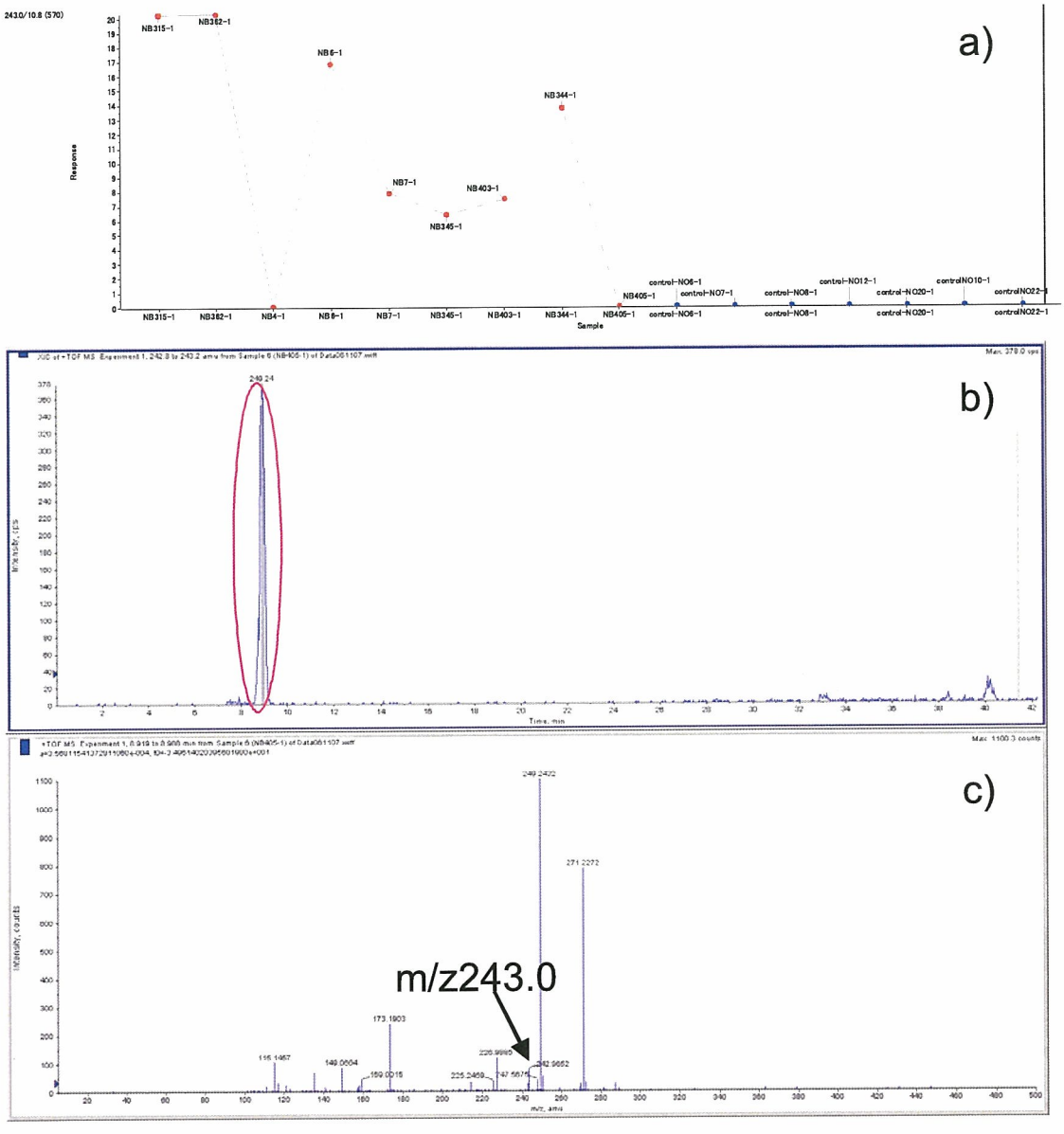

Fig. 12　患者血清分析の MakerView 解析　Ⅳ A 期患者血清（赤）とコントロール血清（青）間で有意な差が見られた物質（10.8 分の m/z243.0 のピーク）
a) t 検定の結果，b) m/z243.0 の MS クロマトグラム，c) MS スペクトル

