Sawada and Dr. Minoru Sakurai, he organized the 30th Annual SIOP meeting in Yokohama as President of the Organizing Committee. Also with Dr. Sawada, he established SIOP-Asia, a step forward to internationalism that should never be forgotten.

Mineko, his wife, said that he was a happy person who devoted himself to his work in which he remained actively engaged until his death.

The worlds of pediatric oncology and pediatric surgery have lost an invaluable senior statesman, educator, investigator, humanitarian, and internationalist, the roles filled by this giant figure that will be impossible to replace.

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Clinical significance of serum NM23-H1 protein in neuroblastoma

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We have previously reported that *NM23* genes are overexpressed in various hematological malignancies and that serum NM23-H1 protein levels are useful for predicting patient outcomes. In this study we assessed the clinical implications of serum NM23-H1 protein on neuroblastoma. We examined serum NM23-H1 protein levels in 217 patients with neuroblastoma, including 131 found by mass-screening and 86 found clinically by an enzyme-linked immunosorbent assay, and determined the association between levels of this protein, and known prognostic factors or the clinical outcome. The serum NM23-H1 protein level was higher in neuroblastoma patients than in control children ($P < 0.0001$). Patients with *MYCN* amplification had higher serum NM23-H1 levels than those with a single copy of *MYCN*. Overall survival was assessed in the 86 patients found clinically, and was found to be worse in patients with higher serum MN23-H1 levels (≥ 250 ng/mL) than in those with lower levels (< 250 ng/mL; $P = 0.034$). The higher level of NM23-H1 was correlated with a worse outcome in patients with a single *MYCN* copy, or in those younger than 12 months of age. Serum NM23-H1 protein levels may contribute to predictions of clinical outcome in patients with neuroblastoma. (*Cancer Sci* 2005; 96: 653–660)

The *NM23* gene was identified by differential hybridization of a cDNA library with total RNA extracted from slightly and highly metastatic melanoma cell lines.[1] The *NM23* gene has been identified as a family of genes encoding different isoforms of nucleoside diphosphate kinase (NDPK).[2] *NM23* genes play critical roles in cellular proliferation, differentiation, oncogenesis, and tumor metastasis.[1,3] The mechanisms for these pleiotropic effects are not well understood. Eight isotypes of the human *NM23* gene (*NM23-H1*, *NM23-H2*, *NM23-H3*/*DR-NM23*, *NM23-H4*, *NM23-H5*, *NM23-H6*, *NM23-H7*, and *NM23-H8*) have been identified.[2] Among these, only *NM23-H1* and *NM23-H2* have been studied extensively in human cancers.

The level of *NM23-H1* expression is inversely correlated with the tumor's metastatic potential in experimental rodent cells and in human tumors such as breast, ovarian, cervical and gastric cancer, hepatocellular carcinoma, and melanomas.[4] Therefore, *NM23-H1* is implicated in the regulation of metastasis in a variety of human cancers. However, overexpression of the *NM23-H1* gene has been reported in various neoplasms including neuroblastoma, hematological malignancies, and pancreatic, lung, ovarian and gastric cancers.[5–8] Overexpression of *NM23-H1* is indicative of a poor patient prognosis for patients with neuroblastoma, acute myelogenous leukemia (AML), or non-Hodgkin's lymphoma (NHL).[8–10]

In neuroblastoma, a gain of 17q is the most frequent genetic abnormality, followed by 1p deletion and *MYCN* amplification, both of which correlate closely with 17q gain. The three genetic events are strong predictors of unfavorable prognosis.[11,12] The *NM23* genes are located at the edge of the common chromosomal region of 17q gain. Godfrid *et al.* identified genes that are activated in the *MYCN* downstream pathway using SAGE libraries of *MYCN*-transfected and control neuroblastoma cell lines.[13] The *NM23-H1* and *NM23-H2* genes are strongly induced in *MYCN*-expressing cells. Neuroblastoma tumor and cell line panels reveal a striking correlation between *MYCN* amplification and mRNA or protein expression of both *NM23* genes. These findings suggest that *NM23-H1* and *NM23-H2* expression may be increased by 17q gain in neuroblastoma, and can be further upregulated by *MYCN* overexpression. These observations suggest a role of *NM23-H1* and *NM23-H2* in the tumorigenesis of an unfavorable type of neuroblastoma.

We previously established an enzyme-linked immunosorbent assay (ELISA) technique for determining the serum level of NM23-H1 protein.[14] Serum levels of NM23-H1 in patients with NHL and AML are significantly higher than those in controls, and elevated NM23-H1 levels correlate with poor prognosis in these patients.[10,15] It has been strongly suggested that serum NM23-H1 protein is produced directly by tumor cells and its level depends on the total mass of malignant cells overexpressing *NM23-H1*.[14,16] These results indicate that the serum level of NM23-H1 protein may be clinically useful as a prognostic factor in NHL and AML. The present study assessed the clinical implications of serum NM23-H1 protein levels in patients with neuroblastoma, in whom tumor samples were used to determine the biological prognostic factors.

Materials and Methods

Patients and controls

Serum NM23-H1 protein was measured in 217 untreated neuroblastoma patients who were admitted to various institutions in Japan and underwent biopsy or surgery between 2000 and 2002. The 217 patients included 131 who were found by

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a mass-screening (MS) program for infants at 6 months of age by measuring urinary catecholamine metabolites and 86 who were found clinically.[17] Of the 86 patients, 29 who were younger than 12 months old were mostly found before MS, and 57 who were 12 months old or older underwent MS with a negative result, or did not undergo MS. Patients were staged according to the International Neuroblastoma Staging System (INSS).[18] Patients of any age with stage 1 or 2 disease, and those younger than 12 months of age with stage 3 disease were treated by surgery or surgery and chemotherapy consisting of cyclophosphamide and vincristine; patients 12 months or older with stage 3 or stage 4 disease and those younger than 12 months of age with stage 4 disease were treated according to the protocol published by the Japanese Neuroblastoma Study Group.[19] Serum samples from 23 children consisting of 22 with inguinal hernias and one with an edematous scrotum before surgery were analyzed for comparison. The median age of the children was 23 months (range: 3–49 months). Informed consent was obtained from patients and/or their parents, and the ethics committee of Saitama Cancer Center approved the study design.

Venous blood samples

Peripheral venous blood samples were collected in sterile test tubes with heparin and placed on ice. The samples were centrifuged at 2000*g* for 15 min at 4°C, and stored at –20°C. As a marker of hemolysis, free serum hemoglobin (Hb) was determined according to the method of Testa *et al.*[20]

ELISA for human NM23-H1 protein

NM23-H1 protein levels in serum were determined using a sandwich ELISA assay, as described previously.[14,15] Recombinant NM23-H1-GST protein was used as a standard.

Examination of *MYCN* copy number, TRKA expression and ploidy

DNA preparation, digestion, and Southern blot analysis using the *MYCN* probe were carried out as described previously.[12] The presence of more than three copies of the *MYCN* gene per haploid genome was considered to indicate amplification.[21] *TRKA* expression was examined by northern blotting as reported previously.[22] DNA index was analyzed on a Becton-Dickinson FACScan flow cytometer by DNA cell-cycle analysis software (version C).

Statistical analysis

The significance of differences in various clinical and biological aspects of the disease among the patient groups was examined by using the Mann–Whitney *U* or Kruskal-Wallis test (non-parametric analysis). Spearman's correlation coefficient (rs) by ranks was used to evaluate the correlation between paired values. Survival analysis was performed according to the Kaplan–Meier method, and the significance of differences in survival was determined by using the generalized Wilcoxon's and log-rank tests. A multivariate analysis of prognostic factors was performed using Cox's proportional-hazards regression model. All statistical analyses were performed with Excell Statcel and Stat Flex software (version 5.0, Artech Co. Ltd, Osaka, Japan), and $P < 0.05$ was taken to indicate significance.

Results

Examination of serum NM23-H1 protein levels in neuroblastoma patients and control children

The serum level of NM23-H1 was examined in 217 neuroblastoma patients and 23 control children. The serum levels of NM23-H1 were significantly higher in patients with neuroblastoma ($n = 217$, mean ± SD 176 ± 280 ng/mL) than in the control children ($n = 23$, 27 ± 41 ng/mL, $P < 0.0001$; Fig. 1a). The serum NM23-H1 levels of the control children were higher than those of the healthy adults (data not shown). The serum NM23-H1 levels in patients with neuroblastoma were significantly higher than those in patients with various hematological malignancies (data not shown). Next, the relationship between serum levels of NM23-H1 and Hb was examined in 217 neuroblastoma patients and 23 control children, because the NM23-H1 protein leaked from red blood cells by hemolysis may have elevated the serum NM23-H1 levels.[23] The results showed a weak correlation (rs = 0.3958, $P = 7.5356 \times 10^{-10}$, Spearman's correlation coefficient by ranks), although some patients had a higher Hb level but a lower NM23-H1 level, or a lower Hb level but a higher NM23-H1 level (Fig. 1b). When we chose samples from 156 patients

