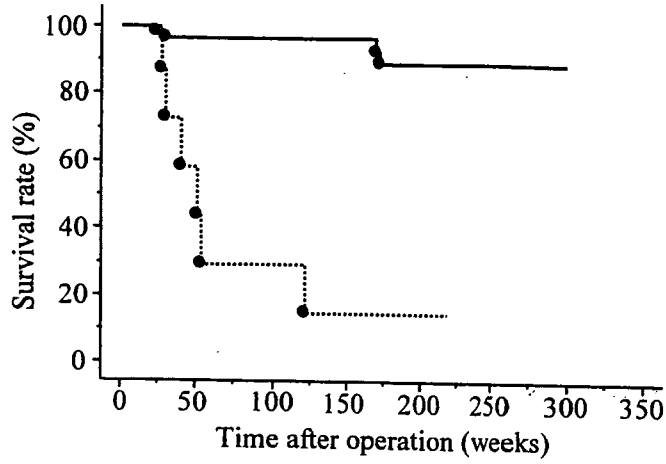

Figure 4 Correlation between the event-free survival rate and Mina53 expression (Kaplan–Meier method). Sixty-four renal cell carcinomas were divided into (·····) Mina53 high-expression tumors ($n = 9$; 14.1%) and (—) Mina53 non-high tumors (Mina53 negative and low-expression tumors; $n = 55$; 85.9%) according to the expression level of Mina53. The patients with Mina53 non-high expression had significantly longer survival ($P < 0.0001$) than those with high Mina53 expression.

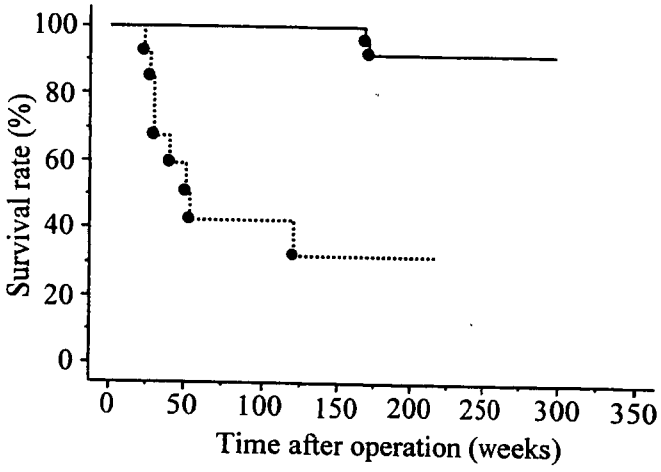

Figure 5 Correlation between the event-free survival rate and Ki-67 expression (Kaplan–Meier method). Sixty-four renal cell carcinomas were divided into a (–) low-Ki-67 labeling index (LI) group ($n = 50$; 78.1%) and a (·····) high-Ki-67 LI group ($n = 14$; 21.9%) according to the expression level of Ki-67. The patients with low Ki-67 LI had significantly longer survival ($P < 0.0001$) than those with high Ki-67 LI.

DISCUSSION

We examined the expression in RCC of *Mina53*, a Myc target gene, on immunohistochemistry, and its significance in cancerous and non-cancerous tissues from 64 patients with known clinicohistopathological information using specific antibody to Mina53 protein as shown on western blot. Previous immunohistochemical studies in esophageal and colonic cancer tissues have reported that Mina53 expression is closely correlated with the expression of Ki-67, a reliable marker of cell proliferation, suggesting the possible involvement of Mina53 in cancer cell proliferation.[14,15] In addition, these studies have indicated that, in esophageal and colorectal cancer cell lines, the induction of Mina53 expression inhibition by a Mina53-specific 21 nt small interfering RNA duplex inhibits cell proliferation *in vitro*, suggesting that Mina53 in these cancer cells is involved in cell proliferation.[14,15]

In the present study Mina53 was diffusely positive in the nuclei of normal renal tubules in the non-cancerous region of all specimens stained. Previous studies have reported that Mina53 is expressed in the nuclei of cells in the basal and suprabasal layers of non-cancerous squamous epithelium of normal human esophageal tissue,[16] but little or no Mina53 is expressed in the nuclei of normal colonic glandular epithelium.[15]

In contrast, Ki-67, a reliable marker of cell proliferation, is expressed in a limited number of cells in the basal and parabasal layers of the esophageal epithelium, with a wide expression of Mina53, and in the nuclei of Mina53-negative, colonic crypt cells.[15] Thus, Mina53 expression in normal cells does not always coincide with Ki-67 expression, and may not be regarded as having the same function as that of involvement in cancer cell proliferation. Otherwise, the function of Mina53 may vary in an organ-specific manner in different organs expressing it, whether in normal or cancerous cells. In the present study Ki-67 was generally negative in renal tubular epithelial cells, suggesting that Mina53 expression in normal renal tubular epithelium does not directly affect cell proliferation.

The expression of Mina53 in RCC varied in level from negative to high. Interestingly, Mina53 expression had disappeared in 21 tumors (32.8%) despite its expression in renal tubular epithelium, from which RCC is considered to arise. Moreover, low Mina53-expressing tumors with a similar level of expression to that in the nuclei of normal renal tubules accounted for 53.1% (34 tumors). These findings suggest that Mina53 expression may possibly have disappeared after malignant transformation, but Mina53 expression is unlikely to be involved in renal carcinogenesis or cancer proliferation in the early stage of RCC. In addition, no significant difference in Ki-67 LI was noted between Mina-negative and low Mina53-expressing tumors, suggesting that a similar level of Mina53 expression to that in renal tubular epithelium does not contribute to the proliferation of RCC cells. In contrast, Ki-67 LI was significantly higher in high Mina53-expressing than in Mina53-negative and low Mina53-expressing tumors, suggesting that Mina53 is not involved in RCC proliferation until the expression level of Mina53 exceeds that in normal renal tubules. Mina53-positive RCC cells was uniformly distributed in the tumor nodule, but in some cases of low-Mina53 expression, Mina53 expression was focally or predominantly observed in the periphery of the tumor. Mina53 expression

Table 2 Statistical analysis of factors that affect event-free survival of 64 RCC patients

Variables	Univariate		Multivariate		
	χ^2	*P*	χ^2	*P*	Hazard ratio
Mina53 high expression	36.698	<0.0001†	0.378	0.5389	2.079 (0.201–21.462)
Gender	0.049	0.8253	0.367	0.5448	2.268 (0.160–32.138)
MVI positive	29.863	<0.0001†	0.350	0.5539	0.604 (0.113–3.213)
Stage IV	56.398	<0.0001†	6.541	0.0105‡	0.061 (0.007–0.520)
Sarcomatoid RCC	5.920	<0.05†	0.039	0.8427	0.727 (0.031–16.868)
Ki-67 LI ≥ 10%	36.062	<0.0001†	6.953	0.0084‡	0.033 (0.201–21.462)

†Identified as significant factors for poor prognosis. ‡Identified as significant independent factors for poor prognosis.
LI, Labeling index; MVI, microvenous invasion; RCC, renal cell carcinoma.

did not always coincide with Ki-67 expression, suggesting that there was no relationship between proliferative activity of tumor cells and Mina53 expression in such cases. Mina53 expression was located in the nucleus, with concentrated amounts in the nucleolus in three high-Mina53 expressing tumors, as we previously demonstrated in HeLa cells and esophageal and colon cancer cells.[14–16] The function of Mina53 in the nucleolus is not certain, but it may play a role in ribosome biogenesis, and so forth.[14]

Considering these results, we examined the relationship of high Mina53 expression or non-high Mina53 expression (no or low mina53 expression) to clinicopathological factors that are generally closely involved in the prognosis of cancers, such as histological type, stage, and MVI. We found that high Mina53-expressing tumors were observed significantly more frequently in sarcomatoid RCC (a histological type that reportedly has the poorest prognosis) than in other histological types, in stage IV than in other stage tumors, and in MVI-positive than in MVI-negative tumors; but there was no relationship between Mina53 expression and metastasis of RCC. Along with these results of the association between Mina53 expression and cancer stage, the reported findings that sarcomatoid component and MVI positivity are often observed in advanced RCC,[23,24] suggest that Mina53 is most likely to be involved in the proliferation of advanced RCC. Moreover, the finding that sarcomatoid RCC were frequently highly Mina53 positive suggests an association between Mina53 expression and RCC dedifferentiation.

In the analysis of Mina53 expression in colon tumors, Mina53 was also expressed in adenomas as precancerous lesions, tended to be more highly expressed in well-differentiated adenocarcinoma, and was expressed in all cancers examined, leading to the speculation that high Mina53 expression in colon cancer is common, representing a relatively early event in colonic carcinogenesis.[15] In colon cancer, APC gene abnormalities are known to induce *c-myc* overexpression,[25] and *Mina53* is a myc target gene; therefore, we speculate that *c-myc* overexpression results in increased expression of *Mina53*. Unlike in colon cancer, overexpression of *c-myc* has been reported in advanced RCC (*c-myc* expression occurs as a late-stage event),[8,26] which is in agreement with the present finding that high Mina53-expressing tumors were observed significantly more frequently in advanced RCC. The expression of c-myc protein in the present RCC series needs to be further studied to confirm this point.

We found that high Mina53 expression was associated with significantly shorter survival than that in non-Mina53-high tumors (Mina53-negative and Mina53-low tumors). In addition, stage IV, Ki-67 LI ≥ 10%, MVI positivity, and sarcomatoid RCC were associated with significantly poorer prognosis. Multivariate analysis showed that stage IV and Ki-67 LI ≥ 10% were independent factors for poor prognosis, but unlike in esophageal cancer, high Mina53 expression was not a significant independent factor for poor prognosis. We speculate that the close association between Ki-67 LI and prognosis influenced Mina53, which is considered a similar growth factor. This is because multivariate analysis failed to identify high Mina53 expression as a significant independent poor prognostic factor.

In conclusion, the present study shows that Mina53 overexpression has an effect on RCC proliferation in the late stage, and that Mina53 is a molecule that may be involved in cancer proliferation or dedifferentiation in the advanced stage, making it an important factor in determining the prognosis of RCC patients.

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Immunohistochemical expressions of Cap43 and Mina53 proteins in neuroblastoma

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Abstract

Background: We studied the expressions of both Mina53, which is a myc target gene and is related to cell proliferation, and Cap43, which is related to metastasis suppression and downregulation of MYCN gene, in neuroblastoma.

Methods: Forty-eight surgically obtained neuroblastoma specimens were immunohistochemically stained. The Cap43 and Mina53 expression levels were determined, and their relationship to clinical prognostic factors, biological prognostic factors, and the patients' prognosis were examined.