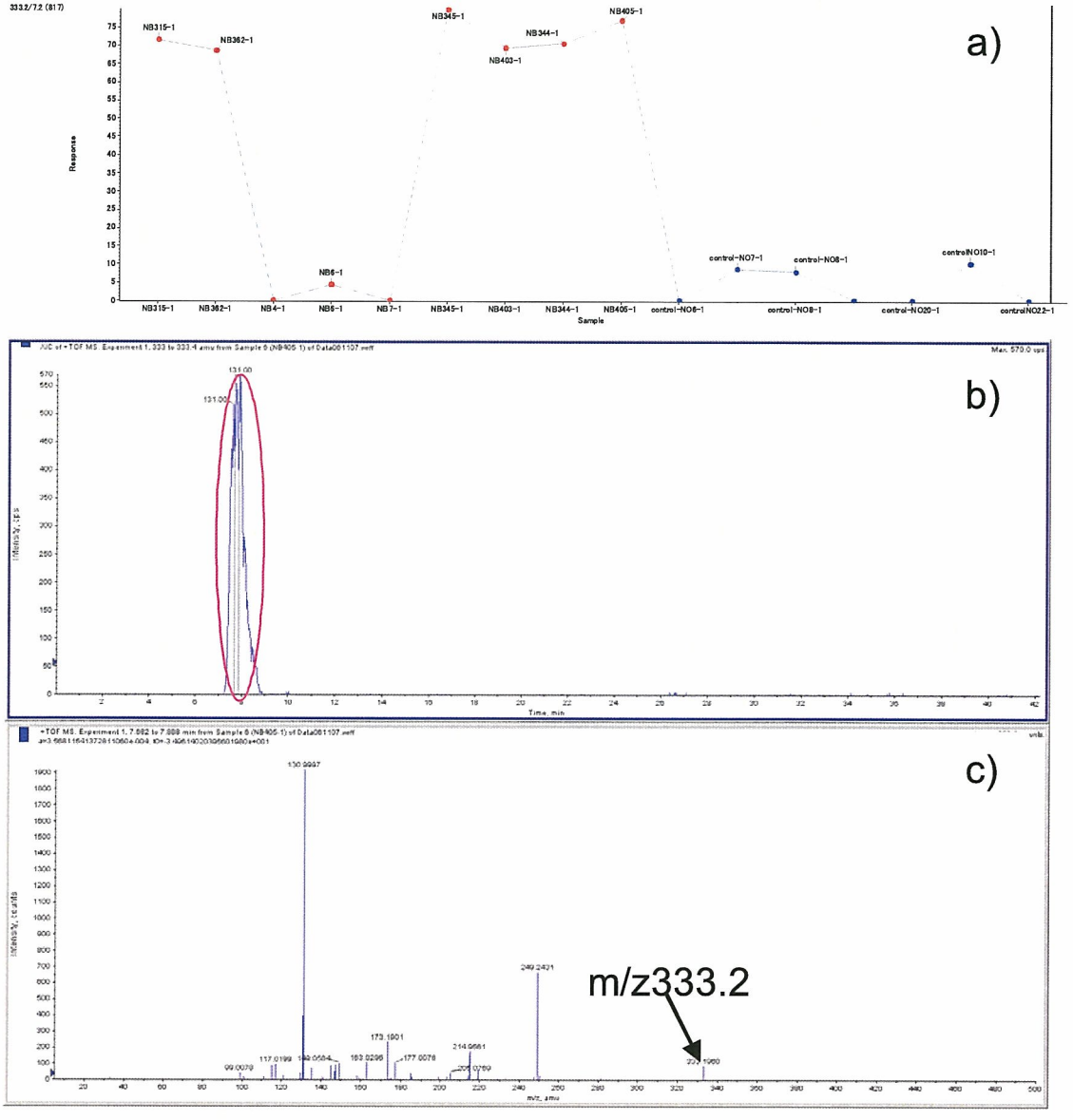

Fig. 13　患者血清分析の MakerView 解析　Ⅳ A 期患者血清（赤）とコントロール血清（青）間で有意な差が見られた物質（7.2 分の m/z333.2 のピーク）
a) t 検定の結果，b) m/z333.2 の MS クロマトグラム，c) MS スペクトル

