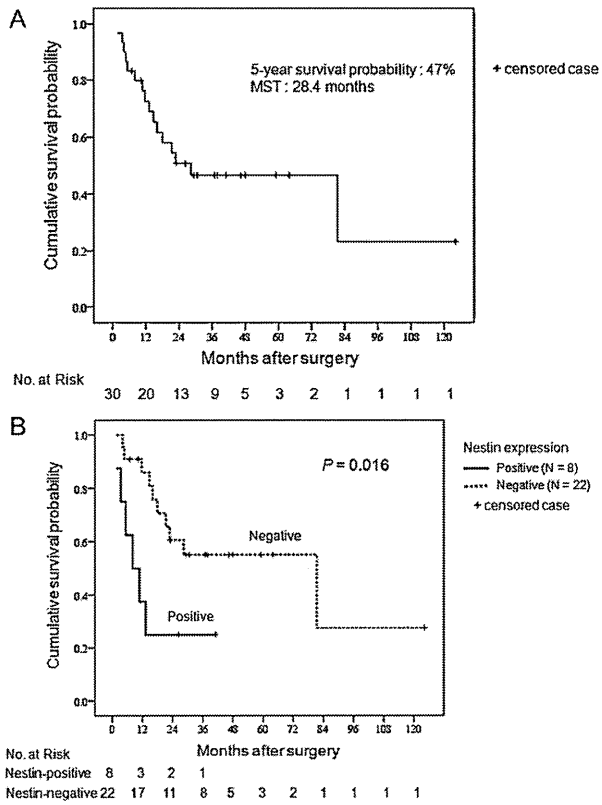

Fig. 2. Cumulative survival of patients with resected LCNEC estimated by the Kaplan–Meier method, treating patients with other causes of death and those lost to follow-up as censored cases. (A) In all 30 patients with resected LCNEC, the 5-year cumulative survival probability was 47%, and the median overall survival time (MST) was 28.4 months. (B) The median overall survival times for patients with nestin-negative LCNEC and nestin-positive LCNEC were 81.4 and 8.1 months, respectively. Nestin expression was significantly associated with a poorer survival in patients with resected LCNEC.

previous reports of pancreatic [17] and prostate cancer [18], including our report of NSCLC [20], suggested that nestin expression may be important for the acquisition of migration and invasion capabilities of tumor cells.

According to these reports, we deduced that nestin-positive tumors might exhibit more aggressive behavior resulting from higher abilities of tumor cell migration and invasion, which subsequently resulted in poorer prognoses in patients with resected LCNEC.

While 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 [12,15]. In the present study, no nestin expression was observed in bronchial or alveolar epithelial cells in non-neoplastic peripheral lung tissues. However, a previous study reported that nestin was expressed in mesenchymal stem cells from human fetal lungs [26]. Thus, it is possible that nestin-positive tumor cells may have more features similar to stem/progenitor cells with more aggressive behavior such as that of migration and invasion. Beachy et al. [27] suggested that Hedgehog (Hh)-dependent tumors may be derived at least partly from cancer progenitor cells. The Hh signaling pathway is considered to be involved in tissue repair and carcinogenesis, and previous studies also reported that nestin could be re-expressed in adult tissues under pathologic conditions, such as formation of the glial scar after injury to the CNS [28], regeneration of injured skeletal muscle tissue [29], and cancers [17–20].

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Several studies have reported that nestin expression is dependent on activation of the Hh signaling pathway in Hh-dependent tumors [30,31], including SCLC [32]. In the present study, although the involvement of the Hh signaling pathway in LCNEC remains unclear, nestin expression may be dependent on the Hh signaling pathway in LCNEC similar to SCLC.

Several studies reported the efficacy of the SCLC-based regimen of adjuvant chemotherapy for patients with resected LCNEC, especially stage I cancer [8–10]. Therefore, customized adjuvant chemotherapy in LCNEC appears to be facilitated by identifying the prognostic biomarker independent of the p-TNM stage. Rossi et al. [9] reported that the tumor size and Met expression are significantly correlated with survival, and the tumor size remains significantly correlated with survival in multivariable analysis. Jones et al. [33] reported that high-grade NE carcinomas of the lung can be classified into two prognostically significant subtypes independent of LCNEC and SCLC by gene expression profiles. Usuda et al. [34] reported that Klotho expression has a significant prognostic value in LCNEC. In the present study, we have revealed nestin expression to be a novel prognostic marker for patients with resected LCNEC; however, the sample size of our retrospective cohort study is relatively small, and the follow-up duration is relatively short. On univariable analyses, the p-TNM stage failed to show significance for the prognosis within the present study population. Only 16 events were observed in this study, so overfitting will exist when performing such a multivariable regression. Because of the exploratory nature of this study, it may not be able to lead to a strong conclusion; however, we believe that the probable prognostic significance of nestin will provide room for further research in this field. A larger, multi-institute cohort study is needed to clarify the finding from our study, especially with stage I cancer, which is more effectively treated with adjuvant chemotherapy.

In conclusion, we have reported that nestin is expressed in a subset of LCNEC, and nestin expression is a prognostic indicator of a poorer survival among patients with resected LCNEC, although its prognostic significance still requires confirmation with larger patient populations. Moreover, new therapeutic targets and anticancer strategies may be established by elucidating the mechanism of nestin expression in LCNEC.

Conflict of interest statement

None declared.

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Author contributions: *Dr Ryuge*: contributed to analysis and interpretation of the data, and drafting the article. *Dr Sato*: contributed to the conception and design of the study, and final approval of the version to be submitted. *Dr Jiang*: contributed to analysis and interpretation of the data, and final approval of the version to be submitted. *Dr Wang*: contributed to analysis and interpretation of the data. *Dr Matsumoto*: contributed to the conception and design of the study. *Dr Katono*: contributed to acquisition of the data. *Dr Inoue*: contributed to the conception and design of the study. *Dr Iyoda*: contributed to acquisition of the data, and final approval of the version to be submitted. *Dr Satoh*: contributed to acquisition of the data, analysis and interpretation of the data analysis. *Dr Yoshimura*: contributed to acquisition of the data, analysis and interpretation of the data analysis. *Dr Masuda*: contributed to the conception and design of the study.

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