death of HCC cells that do not possess AFP production capability, whereas other HCC cells with AFP production capability are not affected, resulting in the upregulation of AFP expression. If this hypothesis is correct, AFP expression would be high or upregulated with time after IFN treatment. In our long-term experiment, AFP mRNA was downregulated at 240 h with PEG-IFN-α2b treatment, and this poses a question as to the correctness of this hypothesis.

There has been much progress in the characterization of cis-acting and trans-acting elements regulating human AFP gene expression. Transcription of human AFP gene is controlled by three regulatory regions (promoter, enhancer, and silencer) in the 5′-flanking sequence.[24–28] The hepatocyte-specific enhancers exist in a far upstream regulatory region (−3.7 and −3.5 kb) of the AFP gene, and the position-dependent silencers are located between the enhancer regions and the hepatocyte-specific promoter region.[25] Several hormones, growth factors, cytokines, and differentiation inducers are reported to be involved in the regulation of AFP in human HCC cells.[23,24,29–33] Dexamethasone elevates AFP mRNA in human hepatoma cells through specific interaction with the glucocorticoid-responsive element in the human AFP gene promoter.[24] On the other hand, epidermal growth factor (EGF) synergistically interacts with phorbol ester to suppress the AFP enhancer activity, resulting in a marked depression of AFP gene transcription,[33] whereas transforming growth factor-β1 (TGF-β1) hepatocyte growth factor, and sodium butyrate repress AFP gene expression through reduction of its promoter activity.[30–32] In a future study, the AFP enhancer and promoter activities of KIM-1 cells cultured with PEG-IFN-α2b should be investigated to elucidate the AFP mRNA upregulation mechanism.

The ratio of AFP-L3 in cell lysate was lower than the ratio in culture medium. It was also lower in the culture with PEG-IFN-α2b than in the cells without PEG-IFN-α2b, and the level tended to decrease in a dose-dependent and time-dependent manner. The molecular basis of AFP-L3 is the fucosylation of the biantennary sugar chain,[8,34] and AFP-L3 is the product of α1-6 fucosyltransferase (α1-6 FucT) in the presence of GDP-fucose.[35] The higher ratio of AFP-L3 in the culture medium (outside the cells) suggests that fucosylated AFP is secreted more readily than nonfucosylated AFP. In addition, it was suggested that PEG-IFN-α2b could reduce the enzymatic activity of α1-6 FucT that is involved in the fucosylation of AFP. Noda et al.[36] found that acyclic retinoid treatment of HCC cells significantly increased the activity and mRNA levels of α1-6 FucT and the relative percentage of fucosylated AFP (AFP-L3) in culture medium. Whether or not PEG-IFN-α2b affects the enzymatic activity of α1-6 FucT is also a theme for future study.

For HCC-directed gene therapy, most investigators have used human AFP regulatory sequences.[37–40] Ido et al.[41] found that a retroviral vector expressing the herpes simplex virus-thymidine kinase gene under the control of a human AFP gene promoter provided the cytotoxicity to ganciclovir in high AFP-producing human HCC cells but not in low AFP-producing cells. Accordingly, they considered that specific enhancement of AFP promoter activity is likely to be required to induce enough cytotoxicity in low AFP-producing HCC cells.[41] If it induces the enhancement of AFP promoter activity, PEG-IFN-α2b could be a potent modulator of such HCC-directed gene therapy.

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REFERENCES

1. Xu D, Erickson S, Szeps M, Gruber A, Sangfelt O, Einhorn S, Pisa P, Grander D. Interferon α down-regulates telomerase reverse transcriptase and telomerase activity in human malignant and nonmalignant hematopoietic cells. *Blood* 2000;96:4313–4318.
2. Stark GR, Kerr IM, Williams BR, Silverman RH, Schreiber RD. How cells respond to interferons. *Annu. Rev. Biochem.* 1998;67: 227–264.
3. Pestka S, Langer JA, Zoon KC, Samuel CE. Interferons and their actions. *Annu. Rev. Biochem.* 1987;56:727–777.
4. Gutterman JU. Cytokine therapeutics: lessons from interferon α. *Proc. Natl. Acad. Sci. USA* 1994;91:1198–1205.
5. Ikeda K, Saitoh S, Arase Y, Chayama K, Suzuki Y, Kobayashi M, Tsubota A, Nakamura I, Murashima N, Kumada H, Kawanishi M. Effect of interferon therapy on hepatocellular carcinogenesis in patients with chronic hepatitis type C: a long-term observation study of 1,643 patients using statistical bias correction with proportional hazard analysis. *Hepatology* 1999;29:1124–1130.
6. Mazzella G, Accogli E, Sottili S, Festi D, Orsini M, Salzetta A, Novelli V, Cipolla A, Fabbri C, Pezzoli A, Roda E. Alpha interferon treatment may prevent hepatocellular carcinoma in HCV-related liver cirrhosis. *J. Hepatol.* 1996;24:141–147.
7. Nishiguchi S, Kuroki T, Nakatani S, Morimoto H, Takeda T, Nakajima S, Shiomi S, Seki S, Kobayashi K, Otani S. Randomised trial of effects of interferon-α on incidence of hepatocellular carcinoma in chronic active hepatitis C with cirrhosis. *Lancet* 1995;346: 1051–1055.
8. Aoyagi Y, Isemura M, Yosizawa Z, Suzuki Y, Sekine C, Ono T, Ichida F. Fucosylation of serum α-fetoprotein in patients with primary hepatocellular carcinoma. *Biochim. Biophys. Acta* 1985; 830:217–223.
9. Taketa K. α-Fetoprotein: reevaluation in hepatology. *Hepatology* 1990;12:1420–1432.
10. Yamashita F, Tanaka M, Satomura S, Tanikawa K. Prognostic significance of Lens culinaris agglutinin A-reactive alpha-fetoprotein in small hepatocellular carcinomas. *Gastroenterology* 1996;111: 996–1001.
11. Tsukuma H, Hiyama T, Tanaka S, Nakao M, Yabuuchi T, Kitamura T, Nakanishi K, Fujimoto I, Inoue A, Yamazaki H, Kawashima T. Risk factors for hepatocellular carcinoma among patients with chronic liver disease. *N. Engl. J. Med.* 1993;328: 1797–1801.
12. Ikeda K, Saitoh S, Koida I, Arase Y, Tsubota A, Chayama K, Kumada H, Kawanishi M. A multivariate analysis of risk factors for hepatocellular carcinogenesis: a prospective observation of 795 patients with viral and alcoholic cirrhosis. *Hepatology* 1993;18: 47–53.
13. Murashima S, Tanaka M, Haramaki M, Nakashima Y, Harada K, Ide T, Kumashiro R, Sata M. A decrease in AFP level related to administration of interferon in patients with chronic hepatitis C and a high level of AFP. *Dig. Dis. Sci.* 2006;51:808–812.
14. Murakami T, Maruiwa M, Fukuda K, Kojiro M, Tanaka M, Tanikawa K. Characterization of a new human hepatoma cell line (KYN-3) derived from the ascites of the hepatoma patient (Ab-