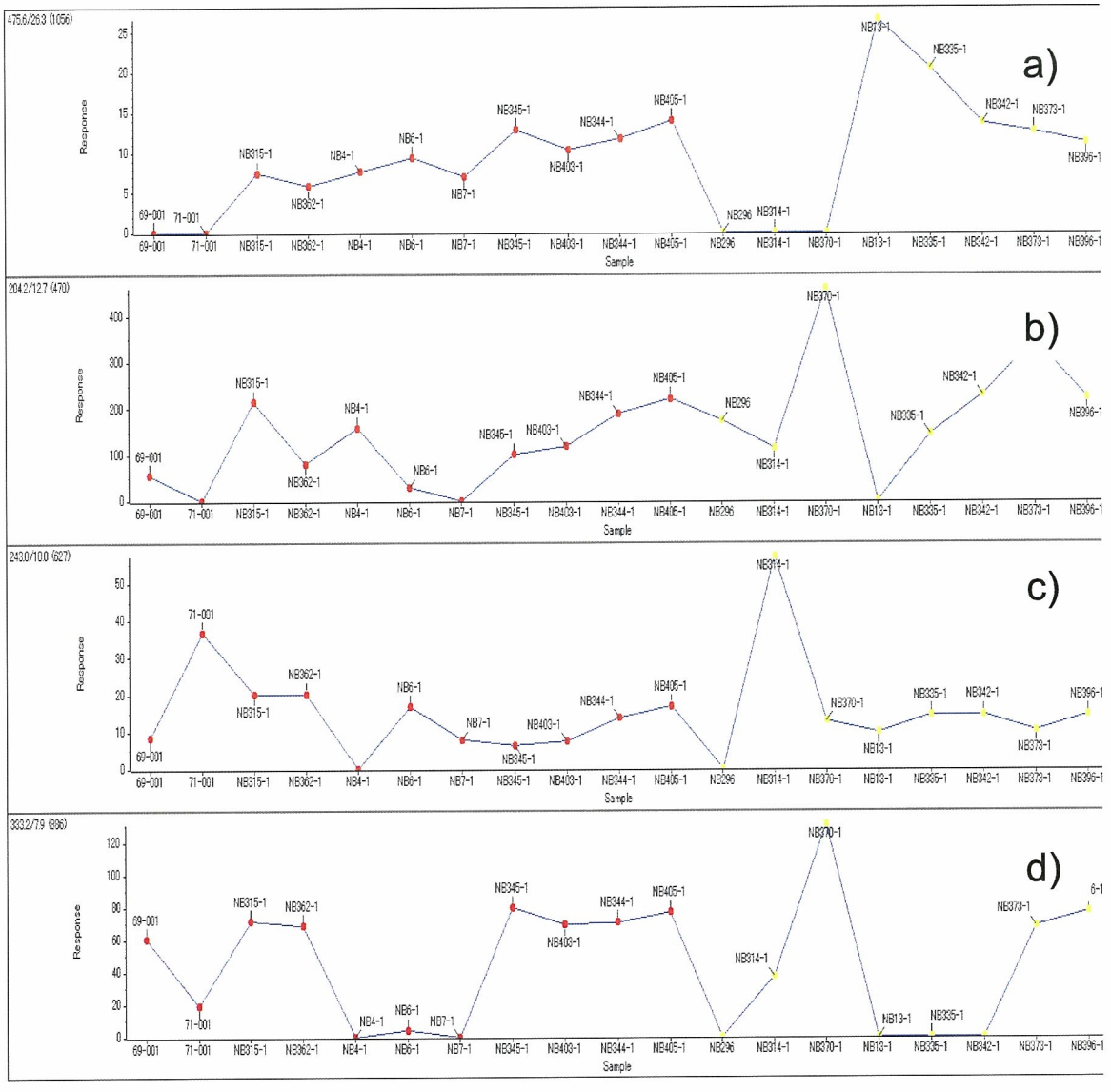

Fig. 14　患者血清分析の MakerView 解析　ⅣA 患者血清（赤）とⅠ患者血清（黄）血清間で有意な差が見られた物質についてⅠ期患者血清とコントロール血清で t 検定を行った結果

