6. Le Naour F, Brichory F, Misek DE, Bréchot C, Hanash SM and Beretta L: A distinct repertoire of autoantibodies in hepatocellular carcinoma identified by proteomic analysis. Mol Cell Proteomics 1: 197-203, 2002.
7. Soussi T: p53 Antibodies in the sera of patients with various types of cancer: a review. Cancer Res 60: 1777-1788, 2000.
8. Stockert E, Jäger E, Chen YT, *et al*: A survey of the humoral immune response of cancer patients to a panel of human tumor antigens. J Exp Med 187: 1349-1354, 1998.
9. Xia Q, Kong XT, Zhang GA, Hou XJ, Qiang H and Zhong RQ: Proteomics-based identification of DEAD-box protein 48 as a novel autoantigen, a prospective serum marker for pancreatic cancer. Biochem Biophys Res Commun 330: 526-532, 2005.
10. Fernández-Madrid F, Tang N, Alansari H, *et al*: Autoantibodies to Annexin XI-A and other autoantigens in the diagnosis of breast cancer. Cancer Res 64: 5089-5096, 2004.
11. Hanash S: Harnessing immunity for cancer marker discovery. Nat Biotechnol 21: 37-38, 2003.
12. Yagihashi A, Asanuma K, Kobayashi D, *et al*: Detection of autoantibodies to livin and survivin in Sera from lung cancer patients. Lung Cancer 48: 217-221, 2005.
13. Vural B, Chen LC, Saip P, *et al*: Frequency of SOX Group B (SOX1, 2, 3) and ZIC2 antibodies in Turkish patients with small cell lung carcinoma and their correlation with clinical parameters. Cancer 103: 2575-2583, 2005.
14. Brichory F, Beer D, Le Naour F, Giordano T and Hanash S: Proteomics-based identification of protein gene product 9.5 as a tumor antigen that induces a humoral immune response in lung cancer. Cancer Res 61: 7908-7912, 2001.
15. Alamowitch S, Graus F, Uchuya M, Reñé R, Bescansa E and Delattre JY: Limbic encephalitis and small cell lung cancer. Clinical and immunological features. Brain 120: 923-928, 1997.
16. Jiang SX, Kameya T, Asamura H, *et al*: hASH1 expression is closely correlated with endocrine phenotype and differentiation extent in pulmonary neuroendocrine tumors. Mod Pathol 17: 222-229, 2004.
17. Nagashio R, Sato Y, Jiang SX, Ryuge S, Kodera Y, Maeda T and Nakajima T: Detection of tumor-specific autoantibodyes in sera of patients with lung cancer. Lung Cancer 62: 364-373, 2008.
18. Sakai K, Gofuku M, Kitagawa Y, *et al*: A hippocampal protein associated with paraneoplastic neurologic syndrome and small cell lung carcinoma. Biochem Biophys Res Commun 199: 1200-1208, 1994.
19. Graus F and Ferrer I: Analysis of a neuronal antigen (Hu) expression in the developing rat brain detected by autoantibodies from patients with paraneoplastic encephalomyelitis. Neurosci Lett 112: 14-18, 1990.
20. Laemmli UK: Cleavage of structural proteins during the assembly of the head of bacteriophage T4. Nature 227: 680-685, 1970.
21. Goshima N, Kawamura Y, Fukumoto A, *et al*: Human protein factory for converting the transcriptome into an *in vitro*-expressed proteome. Nature Methods 5: 1011-1017, 2008.
22. Good PJ: A conserved family of elav-like genes in vertebrates. Proc Natl Acad Sci USA 92: 4557-4561, 1995.
23. Birney E, Kumar S and Krainer AR: Analysis of the RNA-recognition motif and RS and RGG domains: conservation in metazoan pre-mRNA splicing factors. Nucleic Acids Res 21: 5803-5816, 1993.
24. Dalmau J, Furneaux HM, Rosenblum MK, Graus F and Posner JB: Detection of the anti-Hu antibody in specific regions of the nervous system and tumor from patients with paraneoplastic encephalomyelitis/sensory neuronopathy. Neurology 41: 1757-1764, 1991.
25. Grous F, Keime-Guibert F, Rene R, *et al*: Anti-Hu-associated paraneoplastic encephalomyelitis: analysis of 200 patients. Brain 124: 1138-1148, 2001.
26. Anderson NE, Cunningham JM and Posner JB: Autoimmune pathogenesis of paraneoplastic neurological syndromes. Crit Rev Neurobiol 3: 245-299, 1987.
27. Carpentier AF, Voltz R, DesChamps T, Posner JB, Dalmau J and Rosenfeld MR: Absence of HuD gene mutations in paraneoplastic small cell lung cancer tissue. Neurology 50: 1919, 1998.
28. Sekido Y, Bader SA, Carbone DP, Johnson BE and Minna JD: Molecular analysis of the HuD gene encoding a paraneoplastic encephalomyelitis antigen in human lung cancer cell lines. Cancer Res 54: 4988-4992, 1994.
29. Dalmaou J, Furneaux HM, Gralla RJ, Kris MG and Posner JB: Detection of the anti-Hu antibody in the serum of patients with small cell lung cancer-a quantitative Western blot analysis. Ann Neurol 27: 544-552, 1990.
30. Graus F, Dalmou J, Rene R, *et al*: Anti-Hu antibodies in patients with small-cell lung cancer: association with complete response to therapy and improbed survival. J Clin Oncol 15: 2866-2872, 1997.
31. Kazarian M, Calbo J, Proost N, Carpenter CL, Berns A and Laird-Offringa IA: Immune response in lung cancer mouse model mimics human anti-Hu reactivity. J Neuroimmunol 217: 38-45, 2009.
32. Tsou JA, Kazarian M, Patel A, Galler J, Laird-Offringa IA, Carpenter CL and London SJ: Low level anti-Hu reactivity: a risk marker for small cell lung cancer? Cancer Detect Prev 32: 292-299, 2008.
33. Verschuuren JJ, Perquin M, ten Velde G, DeBaets M, van Breda Vriesman P and Twijinstra A: Anti-Hu antibody titre and brain metastases before and after treatment for small cell lung cancer. J Neurol Neurosurg Psychiatry 67: 353-357, 1999.
34. D'Alessandro V, Muscarella LA, La Torre A, *et al*: Molecular analysis of the HuD gene in neuroendocrine lung cancers. Lung Cancer 67: 69-75, 2010.

Investigative report

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Prognostic significance of the hair follicle stem cell marker nestin in patients with malignant melanoma

Nestin is an intermediate filament protein, and serves as a hair follicle stem cell and neural stem cell marker. Recent studies have suggested that nestin expression is also important for tumorigenesis. Previous reports from our laboratory have revealed that nestin is a marker of HMB-45-negative melanoma cells in dermal invasive lesions of nodular malignant melanoma. The present study examines nestin expression in malignant melanoma and investigates the relationship between nestin expression and prognosis in patients. We immunohistochemically stained 78 formalin-fixed and paraffin-embedded malignant melanomas for nestin, HMB-45 and S100 reactivity. We found that nestin, HMB-45 and S100 protein were detected in 56.5%, 88.4% and 100% of malignant melanomas, respectively. The 5-year survival rate of stage I and II nestin-positive cases was significantly decreased compared to the nestin-negative cases ($p < 0.05$). In addition, the 5-year survival rate exceeded 80% in nestin-negative malignant melanomas at all stages of tumor development. We conclude that nestin expression may be a predictor of poor prognosis in patients with malignant melanoma.

Key words: malignant melanoma, hair follicle stem cells, nestin, HMB-45, prognosis

Nestin is a neural stem cell marker protein that is also expressed in the bulge area stem cell compartment of the hair follicle. Nestin-expressing hair follicle stem cells give rise to the outer root sheath and the nestin-expressing interfollicular vascular network. Nestin-expressing stem cells isolated from the hair follicle bulge region are negative for the keratinocyte marker keratin 15 (K15), and are able to differentiate into numerous cell types *in vitro*, including neurons, glia, keratinocytes, smooth muscle cells and melanocytes. This primitive state demonstrated by the nestin-positive stem cells is compatible with their pluripotency [1-9]. Moreover, as various types of cutaneous tumors, including melanoma, originate from the hair follicle and/or epidermal stem cells [10], nestin expression is thought to be important in tumorigenesis.

