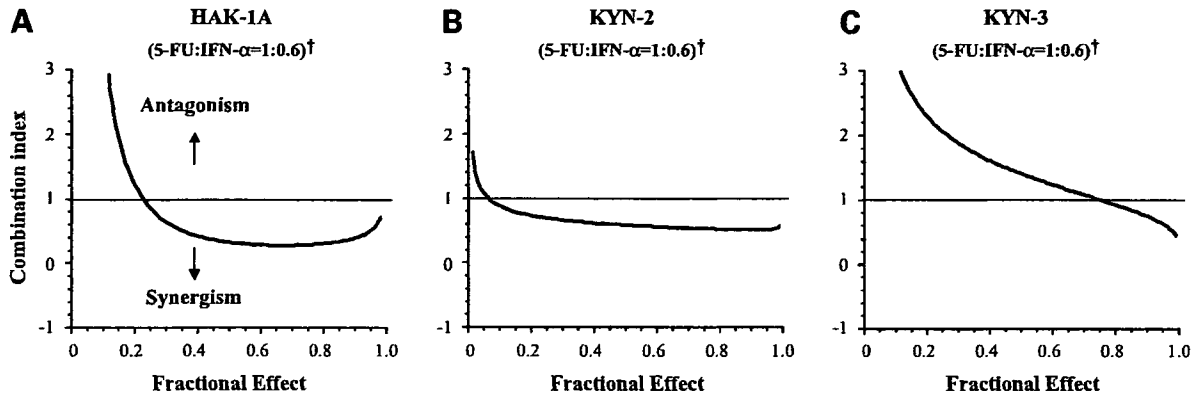

Figure 2. Quantitative analysis of synergy between 5-FU and IFN-α against HAK-1A (**A**), KYN-2 (**B**), and KYN-3 (**C**) cells using the CI method. CI > 1, antagonism; CI = 1, additive; CI < 1, synergistic. †, ratio of RC = (concentration of 5-FU) / (IC_{50} of 5-FU):(concentration of IFN-α) / (IC_{50} of IFN-α).

We next examined whether cotreatment of CDHP could modulate the antiproliferative effect of 5-FU alone and in combination with IFN-α in three HCC cell lines. As seen in Fig. 6A to C, the dose-response curves of 5-FU alone in HAK-1A, KYN-2, and KYN-3 cells and those of 5-FU combined with IFN-α in HAK-1A and KYN-2 cells were not shifted by cotreatment with CDHP. None of these differences were significant at all data points. By contrast, there seemed a marked and significant shift of the 5-FU and IFN-α combined dose-response curve in KYN-3 cells to CDHP: the 5-FU IC_{50} value of 4.6 μmol/L was reduced to 1.1 μmol/L by cotreatment with CDHP in the presence of IFN-α (Fig. 6C).

Discussion

Consistent with the findings of our recent study (27), the combination of 5-FU and IFN-α showed a synergistic antiproliferation effect on two HCC cell lines, HAK-1A and KYN-2, and an additive or antagonistic antiproliferation effect on KYN-3 cells (Figs. 1 and 2). Expression of type 1 IFN receptor was specifically up-regulated by exposure to 5-FU in both HAK-1A and KYN-2, but not KYN-3 cells, suggesting that the modulation of IFN receptor expression by 5-FU could play a pivotal role in therapeutic efficacy (27). In this study, we further examined the molecular events underlying the antiproliferative effects of 5-FU and IFN-α. One of the major mechanisms of antiproliferative activity of 5-FU is the inhibition of TS activity with formation of the ternary complex of FdUMP, TS, and 5,10-methylenetetrahydrofolate. However, we observed a marked IFN-α–induced decrease in TS expression at similar levels in all three cell lines, suggesting that modulation of TS expression itself might not be directly involved in the absence or presence of synergism by the combination of 5-FU and IFN-α.

DPD is a key enzyme involved in 5-FU inactivation, which modulates FdUMP levels and controls formation of

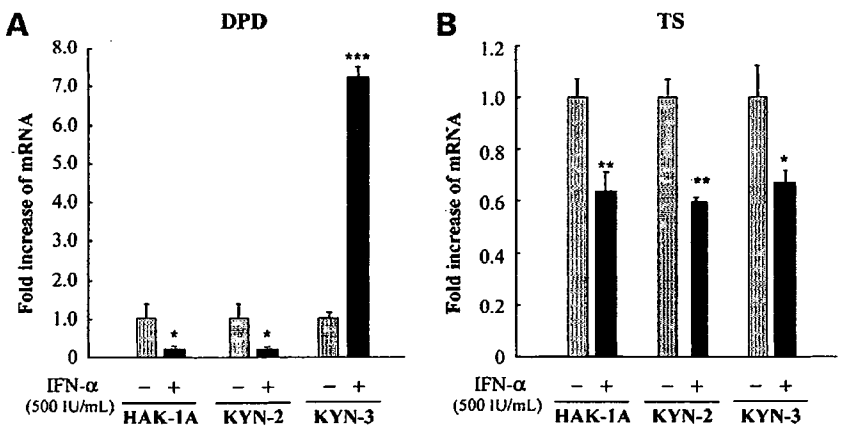

Figure 3. Effects of 500 IU/mL IFN-α on DPD (**A**) and TS (**B**) mRNA and protein expression levels in HAK-1A, KYN-2, and KYN-3 cells. Expression levels were measured by quantitative real-time reverse transcription-PCR and normalized to glyceraldehyde-3-phosphate dehydrogenase. The fold increases are shown relative to the initial level, taken as 1.0. Determinations were carried out in triplicate. *Dotted and black columns,* mean value of relative mRNA levels in HCC cells untreated and treated with IFN-α, respectively; *bars,* SD. *, $P < 0.05$; **, $P < 0.01$; ***, $P < 0.001$, differences are statistically significant by Welch's test, compared with untreated groups.

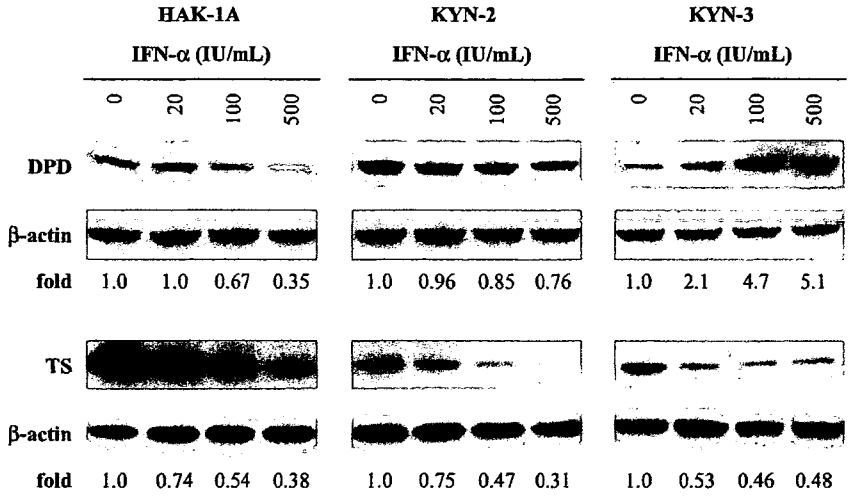

Figure 4. Protein expression levels of DPD and TS in HAK-1A, KYN-2, and KYN-3 cells treated with IFN-α. Protein expressions in HCC cells treated with IFN-α for 48 h were measured by Western blotting. Protein extracts of 10 μg/lane were loaded for KYN-3 cells, and 50 μg/lane were loaded for both HAK-1A and KYN-2 cells. Experiments were repeated twice with similar results. The intensities of immunoblotted bands were quantified by image analyzing methods. The fold increases relative to the initial level, taken as 1.0, are shown under the bands.

the ternary complex. Several clinical studies have shown that the intratumoral expression level of DPD is closely associated with clinical responses to 5-FU in patients with colorectal cancer (1), gastric cancer (34), and non–small cell lung cancer (35). In our present study, expression of DPD protein and mRNA levels in KYN-3 cells were specifically increased 5-fold or more over the basal level after exposure to IFN-α (Figs. 3 and 4). By contrast, down-regulation of DPD by IFN-α was observed in both HAK-1A and KYN-2.

