day [16, 23], and the Japanese group considers that the administration over 3 consecutive days may have an advantage over the others, because they already confirmed protracted plasma concentrations of irinotecan with their 3-day administration schedule [17]. The administration schedule for 5 days developed by Blaney et al. [15] was used widely in the Children's Oncology Group, but the results are not published yet. As a single, independent experience, Rosoff and Bayliff [13] administered irinotecan 50 mg/m^2/day for 5 days every 3–4 weeks in 2 children with desmoplastic round blue cell tumors and saw significant responses. In the ongoing Children's Oncology Group study, ARST 0121, in children with relapsed or progressive rhabdomyosarcoma, patients are randomized to receive irinotecan either on days 1–5 or on days 1–5 and 8–12, though this is not a single-agent study [30].

As the effectiveness as well as the toxicity profile of irinotecan differs depending on the schedule, careful clinical care of the patients will be mandatory. The use of rhG-CSF is advisable to prevent granulocytopenia following the administration of irinotecan 180 mg/m^2/day for 3 days.

There have been significant advances in the treatment of advanced neuroblastoma and rhabdomyosarcoma in recent years [18, 19, 31, 32], but the clinical results are still poor, especially once the tumor relapses. The responses reported by us and by others [27] with various tumors suggest that irinotecan is promising, should be explored further in late phase II trials, and might be included as an active agent in the first-line treatment of pediatric solid tumors with poor prognosis.

REFERENCES

[1] Kaneda N, Nagata H, Furuta T, et al. Metabolism and pharmacokinetics of the camptothecin analogue CPT-11 in the mouse. *Cancer Res.* 1990;50:1715–1720.

[2] Pratt CB, Stewart C, Santana VM, et al. Phase I study of topotecan for pediatric patients with malignant solid tumors. *J Clin Oncol.* 1994;12:539–543.

[3] Tsuchida Y, Shitara T. Topotecan and irinotecan in the treatment of pediatric solid tumors. *Curr Pediatr Rev.* 2005;1:55–61.

[4] Verwyij J. Topoisomerase I inhibitors and other new cytotoxic drugs. *Eur J Cancer.* 1995;5:828–830.

[5] Ohno R, Okada K, Masaoka T, et al. An early phase II study of CPT-11, a new derivative of camptothecin, for the treatment of leukemia and lymphoma. *J Clin Oncol.* 1990;8:1907–1912.

[6] Fukutani K, Wakui A, Nakao M, et al. Late phase II study of irinotecan hydrochloride (CPT-11) in advanced gastric cancer. *Jpn J Cancer Chemother.* 1994; 21:1033–1038.

[7] Masuda N, Fukuoka M, Kusunoki Y, et al. CPT-11: a new derivative of camptothecin for the treatment of refractory or relapsed small cell lung cancer. *J Clin Oncol.* 1992; 10:1225–1229.

[8] Fukuoka M, Niitani H, Suzuki A, et al. A phase II study of CPT-11, a new derivative of camptothecin, for previously untreated non-small cell lung cancer. *J Clin Oncol.* 1992;10:16–20.

[9] Noda K, Nishiwaki Y, Kawahara M, et al. Irinotecan plus cisplatin compared with etoposide plus cisplatin for extensive small-cell lung cancer. *N Engl J Med.* 2002;346: 85–91.

[10] Nishida M, Tsunoda H, Ichikawa Y, Yoshikawa H. Complete response to irinotecan hydrochloride and nedaplatin in a patient with advanced ovarian clear cell carcinoma. *Int J Clin Oncol.* 2004;9:403–405.

[11] Moertel CG, Schut AJ, Reitemeier RJ, et al. Phase II study of camptothecin in the treatment of advanced gastro-intestinal cancer. *Cancer Chemother Rep.* 1992;56:95–101.

[12] Shimada Y, Yoshino M, Wakui A, et al. Phase II study of CPT-11, a new camptothecin derivative, in metastatic colorectal cancer. *J Clin Oncol.* 1993;11:909–913.
[13] Rosoff PM, Bayliff S. Successful clinical response to irinotecan in desmoplastic round blue cell tumor. *Med Pediatr Oncol.* 1999;33:500–503.
[14] Furman WL, Stewart CF, Poquette CA, et al. Direct translation of a protracted irinotecan schedule from a xenograft model to a phase I trial in children. *J Clin Oncol.* 1999;17:1815–1824.
[15] Blaney S, Berg SL, Pratt C, et al. Phase I study of irinotecan in pediatric patients: a Pediatric Oncology Group study. Clin Cancer Res. 2001;7:32–37.
[16] Vassal G, Doz F, Frappaz D, et al. A phase I study of irinotecan as a 3-week schedule in children with refractory or recurrent solid tumors. *J Clin Oncol.* 2003;21:3844–3852.
[17] Mugishima H, Matsunaga T, Yagi K, et al. Phase I study of irinotecan in pediatric patients with malignant solid tumors. *J Pediatr Hematol Oncol.* 2002;24:94–100.
[18] Matthay KK, Villablanca JG, Seeger RC, et al. Treatment of high-risk neuroblastoma with intensive chemotherapy, radiotherapy, autologous bone marrow transplantation, and 13-cis-retinoic acid. *N Engl J Med.* 1999;341:1165–1173.
[19] Kawa K, Ohnuma N, Kaneko M, et al. Long-term survivors of advanced neuroblastoma with MYCN amplification: a report of 19 patients surviving disease-free for more than 66 months. *J Clin Oncol.* 1999;17:3216–3220.
[20] Komuro H, Li P, Tsuchida Y, et al. Effects of CPT-11 (a unique DNA topoisomerase I inhibitor) on a highly malignant xeno-transplanted neuroblastoma. *Med Pediatr Oncol.* 1994;23:487–492.
[21] Kamii Y, Tsuchida Y, Yokomori K. Effects of CPT-11 on a human rhabdomyosarcoma in nude mice and in culture. *Int J Pediatr Hematol Oncol.* 1996;3:201–205.
[22] Choi SH, Yang HW, Tsuchida Y. Oral versus intraperitoneal administration of irinotecan in the treatment of human neuroblastoma in nude mice. *Cancer Lett.* 1998;124:15–21.
[23] Vassal G, Doz F, Frappaz D, et al. A phase II study of irinotecan (CPT-11) in children with relapsed or refractory neuroblastoma. *Med Pediatr Oncol.* 2002;39:257.
[24] Society of Japanese Pharmacopoeia. *Summary Basis of Approval No 1, Irinotecan Hydrochloride.* Tokyo: Yakuji Nippo; 1995.
[25] Oken MM, Greech RH, Tormey DC, et al. Toxicity and response criteria of the Eastern Cooperative Oncology Group. *Am J Clin Oncol.* 1982;5:649–655.
[26] Therasse P, Arbuck SG, Eisenhauer EA, et al. New guidelines to evaluate the response to evaluate the response to treatment in solid tumors. *J Natl Cancer Inst.* 2000;92:205–216.
[27] Cosetti M, Wexler LH, Calleja E, et al. Irinotecan for pediatric solid tumors: the Memorial Sloan-Kettering experience. *J Pediatr Hematol Oncol.* 2002;24:101–105.
[28] Shitara T, Shimada A, Tsuchida Y, et al. Successful clinical response to irinotecan in relapsed neuroblastoma. *Med Pediatr Oncol.* 2003;40:126–128.
[29] Houghton PJ, Cheshire PJ, Hallman JD Jr, et al. Efficacy of topoisomerase I inhibitors, topotecan and irinotecan, administered at low dose levels in protracted schedules to mice bearing xenografts of human tumors. *Cancer Chemother Pharmacol.* 1995;36:393–403.
[30] COG-ARST0121: *http://www.cancer.gov/clinicaltrials/COG-ARST0121*
[31] Raney RB, Anderson JR, Barr FR, et al. Rhabdomyosarcoma and undifferentiated sarcoma in the first two decades of life: a selective review of Intergroup Rhabdomyosarcoma Study Group experience and rationale for Intergroup Rhabdomyosarcoma Study V. *J Pediatr Hematol Oncol.* 2001;23:215–220.
[32] Stevens MCG. Rhabdomyosarcoma, In: Voûte PA, Kalifa C, Barrett A, eds. *Cancer in Children: Clinical Management.* Oxford, UK: Oxford University Press; 1998:193–215.

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MYCN gene amplification is a powerful prognostic factor even in infantile neuroblastoma detected by mass screening

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MYCN is the most powerful prognostic factor in cases of older children. However, how *MYCN* is related to the prognosis of infantile cases is not clear. A mass screening program was carried out by measuring urinary catecholamine metabolites (VMA and HVA) from 6-month-old infants. Of 2084 cases detected by the screening program, *MYCN* amplification (MNA) was examined by Southern blot analyses in 1533 cases from 1987 to 2000. Of the 1533 cases examined, 1500 (97.8%) showed no MNA, 20 cases (1.3%) showed MNA from three to nine copies, and 13 (0.8%) cases showed more than 10 copies. The 4-year overall survival rates of these three groups (99, 89 and 53%, respectively) were significantly different ($P<0.001$), indicating that *MYCN* copy number correlates with the prognosis. Cases with MNA more than 10 copies were more advanced than those without amplification (stage III, IV *vs* I, II, IVs; $P<0.001$). Patients with MNA more than 10 copies had significantly higher serum levels of neuron-specific-enolase (NSE) and ferritin than non-amplified patients ($P=0.049$, $P=0.025$, respectively). *MYCN* amplification was strongly correlated with a poor prognosis in infantile neuroblastoma cases. Therefore, for the selection of appropriate treatment, an accurate determination of MNA is indispensable.

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Neuroblastoma (NB) is characterized by heterogeneous tumours, some of which regress spontaneously while others proliferate and progress (D'angio *et al*, 1971; Evans *et al*, 1971; Look *et al*, 1991). The prognosis for NB in infants is much more favourable than it is in older children. In 1973, the Mass Screening Program for Neuroblastoma (MSPN) was commenced for the early detection of NB in children living in Kyoto, Japan. A nationwide MSPN for 6-month-old infants began in 1985 (Sawada *et al*, 1984). The latter MSPN revealed incidences of infantile NB in the early stages and good biological prognostic factors of tumours increased (Hachitanda *et al*, 1994; Sawada *et al*, 1998). However, it has been argued that MSPN might result in the overdiagnosis of tumours, because some of the tumours might spontaneously regress (Yamamoto *et al*, 2002; Honjyo *et al*, 2003). And, the researcher has concluded that the screening was ineffective, because clustered randomized trials have not shown that screening led to a significant reduction in mortality rate from NB (Woods *et al*, 2002; Kerbl *et al*, 2003). Consequently, criticism has arisen that MSPN might detect only redundant tumours with good prognostic factors. Actually, the prognosis in most NB cases detected by MSPN has proved to be good. However, some cases detected by MSPN have poor prognostic factors resulting in relapsed disease (Kusafuka *et al*, 1995). Moreover, there are reports that the good prognosis has been obtained by early treatment in infantile NB cases with poor prognostic factors (Kusafuka *et al*, 1995; Tanaka *et al*, 1998).

Although *MYCN* is well known to be the most powerful prognostic factor in noninfantile cases of NB, how *MYCN* is related to the prognosis of infantile cases, especially those discovered by MSPN, is not clear. Therefore, we assessed *MYCN* amplification (MNA) in infantile cases. If the prognoses of infantile NB cases detected by mass screening and MNA correlate strongly, it is necessary to evaluate MNA to decide on the appropriate treatment for these cases.

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PATIENTS AND METHODS

Analysis of urine catecholamine

Kits for screening children for urinary chatecholamines were provided to the parents at public health centres throughout Japan

when they brought their child in for a health checkup at 3 months of age. Urine was collected by parents at home and sent to screening centres by mail. Urine samples were assayed for vanillylmandelic acid (VMA) and homovanillic acid (HVA) by high-performance liquid chromatography (HPLC). When children's urinary levels of either VMA or HVA were >2.5 s.d. above normal, the child was given clinical examinations for NB at a hospital. The normal range was based on levels in healthy infants of an age-matched (Sawada, 1988).

Patient population

Between April 1987 and March 2000, the population of the target infants was 17 139 975. Of this number, 14 496 103 (84.6%) were screened for elevated catecholamine levels. Of this number, 2084 children were diagnosed as having NB based on urinary catecholamine levels and were registered with the Committee of Neuroblastoma in the Japanese Society of Pediatric Oncology.

Staging The extent of the disease was evaluated according to the Evans's stage classification (Evans *et al*, 1971). The International Staging System (INSS) (Brodeur *et al*, 1993) had not yet been introduced when the MSPN began.

Biological features The prognosis and clinical features of these cases were evaluated on the basis of the MNA. *MYCN* amplification in tumour samples was detected using a Southern blot analysis with *MYCN* second-exon probe according to standard procedures (Brodeur *et al*, 1984). Although cases with 10 copies or more of the *MYCN* gene are classified into the high-risk group in Japan (Kaneko *et al*, 2002), in this study the *MYCN* gene was considered amplified if there were more than three copies.