Results: The Cap43 expression score was significantly high in the cases that had one of the good prognostic factors (<1 year old, early stage, mass screening case, no MYCN gene amplification), whereas the Mina53 expression score was high in those with poor prognostic factors. Regarding the MYCN expression site, the Cap43 expression score was significantly high in the cases demonstrating cytoplasm expression, whereas the Mina53 expression score was significantly high in the cases demonstrating nucleus expression. A significant relationship was found between Cap43 and TrkA, between Mina53 and Ki-67, and between Mina53 and TrkA. The prognosis was significantly favorable in the Cap43 high-expression cases, whereas it was significantly poor in the Mina53 high-expression cases.

Conclusions: Cap43 and Mina53 are both considered to be important biological and prognostic factors in neuroblastoma.

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Neuroblastoma is one of the most common solid tumors of early childhood [1]. Its incidence is the highest among pediatric solid cancers, with a range of 7% to 10% [2], and the outcome varies [3,4]. This tumor may disappear on its own without any treatment, although it may also develop rapidly into a malignant neoplasm. Important factors that affect the disease course have been reported, that is, clinical factors: age [5] and clinicopathological stage [6]; biological factors relating to an unfavorable prognosis: DNA diploidy [7,8], MYCN gene amplification [9], chromosome 1p deletion [10,11], chromosome 17q gain [12], and a high expression of multidrug resistance-associated protein [13]; and biological factors relating to a favorable prognosis: molecular abnormalities such as TrkA expression [14,15] and CD44 expression [16]. Analyses of the genes and nuclear DNA content have helped to gradually reveal the characteristics of neuroblastoma; however, the amplification of the MYCN gene is considered to be the most important prognostic factor [17,18].

Tsuneoka et al [19] reported that Mina53 (myc-induced nuclear antigen with a molecular weight of 53 kDa) is a novel myc target gene that is related to cell proliferation. The Mina53 gene is located on chromosome 3 (3q12.1), encodes a protein with a molecular weight of 53 kDa, and then localizes in the nucleus while part of the protein is concentrated in the nucleolus. Recent studies have reported that Mina53 is expressed in all pathological grades of colon cancer—it does not appear in nonneoplastic colonic cells or when it does, then it only appears at a low level [20]—and that esophageal cancer with high expression of Mina53 had a significantly unfavorable prognosis [21].

Cap43 (NDRG1) was found by van Belzen et al [22] and Kokame et al [23] almost simultaneously but under different physiological conditions. The NDRG1 gene was mapped to human chromosome 8q24.2, and it encodes a cytoplasmic 43-kDa protein containing a tandem repeat of 10 amino acids. Cap43 is reported to be a molecule that is related to cell differentiation [24] and the inhibition of metastasis [25,26]. The expression of Cap43 was regulated by c-myc and MYCN/Max complex in vitro, thus suggesting a close relationship between the Cap43 and the myc gene family [27]. However, there have so far been no reports suggesting direct interaction between Mina53 and Cap43. The current study first examined the immunohistochemical expression of 2 myc gene–related molecules, namely Mina53 and Cap43, in neuroblastoma and clinicopathologically evaluated whether they could be new prognostic predictors like MYCN, which is a member of the myc gene family and the most reliable prognostic predictor of neuroblastoma. We also examined the relationships among the expressions of Mina53, Cap43, TrkA (a representative gene that is a predictor of a good prognosis of neuroblastoma), and Ki-67 (a marker of cellular proliferation).

1. Materials and methods

1.1. Tissue sample preparation

Tissue samples for immunohistochemistry were obtained from 48 patients with neuroblastoma. Of these patients, 30 were surgically treated at Kurume University Hospital, whereas 18 patients were treated at Kyusyu University Hospital, between 1979 and 2005. All patients in the advanced stage (stages 3 and 4), except for 4 stage 3 cases, received chemotherapy using several antitumor agents. Because this was a retrospective study over a 25-year period, the modality of the chemotherapy after a surgical excision was thus not controlled. Table 1 summarizes the patient characteristics. The patients consisted of 27 boys and 21 girls, and the age at diagnosis ranged from 0 to 189 months (average, 25.7 ± 33.9). The tumors of the 18 patients were identified by a neuroblastoma mass screening (MS) system. The clinical staging was performed according to the International Neuroblastoma Staging System [28], and 9 cases were in stage 1, 6 in stage 2, 15 in stage 3, 17 in stage 4, and 1 in stage 4S. As to histologic type, 4, 43, and 1 cases were classified as undifferentiated, poorly differentiated, and differentiating types, respectively. Histology was also classified according to Shimada's classification [29,30], and it was favorable in 22 cases and unfavorable in 26 cases. Before this study, the number of copies of the MYCN oncogene per haploid genome was independently determined in each tumor by a Southern blot analysis, with a quantitation of the extent of amplification by the serial dilution of DNA. Tumors that have more than 10 copies of the MYCN gene per haploid genome are considered to have MYCN gene amplification [31,32]. As a result, among all cases, except for 4 cases whose records regarding MYCN gene amplification were not available, 14 cases had MYCN amplification from 11 to 180 copies.

1.2. Antibodies

Mouse monoclonal anti-Mina53 antibody and rabbit polyclonal anti-Cap43 antibody were established in our laboratory as previously described [20,33]. Mouse monoclonal anti-MYCN antibody (sc-142, Lot no. L2903) was purchased from Santa Cruz Bio Technology (Santa Cruz, CA); mouse monoclonal anti-Ki-67 antibody (clone MIB-1) was purchased from DAKO A/S (Glostrup, Denmark); and anti-TrkA goat polyclonal antibody (AF175, Lot no. GDB04) was purchased from R&D Systems, Inc (Minneapolis, MN).

1.3. Immunohistochemistry

Formalin-fixed, paraffin-embedded serial sections (4 μm) were mounted on 3-aminopropyltriethoxysilane–coated slides (Matsunami Glass Ind, Ltd, Osaka, Japan) and then were deparaffinized in xylene alcohol and graded

Table 1 Histopathological and immunohistochemical findings of the 48 patients with neuroblastoma

Case	Sex	Age (mo)	INSS	INPC	Shimada	MS	MYCN amplification	Cap43	TrkA	Mina53	MYCN	Location	Ki-67 LI	Outcome
1	F	12	3	Poorly	Unfavorable	(−)	(+)	2	0	8	9	n	11.1	D
2	M	12	3	Poorly	Favorable	(−)	(+)	1	0	6	8	n	39.7	D
3	M	132	4	Poorly	Unfavorable	(−)	(+)	0	0	12	6	n	40.2	D
4	F	20	4	Undifferentiated	Unfavorable	(−)	(+)	2	0	8	6	n	36.1	D
5	M	21	4	Poorly	Unfavorable	(−)	UK	12	8	1	12	c	49.3	A
6	M	63	4	Undifferentiated	Unfavorable	(−)	UK	0	2	12	0		46.3	D
7	M	49	1	Differentiating	Unfavorable	(−)	UK	3	8	0	1		37.9	A
8	F	7	2	Poorly	Favorable	(+)	UK	12	12	2	12		27.8	A
9	M	23	4	Poorly	Unfavorable	(−)	(+)	2	0	12	12	n	14.8	D
10	F	9	1	Poorly	Favorable	(+)	(−)	12	12	1	0		11.0	A
11	M	12	4	Poorly	Unfavorable	(−)	(+)	1	0	8	12	n	45.8	D
12	M	10	1	Poorly	Favorable	(+)	(−)	6	12	2	12	c	44.8	A
13	F	8	2	Poorly	Favorable	(+)	(−)	12	8	1	0		24.4	A
14	F	7	1	Poorly	Favorable	(+)	(−)	12	12	9	12	c	10.6	A
15	M	36	3	Poorly	Unfavorable	(−)	(−)	0	0	12	9	n	27.7	A
16	M	24	3	Poorly	Unfavorable	(−)	(−)	0	0	12	12	n	67.2	A
17	F	6	3	Poorly	Favorable	(+)	(−)	8	8	6	9		19.3	A
18	F	10	3	Poorly	Favorable	(+)	(−)	8	12	12	6	c	16.2	A
19	M	48	4	Poorly	Unfavorable	(−)	(+)	0	0	8	6	n	26.5	D
20	M	8	4s	Poorly	Favorable	(+)	(−)	4	0	12	12	n	44.2	A
21	F	24	4	Undifferentiated	Unfavorable	(−)	(+)	1	0	12	2	c > n	36.2	D
22	M	36	4	Poorly	Unfavorable	(−)	(+)	2	0	6	3	n	18.1	A
23	F	189	3	Poorly	Unfavorable	(−)	(−)	8	3	2	0		42.6	A
24	F	16	3	Poorly	Unfavorable	(−)	(−)	2	0	12	9	n	43.3	D
25	M	8	1	Poorly	Favorable	(+)	(−)	9	6	0	4		5.9	A
26	F	24	4	Poorly	Unfavorable	(−)	(+)	1	6	12	3	n	58.5	A
27	M	26	4	Undifferentiated	Unfavorable	(−)	(−)	0	0	12	0		28.8	A
28	F	8	1	Poorly	Favorable	(+)	(−)	8	4	2	1	c	37.0	A
29	M	21	4	Poorly	Unfavorable	(−)	(+)	0	0	12	12	n	76.9	D
30	M	8	3	Poorly	Favorable	(+)	(−)	12	8	12	12	c	8.4	A
31	F	36	4	Poorly	Unfavorable	(−)	(+)	0	12	9	3	n	67.8	D
32	M	2	3	Poorly	Favorable	(−)	(−)	1	6	1	2	c	41.0	A
33	M	6	1	Poorly	Favorable	(+)	(−)	4	12	4	9	c	38.5	A
34	F	6	2	Poorly	Favorable	(+)	(−)	12	12	2	9	c	18.6	A
35	M	0	2	Poorly	Favorable	(−)	(−)	12	12	1	0		30.1	A
36	M	24	4	Poorly	Unfavorable	(−)	(−)	0	8	12	2	c > n	63.3	D
37	M	6	3	Poorly	Favorable	(+)	(−)	6	8	4	9	c	36.8	D
38	F	7	1	Poorly	Favorable	(+)	(−)	12	12	1	9	c	38.2	A
39	F	12	2	Poorly	Favorable	(+)	(−)	12	12	1	12	c	33.6	A
40	F	7	3	Poorly	Favorable	(+)	(−)	0	6	12	12	c	11.8	A
41	F	8	2	Poorly	Favorable	(+)	(−)	12	8	0	12		5.9	A
42	M	0	1	Poorly	Favorable	(−)	(−)	2	2	1	12	c	38.9	A
43	M	72	4	Poorly	Unfavorable	(−)	(−)	0	6	9	12	c	19.4	D
44	M	73	3	Poorly	Unfavorable	(−)	(−)	8	2	9	2	c	40.7	A
45	F	36	4	Poorly	Unfavorable	(−)	(+)	2	4	8	1	n	70.0	D
46	F	31	4	Poorly	Unfavorable	(−)	(−)	12	8	8	12	c	0.0	A
47	M	12	3	Poorly	Unfavorable	(−)	(+)	1	3	9	3	n	91.3	D
48	M	19	3	Poorly	Unfavorable	(−)	(−)	0	0	1	9	n	27.0	A

INSS indicates International Neuroblastoma Staging System; INPC, International Neuroblastoma Pathology Committee; UK, unknown; n, nuclear; c, cytoplasm; A, alive; D, dead; M, male; F, female.

alcohol. The sections were soaked in 10 mmol/L of sodium citrate buffer (pH 6.9) and treated in a microwave for 20 minutes for antigen retrieval. The immunostaining of Mina53, Cap43, Ki-67, and TrkA were performed using streptavidin-biotin peroxidase kits (Nichirei, Tokyo, Japan). The concentrations of primary antibodies against Mina53, Cap43, N-*myc*, Ki-67, and TrkA were 3.5, 3.5, 2.0, 0.8, and 3.0 μg/mL, respectively.