stract). *Jpn. J. Cancer Res.* Proceedings of the Japanese Cancer Association 1988:292.
15. Murakami T. Establishment and characterization of human hepatoma cell line (KIM-1). *Acta Hepatol. Jpn.* 1984;25:532–539.
16. Yano H, Kojiro M, Nakashima T. A new human hepatocellular carcinoma cell line (KYN-1) with a transformation to adenocarcinoma. *In Vitro Cell. Dev. Biol.* 1986;22:637–646.
17. Yano H, Maruiwa M, Murakami T, Fukuda K, Ito Y, Sugihara S, Kojiro M. A new human pleomorphic hepatocellular carcinoma cell line, KYN-2. *Acta Pathol. Jpn.* 1988;38:953–966.
18. Yano H, Iemura A, Fukuda K, Mizoguchi A, Haramaki M, Kojiro M. Establishment of two distinct human hepatocellular carcinoma cell lines from a single nodule showing clonal dedifferentiation of cancer cells. *Hepatology* 1993;18:320–327.
19. Yano H, Iemura A, Haramaki M, Ogasawara S, Takayama A, Akiba J, Kojiro M. Interferon alfa receptor expression and growth inhibition by interferon alfa in human liver cancer cell lines. *Hepatology* 1999;29:1708–1717.
20. Nakamura K, Kubo A, Hosokawa S, Nagaike K, Hashimoto S. Effect of alpha-interferon on anti-alpha-fetoprotein-monoclonal antibody targeting of hepatoma. *Oncology* 1993;50:35–40.
21. Yano H, Ogasawara S, Momosaki S, Akiba J, Kojiro S, Fukahori S, Ishizaki H, Kuratomi K, Basaki Y, Oie S, Kuwano M, Kojiro M. Growth inhibitory effects of pegylated IFN-α2b on human liver cancer cells *in vitro* and *in vivo*. *Liver Int.* 2006;26:964–975.
22. Grace M, Youngster S, Gitlin G, Sydor W, Xie L, Westreich L, Jacobs S, Brassard D, Bausch J, Bordens R. Structural and biologic characterization of pegylated recombinant IFN-α2b. *J. Interferon Cytokine Res.* 2001;21:1103–1115.
23. Nakabayashi H, Taketa K, Yamane T, Oda M, Sato J. Hormonal control of alpha-fetoprotein secretion in human hepatoma cell lines proliferating in chemically defined medium. *Cancer Res.* 1985; 45:6379–6383.
24. Nakabayashi H, Watanabe K, Saito A, Otsuru A, Sawadaishi K, Tamaoki T. Transcriptional regulation of alpha-fetoprotein expression by dexamethasone in human hepatoma cells. *J. Biol. Chem.* 1989;264:266–271.
25. Nakabayashi H, Hashimoto T, Miyao Y, Tjong KK, Chan J, Tamaoki T. A position-dependent silencer plays a major role in repressing alpha-fetoprotein expression in human hepatoma. *Mol. Cell. Biol.* 1991;11:5885–5893.
26. Sawadaishi K, Morinaga T, Tamaoki T. Interaction of a hepatoma-specific nuclear factor with transcription-regulatory sequences of the human alpha-fetoprotein and albumin genes. *Mol. Cell. Biol.* 1988;8:5179–5187.
27. Sakai M, Morinaga T, Urano Y, Watanabe K, Wegmann TG, Tamaoki T. The human alpha-fetoprotein gene. Sequence organization and the 5′ flanking region. *J. Biol. Chem.* 1985;260: 5055–5060.
28. Watanabe K, Saito A, Tamaoki T. Cell-specific enhancer activity in a far upstream region of the human alpha-fetoprotein gene. *J. Biol. Chem.* 1987;262:4812–4818.
29. Yasuda I, Shiratori Y, Adachi S, Obora A, Takemura M, Okuno M, Shidoji Y, Seishima M, Muto Y, Moriwaki H. Acyclic retinoid induces partial differentiation, down-regulates telomerase reverse transcriptase mRNA expression and telomerase activity, and induces apoptosis in human hepatoma-derived cell lines. *J. Hepatol.* 2002;36:660–671.
30. Tsutsumi T, Ido A, Nakao K, Hamasaki K, Kato Y, Ohtsuru A, Nakata K, Tamaoki T, Nagataki S. Reciprocal regulation of alpha-fetoprotein and albumin gene expression by butyrate in human hepatoma cells. *Gastroenterology* 1994;107:499–504.
31. Hatano M, Nakata K, Nakao K, Tsutsumi T, Ohtsuru A, Nakamura T, Tamaoki T, Nagataki S. Hepatocyte growth factor down-regulates the alpha-fetoprotein gene expression in PLC/PRF/5 human hepatoma cells. *Biochem. Biophys. Res. Commun.* 1992;189: 385–391.
32. Nakao K, Nakata K, Mitsuoka S, Ohtsuru A, Ido A, Hatano M, Sato Y, Nakayama T, Shima M, Kusumoto Y, Koji T, Tamaoki T, Nagataki S. Transforming growth factor beta 1 differentially regulates alpha-fetoprotein and albumin in HuH-7 human hepatoma cells. *Biochem. Biophys. Res. Commun.* 1991;174:1294–1299.
33. Nakata K, Motomura M, Nakabayashi H, Ido A, Tamaoki T. A possible mechanism of inverse developmental regulation of alpha-fetoprotein and albumin genes. Studies with epidermal growth factor and phorbol ester. *J. Biol. Chem.* 1992;267:1331–1334.
34. Aoyagi Y, Suzuki Y, Igarashi K, Saitoh A, Oguro M, Yokota T, Mori S, Suda T, Isemura M, Asakura H. Carbohydrate structures of human alpha-fetoprotein of patients with hepatocellular carcinoma: presence of fucosylated and non-fucosylated triantennary glycans. *Br. J. Cancer* 1993;67:486–492.
35. Nakagawa T, Uozumi N, Nakano M, Mizuno-Horikawa Y, Okuyama N, Taguchi T, Gu J, Kondo A, Taniguchi N, Miyoshi E. Fucosylation of N-glycans regulates the secretion of hepatic glycoproteins into bile ducts. *J. Biol. Chem.* 2006;281:29797–29806.
36. Noda K, Miyoshi E, Kitada T, Nakahara S, Gao CX, Honke K, Shiratori Y, Moriwaki H, Sasaki Y, Kasahara A, Hori M, Hayashi N, Taniguchi N. The enzymatic basis for the conversion of nonfucosylated to fucosylated alpha-fetoprotein by acyclic retinoid treatment in human hepatoma cells: activation of alpha1-6 fucosyltransferase. *Tumour Biol.* 2002;23:202–211.
37. Miao J, Chen GG, Chun SY, Yun JP, Chak EC, Ho RL, Lai PB. Adenovirus-mediated tBid overexpression results in therapeutic effects on p53-resistant hepatocellular carcinoma. *Int. J. Cancer* 2006;119:1985–1993.
38. Kanai F. Transcriptional targeted gene therapy for hepatocellular carcinoma by adenovirus vector. *Mol. Biotechnol.* 2001;18:243–250.
39. Kaneko S, Hallenbeck P, Kotani T, Nakabayashi H, McGarrity G, Tamaoki T, Anderson WF, Chiang YL. Adenovirus-mediated gene therapy of hepatocellular carcinoma using cancer-specific gene expression. *Cancer Res.* 1995;55:5283–5287.
40. Ido A, Uto H, Moriuchi A, Nagata K, Onaga Y, Onaga M, Hori T, Hirono S, Hayashi K, Tamaoki T, Tsubouchi H. Gene therapy targeting for hepatocellular carcinoma: selective and enhanced suicide gene expression regulated by a hypoxia-inducible enhancer linked to a human alpha-fetoprotein promoter. *Cancer Res.* 2001; 61:3016–3021.
41. Ido A, Nakata K, Kato Y, Nakao K, Murata K, Fujita M, Ishii N, Tamaoki T, Shiku H, Nagataki S. Gene therapy for hepatoma cells using a retrovirus vector carrying herpes simplex virus thymidine kinase gene under the control of human alpha-fetoprotein gene promoter. *Cancer Res.* 1995;55:3105–3109.

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Original Article

Overexpression of the *myc* target gene *Mina53* in advanced renal cell carcinoma

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The *myc* target gene *Mina53* was reported to be overexpressed in esophageal cancer with a poor prognosis. The purpose of the present study was to examined Mina53 expression and its relationship to clinicopathological parameters in human renal cell carcinoma (RCC). Mina53 and Ki-67 expression was examined on immunohistochemistry for 64 surgically resected RCC and non-cancerous tissue. In addition, the relationship between Mina53 expression and clinicopathological prognostic factors of RCC such as age, stage, microvenous invasion (MVI), histological subtype, Ki-67 labeling index (LI), and prognosis, was examined. Mina53 was expressed in the nuclei of tumor cells and tubular nuclei of normal renal tissue. The expression level of Mina53 was significantly higher in patients with poor prognostic factors (stage IV, MVI-positive, and sarcomatoid RCC, and high Ki-67 LI). The prognosis of high Mina53-expressing tumors was significantly poorer than that of non-Mina53-high tumors ($P < 0.0001$). In conclusion, Mina53 is overexpressed in RCC tissue from patients with poor prognostic factors, suggesting that Mina53 overexpression is one of the factors for poor prognosis in RCC.