Registry

The hospitals reported the cases to the registration centre within 2 years of the findings of elevated catecholamine levels in the screening process. The hospital reported the outcome of each case 5 years after the initial diagnosis of NB. However, the outcome of the cases diagnosed between 1999 and 2000 has been 2 years since the appearance of disease.

Statistical analysis

The Kaplan–Meier product limit method was used to estimate the event-free survival (EFS) and overall survival (OS) from the time of diagnosis of NB. The log-rank test was performed to compare the OS probabilities between subgroups of patients. The differences between dichotomous variables were analysed by χ^2 test when samples were of sufficient size. The two-tailed *t*-test was carried out to compare the distributions of continuous variables. A two-tailed *P*-value of <0.05 was considered to indicate statistical significance.

RESULTS

Of 1533 infants with elevated urinary catecholamine levels that were examined for MNA, 33 (2.2%) had tumours with MNA. Of these 33 cases, 20 had *MNA* values from three to nine copies of the *MYCN* gene (Table 1). Seventy-seven percent of cases with no MNA had early stage (stages I, II and IVs) tumours. Thirteen cases had more than 10 copies. Of these, only 30% had early stage tumours. The cases without MNA had significantly higher percentage of early stage tumours than cases with MNA over 10 copies ($P<0.001$) (Table 2).

Treatment and survival rates in patients with MNA

All of the 13 cases with MNA of more than 10 copies received megatherapy with stem cell transplantation and radiotherapy. Six of these cases died. None of 20 cases with MNA from three to nine copies received the megatherapy with stem cell transplantation. Sixteen of the 20 cases received mild chemotherapy, and four cases received only surgical resection without chemotherapy. Only two of the 20 cases died (Table 1). Case 15 had the unresectable tumour of stage III and died of progressive disease although he had received chemotherapy. Case 30 had the resectable tumour with *MYCN* 3 copies by the Southern blot analysis and was not classified into the high-risk group. At 3 months after the operation, this patient had relapse with bone and bone marrow metastasis and died of progressive disease. The primary tumour was judged *MYCN* amplification by the FISH method that was performed after the relapse.

Outcome

Of the 2084 cases that were detected NBs by the screening programme, only 15 cases (0.7%) died within 5 years. OS was 99%. Three-year EFS was 99% for cases without MNA ($n=1500$), 88% for cases with MNA from three to nine copies ($n=20$), and 46% for cases with MNA over 10 copies ($n=13$) ($P<0.001$) (Figure 1). The 4-year OS rate was 99% for cases without MNA, 89% for cases with MNA from three to nine copies and 53% for cases with MNA over 10 copies ($P<0.001$). In the cases with MNA over 10 copies, all of the five cases except one died of progressive disease, though they were received chemotherapy.

Characteristics of patients with and without MNA

Table 2 lists the clinical and biological characteristics of patients with and without MNA. The cases with MNA (>10 copies) were found more frequently in advanced stages (stages III and IV), than the cases without MNA (69 and 23%, respectively; $P<0.001$). Of the cases with MNA (>10 copies), a significantly higher percentage of primary tumours was found in the adrenal glands (92%) than in those without MNA (51%; $P=0.002$). The patients with MNA(>10 copies) had significantly higher serum levels of neuron-specific enolase (NSE) and ferritin than the patients without MNA ($P=0.049$, $P=0.025$, respectively). Although the patients with MNA (>10 copies) had significantly higher urinary levels of HVA than the patients without MNA ($P=0.008$), there was no difference in urinary levels of HVA ($P=0.985$).

Characteristics of patients with MNA

The right side of Table 2 shows clinical and biological characteristics of 33 cases with MNA more than three copies. Patients in advanced stages (stage III and IV) had significantly poorer prognoses (3-year EFS; 58.3%) than those in early stages (stage I, II and IVs) (3-year EFS; 93.3%) ($P=0.021$). The patients with primary tumours found in the adrenal gland had significantly poorer prognoses (3-year EFS; 68%) than those with the tumours at other sites (3-year EFS; 100%) ($P=0.021$). The group with high serum levels of NSE also had a significantly poorer prognosis than the group with low levels of NSE ($P=0.0005$). However, urinary levels of VMA and HVA, and serum levels of ferritin, did not correlate with clinical outcomes ($P=0.364$, 0.478 and 0.174, respectively).

DISCUSSION

It is well known that the prognosis for NB in infants is good. Indeed, the prognosis for NB detected by the Japanese MSPN was excellent, with 98% survival. Although most of the cases detected

Table 1 33 screened patients with MYCN amplification

Case No.	MYCN	Stage	Surgery	Chemotherapy	Radio therapy	Mega therapy	Outcome	Follow-up (year)
1	150	3	CE	VCR, CPM	(−)		NED	0.8
2	>100	4	B	VCR, CPM, VP-16, ADR, CDDP, DTI C	(−)		Tumour death	0.3‡
3	55	4	CE	CPM, VP-16, THP-ADR, CDDP, L-PAM, CBDCA	(−)	Auto-BMT	NED	5.3
4	50	4	CE	(+)	(+) 25 gy	PBSCT	Tumour death	2.3‡
5	50	4	CE	(+)	(−)		Tumour death	0.7†
6	29	4	CE	CPM, VP-16, THP-ADR, CDDP	(−)	PBSCT	Therapy complication	1.0†
7	24	2	CE	VCR, CPM, VP-16, THP-ADR, CDDP	(−)		NED	5.9
8	20	2	CE	CPM, VP-16, THP-ADR, CDDP	(+) 20 gy	PBSCT	Tumour death	2.7‡
9	15	4s	CE	(+)	(−)	Auto-BMT	NED	5.1
10	14	4	B	CPM VP-16, THP-ADR CDDP→refuse	(+) 12 gy		Tumour death	2.5‡
11	12	4	CE	(+)	(−)	CBSCT	NED	2.0
12	10	3	CE	VCR, CPM, CDDP, VP-16	(+) 10 gy		NED	3.30
13	10	4s	CE	CPM, VP16, THP-ADR, CDDP	(−)		NED	4.7
14	6	4s	CE	CPM, VP16, THP-ADR, CDDP	(−)		NED	5.0
15	5.7	3	B	VCR, CPM, VP-16, THP-ADR, CDDP	(+) 30 gy		Tumour death	0.9‡
16	5	2	CE	VCR, CPM	(+) 24 gy		NED	10.2
17	5	2	CE	VCR, CPM, ADR, CDDP	(−)		NED	8.1
18	4–5	4	CE	VCR, CPM, THP-ADR, CDDP	(−)		NED	8.8
19	4	1	CE	VCR, CPM	(−)		NED	6.6
20	4	1	CE	VCR, CPM	(−)		NED	8.7
21	4	1	CE	VCR, CPM	(−)		NED	6.1
22	4	3	PE	CPM, VP-16, ADR, CDDP	(−)		NED	7.5
23	3.7	4s	CE	VCR, CPM, ADR, CDDP	(−)		NED	6.8
24	3	1	CE	(−)	(−)		NED	5.7
25	3	1	CE	(−)	(−)		NED	5.0
26	3	2	CE	VCR, CPM, THP-ADR, CDDP	(−)		NED	4.5
27	3	2	CE	(−)	(−)		NED	6.0
28	3	3	B	CPM, VP-16, THP-ADR, CDDP	(−)		NED	5.1
29	3	3	CE	VCR, CPM, VP-16, THP-ADR, CDDP	(−)	Auto-BMT	NED	2.1
30	3	3	CE	(−)	(−)		Tumour death	0.9‡
31	3	4	CE	CPM, THP-ADR, CDDP	(−)		NED	8.7
32	3	4s	CE	(+)	(−)		NED	7.8
33	2–4	4	B	VCR, CPM, THP-ADR, CDDP	(−)		NED	9.7

by the MSPN had biologically favourable factors, such as no-deletion of 1p and low expression of the *TRK-A* gene, some cases with unfavourable prognostic factors have been reported (Matsunaga *et al*, 2000; Tajiri *et al*, 2001). *MYCN* is one of the most important prognostic factors in NB (Rubie *et al*, 1997; Tonini *et al*, 1997). How *MYCN* is related to the prognosis and clinical features of infantile cases, especially those discovered by MSPN, is not clear. Our large-scale study clarified the frequency and clinical features, including the prognoses, of the infantile NB cases with MNA detected that were detected by MSPN.

Among 1533 cases discovered by the MSPN, 33 cases (2.2%) showed MNA. This frequency is much lower than the 15–22% frequency of MNA cases reported in the United States and Europe (Tonini *et al*, 1997; Brodeur, 2003). In addition, in infants that were less than 1-year-old, the frequency of MNA in our study was lower than that reported in Italy (6.8%) (Tonini *et al*, 1997). This suggests that the MSPN detected a greater number of tumours that spontaneously regressed and/or matured than did the clinical examinations.

MYCN is the powerful prognostic factor in infants whose NB was discovered by the MSPN. The 3-year EFS rates (46%) and 4-year OS rates (53%) for patients with MNA were significantly lower than those for patients without MNA (99.3 and 99%, respectively) ($P<0.001$). According to our previous investigation, the 4-year OS rate for cases less than 12 months old with MNA of over 10 copies, which include clinically detected cases, was 41% (Ikeda *et al*, 2002). The prognosis of cases with MNA detected by MSPN might be comparatively good though prognoses cannot be compared because the researches the survival rates of cases detected clinically and cases detected by MSPN did not investigated at the same time.

The infants with MNA that were detected by MSPN might be considered to have benefited from the early detection provided by the screening. Indeed, among patients with MNA, the 3-year EFS rates (93.3%) of patients in stages I, II and IVs were significantly higher than those in stages III and IV (58.3%). If these cases with MNA were not discovered in the early stage by MSPN, some malignant components of tumours would proliferate and progress. As a result, the tumours would be discovered clinically after the patients were 1-year-old. However, the number of cases with MNA is only a very small proportion (2.2%) of the total cases discovered by MSPN. In addition, it is clear that the number of NB patients increased by introduction of MSPN. Therefore, the effectiveness of MSPN discovery of patients with MNA is unclear.

Furthermore, tumours detected by MSPN might regress spontaneously (Yamamoto *et al*, 1998). Several institutions in Japan recently adopted a conservative approach (the 'wait and see' approach), in which children discovered to have stage I, II or IVs tumours by the MSPN were not given any therapeutic treatment in the expectation that the tumour would spontaneously regress (Yamamoto *et al*, 1998). However, a careful follow-up is necessary in cases detected by MSPN, because some of the cases were found to have MNA in the early stage. Most cases with MNA in this study did not have higher urinary VMA levels than without MNA and then, they were not predicted to have a poor clinical outcome at their initial onset. Even in the early stages (stages I, II and IVs), biopsies are required in order to determine the biological prognostic factors of the tumour.

Moreover, in this study, it became clear that patients with MNA of three to nine copies also had poor prognoses. *MYCN* gene has

Table 2 Characteristics of patients with and without *MYCN* amplification detected by mass screening for neuroblastoma

Patient characteristics	Number of cases (%) MNA (>10) ($n=13$)	MNA (3–9) ($n=20$)	MNA (−) ($n=1500$)	*P*-value	MNA (+, >3) 3-yr EFS	*P*-value
Tumour stage						
I	0 (0)	5 (25)	595 (40)	$P<0.001$[a], $P=0.05$[b]	100	$P=0.021$
II	2 (15)	5 (25)	463 (31)	(1,2,4s/3,4)	86	(1,2,4s/3,4)
III	2 (15)	5 (25)	280 (19)		71	
IV	7 (54)	2 (10)	65 (4)		44	
IVs	2 (15)	3 (15)	97 (6)		100	
Gender						
Female	3 (23)	11 (55)	722 (49)	$P=0.043$[a], $P=0.579$[b]	86	
Male	11 (77)	9 (45)	764 (51)		68	
Primary site						
Adrenal gland	12 (92)	13 (65)	764 (51)	$P=0.002$[a], $P=0.131$[b]	68	$P=0.021$
Other abdominal	0 (0)	3 (15)	456 (30)	(adrenal gland/other site)	100	(adrenal gland/other site)
Chest	1 (8)	3 (15)	224 (15)		100	
Pelvis	0 (0)	1 (5)	50 (3)		100	
Neck	0 (0)	0 (0)	6 (0)			
VMA						
$<20\ \mu g\ mgCr^{-1}$	3 (23)	4 (20)	293 (20)		100	
$21-100\ \mu g\ mgCr^{-1}$	7 (54)	15 (75)	982 (67)		82	
$>101\ \mu g\ mgCr^{-1}$	2 (15)	1 (5)	184 (13)		75	
	(mean:			74.6 $\mu g\ mgCr^{-1}$)	(mean: 54.8 $\mu g\ mgCr^{-1}$)	(mean: 54.4 $\mu g\ mgCr^{-1}$)
$P=0.985$[a], $P=0.977$[b]		$P=0.364$				
HVA						
$<20\ \mu g\ mgCr^{-1}$	0 (0)	2 (10)	206 (14)		100	
$21-100\ \mu g\ mgCr^{-1}$	7 (54)	16 (80)	1084 (74)		78	
>101 ng $mgCr^{-1}$	6 (46)	2 (10)	170 (12)		63	
	(mean:			107.1 $\mu g\ mgCr^{-1}$)	(mean: 66.0 $\mu g\ mgCr^{-1}$)	(mean: 55.6 $\mu g\ mgCr^{-1}$)
$P=0.008$a, $P=0.371$[b]		$P=0.478$				
NSE						
$<15\ ng\ ml^{-1}$	5 (38)	9 (45)	526 (47)		93	
$16-100\ ng\ ml^{-1}$	2 (15)	7 (35)	568 (51)		89	
$>101\ ng\ ml^{-1}$	6 (46)	2 (10)	14 (1)		25	
	(mean: 266.9 $ng\ ml^{-1}$)	(mean: 32.6 $ng\ ml^{-1}$)	(mean: 26.2 $ng\ m^{-1}l$)	$P=0.049$[a], $P=0.285$[b]		$P=0.0005$
Ferritin						
$<30\ ng\ ml^{-1}$	2 (15)	5 (25)	506 (54)		100	
$31-100\ ng\ ml^{-1}$	5 (38)	8 (40)	383 (41)		69	
$>101\ ng\ ml^{-1}$	6 (46)	1 (5)	54 (6)		43	
	(mean: 167.3 $ng\ ml^{-1}$)	(mean: 55.9 $ng\ ml^{-1}$)	(mean: 33.7 $ng\ ml^{-1}$)	$P=0.025$[a], $P=0.032$[b]		$P=0.174$