In the present study, we examined nestin expression in malignant melanoma and investigated the relationship between nestin expression levels and the prognosis of patients with malignant melanomas.

Materials and methods

Patients and tumor collection

Formalin-fixed and paraffin-embedded tissues were obtained from 78 (35 male and 43 female) malignant melanoma patients who were observed over a period of at least 5 years and who were surgically resected at the Department of Dermatology or Plastic Surgery of Kitasato University Hospital in Japan. The patients (4 to 85 years old; median, 56.6 years old) between 1988 and 2004 were retrospectively reviewed. And we observed the five year or more passages (the longest is 21 years) of all the cases, excluding the patients who died within five years. All patients underwent subsequent tumor excision. The tumor samples analyzed were obtained from central areas of the primary tumor. Paraffin blocks representing the most typical tumor tissue (mainly central portions of primary tumors) were selected, and 4-μm thick sections were prepared for immunohistochemistry. The samples were histologically analyzed according to the pathological staging criteria of the American Joint Committee on Cancer (AJCC) and subdivided into 6 cases of stage 0, 18 cases of stage I, 33 cases of stage II, 13 cases of stage III and 8 cases of stage IV. The majority of patients in stages II to IV, 51 out of 54 (without three patients in Stage IV), received chemotherapy (DAV-F: intravenous (i.v.) dacarbazine, 160 mg/m^2/day on days 1-5; nimustine, 80 mg/m^2/day i.v. on day 1; vincristine, 0.8 mg/m^2/day i.v. on day 1; and β-feron, 3 million Units s.c. on days 1-5). The survival time was calculated as the date of surgery to the date of death. Informed consent was obtained from all patients prior to admission into the study. This study was approved by the Ethics Committee of Kitasato University School of Medicine.

Immunohistochemical analysis

Indirect immunohistochemical staining was performed using the following primary antibodies: polyclonal anti-nestin (1:20, IBL, Gunma, Japan), monoclonal anti-melanosome (clone HMB-45, undiluted, Dako, Glostrup, Denmark) and polyclonal anti-S100 (1:400, Dako). Bound primary antibodies

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were detected using the dextran polymer reagent (ChemMate Envision, Dako). Following deparaffinization, rehydration and elimination of endogenous peroxidase activity by treatment with 3% hydrogen peroxide for 5 min at room temperature (RT), sections were incubated with 5% fetal calf serum in PBS for 5 min at RT to block non-specific protein binding. The sections were incubated with each antibody for 60 min and then with ChemMate Envision for 30 min at RT. Finally, the color reaction was developed with 8-amino-9-ethylcarbazole solution (Dako) and the sections counterstained with Mayer's hematoxylin (Wako Pure Chemical, Osaka, Japan). S100 protein staining was considered positive when definite expression was observed in the nucleus and/or cytoplasm of > 5% of tumor cells, while HMB-45 and nestin staining was considered positive when definite expression was observed in the cytoplasm of tumor cells. The slide of Nestin staining was evaluated according to staining extent and intensity. Staining extension was assessed by the percentage of stained cells and scored semi-quantitatively, using a 0 to 4 scale for expression: 0 = no expression; 1+ = 1-25%, 2+ = 26-50%, 3+ = 51-75%, 4+ = 76-100%. Staining intensity was categorized into three groups by comparing the staining intensity of tumor cells with vascular endothelial cells: 1+ = weaker than endothelial cells; 2+ = same as endothelial cells; and 3+ = stronger than endothelial cells. By adding the staining extensity and intensity scores, the combined scores were calculated. The combined scores were then divided into 4 groups: Negative = combined score 0; weak staining = combined score 2, moderate staining = combined scores 3-4; strong staining = combine scores 5-7 [10].

Analysis of clinical parameters

The overall survival of the patients among the 6 groups was analyzed using the Kaplan-Meier method, Z-test and Fisher's exact test. Values < 0.05 were considered statistically significant.

Results

Immunohistochemical analysis

The expression of S100 protein was detected in the nucleus and cytoplasm of tumor cells in all cases. The expression of HMB-45 protein was also observed in the cytoplasm of tumor cells. The expression of HMB-45 was detected in 5 out of 6 (83.3%) stage 0 tumors, 17 out of 18 (94.4%) stage I tumors, 29 out of 33 (87.9%) stage II tumors, 10 out of 13 (76.9%) stage III tumors, and 8 out of 8 (100%) stage IV tumors. Nestin expression was observed in the cytoplasm of tumor cells in 59.0% of malignant melanomas (n = 78). Positivity rates were 0 out of 6 (0%) stage 0 tumors, 6 out of 18 (33.3%) stage I tumors, 23 out of 33 (69.7%) stage II tumors, 10 out of 13 (76.9%) stage III tumors and 7 of 8 (87.5%) stage IV tumors. The frequency and combined scores of nestin expression of groups are shown in *table 1*. Average combined score was 0 in stage 0 tumors, 1.5 in stage I tumors, 3.18 in stage II tumors, 3.46 in stage III tumors and 4.57 in stage IV tumors. Average combined score rose gradually as the stage progressed. In addition, some vascular endothelial cells, fibroblasts and peripheral nerve cells were also nestin positive. *Figure 1* indicates the nestin- and HMB-45-positive melanoma cells in the junctional area *(figure 1A)*, dermis *(figure 1B)*, and deep dermis *(figure 1C)*.

Prognostic relevance

Follow-up of patients ranged from 5 to 180 months. During this time, 29.5% of the patients died. The survival curves for each of the different tumor stages are presented in *figure 2*, and the 5-year survival rates of nestin-positive and nestin-negative patients are shown in *figure 3*. The 5-year survival rates were 6 out of 6 (100%) for stage 0 tumors, 16 out of 18 (88.9%) for stage I tumors, 28 out of 33 (84.8%) for stage II tumors, 8 out of 13 (61.5%) for stage III tumors and 1 out of 8 (12.5%) for stage IV tumors. The difference in survival

Table 1. The frequency and combined scores (all patients)

	Stage 0	Stage I	Stage II	Stage III	Stage IV
Nestin expression					
Positive	0 -	6 (33%)	23 (70%)	9 (69%)	7 (88%)
Negative	6 (100%)	12 (67%)	10 (30%)	4 (31%)	1 (13%)
Extension scores					
0	6 (100%)	12 (67%)	10 (30%)	3 (23%)	1 (13%)
1+	0 -	1 (6%)	5 (15%)	1 (8%)	1 (13%)
2+	0 -	4 (22%)	2 (6%)	3 (23%)	0 -
3+	0 -	0 -	9 (27%)	4 (31%)	3 (38%)
4+	0 -	1 (6%)	7 (21%)	2 (15%)	3 (38%)
Intensity scores					
1+	0 -	1 (6%)	8 (24%)	4 (31%)	0 -
2+	0 -	2 (11%)	8 (24%)	2 (15%)	5 (63%)
3+	0 -	3 (17%)	7 (21%)	4 (31%)	2 (25%)
Combined scores					
0 (negative)	6 (100%)	12 (67%)	10 (30%)	3 (23%)	1 (13%)
2 (weak)	0 -	1 (6%)	3 (9%)	1 (8%)	0 -
3-4 (moderate)	0 -	2 (11%)	6 (18%)	3 (23%)	1 (13%)
5-7 (strong)	0 -	3 (17%)	14 (42%)	5 (38%)	6 (75%)
Average scores (0-7)	0 -	1.5	3.18	3.46	4.57