The sections were incubated with primary antibodies for 60 minutes at room temperature after the pretreatment with

avidin and rabbit or goat serum. Immunohistochemistry of MYCN was performed by using the catalyzed signal-amplification system II (Code K1497, DAKO, Ely, UK) according to the manufacturer's protocol. The sections were incubated overnight with primary antibody at 4°C. Peroxidase reaction was developed with the addition of 3,3-diaminobenzitine and H_2O_2 substrate solution with either a 4-minute (Mina53) or 2-minute (Cap43, N-*myc*, Ki-67) incubation time. After light counterstaining with hematoxylin, the slides were dehydrated, coverslipped, and observed under a microscope (Olympus BH-2, Olympus Optical, Tokyo, Japan). Negative controls were prepared by replacing the primary antibody with normal mouse IgG or normal rabbit or goat serum.

1.4. Evaluation of immunohistochemical findings

The results of immunohistochemistry were independently evaluated according to the staining intensity and the percentage of positive cells by 2 pathologists (S.F. and H.Y.) without any knowledge of the patients' information. Briefly, the staining intensity for Cap43, Mina53, MYCN, and TrkA in each specimen was scored on a scale from 0 to 3 (0, negative; 1, weakly positive; 2, moderately positive; 3, strongly positive) (Fig. 1). The intensity level observed in the largest number of positive cells was used for scoring. The percentage of positive cells seen in each specimen was estimated and scored on a scale from 0 to 4 (0, negative; 1, positive in 1%-25% of the cells; 2, positive in 26%-50%; 3, positive in 51%-75%; 4, positive in 76%-100%). After evaluating these parameters, the expression score of each specimen was obtained by multiplying the score of maximum intensity and the figures of the scales. The percentage of Ki-67–positive cells was counted in 5 high-power fields that were randomly chosen on each slide. The Ki-67 labeling index (LI) was calculated as the percentage of positive tumor cell nuclei.

1.5. Statistics

The expression scores of Cap43, Mina53, TrkA, and Ki-67RI were compared according to each of the known prognostic factors (age, clinicopathological stage, MS,

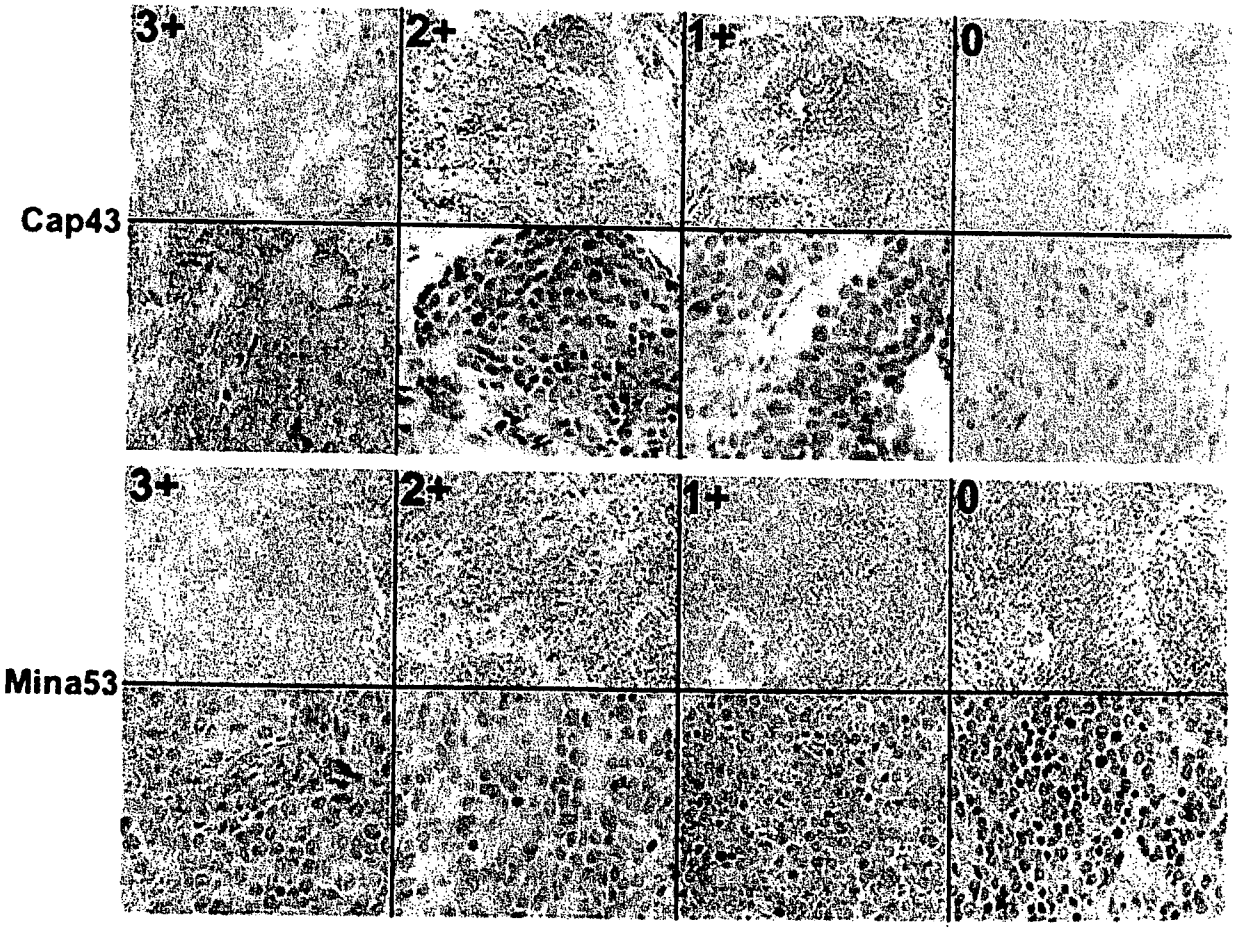

Fig. 1 A panel figure showing representative examples of each staining intensity for Cap43 and Mina53. The staining intensity for Cap43, Mina53, MYCN, and TrkA in each specimen was scored on a scale from 0 to 3 (0 = negative, 1 = weakly positive, 2 = moderately positive, 3 = strongly positive). The maximum intensity was determined as the level observed in the largest number of positive cells.

Shimada's classification, MYCN amplification) using Mann-Whitney *U* test.

The expression scores of Cap43 and Mina53 were compared according to the 3 different locations (nucleus group, cytoplasm group, no expression group) of the MYCN protein expression by means of the Kruskal-Wallis H test. We excluded 2 cases that showed both nuclear and cytoplasmic expression from the comparisons because the number of cases was too small. The relationship among the expressions of TrkA, Cap43, Mina53, and Ki-67 was examined using the Spearman regression analysis using a linear regression.

To examine the importance of Cap43 and Mina53 as biological prognostic factors, all cases, except for 2 cases whose records regarding prognosis were not available, (total, 46 cases) were classified into either the high-expression group (score ≥ 4) or the low-expression group (score ≤ 3), and then their overall survival rates were examined by using the Kaplan-Meier method. The overall survival was measured from the date of diagnosis until death because of neuroblastoma. The overall survival times of the patients that were alive at the last follow-up were censored. The median follow-up time for 46 patients was 5.6 years (range, 8 days-20 years). The *P* values for each survival rate as well as for the prognostic factors (age, clinicopathological stage, MS, MYCN gene amplification, TrkA expression, MYCN protein expression) were analyzed using the log-rank test. An evaluation for MYCN gene amplification was performed in 42 cases (except 4 cases whose record of MYCN gene amplification was not available).

Cox's multivariate analysis was used to examine whether the Cap43 or Mina53 expression was a prognostic factor independent from the other established factors such as MYCN gene amplification.

All statistical analyses were performed with StatMate III (ATMS Co, Ltd, Tokyo, Japan). *P* values less than .05 were considered to be statistically significant.