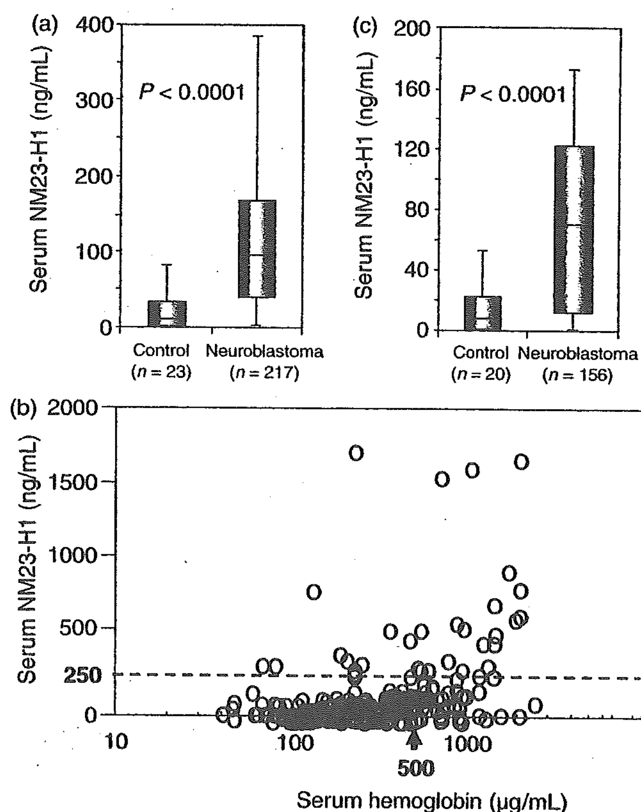

Fig. 1. Serum NM23-H1 levels in patients with neuroblastoma and in control children. (a) Box plots of NM23-H1 serum levels for 217 patients with neuroblastoma and 23 control children with any serum hemoglobin levels. (b) Relationship between the serum levels of NM23-H1 and hemoglobin in all samples examined (black circles, neuroblastoma patients [$n = 217$]; red circles, control children [$n = 23$]). (c) Box plots of NM23-H1 serum levels for 156 patients with neuroblastoma and 20 control children with serum hemoglobin levels less than 500 μg/mL.

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Table 1. Relationship between serum NM23-H1 protein levels and clinicopathological findings in 217 patients with neuroblastoma and 23 control children

Clinicopathological findings	Number of patients (mean ± SD)	Serum NM23-H1 (ng/mL)	P-value (analysis)
Control children	23	27 ± 41	
All patients	217	176 ± 280	< 0.0001 (MW)
Method of detection			
Mass-screening	131	135 ± 206	
Found clinically	86[†]	239 ± 357	0.0595 (MW)
Age of patients			
< 12 months	134	168 ± 292	
≥ 12 months	83	190 ± 260	0.2427 (MW)
Stage of the disease			
1 + 2 + 4s	122	136 ± 159	
3 + 4	95	227 ± 378	0.8088 (MW)
Primary site			
Mediastinum	31	145 ± 212	
Adrenal	101	187 ± 290	
Abdomen	78	184 ± 302	0.3393 (KW)
Others	7	74 ± 82	
MYCN copy number			
1	186	143 ± 204	
> 3	31	378 ± 519	0.0006 (MW)
TRKA expression	173		
Medium or high	125	150 ± 209	
None or low	48	238 ± 373	0.4629 (MW)
Ploidy	168		
Diploid	69	188 ± 273	
Hyperdiploid	99	185 ± 284	0.9012 (MW)
Others	7	112 ± 126	

MW, Mann–Whitney *U*-test; KW, Kruskal–Wallis test. [†]Table 2.

and 20 control children with serum Hb less than 500 µg/mL, the correlation between serum NM23-H1 and Hb levels was negligible (rs = 0.2351, $P = 0.0035$). Even in these patients, the serum levels of NM23-H1 were significantly higher (n = 156, 113 ± 184 ng/mL) than in the control children ($n = 20$, 20 ± 35 ng/mL, $P < 0.0001$; Fig. 1c).

Relationship between serum NM23-H1 protein levels and clinicopathological features in neuroblastoma

The relationship between serum NM23-H1 levels and various clinical and biological features in the 217 patients is shown in Table 1. The serum NM23-H1 levels tended to be higher in patients found clinically than in those found by MS ($P = 0.0595$), and were significantly higher in patients with amplified *MYCN* copies than in those with a single *MYCN* copy ($P = 0.0006$; Table 1). There was a correlation between *MYCN* amplification and the elevated serum NM23-H1 level (≥ 250 ng/mL) in all 217 patients (rs = 0.6970, $P = 0.0005$). However, serum Hb concentrations did not correlate with *MYCN* amplification ($P = 0.6320$), or other factors (data not shown). There was no significant difference in the serum NM23-H1 levels between two groups of patients classified by age of the patients, stage of the disease, expression levels of *TRKA*, or tumor cell ploidy (Table 1).

Serum NM23-H1 levels and overall survival

Of the 217 patients, the 86 patients who were found clinically were included and the 131 patients found by MS were excluded from survival analysis, because all the 131 patients were alive at the last follow-up (18–51 months), and the clinical and biological features are different for the patients found by MS and those found clinically.[12] The relationship between serum NM23-H1 levels and various clinical and biological features in the 86 patients was similar to that found for all 217 patients (Tables 1, 2). The 86 patients were divided into two groups according to various cut-off points over 100 ng/mL, which was the upper limit in control serum (mean + 2 × SD = 20 + 2 × 35 = 90). The cut-off points used here were 100 ng/mL (< 100, $n = 39$, *vs* ≥ 100, $n = 47$), 150 ng/mL (< 150, $n = 54$, *vs* ≥ 150, $n = 32$), 200 ng/mL (< 200, $n = 60$, *vs* ≥ 200, $n = 26$) and 250 ng/mL (< 250, $n = 64$, *vs* ≥ 250, $n = 22$). The cut-off value of greater than 250 ng/mL showed the most significant prognostic effects with generalized Wilcoxon's and log-rank test analysis (data not shown). Therefore, we used 250 ng/mL of serum NM23-H1 as a cut-off value. As shown in Figure 2a, patients with the higher serum NM23-H1 levels had worse overall survival than those with the lower levels ($P = 0.0219$ according to the generalized Wilcoxon test, $P = 0.0340$ according to the log-rank test). Overall survival was significantly worse for patients who were 12 months or older than for those younger than 12 months of age ($P = 0.0364$ and $P = 0.0158$), for patients at stages 3 and 4 than for those at stages 1, 2 and 4S ($P = 0.0157$ and $P = 0.0082$), and for patients with *MYCN* amplification than for those with a single copy of *MYCN* ($P = 0.0195$ and $P = 0.0054$; Fig. 2b,c,d). These results

Table 2. **Relationship between serum NM23-H1 protein levels and clinicopathological findings in 86 patients with neuroblastoma found clinically**

Characteristics	No. of patients (mean ± SD)	Serum NM23-H1 (ng/mL)	*P*-value (analysis)
All patients	86	239 ± 357	
Age			
< 12 months	27	282 ± 471	
≥ 12 months	59	219 ± 294	0.7694 (MW)
Stage			
1 + 2 + 4s	21	154 ± 187	
3 + 4	65	266 ± 394	0.3900 (MW)
Primary site			
Mediastinal	11	124 ± 207	
Adrenal	46	285 ± 383	
Abdominal	26	220 ± 375	0.0982 (KW)
Others	3		
MYCN copy number			
1	59	157 ± 193	
> 3	27	418 ± 534	0.0028 (MW)
TrkA expression	63		
Medium + high	28	154 ± 189	
0 + low	35	296 ± 422	0.1865 (MW)
Ploidy	66		
Diploid	37	255 ± 436	
Hyperdiploid	27	234 ± 352	0.4304 (MW)

MW, Mann–Whitney *U*-test; KW, Kruskal–Wallis test.

indicate that the serum NM23-H1 level serves as a useful prognostic factor for neuroblastoma, as well as the other well-known prognostic factors.

Subsequently, we classified the 86 patients into two groups according to the age of the patients, stage of the disease, or copy numbers of *MYCN*, and evaluated the influence of the serum NM23-H1 levels on the overall survival in each one of the six groups (Fig. 3). Of the 29 patients younger than 12 months of age, the seven patients with higher levels of NM23-H1 had a worse outcome than the 22 patients with the lower levels ($P = 0.0401$ according to the generalized Wilcoxon test and $P = 0.0273$ according to the log-rank test; Fig. 3a). The seven patients with higher levels of NM23-H1 had the following attributes: stage 1 + 2 + 4S ($n = 3$); stage 3 + 4 ($n = 4$); with non-amplified *MYCN* ($n = 4$); with more than three *MYCN* ($n = 3$). Likewise, of the 19 patients with a stage 3 tumor, four patients with higher levels had a worse outcome than the 15 patients with lower levels ($P = 0.0005$ and $P < 0.0001$; Fig. 3c). The four patients with higher levels of NM23-H1 had the following attributes: < 12 months of age ($n = 0$); > 12 months of age ($n = 4$); with non-amplified *MYCN* ($n = 1$); with more than three *MYCN* ($n = 3$). Of the 59 patients with a single copy of *MYCN*, the 11 patients with higher levels had a worse outcome than the 48 patients with lower levels of serum NM23-H1 ($P = 0.0301$ and $P < 0.0366$; Fig. 3e). The 11 patients with higher levels of NM23-H1 had the following attributes: < 12 months of age ($n = 4$); > 12 months of age ($n = 7$); stage 1 + 2 + 4S ($n = 2$); stage 3 + 4 ($n = 9$). In contrast, a higher serum NM23-H1 level did not influence overall survival in the 57 patients 12 months old or older, in the 46 patients with stage 4 disease, or in the 27 patients with *MYCN* amplification (Fig. 3b,d,f).