In these two HCC cell lines, we previously proposed that 5-FU–induced up-regulation of the IFN receptor was the main mechanism underlying the synergistic antiproliferative effect of 5-FU and IFN-α (27). Moreover, down-regulation of DPD by IFN-α in these two cell lines might be involved in the synergistic effect. By contrast, up-regulation of DPD by IFN-α might account for the antagonism between IFN-α and 5-FU in KYN-3 cells.

Shestopal et al. (36) reported that 5′ flanking region of *DPYD* gene lacks the canonical TATA and CCAAT boxes, however, contains several *cis*-acting regulatory elements including binding sites for activator protein-2, nuclear factor-κB, Sp1, and Egr families. About the regulatory mechanism for IFN-α modulation of *DPYD* expression, we

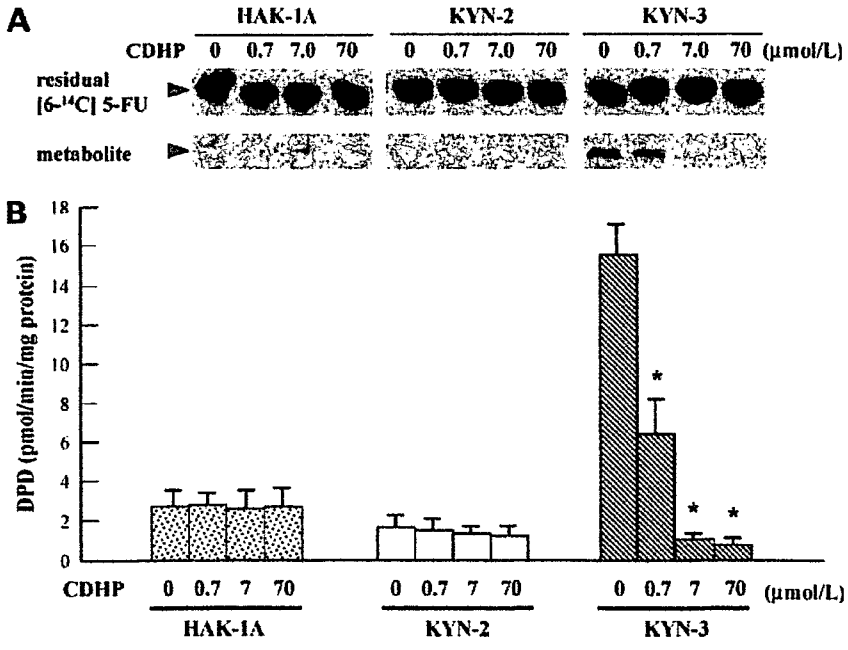

Figure 5. Inhibitory effect of CDHP on DPD activity. DPD activity was measured using [6-^{14}C]5-FU as a substrate. Residual [6-^{14}C]5-FU and metabolites were separated by TLC and visualized with an imaging analyzer (**A**). Each sample was developed on separated and independent TLC plate. Assays were carried out in triplicate. *Columns*, DPD; *bars*, SD (**B**). *, $P < 0.001$, differences are statistically significant by Welch's test, compared with CDHP untreated group.

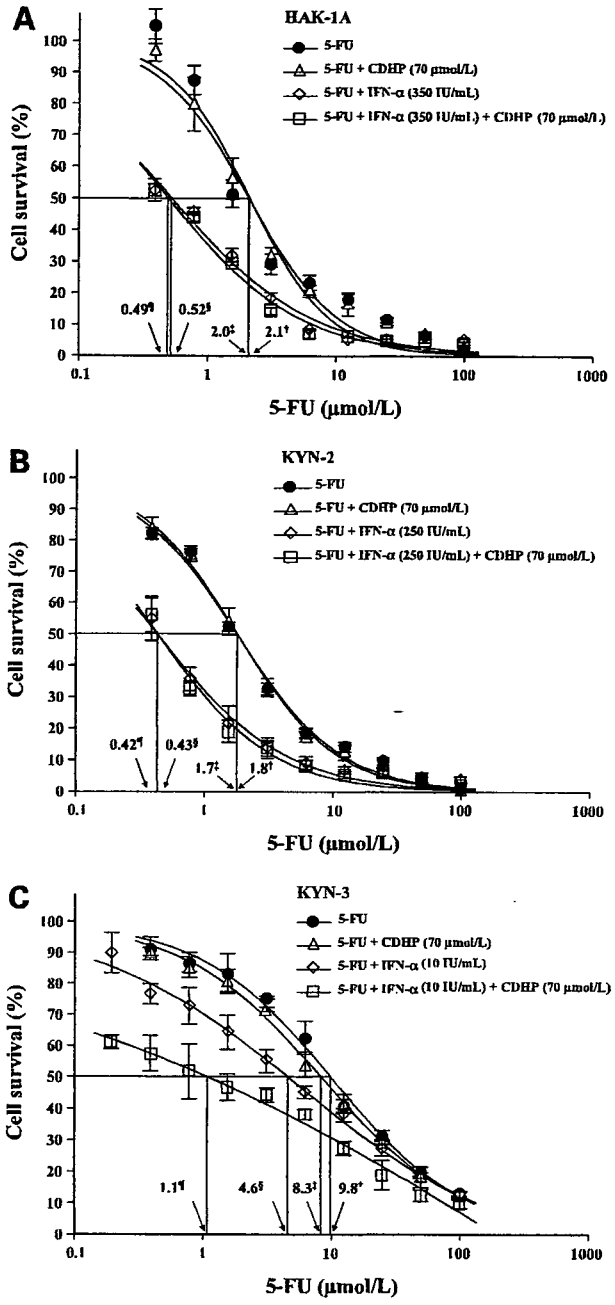

Figure 6. Effect of CDHP on combined antiproliferative effect of IFN-α and 5-FU in HAK-1A (**A**), KYN-2 (**B**), and KYN-3 (**C**) cells. Assays were carried out in quadruplicate. Experiments were repeated twice with similar results. IC_{50} values of 5-FU against HCC cells treated with 5-FU alone (†), 5-FU/CDHP (‡), 5-FU/IFN-α (§), and 5-FU/IFN-α/CDHP (¶).

analyzed the sequence of 1.2 kb of 5′ flanking region of *DPYD* and found that this region contains two putative consensus binding sites for signal transducer and activator of transcription families (data not shown), suggesting that gene expression of *DPYD* is highly susceptible to IFN-α, which strongly activates signal transducer and activator of transcription 1 and signal transducer and activator of transcription 2 through the Janus-activated kinase-signal transducer and activator of transcription pathway. However, it remains unclear why *DPYD* expression is differentially controlled by IFN-α in HCC cell lines. Further elucidation of this differential regulatory mechanism at the molecular basis is required.