[a]*P*-value between MNA (>10) and MNA (−). [b]*P*-value between MNA (3–9) and MNA (−).

been analysed by the Southern blotting method for whole tumours, but this method is not able to evaluate the status MNA in individual NB cells. While, the FISH method is able to evaluate MNA individual tumour cells, however, it is difficult to determine the copy number of MNA by the FISH method. *MYCN* amplification was defined as a more than the fourfold increase of *MYCN* signals in relation to the number of chromosomes 2 in FISH method. Moreover, additional copies up to the fourfold were defined as *MYCN* gain (Spitz *et al*, 2004). Spitz reported that 6% of tumours displayed *MYCN* gain and this *MYCN* gain was associated only with a poor outcome in localized or 4s NB cases (Spitz *et al*, 2004). In our study, these patients with MNA of three to nine copies might suggest the *MYCN* gain rather than *MYCN* amplification. In cases 4 and 30, MNA were confirmed by FISH method, however, in all the cases MNA were not confirmed by it. *MYCN* amplification must be determined by adding the FISH method in these cases (Mathew *et al*, 2001).

In the studies of the USA group (COG) and the German group, the therapeutic strategy of surgical resection or observation is recommended for NB patients in stages I or II, regardless of the presence of MNA (Cohn *et al*, 1995; Kawa *et al*, 1999; Berthold and Hero, 2000; Perez *et al*, 2000). However, in Japan, patients with MNA of more than 10 copies are classified as being in a high-risk group. In the protocol for high-risk NB, patients receive intensive chemotherapy combined with stem cell transplantation (Kawa *et al*, 1999; Kaneko *et al*, 2002). Infantile NB patients with MNA as well as patients in the high-risk group more than 1-year-old with MNA of over 10 copies have been receiving intensive chemotherapy (Matsumura and Michon, 2000). In our study 29 of 33 cases with MNA received chemotherapy regardless of the stage. The use of chemotherapy might improve the prognosis of patients with MNA. In the cases with MNA over 10 copies, the treatment strategy including more intensive chemotherapy might be necessary, because five cases except one died of progressive disease. For cases with

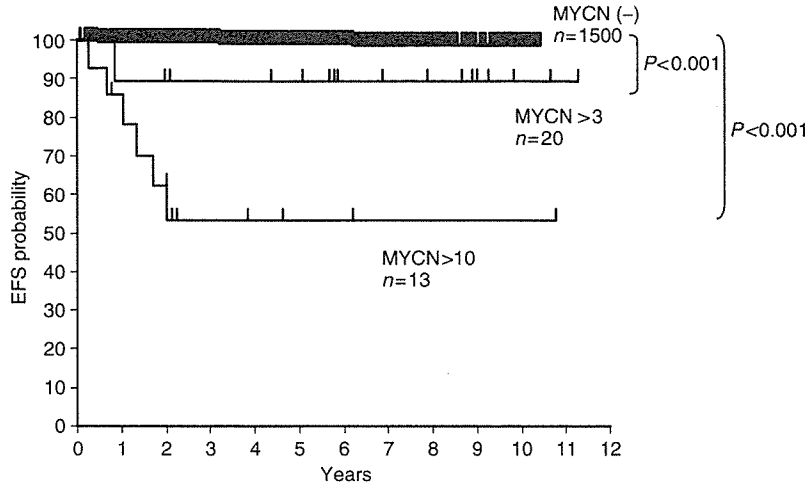

Figure 1 Four-year event-free survival of neuroblastoma infants detected by mass screening based on *MYCN* amplification. The curve was generated with the Kaplan and Meier product limit method. The 4-year OS rate was 99% for patients without MNA, 89% for patients with amplification from three to nine copies, and 53% for patients with more than 10 copies ($P<0.001$).

MNA, it is necessary to establish and perform the appropriate treatment, including not only surgical resection but also chemotherapy.

MYCN amplification was strongly and inversely correlated with the prognosis in infantile cases, although the frequency of MNA in the cases discovered through the MSPN was small (2.2%). Prediction of the presence of MNA in the tumour based on urinary levels of HVA and VMA and stage of the tumour was difficult in the cases we encountered. Our results demonstrate that evaluation of MNA is important for the selection of appropriate treatment for infantile NB.

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REFERENCES

Berthold F, Hero B (2000) Neuroblastoma: current drug therapy recommendations as part of the total treatment approach. *Drugs* **59:** 1261–1277

Brodeur GM (2003) Neuroblastoma: biological insights into a clinical enigma. *Nat Rev Cancer* **3:** 203–216

Brodeur GM, Pritchrd J, Berthold F, Carlsen NL, Castel V, Castelberry RP, De Bernardi B, Evans AE, Favrot M, Hedborg F (1993) Revisions of the international criteria for neuroblastoma diagnosis, staging, and response to treatment. *J Clin Oncol* **11:** 1466–1477

Brodeur GM, Seeger RC, Schwab M, Varmus HE, Bishop JM (1984) Amplification of *N-myc* in untreated human neuroblastomas correlates with advanced disease stage. *Science* **224:** 1121–1124

Cohn SL, Look AT, Joshi VV, Holbrook T, Salwen H, Chagnovich D, Chesler L, Rowe ST, Valentine MB, Komuro H (1995) Lack of correlation of *N-myc* gene amplification with prognosis in localized neuroblastoma: A Pediatric Oncology Group study. *Cancer Res* **55:** 721–726

D'angio GJ, Evans AE, Koop CE (1971) Special pattern of widespread neuroblastoma with a favorable prognosis. *Lancet* **1:** 1046–1049

Evans AE, D'angio GJ, Randolph J (1971) A proposal staging for children with neuroblastoma. *Cancer* **27:** 374–378

Hachitanda Y, Ishimoto K, Hata J, Shimada H (1994) One hundred neuroblastomas detected through a mass screening system in Japan. *Cancer* **74:** 3223–3226

Honjyo S, Doran H, Stiler C, Ajiki W, Tsukuma H, Oshima A, Coleman MP (2003) Neuroblastoma trends in Osaka, Japan, and Great Britain 1970–1994, in relation to screening. *Int J Cancer* **103:** 538–543

Ikeda H, Iehara T, Tsuchida Y, Kaneko M, Hata J, Naito H, Iwafuchi M, Ohnuma N, Mugishima H, Toyoda Y, Hamazaki M, Mimaya J, Kondo S, Kawa K, Okada A, Hiyama E, Suita S, Takamatsu H (2002) Experience with International Neuroblastoma Staging System and Pathology Classification. *Br J Cancer* **86:** 1110–1116

Kaneko M, Tsuchida Y, Mugishima H, Ohnuma N, Yamamoto K, Kawa K, Iwafuchi M, Sawada T, Suita S (2002) Intensified chemotherapy increases the survival rates in patients with stage 4 neuroblastoma with *MYCN* amplification. *J Pediatr Hematol Oncol* **24:** 613–621

Kawa K, Ohnuma N, Kaneko M, Yamamoto K, Etoh T, Mugishima H, Ohhira M, Yokoyama J, Bessho F, Honna T, Yoshizawa J, Nakada K, Iwafuchi M, Nozaki T, Mimaya J, Sawada T, Nakamura T, Miyata H, Yamato K, Tsuchida Y (1999) Long-term survivors of advanced neuroblastoma with *MYCN* amplification: A report of 19 patients surviving disease-free for more than 66 months. *J Clin Oncol* **17:** 3216–3220

Kerbl R, Urban CE, Ambros IM, Dornbusch HJ, Schwinger W, Lackner H, Ladenstein R, Strenger V, Gadner H, Ambros PF (2003) Neuroblastoma mass screening in late infancy: insights into the biology of neuroblastic tumors. *J Clin Oncol* **21:** 4228–4234

Kusafuka T, Nagahara N, Oue T, Imura K, Nakamura T, Kobayashi Y, Komoto Y, Fukuzawa M, Okada A, Nakayama M (1995) Unfavorable DNA ploidy and Ha-ras p21 findings in neuroblastomas detected through mass screening. *Cancer* **76:** 695–699

Look AT, Hayes FA, Shuster JJ, Douglass EC, Castleberry RP, Bowman LC, Smith EI, Brodeur GM (1991) Clinical relevance of tumor cell ploidy and N-myc gene amplification in childhood neuroblastoma: a pediatric oncology group study. *J Clin Oncol* **9:** 581–591

Mathew P, Valentine MB, Bowman LC, Rowe ST, Nash MB, Valentine VA, Cohn SL, Castleberry RP, Brodeur GM, Look AT (2001) Detection of *MYCN* gene amplification in neuroblastoma by fluorescence *in situ* hybridization: a pediatric oncology group study. *Neoplasia* **3:** 105–109

Matsumura T, Michon J (2000) Treatment of localized neuroblastoma. In *Neuroblastoma*, Brodeur GM, Sawada T, Tsuchida Y and Voute PA (eds) pp 403–415. Amsterdam: Elsevier

Matsunaga T, Shirasawa H, Hishiki T, Yoshida H, Kouchi K, Ohtsuka Y, Kawamura K, Etoh T, Ohnuma N (2000) Enhanced expression of *N-myc* messenger RNA in neuroblastomas found by mass screening. *Clin Cancer Res* **6:** 3199–3204

Perez CA, Matthy KK, Atkinsin JB, Seeger RC, Shimada H, Haase GM, Stram DO, Gerbing RB, Lukens JN (2000) Biologic variable in the outcome of stages I and II neuroblastoma treated with surgery as primary therapy: a Children's Cancer Group study. *J Clin Oncol* **18:** 18–26

Rubie H, Hartmann O, Michon J, Frappaz D, Coze C, Chastagner P, Baranzelli MC, Plantaz D, Avet-Loiseau H, Benard J, Delattre O, Favrot M, Peyroulet MC, Thyss A, Perel Y, Bergeron C, Courbon-Collet B, Vannier JP, Lemerle J, Sommelet D (1997) *N-myc* gene amplification is a major prognostic factor in localized neuroblastoma: Results of the French NBL 90 study. *J Clin Oncol* **15:** 1171–1182

Sawada T (1988) Laboratory techniques and neuroblastoma screening. *Lancet* **2:** 1134–1135

Sawada T, Hirayama M, Nakata T, Takeda T, Takasugi N, Mori T, Maeda K, Koide R, Hanawa Y, Tsunoda A (1984) Mass screening for neuroblastoma in infants in Japan. Interim report of a mass screening group. *Lancet* **2:** 271–273

Sawada T, Nishi M, Takeda T, Iehara T (1998) Mass screening for neuroblastoma in Japan. *Med Pediatr Oncol* **31:** 429–434

Spitz R, Hero B, Skowron M, Ernestus K, Berthold F (2004) *MYCN*-status in neuroblastoma: characteristics of tumours showing amplification, gain, and non-amplification. *Eur J Cancer* **40:** 2753–2759

Tajiri T, Suita S, Sera Y, Takamatsu H, Mizote H, Nagasaki A, Kurosaki N, Handa N, Hara T, Okamura J, Miyazaki S, Sugimoto T, Kawakami K, Eguchi H, Tsuneyoshi M (2001) Clinical and Biologic Characteristics for recurring neuroblastoma at mass screening cases in Japan. *Cancer* **92:** 349–353

Tanaka T, Sugimoto T, Sawada T (1998) Prognostic discrimination among neuroblastomas according to *Ha-ras/trk A* gene expression: a comparison of the profiles of neuroblastomas detected clinically and those detected through mass screening. *Cancer* **83:** 1626–1633