Four prognostic factors, including the age of the patients, stage of the disease, *MYCN* copy number, and the serum NM23-H1 level, were available for multivariate analysis in the 217 patients (Table 3a) and 86 patients (Table 3b). According to multivariate analysis, the serum NM23-H1 level provided no significant influence on overall survival in either group of patients (Table 3).

Discussion

The *NM23-H1* gene is overexpressed in various hematological malignancies and other neoplasms including neuroblastoma. Overexpression of *NM23-H1* mRNA is indicative of a poor prognosis in patients with neuroblastoma, and mutations and increased copy numbers of *NM23-H1* have been reported in advanced neuroblastoma.[6,24] In the present study, we found that the serum NM23-H1 level was significantly higher in patients with neuroblastoma than in the control children (Fig. 1), and that the serum NM23-H1 level predicted a poor outcome for patients with tumors (Fig. 2a). Furthermore, the higher level of NM23-H1 was correlated with a worse outcome in patients younger than 12 months of age, in those with stage 3 disease, or in those with a single *MYCN* copy (Fig. 3). In contrast, a higher serum NM23-H1 level did not influence overall survival in patients who were 12 months old or older, in those with stage 4 disease, or in those with *MYCN*

Table 3. **Univariate and multivariate analysis for predictors of survival in neuroblastoma**

Prognostic factors	Univariate (χ^2, log-rank)	*P*-value	Multivariate (relative risk & 95% CI)	*P*-value
Patients found by mass-screening or clinically ($n = 217$)				
Serum NM23-H1(< 250/> 250 ng/mL)	11.211	0.0008	1.7294 (0.7997–3.7398)	0.1639
Age (< 12/≥ 12 months)	32.353	< 0.00001	3.8979 (1.3818–10.996)	0.0101
Stage (1, 2, 4s/3, 4)	33.142	< 0.00001	8.2514 (1.8173–37.466)	0.0063
NMYC amplification (–/+)	43.997	< 0.00001	2.3253 (1.0541–5.1297)	0.0366
Patients found clinically ($n = 86$)				
Serum NM23-H1(< 250/> 250 ng/mL)	4.493	0.0340	1.6143 (0.7386–3.5282)	0.2299
Age (< 12/≥ 12 months)	5.825	0.0158	1.4742 (0.4877–4.4563)	0.4916
Stage (1, 2, 4s/3, 4)	6.994	0.0082	3.5721 (0.7158–17.826)	0.1206
NMYC amplification (–/+)	7.749	0.0054	1.9682 (0.9016–4.2967)	0.0892

CI, confidence interval.

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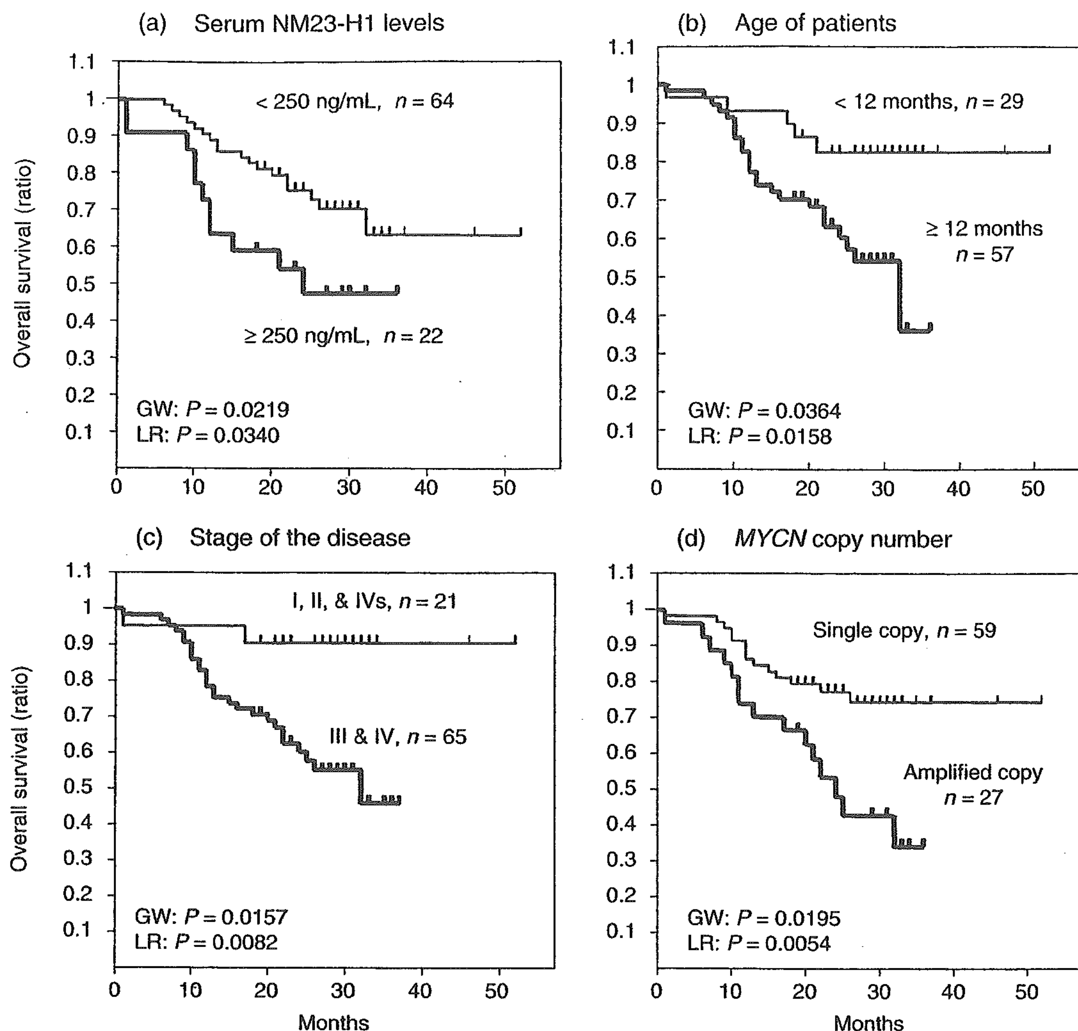

Fig. 2. Overall survival curves for 86 patients with neuroblastoma who were found clinically. (a) Overall survival curves for 22 patients with a serum NM23-H1 level ≥ 250 ng/mL, and for 64 patients with a level < 250 ng/mL. (b) Overall survival curves for 57 patients 12 months of age or older, and for 29 patients younger than 12 months. (c) Overall survival curves for 65 patients at stages 3 and 4 of the disease, and for 21 patients at stages 1, 2 and 4s. (d) Overall survival curves for 27 patients with *MYCN* amplification, and for 59 patients with a single copy of *MYCN*. GW, generalized Wilcoxon's test; LR, log-rank test.

amplification (Fig. 3). These findings suggest that the NM23-H1 level may be an important factor for predicting the outcome of patients in these low or intermediate risk groups (i.e. patients younger than 12 months of age, with stage 3 disease, or with a single copy of *MYCN*). In addition, the serum NM23-H1 level may be a clinically useful prognostic factor, because the measurement of serum NM23-H1 protein is easily and quickly carried out prior to treatment.

According to multivariate analysis, the serum NM23-H1 level provided no significant influence on overall survival in either group of patients shown in Table 3. These results might be due to the short observation time, the small number of cases, or the strong correlation between *MYCN* amplification and the elevated serum NM23-H1 level.

Although all the 131 patients found by MS were alive at the last follow-up (18–51 months) and were excluded from survival analysis, they contained 15 patients (the last follow-up: 19–37 months) with higher levels than 250 ng/mL of serum NM23-H1. It might be interesting to follow up these patients to clarify the clinical significance of serum NM23-H1 in the MS group.