Key words: immunohistochemistry, Ki-67, Mina53, renal cell carcinoma

In Japan, 6358 individuals (4372 men and 1986 women) developed renal cell carcinoma (RCC) in 1997. The crude incidence rates per 100 000 people for men and women were 7.1 and 3.1, respectively, and the age-standardized incidence rates per 100 000 people for men and women were 4.9 and 1.8, respectively.[1]

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Recently, the 10 year survival rate of all RCC patients has exceeded 65% due to improvements in diagnostic imaging and therapy.[2,3] Because RCC is known to be resistant to chemotherapy, radiotherapy and surgery remain the only effective treatments.[4,5] Tumor stage, tumor grade, and Ki-67 status, together with clinical parameters, can best predict prognosis; but even a small or T1 RCC sometimes metastasizes to distant sites and, in rare cases, distant metastatic lesions spontaneously regress or disappear.[6,7] RCC often has an unpredictable outcome. While much is unknown about RCC, various genes reportedly involved in the progression, recurrence, and proliferation of RCC, such as *epidermal growth factor receptor* (*EGFR*), *p53*, and *c-myc*, have been identified.[8–11] Among these, *c-myc* has been shown to be an oncogene that interacts with various cellular factors to stimulate proliferation, apoptosis induction, and lymphangiogenesis.[11,12] The oncogene *myc* with such diverse functions plays an important role, especially in cell proliferation, and is expressed in many cancers. The *myc* product directly binds to the E-box sequence of genomic genes to increase their expression.[13] Previous studies have shown that genes closely involved in cell proliferation are *myc* target genes, that is, genes whose expression is directly increased by *myc*.[14] Tsuneoka *et al.* reported that *Mina53* (myc-induced nuclear antigen with a molecular weight of 53 kDa) is a novel *myc* target gene involved in cell proliferation. The *Mina53* gene is located on chromosome 3q12.1,[14] and encodes a protein with a molecular weight of 53 kDa, which is localized in the nucleus, and is concentrated, in part, in the nucleolus. Recent studies have reported that Mina53 is expressed in all pathological grades of colon cancer, but not or slightly in non-neoplastic colonic cells,[15] and esophageal cancer with a high expression of Mina53 has a very poor prognosis.[16] The purpose of the present study was to examine the expression of myc gene-related molecules, Mina53 in RCC, on

immunohistochemistry, and clinicopathologically evaluate the potential use of Mina53 expression as a new prognostic factor.

MATERIALS AND METHODS

Antibodies

Mouse monoclonal anti-Mina53 antibody (IgG2a, clone M532) was established at Division of Human Genetic Department of Forensic Medicine, Kurume University School of Medicine, as previously described.[15] Mouse anti-Ki-67 antibody (clone MIB-1) and peroxidase-labeled goat antimouse IgG Fab' were purchased from Dako (Glostrup, Denmark) and Nichirei (Tokyo, Japan), respectively.

Immunoblotting

A renal cell carcinoma cell line (ACHN) that was purchased from the American Type Culture Collection (Manassas, VA, USA) was maintained in modified Eagle's medium (Gibco BRL/Life Technologies, Gaithersburg, MD, USA) supplemented with 10% fetal bovine serum (Bioserum, Victoria, Australia), 100 U/mL penicillin, and 100 μg/mL streptomycin (Gibco BRL/Life Technologies). For western blotting, cells were collected by treatment with trypsin and EDTA in PBS and washed with PBS. Cells were then suspended in 0.125 mol/L Tris-HCl buffer, pH 6.8, containing 3% sodium dodecylsulfate, 50 mmol/L dithiothreitol, and 20% glycerol and boiled for 10 min before separation on a gradient sodium dodecylsulfate–polyacrylamide gel (4–20%). Proteins were transferred to a polyvinylidene difluoride microporous membrane (Millipore, Bedford, MA, USA), and non-specific binding sites were blocked with 1% skim milk in PBS. After treatment with mouse monoclonal anti-Mina53 and HRP-conjugated goat antimouse IgG, signals were detected using an enhanced chemiluminescence western blotting detection reagent system (Amersham Biosciences, Buckinghamshire, UK).

Patients and specimens

Preserved specimens from 64 consecutive RCC patients who had received surgical treatment at Kurume University Hospital between 2000 and 2005 were analyzed and their clinical records were reviewed. All patients underwent radical nephrectomy or partial nephrectomy with para-aortic lymphadenectomy and renal hilar lymphadenectomy. No patients received preoperative treatment. The patients were staged in accordance with the 1997 Union Internationale Contre le Cancer (UICC)/American Joint Committee on Cancer (AJCC) consensus classification.[17] Of the 64 patients, 36, four, 13, and 11 were in stage I, II, III, and VI, respectively (pT1 = 37, pT2 = 4, pT3 = 21, pT4 = 2). Tumors were grouped according to the histological typing of the World Health Organization (WHO). Nuclear grade was assigned according to the Fuhrman nuclear grading system.[18] Microvenous invasion (MVI), stage of cancer, histological subtype, and nuclear grade were determined by two pathologists, H.I. and H.Y. Survival time was defined as the period from the date of surgical resection of the primary tumor to the date of death or last follow up. For disease-specific survival, the survival time of patients who died from causes other than RCC was defined as the period from the date of surgical resection to the date of death. The cause of death was determined by correspondence with the patient's family or local physician, or by reference to the death certificate. During the follow-up period, ranging from 0.9 to 301 weeks (mean, 147 weeks), two patients died soon after surgery of intraoperative or postoperative complications, making the period of follow up extremely short. There were three recurrences and 10 carcinoma-related deaths. The mean age at surgery was 60.4 years (range, 24–86 years). Forty-four patients were men, and 20 were women. Of the 64 patients, 53 had clear cell RCC, five had papillary RCC, five had sarcomatoid RCC, and one had chromophobe RCC (Table 1). Sarcomatoid RCC was first described by Farrow *et al.* as a tumor exhibiting marked cytological atypia and containing enlarged pleomorphic or malignant spindle cells suggestive of sarcoma;[19] but sarcomatoid RCC is currently regarded as a common, dedifferentiated process in the different subtypes of RCC.[20] In the present study we defined sarcomatoid RCC as an RCC with a sarcomatoid component, and classified all tumors with a sarcomatoid component as nuclear grade 4. Of the five sarcomatoid RCC, three and two contained clear cell and papillary cell carcinoma components, respectively. Their sarcomatoid components occupied >60% of tumor tissue. Pure sarcomatoid tumors, whose histological subtypes could not be identified, were not included in the specimens examined.

Immunostaining

Routinely processed, formalin-fixed, paraffin-embedded serial sections (4 μm) containing cancerous and non-cancerous areas were mounted on 3-aminopropyltriethoxysilane-coated slides (Matsunami Glass, Osaka, Japan), and deparaffinized in xylene/alcohol and graded alcohol. The sections were soaked in 10 mmol/L sodium citrate buffer (pH 6.9), and treated in a microwave oven for 50 min for antigen retrieval. Immunostaining for Mina53 and Ki-67 was performed using streptavidin–biotin peroxidase (SAB-PO) kits (Nichirei,

Table 1 Mina53 expression and clinicopathological factors in RCC

Factors	Mina53 expression		Total
	Negative and low-expression tumors	High-expression tumors	
No. patients (%)	55	9	64
Average age (years)	60.9	57.3	60.4
Gender			
Male	38	6	44
Female	17	3	20
MVI			
Negative	49	3	52
Positive	6	6‡	12
Stage			
Stage I	36	0	36
Stage II	4	0	4
Stage III	10	3	13
Stage IV	5	6§	11
Histological subtype			
Clear cell RCC	49	4	53
Papillary RCC	4	1	5
Chromophobe RCC	1	0	1
Sarcomatoid RCC	1	4*	5
Nuclear grade			
1	9	0	9
2	30	1	31
3	14	3	17
4	2	5**	7
Ki-67 LI			
<10%	50	0	50
≥10%	5	9***	14
Lymph-node metastasis			
Negative	53	5	58
Positive	2	4††	6
Distant metastasis			
Negative	54	4	58
Positive	1	5††	6

*$P < 0.001$–0.0001, vs sarcomatoid RCC or the other subtypes. **$P < 0.01$–0.0001, versus nuclear grade 1, or nuclear grade 2, or nuclear grade 3, or nuclear grades 1–3. ***$P < 0.0001$, vs <10%.

‡$P < 0.0001$, versus MVI negative. §$P < 0.05$–0.0001, vs stage I, stage III, or stages I–III. ††Not significant, vs negative.

LI, labeling index; MVI, microvenous invasion; RCC, renal cell carcinoma.