Our present study showed that cotreatment with the DPD inhibitor CDHP further synergistically enhanced the antiproliferative effect of 5-FU and IFN-α in KYN-3 cells only and not in HAK-1A and KYN-2 cells (Fig. 6). The antiproliferative effect of 5-FU alone was only slightly altered by CDHP cotreatment in KYN-3 cells, if any, and not at all in the other two cell lines (Fig. 6). This IC_{50} reduction, however, was not statistically significant. Basal DPD activity and expression levels were much higher in KYN-3 cells than in HAK-1A and KYN-2 cells. Moreover, cellular DPD levels were specifically up-regulated >5-fold in KYN-3 cells by IFN-α, but this was not observed in HAK-1A and KYN-2 cells. It was presumed that 15 pmol/min/mg protein of basal DPD activity in KYN-3 cells was up-regulated to 80 to 110 pmol/min/mg protein. Taken together, this suggests that a relatively high DPD activity might be more susceptible to inhibition by CDHP, resulting in an apparent synergistic effect of CDHP on the antiproliferative effect by 5-FU and IFN-α.

Certain levels of DPD in cancer cells could be sensitive to CDHP-induced inhibition. A relevant study by Takechi et al. (37) showed that the antiproliferative activity of 5-FU could be markedly enhanced by cotreatment with 69 μmol/L CDHP in two human tumor cell lines with relatively high DPD activities, approximately 101 and 153 pmol/min/mg protein, respectively, but not those with low enzyme activity, 33 pmol/min/mg protein. However, this plausible mechanism why CDHP did induce synergism of 5-FU and IFN-α against only KYN-3 cells requires further study to validate these findings.

CDHP has been applied as a modulator in the newly developed antimetabolite TS-1 (Taiho Pharmaceutical). TS-1 consists of tegafur, CDHP, and potassium oxonate in a molar ratio of 1:0.4:1 (38). Potassium oxonate is a competitive inhibitor for orotate phosphoribosyltransferase that activates 5-FU. Potassium oxonate is mainly distributed in the gastrointestinal tract after p.o. administration and prevents gastrointestinal toxicity induced by 5-FU without reducing 5-FU activity in tumor (38, 39). In Japan, TS-1 has been used to treat patients with gastric, head and neck, and pancreatic cancers and shows potent therapeutic efficacy against gastric tumors, with a response rate of 46.5% (40–42). Nakamura et al. (43) applied a new combination regimen of TS-1 and IFN-α to advanced HCC patients with portal vein thrombus and multiple pulmonary metastases and observed some improvement in therapeutic efficacy. The combination of TS-1 and IFN-α could therefore be effective against patients with advanced HCC. However, the side effects of these combination therapies should be seriously considered before their implementation. Further studies

are required to determine how DPD could be differentially controlled between normal cells including normal hepatic cells and malignant hepatic cells in patients with HCC when DPD inhibitory drugs are introduced.

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Immunohistochemical expressions of Cap43 and Mina53 proteins in neuroblastoma

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Index words:
Neuroblastoma;
Cap43;
Mina53;
MYCN;
TrkA;
Immunohistochemistry

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,

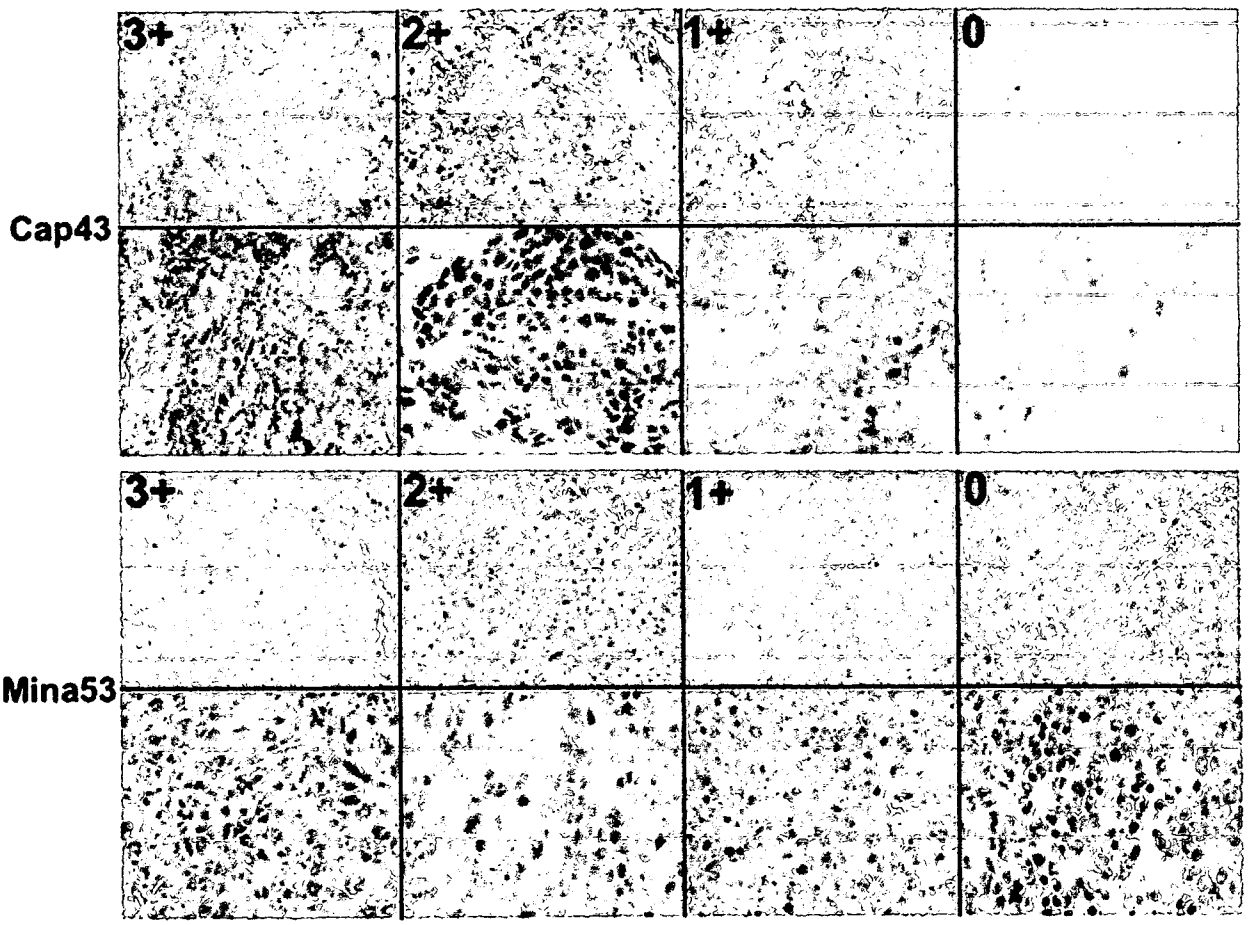

Fig. 1 A panel figure showing representative examples of each staining intensity for Cap43 and Mina53. The staining intensity for Cap43, Mina53, MYCN, and TrkA in each specimen was scored on a scale from 0 to 3 (0 = negative, 1 = weakly positive, 2 = moderately positive, 3 = strongly positive). The maximum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