Prognostic factors in neuroblastoma have been thoroughly investigated and include *MYCN* copy number, *TRKA* expression level, chromosomal ploidy, 1p loss, and 17q gain in tumor cells. Laborious and time-consuming work is required to examine these biological factors in tumor tissues. Therefore, serum markers that are easily measurable and can predict a clinical outcome are desired. Serum levels of lactate dehydrogenase (LDH) and ferritin are high in advanced stage neuroblastomas, but both may reflect a rapid cellular turnover or a large tumor burden.[25,26] Neuron-specific enolase (NSE) is a cytoplasmic protein that is associated with neural cells,

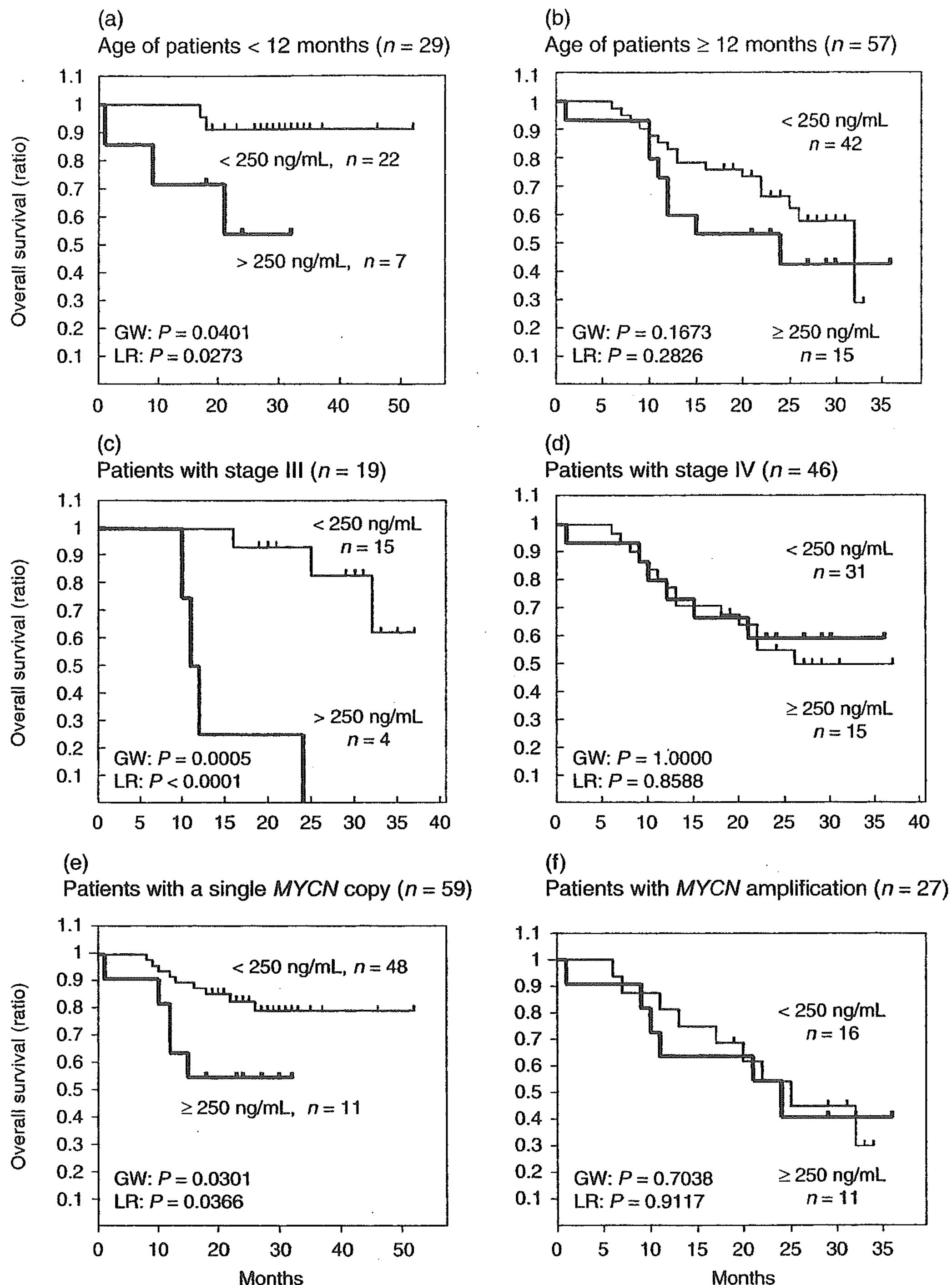

Fig. 3. Clinical significance of the serum NM23-H1 levels in the groups classified according to the age of the patients, or stage of the disease, or copy number of *MYCN*. (a) Survival curves for seven patients with a serum NM23-H1 level of ≥ 250 ng/mL, and for 22 patients with a level < 250 ng/mL. Both groups of patients were younger than 12 months of age. (b) Survival curves for 15 patients with a serum NM23-H1 level of ≥ 250 ng/mL, and for 42 patients with a level < 250 ng/mL. Both groups of patients were 12 months old or older. (c) Survival curves for four patients with a serum NM23-H1 level of ≥ 250 ng/mL, and for 15 patients with a level < 250 ng/mL. Both groups of patients were at stage 3 of the disease. (d) Survival curves for 15 patients with the serum NM23-H1 level ≥ 250 ng/mL, and for 31 patients with the level < 250 ng/mL. Both groups of patients were at stage 4 of the disease. (e) Survival curves for 11 patients with a serum NM23-H1 level of ≥ 250 ng/mL, and for 48 patients with a level < 250 ng/mL. Both groups of patients had a single copy of *MYCN*. (f) Survival curves for 11 patients with a serum NM23-H1 level of ≥ 250 ng/mL, and for 16 patients with a level < 250 ng/mL. Both groups of patients had *MYCN* amplification in the tumor. GW, generalized Wilcoxon's test; LR, log-rank test.

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and serum NSE is a useful marker for patients with advanced neuroblastoma in whom the elevated levels are associated with a poor outcome.[27] The disialoganglioside GD2 is found on the surface of most neuroblastoma cells, and elevated plasma levels have been found in patients.[28] Nevertheless, none of these markers is used at present to predict clinical outcomes or to choose treatment protocols. Therefore, serum NM23-H1 levels might be useful for clinical purposes.

The elevated serum level of NM23-H1 was correlated with a poor prognostic feature, namely, *MYCN* amplification (Table 1). Godfrid *et al.* identified genes that are part of the *MYCN* downstream pathway using SAGE libraries of *MYCN* transfected and control neuroblastoma cell lines.[13] The chromosome 17q genes *NM23-H1* and *NM23-H2* were strongly induced in *MYCN*-expressing cells. A striking correlation between *MYCN* amplification and mRNA or protein expression of both *NM23* genes was found in the cell lines. The present multivariate analysis showed no influence of serum NM23-H1 level on overall survival, and this finding might be caused by the overlap of patients with *MYCN* amplification with those with a high serum level of NM23-H1. However, within the group of patients with a single copy of *MYCN*, patients with a higher level of NM23-H1 had a worse outcome (Fig. 3e). The findings suggest that *NYCN* amplification may influence serum NM23-H1 levels as well as clinical outcome, and that neuroblastomas with a single copy of *MYCN* and a higher serum NM23-H1 level may have had a mutation or an increased copy number of the *NM23-H1* gene.[6,24,29] *MYCN* overexpression in some neuroblastomas with a single copy of *MYCN* may have resulted in higher serum NM23-H1 levels and a poor outcome; however, a recent study showed that *MYCN* overexpression did not affect the prognosis of advanced-stage neuroblastomas with a single *MYCN* copy.[30]

In patients with NHL and AML, it is thought that serum NM23-H1 protein is produced directly by the tumor cells, and its serum level depends on the total mass of malignant cells overexpressing *NM23-H1*.[14] High concentrations of NM23 protein were found in the serum and body fluid of patients with lung cancer overexpressing the *NM23* genes.[31] Tumor cells may secrete this protein through some unknown mechanism, because there is no signal peptide sequence for secretion in the NM23 molecule. Serum NM23-H1 in patients with neuroblastoma might be derived from tumor cells and might be induced by *MYCN* amplification/overexpression or by *NM23-H1* overexpression independent of *MYCN*.

The serum level of NM23-H1 protein is clinically useful as an important prognostic factor in NHL or AML, and the present study showed that the protein could be a factor predicting an outcome of patients with neuroblastoma. It would be interesting to examine whether the serum NM23-H1 level generally predicts a poor outcome for patients with other tumors. The mechanisms by which the NM23-H1 protein is secreted into the serum and how it affects patient outcome are unclear. We are now studying the possibility that a high concentration of serum NM23-H1 may positively affect tumor cell growth or negatively affect normal cells.

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Review Article

p73, a sophisticated p53 family member in the cancer world

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p73 belongs to a family of p53-related nuclear transcription factors that includes p53, p73 and p63. The overall structure and sequence homology indicates that a *p63/p73*-like protogene is the ancestral gene, whereas *p53* evolved later in higher organisms. In accordance with their structural similarity, p73 functions in a manner analogous to p53 by inducing tumor cell apoptosis and participating in the cell cycle checkpoint control through transactivating an overlapping set of p53/p73-target genes. In sharp contrast to p53, however, p73 is expressed as two NH_2-terminally distinct isoforms including transcriptionally active (TA) and transcriptionally inactive (ΔN) forms. ΔNp73, which has oncogenic potential, acts in a dominant negative manner against TAp73 as well as p53. p73 is induced to be stabilized in response to a subset of DNA-damaging agents in a way that is distinct from that of p53, and exerts its pro-apoptotic activity. Several lines of evidence suggest that p73 can induce tumor cell apoptosis in a p53-dependent and p53-independent manner. Some tumors exhibit resistance to the p53-dependent apoptotic program, therefore p73, which can induce apoptotic cell death by p53-independent mechanisms, is particularly useful. In this review, we discuss the regulatory mechanisms of p73 activity, and also the functional significance of p73 in the regulation of cellular processes including tumorigenesis, apoptosis and neurogenesis. (*Cancer Sci* 2005; 96: 729–737)