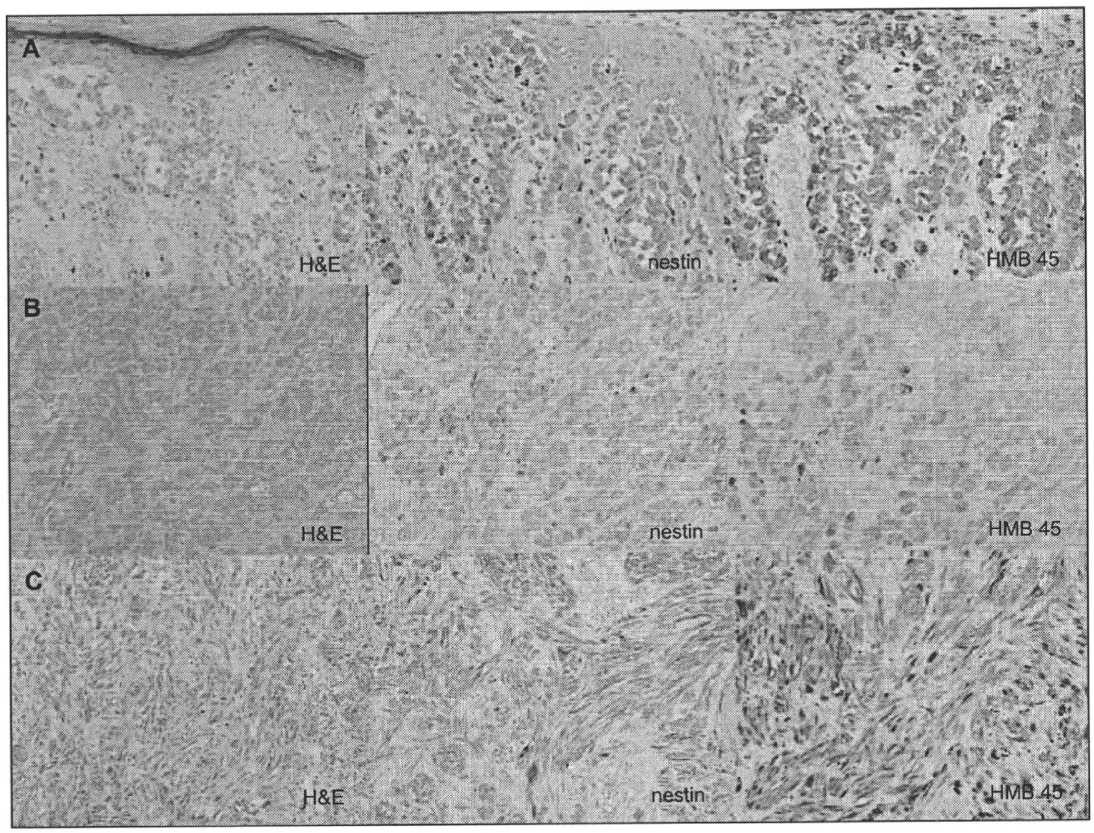

Figure 1. Based on immunohistochemical staining using anti-nestin and anti-HMB-45 antibodies, malignant melanoma tissues were evaluated and scored as negative (< 5% of positive cells) or positive (> 5% of positive cells). Melanoma cells in the junctional area **(A)**, melanoma cells in the dermis **(B)** and melanoma cells in the deep dermis **(C)** are presented. Melanoma cells in the junctional area, dermis, and deep dermis in these samples are both nestin- and HMB-45-positive.

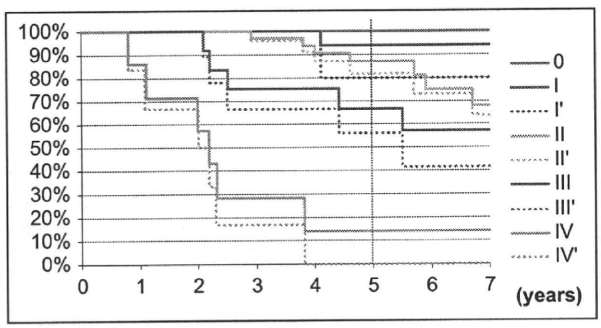

Figure 2. Relationship between nestin expression and survival rate in malignant melanoma. Nestin-positive patients (hashed lines) are compared to nestin-negative patients (solid lines) for each tumor stage.

rate among the stages was significant *(figure 2)*. Comparison of the survival rate between nestin-positive patients and nestin-negative patients for each stage also showed significant differences in all cases ($P < 0.001$ Fisher's exact test). That is, the 5-year survival rate was 100% in all patients exhibiting nestin-negative tumors (stage 0: 6 cases, stage I: 12 cases, stage II: 10 cases, stage III: 3 cases and stage IV: 1 case). In stage I tumors, nestin-positive patients demonstrated a decreased 5-year survival rate (66.7%) compared to the nestin-negative patients (100%, $P < 0.05$, Z-test). Nestin-positive patients also exhibited a decreased 5-year survival rate (78.3%) in patients with stage II tumors compared to the nestin-negative patients with stage II tumors (100%, $P < 0.05$ Z-test) *(figure 3)*. And the frequency and combined scores of nestin expression of groups, divided 5-year survival groups and death groups, are shown in *table 2*. In each stage, average combined scores in 5-year survival groups were higher than in death groups.

Discussion

Nestin is a neural stem cell marker protein that is also expressed in hair follicle stem cells in the bulge region [5-9]. The nestin-expressing hair follicle stem cells give rise to the outer root sheath and the nestin-expressing interfollicular vascular network in nestin-GFP transgenic mice [6]. We have recently demonstrated that nestin-expressing stem cells isolated from the hair follicle stem cell region in mice that were negative for the keratinocyte marker K15 were able to differentiate into neurons, glia, keratinocytes, smooth muscle cells and melanocytes *in vitro* [5-9]. In addition to being K15-negative, the pluripotent nestin-expressing stem cells are also positive for the stem cell marker CD34, demonstrating their relatively undifferentiated state. This primitive state of the nestin-expressing

