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Biology of Neuroblastomas That Were Found by Mass Screening at 6 Months of Age in Japan

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Background. Mass screening (MS) of neuroblastoma has been carried out by measuring the urinary catecholamine metabolites in infants at the age of 6 months in Japan. We assessed the incidence of neuroblastoma that may be a target for MS by studying tumor biology. ***Procedure.*** FISH on chromosome 1 and MYCN analysis was performed on 453 patients that were classified into three clinical groups (287 infants found by MS, 51 infants <12 months diagnosed clinically, and 115 children ≥12 months diagnosed clinically). The relationship between the biological types of tumors and the clinical outcome was examined. ***Results.*** Type 1 (trisomy 1 and normal MYCN), type 2 (disomy 1/tetrasomy 1 and normal MYCN), and type 3 (disomy 1/tetrasomy 1 and amplified MYCN) tumors were found in 88.2%, 10.5%, and 1.4% of infants found by MS, in 68.0%, 24.0%, and 8.0% of infants diagnosed clinically, and in 23.4%, 42.3%, and 34.2% of children diagnosed clinically ($P < 0.001$). Infants with type 1 tumors found by MS or diagnosed clinically had earlier stages of the disease ($P < 0.0001$ and $P = 0.0005$) and better overall survival ($P < 0.001$ and $P = 0.005$) than children with type 1 tumors diagnosed clinically. Infants with type 2 tumors found by MS, had earlier stages ($P = 0.06$ and $P < 0.0001$) and better overall survival ($P = 0.014$ and $P < 0.001$) than infants or children with type 2 tumors diagnosed clinically. All three clinical groups of patients with type 3 tumors had advanced stages and dismal prognoses. ***Conclusions.*** About 12% of tumors found by MS showed unfavorable biological (types 2 and 3) characteristics. Pediatr Blood Cancer 2006;46:285–291.

Key words: constitution of chromosome 1; mass screening; neuroblastoma; ploidy

INTRODUCTION

Neuroblastoma is one of the most common solid tumors in childhood and is characterized by a broad spectrum of clinical behaviors [1]. Because of the favorable prognosis of neuroblastomas in infants and the difficulty in curing disseminated neuroblastomas in children 12 months or over, a mass screening (MS) program has been carried out for infants in Japan and some other countries by measuring urinary catecholamine metabolites based on the assumption that early detection of the tumor in infants could improve the overall prognosis of patients [2–4].

Epidemiological studies have recently been published from Quebec, Germany, and Japan [3–6]. The methods of detecting urinary catecholamine metabolites, the time of screening, and the study designs were different among the programs. Although an increase in the incidence of neuroblastoma was seen in all the screening programs, no reduction of mortality was seen in the Quebec and German studies, and a modest decrease in mortality was reported in the Japanese studies.

We previously reported that while neuroblastomas found by MS were characterized by early stages of the disease, triploidy, and a low incidence of *MYCN* gene amplification or 1p deletion, those found clinically at 12 months or over were characterized by advanced stages, diploidy or tetraploidy, and a high incidence of *MYCN* gene amplification and 1p deletion [7,8]. These findings may suggest the limited efficacy of the program.

To clarify the biological characteristics of neuroblastomas that were found by MS, we extended the previous study using interphase fluorescent in situ hybridization (FISH) analysis by increasing the number of tumors, by newly including the tumors found clinically before 12 months of age, and by adding flow cytometric analysis. We here report the biological features of the largest number of neuroblastomas that were found by MS, and that about 12% of tumors found by MS showed poor

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prognostic factors that characterize the tumors found in older children with unfavorable prognoses [7,8].