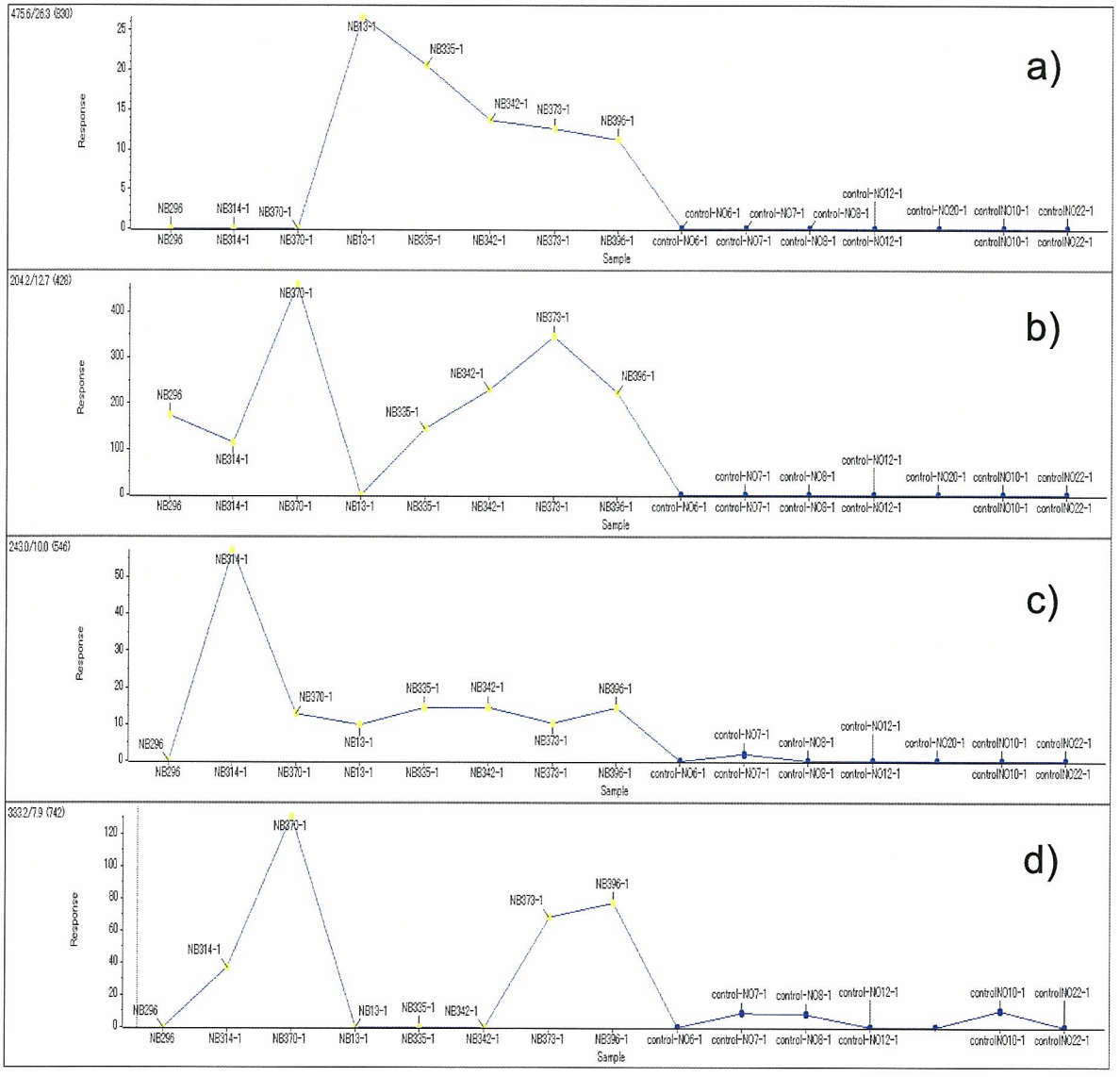

Fig. 15　患者血清分析の MakerView 解析　Ⅰ期患者血清（黄）とコントロール（青）血清間で有意な差が見られた物質についてⅣ A 期患者血清とⅠ期患者血清で t 検定を行った結果

	4A-control	1-control	4A-1
475.2/26.2	99.999961	98.417156	-56.268898
204.2/12.0	99.894408	97.528707	-94.431961
243.0/10.8	99.607239	99.829179	-32.314386
333.2/7.2	99.454352	91.870757	32.904877

Table. 10　患者血清分析の MakerView 解析
ⅣA患者血清とコントロール血清間で有意な差が見られた物質についてⅠ患者血清とコントロール血清，ⅣA患者血清とⅠ血清でｔ検定を行った結果のｔ値の比較

D. 結論

本研究によりこれまでなされていなかった中間代謝物を含めたカテコールアミン類のLC/MS及びLC/MS/MSによる一斉分析法を確立することができた。また尿中及び血清中のカテコールアミン類を夾雑物質と分離・濃縮することも可能となった。さらに血清中の低分子の網羅的解析により、神経芽細胞腫に特異的な物質、新規マーカーの候補を発見することができた。今後、それらの物質について検証を進めるとともに予後に関与する物質などの検証も進めていく。

G. 研究発表

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H．知的財産権の出願・登録状況(予定を含む.)

1．特許取得

なし

2．実用新案登録

なし

3．その他

なし

Ⅴ．研究成果の刊行に関する一覧表

研究成果の刊行に関する一覧表

雑誌

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厚生労働科学研究費補助金

（子ども家庭総合研究事業）

登録症例に基づく
神経芽細胞腫マススクリーニングの
効果判定と医療体制の確立

平成18年度　総括・分担研究報告書（２／２冊）

主任研究者　檜山　英三

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VI. 研究成果の刊行物・別刷

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Single nucleotide polymorphism array analysis to predict clinical outcome in neuroblastoma patients☆

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Index words:
Neuroblastoma;
Mass screening;
Genetic alteration;
Microarray;
Prognosis

Abstract

Purpose: Neuroblastoma (NB) is a heterogeneous tumor and demonstrates favorable or unfavorable outcomes. In Japan, a nationwide NB mass screening (MS) had been performed on 6-month-old infants for approximately 20 years, which might have detected almost all NB including regressing/maturing tumors. To clarify the heterogeneity of this tumor, we examined genetic alterations in the representative cases using genomewide microarrays.

Methods: Genomic DNA was extracted from 198 NB tissue samples and paired blood samples including 76 MS-detected cases and analyzed by single nucleotide polymorphism arrays.

Results: The single nucleotide polymorphism array classified the genetic aberrations into 4 types: whole gain/loss type, partial gain/loss type, *MYCN*-amplified type, and silent type. Most MS-detecting cases belonged to the whole gain/loss type, whereas unfavorable cases who died of disease showed partial gain/loss, *MYCN*-amplified, or silent types.

Conclusions: Genomewide genetic analysis is useful to predict the outcome of patients. Although the cases whose tumors showed whole gain/loss may respond well to contemporary therapy, sparing intensive surgery, current therapeutic strategy may be insufficient for the subgroups with partial gain/loss, *MYCN*-amplified, or silent type. Validation of these results would provide new tools to predict clinical outcome of children with NB.