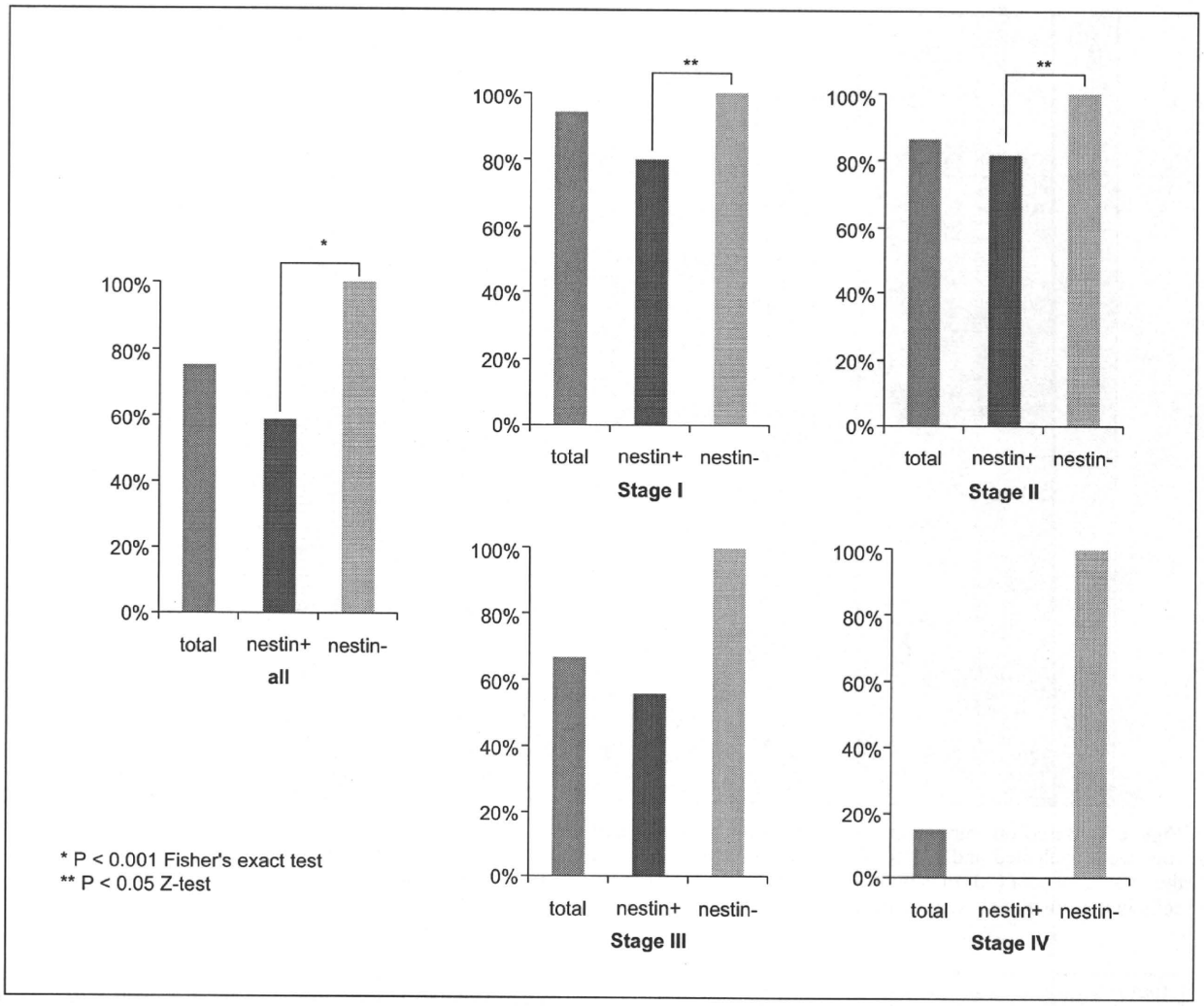

Figure 3. Five-year survival rate of nestin-positive and -negative patients for each tumor stage.

stem cells appears to be compatible with their pluripotency [6-9]. Recently, numerous studies have suggested that various forms of cutaneous tumors, including melanoma, originate from the hair follicle and epidermal stem cells. Brychtova *et al.* [11] demonstrated via immunohistochemical analysis that nestin is expressed in malignant melanoma and melanocytic nevi. They also demonstrated that nestin immunoreactivity was significantly increased in malignant melanomas, and that the precise levels of nestin correlated with the clinical stage of the tumor. Moreover, immunohistochemical analysis demonstrated nestin-positive cells in 35 of 42 (83.3%) nodular melanomas, 10 of 32 (31.3%) superficial spreading melanomas, 10 of 12 (83.3%) metastatic melanomas, 2 of 10 (20.0%) dysplastic nevi and 20 of 43 (46.5%) nevomelanocytic nevi [12]. Thus, the expression levels of nestin significantly correlate with the aggressiveness of malignant melanoma [13]. In addition, a significantly greater percentage of CD166-, CD133- and nestin-positive tumor cells were identified in malignant melanomas compared to nevomelanocytic nevi, while all cases of metastatic melanoma expressed at least one stem cell marker. However, statistical significance for nestin expression was only detected between the primary and metastatic melanomas [13]. Moreover, nestin expression has not been observed in HMB-45-negative melanotic and amelanotic malignant melanomas [12].

As the precise diagnosis of HMB-45-negative malignant melanoma is clinically most important, we investigated the relationship between nestin expression and melanoma class in the present study. We found that nestin was a useful marker for the diagnosis of HMB-45-negative malignant melanoma. Tumor cells in epidermal lesions that also expressed melanin failed to express nestin. This result correlated with previous studies demonstrating that tumor cells proliferating into the dermis, especially within the invasive front, lacked melanin and expressed nestin [12]. Xu *et al.* evaluated the reactivity of a panel of antibodies against markers associated with melanoma and melanocytic differentiation in HMB-45-negative, non-desmoplastic melanomas. They concluded that melanoma antigen 1 (MAGE-1), melanocyte-specific transcription factor (MITF), tyrosinase and Melan-A served as useful markers for the diagnosis of

Table 2. The frequency and combined scores (divided 5 year survival groups and death groups)

	Stage 0		Stage I		Stage II		Stage III		Stage IV	
	Survive	Death	Survive	Death	Survive	Death	Survive	Death	Survive	Death
Nestin expression										
Positive	0	0	4	2	18	5	4	5	0	7
Negative	6	0	12	0	10	0	4	0	1	0
Extension scores										
0	6	0	12	0	10	0	4	0	1	0
1+	0	0	1	0	4	1	1	0	0	1
2+	0	0	2	2	2	0	0	3	0	0
3+	0	0	0	0	7	2	3	1	0	3
4+	0	0	1	0	5	2	1	1	0	3
Intensity scores										
1+	0	0	1	0	6	2	3	1	1	0
2+	0	0	1	1	7	1	1	1	0	5
3+	0	0	2	1	5	2	1	3	0	2
Combined scores										
0 (negative)	6	0	12	0	10	0	3	0	1	0
2 (weak)	0	0	1	0	2	1	1	0	0	0
3-4 (moderate)	0	0	1	1	6	0	1	2	0	1
5-7 (strong)	0	0	2	1	10	4	2	3	0	6
Average scores (0-7)	0	0	1.13	4.5	2.85	5	2.5	5	0	5.43

malignant melanotic lesions when HMB-45 is not present [14]. In contrast, we reported the presence of Melan-A in only 6 out of 10 cases of dermal lesions in nodular malignant melanoma, and only 2 out of 5 cases of dermal lesions of amelanotic malignant melanoma. In addition, MITF immunoreactivity was only observed in 5 out of 10 cases of dermal lesions of nodular malignant melanoma and 4 out of 5 cases of dermal lesions of amelanotic malignant melanoma, while MAGE-1 immunoreactivity was only observed in 1 case of dermal lesion, 10 cases of nodular malignant melanoma and 5 cases of amelanotic malignant melanoma. Thus, nestin may be a useful marker of HMB-45-negative melanoma cells in dermal lesions of melanotic and amelanotic nodular malignant melanomas [12].

Recently, Flammiger *et al.* described that the intermediate filament protein and stem cell marker nestin, as well as the lineage restricted transcription factors BRN2, SOX9, and SOX10, are expressed in melanoma cell lines of all progression stages as well as in melanoma tissues, and that SOX9 and SOX10 but not BRN2 can alter nestin expression in melanoma [15]. And Bakos *et al.* demonstrated that nestin and SOX9 expression are increased, respectively, in ulcerated melanomas and advanced-stage melanoma, and may be markers of tumor aggressiveness [16]. In addition, Yang *et al.* suggested that the expression of nestin may play an important role in the development of some neoplasms, such as GIST and angiosarcoma [17].

In the present study, we investigated the relationship between nestin expression and patient outcome in malignant melanomas. We found that nestin, HMB- 45 and S100 protein were detected in 56.5%, 88.4% and 100% of malignant melanomas, respectively. The 5-year survival rates of stages I and II nestin-positive cases was significantly decreased compared to the nestin-negative cases ($p < 0.05$). In addition, the 5-year survival rates were 100% in nestin-negative malignant melanomas at all stages of tumor development. We conclude that nestin expression may be a predictor of poor prognosis in patients with malignant melanoma. ■

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References

1. Hoffman RM. The hair follicle as a gene therapy target. *Nat Biotechnol* 2000; 18: 20-1.
2. Oshima H, Rochat A, Kedzia C, Kobayashi K, Barrandon Y. Morphogenesis and Renewal of Hair Follicles from Adult Multipotent Stem Cells. *Cell* 2001; 104: 233-45.
3. Cotsarelis G, Sun T-T, Lavker RM. Label-retaining cells reside in the bulge area of pilosebaceous unit: implications for follicular stem cells, hair cycle, and skin carcinogenesis. *Cell* 1990; 61: 1329-37.
4. Taylor G, Lehrer MS, Jensen PJ, Sun TT, Lavker RM. Involvement of Follicular Stem Cells in Forming Not Only the Follicle but Also the Epidermis. *Cell* 2000; 102: 451-61.
5. Li L, Mignone J, Yang M, *et al.* Nestin expression in hair follicle sheath progenitor cells. *Proc Natl Acad Sci USA* 2003; 100: 9958-61.
6. Amoh Y, Li L, Yang M, *et al.* Nascent blood vessels in the skin arise from nestin-expressing hair-follicle cells. *Proc Natl Acad Sci USA* 2004; 101: 13291-5.
7. Amoh Y, Li L, Yang M, *et al.* Multipotent nestin-positive, keratin-negative hair-follicle bulge stem cells can form neurons. *Proc Natl Acad Sci USA* 2005; 102: 5530-4.
8. Amoh Y, Li L, Campillo R, *et al.* Implanted hair follicle stem cells form Schwann cells which support repair of severed peripheral nerves. *Proc Natl Acad Sci USA* 2005; 102: 17734-8.