Tonini GP, BOni L, Pession A, Rogers D, Iolascon A, Basso G, Cordero di Montezemolo L, Casale F, Pession A, Perri P, Mazzocco K, Scaruffi P, Lo Cunsolo C, Marchese N, Milanaccio C, Conte M, Bruzzi P, De Bernardi B (1997) *MYCN* oncogene amplification in neuroblastoma is associated with prognosis, except in stage 4s: The Italian experience with 295 children. *J Clin Oncol* **15:** 85–93

Woods WG, Gao RN, Shuster JJ, Robison LL, Bernstein M, Weitzman S, Bunin G, Levy I, Brossard J, Dougherty G, Tuchman M, Lemieux B (2002) Screening of infants and mortality due to neuroblastoma. *N Engl J Med* **346:** 1041–1046

Yamamoto K, Hanada R, Kikuchi A, Ichikawa M, Aihara T, Oguma E, Moritani T, Shimanuki Y, Tanimura M, Hayashi Y (1998) Spontaneous regression of localized neuroblastoma detected by mass screening. *J Clin Oncol* **16:** 1265–1269

Yamamoto K, Ohta S, Ito E, Hayashi Y, Asami T, Mabuchi O, Higashigawa M, Tanimura M (2002) Marginal decrease in mortality and marked increase in incidence as result of neuroblastoma screening at 6 months of age: Cohort study in seven prefectures in Japan. *J Clin Oncol* **20:** 1209–1214

Appendix A1

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Biological diagnosis for neuroblastoma using the combination of highly sensitive analysis of prognostic factors

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Index words:
Neuroblastoma;
MYCN;
Survivin;
BIN1;
Quantitative PCR

Abstract

Background/Purpose: To select the optimal treatment according to the degree of malignancy of neuroblastoma, it is essential to accurately and rapidly identify any genetic abnormalities associated with the prognosis. This study aims to assess the correlation between the combination of prognostic factors and the biologic findings of neuroblastoma using a highly sensitive analysis of prognostic factors.

Methods: In 44 neuroblastoma primary samples, we determined the gene dosages of *MYCN* and *Survivin* (as the target of 17q gain) and the expression levels of *MYCN*, *Survivin*, and *BIN1* using highly sensitive analysis (the quantitative polymerase chain reaction method); furthermore, we assessed the correlation between the combination of their prognostic factors and the biology of neuroblastoma.

Results: The gene dosage of *MYCN* or *Survivin* was significantly associated with all known prognostic factors. The expression level of *MYCN* or *Survivin* was not significantly associated with any prognostic factors, whereas the expression level of *BIN1* was significantly associated with 5 of 6 prognostic factors. Regarding the combination of *MYCN* amplification and 17q gain (the gene dosage of *Survivin*), and the low expression of *BIN1*, the rates of advanced stages (stage III or IV) were 100% for the cases with 3 factors, 63% for the cases with 2 factors, 42% for the cases with 1 factor, and 0% for the cases with null factor. Furthermore, the survival rates were 20% for the cases with 3 factors, 50% for the cases with 2 factors, 100% for the cases with 1 factor, and 100% for the cases with null factor.

Conclusion: The combination of gene dosages of *MYCN* and *Survivin* and the expression level of *BIN1* using the quantitative polymerase chain reaction method was significantly correlated with the clinical stage and the patients' outcome. This combination of biologic factors may enhance the accuracy to the conventional criteria, but this would have to be shown in a much larger study that is adequately powered to detect such an advantage.

Neuroblastoma is the most common solid tumor in children, and its development is still unclear [1]. The prognosis in neuroblastoma tends to vary greatly, and many studies have demonstrated both clinical and biologic

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factors to be correlated with the outcome [2]. To select the optimal treatment according to the degree of malignancy of neuroblastoma, it is essential to accurately and rapidly identify any genetic abnormality associated with the prognosis.

The amplification of the *MYCN* gene is the most unfavorable prognostic factor in neuroblastoma [3]. Approximately 20% to 30% of all patients presenting at advanced stages show an amplification of the *MYCN* gene, using the Southern blot method [4]. Regarding the *MYCN* gene, it is easy to consider that the amplification of *MYCN* gene results in an enhanced expression of *MYCN*, which activates the transcription of genes associated with the cell proliferation [5]. However, the clinical significance of *MYCN* expression in children with neuroblastoma remains controversial [6,7]. On the other hand, a gain of the chromosome 17q region has recently been implicated in close correlation with the aggressiveness of neuroblastoma, using either a comparative genomic hybridization study or the fluorescence in situ hybridization (FISH) method [8]. In particular, the gain of the q arm only has also been reported to play a role in an unfavorable outcome [9]. The region has been narrowed down to the 17q21-terminal, which is also considered to include the *Survivin* gene [10]. *Survivin* is a family member of an inhibitor of apoptosis proteins, and its expression is also cell cycle–regulated [11]. Recently, a high-level expression of *Survivin* in advanced stages of neuroblastoma has been shown, and it is thus considered to be one of the candidate genes for 17q gain [12]. We preliminary reported the quantitative polymerase chain reaction (PCR) method (TaqMan method) to be useful as a quick and accurate modality for evaluating for the status of *MYCN* amplification and the gene dosage of *Survivin* as the target of 17q gain in 25 neuroblastoma samples [13].

BIN1 (2q14) encodes multiple tissue-specific isoforms of an Myc-interacting adaptor protein that has features of a tumor suppressor, including the ability to inhibit Myc-mediated cell transformation and promote apoptosis [14]. We previously hypothesized that *BIN1* may function as a suppressor gene in neuroblastoma because *BIN1* is highly expressed in neural tissues and binds Myc within a region with 100% identity to *MYCN*, and reported data correlating reduced expression of *MYCN*-interacting *BIN1* isoforms with unfavorable features in primary neuroblastoma [15].

The quantitative TaqMan PCR System determines the initial copy number of the target gene by a kinetic analysis of the cycle-to-cycle change in the fluorescence signal as a result of the amplification of the template during PCR. Using this system, the detection of a loss of the 18q21 region in colon carcinoma tissue [16] and sex determination by defining the copy number of X chromosome have been reported. The advantage of quantitative PCR over Southern blotting, FISH, and comparative genomic hybridization is its speed (it takes about 4 hours from DNA extraction to the end of data analysis). Furthermore, this method is considered to have a high sensitivity [17].

In this study, we determined altogether the gene dosages of *MYCN* and *Survivin* (as the target of 17q gain) and the expression levels of *MYCN*, *Survivin*, and *BIN1* using highly sensitive analysis (the quantitative PCR method) in 44 neuroblastoma primary samples; furthermore, we assessed the correlation between the combination of their prognostic factors and the biology of neuroblastoma.

1. Materials and methods

1.1. Clinical data of patients and biologic data of neuroblastoma samples

Patients with neuroblastoma evaluated at the Department of Pediatric Surgery, Kyushu University, were diagnosed and staged according to the International Neuroblastoma Staging System [18]. Forty-four frozen tumor samples were obtained from untreated patients with neuroblastoma. The characteristics of the patients were shown to be as follows. The sex of the patients was 26 males and 18 females, and the age at diagnosis ranged from 19 days after birth to 11 years of age. Of the 44 cases, 15 patients were diagnosed at older than 1 year, whereas the remaining 29 were diagnosed at younger than 1 year. Twenty-four patients were identified by a neuroblastoma mass screening system. Of the 44 samples, 29 were tumors from patients who were stage I, II, or IVS, whereas 3 were stage III and 12 were stage IV. Thirty-six patients are still alive, of whom 3 cases are still under treatment, whereas 8 patients have died of the disease. The follow-up period after treatment ranged from 1 month to 12 years. In all 44 samples, the status of *MYCN* amplification was also previously determined by the Southern blotting method. In 36 of 44 cases with a single copy of *MYCN* identified by Southern blotting. DNA ploidy was previously examined using flow cytometry in 32 cases. Twenty-three cases were triploid, whereas 9 cases were diploid or tetraploid. Regarding the histologic findings, all 44 cases were classified based on the Shimada classification [19]. Thirty-two cases showed a favorable histology, whereas the remaining 12 cases showed an unfavorable histology. Regarding International Neuroblastoma Risk Group (INRG), all 44 cases were classified by age, stage (International Neuroblastoma Staging System), the status of *MYCN* amplification (Southern blot), DNA ploidy, and Shimada classification. Thirty cases showed a not high-risk group, whereas the remaining 14 cases showed a high-risk group.

1.2. DNA or RNA extraction and complementary DNA synthesis

DNA was extracted from the frozen tumor samples using proteinase K and phenol. Isogen LS (Nippon Gene, Osaka, Japan) was used to extract total RNA, and reverse transcription was performed with a First-strand complementary DNA synthesis kit (Amersham Pharmacia, Uppsala, Sweden) using random hexanucleotide primers.

Table 1 The sequences of the PCR primers and TaqMan probes for quantitative PCR

Target gene	Forward primer	Reverse primer	TaqMan probe
MYCN	5′-CCC AGC GTG GTA GTC AAT GA-3′	5′-TTA ATG ACA AAG CCA TAA TCC ACA G-3′	5′-AGA ATG CGC ACA TGA TGC TAC ACG TTT CT-3′
Survivin	5′-GGG CTG CCA CGT CCA C-3′	5′-GTC GTC ATC TGG CTC CCA-3′	5′-TTC ATC CAC TGC CCC ACT GAG AAC GA-3′
p53	5′-GCC CTT ACT TGT CAT GGC GA-3′	5′-ATC CCA CAA CCC CTG CG-3′	5′-TGT CCA GCT TTG TGC CAG GAG CC-3′

1.3. Quantitative PCR (TaqMan)

As previously described, the *p53* gene was used as an internal control gene to obtain the gene dosage (*MYCN/p53*, *Survivin/p53*). The p53 gene is a tumor suppressor gene in which mutations or deletions are found in a variety of malignant tumors. However, no aberration of the p53 gene in neuroblastoma has ever been found, and the gene status in neuroblastoma is known to be stable [20]. The corrected gene dosage of the *MYCN* gene and *Survivin* gene was obtained based on the assumption that the mean gene dosage of 20 normal individual lymphocytes was 1.00. The mean ± 2SD of *MYCN* gene dosage of 20 normal individual lymphocytes was 1.00 ± 0.58. In this study, we evaluated that the *MYCN* amplified cells apparently present in the samples with a corrected gene dosage (*MYCN/p53*) of more than 2.0. The mean ± 2SD of *Survivin* gene dosage of 20 normal individual lymphocytes was 1.00 ± 0.40. In this study, we evaluated that the *Survivin* amplified cells apparently present in the samples with a corrected gene dosage (*Survivin/p53*) of more than 1.50. The primers and TaqMan probes for the *MYCN* gene, *Survivin* gene, and the *p53* gene were designed using the application-based primer design software Primer Express (Applied Biosystems, Foster City, Calif). The sequences of the PCR primers and TaqMan probes were shown in Table 1. Quantitative PCR was performed in a final volume 25 μL, and each sample was analyzed in duplicate. Each reaction mixture contained 0.1 pmol/μL TaqMan probe, 0.2 pmol/μL each primer, 1× TaqMan PCR master mix, and 10 to 50 ng DNA. Thermal cycling was started with a 2 minutes incubation at 50°C, followed by a first denaturation step of 10 minutes at 95°C, and then 40 cycles of 2-step PCR consisting of 95°C for 5 seconds and 60°C for 1 minute. The quantification of the *MYCN* gene was achieved by means of the ABI Prism 7700 Sequence Detection System (Applied Biosystems). Genomic DNA from 1 neuroblastoma with 90 copies of *MYCN* by Southern blotting method was serially diluted to establish the calibration curve.

1.4. Quantitative reverse transcriptase PCR (TaqMan)

The primers and TaqMan probes were designed to be located on exons 2 to 3 for *MYCN* messenger RNA (mRNA), exons 2 to 3 for *Survivin* mRNA, and on exons 9 to 11 for *BIN1* mRNA, hereby avoiding the amplification contaminating genomic DNA. *GAPDH* was used as an internal control gene to analyze the *MYCN* gene expression (*MYCN/GAPDH*). The sequences of the PCR primers and TaqMan probe were shown in Table 2. Polymerase chain reaction primer and TaqMan probe for GAPDH were purchased from ABI as a kit of TaqMan GAPDH Control Regent and Predeveloped TaqMan Assay Regents Control Kit. The quantitative reverse transcriptase polymerase chain reaction (RT-PCR) system was performed in the same manner as that for the quantitative PCR.

1.5. Statistical analysis

Fisher's Exact test was used to test the association between *MYCN* amplification (*MYCN/p53*, ≥2.0) or 17q gain (*Survivin/p53*, ≥1.50) and other prognostic factors. The expression levels of *MYCN* (*MYCN/p53*), *Survivin* (*Survivin/GAPDH*), and *BIN1* (*BIN1/GAPDH*) in the subgroups were represented by percentile (50%). A comparison of the gene dosage and expression in relation to clinical and genetic parameters was made using Mann-Whitney *U* test. Kruskal-Wallis exact test was used to test the association between the clinical stage or the patients' outcome and the combination of 3 prognostic factors.