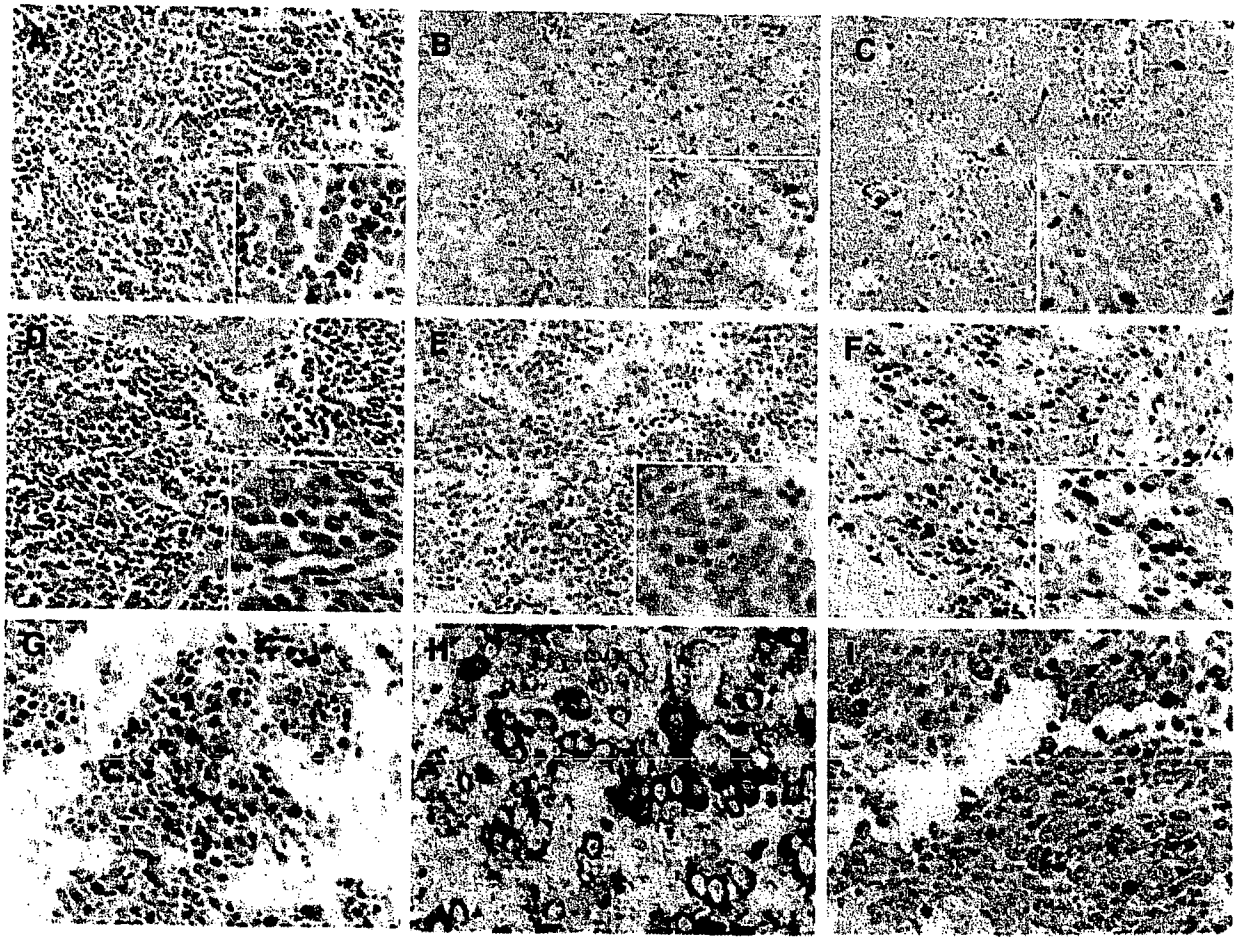

Fig. 2 Immunohistochemical staining of Cap43, Mina53, TrkA, Ki-67, and N-*myc* in neuroblastoma. A, Neuroblastoma (case 39) stained with H&E (×200). B, Cap43 expression in cytoplasm (×200). Inset: high-power view (×400). C, TrkA expression in cytoplasm (×200). Inset: high-power view (×400). D, Neuroblastoma (case 24) (H&E staining, ×200). E, Mina53 expression in nucleus (×200). Inset: high-power view (×400). F, Ki-67 expression in nucleus (×200). Inset: high-power view (×400). G, N-*myc* expression in nucleus (×400). H, N-*myc* expression in cytoplasm (×400). I, N-*myc* expression in nucleus and cytoplasm (×400).

2. Results

2.1. Immunohistochemistry for Cap43, Mina53, TrkA , Ki-67, and MYCN

Cap43 and TrkA were expressed in the cytoplasm, membrane, and neurofilament of tumor cells (Fig. 2A-C). Mina53 and Ki-67 were expressed only in the nucleus (Fig. 2D-F). MYCN protein expression presented 4 different patterns: in the nucleus only (Fig. 2G), in the cytoplasm only (Fig. 2H), in the nucleus and cytoplasm (Fig. 2L) and no expression, and their frequencies were 18 cases (40.9%), 22 (45.8%), 2 (4.6%), and 6 (12.5%), respectively. The cases with MYCN gene amplification showed a significantly higher incidence of the nucleus expression (14/14, 100%; $P < .001$), whereas those without MYCN gene amplification showed a significantly higher incidence of cytoplasmic expression (19/30, 63.3%; $P < .001$). Table 1 summarizes the histopathological and immunohistochemical findings. In all cases, the expression score (mean ± SD) was 4.92 ± 4.87 for Cap43, 5.29 ± 4.74 for Mina53, and 6.63 ± 4.64 for TrkA; mean Ki-67 LI was 35.0 ± 20.0%.

2.2. Relationship between the expression of Cap43, Mina53, TrkA, or Ki-67 and the prognostic factors

Table 2 summarizes the relationship between each of the known prognostic factors and the expression of Cap43, Mina53, TrkA, or Ki-67. The expression scores of Cap43 and TrkA were significantly higher in the cases that had one of the good prognostic factors, that is, younger than 1 year, early stage (stages 1, 2, and 4S), MS case, favorable histology by Shimada's classification ($P < .001$), and no MYCN gene amplification ($P < .01$). On the other hand, the expression score of Mina53 was significantly higher in the cases that had one of the poor prognostic factors, that is, age more than 1 year ($P < .01$), advanced stage (stages 3 and 4) ($P < .001$), non-MS case ($P < .05$), an unfavorable histology by Shimada's classification ($P < .001$), and amplification of the MYCN gene ($P < .05$). Ki-67 LI was significantly higher in a manner similar to that for Mina53 except for the stage ($P = .117$) and MYCN gene amplification ($P = .054$), that is, $P < .05$ for age and Shimada's classification and $P < .01$ for MS.

2.3. Relationship between the MYCN protein expression and the expression of Cap43 or Mina53

Fig. 3 shows comparisons of the expression scores of Cap43 and Mina53 according to the 3 different locations of MYCN protein expression. The Cap43 expression score was 1.11 ± 1.13 in the nucleus group, 7.77 ± 4.40 in the cytoplasm group, and 7.33 ± 5.89 in the no expression group. A significant difference was observed between the nucleus group and the cytoplasm group ($P < .001$). On the other hand, the Mina53 expression score was 9.28 ± 3.04 in the nucleus group, 4.45 ± 4.32 in the cytoplasm group, and 4.83 ± 5.56 in the no expression group. A significant difference was also found between the nucleus group and the cytoplasm group ($P < .05$).

2.4. Relationship among the scores of Cap43, Mina53, and TrkA, and Ki-67 LI

As shown in Table 3, a significant correlation was observed between Cap43 and Mina53 ($rs = -0.563$, $P < .001$), Cap43 and TrkA ($rs = 0.643$, $P < .001$), Cap43 and Ki-67 LI ($rs = -0.421$, $P < .01$), and Mina53 and TrkA ($rs = -0.439$, $P < .01$). However, no significant correlation was observed between Mina53 and Ki-67 LI ($rs = 0.211$, $P = .148$).

Table 2 Relationship between the Cap43 or Mina53 expression and the prognostic factors

Category	Cap43			Mina53		TrkA		Ki-67 LI	
	n	mean ± SD	P	mean ± SD	P	mean ± SD	P	mean ± SD	P
Age									
<1	20	8.20 ± 4.19	<.001	4.25 ± 4.51	<.01	8.60 ± 3.73	<.001	25.5 ± 13.8	<.05
≥1	28	2.57 ± 3.91		8.32 ± 4.00		2.93 ± 3.93		41.4 ± 21.2	
Stage									
1 + 2 + 4S	16	9.00 ± 3.88	<.001	2.44 ± 3.35	<.001	9.00 ± 4.07	<.001	28.0 ± 13.6	.117
3 + 4	32	2.88 ± 3.97		8.72 ± 3.69		3.44 ± 3.93		38.2 ± 21.9	
MS									
(+)	18	8.94 ± 3.70	<.001	4.61 ± 4.62	<.05	9.11 ± 3.51	<.001	24.1 ± 13.7	<.01
(−)	30	2.50 ± 3.79		7.83 ± 4.28		3.00 ± 3.84		41.2 ± 20.5	
Shimada's classification									
Favorable	22	8.05 ± 4.36	<.001	4.18 ± 4.36	<.001	8.36 ± 4.08	<.001	26.5 ± 13.6	<.05
Unfavorable	26	2.27 ± 3.57		8.69 ± 3.84		2.69 ± 3.61		41.8 ± 21.9	
N-*myc* amplification									
(+)	14	1.07 ± 0.83	<.01	9.29 ± 2.27	<.05	1.79 ± 3.51	<.01	45.2 ± 24.5	.054
(−)	30	6.47 ± 4.91		5.77 ± 4.90		6.63 ± 4.55		29.2 ± 16.6	

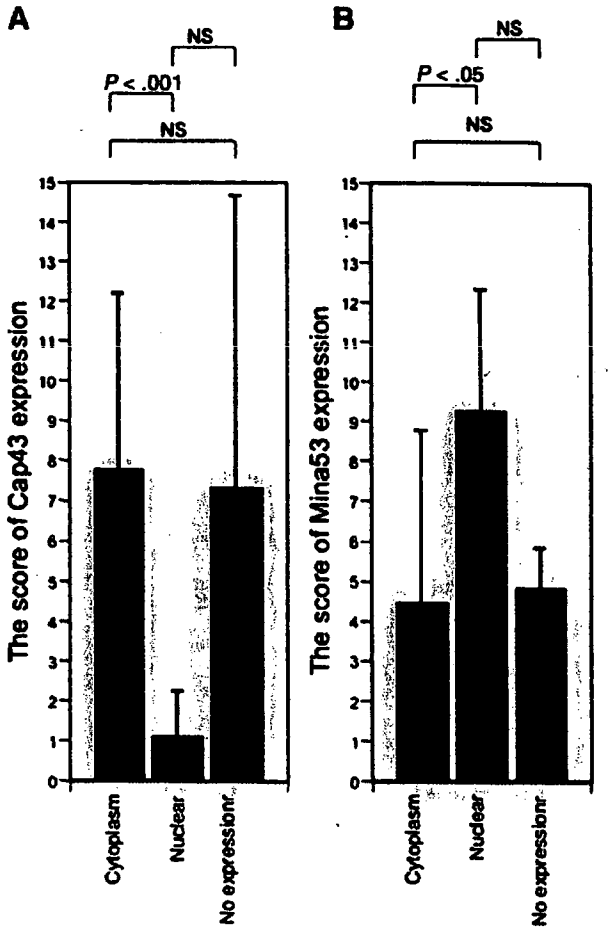

Fig. 3 The relationship between N-*myc* protein expression and the expression of Cap43 or Mina53. The expression scores of Cap43 and Mina53 are compared according to the 3 different groups of N-*myc* protein expression, that is, nucleus group, cytoplasm group, and no expression group. The relationship between Cap43 or Mina53 expression score and the pattern of N-*myc* expression was analyzed by using the Kruskal-Wallis H test.