Neuroblastoma (NB), one of the common malignant childhood tumors, arises from neuroblast cells derived from the neural crest and destined for the adrenal medulla and the sympathetic nervous system and affects approximately 1 in 7000 individuals [1]. It has been known for many years that NBs show remarkable biologic heterogeneity, resulting in favorable prognosis in some instances and unfavorable prognosis owing to aggressive growth despite multimodal

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Table 1 Age at diagnosis and INSS

Age at diagnosis (mo)	MS cases (deceased)	Clinical cases (deceased)	INSS	MS cases (deceased)	Clinical cases (deceased)
0-5	0	19 (3)	1	30	10
6-11	65 (2)	8 (2)	2A	8	7 (2)
12-17	10	17 (7)	2B	10	13 (2)
18-23	1	28 (20)	3	15	36 (23)
24-35	0	26 (21)	4	5 (2)	49 (41)
36 and above	0	24 (17)	4S	8	7 (2)

therapy in other instances. Because more than 80% of NBs produce catecholamine metabolites (vanillylmandelic acid and homovanillic acid) that are detectable in the urine, and aggressive NBs usually occur after 1 year of age, a nationwide mass screening (MS) project intending to detect NBs at earlier stages was carried out in Japan between 1985 and 2003. Surprisingly, the incidence of this disease increased approximately 2-fold, whereas that of advanced NB in older patients did not change so much, indicating that a large number of NBs occur in infants without clinical detection and spontaneously regress or mature behind the scenes. Thus, this project to decrease the incidence of advanced NBs resulted in disappointment but gave us suggestive insights in solving the biologic problems in NB [2-4]. These phenomena raised the question whether advanced stage tumors develop from early stage tumors or represent a de novo subgroup. Transition from a favorable type to an unfavorable type has not been clearly evaluated but seems to occur rarely [5], suggesting that NB may consist of at least 2 distinct subtypes [6], which may be distinguishable by genetic characteristics.

To estimate the malignant grade and predict the biologic behavior of an individual tumor, we have proposed several prognosis-predicting markers, such as *MYCN* gene amplification, loss of chromosome 1p, loss of 11q, and gain of 17q, and DNA diploidy/tetraploidy [7]. However, each of these parameters appears to be insufficient to predict the prognosis of individual patient. Recently, microarray techniques have been developed and have rapidly become a fundamental tool in genomic research. Highly multiplexed microarray systems for single nucleotide polymorphism (SNP) genotyping can genotype hundreds of thousands of SNPs at one time to detect gene dose imbalances in a whole genome [8,9]. This powerful tool permits detection of genomic aberrations and maps these directly onto the sequence of the human genome.

Table 2 Pattern of genetic alterations in NB

Pattern	Total cases (deceased)	MS cases (deceased)	Aneuploid cases
W	58 (2)	52	52
P	52 (20)	7	9
M	39 (34)	4 (2)	11
S	49 (16)	13	5
Total	198 (72)	76 (2)	77

In this study, to clearly divide NB cases into subtypes by the difference of genomic alterations, we applied the Affymetrix GeneChip Mapping 100K array set (Affymetrix, Inc, Santa Clara, Calif) to NBs detected by MS and by clinical symptoms.

1. Materials and methods

1.1. Samples

In Japan, approximately 5000 children, including 2500 MS-detected children, were registered between 1981 and 2004 in a database maintained by the Japanese Society of Pediatric Surgeons [10]. In the present study, genomic DNAs were extracted from 198 NB samples including 76 MS-detected cases. Ages at diagnosis and stages at surgery according to the International Neuroblastoma Staging System (INSS) are shown in Table 1. All patients were diagnosed as having NB between 1991 and 1998 at the Hiroshima University Hospital, Hiroshima, Japan, or affiliated hospitals, and most patients were treated according to the Japanese NB protocols for infants or advanced stage NB (A1, new A1, or A3) [11]. The follow-up period of all patients was more than 5 years. This research was approved by the ethical committee of the Hiroshima University (Hiro-Rin-20). Written informed consent for this research was obtained from parents of all patients. None of the patients had prior therapy before surgery or biopsy to obtain tumor specimens. Venous blood (5-7 mL) was taken from patients before surgery. Tumor DNA and constitutive DNA in each patient were extracted and purified using standard methods.