Until recently, the tumor suppressor p53 has been believed to be encoded by a single gene which lacks any structural or functional homologs. The identification of two p53-related proteins, termed p73 and p63, revealed that p53 belongs to a small family of sequence-specific nuclear transcription factors.[1–3] p53 family members share three major functional domains: the NH_2-terminal transactivation domain; the central core sequence-specific DNA-binding domain; and the COOH-terminal oligomerization domain. Of these, the central DNA-binding domain is highly conserved across the family. As expected from their structural similarities, p73 can bind to the p53-responsive elements, and transactivate an overlapping set of p53-target genes implicated in G1/S cell cycle arrest and apoptotic cell death.[1,4] Recent studies demonstrated that p73 is required for p53-dependent apoptosis.[5] Unlike p53, p73 is expressed as at least six variants with different COOH-terminal ends, arising from the alternative splicing at the 3′ portion of the primary transcript.[1,6,7] Each of these splicing variants (TAp73) contains an intact NH_2-terminal transactivation domain, and exerts its transcriptional activity to various degrees. Additionally, *p73* contains a second transcriptional start site within intron 3, giving rise to the NH_2-terminally truncated form of p73 (ΔNp73) which has little transcriptional activity.[8] Similar to p73, ΔNp63 is also generated by an alternative promoter (Fig. 1a,b).[2] ΔNp73 displays dominant negative behavior toward p73 as well as wild-type p53, and has oncogenic potential.[9,10] Of note, we and others found that ΔNp73 is a direct transcriptional target of p73, suggesting that there exists a negative feedback regulation of p73 by ΔNp73, to modulate cell survival and death.[11–13]

Steady-state expression levels of endogenous p73 are kept extremely low under physiological conditions. Similar to p53, p73 is induced to be stabilized at the protein level in response to a subset of DNA-damaging agents, and exerts its pro-apoptotic activity.[14] Accumulating evidence suggests that p73 turnover is regulated through a ubiquitination-dependent and ubiquitination-independent degradation pathway. MDM2 acts as an E3 ubiquitin protein ligase for p53, and promotes the proteasome-mediated proteolytic degradation of p53.[15–17] On the other hand, MDM2 increases the stability of p73,[18] indicating that p73 stability is regulated through a pathway distinct from that of p53. Alternatively, Ohtsuka *et al.* reported that cyclin G binds to p73 and stimulates its proteolytic degradation in a ubiquitination-independent manner, however, the precise molecular mechanism of cyclin G-mediated degradation of p73 remains unknown.[19]

Considering that p73 has a p53-like property and is mapped to the human chromosome 1p36.2-3, a region which is frequently lost in a wide variety of human tumors including neuroblastoma, it is likely that p73 could be one of the classic Knudson-type tumor suppressors.[1] In spite of extensive mutation searches, *p73* was rarely mutated in primary tumors.[20] Additionally, initial genetic studies demonstrated that p73-deficient mice exhibit severe developmental defects, however, they do not develop spontaneous tumors, suggesting that p73 might participate in the regulation of normal development *in vivo*, and that p73 does not link directly to tumor suppression.[8] Indeed, p73 has the ability to induce neuronal differentiation of undifferentiated neuroblastoma cells.[21] However, this viewpoint has been challenged by the observation that mice mutant for p73 and p63 develop spontaneous tumors, and their spectrum is quite different from that of p53-deficient mice.[22] Thus, it is likely that p73 and

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Abbreviations: EEC, ectrodactyly, ectodermal dysplasia, and facial clefts (syndrome); OPC, oligodendrocyte precursor cell; SAM, sterile α motif; YAP, Yes-associated protein.

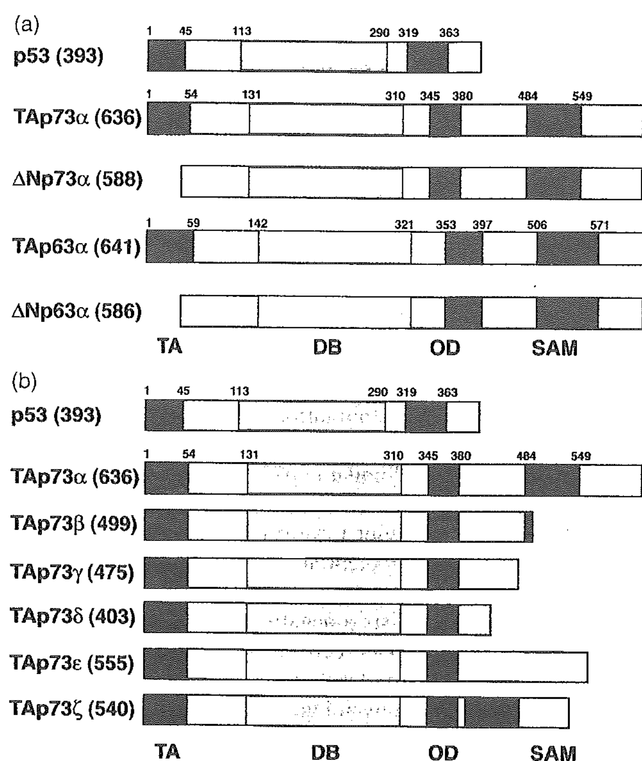

Fig. 1. (a) Structural comparison between TA and ΔN variants. The transactivation domain (TA), DNA-binding domain (DB), oligomerization domain (OD) and sterile α motif domain (SAM) are indicated. ΔN variant, which lacks the NH_2-terminal transactivation domain, is generated by alternative promoter usage. The numbers in parentheses are the amino acid lengths of each protein. (b) Schematic drawing of the splicing variants of p73. p73 is expressed as multiple variants with different COOH-terminal ends arising from an alternative splicing at the 3′ portion of the primary transcript. The numbers in parentheses are the amino acid lengths of each protein.

p63 exert their tumor suppressor activity in specific tissues. In this review, we will discuss the functional significance of p73 in the regulation of cellular processes such as tumorigenesis, apoptotic cell death and neuronal differentiation.

Splicing variants of p73

The overall genomic organization of *p73* is quite similar to that of *p53*. The *p53* gene is 20 kb in length and contains 11 exons. The *p73* gene is larger than 60 kb in length and contains 14 exons.[23] In sharp contrast to p53, p73 is expressed as multiple variants arising from an alternative splicing of the primary *p73* transcript including p73α, p73β, p73γ, p73δ, p73ε, and p73ζ.[1,6,7] Among them, p73α is the longest form, which contains a sterile α motif domain (SAM domain) and an extreme COOH-terminal region, whereas p73β lacks the extreme COOH-terminal tail and most of the SAM domain. We and others revealed that these COOH-terminal splicing variants display different transcriptional and biological properties.[6,7,24] Indeed, the ability of p73β to transactivate a variety of p53/p73 target genes and to induce apoptotic cell death in certain cancerous cells was stronger than those of the full-length p73α.[7,25] This indicated that the COOH-terminal region of p73 might possess a regulatory role, which modulates its transcriptional and pro-apoptotic activity[24,25] These splicing variants with different COOH-terminal extensions were expressed differentially among normal human tissues and cell lines, suggesting that they have distinct physiological functions.[6,7]

TAp73 and ΔNp73

In addition to the differential splicing variants of p73, there exist the ΔN variant forms of p73 (ΔNp73α and ΔNp73β) which are transcribed from an internal promoter located within an extra exon (exon 3′) of the full-length *p73* gene, and lacks the NH_2-terminal transactivation domain in TAp73.[8] Like p73, ΔNp63 is also generated using an alternative promoter.[2] As expected, ΔNp73 has little transactivation activity. Furthermore, ΔNp73 displays dominant negative behavior toward TAp73 as well as wild-type p53,[9] and also has oncogenic potential.[10] ΔNp73-mediated inhibition of TAp73 and p53 occurs at the oligomerization level or by the competition for binding to the same p53/p73-responsive element, with ΔNp73 displacing TAp73 and p53 from the DNA binding site.[26,27] For example, ΔNp73 was expressed predominantly in sympathetic neurons, and inhibited p53-dependent neuronal apoptosis.[9] ΔNp73-dependent repression of apoptosis induced by p53 is critical for the normal development of the neural system. In addition, the endogenous expression levels of ΔNp73 were significantly associated with poor prognosis in human cancers such as neuroblastoma.[28] Thus, it is likely that a balance between the intracellular expression levels of pro-apoptotic TAp73 or p53 and anti-apoptotic ΔNp73 plays an important role in regulating cell fate determination. Intriguingly, we and others demonstrated that there exists a functional p53/p73-responsive element within the *ΔNp73* promoter region, and indeed the expression of ΔNp73 is directly transactivated by TAp73 and/or wild-type p53, creating a dominant negative feedback loop which regulates the pro-apoptotic activities of both TAp73 and wild-type p53[11–13] (Fig. 2).

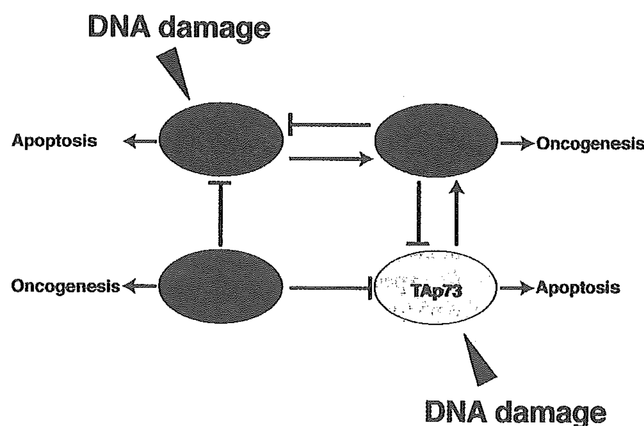

Fig. 2. Functional interactions between TAp73, ΔNp73, wild-type p53 and a mutant form of p53. DNA damage induces TAp73 and wild-type p53 through distinct pathways. The mutant form of p53 inhibits the pro-apoptotic activity of TAp73 and wild-type p53. ΔNp73, which is directly transactivated by TAp73 and wild-type p53, displays dominant negative behavior toward TAp73 and wild-type p53.