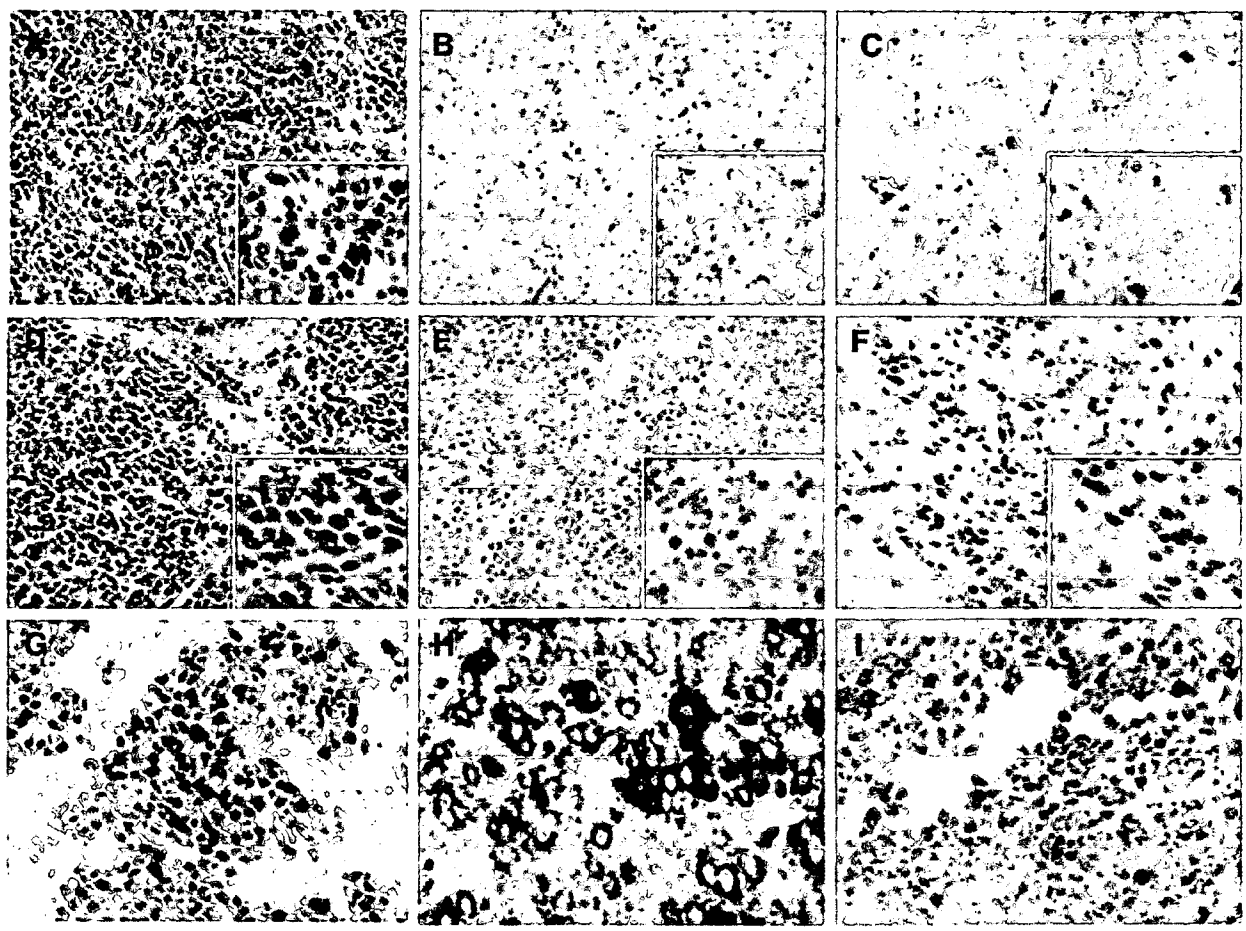

Fig. 2 Immunohistochemical staining of Cap43, Mina53, TrkA, Ki-67, and N-*myc* in neuroblastoma. A, Neuroblastoma (case 39) stained with H&E (×200). B, Cap43 expression in cytoplasm (×200). Inset: high-power view (×400). C, TrkA expression in cytoplasm (×200). Inset: high-power view (×400). D, Neuroblastoma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

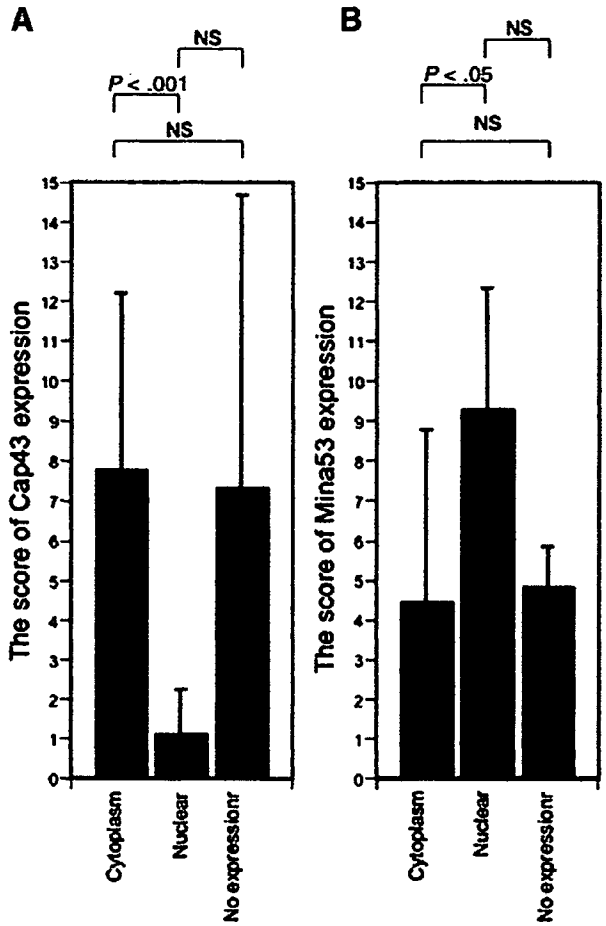

Fig. 3 The relationship between N-*myc* protein expression and the expression of Cap43 or Mina53. The expression scores of Cap43 and Mina53 are compared according to the 3 different groups of N-*myc* protein expression, that is, nucleus group, cytoplasm group, and no expression group. The relationship 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

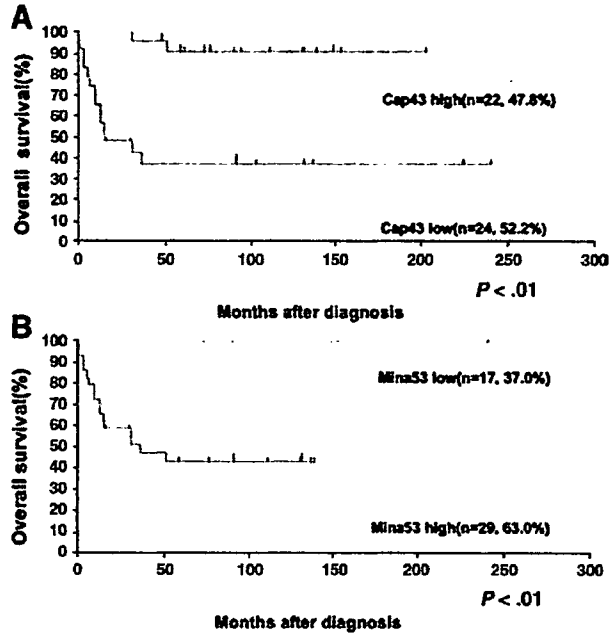

Fig. 4 Correlation between the overall survival rate and the expression of Cap43 or Mina53 analyzed by the Kaplan-Meier method. Forty-six neuroblastoma tumors were divided into high-expression group or low-expression group according to the expression scores of Cap43 and Mina53. The survival rate was 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