Table 2 The sequences of the PCR primers and TaqMan probes for quantitative RT-PCR

Target gene	Forward primer	Reverse primer	TaqMan probe
MYCN	5′-GAC CAC AAG GCC CTC AGT ACC-3′	5′-TGA CCA CGT CGA TTT CTT CCT-3′	5′-CCG GAG AGG ACA CCC TGA GCG A-3′
Survivin	5′-GAC GAC CCC ATA GAG GAA CAT AA-3′	5′-GGG TTA ATT CTT CAA ACT GCT TCT TG-3′	5′-CGT CCG GTT GCG CTT TCC TTT CT-3′
BIN1	5′-AAG GCC CAG CCC AGT GAC-3′	5′-GAG CCA TCT GGA GGC GAA G-3′	5′-CGC GCC TGC AAA AGG GAA CAA GA-3′

2. Results

2.1. The gene dosages of MYCN and Survivin by the quantitative PCR method

Of the 36 samples with a single copy of *MYCN* based on the Southern blotting method, 33 samples showed the corrected gene dosage (*MYCN/p53*) to be less than 2.0, whereas the remaining 3 samples with more than 2.0 had tumors from patients with an advanced stage of disease (stages III and IV). Of the 3 samples with a dosage of more than 2.0, 2 cases died of the disease. In 8 cases with more than 2 copies of *MYCN* based on the Southern blotting method, the corrected *MYCN* gene dosages by the quantitative PCR were all more than 10.0. In most of these cases, the analytic value based on the quantitative PCR was shown to be a higher than based on a Southern blotting analysis. The relationship between the *MYCN* gene dosage and the known prognostic factors (age, clinical stage, Shimada classification, INRG) is shown in Table 3. The group of cases with a gene dosage of more than 2.0 were strongly associated with an age of older than 1 year at diagnosis ($P < .001$), advanced stage ($P < .001$), a Shimada unfavorable histology ($P < .001$), and a high-risk group ($P < .001$), which are all unfavorable factors.

The corrected *Survivin* gene dosages ranged from 0.55 to 4.00. Ten cases showed that the *Survivin* gene dosages were more than 1.50-fold, and 6 of 10 cases were dead of disease. On the other hand, 32 of 34 cases with the *Survivin* gene dosages of less than 1.50-fold were free of disease. The relationship between the *Survivin* gene dosage and the known prognostic factors (age, clinical stage, Shimada classification, INRG) is shown in Table 3. The group of cases with a gene dosage of more than 2.0 was strongly associated with an age of older than 1 year at diagnosis ($P < .001$), advanced stage ($P < .001$), a Shimada unfavorable histology ($P < .001$), and a high-risk group ($P < .001$), which are all unfavorable factors.

Furthermore, we analyzed 20 samples that are clinically detected but not detected through mass screening. The relationship between the gene dosages *MYCN* or *Survivin* and the known prognostic factors was with the same trends compared with the results for all 44 samples. The gene dosage of *MYCN* was significantly associated with all 4 factors ($P < .01$), and the gene dosage of *Survivin* was significantly associated with 2 factors (age and Shimada, $P < .05$).

2.2. The expression level of MYCN, Survivin, and BIN1 by the quantitative RT-PCR method

The relationship between the *MYCN* gene, *Survivin* gene, or *BIN1* gene expression level and prognostic factors is shown in Table 4.

The level of *MYCN* expression in cases with *MYCN* amplification (*MYCN/p53*, ≥2.0) had a trend toward higher than that of cases with no *MYCN* amplification (*MYCN/p53*, <2.0); however, this finding was not statistically significant ($P = .15$). Furthermore, the expression level of *MYCN* was not significantly associated with any other prognostic factor (age, clinical stage, Shimada classification, the gene dosage of *MYCN* and *Survivin*, and INRG).

The level of *Survivin* expression was not significantly associated with the gene dosage of *Survivin*. In addition, the expression level of *Survivin* was not significantly associated with any other prognostic factor (age, clinical stage, Shimada classification, the gene dosage of *MYCN* and *Survivin*, and INRG).

The expression level of *BIN1* was significantly associated with 5 of 6 prognostic factors. Regarding 5 prognostic factors except the factor of age, the level of *BIN1* expression in neuroblastoma with the unfavorable factor was significantly lower than that in neuroblastoma with the favorable factor.

In addition, we analyzed 20 samples that are clinically detected but not detected through mass screening. The

Table 3 Gene dosage of *MYCN* and *Survivin* in relation to clinical and biologic prognostic factors

Category	n	*MYCN/p53*		*P*	*Survivin/p53*		*P*
		<2.0	≥2.0		<1.5	≥1.5	
Age (y)							
<1	29	27 (93.1)	2 (6.9)	<.01	28 (96.6)	1 (3.4)	<.01
≥1	15	6 (40.0)	9 (60.0)		6 (40.0)	9 (60.0)	
Stage							
Stage I, II, IVS	29	28 (96.6)	1 (3.4)	<.01	27 (93.1)	2 (6.9)	<.01
Stage III, IV	15	5 (33.3)	10 (66.7)		7 (46.7)	8 (53.3)	
Shimada							
Favorable	32	30 (93.8)	2 (6.2)	<.01	30 (93.8)	2 (6.2)	<.01
Unfavorable	12	3 (25.0)	9 (75.0)		4 (33.3)	8 (66.7)	
INRG							
Not high risk	30	29 (96.7)	1 (3.3)	<.01	28 (93.3)	2 (6.7)	<.01
High risk	14	4 (28.6)	10 (71.4)		6 (42.9)	8 (57.1)	

Values are presented as n (%). *P* value was determined by Fisher's Exact test.

Table 4 Expression of *MYCN*, *Survivin*, and *BIN1* in relation to clinical and biologic prognostic factors

Category	n	*MYCN*/GAPDH 50 percentile	*P*	*Survivin/GAPDH* 50 percentile	*P*	*BIN1/GAPDH* 50 percentile	*P*
Age (y)							
<1	29	0.22	.78	0.30	.79	1.17	.08
≥1	15	0.25		0.32		0.41	
Stage							
Stage I, II, IVS	29	0.15	.36	0.28	.42	1.36	<.01
Stage III, IV	15	0.52		0.5		0.41	
Shimada							
Favorable	32	0.19	.51	0.29	.43	1.21	<.01
Unfavorable	12	0.44		0.41		0.21	
Survivin/p53							
<1.5	34	0.31	.20	0.40	.94	1.15	<.05
≥1.5	10	0.08		0.27		0.38	
MYCN/p53							
<2.0	33	0.15	.15	0.34	.79	1.50	<.01
≥2.0	11	0.52		0.25		0.07	
INRG							
Not high risk	30	0.15	.33	0.29	.35	1.31	<.01
High risk	14	0.49		0.41		0.21	

P value was determined by Mann-Whitney *U* test.

relationship between the expression level of *MYCN*, *Survivin*, and *BIN1*, and the known prognostic factors was with the same trends compared with the results for all 44 samples. The expression level of *BIN1* was significantly associated with 4 factors (clinical stage, Shimada classification, the gene dosage of *MYCN*, and INRG; $P < .05$).

2.3. Evaluation of biology for neuroblastomas using the combination of 3 prognostic factors

In the highly sensitive analysis of prognostic factors in this study, the gene dosage of *MYCN*, the gene dosage of *Survivin*, and the level of *BIN1* expression were significant prognostic factors. The relationship between these 3 unfavorable prognostic factors (*MYCN* amplification—*MYCN/p53*, ≥2.0; 17q gain—*Survivin/p53*, ≥1.50; the low expression of *BIN1*—*BIN1/GAPDH*, <1.0) and clinical behavior (clinical stage and outcome) is shown in Fig. 1. Regarding the combination of *MYCN* amplification and 17q gain, and the low expression of *BIN1*, the rates of advanced stages (stages III and IV) were 100% for the cases with 3 factors, 63% for the cases with 2 factors, 42% for the cases with 1 factors, and 0% for the cases with null factor ($P < .001$, trend test using Kruskal-Wallis exact test). Furthermore, the survival rates were 20% for the

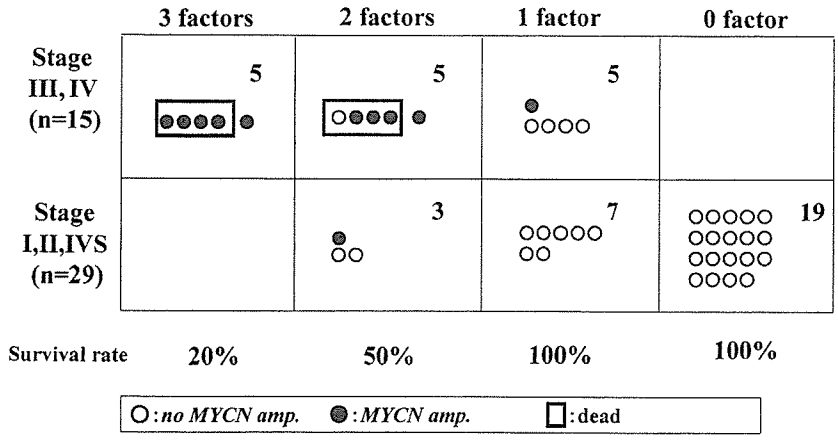

Fig. 1 The correlation of 3 unfavorable factors (*MYCN* amplification—*MYCN/p53*, ≥2.0; 17q gain—*Survivin/p53*, ≥1.50; the low expression of *BIN1*—*BIN1/GAPDH*, <1.0) and the clinical behavior in neuroblastomas. The rates of advanced stages (stages III and IV) were 100% for the cases with 3 factors, 63% (5/8) for the cases with 2 factors, 42% (5/12) for the cases with 1 factors, and 0% (0/19) for the cases with null factor ($P < .001$, trend test using Kruskal-Wallis exact test). The survival rates were 20% for the cases with 3 factors, 50% for the cases with 2 factors, 100% for the cases with 1 factor, and 100% for the cases with null factor ($P < .001$, trend test using Kruskal-Wallis exact test).

cases with 3 factors, 50% for the cases with 2 factors, 100% for the cases with 1 factor, and 100% for the cases with null factor ($P < .001$, trend test using Kruskal-Wallis exact test).

3. Discussion

Neuroblastomas have a variety of genetic variables that might predict the clinical behavior [2]. To select the optimal treatment according to the degree of malignancy of neuroblastoma, it is essential to accurately and rapidly identify any genetic heterogeneity associated with the prognosis. Generally, the gene dosage was analyzed by Southern blot method or FISH, whereas the expression level of gene was assessed by Northern blot method or semiquantitative PCR method. We previously reported that the quantitative PCR method may be considered to be the most effective methods for quickly and accurately evaluating any aberration in the gene dosages associated with the patients' outcomes [13,21]. In this study, we determined altogether the gene dosages of *MYCN* and *Survivin* (as the target of 17q gain) and the expression levels of *MYCN*, *Survivin*, and *BIN1* using highly sensitive analysis (the quantitative PCR method) in 44 neuroblastoma primary samples.

Regarding the *MYCN* gene, the amplification of the *MYCN* gene is strongly associated with rapid tumor progression [3,4]; however, the clinical significance of *MYCN* expression in children with neuroblastoma remains controversial [6,7]. In the present study, the gene dosage of *MYCN* was significantly associated with all prognostic factors, whereas the expression level was not significantly associated with any prognostic factor. Furthermore, the significant association between the gene dosage and the expression level was not observed. These findings are suggesting that the only gene dosage of *MYCN* does not always contribute to the level of *MYCN* expression in neuroblastoma, and the expression level of *MYCN* does not seem to be an independently significant prognostic factor in this highly sensitive analysis.

Regarding the *Survivin* gene, we assumed the *Survivin* gene could be one of the candidate genes for the 17q gain in neuroblastoma. In the present study, the gene dosage of *Survivin* was significantly associated with all prognostic factors, whereas the expression level was not significantly associated with any prognostic factor. In addition, there was no correlation between the *Survivin* gene dosage and the expression level. These results are demonstrating that analysis of the gene dosage of *Survivin* is useful for evaluating the 17q gain; however, the *Survivin* was not the candidate gene for 17q gain in neuroblastoma.

It is unclear why gene dosage is more correlative with risk than expression level. The chromosomal gain or loss may be correlated with the genomic instability, which is associated with poor prognosis in adult cancers [22]. The genomic instability may generate *MYCN* amplification or 17q gain as one of chromosomal alteration in neuroblastoma.