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As described above, ΔNp73 is transcriptionally inactive due to a lack of the NH_2-terminal transactivation domain in TAp73. However, this viewpoint has been challenged by the recent finding of Liu *et al.* showing that ΔNp73β has weak but distinct transcriptional activity, thereby inducing cell cycle arrest and/or apoptosis.[29] In contrast to ΔNp73β, ΔNp73α failed to induce cell cycle arrest and/or apoptosis under their experimental conditions. According to their results, the NH_2-terminal 13 unique amino acid residues as well as PXXP motifs of ΔNp73β might be a novel activation domain. Thus, it is possible that ΔNp73β might exert a distinct function under certain cellular processes.

Transcriptional regulation of the main promoter of p73

E2F1 transcription factor plays an important role in the regulation of cell cycle progression by inducing the transcription of genes whose products are directly or indirectly required for entry into the S phase.[30] In addition to the proliferative effect of deregulated E2F1 activity, unscheduled E2F1 activation leads to apoptosis to protect cells from cellular transformation.[31] Consistent with this notion, E2F1-deficient mice exhibited a high incidence of unusual tumors.[32,33] E2F1-induced apoptosis is regulated in a p53-dependent or p53-independent manner. It is interesting that the *p73* promoter region contains a TATA-like box and at least three E2F1-binding sites, and indeed the enforced expression of E2F1 strongly stimulates the transcription of *p73* through the direct binding to the E2F1-responsive elements in the *p73* promoter.[34,35] The E2F1-mediated up-regulation of p73 results in a significant induction of apoptosis. Other studies demonstrated that T cell receptor-mediated apoptosis is dependent on both E2F1 and p73.[36] Thus, E2F1-mediated apoptosis requires p73, at least in part. Alternatively, E2F1 might also contribute to the up-regulation of *p73* mRNA levels during muscle and neuronal differentiation of murine C2C12 myoblasts and P19 cells, respectively.[37] It is worth noting that Chk1 and Chk2 are required for the induction of p73 in response to DNA damage, and E2F1 contributes to the Chk kinase-dependent transcriptional regulation of p73.[38] In addition to E2F1, cellular and viral oncogene products such as c-Myc and E1A indirectly activated the transcription of p73.[39]

Recently, Fontemaggi *et al.* identified a 1 kb negative regulatory fragment within the first intron of *p73* gene.[37] Under their experimental conditions, this intronic fragment significantly reduced the activity of the *p73* promoter upon E2F1 overexpression. Of note, the *p73* intronic fragment contained six consensus binding sites for transcriptional repressor ZEB. Ectopic expression of ZEB in C2C12 myoblasts attenuated myotube formation, and repressed the transcription of *p73*. In accordance with these results, the dominant negative form of ZEB had an ability to restore the expression levels of p73 in proliferating cells.

Because DNA hypermethylation contributes to the alteration of the entry of transcription factors into the regulatory region, the epigenetic modification of the *p73* promoter region through aberrant hypermethylation could be an alternative molecular mechanism for silencing the *p73* gene. Corn *et al.* described the aberrant promoter methylation of *p73* as occurring frequently in primary acute lymphoblastic leukemias and Burkitt's lymphomas, whereas the *p73* promoter methylation was not detected in normal lymphocytes or bone marrow.[40] Similar results were also reported by Kawano *et al.*[41] In contrast, hypermethylation of the *p73* promoter region was not observed in solid tumors including breast, renal, colon cancers or neuroblastomas,[42] suggesting that the methylation-dependent silencing of *p73* transcription might be specific to hematological malignancies.

p73 is rarely mutated in human cancers

The *p73* gene has been mapped to human chromosome 1p36.2-3, a region which exhibits frequent loss of heterozygosity in a wide variety of human cancers including neuroblastoma, and its gene product has an ability to promote G1/S cell cycle arrest and/or cell death through apoptosis in certain cancerous cells. Therefore, p73 could act as a tumor suppressor.[1] In spite of an extensive search of the *p73* status in human primary tumors, *p73* was infrequently mutated in many human tumors.[20,43–48] *p73* mutations were detected in fewer than 0.5% of human cancers, whereas over 50% of cancers carry *p53* mutations. For example, only two types of *p73* mutations with amino acid substitution (P405R and P425L) were found in primary neuroblastoma and lung cancer.[20] In addition to the NH_2-terminal transactivation domain, Takada *et al.* found a potential second transactivation domain within the COOH-terminal portion of p73α (amino acid residues 380–513), albeit to a lesser extent than the NH_2-terminal transactivation domain.[49] This region is rich in glutamine and proline residues. Among the two types of p73 mutations, the P425L substitution significantly reduced the transcriptional and growth-suppressive activity of p73α, whereas the P405R substitution had a negligible effect on p73α.[50]

In sharp contrast to p53-deficient mice, which develop tumors with high frequency,[51] p73-deficient mice were viable, but the loss of p73 did not predispose mice to cancer, suggesting that p73 does not function as a classic Knudson-type tumor suppressor,[8] and its possible contribution to tumor suppression is still unclear. Instead, mice lacking p73 displayed severe developmental defects, including hydrocephalus, hippocampal dysgenesis, and abnormalities in the pheromone sensory pathways. These observations strongly suggest that p73 and p53 have distinct biological functions, and p73 plays an important role in normal development, especially in neural development and apoptosis. Because those p73-deficient mice lacked both TAp73 and ΔNp73 variants, further studies of variant-specific knockout mice might provide an insight into the unique role of each variant in tumorigenesis.

As mentioned above, initial genetic studies revealed that p73-deficient mice do not display an increased susceptibility to spontaneous tumorigenesis. More recently, Flores *et al.* examined whether synergistic effects of p73 and p63 could exist, alone or in combination with p53, in tumor suppression.[22] Strikingly, they found that p73 and p63 heterozygous mice ($p73^{+/-}$ and $p63^{+/-}$) developed malignant tumors at high frequency including various tumor types not observed in $p53^{+/-}$ mice, and $p53^{+/-}$; $p73^{+/-}$ and $p53^{+/-}$; $p63^{+/-}$ mice

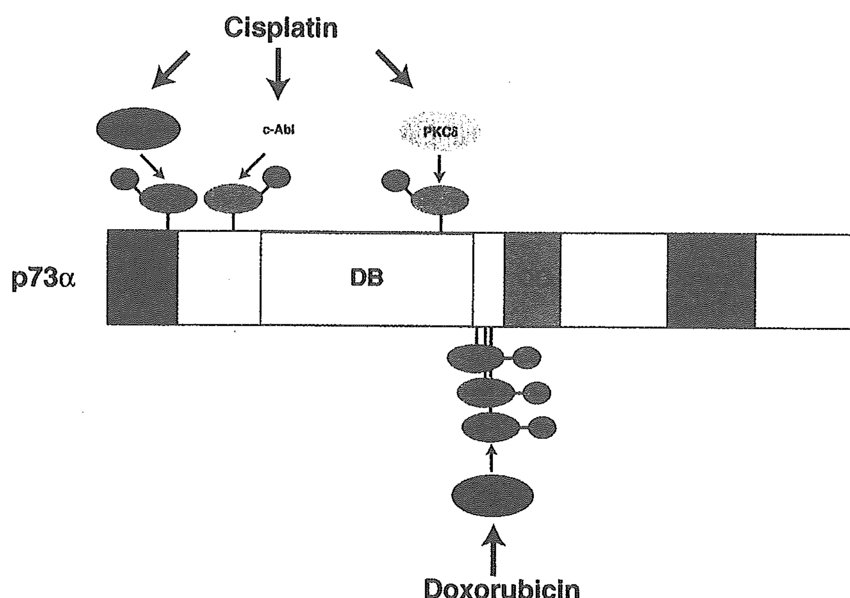

Fig. 3. DNA damage-induced activation of p73 is mediated by post-translational modifications including phosphorylation and acetylation. During cisplatin-mediated apoptosis, p73 is induced to be phosphorylated at Ser-47, Tyr-99 and Ser-289 by Chk1, c-Abl and PKCδ, respectively. In response to doxorubicin, p300 acetylates p73 at Lys-321, Lys-327 and Lys-331.

developed a more aggressive tumor phenotype.[22] In addition, tumors derived from $p73^{+/-}$ and $p63^{+/-}$ mice exhibited loss of the remaining wild-type allele at high frequency. These results strongly suggest that loss of p73 and/or p63 function causes tumor development, and their tumor suppressor activities play a pivotal role in specific tissues distinct from those of p53.