PATIENTS AND METHODS

Patients and Specimens

Tumors that were obtained from 453 Japanese infants or children with neuroblastoma who underwent biopsy or surgery between January 1985 and December 1998. The tumors were sent to the Saitama Cancer Center for cytogenetic and FISH analysis. Two hundred and eighty-seven infants were found by MS to have neuroblastoma at 6 months of age, 51 infants less than 12 months of age, and 115 children 12 months or over were diagnosed clinically. During the same period, 1,896 infants were found by MS to have the tumor in Japan [9], and the 287 MS-positive patients constituted 15.1% of these 1,896 patients. Infants found by MS ranged in age from 6 to 18 months with a median age of 7 months; it took at least 1 month from the screening to the diagnosis of neuroblastoma, infants diagnosed clinically from 6 days to 10 months with a median age of 2 months, and children diagnosed clinically from 13 months to 21 years with a median age of 34 months. Most infants diagnosed clinically did not undergo MS, and most children diagnosed clinically underwent MS with a negative result, but the exact numbers of each category of patients could not be determined. Of the 287 infants found by MS, 269 (94%) were examined by the quantitative measurement of VMA/creatinine (Cre) and homovanillic acid (HVA)/Cre by high-performance liquid chromatography (HPLC) that was used after 1988, and 18 by qualitative assessment of urinary vanillylmandelic acid (VMA) used before 1988. Informed consent was obtained from patients and/or their parents, and the study was approved by the ethics committee of the Saitama Cancer Center. All tumors were histologically classified as neuroblastoma or ganglioneuroblastoma. Patients were staged according to the INSS staging system [10]. Patients of any age with stage 1 or 2 disease, and those less than 12 months with stage 3 disease were treated with either surgery or surgery plus chemotherapy consisting of cyclophosphamide and vincristine, and those 12 months or older with stage 3 or 4 disease, and those less than 12 months with stage 4 disease were treated according to the protocol by the Japanese Neuroblastoma Study Group [11].

Interphase FISH and MYCN Copy Number Analyses

Pathologists in each institution verified that each sample contained 50% or more tumor cells. One half of each sample was used for cytogenetic, FISH, and *MYCN* copy number analyses. The results of the cytogenetic analysis were incorporated in the results of the FISH analysis. To detect the copy number of chromosome 1 s and the status of 1p, we used repetitive DNA probes, D1Z1 (pUC1.77) and D1Z2 (p1-79), specific for the pericentromeric region (1q12) and the sub-telomeric region (1p36.33), respectively. Two-color FISH using the two probes was performed as described previously [8]. Disomy 1, trisomy 1, tetrasomy 1, or pentasomy 1 was determined on the basis of the number of the D1Z1 signals, and 1p deletion was defined when the number of the D1Z2 signals was less than the number of the D1Z1 signals. The results of FISH analysis on 170 of the 453 tumors have been reported previously [8].

Tumors were classified into four cytogenetic groups on the basis of the constitution of chromosome 1, i.e., disomy 1 with no deletion of the short arm (Dis1Norm1p group), disomy 1 with the short-arm deletion (Dis1Del1p group), trisomy 1 with no deletion of the short arm (Tris1Norm1p group), and trisomy 1 with the short-arm deletion (Tris1Del1p group). Tris1 tumors included tumors with trisomy 1 and those with a mixed population of cells with trisomy 1 and cells with tetrasomy 1, with or without cells having pentasomy 1 (Fig. 1). Dis1 tumors included tumors with disomy 1 and those with tetrasomy 1. The tumors were assigned to one of the four cytogenetic groups on the basis of the abnormal tumor-cell population dominating in the 100 cells examined (i.e., $\geq$50%). If tumors had a mixed population of cells with trisomy 1 and cells with tetrasomy 1, each group of cells should occupy at least 25% of the 100 cells counted.