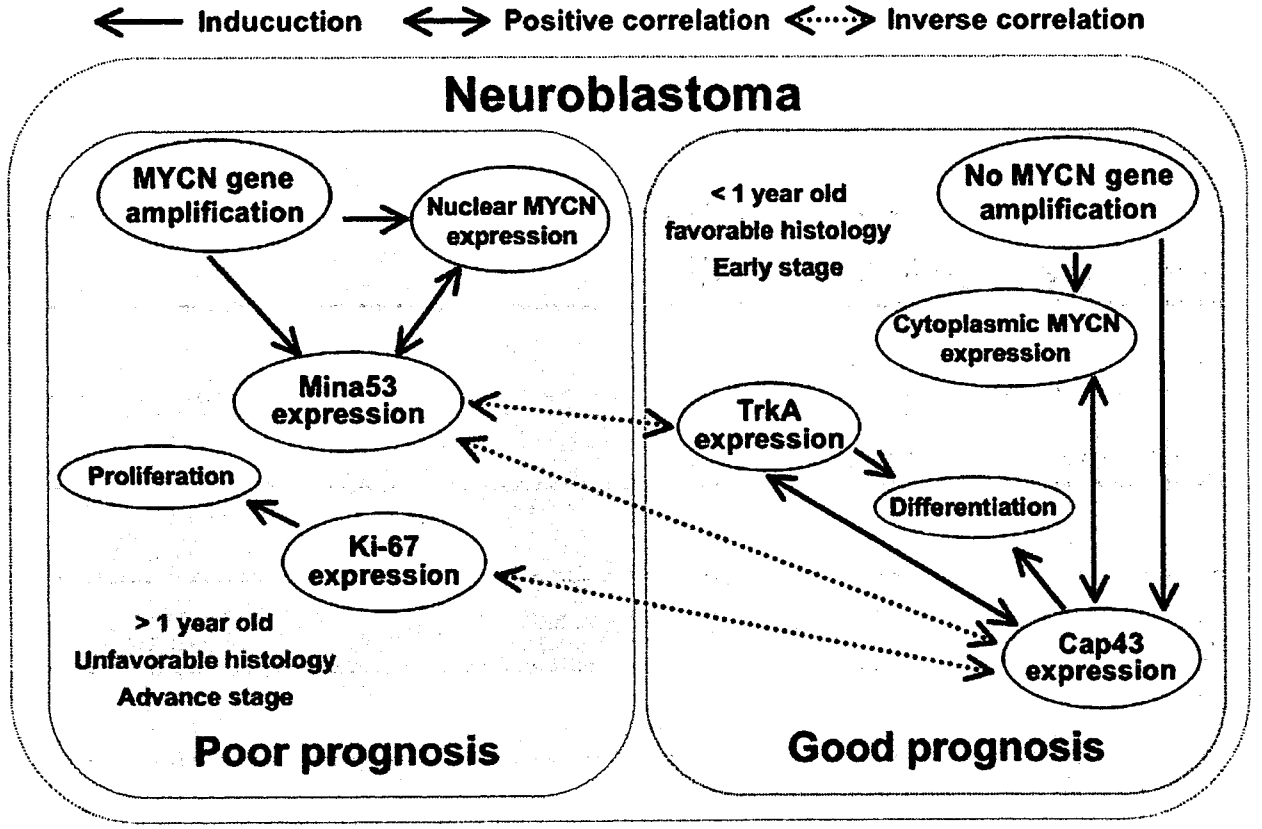

Fig. 5 A schematic drawing showing the possible association of Cap43, Mina53, TrkA, Ki-67, MYCN gene and protein, cell proliferation and differentiation, and prognosis in neuroblastoma. There is an inverse correlation between Ca43 and Mina53, between TrkA and Mina53, and between Cap43 and Ki-67, and 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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Expression profile of class I histone deacetylases in human cancer tissues

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Abstract. Histone deacetylase (HDAC) activity is one of the widely used and well-established mechanisms for regulation of various genes in cancer. To identify which subtype of class I HDACs are overexpressed in cancers, we analyzed the expression of class I HDAC isotypes composed of HDAC1, 2, 3 and 8 in several cell lines and human cancer tissues, including cancer of the stomach, esophagus, colon, prostate, breast, ovary, lung, pancreas and thyroid. The results showed that >75% of human cancer tissues and their corresponding non-cancerous epithelium showed high expression of these class I HDACs. However, the immunoreactivity of HDAC8 in both prostatic cancer tissue and non-cancerous prostate glands was lower than that in other cancer tissues. Furthermore, 5-40% of cancer tissues overexpressed class I HDACs, when compared with normal epithelium. The results suggest the potential usefulness of HDAC inhibitors for the treatment of a wide variety of human cancers.

Introduction

Histone acetylation-deacetylation is considered to be the best understood of the post-translational modifications of the core histones, and occurs by the opposing action of histone acetylases (HATs) and histone deacetylases (HDACs) (1-7). To date, 18 HDAC enzymes have been identified in humans and divided into three subclasses based on amino acid sequence homology in their catalytic domains (8). Class I enzymes, which include HDACs 1, 2, 3 and 8, are related to yeast reduced potassium dependency 3 deacetylase and share homology in their catalytic sites (8,9). Class I HDACs play an important role in the regulation of cell proliferation (10) and are expressed almost exclusively in the nucleus of most cell types (8,11).

Overexpression of class I HDACs have been reported in several cancer tissues, such as stomach (12-14), esophagus (15), colorectal (16,17), prostate (18,19), breast (21) and lung (22,23). However, systematic analysis of expression profiles of class I HDACs in various human cancer tissues has not yet been documented.

Currently, several HDAC inhibitors are in clinical trials, and are showing promising anticancer effects in phase I and II trials. To identify the HDAC inhibitors with specificity for cancer types and for specific HDAC classes or isoforms is of interest, and it is important to consider that HDACs have varying expression patterns in different cell types, as well as diverse functions (24).

To investigate expression of class I HDACs in cancer tissues, we first confirmed the expression profiles of class I HDACs in 11 major cancer cell lines. Next, we evaluated immunohistochemical nuclear staining of class I HDACs in nine types of carcinoma, including stomach, esophagus, colon, prostate, breast, ovary, lung, pancreas and thyroid.

Materials and methods

Cell lines and Western blot analysis. The following human cancer cell lines were purchased from the American Type Culture Collection (ATCC) (Manassas, VA, USA): lung (A549), breast (MCF-7 and MDA-MB-231), ovary (SKOV3), prostate (LNCap, PC-3 and DU145), pancreas (MiaPaCa-2 and PANC-1) and colon (SW480 and SW620). All cell lines were cultured in medium recommended by ATCC, supplemented with 10% fetal bovine serum under standard cell culture condition at 37°C and 5% CO_2 in a humid environment. Cells were rinsed with ice-cold PBS and lysed in buffer containing 50 mmol/l Tris/HCl, 350 mmol/l NaCl, 0.1% NP40, 5 mmol/l EDTA, 50 mmol/l NaF, 1 mmol/l phenylmethyl-

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sulfonyl fluoride, 10 μg/ml aprotinin, 10 μg/ml leupeptin, and 1 mmol/l Na_3VO_4. Cell lysates were subjected to SDS-PAGE and blotted onto Immobilon membranes (Millipore, Bedford, MA, USA), as described previously (25). After transfer, blots were incubated with the blocking solution and probed with primary polyclonal antibodies, including anti-HDAC1 antibody (1:25 dilution; Cell Signaling Technology, Danvers, MA, USA), anti-HADC2 antibody (1:1250 dilution; Affinity BioReagents, Golden, CO, USA), anti-HDAC3 antibody (1:500 dilution; Sigma, St. Louis, MO, USA), anti-HDAC8 antibody (1:100 dilution; Santa Cruz Biotechnology, Santa Cruz, CA, USA) and anti-ß-actin antibody (1:5000 dilution; Abcam, Cambridge, MA, USA). The protein content was visualized using horseradish peroxidase-conjugated secondary antibodies followed by enhanced chemiluminescence (Amersham, Piscataway, NJ, USA).