Tokyo, Japan) according to the manufacturer's protocol. After treatment with avidin and rabbit serum, the sections were incubated with primary antibodies at 4°C overnight. The peroxidase reaction was developed by the addition of 3,3-diaminobenzidine-H_2O_2 substrate solution, followed by incubation for 5 min (Mina53) or 2 min (Ki-67). After light counterstaining with hematoxylin, the slides were dehydrated, coverslipped, and observed under a microscope (Olympus BX41, Olympus Optical, Tokyo, Japan). Negative controls were prepared by replacing the primary antibody with normal mouse IgG. The Mina53 expression level in the nuclei of renal tubular epithelium in non-tumorous areas was used as an internal positive control.

Microscopic assessment of Mina53 and Ki-67 expression

Two pathologists, H.I and H.Y., who did not know the clinical status of each patient, independently evaluated and interpreted the results of immunostaining. The expression (staining) levels of Mina53 were classified into three categories. Tumors containing no identifiable Mina53-positive tumor cells were classified as Mina53-negative; tumors that contained focally or diffusely Mina53-positive tumor cells stained as intensely as, or less intensely than, non-cancerous normal renal tubules, were classified as low Mina53-positive; and tumors containing diffusely Mina53-positive tumor cells that were clearly more intensely stained than non-cancerous normal renal tubules were classified as high Mina53-positive. Proliferative activity was assessed by detecting Ki-67 protein. The Ki-67 antigen represents a nuclear cell proliferation-associated protein expressed in the G_1, S, G_2, and M phases of the cell cycle, but not in non-proliferative G_0 cells. The Ki-67 staining results of tumor cells, as expressed by the Ki-67 labeling index (LI), were evaluated as follows. In Mina53-positive tumors, Ki-67 expression was assessed in the area containing Mina53-positive cells and having the highest Ki-67 LI. In Mina53-negative tumors, Ki-67 expres-

sion was assessed in the area having the highest Ki-67 LI in tumor tissue. In each tumor an appropriate area was photographed at a magnification of ×200 with a digital camera, and printed. To estimate the percentage of stained cells, the numbers of positive and negative tumor cells in a field were counted, and the ratio of positive to total cells was expressed as a percentage.

Statistics

The relationships between Mina53 expression levels in negative, low-positive, and high-positive tumors and Ki-67 LI were analyzed on Mann–Whitney *U*-test. In addition, associations between Mina53 expression in RCC and poor prognostic factors such as MVI, stage IV, sarcomatoid RCC, nuclear grade 4, and Ki-67 LI ≥ 10% were examined on χ^2 test. Disease-free survival rates were calculated using the Kaplan–Meier method. *P* for each survival rate as well as prognostic factors (high Mina53 expression, MVI-positive, stage IV, sarcomatoid RCC, nuclear grade 4, and Ki-67 LI ≥ 10%) were analyzed using the log–rank test.[21] Cox's multivariate analysis was used to examine whether Mina53 expression is a prognostic factor independent of other established factors such as cancer stage.[22] All statistical analyses were performed using StatView software (SAS Institute, Cary, NC, USA). $P < 0.05$ were considered significant.

RESULTS

Expression of Mina53 in renal cell carcinoma cells on western blot

Anti-Mina53 mouse monoclonal antibody (M532) recognized a single band with a molecular weight of 53 kDa on western blot (Fig. 1). The result indicates that ACHN cells express Mina53 and that anti-Mina53 antibody specifically recognizes Mina53 protein in RCC cells with no cross-reactivity to other proteins.

Immunohistochemical expression of Mina53 and Ki-67 in RCC and non-cancerous tissues

Low level of Mina53 expression was observed in the nuclei of normal tubules in all non-cancerous renal tissues (Fig. 2a,b). Mina53 expression in the nuclei of RCC cells varied from negative to high, and Mina53-negative (Fig. 2d,e), low Mina53-expressing (Fig. 2g,h), and high Mina53-expressing tumors (Fig. 2j,k) accounted for 32.8% (21 tumors), 53.1% (34 tumors), and 14.1% (nine tumors) of all cancers. The distribution of Mina53-positive RCC cells was uniform in the

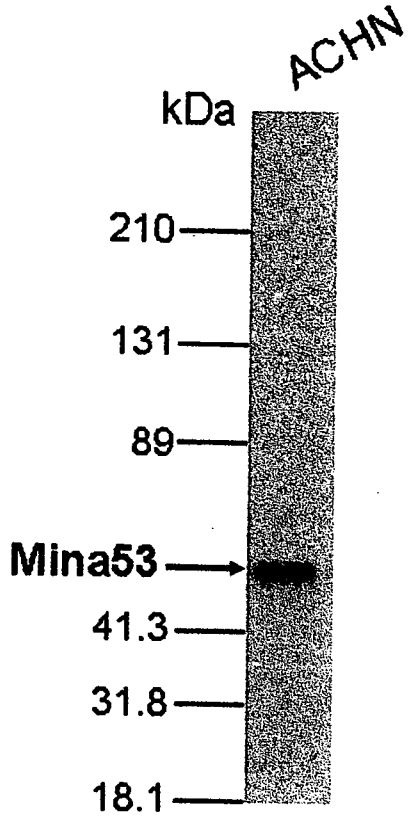

Figure 1 Western blot analysis of Mina53 protein in cultured renal cell carcinoma (RCC) cells. Proteins were subjected to electrophoresis in the 4–20% gradient sodium dodecylsulfate–polyacrylamide gel, transferred to polyvinylidene difluoride transfer membrane, and probed with anti-Mina53 (clone M532) monoclonal antibody. Anti-Mina53 mouse monoclonal antibody (M532) recognized a single band with a molecular weight of 53 kDa on western blot. The result indicates that ACHN cells express Mina53 and that anti-Mina53 antibody specifically recognizes Mina53 protein in RCC cells with no cross-reactivity to other proteins.

tumor nodule but in some cases of low-Mina53 expression, Mina53 expression was focally or predominantly observed in the periphery of the tumor. In all cases the expression level of Mina53 was uniform in the tumor nodule. In three high-Mina53 expressing tumors (two sarcomatoid RCC and one papillary RCC cases), Mina53 expression was observed in the nucleus with concentrated amounts in the nucleolus. On histology Mina53 was expressed in 33 clear cell RCC (62.3%), four papillary RCC (80%), five sarcomatoid RCC (100%), and one chromophobe RCC (100%; Table 1). Nuclear Ki-67 expression was observed in all 64 RCC at various levels (Fig. 2f,i,l), but few or no renal tubules were positive for Ki-67 (Fig. 2c).

Association between Mina53 expression and clinicopathological parameters in RCC

The patients were 44 men and 20 women. There was no association between Mina53 expression and gender or age.