DNA preparation, digestion, and Southern blot analysis using the *MYCN* probe were performed as described previously [8]. More than three copies of the *MYCN* gene

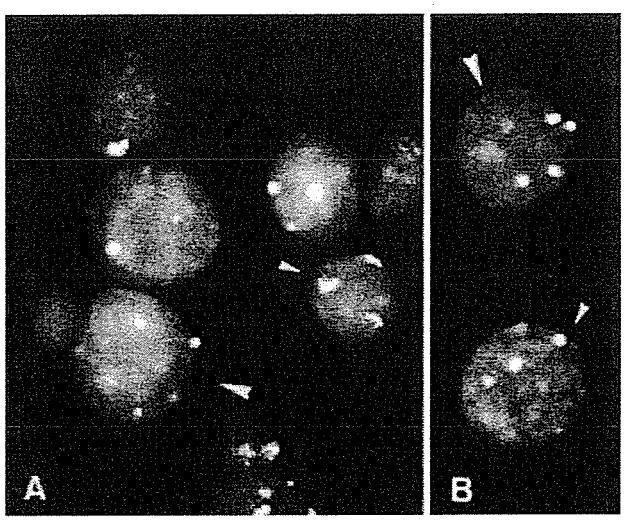

Fig. 1. Interphase FISH analysis using a chromosome 1 probe (D1Z1) on Tris1 tumors with a mixed population of cells with trisomy 1 and tetrasomy 1, with or without pentasomy 1. Cells in A and those in B were derived from 2 different tumors. Large and small arrowheads show cells with tetrasomy 1 and cells with trisomy 1, respectively.

per haploid genome were considered to indicate amplification. Tumors were also classified into three biologic types by the number of chromosome 1 and *MYCN* status; i.e., type 1 (Tris1 and normal *MYCN* copy number), type 2 (Dis1 and normal *MYCN* copy number), and type 3 (Dis1 and amplified *MYCN* copy number) [12,13].

Flow Cytometry

Of 453 neuroblastomas examined by FISH, 134 were also examined by flow cytometry. The DNA index was analyzed on the Becton-Dickinson FACScan flow cytometer by DNA cell-cycle analysis software-version C.

Statistical Analysis

The significance of the differences in various biological and clinical aspects of the disease among the patient groups was examined by the chi-square or Fisher's exact test. The overall survival (OS) for each group of patients was estimated on August 30, 2003 by the Kaplan–Meier method, and compared using log-rank tests. The survival time was defined as the interval between remission induction or surgery and death from any cause.

RESULTS

We examined chromosome 1 by FISH and *MYCN* copy number by Southern blot in all 453 tumors.

Grouping of Neuroblastomas by Constitution of Chromosome 1 and Frequencies of *MYCN* Amplification in Four Cytogenetic Groups

Of the 453 tumors, 56, including 55 with disomy 1 and 1 with tetrasomy 1, were classified as the Dis1 Norm1p tumor, 79, including 58 with disomy 1 and 21 with tetrasomy 1, as the Dis1Del1p tumor, 283, including 192 with trisomy 1 and 91 with a mixed population of the various cells as the Tris1Norm1p tumor, and 35 including 23 with trisomy 1 and 12 with a mixed population of the various cells, as the Tris1Del1p tumor (Table I).

MYCN amplification was found in none of 56 Dis1-Norm1p tumors, 46 of 79 Dis1Del1p tumors, 4 of 283 Tris1Norm1p tumors, and 1 of 35 Tris1Del1p tumors (Table I). Thus, *MYCN* amplification was closely associated with Dis1Del1p tumors. Cytogenetic analysis was successful in two of the five Tris1 tumors with *MYCN* amplification; one showed a modal chromosome number of 62 with three normal chromosome 1 s and the other

TABLE I. Clinical and *MYCN* Features of Four Cytogenetic Groups of Patients Classified by the Constitution of Chromosome 1 in Three Groups of Patients Classified by the Age of Patients and the Method of Tumor Detection