Taken together, the gene dosage of *MYCN* (*MYCN* amplification), the gene dosage of *Survivin* (17q gain), and the level of *BIN1* expression were significant prognostic factors in the highly sensitive analysis using the quantitative PCR method. Furthermore, the combination of gene dosages of *MYCN* and *Survivin* and the expression level of *BIN1* using the quantitative PCR method was substantially correlated with the clinical stage and the patients' outcome. The current protocol for neuroblastoma in the world is mainly based on the age, clinical stage, and *MYCN* amplification. In the Study Group of Japan for Advanced Neuroblastoma, 2 chemotherapeutic regimens for advanced neuroblastoma have been designed based on the *MYCN* amplification status since 1991 [23]. However, the status of *MYCN* amplification does not necessarily predict the patients' outcome. This combination of biologic factors may enhance the accuracy to the conventional criteria (*MYCN*, Shimada classification), but this would have to be shown in a much larger study that is adequately powered to detect such an advantage.

References

[1] Crist WM, Kun LE. Common solid tumors of childhood. N Engl J Med 1991;324:461-71.
[2] Brodeur GM. Molecular basis for heterogeneity in human neuroblastoma. Eur J Cancer 1995;31:505-10.
[3] Brodeur GM, Maris JM, Yamashiro DJ, et al. Biology and genetics of human neuroblastomas. J Pediatr Hematol Oncol 1997;19:93-101.
[4] Brodeur GM, Seeger RC, Schwab M, et al. Amplification of *MYCN* in untreated human neuroblastomas correlates with advanced disease stage. Science 1984;224:1121-4.
[5] Lutz W, Stohr M, Schurmann J, et al. Conditional expression of N-*myc* in human neuroblastoma cells increases expression of alpha-prothymosin and ornithine decarboxylase and accelerates progression into S-phase early after mitogenic stimulation of quiescent cells. Oncogene 1996;13:803-12.
[6] Bordow SB, Norris MD, Haber PS, et al. Prognostic significance of *MYCN* oncogene expression in childhood neuroblastoma. J Clin Oncol 1998;16:3286-94.
[7] Seeger RC, Wada R, Brodeur GM, et al. Expression of N-*myc* by neuroblastomas with one or multiple copies of the oncogene. Prog Clin Biol Res 1998;271:41-9.
[8] Caron HN. Allelic loss of chromosome 1 and additional chromosome 17 material are both unfavorable prognostic markers in neuroblastoma. Med Pediatr Oncol 1995;24:215-21.
[9] Lastowska M, Cotterill S, Pearson ADJ, et al. Gain of chromosome arm 17q predicts unfavorable outcome in neuroblastoma patients. Eur J Cancer 1997;33:1627-33.
[10] Plantaz D, Mohapatra G, Matthay KK, et al. Gain of chromosome 17 is the most frequent abnormality detected in neuroblastoma by comparative genomic hybridization. Am J Pathol 1997;150:81-9.
[11] Adida C, Crotty PL, McGrath J, et al. Developmentally regulated expression of the novel cancer anti-apoptosis gene *Survivin* in human and mouse differentiation. Am J Pathol 1998;19:617-23.
[12] Islam A, Kageyama H, Takada N, et al. High expression of *Survivin*, mapped to 17q25, is significantly associated with poor prognostic factors and promotes cell survival in human neuroblastoma. Oncogene 2000;19:617-23.

[13] Tajiri T, Tanaka S, Shono K, et al. Quick quantitative analysis of gene dosages associated with prognosis in neuroblastoma. Cancer Lett 2001;166:89-94.
[14] Hogarty MD, Liu X, Thompson PM, et al. *BIN1* inhibits colony formation and induces apoptosis in neuroblastoma cell lines with *MYCN* amplification. Med Pediatr Oncol 2000;35:559-62.
[15] Tajiri T, Tanaka S, Suita S, et al. Expression of a *MYCN*-interacting isoform of the tumor suppressor *BIN1* is reduced in neuroblastomas with unfavorable biological features. Clin Cancer Res 2003;9:3345-55.
[16] Laurendeau I, Bahuau M, Vodovar N, et al. TaqMan PCR–based gene dosage assay for predictive testing in individuals from a cancer family with INK 4 locus haploinsufficiency. Clin Chem 1999;45: 982-6.
[17] Kariyazono H, Ihara K, Ohno T, et al. Rapid detection of 22q11.2 deletion with quantitative real-time PCR. Mol Cell Probes 2001; 15:71-3.
[18] Brodeur GM, Pritchard J, Berthold F, et al. Revision of the international criteria for neuroblastoma diagnosis, staging, and response to treatment. J Clin Oncol 1993;11:1466-77.
[19] Shimada H, Chatten J, Newton WA. Histopathologic prognostic factors in neuroblastic tumors: definition of subtypes of ganglio-neuroblastoma and an age-linked classification of neuroblastomas. J Natl Cancer Inst 1984;73:405-16.
[20] Kusafuka T, Fukuzawa M, Oue T, et al. Mutation analysis of p53 gene in childhood malignant solid tumors. J Pediatr Surg 1997;32:1175-80.
[21] Tanaka S, Tajiri T, Suita S, et al. Clinical significance of a highly sensitive analysis for gene dosage and the expression level of *MYCN* in neuroblastoma. J Pediatr Surg 2004;39:63-8.
[22] Diaz Jr LA. The current clinical value of genomic instability. Semin Cancer Biol 2005;15:67-71.
[23] Kaneko M, Nishihira H, Mugishima H, et al. Stratification of treatment of stage 4 neuroblastoma patients based on N-*myc* amplification status. Med Pediatr Oncol 1999;31:1-7.

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ORIGINAL ARTICLE

Increased expression of proapoptotic *BMCC1*, a novel gene with the *BNIP2* and *Cdc42GAP* homology (BCH) domain, is associated with favorable prognosis in human neuroblastomas

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Differential screening of the genes obtained from cDNA libraries of primary neuroblastomas (NBLs) between the favorable and unfavorable subsets has identified a novel gene *B*CH *m*otif-containing molecule at the *c*arboxyl terminal region *1* (*BMCC1*). Its 350 kDa protein product possessed a Bcl2-/adenovirus E1B nineteen kDa-interacting protein 2 (BNIP2) and Cdc42GAP homology domain in the COOH-terminus in addition to P-loop and a coiled-coil region near the NH_2-terminus. High levels of *BMCC1* expression were detected in the human nervous system as well as spinal cord, brain and dorsal root ganglion in mouse embryo. The immunohistochemical study revealed that BMCC1 was positively stained in the cytoplasm of favorable NBL cells but not in unfavorable ones with *MYCN* amplification. The quantitative real-time reverse transcription–PCR using 98 primary NBLs showed that high expression of *BMCC1* was a significant indicator of favorable NBL. In primary culture of newborn mice superior cervical ganglion (SCG) neurons, m*BMCC1* expression was downregulated after nerve growth factor (NGF)-induced differentiation, and upregulated during the NGF-depletion-induced apoptosis. Furthermore, the proapoptotic function of BMCC1 was also suggested by increased expression in CHP134 NBL cells undergoing apoptosis after treatment with retinoic acid, and by an enhanced apoptosis after depletion of NGF in the SCG neurons obtained from newborn mice transgenic with *BMCC1* in primary culture. Thus, BMCC1 is a new member of prognostic factors for NBL and may play an important role in regulating differentiation, survival and aggressiveness of the tumor cells.
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Keywords: BMCC1; neuroblastoma; apoptosis; BNIP2; Cdc42GAP; BCH domain

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Introduction

Neuroblastoma (NBL) is one of the most common pediatric neoplasms and originates from the sympathoadrenal lineage of neural crest. However, its biological as well as clinical behavior is highly heterogeneous. The tumors occurred in the patients under 1 year of age have a tendency to spontaneously regress or differentiate (Evans *et al.*, 1976). On the other hand, the tumors found in the patients more than 1 year of age are usually aggressive and often kill the patients. The latter subsets of the tumor frequently have multiple genomic aberrations which include frequent loss of the distal part of the short arm of chromosome 1, amplification of the *MYCN* oncogene, and gain of chromosome 17q, all of which are associated with unfavorable prognosis (Brodeur *et al.*, 1984; Caron, 1995).

Although the molecular mechanism underlying regression of NBL is still unclear, accumulating evidence suggests that the signals from neurotrophic factors and their receptors play an important role in regulating growth, differentiation and programmed cell death. High expression of *TrkA*, a high affinity receptor for nerve growth factor (NGF), is associated with the favorable outcome, and there is an inverse correlation between *TrkA* expression and *MYCN* amplification. Cells expressing functional TrkA may be susceptible to either programmed cell death leading to tumor regression, especially in infants, or to differentiation to a benign ganglioneuroma. Thus, like normal sympathetic neurons, a limited amount of NGF may be supplied from the stromal cells such as Schwann cells and fibroblasts, that at least partly regulates differentiation and programmed cell death of the NBL cells. In contrast, TrkB, another family member, is preferentially expressed in aggressive NBL cells together with its preferred ligands BDNF and NT-4/5 which stimulate proliferation in an autocrine/paracrine manner, conferring potency to invade and/or metastasize on the tumor cells (Nakagawara *et al.*, 1993, 1994).

The proto-oncogene *bcl-2* encodes a 25-kDa mitochondrial membrane protein that inhibits programmed cell death (Hockenbery *et al.*, 1990; Garcia *et al.*, 1992;

Oltvai *et al*., 1993). The recent reports have suggested that *bcl-2* protein is expressed at relatively high levels in both NBLs and neural crest cells. However, the role of *bcl-2* in the regulation of differentiation and survival of NBL cells is still elusive.

In order to clarify the molecular mechanism of cellular signaling related to regression of NBL, we have cloned a large number of genes from full-length-enriched oligo-capping cDNA libraries constructed from two different subsets of NBL with favorable and unfavorable biology (Ohira *et al*., 2003a, b). Sequence analysis of the genes from those libraries has revealed that the expression profile is significantly different between the both subsets. Screening by using semiquantitative RT–PCR has shown that more than 500 genes are differentially expressed between them. In the present paper, we report cloning and functional characterization of a novel gene termed as *B*cl2-/adenovirus E1B *n*ineteen kDa-*i*nteracting *p*rotein *2* (*B*NIP2) and *Cdc42GAP* homology *B*CH *m*otif-*c*ontaining molecule at the *c*arboxyl terminal region *1* (*BMCC1*), which is preferably expressed in favorable NBL.

Results

Full-length cDNA cloning and structural analysis of the BMCC1 *gene*

As reported previously, we constructed oligo-capping cDNA libraries from different subsets of primary NBLs (Ohira *et al*., 2003b). After DNA sequencing both ends of about 10 000 clones randomly picked up, we obtained 5000 independent genes, among which about 2000 were found to be novel by homology search. They were then subjected to semi-quantitative RT–PCR to examine if they are differentially expressed between favorable (stage 1, less than 1-year-old, single copy of *MYCN* and high expression of *TrkA*) and unfavorable (stage 3 or 4, more than 1-year-old, amplified *MYCN* and low expression of *TrkA*) subsets of NBL. The differential screening in a panel of template cDNAs obtained from 16 favorable and 16 unfavorable primary NBLs demonstrated an interesting novel gene (*Nbla00219*) which had a BNIP2 and BCH domain, a recently reported new motif which might interact with Bcl-2 protein, at the COOH-terminus. It was preferentially expressed in favorable NBLs.

Sequencing of the *Nbla00219* clone showed that the insert size was 2277 bp with a putative open reading frame (ORF) of 1452 bp (484 amino acids) localized at the 5′-end region. The database search demonstrated that the *Nbla00219* sequence matched to the *KIAA0367* cDNA (accession no.: AB002365) with 95% identity as well as a part of the genomic sequence within the BAC clone RP11-146P9 (GenBank accession no.: AL161625) which was mapped to chromosome 9p13. However, there was no in-frame stop codon in the upstream region of the putative initiation site of *KIAA0367*, suggesting that the coding region of the gene extended over the 5′-end. In fact, Northern blot analysis of human fetal brain mRNA using *nbla00219* cDNA as a probe demonstrated that the transcript size was approximately 12 kb (Figure 1d). In order to determine a full-length cDNA of this gene, we performed gene prediction according to the sequence information from the BAC clone RP11-146P9 by using several algorithm. The exons expected in the upstream region of the gene were confirmed by RT–PCR using cDNA libraries constructed from human fetal brain and/or NBL tissues with favorable prognosis as template with subsequent DNA sequencing. It revealed that the gene contained a large exon of about 6.5 kb within the extended 5′-coding region. The predicted 5′-side ORF was also confirmed by matching to the several mouse ESTs. Then, we finally identified the full-length *Nbla00219* cDNA (Figure 1a) with a 5′-untranslated region of 323 bp (nt. no. 1-323), an ORF of 8355 bp (nt. no. 324-8497), and a 3′-untranslated region of 3196 bp (nt. no. 8498-11 690) (accession no.: AB050197). The Kozak consensus sequence for translation initiation site (Kozak, 1987) was found at the putative ATG start codon (at position 324), though no in-frame stop codon was found in the upstream region. The blast search against public databases showed no significant homology except BNIP2 (accession no.: XM007602, 52% identity) and Cdc42GAP (accession no.: NM004308, 38% identity) at the COOH-terminal end of the full-length *Nbla00219* (Figure 1a and b). Since the region had been termed as the BCH domain which was highly conserved among the three genes (Figure 1c), we named the full-length *Nbla00219* gene as *BMCC1*.