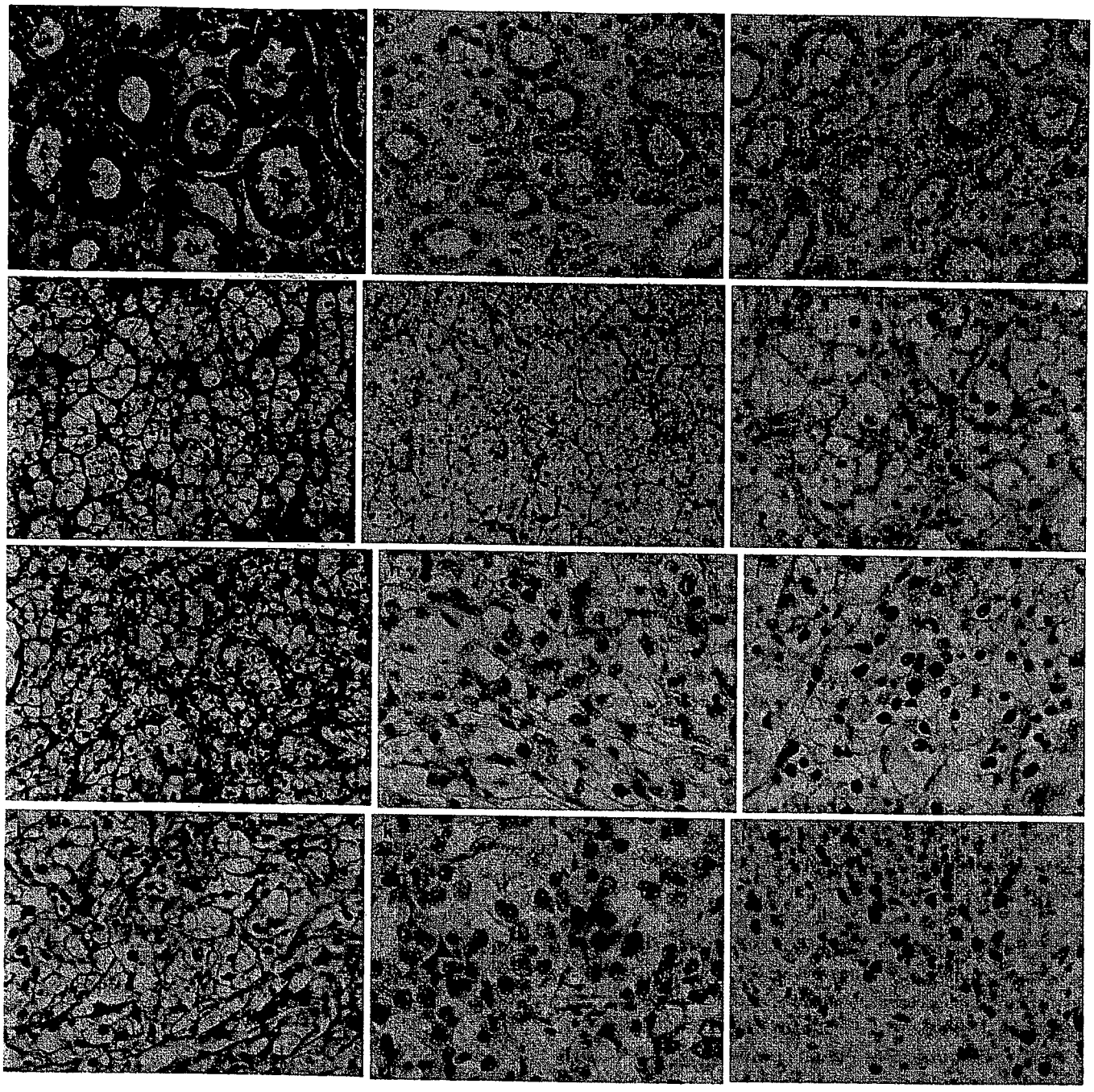

Figure 2 Immunohistochemical staining of Mina53 and Ki-67 in renal cell carcinoma (RCC) and non-cancerous tissues. (**a**) Normal tubules in the non-cancerous area of clear cell RCC (HE). (**b**) Low level of Mina53 expression in the nuclei of tubules in a non-tumorous area (counterstained with hematoxylin). (**c**) Ki-67 expression in the nuclei of tubules in a non-tumorous area. Few or no renal tubules were positive for Ki-67 (counterstained with hematoxylin). (**d**) Mina53-negative clear cell RCC (HE). (**e**) No Mina53 expression in tumor cells (counterstained with hematoxylin). (**f**) Ki-67 expression in the same tumor as in (**d**). Ki-67-positive cells were seen scattered (counterstained with hematoxylin). (**g**) Low Mina53-expressing clear cell RCC (HE). (**h**) Mina53 expression in the same tumor as in (**g**). Tumor cells stained for Mina53 as intensely as renal tubules were noted (counterstained with hematoxylin). (**i**) Ki-67 expression in the same tumor as in (**g**). Ki-67-positive cells were seen scattered (counterstained with hematoxylin). (**j**) High Mina53-expressing sarcomatoid RCC (HE). (**k**) Mina53 expression in the same tumor as in (**j**). Strong Mina53 expression was observed in the nuclei with concentrated amounts in the nucleoli of tumor cells (counterstained with hematoxylin). (**l**) Ki-67 expression in the same tumor as in (**j**). Many Ki-67-positive cells were observed (counterstained with hematoxylin).

Of all 64 tumors, 12 (18.8%) were MVI positive. Of these 12 tumors, six (50%) were high Mina53 positive. The percentage of high Mina53-expressing tumors was significantly higher in MVI-positive than in MVI-negative tumors ($P < 0.0001$, χ^2 test; Table 1).

The numbers of stage I, II, III, and IV tumors were 36, four, 13, and 11, respectively. Of the 11 stage IV tumors, six (54.5%) were high Mina53 positive. The percentage of high Mina53-expressing tumors was significantly higher in stage IV than in stages I–III tumors ($P < 0.0001$, χ^2 test; Table 1). In addition there were significant differences in the percentage of high Mina53-positive tumors between stage I and IV tumors ($P < 0.0001$, χ^2 test) and between stage III and IV tumors ($P < 0.05$, χ^2 test; Table 1).

Of the five sarcomatoid RCC, four (80%) were high Mina53 positive. The percentage of high Mina53-expressing tumors was significantly higher in sarcomatoid RCC than in other histological subtypes of RCC (clear cell RCC, papillary RCC, and chromophobe RCC; $P < 0.0001$, χ^2 test). In addition, a significant difference was noted in the percentage of high Mina53-expressing tumors between sarcomatoid and clear cell RCC ($P < 0.001$, χ^2 test; Table 1).

The numbers of nuclear grade 1, 2, 3, and 4 tumors were 9, 31, 17, and 7, respectively. Of the seven nuclear grade 4 tumors, five (71.4%) were high Mina53 positive. The percentage of high Mina53-expressing tumors was significantly higher in nuclear grade 4 tumors than in nuclear grades 1–3 tumors ($P < 0.0001$, χ^2 test; Table 1). In addition, there were significant differences in the percentage of high Mina53-expressing tumors between grade 1 and 4 tumors, between grade 2 and 4 tumors, and between grade 3 and 4 tumors ($P < 0.01$, $P < 0.001$, and $P < 0.01$, respectively, χ^2 test; Table 1).

Comparison of Mina53 and Ki-67 expression in RCC tissue

Ki-67 expression was observed in the nucleus of RCC at various levels. The mean Ki-67 LI for all tumors was 9.6%, and the mean LI for Mina53-negative, low Mina53-expressing, and high Mina53-expressing tumors was 5.2%, 5.2%, and 34.7%, respectively. When tumors were divided into high and low Ki-67-expressing groups by the approximate mean Ki-67 LI of 10%, the frequency of high Mina53 expression was significantly higher in the high than in the low Ki-67-expressing group ($P < 0.0001$, χ^2 test; Table 1). Moreover, significant differences in Ki-67 LI were noted between Mina53-negative and high Mina53-expressing tumors ($P < 0.0001$), and between low and high Mina53-expressing tumors ($P < 0.0001$, Mann–Whitney *U*-test; Fig. 3).

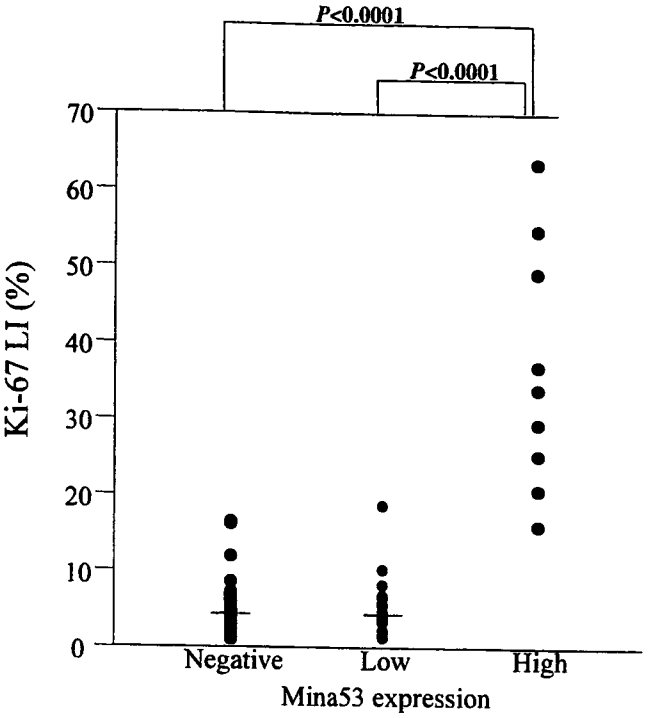

Figure 3 Relationship between Mina53 and Ki-67 expression. Ki-67 labeling indices (LI) were compared in three different groups: Mina53-negative group, Mina53 low-expression group, Mina53 high-expression group. Their respective mean LI were 5.2%, 5.2%, and 34.7%. Their means are indicated by transverse lines. Significant differences in Ki-67 LI were noted between Mina53-negative and Mina53 high-expression groups, and between Mina53 low-expression and Mina53 high-expression groups.