9. Amoh Y, Li L, Katsuoka K, Hoffman RM. Chemotherapy targets the hair-follicle vascular network but not the stem cells. *J Invest Dermatol* 2007; 127: 11-5.
10. Levent Y, Mehmet K, Oguz A, Bedri K. Fascin expression in melanocytic lesions of the skin. *Eur J Dermatol* 2009; 19: 445-50.
11. Brychtova S, Fiuraskova M, Hlobilková A, Brychta T, Hirnak J. Nestin expression in cutaneous melanomas and melanocytic nevi. *J Cutan Pathol* 2007; 34: 370-5.
12. Kanoh M, Amoh Y, Tanabe K, Maejima H, Takasu H, Katsuoka K. Nestin is expressed in HMB-45 negative melanoma cells in dermal lesion of nodular malignant melanoma. *Eur J Derm* 2008; 18: 518-23.
13. Klein WM, Wu BP, Zhao S, Wu H, Klein-Szanto AJ, Tahan SR. Increased expression of stem cell markers in malignant melanoma. *Mod Pathol* 2007; 20: 102-7.
14. Xu X, Chu AY, Pasha TL, Elder DE, Zhang PJ. Immunoprofile of MITF, Tyrosinase, Melan-A, and MAGE-1 in HMB-45 negative Melanomas. *Am J Surg Pathol* 2002; 26: 82-7.
15. Flammiger A, Besch R, Cook AL, Maier T, Sturm RA, Berking C. SOX9 and SOX10 but Bot BRN2 Are Required for Nestin Expression in Human Melanoma Cells. *J Invest Dermatol* 2009; 129: 945-53.
16. Bakos RM, Maier T, Besch R, Mestel DS, Ruzicka T, Sturm RA, Berking C. Sturm and Carola Berking. Nestin and SOX9 and SOX10 transcription factors are coexpressed in melanoma. *Exp Dermatol* 2009 Oct 21 (Epub ahead of print).
17. Yang XH, Wu QL, Yu XB, *et al.* Nestin expression and malignant grade for different tumors. *J Clin Pathol* 2008; 61: 467-73.

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

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

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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

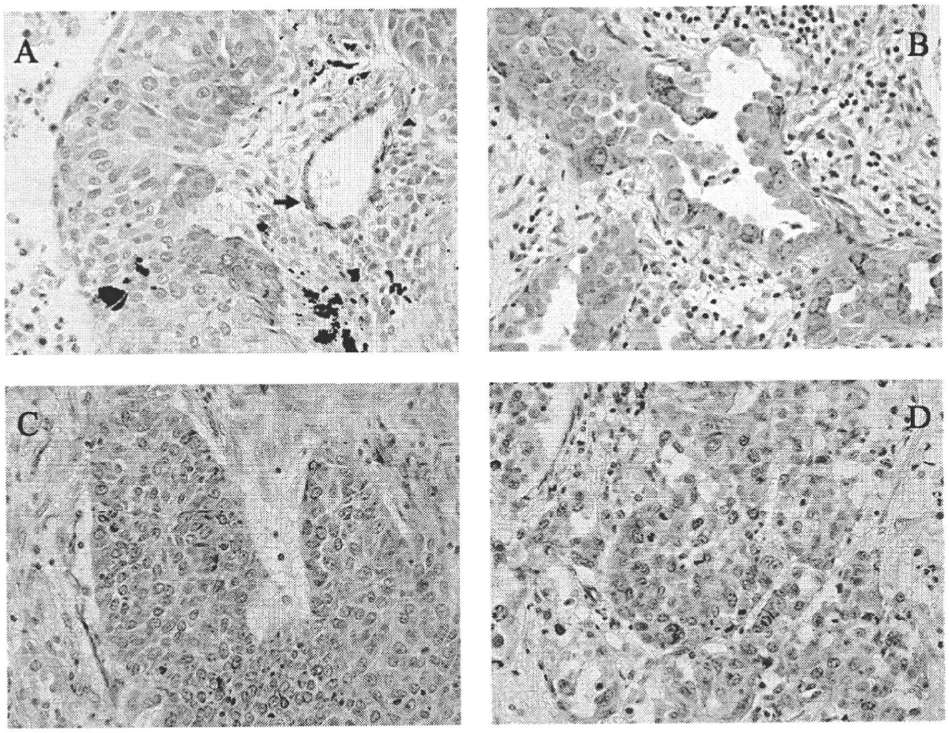

FIGURE 1. Immunohistochemical stain analysis of nestin expression in non-small cell lung cancer (NSCLC). Nestin was expressed in vascular endothelial cells (arrows). The stainability of peritumoral vascular endothelial cells was used as an internal control. A, Some tumor cells were observed in weak to 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]

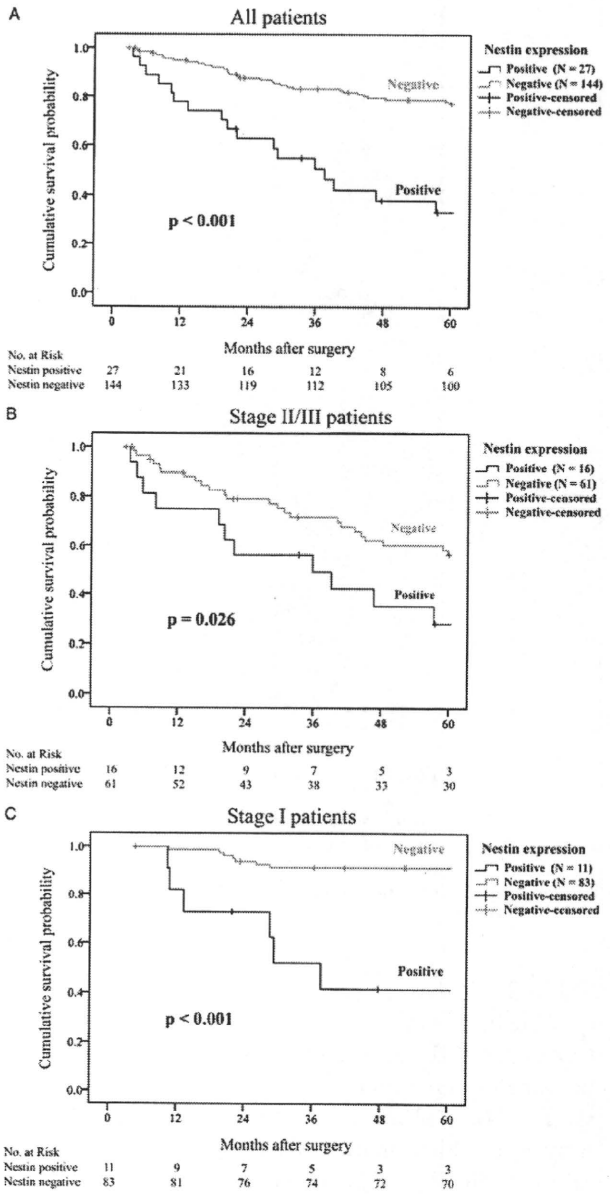

FIGURE 2. Cumulative survival of patients with NSCLC according to nestin expression estimated by the Kaplan-Meier method. Nestin expression was significantly associated with poorer survival in resected NSCLC. A, For all patients. B, For patients with stage II cancer and stage III cancer. C, For patients 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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References