		Stage of disease					*MYCN* amplification		Overall survival at 6 years	
Group of patients	Number of patients	1	2	4S	3	4	+	−	%	Standard error
Infants found by mass-screening										
Dis1Norm1p	18	6	5	4	1	2	0	18	100	
Dis1Del1p	16	5	2	5	0	4	4	12	75.0	10.8
Tris1Norm1p	228	95	62	19	38	14	0	228	99.5	0.4
Tris1Del1p	25	16	4	2	1	2	0	28	100	
Total	287	122	73	30	40	22	4	283	98.2	0.8
Infants diagnosed clinically (<12 months)										
Dis1Norm1p	8	1	1	3	1	2	0	8	87.5	11.7
Dis1Del1p	8	0	1	3	2	2	4	4	37.5	17.1
Tris1Norm1p	34	14	6	7	6	1	1	33	97.1	2.9
Tris1Del1p	1	1	0	0	0	0	0	1	Alive	
Total	51	16	8	13	9	5	5	46	88.0	4.9
Children diagnosed clinically (≥12 months)										
Dis1Norm1p	30	2	3	0	4	21	0	30	58.7	9.1
Dis1Del1p	55	1	3	0	9	42	38	17	38.1	6.8
Tris1Norm1p	21	5	6	0	2	8	3	18	71.4	9.8
Tris1Del1p	9	0	0	0	2	7	1	8	33.3	15.7
Total	115	8	12	0	17	78	42	73	49.0	4.8

Dis1Norm1p, disomy 1/tetrasomy 1 with no 1p deletion; Dis1Del1p, disomy 1/tetrasomy 1 with 1p deletion; Tris1Norm1p, trisomy 1 with no 1p deletion; Tris1Del1p, trisomy 1 with 1p deletion. See the text for more detailed definition. Infants found by MS: Dis1Norm1p, Tris1Norm1p or Tris1Del1p vs. Dis1Del1p ($P = 0.014$, $P < 0.001$, or $P = 0.003$). Infants diagnosed clinically: Dis1Norm1p or Tris1Norm1p vs. Dis1Del1p ($P = 0.079$ or $P < 0.001$). Children diagnosed clinically: Dis1Norm1p or Tris1Norm1p vs. Dis1Del1p ($P < 0.006$ or $P < 0.006$).

showed that of 94 with 3 or 4 normal chromosome 1 s, and both had many double minutes.

Three Biological Types of Neuroblastomas Defined by the Number of Chromosome 1 and *MYCN* Status, and Survivals and Stage of the Disease in Patients With Each Type of the Tumors

The OS was excellent in infants found by MS, dismal in children diagnosed clinically, and intermediate in infants diagnosed clinically (Table I). Because there was no significant difference in OS between patients with Tris1Norm1p tumors and those with Tris1Del1p tumors in all three clinical groups (infants found by MS, infants diagnosed clinically, and children diagnosed clinically), OS of Tis1Norm1p patients was combined with that of Tris1Del1p patients. The OS of patients with Dis1Del1p tumors was worse than that of patients with Dis1Norm1p tumors in all three clinical groups (Table I). The OS was better for the 12 patients with Dis1Del1p tumors with no *MYCN* amplification than for the four patients with Dis1Del1p tumors with *MYCN* amplification in infants found by MS ($P < 0.001$). In contrast, the OS did not differ between the patients with Dis1Del1p tumors with no *MYCN* amplification and those with Dis1Del1p tumors with *MYCN* amplification in infants or children diagnosed clinically. In addition, there was no significant difference in OS between the patients with Dis1Norm1p tumors and those with Dis1Del1p tumors with no *MYCN* amplification in infants found by MS or infants diagnosed clinically. Children diagnosed clinically with Dis1Norm1p tumors tended to show the better OS than those with Dis1Del1p tumors with no *MYCN* amplification ($P = 0.078$).

Thus, the 453 tumors were classified into three biological types: type 1, 313 tumors with Tris1Norm/Del1p with no *MYCN* amplification; type 2, 89 tumors with Dis1Norm/Del1p with no *MYCN* amplification; type 3, 46 tumors with Dis1Del1p with *MYCN* amplification (Table II). Of five patients with Tris1Norm/Del1p tumors with *MYCN* amplification, an infant diagnosed clinically with five copies of *MYCN* at stage 2 and a child diagnosed clinically with 10 copies at stage 3 were alive, and the other three children diagnosed clinically with more than 10 copies at stage 4 died of the disease. The five tumors with Tris1 with *MYCN* amplification were excluded in the subsequent analysis because of the rare incidence of the combination.