Clinical specimens and immunohistochemistry. Surgical specimens from advanced cancer of the stomach, esophagus, colon, prostate, breast, ovaries, lung, pancreas and thyroid were retrieved from the files of the Department of Anatomic Pathology, Kyushu University, Japan, for the period 1995-2005. For each of the nine types of carcinoma, 20 cases were selected from representative formalin-fixed paraffin-embedded materials. Consequently, 180 paraffin-embedded blocks were prepared for immunohistochemistry. Histological characteristics of each type of carcinoma are summarized in Table I. The specimens were immersed in 10% neutral-buffered formalin immediately after surgery, and embedded in paraffin blocks. Blocks containing both carcinoma and the corresponding normal epithelium were chosen for stomach, esophagus, colon and prostate cancer. After deparaffinization of 4-μm-thick sections, inhibition of endogenous peroxidase activity was achieved with methanol containing 3% H_2O_2. Tissue sections were boiled in 0.1 M sodium citrate buffer (pH 6.0) in a pressure cooker inside a microwave oven for 40 min, to retrieve the antigen. The slides were incubated at 4˚C overnight, with the same primary polyclonal antibodies as used in Western blotting. The working dilutions were as follows: HDAC1 (1:25), HDAC2 (1:1250), HDAC3 (1:500) and HDAC8 (1:100). The above procedures were followed by staining with a streptavidin/biotin/peroxidase kit (Nichirei, Tokyo, Japan). The sections were then reacted with 3,3-diaminobenzidine as a chromogen, and counterstained with hematoxylin. The localization of HDAC1 and HDAC2 is exclusively nuclear (26). Although HDAC3 (27) and HDAC8 (28) are mainly localized in the nucleus, but can also localize in the cytoplasm, we evaluated only nuclear immunostaining.

Immunohistochemical evaluation of class I HDACs was subtle, because some degree of heterogeneity of class I HDAC expression was detected, even within some morphologically similar areas of the tumor, as previously described in gastric cancer tissues (13). Furthermore, there were no significant differences in nuclear staining intensity between cancer tissues and the corresponding normal epithelium in any of the nine types of tumor. We evaluated the extent of nuclear staining on a four-tiered scale (1+, 0-25%; 2+, 26-50%; 3+, 51-75%; 4+, 76-100%). The staining score was evaluated in 10 fields by low-power magnification, and only one stained spot was examined at high power (x400). Each case was scored independently by three pathologists (M.N., O.Y. and S.A.). Samples with a score of 3+ or 4+ were defined as 'HDAC-positive'. For negative controls, the slides were treated with non-immune serum rather than primary antibody.

Table I. Histopathological characteristics of several carcinomas.

Stomach	Cases
Well-differentiated adenocarcinoma	7
Moderately differentiated adenocarcinoma	7
Poorly differentiated adenocarcinoma	6
Esophagus	
Well-differentiated squamous cell carcinoma	8
Moderately differentiated squamous cell carcinoma	12
Colon	
Well-differentiated adenocarcinoma	10
Moderately differentiated adenocarcinoma	10
Prostate	
Well-differentiated adenocarcinoma	7
Moderately differentiated adenocarcinoma	6
Poorly differentiated adenocarcinoma	7
Breast	
Papillotubular carcinoma	7
Solid tubular carcinoma	7
Scirrhous carcinoma	6
Ovary	
Clear cell adenocarcinoma	3
Mucinous adenocarcinoma	3
Endometrial adenocarcinoma	3
Serous adenocarcinoma	11
Lung	
Squamous cell carinoma	10
Adenocarcinoma	10
Pancreas	
Well-differentiated adenocarcinoma	8
Moderately differentiated adenocarcinoma	10
Poorly differentiated adenocarcinoma	1
Undifferentiated carcinoma	1
Thyroid	
Papillary carcinoma	20

Breast tumors were classified according to the general rules for clinical and phathological records for breast cancer in Japan.

Results

Western blot analysis. All class I HDACs, including HDAC1, 2, 3 and 8, were ubiquitously expressed in all 11 cancer cell lines (Fig. 1). This feature indicates that class I HDACs are expressed in many cancer tissues. Class I HDACs were

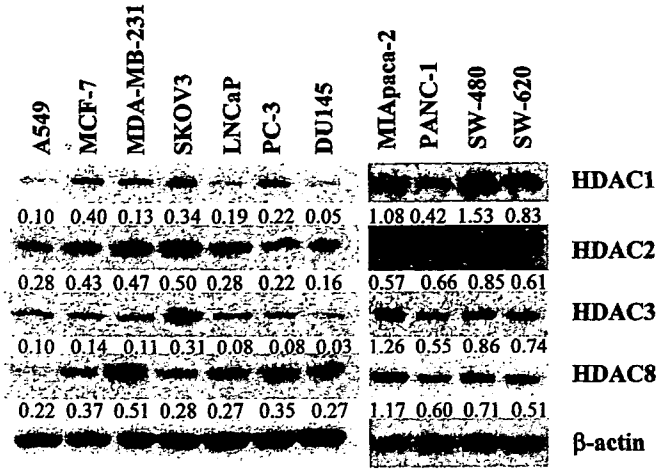

Figure 1. Protein expression of class I HDAC family members HDAC1, HDAC2, HDAC3 and HDAC8 in 11 human cancer cell lines, as assessed by Western blot analysis with specific antibodies. Relative expression of class I HDACs in each cell line was present as the ratio of HDAC expression to that of ß-actin.

Table II. The number and rate of 'HDAC-positive' cases in each 20 examined carcinomas.

Cancers	HDAC1	HDAC2	HDAC3	HDAC8
Stomach				
C	17 (85)	20 (100)	20 (100)	19 (95)
N	17 (85)	20 (100)	17 (85)	15 (75)
Esophagus				
C	19 (95)	20 (100)	19 (95)	18 (90)
N	20 (100)	19 (95)	16 (80)	15 (75)
Colon				
C	19 (95)	20 (100)	19 (95)	17 (85)
N	18 (90)	20 (100)	19 (95)	18 (90)
Prostate				
C	15 (75)	20 (100)	20 (100)	7 (35)
N	17 (85)	17 (85)	19 (95)	6 (30)
Breast	17 (85)	20 (100)	20 (100)	17 (85)
Ovary	19 (95)	20 (100)	16 (80)	16 (80)
Lung	20 (100)	20 (100)	20 (100)	17 (85)
Pancreas	17 (85)	18 (90)	20 (100)	18 (90)
Thyroid	20 (100)	20 (100)	20 (100)	20 (100)

Percentage values shown in parentheses; C, cancer tissue; N, non-cancerous tissue.

overexpressed in MIApaca-2, SW-480 and SW-620 cancer cell lines, compared with the other cancer cell lines.

Immunohistochemistry. The number and rate of each 'HDAC-positive' case in each cancer tissue are summarized in Table II. The score for class I HDAC expression in the cancer tissues and normal epithelium was almost equal in four types of cancer, including stomach, esophagus, colon and prostate, therefore, expression of class I HDACs in four types of cancer tissues were compared with that in normal epithelium (Table III). Immunohistochemical findings in five types of human cancer and four types of normal epithelium are shown in Fig. 2.