1. Jemal A, Siegel R, Ward E, Murray T, Xu J, Thun MJ. Cancer statistics, 2007. *CA Cancer J Clin*. 2007;57(1):43-66.
2. van Rens MT, de la Rivière AB, Elbers HR, van Den Bosch JM. Prognostic assessment of 2,361 patients who underwent pulmonary resection for non-small cell lung cancer, stage I, II, and IIIA. *Chest*. 2000;117(2):374-379.
3. Scott WJ, Howington J, Feigenberg S, Movsas B, Pisters K; American College of Chest Physicians. Treatment of non-small cell lung cancer stage I and stage II: ACCP evidence-based clinical practice guidelines (2nd edition). *Chest*. 2007; 132(3 Suppl):234S-242S.
4. Robinson LA, Ruckdeschel JC, Wagner H Jr, Stevens CW; American College of Chest Physicians. Treatment of non-small cell lung cancer-stage IIIA: ACCP evidence-based clinical practice guidelines (2nd edition). *Chest*. 2007;132(3 Suppl): 243S-265S.
5. Arriagada R, Bergman B, Dunant A, Le Chevalier T, Pignon JP, Vansteenkiste J; International Adjuvant Lung Cancer Trial Collaborative Group. Cisplatin-based adjuvant chemotherapy in patients with completely resected non-small-cell lung cancer. *N Engl J Med*. 2004;350(4):351-360.
6. Winton T, Livingston R, Johnson D, et al; National Cancer Institute of Canada Clinical Trials Group; National Cancer Institute of the United States Intergroup JBR.10 Trial Investigators. Vinorelbine plus cisplatin vs. observation in resected non-small-cell lung cancer. *N Engl J Med*. 2005;352(25): 2589-2597.
7. Lendahl U, Zimmerman LB, McKay RD. CNS stem cells express a new class of intermediate filament protein. *Cell*. 1990;60(4):585-595.
8. Bieberich E, MacKinnon S, Silva J, Noggle S, Condie BG. Regulation of cell death in mitotic neural progenitor cells by asymmetric distribution of prostate apoptosis response 4 (PAR-4) and simultaneous elevation of endogenous ceramide. *J Cell Biol*. 2003;162(3):469-479.
9. Sahlgren CM, Mikhailov A, Vaittinen S, et al. Cdk5 regulates the organization of Nestin and its association with p35. *Mol Cell Biol*. 2003;23(14):5090-5106.
10. Sejersen T, Lendahl U. Transient expression of the intermediate filament nestin during skeletal muscle development. *J Cell Sci*. 1993;106(Pt 4):1291-1300.
11. Frisén J, Johansson CB, Török C, Risling M, Lendahl U. Rapid, widespread, and longlasting induction of nestin contributes to the generation of glial scar tissue after CNS injury. *J Cell Biol*. 1995;131(2):453-464.
12. Vaittinen S, Lukka R, Sahlgren C, et al. The expression of intermediate filament protein nestin as related to vimentin and desmin in regenerating skeletal muscle. *J Neuropathol Exp Neurol*. 2001;60(6):588-597.
13. Dahlstrand J, Collins VP, Lendahl U. Expression of the class VI intermediate filament nestin in human central nervous system tumors. *Cancer Res*. 1992;52(19):5334-5341.
14. Kawamoto M, Ishiwata T, Cho K, et al. Nestin expression correlates with nerve and retroperitoneal tissue invasion in pancreatic cancer. *Hum Pathol*. 2009;40(2):189-198.
15. Kleeberger W, Bova GS, Nielsen ME, et al. Roles for the stem cell associated intermediate filament Nestin in prostate cancer migration and metastasis. *Cancer Res*. 2007;67(19):9199-9206.
16. Liu C, Chen B, Zhu J, et al. Clinical implications for nestin protein expression in breast cancer. *Cancer Sci*. 2010;101(3): 815-819.
17. Lobo MV, Arenas MI, Alonso FJ, et al. Nestin, a neuroectodermal stem cell marker molecule, is expressed in Leydig cells of the human testis and in some specific cell types from human testicular tumours. *Cell Tissue Res*. 2004;316(3):369-376.
18. Watkins DN, Berman DM, Burkholder SG, Wang B, Beachy PA, Baylin SB. Hedgehog signalling within airway epithelial progenitors and in small-cell lung cancer. *Nature*. 2003;422(6929):313-317.
19. Chen Z, Wang T, Luo H, et al. Expression of nestin in lymph node metastasis and lymphangiogenesis in non-small cell lung cancer patients. *Hum Pathol*. 2010;41(5):737-744.
20. Travis WD, Branbilla E, Muller-Hermelink HK, et al. *World Health Organization Classification of Tumors; Pathology and Genetics of Tumors of the Lung, Pleura, Thymus and Heart*. Lyon, France: IARC Press; 2004.
21. Goldstraw P, Crowley J, Chansky K, et al; International Association for the Study of Lung Cancer International Staging Committee; Participating Institutions. The IASLC Lung Cancer Staging Project: proposals for the revision of the TNM stage groupings in the forthcoming (seventh) edition of the TNM Classification of malignant tumours. *J Thorac Oncol*. 2007;2(8):706-714.
22. Sugawara K, Kurihara H, Negishi M, et al. Nestin as a marker for proliferative endothelium in gliomas. *Lab Invest*. 2002;82(3):345-351.
23. Rubin DB. Estimating causal effects from large data sets using propensity scores. *Ann Intern Med*. 1997;127(8 Pt 2): 757-763.
24. Hua J, Yu H, Dong W, et al. Characterization of mesenchymal stem cells (MSCs) from human fetal lung: potential differentiation of germ cells. *Tissue Cell*. 2009;41(6):448-455.
25. Beachy PA, Karhadkar SS, Berman DM. Tissue repair and stem cell renewal in carcinogenesis. *Nature*. 2004;432(7015): 324-331.
26. Karhadkar SS, Bova GS, Abdallah N, et al. Hedgehog signalling in prostate regeneration, neoplasia and metastasis. *Nature*. 2004;431(7009):707-712.
27. Berman DM, Karhadkar SS, Hallahan AR, et al. Medulloblastoma growth inhibition by hedgehog pathway blockade. *Science*. 2002;297(5586):1559-1561.
28. Yuan Z, Goetz JA, Singh S, et al. Frequent requirement of hedgehog signaling in non-small cell lung carcinoma. *Oncogene*. 2007;26(7):1046-1055.
29. Tsao MS, Aviel-Ronen S, Ding K, et al. Prognostic and predictive importance of p53 and RAS for adjuvant chemotherapy in non small-cell lung cancer. *J Clin Oncol*. 2007;25(33): 5240-5247.
30. Olaussen KA, Dunant A, Fouret P, et al; IALT Bio Investigators. DNA repair by ERCC1 in non-small-cell lung cancer and cisplatin-based adjuvant chemotherapy. *N Engl J Med*. 2006;355(10):983-991.
31. Potti A, Mukherjee S, Petersen R, et al. A genomic strategy to refine prognosis in early-stage non-small-cell lung cancer. *N Engl J Med*. 2006;355(6):570-580.
32. Dean M, Fojo T, Bates S. Tumour stem cells and drug resistance. *Nat Rev Cancer*. 2005;5(4):275-284.

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RESEARCH ARTICLE

Peroxiredoxin 2 as a chemotherapy responsiveness biomarker candidate in osteosarcoma revealed by proteomics

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Purpose: We aimed to identify novel chemotherapy responsiveness biomarkers for osteosarcoma (OS) by investigating the global protein expression profile of 12 biopsy samples from OS patients.

Experimental design: Six patients were classified as good responders and six as poor responders, according to the Huvos grading system. The protein expression profiles obtained by 2-D DIGE consisted of 2250 protein spots.

Results: Among them, we identified 55 protein spots whose intensity was significantly different (Bonferroni adjusted p-value < 0.01) between the two patient groups. Mass spectrometric protein identification demonstrated that the 55 spots corresponded to 38 distinct gene products including peroxiredoxin 2 (PRDX 2). Use of a specific antibody against PRDX 2 confirmed the differential expression of PRDX 2 between good and poor responders, while PRDX 2 levels as measured by Western blotting correlated highly with their corresponding 2-D DIGE values. The predictive value of PRDX 2 expression was further confirmed by examining an additional four OS cases using Western blotting.