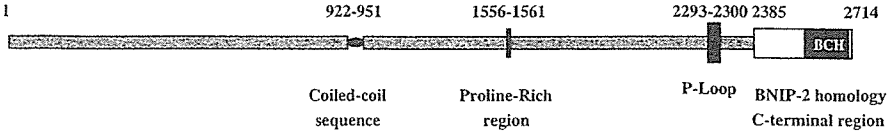
The BCH domain acts as the GTPase activating protein (GAP) in BNIP2. There are two critical arginine residues, Arg-236 and Arg-238, which are important for conferring the GAP activity to the Cdc42 homodimers (Zhang and Zheng, 1998; Zhang *et al*., 1999; Low *et al*., 2000). In BMCC1, both critical arginine residues were well conserved. Using several algorithms to predict the secondary structure of amino acids and the intracellular localization, we found the coiled-coil motif (amino acids 918–941) and P-loop (amino acids 2293–2300) within the BMCC1 protein (Figure 1a). Three putative transmembrane domains (amino acids 2545–2563, 2573–2597 and 2632–2653) were also suggested.

Although *BMCC1* was expressed significantly at higher levels in favorable than unfavorable NBLs, the expression levels of *BNIP2 family* were similar between the NBL subsets (Figure 2a).

Expression of BMCC1 *in human tissues and cell lines*

To study the expression pattern of *BMCC1* mRNA in human tissues, we performed semiquantitative RT–PCR. *BMCC1* was expressed in many tissues examined except for bone marrow, thymus and spleen (Figure 2c). The high levels of expression were seen in the nervous system (brain, cerebellum and spinal cord) as well as adrenal gland which were the tissues NBL originated from. We further performed semiquantitative RT–PCR to examine the expression levels of *BMCC1* in cultured cell lines including NBL and other cancers. *BMCC1* was expressed in most of 17 NBL cell lines tested (Figure 2b). Among the other cancer lines, high expression of *BMCC1* was observed in rhabdomyosar-

a



b

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HTLLPGLDSYSPIPEGAVAEEHAWSGEHGEHFDLFNFDPAPMASGQSQQSSHSADYSPADDFFPNSDLSEGQLPAGPEGLDGMGTNMSNYSSSSLLSGAG 200
KDSLVEHDEEFVQRQDSPRDNSERNLSLTDFVGDESPSPERLKNTGKRIPPTPMNSLVESSPSTEEPASLYTEDTTQKATDTGHMGPPQTHARRSSWWGG 300
LEIDSKNIADAWSSSEQESVFQSPESWKEHKPSSIDRRASDSVFQPKSLEFTKSGPWESEFGQPELGSNDIQDKNEESLPFQNLPMEKSPLPNTSPQGTN 400
HLIEDFASLWHSGRSPTAMPEPWGNPTDDGEPAAVAPFPAWSAFGKEDHDEALKNTWDLHPTSSKTPSVRDPNEWAMAKSGFAFSSSELLDNSPSEINNE 500
AAPEIWGKKNNDSRDHIFAPGNPSSDLDHTWTNSKPPKEDQNGLVDPKTRGKVYEKVDSWNLFEENMKKGGSDVLVPWEDSFLSYKCSDYSASNLGEDSV 600
PSPLDTNYSTSDSYTSPTFAGDEKETEHKPFAKEEGFESKDGNSMAEETDIPPQSLQQSLQQSSRNRISSGPGNLDMWASPHTDNSSEINTTHNLDENEL 700
KTEHTDGKNISMEDDVGESSQSSYDDPSMMQLYNETNRQLTLLHSSTNSRQTAPDSLDLWNRVILEDTQSTATISDMDNDLDWDDCSGGAAIPSDGQTEG 800
YMAEGSEPETRFTVRQLEPWGLEYQEANQVDWELPASDEHTKDSAPSEHHTLNEKSGQLIANSIWDSVMRDKDMSSFMLPGSSHITDSEQRELPPEIPSH 900
SANVKDTHSPDAPAASGTSESEALISHLDKQDTERETLQSDAASLATRLENPGYFPHPDPRKGHGDGQSESEKEAQGATDRGHLDEEEVIASGVENASGI 1000
SEKGQSDQELSSLVASEHQEICIKSGKISSLAVTFSPQTEEPEEVLEYEEGSYNLDSRDVQTGMSADNLQPKDTHEKHLMSQRNSGETTETSDGMNFTKY 1100
VSVPEKDLEKTEECNFLEPENVGGGPPHRVPRSLDFGDVPIDSDVHVSNTRSEITKNLDVKGSENSLPGAGSSGNFDRDTISSEYTHSSASSPELNDSSV 1200
ALSSWGQQPSSGYQEENQGNWSEQNHQESELITTDGQVEIVTKVKDLEKNRINEFEKSFDRKTPTFLEIWNDSVDGDSFSSLSSPETGKYSEHSGTHQES 1300
NLIASYQEKNEHDISATVQPEDARVISTSSGSDDDSVGGEESIEEEIQVANCNVAEDESRAWDSLNESNEFLVTADPKSENIYDYLDSSEPAENENKSNP 1400
FCDNQQSSPDPWTFSPLTETEMQITAVEKEKRSSPETGTTGDVAWQISPKASFPKNEDNSQLEMLGFSADSTEWWTSPQEGRLIESPFERELSDSSGVLE 1500
INSSVHQNASPWGVPVQGDIEPVETHYTNPFSDNHQSPFLEGNGKNSHEQLWNIQPRQPDPDADKFSQLVKLDQIKEKDSREQTFVSAAGDELTPETPTQ 1600
EQCQDTMLPVCDHPDAAFTHAEENSCVTSNVSTNEGQETNQWEQEKSYLGEMTNSSIATENFPAVSSPTQLIMKPGSEWDGSTPSEDSRGTFVPDILHGN 1700
FQEGGQLASAAPDLWMDAKKPFSLKADGENPDILTHCEHDSNSQASDSPDICHDSEAREETEKHLSACMGPEVESSELCLTEPEIDEEPIYEPGREFVPS 1800
NAELDSENATVLPPIGYQADIKGSSQPTSHKGSPEPSEINGDNSTGLQVSEKGASPDMAPILEPVDRRIPRIENVATSIFVTHQEPTPEGDGSWISDSFS 1900
PESQPGARALFDGDPHLSTENPALVPDALLASDTCLDISEAAFDHSFSDASGLNTSTGTIDDMSKLTLSEGHPETPVDGDLGKQDICSSEASWGDFEYDV 2000
MGQNIDEDFLREPEHFLYGGDPPLEEDSLKQSLAPYTPPFDLSYLTEPAQSAETIEEAGSPEDESLGCRAAEIVLSALPDRRSEGNQAETKNRLPGSQLA 2100
VLHIREDPESVYLPVGAGSNILSPSNVDWEVETDNSDLPAGGDIGPPNGASKEIPELEEEKTIPTKEPEQIKSEYKEERCTEKNEDRHALHMDYILVNRE 2200
ENSHSKPETCEERESIAELELYVGSKETGLQGTQLASFPDTCQPASLNERKGLSAEKMSSKGDTRSSFESPAQDQSWMFLGHSEVGDPSLDARDSGPGWS 2300
GKTVEPPSELGLGEGPQPQILEEMKPLESLALEEASGPVSQSQKSKSRGRAGPDAVTHDNEWEMLSPQPVQKTMIPDTEMEEETEFLELGTRISRPNGLL 2400
SEDVGMDIPFEEGVLSPSAADMRPEPPNSLDLNDTHPRRIKLTAPNINLSLDQSEGSILSDDNLDSPDEIDINVDELDTPDEADSFEYTGHDPTANKDSG 2500
QESESIPEYTAEEEREDNRLWRTVVIGEQEQRIDMKVIEPYRRVISHGGYYGDGLNAIIVFAACFLPDSSRADYHYVMENLFLYVISTLELMVAEDYMIV 2600
YLNGATPRRRMPGLGWMKKCYQMIDRRLRKNLKSFIIVHPSWFIRTILAVTRPFISSKFSSKIKYVNSLPELSGLIPMDCIHIPESIIKTSCLYNDPEMS 2700
SMEKDIDLKLKEKP 2714
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c

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BMCC1       2542:RRVISHGG------YYGDGLNAIIVFAACFLPDSSRADYHYVMENLFLYVISTLE LMVAEDYMIVYLNGATPRRRMPGLGWMKKCYQMI
BNIP2        151:KKVISHGG------YYGDGLNAIVVFAVCFMPESSQPNYRYLMDNLFKYVIGTLE LLVAENYMIVYLNGATTRRKMPSLGWLRKCYQQI
BNIP-Salpha  117:KKVLSHGG------YHGDGLNAVILFASCYLPRSSIPNYTYVMEHLFRYMVGTLE LLVAENYLLVHLSGGTSRAQVPPLSWIRQCYRTL
BNIP-Sbeta   117:KKVLSHGG------YHGDGLNAVILFASCYLPRSSIPNYTYVMEHLFRYMVGTLE LLVAENYLLVHLSGGTSRAQVPPLSWIRQCYRTL
Cdc42GAP      85:----------------------IIVFSACRMPPSHQLDHSKLLG----YLKHTLD QYVESDYTLLYLHHGLTSDNKPSLSWLRDAYREF

BMCC1       2646:DRRLRKNLKSFIIVHPSWFIRTILAVTRPFISSKFSSKIKYVNSLPELSGLIPMDCIHIPESIIKTSCLYNDPEMS SMEKDIDLKLKEKP 2714 (100%)
BNIP2        234:DRRLRKNLKSLIIVHPSWFIRTLLAVTRPFISSKFSQKIRYVFNLAELAELVPMEYVGIPECI-KQVDQELNG-KQ DEPKNEQ------- 314  (57%)
BNIP-Salpha  200:DRRLRKNLRALVVVHATWYVKAFLALLRPFISSKFTRKIRFLDSLGELAQLISLDQVHIPEAVRQLDRDLHGSGGT -------------- 275  (41%)
BNIP-Sbeta   200:DRRVL---------------------------------------------------------------------- -------------- 204  (42%)
Cdc42GAP     148:DRKYKKNIKALYIVHPTMFIKTLLILFKPLISFKFGQKIFYVNYLSELSEHVKLEQLGIPRQVLK----YDDFLKS TQKSP--------- 224  (34%)
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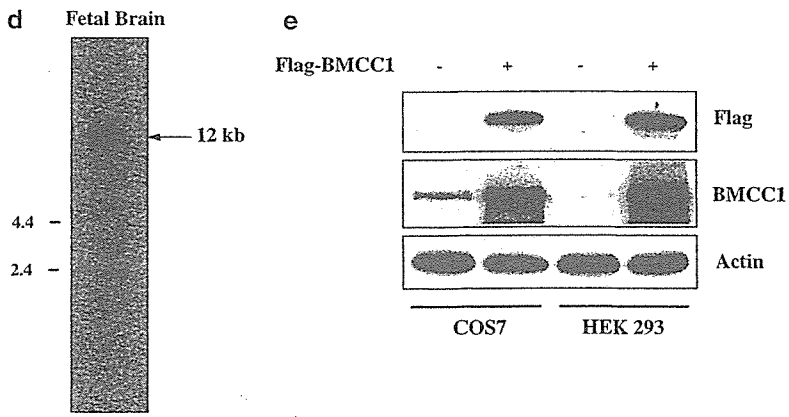

Figure 1 Molecular cloning of BMCC1. (a) Schematic structure of BMCC1. BMCC1 contains coiled-coil sequence, proline-rich region, P-loop and BCH domain in its concurrent position. (b) Full-length amino-acid sequence of human BMCC1. Coiled-coil, proline-rich and P-loop regions were underlined and BCH domain was indicated in box. (c) Alignment of C-terminal regions of BMCC1, BNIP-2, BNIP-Salpha, BNIP-Sbeta, and Cdc42GAP homologous to BCH domain. Total number of amino-acid residues of each protein and their percent homology were described at the end of each sequence. RRKMP (homophilic/heterophilic dimerized sequence), EYV (binding to switch I and insert region of Cdc42) (Low *et al.*, 2000) and RRLRK (arginine patch of BCH domain), ATWYVKA (binding motif for homophilic complex and critical for proapoptotic activity) (Zhou *et al.*, 2002), were underlined. (d) Northern blot analysis of *BMCC1* transcript in fetal brain tissue. Total RNA (25 μg) purchased from Clontech was loaded for Northern blotting. Left; size markers showing 2.4 and 4.4 kb. (e) BMCC1 expression in COS7 and HEK293 cells. pCAGGS-BMCC1-Flag was transfected into COS7 and HEK 293 cells and lysed after 48 h. Cell lysates were run into 8% SDS–PAGE in 35 mA for more than 4 h, transferred to immobilon-P membrane (MILLIPORE) and probed by anti-Flag, anti-BMCC1 (C-terminal end epitope), and antiactin antibodies.