Table III. Comparison of the proportion score of class I HDACs in cancerous and non-cancerous tissues from four types of cancer.

Cancers	HDAC1	HDAC2	HDAC3	HDAC8
Stomach				
C>N	5 (25)	1 (5)	6 (30)	5 (25)
C=N	9 (45)	16 (80)	14 (70)	14 (70)
C<N	6 (30)	3 (15)	0 (0)	1 (5)
Esophagus				
C>N	3 (15)	5 (25)	7 (35)	6 (30)
C=N	10 (50)	11 (55)	8 (40)	10 (50)
C<N	7 (35)	4 (20)	5 (25)	4 (20)
Colon				
C>N	2 (10)	0 (0)	1 (5)	4 (20)
C=N	13 (65)	19 (95)	11 (55)	12 (60)
C<N	5 (25)	1 (5)	8 (40)	4 (20)
Prostate				
C>N	6 (30)	8 (40)	7 (35)	7 (35)
C=N	7 (35)	12 (60)	13 (65)	10 (50)
C<N	7 (35)	0 (0)	0 (0)	3 (15)

Percentage values shown in parentheses; C, cancer tissue; N, non-cancerous tissue. Class I HDAC expression was compared between cancer tissues and normal epithelium. C>N, C=N, C<N mean that class I HDAC expression in the cancer cells was more, equal to or less than that in the normal epithelial cells, respectively.

Cancer and non-cancerous epithelium of the stomach. Immunoreactivity showed expression for HDAC1, HDAC2, HDAC3 and HDAC8 in 17 (85%), 20 (100%), 20 (100%) and 19 (95%) of 20 cases of gastric cancer, respectively (Fig. 2A). In addition, HDAC1, HDAC2, HDAC3 and HDAC8 were highly expressed in 17 (85%), 20 (100%), 17 (85%) and 15 (75%) of 20 corresponding samples of non-cancerous gastric mucosa, respectively (Fig. 2B). HDAC1, HDAC2, HDAC3 and HDAC8 was overexpressed more in the cancer tissues than the normal epithelium in 5 (25%), 1 (5%), 6 (30%) and 5 (25%) cases, respectively (Table III).

Cancer and non-cancerous epithelium of the esophagus. Immunoreactivity for HDAC1, HDAC2, HDAC3 and HDAC8 was observed in 19 (95%), 20 (100%), 19 (95%) and 18 (90%) of 20 cases of esophageal cancer, respectively (Fig. 2C).