Conclusions and clinical relevance: These results establish PRDX 2 as a candidate for chemotherapy responsiveness marker in OS. Measuring PRDX 2 in biopsy samples before treatment may contribute to more effective management of OS.

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1 Introduction

Osteosarcoma (OS) is the most common primary malignant bone tumor in children and adolescents [1, 2]. In the mid-1970s, Rosen *et al.* noted that the response to the initial chemotherapy for OS was predictive of long-term outcome [3]; others have subsequently confirmed this finding [4, 5]. During the past three decades, the standard treatment for OS has been surgery and neo- and adjuvant chemotherapy. Chemotherapy has improved the cure rate of patients with localized OS from 15 to 20% achieved with surgery alone to approximately 70% [6]. However, OS patients whose tumors respond poorly to chemotherapy are at a higher risk of relapse and poor outcome, and chemotherapy response has always been the most important, and most consistently reported, predictor for survival [7–13]. Therefore, it is

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Abbreviation: ADR, doxorubicin; **CDDP,** cisplatin; **IFO,** ifosfamide; **OS,** osteosarcoma; **PRDX 2,** peroxiredoxin 2

imperative to identify chemotherapy responsiveness biomarkers at the time of diagnosis and prior to the initiation of therapy to detect chemotherapy-resistant tumors and to modify the treatment regimen appropriately.

Response to chemotherapy following the induction chemotherapy is evaluated using the Huvos grading system, where the levels of tumor necrosis achieved reflect the effectiveness of the given chemotherapy. Patients with $\geq$90% tumor necrosis following induction therapy are classified as good responders and patients with <90% tumor necrosis as poor responders [2]. Although this grading system is a powerful predictor of chemotherapy responsiveness, it can only be determined after chemotherapy.

In recent years, high-throughput screening technologies such as cDNA microarray technology have been used to develop biomarkers to predict the response to chemotherapy. A small number of comprehensive studies using these technologies suggested the presence of a chemotherapy resistance signature before treatment and identified a number of genes that were involved in the process of chemotherapy responsiveness in OS [14, 15]. However, none of the identified genes and signatures has been used in a clinical setting.

Emerging technologies that examine the overall features of the expressed proteins, namely proteomics, have identified many candidate proteins associated with early diagnosis [16], differential diagnosis [17], prognosis [18, 19], and response to chemotherapy [20] in various diseases. However, there is no proteomic study on predicting the response to chemotherapy in OS to date.

In this study, using biopsy samples taken prior to therapy, we found that peroxiredoxin 2 (PRDX 2) expression significantly correlated with the response to chemotherapy resistance of OS, and thus establishing PRDX 2 expression as a novel biomarker in OS.

2 Materials and methods

2.1 Patients and clinical information

Sixteen OS cases, including 12 cases used for protein expression profiling and Western blotting and four cases used in the validation study using Western blotting, were diagnosed and treated in the National Cancer Center Hospital between 2003 and 2008. Biopsy samples prior to any treatment were obtained from all 16 OS cases. The clinical information of the patients is summarized in Table 1. These 16 cases were diagnosed as conventional type OS that had developed in the extremities, in younger patients. All cases were treated with the identical induction chemotherapy protocol that included ifosfamide (IFO), cisplatin (CDDP) and doxorubicin (ADR), all of which are key drugs in the recent protocols. According to the Huvos grading system [2], chemotherapy responsiveness was defined based on histological observations on the resected specimens. Using the Huvos grading system, the cases where a tumor contained at least 90% of necrotic regions were defined as good responders (Huvos 3/4), while cases in which the necrotic regions covered <90% of the tumor area examined (Huvos 1/2) were defined as poor responders. Samples were snap frozen in liquid nitrogen and stored at –80°C until use. This project was approved by the ethical review board of the National Cancer Center, after signed informed consent was obtained from all patients. All cases were reviewed and histopathologically diagnosed by certified pathologists (N. T. and T. S.).

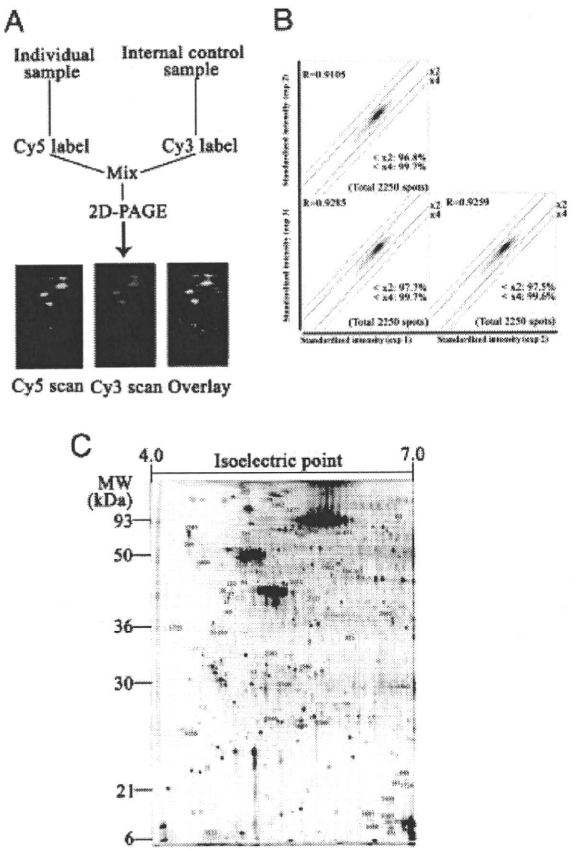

Figure 1. Identification of proteins differentially expressed in OS. (A) Schematic workflow of sample preparation for quantitative analysis. Protein lysates are labeled with fluorescent dyes of different wavelengths of excitation and emission. Cy3-labeled samples are simultaneously mixed and divided into Cy5-labeled samples. Then, the Cy3- and Cy5-labeled lysates are co-separated by 2-D DIGE. The gel is scanned at two wavelengths, each specific for each dye. (B) Scattergram of the expression profile of OS tissues. Comparison of data from three independent experiments revealed the high reproducibility of protein expression profiling. (C) Representative 2-D image of proteins detected in OS tissues. The 55 spots identified in this study are circled and numbered. The spot numbers correspond to those in Fig. 2, Table 2, and Supporting information Table 1.

2.2 Protein expression profiling

Frozen samples were crushed to powder with a Multi-beads shocker (Yasui Kikai, Osaka, Japan) with liquid nitrogen. The frozen powder was then treated with urea lysis buffer (6 M urea, 2 M thiourea, 3% CHAPS, 1% Triton X-100). After centrifugation at 15 000 rpm for 30 min, the supernatant was recovered and used in the subsequent protein expression studies.

2-D DIGE was performed as described previously [21]. In brief, the internal control sample was prepared by mixing a portion of all individual samples. Five micrograms of the internal control sample and each of the individual samples were labeled with Cy3 and Cy5, respectively (CyDye DIGE Fluor saturation dye, GE Healthcare Biosciences, Uppsala, Sweden) according to the manufacturer's instructions. The differently labeled protein samples were mixed and separated by 2-D gel electrophoresis. The first-dimension separation was achieved using IPG DryStrip gels (24 cm length, p*I* range between 4 and 7, GE Healthcare Biosciences). The second-dimension separation was achieved by SDS-PAGE on large-format gels (38 cm length, Bio-craft, Itabashi, Tokyo, Japan) [21]. The gels were scanned using laser scanners (Tyhoon Trio, GE Healthcare Biosciences) at appropriate wavelengths (Fig. 1A). For all spots, the intensity of the Cy5 image was normalized by that of the Cy3 image in the identical gel so that gel-to-gel differences were compensated, using the Progenesis PG240 software (Nonlinear Dynamics, Newcastle upon Tyne, UK). System reproducibility was verified by comparing the protein profiles obtained from three independent separations of the same sample (case 1, Table 1). Scatter plot analysis revealed that the standardized intensity of more than 96.8% of the spots ranged within a two-fold difference (Fig. 1B, $R = 0.9105$).