Activation of p73 at protein level by genotoxic stresses

Under normal physiological conditions, the expression levels of the p73 protein are maintained at an extremely low level, keeping this pro-apoptotic protein in an inactive state. The initial studies demonstrated that, unlike p53, p73 is not induced at the protein level in response to DNA damage.[1] Indeed, the exposure to either actinomycin D or ultraviolet radiation had no significant effects on p73 protein levels, whereas p53 and one of its target $p21^{WAF1}$ levels were markedly elevated in response to actinomycin D or ultraviolet radiation. However, recent studies revealed that p73 is induced to be accumulated in response to a subset of DNA-damaging agents, including cisplatin, adriamycin, camptothecin and etoposide.[14] p73 is predominantly regulated at the post-translational level, and the stabilization of p73 results in either G1/S cell cycle arrest or cell death through apoptosis. Therefore, the stabilization of p73 is directly linked with its activity.

Accumulating evidence strongly suggests that chemical modifications of p73, such as phosphorylation and acetylation, prolong its half-life, which, in turn, enhance its transcriptional and pro-apoptotic activity. During the cisplatin-mediated apoptotic process, p73 is phosphorylated at Tyr-99 and stabilized in a pathway dependent on nuclear non-receptor tyrosine kinase c-Abl.[52–54] In addition to c-Abl, exposure to cisplatin promoted a complex formation between p73 and a protein kinase Cδ catalytic fragment, which phosphorylated p73 at Ser-289 and increased its stability and transcriptional activity.[55] Recently, it has been shown that cisplatin-induced apoptosis is associated with p73 phosphorylation at Ser-47 mediated by Chk1.[56] Chk1-dependent phosphorylation resulted in an increase in the transcriptional activity of p73 (Fig. 3). In contrast, CDK-mediated phosphorylation of p73 led to significant inhibition of its transcriptional activity,[57] indicating that the phosphorylation of p73 might not always convert a latent form of p73 to an active one.

Alternatively, p73 is regulated by acetylation. p73 was previously found to be associated with p300 histone acetyltransferase through its NH_2-terminal transactivation domain, and this interaction resulted in a significant enhancement of p73-mediated transcriptional activation as well as apoptosis.[58] Costanzo *et al.* reported that p300 acetylates p73 at Lys-321, Lys-327 and Lys-331 in response to doxorubicin in a c-Abl-dependent manner, and the acetylated forms of p73 have pro-apoptotic activity[59] (Fig. 3). Intriguingly, the p300-mediated acetylation of p73 was stimulated by prolyl isomerase Pin1, thereby stabilizing p73.[60] It is likely that p73 acetylation catalyzed by p300 reduces its ubiquitination levels by competition between acetylation and ubiquitination.

Regulation of p73 turnover

Lee and La Thangue described p73 as being stabilized in cells treated with proteasome inhibitor such as LLnL.[25] They also showed that p73β is much more stable than p73α, indicating that the COOH-terminal extension of p73α might be involved in the stability control of p73. In support of this notion, we have found that RanBPM binds to the extreme COOH-terminal region of p73α, and prolongs the half-life of p73α.[61] Subsequently, several lines of evidence suggest that p73 turnover is regulated by a ubiquitination-dependent and a ubiquitination-independent proteasome pathway. MDM2, which is transcriptionally activated by p53, acts as an E3 ubiquitin protein ligase for p53. MDM2 promotes the ubiquitination of p53 through physical interaction with its

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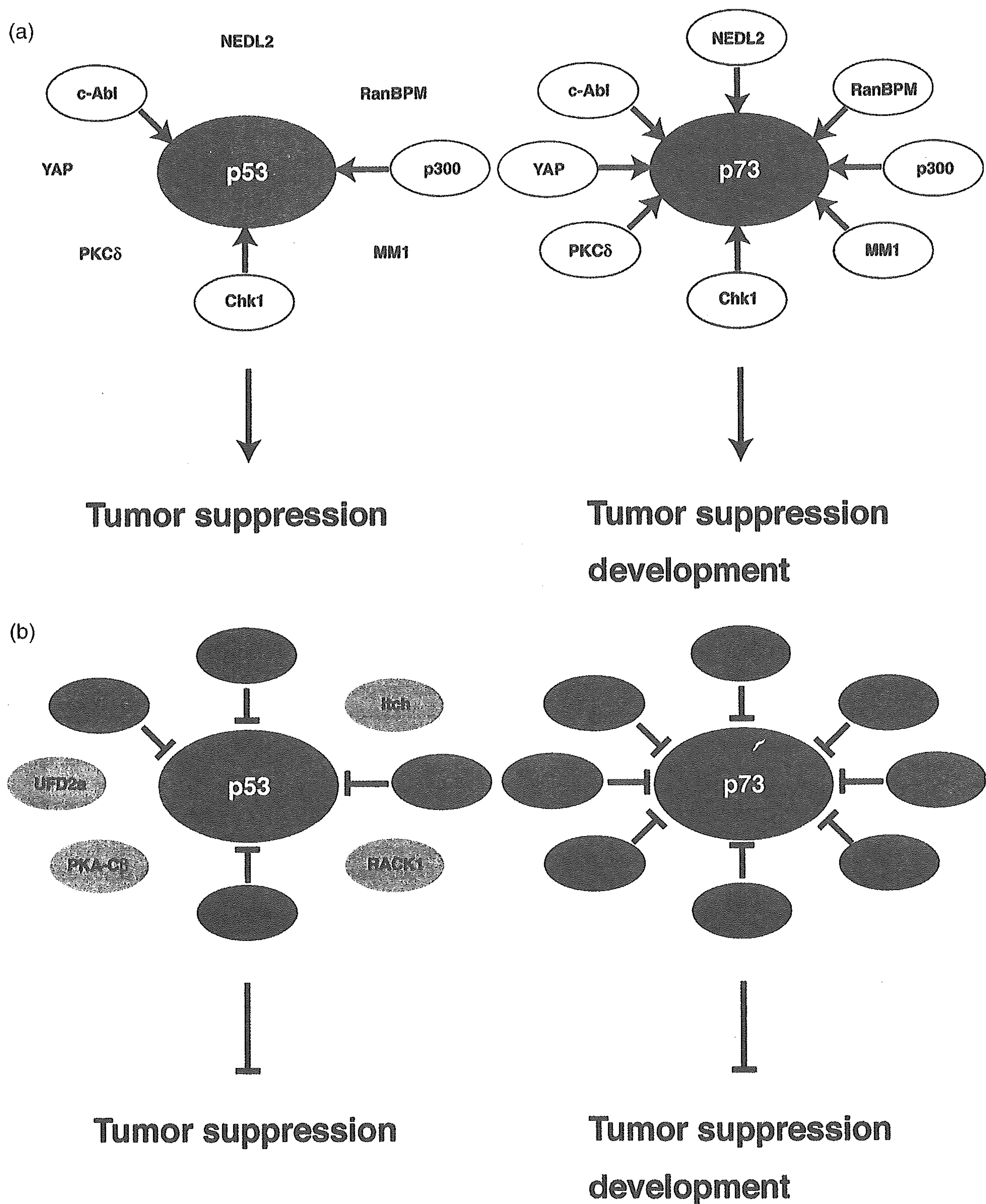

Fig. 4. Positive (a) and negative (b) regulation of p73 or p53 activity through the physical and functional interaction with various cellular proteins. Uncircled proteins have an undetectable effect on p53.

NH_2-terminal transactivation domain, and subsequent proteolytic degradation of p53.[15–17] Similar to p53, MDM2 was also a direct transcriptional target of p73, bound to its NH_2-terminal transactivation domain and thereby inhibiting p73-mediated transcriptional activation and apoptosis. However, MDM2 failed to ubiquitinate p73, and this interaction resulted in an increase in p73 protein stability.[18] Additionally, a newly identified p53-induced E3 ubiquitin

p53, p73 is induced to be accumulated in response to a subset of DNA-damaging agents, however, the regulatory mechanisms of the pro-apoptotic activity of p73 are distinct from those used for p53. p73 has the ability to induce apoptotic cell death in a p53-independent manner. Indeed, p73 can promote apoptotic cell death in tumor cells that lack functional p53. In addition, p73 might enhance the chemosensitivity of tumor cells. Therefore, p73 alone or in combination with the other p53 family members might provide a clue to overcoming chemoresistance in tumor cells.

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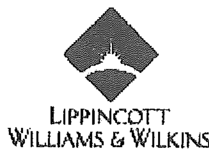

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Unusual Chromaffin Cell Differentiation of a Neuroblastoma After Chemotherapy and Radiotherapy: Report of an Autopsy Case With Immunohistochemical Evaluations

[Case Report]

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Recent History

Unusual Chromaffin Cell D... GO

Abstract:

Neuroblastomas are derived from neural crest cells that are capable of multilineage differentiation. Ganglionic neuronal differentiation of childhood neuroblastoma is seen with increasing age, leading to more differentiated tumors called ganglioneuroblastomas or ganglioneuromas. Despite the fact that neuroblastomas most often arise from the adrenal medulla, chromaffin-cell differentiation in neuroblastomas is not widely recognized. Tumor cells with a chromaffin-cell nature have only been detected using histochemical techniques in neuroblastoma cell lines or focal areas of certain in vivo tumors. We describe a neuroblastoma that exhibited an unusual differentiation toward chromaffin cells in a patient that had been treated with surgery, intensive chemotherapy, and radiotherapy. Although a biopsy specimen of the retroperitoneal primary tumor was extensively necrotic, possibly because of a previous chemotherapy regimen, surgically resected metastatic tumors of bilateral ovaries were viable and diagnosed as poorly differentiated neuroblastomas according to the International Neuroblastoma Pathology Classification system. However, metastatic tumors of bilateral lungs

examined at the time of autopsy exhibited histologic features similar to those of a pheochromocytoma/paraganglioma, and immunohistochemical examinations demonstrated that these tumors were composed of extra-adrenal chromaffin cells. This case confirms that neuroblastomas in childhood can transform into pheochromocytoma/paraganglioma-like tumors under special conditions.