2.5. Relationship between the survival rate and the expression of Cap43 or Mina53

As shown in Fig. 4, the Cap43 high-expression group (n = 22) had a significantly better prognosis than the Cap43 low-expression group (n = 24, $P < .01$). The Mina53 high-expression group (n = 29) had a significantly poorer prognosis than the Mina53 low-expression group (n = 17, $P < .01$). Regarding the prognostic factors, the prognosis was significantly better in the cases with age less than 1 year ($P < .001$) and in those with a TrkA high expression ($P < .01$). A significantly poorer prognosis was observed in the cases in the advanced stage ($P < .001$) and in those with the unfavorable histology ($P < .001$) and the MYCN gene amplification ($P < .001$). According to Cox's multivariate analysis shown in Table 4, MYCN gene amplification was a significant prognostic factor ($P < .05$), whereas Cap43 ($P = .641$), Mina53 ($P = .104$), TrkA ($P = .288$), age ($P = .351$), and stage ($P = .537$) were not.

Table 3 Relationship among the scores of Cap43, Mina53, TrkA, and the Ki-67 LI

Factors	rs	P
Cap43 and Mina53	−0.563	<.001
Cap43 and TrkA	0.643	<.001
Cap43 and Ki-67 RI	−0.421	<.01
Mina53 and TrkA	−0.439	<.01
Mina53 and Ki-67 RI	0.211	.148

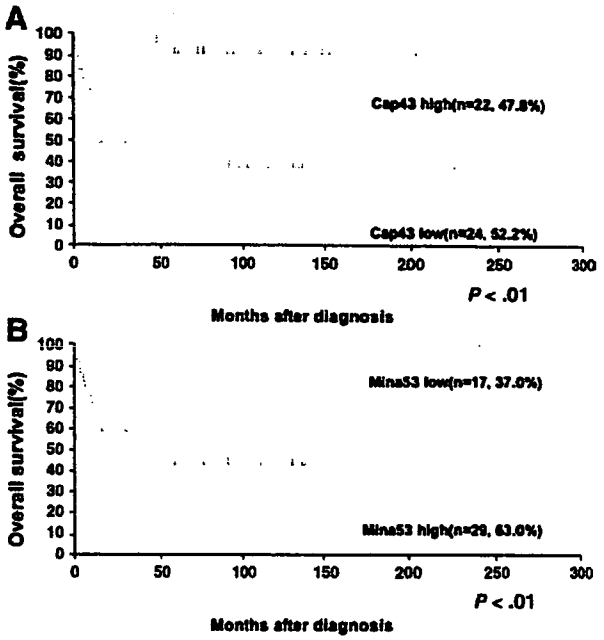

Fig. 4 Correlation between the overall survival rate and the expression of Cap43 or Mina53 analyzed by the Kaplan-Meier method. Forty-six neuroblastoma tumors were divided into high-expression group or low-expression group according to the expression scores of Cap43 and Mina53. The survival rate was significantly higher in the cases with a high Cap43 expression (n = 22, 47.8%), whereas the rate was significantly lower in the cases with a high Mina53 expression (n = 29, 63.0%).

3. Discussion

Regarding the relationship between expression of Cap43 or Mina53 and the known prognostic factors of neuroblastoma, the Mina53 expression was significantly higher in the cases that had one of the poor prognostic factors, that is, age more than 1 year, advanced stage, non-MS case, unfavorable histology according to Shimada's classification, MYCN gene amplification, whereas Cap43 expression was significantly higher in the cases that had one of the good prognostic factors, that is, age of less than 1 year, early stage, MS case, a favorable histology, and no MYCN gene amplification. Shimono et al [27] reported that Ndr1 (Cap43) was

Table 4 Univariate and multivariate analyses on the factors that affected the overall survival of the 46 neuroblastoma patients

Variables	Univariate		Multivariate		Hazard ratio
	χ^2	*P*	χ^2	*P*	
Age	14.784	<.001	0.871	.351	2.840 (0.317-25.413)
Disease stage	12.142	<.001	0.380	.537	0.410 (0.024-6.963)
Shimada's classification	15.598	<.001		ND	
N-*myc* amplification	36.59	<.001	5.120	.023	2.733 (1.144-6.529)
Cap43 expression	18.881	<.001	0.217	.641	0.574 (0.055-5.945)
Mina53 expression	12.266	<.001	2.639	.104	4.969 (0.718-34.386)
TrkA expression	9.496	<.01	1.129	.288	0.510 (0.147-1.766)

ND indicates not done.

suppressed by the combination of MYCN and Max, which agrees with the findings in our current study. On the other hand, previous studies have reported a close relationship between the expressions of Mina53 and c-myc in esophageal cancer and colon cancer [20,21], and our findings showed the presence of a close relationship between MYCN and Mina53. C-myc is expressed in most differentiated cells, and its deregulated expression is related to the proliferation of many classes of tumors, whereas MYCN is only expressed in neurogenic tumors, such as neuroblastoma [34]. Our findings indicate that Mina53 expression plays a significant role not only in the MYCN gene expression but also in the prognosis of neuroblastoma.

The present study showed a significantly higher Mina53 expression in the cases that had MYCN protein expression in the nucleus and a significantly higher Cap43 expression in the cases that had MYCN protein expression in the cytoplasm. MYCN gene amplification is an established indicator of a poor prognosis in neuroblastoma; however, no consensus has yet been reached on the meaning of MYCN protein expression [35,36], and the significance of the cellular location of MYCN protein in neuroblastoma cells

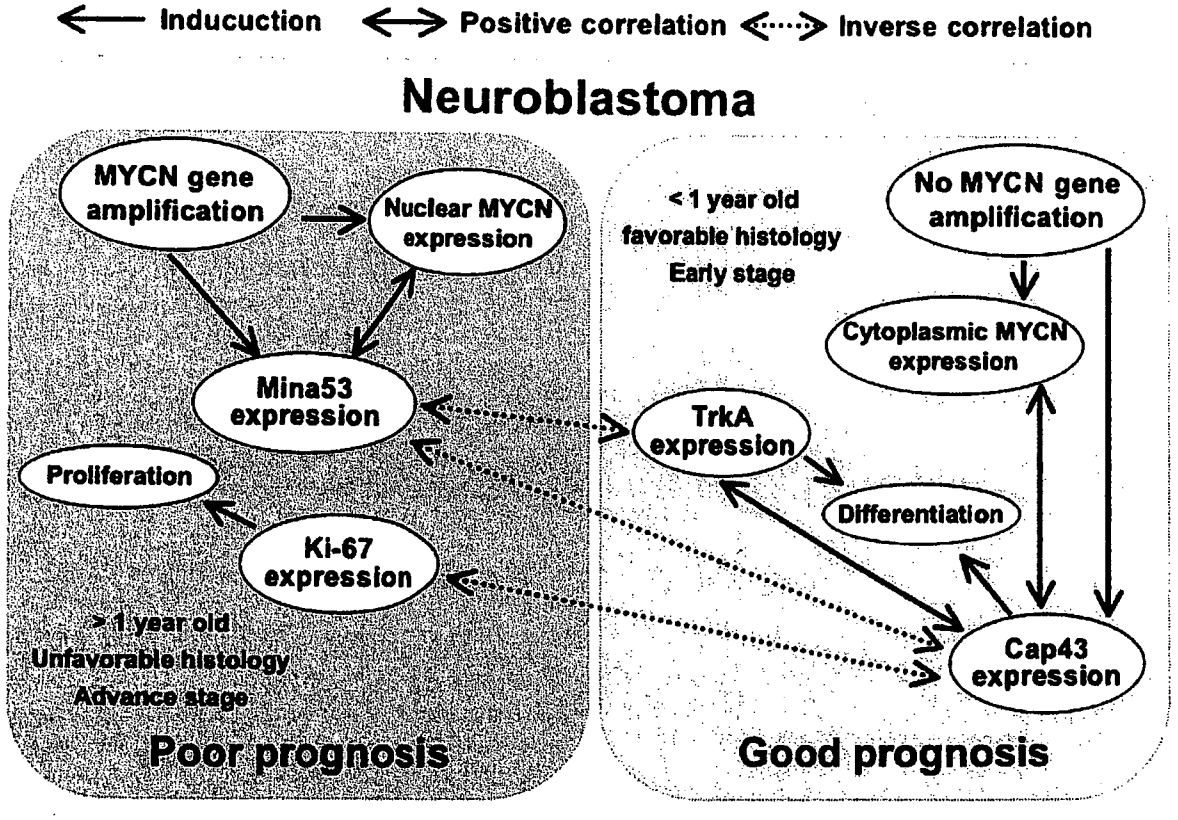

Fig. 5 A schematic drawing showing the possible association of Cap43, Mina53, TrkA, Ki-67, MYCN gene and protein, cell proliferation and differentiation, and prognosis in neuroblastoma. There is an inverse correlation between Ca43 and Mina53, between TrkA and Mina53, and between Cap43 and Ki-67, and a positive correlation between Cap43 and TrkA. Representative good prognostic factors include Cap43 and TrkA expressions, whereas representative poor prognostic factors include MYCN gene amplification and Mina53 expression.

has not yet been fully investigated. Hiyama et al [37] studied the cellular location of MYCN protein in neuroblastoma cases with MYCN gene amplification. They found that some cases showed a cytoplasmic expression, whereas almost all cases showed a strong nuclear expression, suggesting that the cytoplasmic MYCN protein expression may reflect the de novo synthesis of MYCN. Wakamatsu et al [38] reported that the MYCN mRNA level generally decreases in the process of nerve differentiation, whereas MYCN protein is translocated from the nucleus to the cytoplasm. MYCN is a multifunctional gene that is mainly related to cell proliferation, differentiation, and apoptosis. In the current study, MYCN protein was localized in the nucleus in all cases of MYCN gene amplification, whereas it was often observed in the cytoplasm in the cases without MYCN gene amplification. Referring to the characteristics of Mina53 and Cap43 genes described previously [19-21,24-26], it is presumed that nuclear and cytoplasmic MYCN protein expressions may be related to cell proliferation and differentiation, respectively.

Among the expression scores of Cap43, Mina53 and TrkA, and Ki-67 LI, a positive correlation was obtained between Cap43 and TrkA, whereas a negative correlation was obtained between Cap43 and Mina53, between Cap43 and Ki-67 LI, and between Mina53 and TrkA. Nakagawara et al [14] reported that the TrkA gene has been implicated to show a negative correlation with MYCN gene amplification, and it is regarded as a favorable prognostic factor for neuroblastoma. Hirata et al [39] reported that Cap43 plays an important role in the differentiation of Schwann cells, suggesting it to be related with the differentiation process of normal nerve cells. Our results suggest that Cap43 is also identified as a favorable prognostic factor and could be involved in the differentiation process of neuroblastoma cells.