Mina53 and Ki-67 expression in relation to survival time

The survival rates of patients with Mina53-negative, Mina53-low, and Mina53-high tumors were 95.2% (20/21), 91.1% (31/34), and 33.3% (3/9), respectively. The patients were divided into a Mina53-high group and a non-Mina53-high group (consisting of Mina53-negative and Mina53-low groups). Crude survival curves were estimated for each group using the Kaplan–Meier method. The patients with non-Mina53-high tumors had longer survival than those with Mina53-high tumors (Fig. 4). The survival rate was significantly higher in patients with non-Mina53-high tumors than in those with Mina53-high tumors ($P < 0.0001$, log–rank test). The patients with low-Ki-67 LI tumors had longer survival than those with high-Ki-67 LI tumors (Fig. 5). The survival rate was significantly higher in patients with low-Ki-67 LI tumors than those with high-Ki-67 LI tumors ($P < 0.0001$, log–rank test). Possible prognostic factors, including gender, MVI-positive, stage IV, sarcomatoid RCC, and high Mina53 expression, were analyzed using Cox's proportional hazards method. As shown in Table 2, only stage IV and high-Ki-67 LI were found to reflect the survival rate (Table 2).

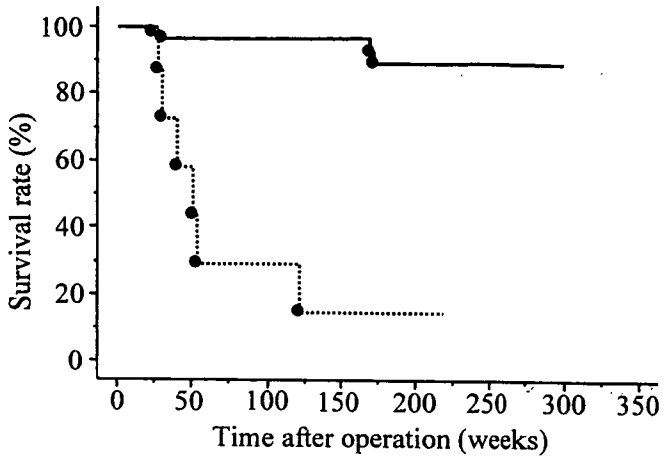

Figure 4 Correlation between the event-free survival rate and Mina53 expression (Kaplan–Meier method). Sixty-four renal cell carcinomas were divided into (·····) Mina53 high-expression tumors ($n = 9$; 14.1%) and (—) Mina53 non-high tumors (Mina53 negative and low-expression tumors; $n = 55$; 85.9%) 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.

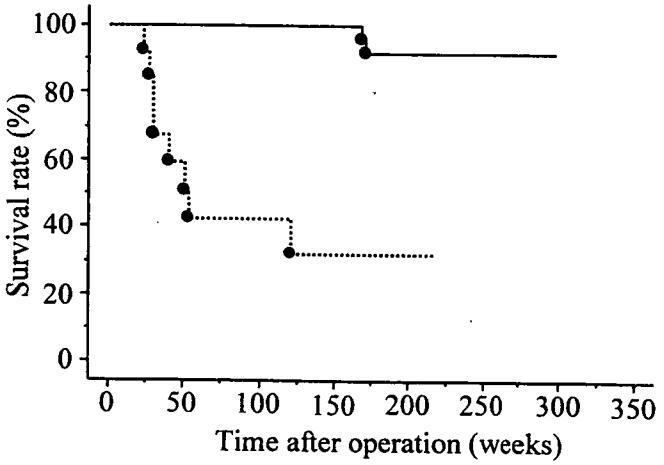

Figure 5 Correlation between the event-free survival rate and Ki-67 expression (Kaplan–Meier method). Sixty-four renal cell carcinomas were divided into a (–) low-Ki-67 labeling index (LI) group ($n = 50$; 78.1%) and a (·····) high-Ki-67 LI group ($n = 14$; 21.9%) according to the expression level of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

1 Marumo K, Satomi Y, Miyao N *et al.* The prevalence of renal cell carcinoma: A nation-wide survey in Japan in 1997. *Int J Urol* 2001; **8**: 359–65.
2 Homma Y, Kawabe K, Kitamura T *et al.* Increased incidental detection and reduced mortality in renal cancer: Recent retrospective analysis at eight institutions. *Int J Urol* 1995; **2**: 77–80.
3 Kirkali Z, Tuzel E, Mungan MU. Recent advances in kidney cancer and metastatic disease. *BJU Int* 2001; **88**: 818–24.

4 Ficarra V, Novara G, Galfano A, Artibani W. Neoplasm staging and organ-confined renal cell carcinoma: A systematic review. *Eur Urol* 2004; **46**: 559–64.
5 Kirkali Z, Lekili M. Renal cell carcinoma: New prognostic factors? *Curr Opin Urol* 2003; **13**: 433–8.
6 Takahashi M, Yang XJ, Sugimura J *et al.* Molecular subclassification of kidney tumors and the discovery of new diagnostic markers. *Oncogene* 2003; **22**: 6810–18.
7 Lenburg ME, Liou LS, Gerry NP, Frampton GM, Cohen HT, Christman MF. Previously unidentified changes in renal cell carcinoma gene expression identified by parametric analysis of microarray data. *BMC Cancer* 2003; **3**: 31.
8 Lovisolo JA, Casati B, Clerici L *et al.* Gene expression profiling of renal cell carcinoma: A DNA macroarray analysis. *BJU Int* 2006; **98**: 205–16.
9 Moch H, Sauter G, Buchholz N *et al.* Epidermal growth factor receptor expression is associated with rapid tumor cell proliferation in renal cell carcinoma. *Hum Pathol* 1997; **28**: 1255–9.
10 Shvarts O, Seligson D, Lam J *et al.* p53 is an independent predictor of tumor recurrence and progression after nephrectomy in patients with localized renal cell carcinoma. *J Urol* 2005; **173**: 725–8.
11 Yao M, Shuin T, Misaki H, Kubota Y. Enhanced expression of c-myc and epidermal growth factor receptor (C-erbB-1) genes in primary human renal cancer. *Cancer Res* 1988; **48**: 6753–7.
12 Amundadottir LT, Nass SJ, Berchem GJ, Johnson MD, Dickson RB. Cooperation of TGF alpha and c-Myc in mouse mammary tumorigenesis: Coordinated stimulation of growth and suppression of apoptosis. *Oncogene* 1996; **13**: 757–65.
13 Levens DL. Reconstructing MYC. *Genes Dev* 2003; **17**: 1071–7.
14 Tsuneoka M, Koda Y, Soejima M, Teye K, Kimura H. A novel myc target gene, mina53, that is involved in cell proliferation. *J Biol Chem* 2002; **277**: 35 450–59.
15 Teye K, Tsuneoka M, Arima N *et al.* Increased expression of a Myc target gene Mina53 in human colon cancer. *Am J Pathol* 2004; **164**: 205–16.
16 Tsuneoka M, Fujita H, Arima N *et al.* Mina53 as a potential prognostic factor for esophageal squamous cell carcinoma. *Clin Cancer Res* 2004; **10**: 7347–56.
17 Storkel S, Eble JN, Adlakha K *et al.* Classification of renal cell carcinoma: Workgroup No. 1. Union Internationale Contre le Cancer (UICC) and the American Joint Committee on Cancer (AJCC). *Cancer* 1997; **80**: 987–9.
18 Fuhrman SA, Lasky LC, Limas C. Prognostic significance of morphologic parameters in renal cell carcinoma. *Am J Surg Pathol* 1982; **6**: 655–63.
19 Farrow GM, Harrison EG Jr, Utz DC, ReMine WH. Sarcomas and sarcomatoid and mixed malignant tumors of the kidney in adults. I. *Cancer* 1968; **22**: 545–50.
20 Delahunt B. Sarcomatoid renal carcinoma: The final common dedifferentiation pathway of renal epithelial malignancies. *Pathology* 1999; **31**: 185–90.
21 Kaplan EL, Meier P. Nonparametric estimation from incomplete observations. *J Am Stat Assoc* 1958; **53**: 457–81.
22 Cox DR. Regression models and life tables. *J R Stat Soc Series B* 1972; **34**: 187–220.
23 de Peralta-Venturina M, Moch H, Amin M *et al.* Sarcomatoid differentiation in renal cell carcinoma: A study of 101 cases. *Am J Surg Pathol* 2001; **25**: 275–84.
24 Mrstik C, Salamon J, Weber R, Stogermayer F. Microscopic venous infiltration as predictor of relapse in renal cell carcinoma. *J Urol* 1992; **148**: 271–4.
25 He TC, Sparks AB, Rago C *et al.* Identification of c-MYC as a target of the APC pathway. *Science* 1998; **281**: 1509–12.
26 Jacqmin D, van Poppel H, Kirkali Z, Mickisch G. Renal cancer. *Eur Urol* 2001; **39**: 361–9.