2.3 Data analysis

The numerical data in the XML files were imported to Expressionist software (GeneData, Basel, Switzerland) for scatter-plotting and hierarchical clustering. The Kruskal–Wallis test and Bonferroni adjustment were used to identify the protein spots that showed different intensities between the good and the poor responders.

2.4 Protein identification by mass spectrometry

The proteins corresponding to the detected spots were identified using mass spectrometry, according to our previous report [21]. In brief, 100 μg of Cy5- or Cy3-labeled proteins were separated by 2-D PAGE, recovered as gel plugs and digested with modified trypsin (Promega, Madison, WI, USA). The trypsin digests were subjected to LC (Paradigm MS4 dual solvent delivery system, Michrom BioResources, Auburn, CA) and MS using a Finnigan LTQ linear ion trap mass spectrometer (Thermo Electron, San Jose, CA, USA) equipped with a nano-electrospray ion source (AMR, Megro, Tokyo, Japan). The MASCOT software

Table 1. Clinicopathologic features of the cases frozen samples of which were examined by proteomics

Case no.	Age (years)	Sex	Histological subtype	Site	Huvos grading	Metastasis at diagnosis	Preoperation chemotherapy agents	Dose of chemotherapy IFO (g)/CDDP (mg)/ADR (mg)
1	38	Female	Osteoblastic	Distal femur	3	–	IFO, CDDP/ADR	14/100/60
2	16	Male	Fibroblastic	Proximal tibia	1	–	IFO, CDDP/ADR	14/120/60
3	14	Male	Osteoblastic	Distal femur	1	Metastasis (skip)	IFO, CDDP/ADR	14/120/60
4	23	Female	Osteoblastic	Proximal humerus	1	Metastasis (lung)	IFO, CDDP/ADR	14/120/60
5	11	Male	Osteoblastic	Proximal tibia	1	–	IFO, CDDP/ADR	14/120/60
6	25	Male	Osteoblastic	Diaphesis of humerus	3	–	IFO, CDDP/ADR	16/120/60
7	15	Female	Teleangiectatic	Distal femur	4	–	IFO, CDDP/ADR	14/120/60
8	13	Male	Osteoblastic	Proximal tibia	1	–	IFO, CDDP/ADR	14/120/60
9	18	Male	Osteoblastic	Distal tibia	3	–	IFO, CDDP/ADR	14/120/60
10	16	Male	Chondroblastic	Proximal tibia	4	–	IFO, CDDP/ADR	14/120/60
11	14	Male	Chondroblastic	Proximal femur	1	Metastasis (lung)	IFO, CDDP/ADR	14/120/60
12	8	Male	Osteoblastic	Proximal humerus	3	–	IFO, CDDP/ADR	14/120/60
13	20	Male	Osteoblastic	Distal femur	3	–	IFO, CDDP/ADR	14/120/60
14	7	Female	Osteoblastic	Distal femur	3	–	IFO, CDDP/ADR	14/120/60
15	11	Female	Chondroblastic	distal femur	1	–	IFO[a)]	14[a)]
16	14	Male	Osteoblastic	distal femur	1	–	IFO, CDDP/ADR	14/120/60

a) Severe myelosuppression and could not continue IFO, CDDP/ADR protocol.

(version 2.1, Matrix Science, London, UK) was used to search for the mass of the peptide ion peaks against the Swiss-Prot database (*Homo sapiens*, 12867 sequence in Sprot_47.8 fasta file).

2.5 Western blotting

Protein samples were separated by SDS-PAGE. The separated proteins were subsequently blotted on a nitrocellulose membrane. The membrane was incubated with the polyclonal antibody against PRDX 2 (1:1000 dilution, Protein Tec Group, Chicago, IL, USA). The membrane was reacted with HRP-conjugated secondary antibody (1:1000 dilution, GE Healthcare Biosciences, Chicago, IL, USA). PRDX 2 was detected using an enhanced chemiluminesence system (GE Healthcare Biosciences, Chicago, IL, USA) and the LAS 3000 luminescent image analyzer (Fujifilm, Tokyo, Japan).

3 Results

We generated and compared the protein expression profiles between six good responders (Huvos grade 3/4) and six poor responders (Huvos grade 1) using 2-D DIGE. The clinicopathological parameters other than the responsiveness were not significantly different between these two groups. We detected 2250 protein spots that appeared in all the images of the Cy3-labeled internal control sample (Fig. 1C). The use of a large format 2-D system enabled to observe a relatively large number of protein spots. We identified 55 protein spots that are differentially expressed between the good and poor responders, performing a Kruskal–Wallis test with an applied Bonferroni adjusted p-value of <0.01. The localization of the 55 spots on the 2-D image is shown in Fig. 1C, whereas their intensity is shown in Fig. 2.

Mass spectrometric analysis resulted in the identification of 38 distinct gene products corresponding to the 55 protein spots (Fig. 2, Table 2, Supporting information Table 1).

We examined the correlation between the identified proteins and the prognostic information using several antibodies. After trials, we found that the use of a specific antibody against PRDX 2 confirmed the higher expression of PRDX 2 in the biopsy samples than from poor responders. In 2-D DIGE experiments, the PRDX 2 expression level inversely correlated with response to chemotherapy (Fig. 2), and the PRDX 2 levels measured by 2-D DIGE and Western blotting highly correlated (Fig. 3A). In an additional four cases, Western blotting showed that the expression of PRDX 2 was higher in the poor responders when compared with the good responders (Fig. 3B), thus confirming the results using the initial set of cases. Aberrant expression of PRDX 2 has been implicated in certain types of cancer such as human breast cancer [22]. PRDX 2 inhibited apoptosis, probably rendering tumor cells resistant

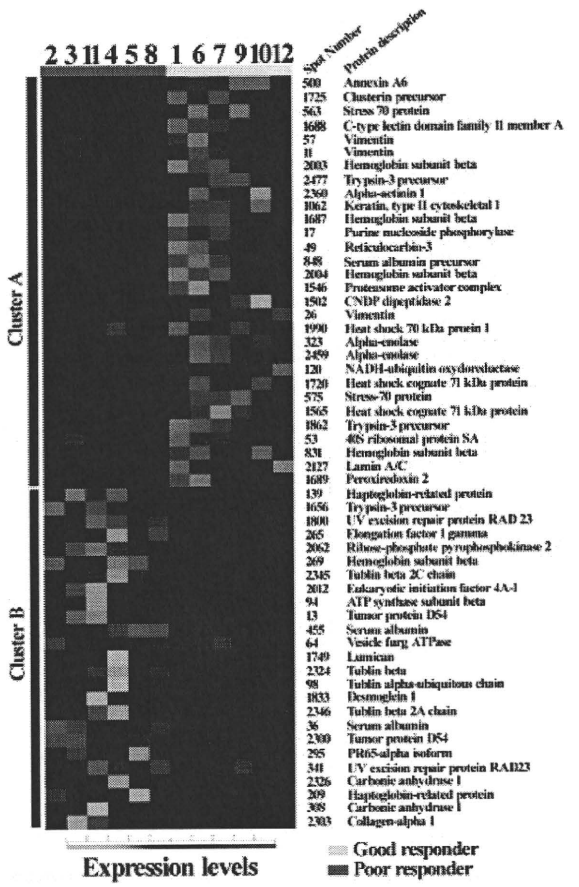

Figure 2. Hierarchical clustering of the 12 OS cases examined based on the intensity of the 55 protein spots detected. The cases are color-coded as yellow (good responder) or blue (poor responder) as indicated in the panel. The spot numbers and the protein names related proteins are shown on the right side.

to chemotherapeutic agents [23–25]. However, its association with OS has not been reported previously.

4 Discussion

High-throughput screening technologies such as the cDNA microarray technology have recently been employed to develop chemotherapy responsiveness biomarkers in OS. However, proteomic studies have not been performed using OS biopsy samples to date. Proteomic studies have unique advantages over other so-called -omics studies. The proteome is a functional translation of the genome, directly regulating cell phenotypes and is thus a rich source of biomarkers. With this notion, we have established a gel-based proteomics system for cancer research [21] and applied it to the OS proteomic study presented here in the first report using a proteomic approach to develop chemotherapy responsiveness biomarkers for OS.