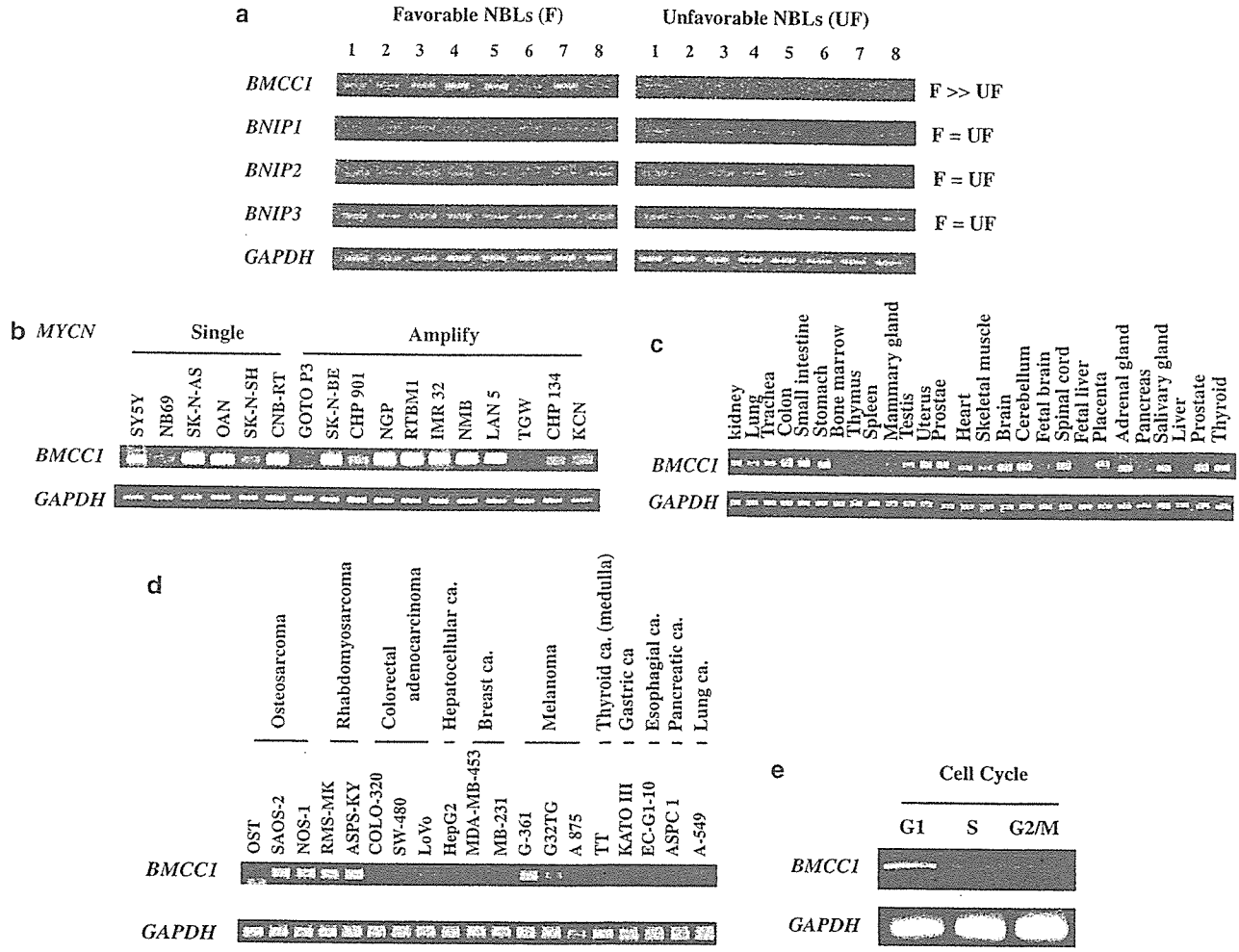

Figure 2 Expression of *BMCC1* mRNA. (**a**) Differential expression of *BMCC1* in favorable and unfavorable neuroblastomas. mRNA expression patterns for *BMCC1* and *BNIP* gene family members were detected by semiquantitative RT–PCR procedure. Results for eight favorable and eight unfavorable NBLs are shown. The expression of *GAPDH* is also shown as a control. Lanes 1–8: favorable NBLs (F; stage 1 or 2, with a single copy of *MYCN*), lanes 9–6: unfavorable NBLs (UF; stage 3 or 4, with *MYCN* amplification). (**b**) Expression of *BMCC1* mRNA in neuroblastoma cell lines. In all, 11 NBL cell lines with *MYCN* amplification and six cell lines with a single copy of *MYCN* were used for semiquantitative RT–PCR as templates. (**c**) Semiquantitative RT–PCR of *BMCC1* in multiple human tissues. Total RNA of 25 adult tissues and two fetal tissues were purchased from Clontech Co. Ltd. As a control, same cDNA templates were amplified by *GAPDH* primers. (**d**) Expression of *BMCC1* mRNA in the other cancer cell lines. Semiquantitative RT–PCR analysis was performed using cDNA primers and control *GAPDH* primers. Tumor origins were shown on the top. (**e**) The changes in expression of *BMCC1* at the cell cycle stages. HeLa cells were synchronized by treatment with 400 μM mimosine for 18 h (G1-phase arrest), with 2 mM thymidine for 20 h (S-phase arrest), or with 0.6 μg/ml nocodazole for 18 h (G2/M-phase arrest) and collected for RNA isolation. Semiquantitative RT–PCR was conducted by using *BMCC1* primers and *GAPDH* control primers.

coma, melanoma and some osteosarcoma cell lines, whereas only low levels of expression were found in cancer cell lines of liver, breast, thyroid and colon (Figure 2d). We further examined the cell cycle-dependent expression of *BMCC1* mRNA in HeLa cells by using semiquantitative RT–PCR. As shown in Figure 2e, *BMCC1* was predominantly expressed in G1 phase of the cell cycle.

In situ *hybridization of* BMCC1 *in mouse embryo*

In situ hybridization in mouse embryo showed that *BMCC1* was specifically expressed in neural tube and neural crest-related tissues. In E10.5 mouse embryo, *BMCC1* was highly expressed in neural tube and pharyngeal arches which are derived from neural crest. The expression of *BMCC1* seemed to be more restricted in the later stages of development (Figure 3). In E12.5 mouse embryo (Figure 3d), *BMCC1* was expressed in spinal cord, hindbrain, midbrain, forebrain and dorsal root ganglia (DRG). Although the expressions of *BMCC1* in E14.5 mouse embryos (Figure 3a and b) were similar to those in E12.5, the regions expressing *BMCC1* in hindbrain (Figure 3a), spinal cord and forebrain at E14.5 (Figure 3b) were more dorsally restricted than at E12.5.

Immunohistochemical staining of BMCC1 in primary NBLs

The favorable NBLs occasionally expressed BMCC1 in the cytoplasm of the tumor cells (Figure 4b). In contrast,

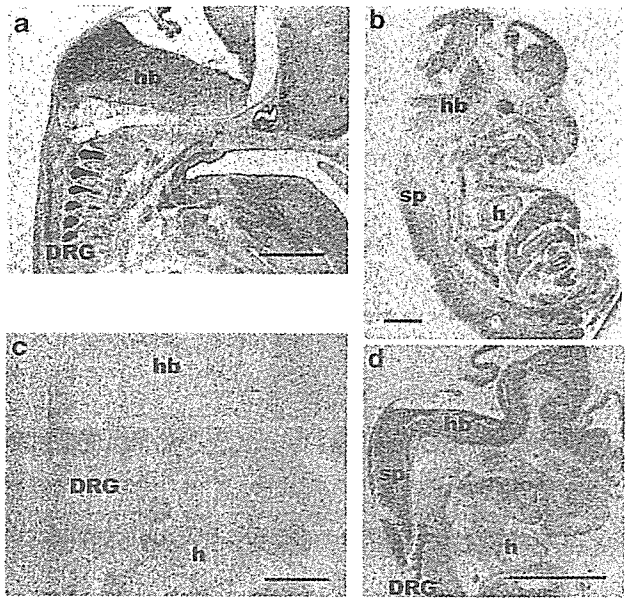

Figure 3 Section *in situ* hybridization of embryos with the *BMCC1* probe. Sagittal sections of embryos at E14.5 (**a**–**c**) and E12.5 (**d**) were prepared and the *BMCC1* expression was examined by section *in situ* hybridization. (**a**), (**b**) and (**d**) Antisense probes. (**c**) Sense probe (control). The *BMCC1* probe used is described in the Experimental procedures. DRG, dorsal root ganglion; sp, spinal cord; hb, hindbrain; h, heart. Scale bar, 200 μm.

in the unfavorable neuroblastomas the tumor cells were entirely negative for BMCC1 or only a few positive cells were observed (Figure 4d).

Prognostic significance of BMCC1 *mRNA expression in human NBLs*

The levels of *BMCC1* mRNA expression were measured in 98 primary NBLs by using quantitative real-time RT–PCR. The high levels of *BMCC1* expression were significantly associated with favorable NBL in stages 1, 2 and 4 (Figure 4e). The high levels of *BMCC1* expression was significantly associated with young age ($P<0.00005$), favorable stages ($P<0.00005$), high expression of *TrkA* mRNA ($P<0.00005$), single copy of *MYCN* ($P<0.00005$), tumors found by mass screening (MS) ($P<0.00005$), nonadrenal origin ($P=0.0025$) according to the Student's *t*-test. The log-rank test showed that the high expression of *BMCC1* was significantly correlated with a favorable outcome ($P=0.0008$) as shown in the Kaplan–Meier cumulative survival curves (Table 1 and Figure 4f).

The multivariate Cox regression analysis also demonstrated that *BMCC1* expression (high vs low), age (< 1 year vs ⩾ 1 year), *MYCN* copy number (1 copy vs > 1 copy), and MS (positive tumors vs sporadic tumors) had prognostic significance ($P<0.0005$) (Table 2). *BMCC1* expression was significantly related to survival ($P=0.007$) after controlling age ($P=0.018$). However, it lost significance in a model including jointly with *MYCN* amplification or MS. Furthermore, *BMCC1* expression was significantly related to survival ($P=0.027$) after controlling age ($P=0.014$) and origin ($P=0.403$).

Changes in BMCC1 *mRNA expression during neuronal differentiation and apoptosis*

To examine whether exogenous expression of BMCC1 affects the cell growth of neuronal PC12 cells, a rat pheochromocytoma cell line, we transfected the cells with a full-length *BMCC1* cDNA. The overexpression of BMCC1 appeared to decrease the cell growth but the result was not statistically significant (data not shown). We then tested if expression of *BMCC1* mRNA was changed during neuronal differentiation and/or apoptosis. For that purpose, we used three different neuronal cell lines. The NT2 cell line, which was established from human immature teratocarcinoma and the cells show astrocytic differentiation after treatment with retinoic acid (RA) (Moasser *et al*., 1996). The CHP134 NBL cells undergo apoptosis after 3 days of the treatment with RA (Islam *et al*., 2000). On the other hand, the RTBM1 human NBL cells are induced to differentiate after the treatment with RA (Nakamura *et al*., 1998). We have confirmed that caspase 3 expression was increased in CHP134 cells but decreased in RTBM1 cells at day 7 after treatment with RA by semiquantitative RT–PCR. On the other hand, nestin expression was not changed in the former and slightly increased in the latter (Figure 5a). Expression of *BMCC1* mRNA was downregulated during RA-induced neuronal differentiation in both NT2 and RTBM1 cells, whereas it was rather upregulated in CHP134 cells on day 7 after the treatment with RA when many cells were undergoing apoptosis (Figure 5a).

To further confirm the above observation seen in neuronal cell lines, we examined the changes in *BMCC1* expression in superior cervical ganglion (SCG) neurons obtained from newborn mice in primary culture. The cultured cells were treated with 50 ng/ml NGF for 5 days (induction of neuronal differentiation) and then depleted NGF from the medium and added anti-NGF antibodies to induce neuronal apoptosis. As shown in Figure 5b, induction of differentiation by NGF decreased expression of *BMCC1*, whereas the NGF-depletion-induced apoptosis was accompanied with increase in *BMCC1* expression. This was very similar to the changes in expression of *c-jun* and *Bim* which had already been reported (Whitfield *et al*., 2001). Thus, the levels of *BMCC1* mRNA are changed during neuronal differentiation and apoptosis in an opposite manner.

Enhanced NGF-depletion-induced apoptosis in SCG neurons obtained from BMCC1 transgenic mice

We next generated *BMCC1* transgenic mice by using the expression construct with the tyrosine hydroxylase promoter-driven promoter to examine the functional role of BMCC1 in the sympathetic neurons. The SCG neurons obtained from either control or transgenic newborn mice were subjected to primary culture. The integration of the BMCC1 in the mouse genome and its overexpression in SCG neurons were confirmed by both