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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,

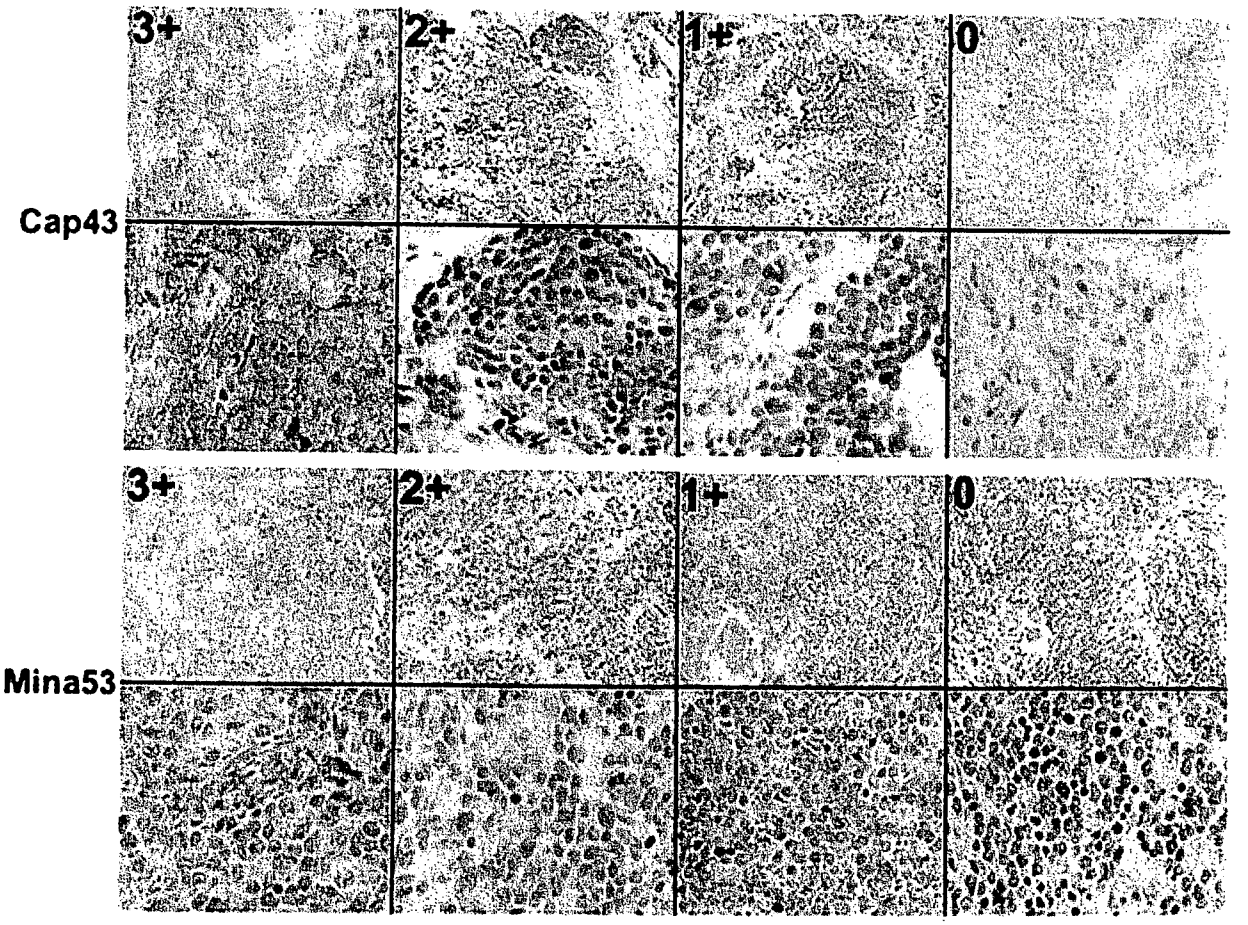

Fig. 1 A panel figure showing representative examples of each staining intensity for Cap43 and Mina53. The staining intensity for Cap43, Mina53, MYCN, and TrkA in each specimen was scored on a scale from 0 to 3 (0 = negative, 1 = weakly positive, 2 = moderately positive, 3 = strongly positive). The maximum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

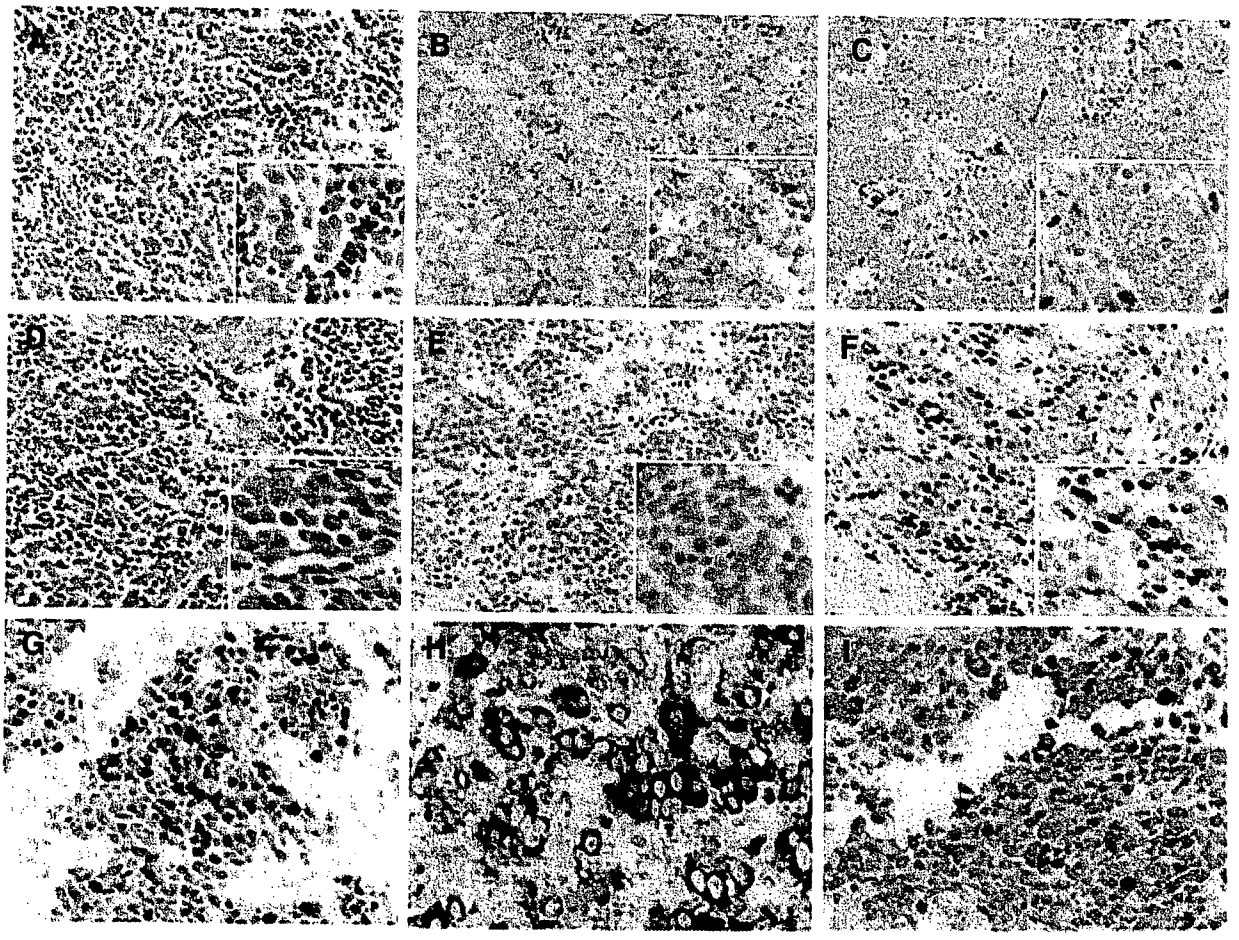

Fig. 2 Immunohistochemical staining of Cap43, Mina53, TrkA, Ki-67, and N-*myc* in neuroblastoma. A, Neuroblastoma (case 39) stained with H&E (×200). B, Cap43 expression in cytoplasm (×200). Inset: high-power view (×400). C, TrkA expression in cytoplasm (×200). Inset: high-power view (×400). D, Neuroblastoma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