1.2. Affymetrix platform

Array experiments were done according to the standard protocols for Affymetrix GeneChip Mapping 100K arrays [12]. Briefly, total genomic DNA was digested with a restriction enzyme (*Xba*I or *Hin*dIII), ligated to an appropriate adapter for each enzyme, and subjected to polymerase chain reaction amplification. After digestion with DNase I, the polymerase chain reaction products were labeled with a biotinylated nucleotide analogue using

terminal deoxynucleotidyl transferase and hybridized to the 100K SNP array [13]. This 100K microarray set consists of 2 microarrays: the 100K *Xba*I microarray genotyping 58,960 SNPs and the *Hin*dIII microarray genotyping 57,244 SNPs, covering 92% of the genome with an SNP for every 100 kb. The 100K SNP arrays were scanned with the Affymetrix GeneChip Scanner 3000 using GeneChip Operating System 1.2 (Affymetrix). Genotype calls and intensity of the SNP probes were processed by GeneChip DNA Analysis Software. Individual SNP copy numbers and chromosomal regions with gains or losses were evaluated with the Affymetrix GeneChip Chromosome Number Tool 2.0.

1.3. DNA ploidy

Frozen samples were cut into small pieces with scissors. Suspensions of single nuclei were prepared using the detergent-trypsin procedure of Vindelov et al [14] and stained with propidium iodide (Becton Dickinson, Mountain View, Calif). Measurement of DNA cellular content was performed using the FACScan or FACS caliber flow cytometer (BD, Franklin Lakes, NJ). The DNA index (DI) was determined by calculating the ratio of the modal channel number for tumor G0/G1-phase cells to that for normal diploid cells. Hence, the DI of diploid NB cells was 1.0; tumors with a distinct population with DI higher than 1.0 were defined as aneuploid.

1.4. Statistical analysis

Tests of association were performed with the use of Fisher's Exact test. Survival curves were constructed according to the methods of Kaplan and Meier [15], and comparisons of the survival curves were performed with a 2-sided log-rank test.

2. Results

Good hybridization signal intensities were obtained, and genotype calls were obtained for more than 95% SNPs on all *Xba*I and *Hin*dIII microarrays. This confirms that the assay was performed correctly, and that no sample contamination occurred. The loci with allelic imbalance

Table 3 Chromosome 1p loss, 11q loss, and 17q gain in each pattern of genetic alterations in NB

Pattern	1p loss*	2p gain	11q loss	17q gain
W (n = 58)	0	0	0	0
P (n = 52)	3	29	27	32
M (n = 39)	33	–	2	21
S (n = 49)	0	0	0	0
Total (deceased)	36 (35)	29 (17)	29 (18)	53 (34)

* This deletion was defined as the large deletion of 1p including 1p32-36.

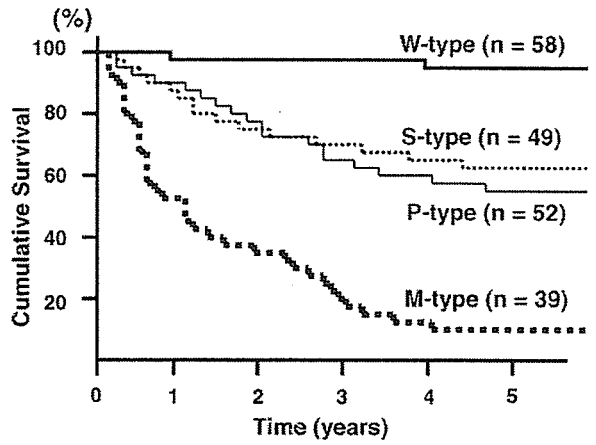

Fig. 1 Kaplan-Meier cumulative survival spots for patients with NB. Survival curves of the cases with W-type, P-type, M-type, and S-type NBs. The survival of patients with W-type tumors showed significantly better than others ($P < .001$), whereas those with M-type tumors showed significantly worse than others ($P < .001$). The survival rates of patients with P-type or S-type tumors were intermediate.