Tsuneoka et al [21] reported that the expression of Mina53 significantly correlated with Ki-67 LI in esophageal cancer, suggesting that Mina53 plays an important role in cancer cell proliferation. We also examined the relationship between the Mina53 expression score and Ki-67 LI; no significant correlation was observed. This is probably because of the presence of such cases with high Ki-67 LI level but no MYCN gene amplification and a low Mina53 expression score in the early stage (Table 2). Another molecule related to cell proliferation could be involved in such cases. Ki-67 LI has been shown to correlate with some established prognostic factors for neuroblastoma and its prognosis [2,40]. In the current study, Ki-67 LI was significantly higher in the cases with poor prognostic factors, such as age (older than 1 year), non-MS case, unfavorable histology, and MYCN gene amplification, whereas there was no significant difference regarding the stage. In comparison with the cases of Krams et al [2], the average Ki-67 LI was higher in our cases in the early stage. The proliferation capability has been reported to increase temporally in some young patients with neuroblastoma. This temporal increase in the proliferation capability occurred and induced a high level of Ki-67 LI in some of our cases in the early stage, thus resulting in no significant difference.

In a univariate analysis for prognosis, a significantly high Mina53 expression was observed in the cases with a poor prognosis, whereas the Cap43 expression was significantly high in the cases with a good prognosis. However, according to a multivariate analysis, only the MYCN amplification was identified to be an independent prognostic factor. This suggests that these 2 genes are strongly influenced by MYCN gene amplification. The current study included only 4 fatal cases without MYCN gene amplification, and they are therefore probably insufficient in number to evaluate whether Cap43 and Mina53 are independent prognostic factors. This question thus requires further study.

Finally, a schematic drawing regarding the possible association of Cap43, Mina53, TrkA, Ki-67, MYCN gene and protein, cell proliferation and differentiation, and prognosis in neuroblastoma is shown in Fig. 5. In relation to prognosis, both the Cap43 and Mina53 genes were closely related to the MYCN gene amplification as well as protein expression and location. Although these genes were not identified to be independent prognostic factors based on a multivariate analysis, the results did indicate that Cap43 may be a favorable prognostic factor, and Mina53, an adverse prognostic factor.

Acknowledgments

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総　説　— 医学・医療の最前線シリーズ —

インターフェロンによる肝発癌抑制機序に関する基礎的研究

久留米大学医学部病理学講座
矢野博久・神代正道

はじめに

インターフェロン（IFN）は，長野らによりウイルス抑制因子として1954年に見出され，1957年にIsaacsらにより，ウイルス増殖における干渉現象を担う因子の意味を込めてIFNと命名された．IFNは，抗ウイルス作用の他，細胞増殖抑制作用，血管新生抑制作用，免疫応答調節作用，抗テロメラーゼ作用など，多彩な生物活性を有するサイトカインであり，α，β，ωからなるⅠ型IFNと，γからなるⅡ型IFNに大きく分類される[1]．それぞれの型のIFNは細胞表面の特異的なレセプターに結合する事によりシグナルを細胞内に伝え，共通あるいは別々の遺伝子の発現を誘導する．αやβなどのⅠ型IFNは，C型慢性肝炎などのウイルス性疾患，慢性骨髄性白血病，メラノーマ，腎細胞癌などの腫瘍性疾患の治療薬として臨床的に使用されている[2]．

C型慢性肝炎は，長期的経過で肝硬変，更に肝細胞癌へと進展する例が多く，肝病変の進展阻止は重要な問題である．近年，C型慢性肝炎あるいは肝硬変に対してIFN療法を行うことにより，ウイルスの完全排除と関係なく，肝機能の改善や肝発癌率の低下が誘導されることが報告されている[3)4)5)]．更に，肝細胞癌の切除術後の再発防止に対するIFN投与の有用性や[6)7)]，進行肝癌の治療に対するIFNと抗癌剤の併用療法の有用性[8)9)]も報告されている．このように肝癌発生予防や治療に対するIFNの有用性が臨床的に明らかにされつつあるが，その作用メカニズムは未だ十分に解明されていないのが現状である．IFNには，細胞増殖抑制作用があることから，肝癌細胞に対して直接的に作用して，発癌抑制や抗癌作用を示している可能性も考えられる．

筆者らは，IFNの肝癌細胞に対する作用を明らかにするために，肝癌細胞のIFNレセプターの発現や，種々のIFNによる増殖抑制作用とその作用機序などに関して，培養肝癌細胞を使用し*in vitro*や*in vivo*で検討を行って来たが[10)-16)]，本稿では，その研究結果の一部を以下に紹介する．

肝癌におけるⅠ型IFNレセプターの発現

Ⅰ型IFNの作用発現には，標的細胞上におけるⅠ型IFNレセプターの発現が必須である．我々は，当教室で独自に樹立・維持されている11種類の分化度の異なる肝細胞癌細胞株と2種類の混合型肝癌株の合計13株の肝癌の細胞株を使用して，まずレセプターの発現の検討を行った．Ⅰ型IFNレセプターは，AR-1鎖とAR-2鎖の2つから構成されており，AR-2鎖には，AR-2a，-2b，-2cの3種類があるが，AR-2cが，インターフェロンとの結合ユニットでありIFNの作用発現には最も重要と言われている．AR-1鎖は，高親和性のレセプターを形成するために必要なユニットであると言われている[1)17)18)]．RT-PCR法を用いてmRNAレベルのAR-1鎖とAR-2c鎖の発現を検討したところ，全ての細胞株でこれらの発現が確認された．細胞表面のAR-2鎖の発現を蛋白レベルで検討すると13株中12株で種々の程度に発現が確認された[10)]．更に，我々は69例の手術切除肝癌及びその非癌部組織におけるAR-2鎖の発現に関しても免疫組織化学的に検討を行った．その結果，癌部では69例中53例（77％）に，

H. Yano and M. Kojiro. Basic studies on the suppressive mechanisms of interferon on hepatocellular carcinogenesis.

非癌部では61例（88％）に発現を認めた．肝細胞癌の分化度・被膜侵襲・肝内転移などの病理学的なパラメーターとAR-2鎖の発現との間に関連性は認めず，非癌部に関しても慢性肝炎と肝硬変で発現の差を認めなかった[15]．このように，肝癌細胞では比較的高頻度にIFNのレセプターを発現していることが明らかとなった．

ヒト天然型IFN-α の肝癌細胞株に対する *in vitro* の増殖抑制作用

リンパ芽球由来の天然型IFN-α（1-1024 U/mL，OIF® を13種類の肝癌細胞株に対し24～96時間接触させると，大部分の細胞株で時間依存性の細胞増殖抑制作用が認められた[10]．また，IFN-α 接触後96時間目では，すべての細胞株で種々の程度に濃度依存性に細胞増殖が抑制された（図1）．IFN-α の増殖抑制作用に対する感受性と細胞株のオリジナル腫瘍の組織学的異型度との間に関連性は認めなかった．また，細胞表面のAR-2鎖の発現とIFN-α による増殖抑制作用との関連性に関しては，AR-2鎖の発現が極端に低い細胞は，増殖抑制作用が乏しかったが，ある程度以上の発現がある細胞では，発現と増殖抑制作用とは必ずしも相関していなかった[10]．

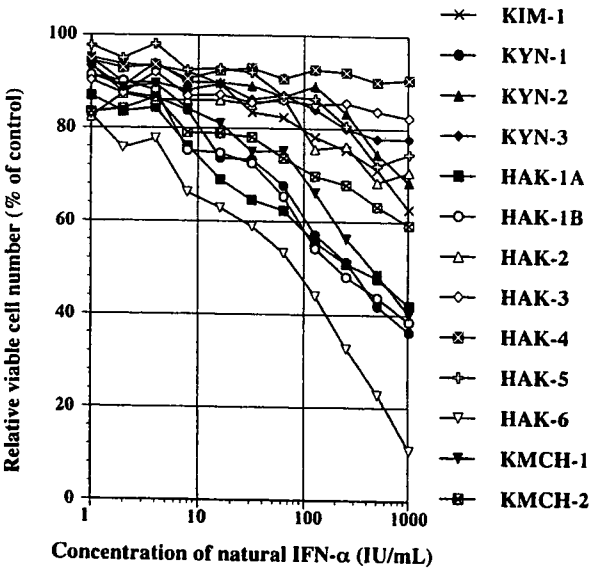

図1　ヒト天然型IFN-α 製剤（OIF®）の13種類の肝癌細胞株に対する増殖抑制作用．13種類の肝癌細胞株を1～1024 U/mL のIFN-α 添加培地で培養し，96時間目にIFN-α 非添加培養（コントロール）と比較した生細胞数の割合（％）を示す．

IFN-α サブタイプの肝癌細胞株に対する *in vitro* の増殖抑制作用

IFN-α には，少なくとも13種類の活性強度の異なるサブタイプ遺伝子（α1, 2, 4, 5, 6, 7, 8, 10, 13, 14, 16, 17, 21）がある．各サブタイプ分子が構造的に極めて類似しており，共通のレセプター分子を介して細胞内にシグナルを送るにもかかわらず，抗ウイルス作用，細胞増殖抑制作用などの生物学的作用や標的細胞特異性にはサブタイプ間で差があると報告されている[19]．天然型IFN-α は，複数のサブタイプから構成されるのが特徴であり，上記実験に使用した天然型IFN-α の成分は，約75％がα2で，残り25％がα8で構成されている．肝癌細胞においても，IFN-α サブタイプの種類によって増殖抑制作用が異なるかどうかを明らかにするため，5種類のリコンビナントIFN-α1, 2, 5, 8, 10を使用し，その増殖抑制作用を検討した．その結果，各IFN-α サブタイプの増殖抑制作用に対して13種類の細胞株は，それぞれ異なる感受性を示したが，特にα5とα8に高感受性を示す細胞株が多いことが判明した[14]．サブタイプ別の増殖抑制効果を13株の平均値で比較すると，α5が終始効果が最も強く，次いでα8，α10，α2，α1の順であった．α5の増殖抑制効果は，他のサブタイプより早期に出現し接触後24時間目から認められた．