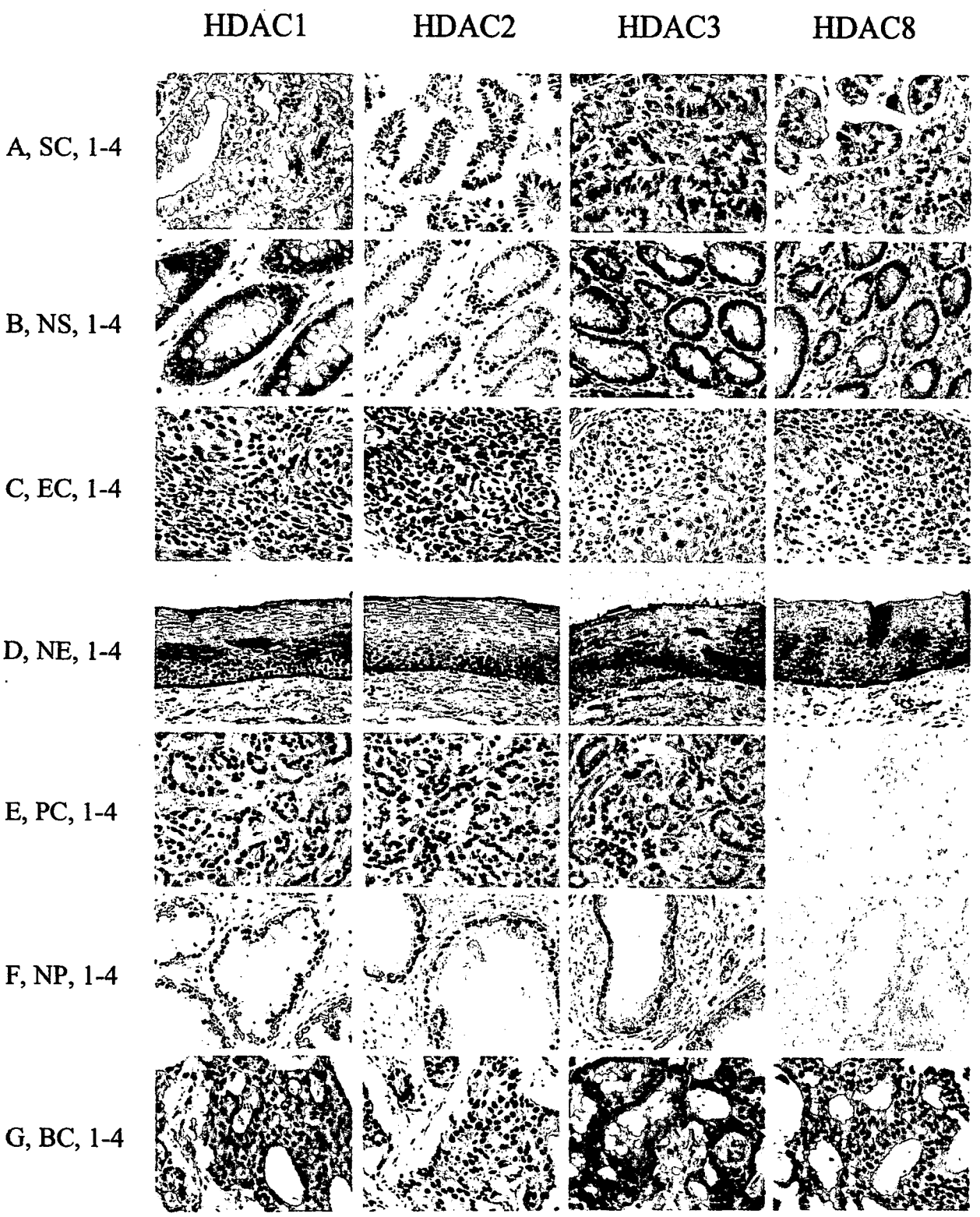

Figure 2. Immunohistochemistry of class I HDAC expression in several types of human cancer tissues and corresponding non-cancerous epithelium. (SC, stomach carcinoma; NS, normal stomach epithelium; EC, esophagus carcinoma; NE, normal esophagus epithelium; PC, prostate carcinoma; NP, normal prostate epithelium; BC, breast carcinoma) (A). Immunostaining of well-differentiated gastric adenocarcinoma with HDAC1 (A1), HDAC2 (A2), HDAC3 (A3) and HDAC8 (A4), showing strong nuclear expression. Cytoplasmic staining was also recognized for HDAC8. (B) Immunostaining of gastric non-cancerous epithelium with HDAC1 (B1), HDAC2 (B2), HDAC3 (B3) and HDAC8 (B4), showing strong nuclear expression. (C) Immunostaining of well-differentiated esophageal squamous cell carcinoma with HDAC1 (C1), HDAC2 (C2), HDAC3 (C3) and HDAC8 (C4), showing strong nuclear expression. Cytoplasmic staining was also recognized for HDAC8. (D) Immunostaining of non-cancerous esophageal epithelium with HDAC1 (D1), HDAC2 (D2), HDAC3 (D3) and HDAC8 (D4), showing strong nuclear expression. The expression of class I HDACs was mainly localized at the basal layer, whereas no immunostaining was observed in the keratinizing superficial layer. (E) Immunostaining of well-differentiated prostate adenocarcinoma with HDAC1 (E1), HDAC2 (E2), HDAC3 (E3) and HDAC8 (E4), showing strong nuclear expression. Cytoplasmic staining was also recognized for HDAC8. Immunoreactivity for HDAC 8 was lower than that in other cancer tissues. (F) Immunostaining of non-cancerous prostate epithelium with HDAC1 (F1), HDAC2 (F2), HDAC3 (F3) and HDAC8 (F4), showing strong nuclear expression. Immunoreactivity for HDAC 8 was lower than that in other normal epithelium. (G) Immunostaining of breast papillotubular carcinoma with HDAC1 (G1), HDAC2 (G2), HDAC3 (G3) and HDAC8 (G4), showing strong nuclear expression. Cytoplasmic staining was also recognized for HDAC8.

HDAC1, HDAC2, HDAC3 and HDAC8 was immunopositive in 20 (100%), 19 (95%), 16 (80%) and 15 (75%) of 20 samples of corresponding non-cancerous squamous epithelium of the esophagus, respectively (Fig. 2D). In the corresponding non-cancerous esophageal epithelium, the expression of class I HDACs was mainly localized at the basal and intermediate layers, whereas no immunostaining was observed in keratinizing superficial layers (Fig. 2D). HDAC1, HDAC2, HDAC3 and HDAC8 were overexpressed more in the cancer tissues than the normal epithelium in 3 (15%), 5 (25%), 7 (35%) and 6 (30%) cases, respectively (Table III).

Cancer and non-cancerous epithelium of the colon. Immunoreactivity for HDAC1, HDAC2, HDAC3 and HDAC8 was observed in 19 (95%), 20 (100%), 19 (95%) and 17 (85%) of 20 cases of colon cancer, respectively. HDAC1, HDAC2, HDAC3 and HDAC8 was positive in 18 (90%), 20 (100%), 19 (95%) and 18 (90%) of 20 specimens of corresponding non-cancerous colon mucosa, respectively. HDAC1, HDAC2, HDAC3 and HDAC8 were overexpressed more in cancer tissues than in normal colon epithelium in 2 (10%), 0 (0%), 1 (5%) and 4 (20%) cases, respectively (Table III).

Cancer and non-cancerous epithelium of the prostate. Immunoreactivity for HDAC1, HDAC2, HDAC3 and HDAC8 was observed in 15 (75%), 20 (100%), 20 (100%) and 7 (35%) of 20 cases of prostate cancer, respectively (Fig. 2E). HDAC1, HDAC2, HDAC3 and HDAC8 was positive in 17 (85%), 17 (85%), 19 (95%) and 6 (30%) of 20 specimens of non-neoplastic prostate, respectively (Fig. 2F). Both the epithelial and myoepithelial cells of the prostate equally expressed these class I HDACs. Immunoreactivity for HDAC8 in both cancer tissue (35%) and non-cancerous prostate (30%) was lower than that in other cancer tissues. Generally, overexpression of class I HDACs is observed in hyperplastic glands, whereas atrophic glands show reduced expression. HDAC1, HDAC2, HDAC3 and HDAC8 was overexpressed more in the cancer tissues than the normal epithelium in 6 (30%), 8 (40%), 7 (35%) and 7 (35%) cases, respectively (Table III).

Breast cancer. Immunoreactivity for HDAC1, HDAC2, HDAC3 and HDAC8 was positive in 17 (85%), 20 (100%), 20 (100%) and 17 (85%) of 20 cases of breast cancer, respectively (Fig. 2G).

Ovarian cancer. Immunoreactivity for HDAC1, HDAC2, HDAC3 and HDAC8 was observed in 19 (95%), 20 (100%), 16 (80%) and 16 (80%) of 20 cases of ovarian cancer, respectively. There was no significant difference in the expression profiles between histological subtypes.

Lung cancer. Immunoreactivity for HDAC1, HDAC2, HDAC3 and HDAC8 was observed in 20 (100%), 20 (100%), 20 (100%) and 17 (85%) of 20 cases of lung cancer, respectively. No significant difference in immunoreactivity was observed between adenocarcinoma and squamous cell carcinoma.

Pancreas cancer. Immunoreactivity for HDAC1, HDAC2, HDAC3 amd HDAC8 was observed in 17 (85%), 18 (90%), 20 (100%) and 18 (90%) of 20 cases of pancreas cancer, respectively.

Thyroid cancer. Immunoreactivity for HDAC1, HDAC2, HDAC3 and HDAC8 was observed in 20 (100%), 20 (100%), 20 (100%) and 20 (100%) of 20 cases of thyroid cancer, respectively. Twenty cases of thyroid papillary carcinoma were all immunoreactive for class I HDACs.

Discussion

There have been several reports that the class I HDACs are upregulated in many cancer cell lines and tissues, at both the transcriptional and translational levels. However, systematic analysis of the expression profiles of class I HDACs in human cancer tissues has not been carried out. Overexpression of class I HDACs have been reported in several cancer tissues (12-23). In gastric cancer, overexpression of HDAC1 (13,14) and HDAC2 (12) has been investigated and 76% (38/50) of advanced gastric cancer shows a high rate of HDAC2 expression (12). In breast cancer, overexpression of HDAC1 has been observed in 39.8% (70/176), and HDAC3 in 43.9% (76/173) of patients (21). In lung cancer, 62% (28/45) of cancer tissues were immunohistochemically stained for HDAC1 in more than 10% of tumor cells (22). Halkidou *et al* have assessed HDAC1 immunostaining in prostate cancer, with both a proportion and intensity score (20). In their study, 100% of the hormone-refractory (14/14) and low-grade prostate (5/5) cancer showed moderate to strong nuclear expression, and benign prostatic hyperplasia showed weak nuclear expression. As for colon cancer, Zhu *et al* (16) have shown that 82% (47/57) of colon cancer patients overexpress HDAC2.

To evaluate the expression of class I HDAC proteins in several types of human cancer, we first analyzed the expression of class I HDAC isotypes consisting of HDAC1, 2, 3 and 8 in 11 cell lines from six different types of cancer, by Western blotting. Class I HDACs are ubiquitously expressed in many cancer cell lines, and the expression level of class I HDACs seems to be different in each cancer tissue and each class I HDAC.

We next investigated class I HDAC proteins by immunohistochemistry. Although most previous studies have evaluated class I HDACs by intensity of nuclear staining, we evaluated the expression in cancer tissues by the proportion of nuclei stained, because class I HDAC nuclear staining intensity was strong in both cancer tissues and corresponding normal epithelium. In the current study, some degree of heterogeneity of class I HDAC staining was detected in our nine types of cancer tissue, as previously described in gastric cancer tissues (13). Therefore, we employed conventional evaluation of grossly mounted and immunostained slides, instead of the tissue microarray method.

In the current study, class I HDACs were highly expressed in tissues from stomach, esophagus, colon, breast, ovaries, lung, pancreas and thyroid cancer, but expression of HDAC8 in prostate cancer was definitely lower than that in the other cancer tissues. Waltregny *et al* found that HDAC8 immunoreactivity was not detected in either normal or malignant epithelial cells in any of 24 prostate cancer tissues (19).