were determined for all chromosomes in each case. A case of whole chromosomal gain/loss was determined when SNP signals in each chromosome were gain (upward signals) or loss (downward signals). Such chromosomal aberrations were defined as W type. On the other hand, a case of partial chromosomal gain/loss in several chromosomes was defined as P type. In other instances, we defined M type as the cases with *MYCN*-amplified tumors. These tumors showed 1 or several amplicons including *MYCN* region. In 198 cases examined, 39 (19.6%) showed *MYCN* amplification. Two or more amplicons in 2p region were detected in 23 cases. Finally, the remaining cases that had no large chromosomal aberrations were defined as S type (silent). All tumors belonged to one of these 4 types (Table 2). In 58 W-type tumors, 52 were MS-detected tumors and only 2 cases showed unfavorable outcome. On the other hand, in 52 P-type tumors and 39 M-type tumors, most cases were detected by clinical symptoms. And the outcome of the patients with M-type tumors was very poor and those with P- or S-type tumors were intermediate.

Then, we analyzed the well-known chromosomal abnormalities in this series (Table 3). Large deletion in chromosome 1p was detected in 36 samples (18%), including the common region of deletion at 1p36 [16]. There was a significant association between 1p loss and *MYCN* amplification (M type) ($P < .001$). On the other hand, 2q gain and loss of 11q were detected in 29 samples (15%) and showed significant association with P type ($P < .001$). Partial gain of 17q was detected in 53 cases (27%), including both P-type and M-type tumors. There were significant correlations between each of these chromosomal aberrations and poor prognosis ($P < .01$).

We also examined the correlation between these genetic alterations and DNA ploidy. In 58 W-type tumors, 52 (90%)

showed aneuploid, whereas 25 (18%) of other type tumors showed also aneuploidy. Thus, incidence of aneuploid was significantly higher in the W-type tumors, but the data of DNA ploidy were not always compatible with those of SNP array.

The median follow-up period in the series of patients examined was 82 months (range, 1-186 months). Kaplan-Meier event-free survival curves of all patients (Fig. 1) show that the 5-year event-free survival rate in the patients with W-type tumors was 88%, which was significantly better than the remaining cases.

3. Discussion

Clinical, biologic, and genetic observations have clearly demonstrated that NB encompasses several different diseases [6,7]. An Affymetrix platform to survey genomewide genetic alterations revealed that more than 4 subtypes exist in 1 disease entity of NB. And W-type tumor is rare in the elder patients but accounts for more than half of MS-detected tumors, including most of the regressing/maturing tumors. Because the outcome of the patients with W-type tumor is excellent regardless of stages of tumor, these W-type tumors are considered as regressing/maturing NBs. Thus, the treatment strategies for W-type tumors should be less aggressive to minimize side effects. In the remaining 3 types, in which most patients had been diagnosed by clinical symptoms, *MYCN*-amplified tumors showed poorest prognosis, whereas the other 2 types showed intermediate mortality rates. Among the well-known chromosomal alterations, 1p loss was frequently detected in M type, whereas 2p gain and 11q loss were in P type. Gain of 17q was detected in both M and P types. These results are consistent to the previous reports that demonstrated these chromosomal aberrations as prognosis-associated factors [17-23]. This platform successfully evaluated these alterations at once.

In the partial gain/loss tumors and silent tumors mainly diagnosed by clinical symptoms, 30% to 40% of the patients showed poor prognosis. There is a possibility that critical genetic alterations that determine a malignant grade of tumors may exist in small lesions, especially in silent type of tumors. The genomewide gene expression profile is one of the most promising tools to reveal such critical genes. We are now analyzing the combination of genetic alterations and gene expression profiles in these tumors [24,25]. These studies might point out the key genes as candidates of risk assessment markers as well as therapeutic targets in NB.

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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.

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

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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