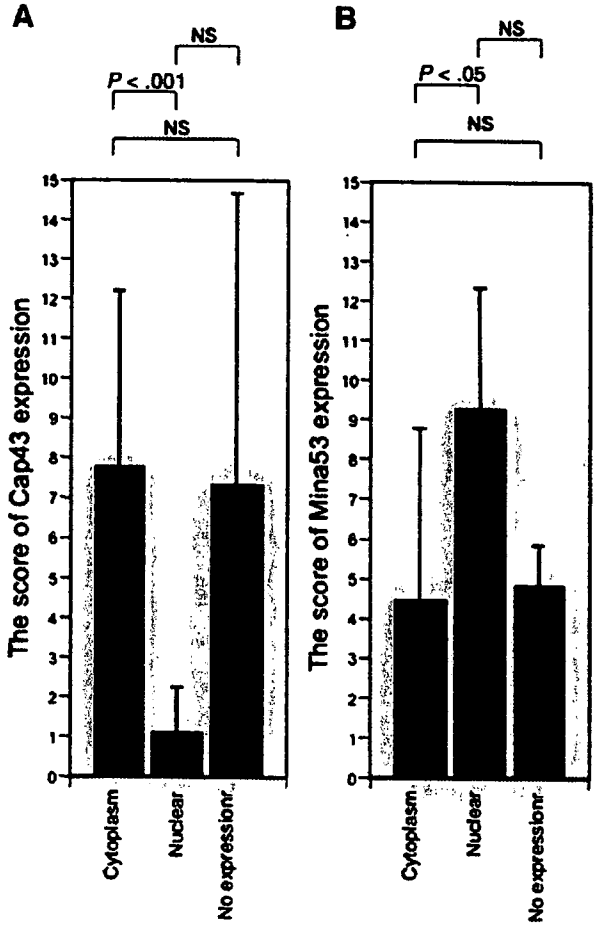

Fig. 3 The relationship between N-*myc* protein expression and the expression of Cap43 or Mina53. The expression scores of Cap43 and Mina53 are compared according to the 3 different groups of N-*myc* protein expression, that is, nucleus group, cytoplasm group, and no expression group. The relationship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

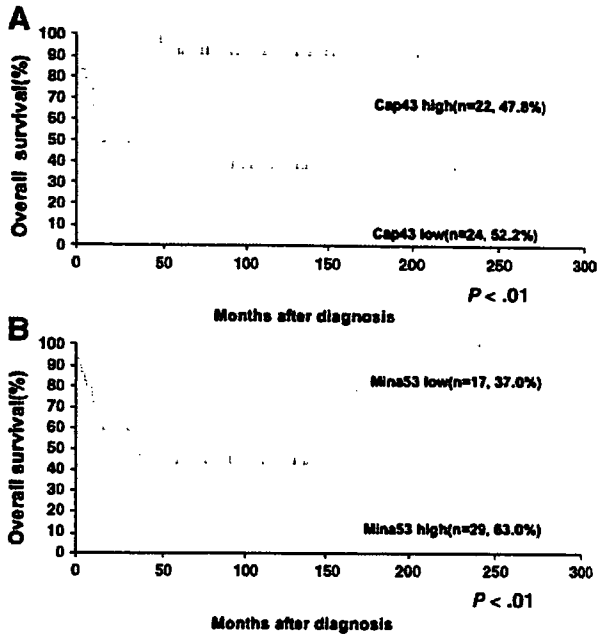

Fig. 4 Correlation between the overall survival rate and the expression of Cap43 or Mina53 analyzed by the Kaplan-Meier method. Forty-six neuroblastoma tumors were divided into high-expression group or low-expression group according to the expression scores of Cap43 and Mina53. The survival rate was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

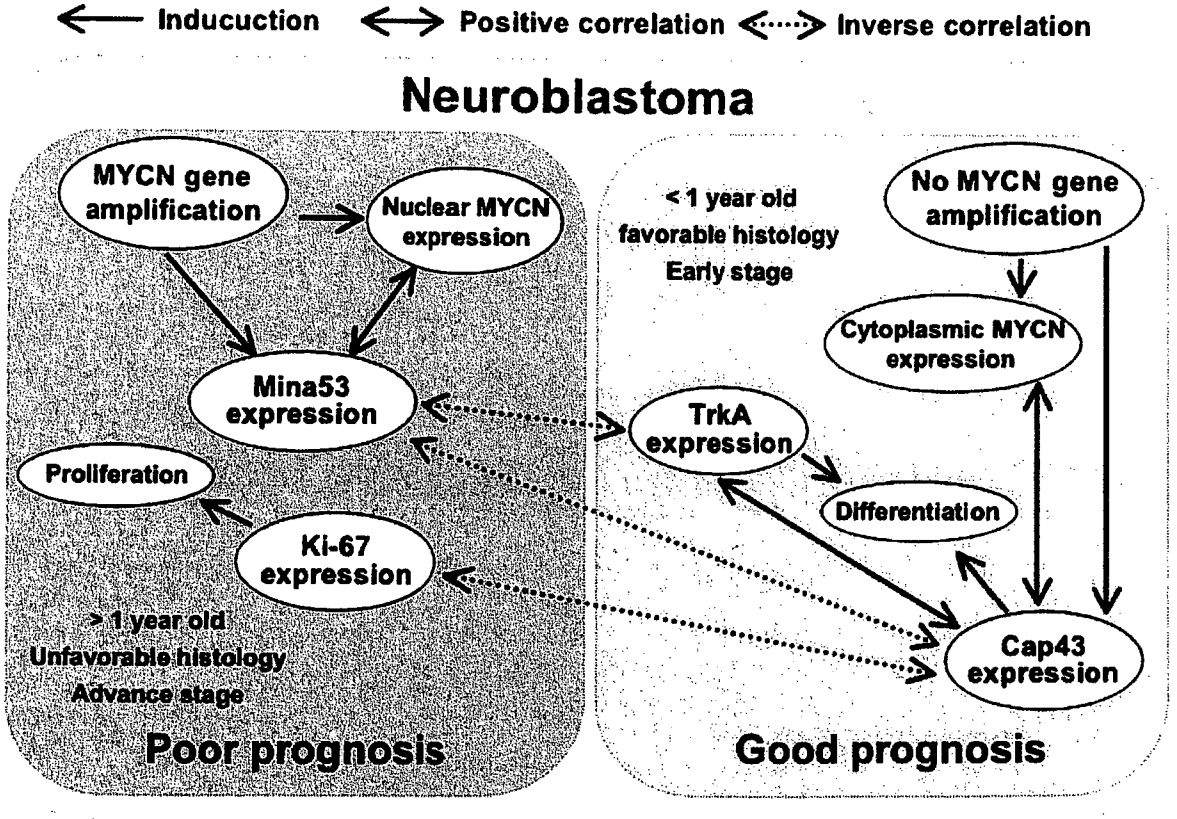

Fig. 5 A schematic drawing showing the possible association of Cap43, Mina53, TrkA, Ki-67, MYCN gene and protein, cell proliferation and differentiation, and prognosis in neuroblastoma. There is an inverse correlation between Ca43 and Mina53, between TrkA and Mina53, and between Cap43 and Ki-67, and 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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References

[1] Crist WM, Kun LE. Common solid tumors of childhood. N Engl J Med 1991;324:461-71.
[2] Krams M, Hero B, Berthold F, et al. Proliferation marker KI-S5 discriminates between favorable and adverse prognosis in advanced stages of neuroblastoma with and without MYCN amplification. Cancer 2002;94:854-61.
[3] Brodeur GM, Nakagawara A. Molecular basis of clinical heterogeneity in neuroblastoma. Am J Pediatr Hematol Oncol 1992;14:111-6.
[4] Tonini GP. Neuroblastoma: a multiple biological disease. Eur J Cancer 1993;29A:802-4.
[5] Morris JA, Shcochat SJ, Smith EI, et al. Biological variables in thoracic neuroblastoma: a Pediatric Oncology Group study. J Pediatr Surg 1995;30:296-302 [discussion 302-3].
[6] Evans AE, Silber JH, Shpilsky A, et al. Successful management of low-stage neuroblastoma without adjuvant therapies: a comparison of two decades, 1972 through 1981 and 1982 through 1992, in a single institution. J Clin Oncol 1996;14:2504-10.