IFN-α 製剤及びIFN-β 製剤間の肝癌細胞株に対する *in vitro* の増殖抑制作用と増殖抑制機序の比較

現在日本において臨床的に使用されているⅠ型IFN製剤には，天然型には，上記実験で使用したリンパ芽球由来の天然型IFN-α（OIF®）や線維芽細胞由来の天然型IFN-β（FERRON®）がある．遺伝子組換え型のIFNとしては，IFN-α2b（Intron® A）や，特殊なものとして，IFN-α の13種類のサブタイプ遺伝子のそれぞれのアミノ酸配列について各位置で最も出現頻度が高いアミノ酸を選択することによりアミノ酸配列を決定し，人工的に作成したコンセンサスIFN（rIFN-α Con1, Advaferon®）がある．最近開発された

IFNとしては，遺伝子組換え型IFN-αにメトキシポリエチレングリコール（PEG）を結合させることにより生物学的半減期を延長させ，少ない投与回数で高い効果が期待可能なPEG-IFN-α2b（PegIntron®）やPEG-IFN-α2a（PEGASYS®）がある．これらのIFNの中からPEG-IFN-α2aを除く，4種類のIFN-α製剤と，1種類のIFN-β製剤を用いて13種類に肝癌細胞株に対する増殖抑制作用について比較検討を行った．1024 IU/mLの各種IFN添加培地で96時間培養後に，IFN非添加培養（コントロール）と比べ生細胞数の割合が50％以下まで低下した細胞株の数は，ヒト天然型IFN-αでは，5株[10]，コンセンサスIFNでは，7株[16]，IFN-α2bとPEG-IFN-α2bでは，いずれも2株[12]，天然型IFN-βでは，10株であった[20]．これらの細胞株の50％増殖抑制濃度（IC50）は，ヒト天然型IFN-αでは，86～466 IU/mL，コンセンサスIFNでは，128～804 IU/mL（0.128～0.804 ng/mL），IFN-α2bでは，628～919 IU/mL，PEG-IFN-α2bでは，832～839 IU/mL，天然型IFN-βでは，15～153 IU/mLであり，IFN-α2bとPEG-IFN-α2bのIC50値は，他より高く，逆にIFN-βのIC50値は最も低かった．IFNの製剤別の増殖抑制作用を13株の平均値で比較すると，天然型IFN-β，コンセンサスIFN，天然型IFN-α，PEG-IFN-α2b・IFN-α2bの順に強い作用を認めた（図2）．特に，天然型IFN-βでは，経時的に増殖抑制作用が増大し，接触96時間後では，低濃度でも比較的強い増殖抑制効果が見られた．PEG-IFN-α2bやIFN-α2bの増殖抑制作用が比較的低いと言う結果は，上記の5種類のIFN-αのサブタイプの中でIFN-α2の増殖抑制作用が比較的低いという結果とよく一致していた．

各種IFNを肝癌細胞の培地に添加し，48から72時間培養し細胞形態を観察すると細胞質の縮小や核の濃縮・核の断片化など，アポトーシスに特徴的な細胞像の出現が認められた（図3A）．アポトーシス誘導は，使用したIFNの種類，濃度，そして細胞株により差をみとめるものの，最低でも13株中10株で認められた[10)12)16)20)]．IFN-α誘導性アポトーシスでは，caspase-9，

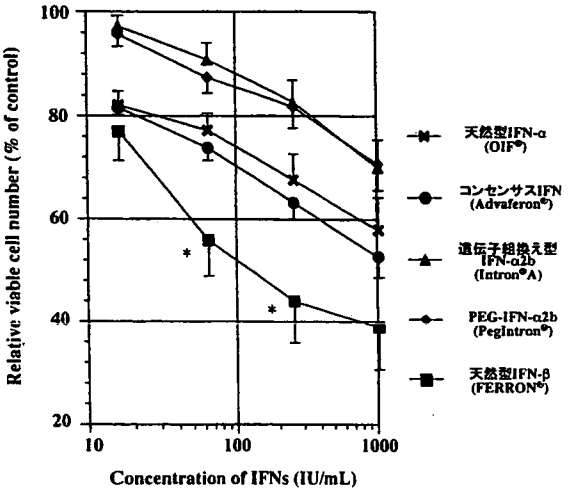

図2　4種類のIFN-α製剤（ヒト天然型IFN-α，コンセンサスIFN，IFN-α2b，PEG-IFN-α2b）と1種類の天然型IFN-β製剤の肝癌細胞株に対する増殖抑制作用．13種類の肝癌細胞株を4～1024 IU/mLの各種IFN製剤添加培地で96時間培養し，各種IFN製剤に関してIFN-α非添加培養（コントロール）と比較した生細胞数の割合（％）を算出した．更に，IFN-α製剤毎に13株の生細胞数の割合の平均値を算出しプロットしたものを図に示す．IFN-βの平均値がいずれの測定時間でもほかのIFNに比べ有意に（*$P<0.05$）低い値を示し，最も強い増殖抑制作用を示していた．値は，平均値±標準誤差を示す．

caspase-8，caspase-7，caspase-3の活性化と伴にcytochrome cやSmac/DIABLOのミトコンドリアから細胞質への放出がみられ，ミトコンドリア系のアポトーシス誘導経路の関与が示唆されるが[13]，TRAILやTRAIL-R1，-R2などの発現亢進も見られており（未発表データ），デスリガンド-デスレセプターを介した経路の関与も考えられ，今後更なる検討が必要である．アポトーシス誘導以外の増殖抑制の機序としてすべての細胞株で細胞周期の進行停止誘導が認められ，S期での停止誘導が11株，G_2/M期での停止誘導が1株，G_1期での停止誘導が1株で認められた[10)12)]（図3B）．

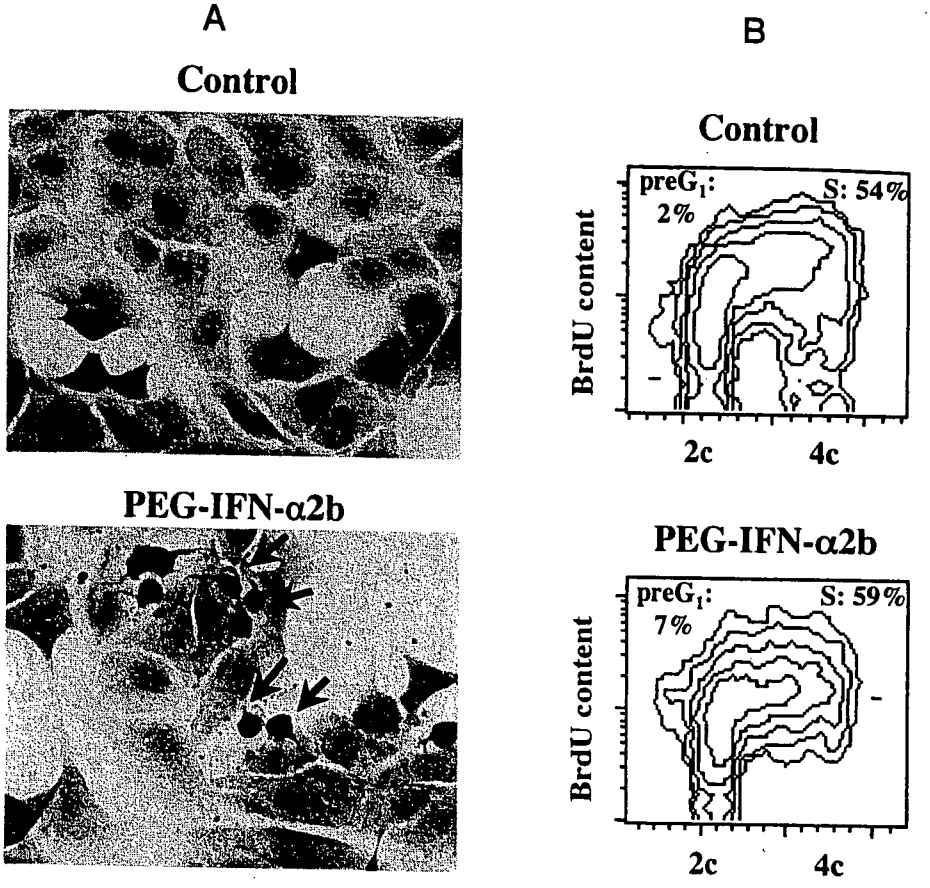

図3 （A）ヒト天然型 PEG－IFN-α2b 添加あるいは非添加培地で 72 時間培養後の肝癌細胞株 HAK-B の細胞像（上：PEG-IFN-α2b 非添加培養，200倍；下：1,000 IU/mL PEG-IFN-α2b 添加培養，200 倍，ヘマトキシリン・エオジン染色）．（B）PEG-IFN-α2b 添加あるいは非添加培地で 72 時間培養後の肝癌細胞株 HAK-1B の細胞周期の解析結果（上：PEG-IFN-α2b 非添加培養；下：1,000 IU/mL PEG-IFN-α2b 添加培養）．アポトーシス集団を示す $preG_1$ 期の細胞集団の増加と S 期の細胞集団の増加を PEG -IFN-α2b 添加培養細胞で認める．

IFN-α 製剤及び IFN-β 製剤の肝癌細胞株に対する *in vivo* の増殖抑制作用と増殖抑制機序

肝細胞癌細胞株 HAK-1B[21] をヌードマウスの皮下に接種し，約 1 週間後 5 - 10 mm の腫瘍径の腫瘍が形成された時点から，各種 IFN 製剤を投与し *in vivo* における増殖抑制作用の検討を行った．天然型 IFN-α は，C 型慢性肝炎患者の治療に使用される投与量にほぼ相当する量（臨床量）(4,000 IU/mouse，2.0×10^5 IU/kg)，その 10 倍量あるいは 100 倍量を 14 日間連日マウスの皮下に接種し，腫瘍の経時的な推定体積や，15 日目に摘出された腫瘍の組織像を比較検討した．コンセンサス IFN-α は，臨床量の約 1.4 倍量 (0.01 μg/mouse，0.5 μg/kg)，その10倍量，100 倍量を同様に投与し，天然型 IFN-β は，臨床量の約半量（1,000 IU/mouse，5.0×10^4 IU/kg)，その 10 倍量，100 倍量を腹腔内に投与し同様に検討した．その結果，14 日目の腫瘍体積は，最小量の IFN 投与により IFN を投与しなかったマウス（コントロール）に比べ，天然型 IFN で 30 %前後（未発表データ)，コンセンサス IFN で 40 %前後[16]，天然型 IFN-β で 15 %前後[20]減少した．このように，臨床量前後の IFN 製剤の投与は，*in vivo* において肝癌細胞の増殖を抑制した．臨