Neuroblastoma is the most common extracranial solid cancer that occurs during infancy and childhood.[4] Neuroblastomas are derived from embryonic neural crest cells [20] and can differentiate along the sympathetic neuronal cell pathway with increasing age. Depending on the degree of differentiation and the amount of Schwannian stromal components, neuroblastic tumors are classified into three major categories: neuroblastoma, ganglioneuroblastoma, and ganglioneuroma.[22,23] In contrast to these well-known neuronal differentiation patterns, chromaffin-cell differentiation in neuroblastomas is not widely recognized, although some investigators have histochemically demonstrated a chromaffin nature in neuroblastoma cell lines [7] as well as in focal areas of extra-adrenal tumors.[9,12,13] Despite these observations, the differentiation of neuroblastomas exclusively toward chromaffin cells is extremely rare, and only one such tumor has been previously described.[15] Here, we report a case of childhood neuroblastoma originating from the retroperitoneum with bilateral ovary metastases that were histologically diagnosed as ordinary neuroblastoma. An autopsy, performed after intensive chemotherapy and radiotherapy, revealed metastatic tumors of the lung consisting of differentiated chromaffin cells.

CASE REPORT

The patient was a 4-year-old girl who was admitted to a hospital in China complaining of abdominal distension. Histologic examinations of needle biopsy specimens from the abdominal tumor, serum analysis data (including an elevation in vanillylmandelic acid), and radiographic evaluations suggested the presence of a stage 3 neuroblastoma (International Neuroblastoma Staging System [5]). A urinary test performed in the neuroblastoma mass screening program in Japan when the patient was 6 months old had been negative. After receiving one course of chemotherapy (cyclophosphamide, 0.53 g/m^2; vincristine, 0.66 mg/m^2 ×2; THP-adriamycin, 40 mg/m^2), she was transferred to our hospital about 1 month after the onset.

A meta-iodobenzyl-guanidine scintigram revealed accumulations of radioactivity in the left renal hilus, pelvic cavity (later determined to be the left ovary), paraaortic lymph nodes, and spinal bone marrow. A computed tomographic (CT) scan revealed a retroperitoneal primary tumor and a large mass lesion in the pelvic cavity. The tumor markers were elevated as follows: neuron specific enolase = 12.3 ng/mL (normal range, <=6.0 ng/mL), vanillylmandelic acid = 255.6 μg/mg Cr (normal range, 3.5–15 μg/mg Cr), and homovanillic acid = 121.9 μg/mg Cr (normal range, 4.5–20 μg/mg Cr). Her left ovary was surgically resected because of massive enlargement, suggesting tumor metastasis, and a biopsy specimen was taken from the retroperitoneal primary tumor. The N-*myc* gene was not amplified in the resected tumor sample. After surgery, four courses of chemotherapy according to the Regimen 98A$_3$ protocol (cyclophosphamide, 1.2g/m^2 ×2; vincristine, 1.5 mg/m^2 ×1; THP-adriamycin, 40 mg/m^2 ×1; cisplatin, 25 mg/m^2 ×5 *c.i.*) of the Study Group of Japan [21] were performed, but the meta-iodobenzyl-guanidine scintigram remained positive. Following the fifth course of chemotherapy, the retroperitoneal primary tumor, the metastatic tumor in the right ovary, and the lymph nodes were surgically removed 6 months after the initial surgery. Although the values of all the

tumor markers decreased to within a normal range thereafter, the meta-iodobenzyl-guanidine scintigram still revealed an uptake of radioactivity, so an additional four courses of chemotherapy and radiation therapy were performed. The patient was scheduled to receive a bone marrow transplantation, but a chest x-ray and CT scan revealed multiple, fine, nodular lesions in bilateral lungs, and values of her tumor markers began to increase once again. After treatment with total body irradiation (12 Gy), the patient received a stem cell transplantation using umbilical cord blood, but she died of complications from the transplantation, including graft-versus-host disease and a brain hemorrhage, at the age of 5 years.

METHODS

Immunohistochemistry

An indirect immunohistochemical analysis was performed using formalin-fixed, paraffin-embedded tissue sections and the standardized streptavidin-biotin peroxidase complex method (DAKO-LSAB; Dako Japan, Kyoto, Japan) with 3,3'-diaminobenzidine as a chromogen. When required, antigen retrieval was performed according to the manufacturer's instructions. The sources and clones of the primary antibodies that were used are listed in Table 1.

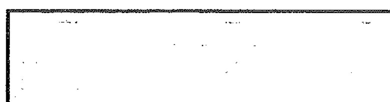

Table 1. Panel of Primary Antibodies Used in Immunohistochemistry

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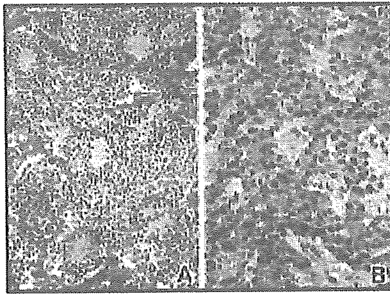
RESULTS

Pathologic Findings of the Surgical Materials

The left ovarian tumor, surgically resected when the patient was 4 years old, measured 12.5 × 11.5 × 8.0 cm in diameter and weighed 610 g. The cut surface of the tumor was solid, grayish brown in color, with scattered foci of necrosis. The tumor consisted of small neuroblasts with round nuclei, forming neuropils and rosettes (Fig. 1A), corresponding to a poorly differentiated neuroblastoma according to the International Neuroblastoma Pathology Classification system,[22,23] although this evaluation was made after the patient had received chemotherapy. The tumor contained thin fibrovascular stroma that partially formed a lobular architecture. The biopsy material from the retroperitoneal primary tumor, obtained simultaneously at the time of surgery, consisted of necrotic tissue with no viable tumor cells, possibly due to the chemotherapy performed in China.

FIGURE 1. Histology of the tumors. **A:** The metastatic left ovarian tumor consists of small round cells forming neuropils and rosettes, a typical histologic appearance of neuroblastoma (original magnification, ×36). **B:** The metastatic lung tumor observed at autopsy consists of compact sheets of cells with a



deeply basophilic cytoplasm and fibrovascular stroma, a histologic appearance similar to that of pheochromocytoma/paraganglioma (original magnification, ×60).

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The right ovarian tumor, resected 6 months later, measured 4.0 × 2.7 × 2.5 cm in diameter and weighed 18 g, with its cut surface being solid and grayish brown in color. The tumor consisted of compact nests of small round neuroblasts forming neuropils, with few rosettes, and was histologically similar to the left ovarian tumor. The retroperitoneal primary tumor, resected together with the right ovary, measured 4 × 3 cm in diameter on the cut surface, exhibited extensive necrosis with calcification and hemosiderosis, and contained only tiny nests of viable neuroblastoma cells at the periphery. The left adrenal gland adjacent to the primary tumor was intact with no tumor involvement, indicating that the tumor had arisen from the retroperitoneum.

Autopsy Findings

An autopsy was performed about 8 hours after the death of the patient. Extensive dissemination of the tumor was present in bilateral lungs. The metastatic lung tumors consisted of small nodules, measuring up to 5 mm in diameter, located mainly on the pleura and in the interlobular connective tissue. The tumors were composed of solid, compact sheets of epithelial-like cells with a deeply basophilic cytoplasm and single, round to oval nuclei (Fig. 1B). The tumor stroma consisted of fine vascular channels surrounded by a small amount of fibrous tissue. These characteristics are similar to those for pheochromocytoma and paraganglioma. Neuroblasts, ganglion cells, and Schwann cells were absent. Microscopic tumor metastases were also found in the left kidney, pancreatic head, and paraaortic lymph nodes. The histologies of these metastases were basically similar to those in the lung, although degenerative changes and/or necrosis made a definite histologic diagnosis difficult.

Immunohistochemistry

Immunohistochemistry showed that the left ovarian metastatic tumor, resected during the initial surgery, was positive for Bcl-2. The tumor was also positive for chromogranin A (CGA) (Fig. 2A), synaptophysin (Syn), and CD57 (HNK-1), but only in the neuropils at the center of the rosettes. Dopamine [beta]-hydroxylase (D[beta]H), tyrosine hydroxylase (TH), and insulin-like growth factor II (IGF-II) stained weakly and/or focally positive, while phenylethanolamine N-methyltransferase (PNMT) stained negative (Table 2). These results are consistent with the characteristics of a neuroblastoma. On the other hand, the metastatic lung tumors exhibited strong and diffuse positive staining for CGA (Fig. 2B), Syn, and TH, weak to moderate positive staining for D[beta]H and IGF-II, a very weak staining reaction for CD57, and negative staining