図4　ヌードマウス皮下にヒト肝癌腫瘍を作成し，1μg（1.0X10^6 IU/mouse）のコンセンサスIFN（左），あるいはリン酸緩衝液（コントロール）（右）を毎日1回，14日間連日マウスに皮下接種し15日目の皮下腫瘍の状態を示す．左のコンセンサスIFN投与マウスの皮下腫瘍は消失した．右のコントロールマウスには皮下腫瘍（矢印）を認める．

床量の140倍のコンセンサスIFNを投与されたマウスでは，腫瘍がほぼ消失した[16]（図4）．IFNを投与されたマウスの腫瘍組織では，IFNの濃度依存性に肝癌細胞のアポトーシス数の増加を認めた，コンセンサスIFNを投与されたマウスの腫瘍では，腫瘍内血管の減少も認められた．

PEG-IFN-α2bは，前述のごとくPEG化により吸収・排泄速度が低下し，通常のIFNに比べ生物学的半減期が数倍延長する結果，長時間IFN-α2bの血液濃度が維持されるという特徴を有する．臨床量の1/3量（640 IU/mouse，3.2×10^4 IU/kg），その10倍量，100倍量，1,000倍量を1週間に2回，合計4回皮下に投与し腫瘍の経時的な推定体積や，15日目に摘出された腫瘍の重量や組織像を比較した．その結果，臨床量の1/3量の投与でコントロールに比べ約50％前後の腫瘍の体積及び重量の減少が認められた[12]（図5）．増殖抑制機序としては，アポトーシスの誘導を認めたが，血管新生抑制は確認できなかった（図6）．PEG-IFN-α2bと同じ活性（IU）のIFN-α2bを同様の方法で投与し，抗腫瘍作用をPEG-IFN-α2bを投与した場合と比較すると，PEG-IFN-α2bを投与した方が，腫瘍のアポトーシス誘導は高度であり，有意により強い抗腫瘍作用を認めた[12]（図5，6）．*in vitro*では，PEG-IFN-α2bはIFN-α2bと同程度に増殖抑制効果が最も低いIFN-α製剤であったが，PEG化により長時間血中IFN-α2bの濃度が維持された事により，肝癌細胞に持続的に作用し，非PEG化IFN-α2bや他のIFN-α製剤より強い増殖抑制作用を発揮したと推察される．

PEG-IFN-α2bの肝癌細胞のα-fetoprotein（AFP）産生に及ぼす作用

臨床的に明らかな肝細胞癌の合併を認めないにもかかわらず，血清のAFPの持続高値を呈するC型慢性肝炎患者は，肝細胞癌発症のハイリスク群に位置すると報告されている[22]．最近，これらの患者に対して，肝庇護剤を投与した場合，肝機能の改善はみられても，血清AFP値の低下は見られないのに対し，IFNを投与すると肝機能の改善と血清AFP値も有意に低下すると報告されている[23]．このIFNによるAFP低下の機序は解明されていないが，以下の二つの可能性が考えられる．一つ目は，不顕性なAFP産生性の肝癌細胞が存在し，IFNが直接的にそれらの細胞に対し抗腫瘍作用を示し，細胞が減少あるいは消失した結果AFPも低下すると言う可能性．二つ目は，一つ目同様不顕性なAFP産生性の肝癌細胞が存在するが，IFNが細胞に作用してAFPの産生を機能的に抑制する可能性である．ホルモンや増殖因子によるAFPの発現制御に関しては，Dexamethasoneが，promoter領域のglucocorticoid responsive elementを介してAFP発現を増加させる事や，逆に，上皮成長因子，transforming growth factor-β1，肝細胞増殖因子などがAFP発現を低下させる事が報告されている[24][25][26][27]．13種類の肝癌細胞の内AFP産生性の肝癌細胞株KIM-1を使用して，PEG-IFN-α2bのAFP発現に対する作用を蛋白及びmRNAレベルで検討した結果，PEG-IFN-α2bとの接触8日目まではAFPの蛋白及びmRNAの発現を種々の程度に上昇させる事が判明した[11]．従って，IFN投与によるAFP減少の機序としては，上記の一つ目の可能性がより考えられる．

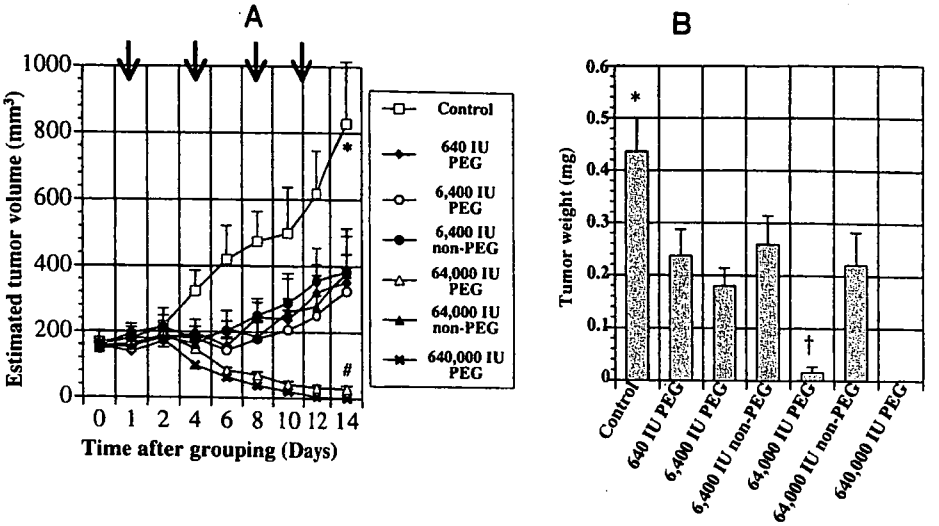

図5　PEG-IFN-α2b と IFN-α2b の *in vivo* における抗腫瘍効果の比較．ヌードマウス皮下にヒト肝癌腫瘍を作成し，640，6,400，64,000，640,000 IU の PEG-IFN-α2b，6,400 IU，64,000 IU の IFN-α2b あるいは培養液（コントロール）を1週間に2回，合計4回ヌードマウスの皮下に接種し（矢印），腫瘍体積の経時的変化（A）及び15日目に切除された腫瘍重量（B）を示す．IFN を投与されたマウスの腫瘍の体積・重量は，コントロールに比べ有意（$^*P<0.05 \sim 0.001$）に低値であった．同じ活性の PEG-IFN-α2b と IFN-α2b 投与では，PEG-IFN-α2b 投与の方が腫瘍の体積及び重量ともに低い値を呈した（$\dagger P<0.01$，vs 64,000 IU non-PEG）．640 IU PEG-IFN-α2b のヌードマウスへの投与は，C型慢性肝炎の治療にヒトに使用する量の1/3の量に相当 PEG は，PEG-IFN-α2b，non-PEG は，IFN-α2b の略．値は，平均値±標準誤差を示す．

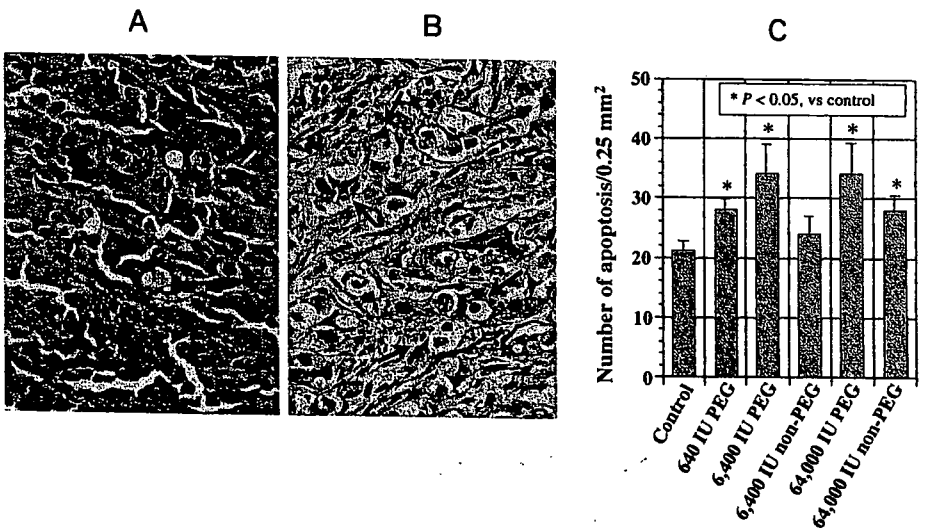

図6　PEG-IFN-α2b あるいは IFN-α2b のヌードマウス皮下移植ヒト肝癌腫瘍組織に対する作用．ヌードマウス皮下にヒト肝癌腫瘍を作成し，640，6,400，64,000，640,000 IU の PEG-IFN-α2b，6,400 IU，64,000 IU の IFN-α2b あるいは培養液（コントロール）を1週間に2回，合計4回ヌードマウスの皮下に接種し，15日目に切除した腫瘍から HE 染色標本を作製し，腫瘍細胞のアポトーシスを測定した．A，B に，腫瘍の組織像（200倍，ヘマトキシリン・エオジン染色）を示す．コントロール（A）に比べ，6,400 IU PEG-IFN-α2b 投与マウス（B）の腫瘍組織にアポトーシスの出現が目立つ．C には，各種活性の PEG-IFN-α2b あるいは IFN-α2b を投与されたマウス及びコントロールマウスの皮下腫瘍の癌細胞のアポトーシス数の測定結果を示す．PEG-IFN-α2b あるいは IFN-α2b の投与量が増えるとアポトーシス数は増加し，同じ活性では，IFN-α2b に比べ PEG-IFN-α2b の方がアポトーシス数は増加している．640,000 IU PEG-IFN-α2b 投与マウスは，腫瘍が消失したため測定値なし．値は，平均値±標準誤差を示す．

おわりに

これまでの我々の検討により，ヒトの肝癌細胞がI型IFNの作用発現に重要なI型IFNのレセプターを発現していること，IFNは，*in vitro*では，肝癌細胞株に対しアポトーシス・細胞周期の進行停止などを誘導し直接的に増殖を抑制するが，その増殖抑制作用はIFN製剤により異なること，*in vivo*では，臨床量のIFN投与でも，アポトーシス誘導などにより抗腫瘍作用がもたらされる事が明らかとなった．また，血清AFP持続高値のC型慢性肝炎患者がIFN投与後に血清AFP値が低下する機序が，不顕性な初期段階の肝癌細胞に対するIFNの直接的排除である可能性も示唆された．以上より，IFN投与による肝発癌・再発抑制機序の一つとしてIFNによる直接的抗腫瘍作用が関与している可能性が十分考えられる．

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