Type 1 tumors in infants found by MS or diagnosed clinically had earlier stages of the disease than type 1 tumors in children diagnosed clinically ($P < 0.0001$ and $P = 0.0007$) (Table II). Type 2 tumors in infants found by MS tended to have earlier stages than type 2 tumors in infants diagnosed clinically ($P = 0.06$) and showed earlier stages than type 2 tumors in children diagnosed clinically ($P < 0.0001$). There was no significant difference in the stage distribution of type 3 tumors between any 2 of the three clinical groups (infants found by MS, infants diagnosed clinically and children diagnosed clinically).

The OS was better for infants with type 1 tumors found by MS ($P < 0.001$) or for infants with type 1 tumors diagnosed clinically ($P = 0.005$) than for children with type 1 tumors diagnosed clinically (Fig. 2). There was no significant difference in the OS between type 1 infants detected by MS and type 1 infants diagnosed clinically. The OS was better for infants with type 2 tumors found by MS than for infants with type 2 tumors diagnosed

TABLE II. Three Biological Types of Neuroblastoma Classified by the Number of Chromosome 1 and *MYCN* Status in Three Groups of Patients Classified by the Age of Patients and the Method of Tumor Detection

Group of patients	Number of patients	Stage of disease					Overall survival at 6 years	
		1	2	4S	3	4	%	Standard error
Infants found by mass-screening								
Type 1 (Tris1 with normal *MYCN*) tumor	253	111	66	21	39	16	99.6	0.4
Type 2 (Dis1 with normal *MYCN*) tumor	30	11	7	9	1	2	100	
Type 3 (Dis1 with amplified *MYCN*) tumor	4	0	0	0	0	4	0	
Total	287	122	73	30	40	22	98.2	0.8
Infants diagnosed clinically (<12 months)								
Type 1 (Tris1 with normal *MYCN*) tumor	34	15	5	7	6	1	97.1	2.9
Type 2 (Dis1 with normal *MYCN*) tumor	12	1	2	4	2	3	72.9	13.5
Type 3 (Dis1 with amplified *MYCN*) tumor	4	0	0	2	1	1	25.0	21.7
Total	50	16	7	13	9	5	85.3	5.1
Children diagnosed clinically (≥12 months)								
Type 1 (Tris1 with normal *MYCN*) tumor	26	5	6	0	4	11	68.6	9.2
Type 2 (Dis1 with normal *MYCN*) tumor	47	3	4	0	6	34	53.5	7.4
Type 3 (Dis1 with amplified *MYCN*) tumor	38	0	2	0	7	29	35.6	8.0
Total	111	8	12	0	17	74	50.9	4.9

Tris1, trisomy 1; Dis1, disomy 1. See the text for more detailed definition.

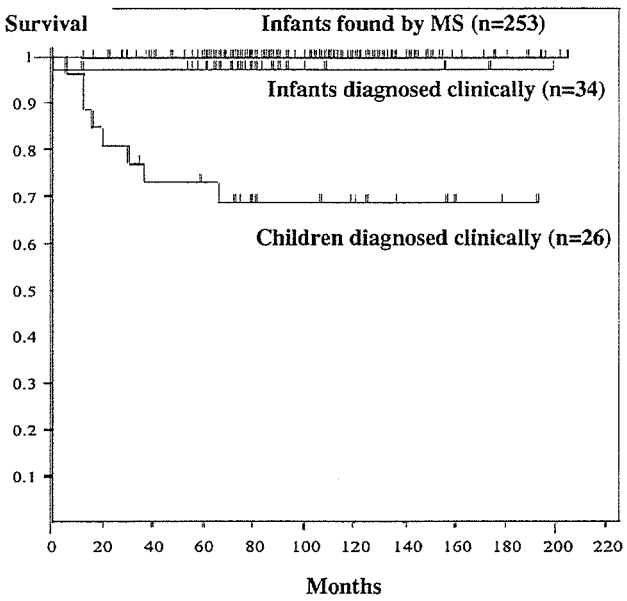

Fig. 2. The OS curve for 253 type 1 infants found by MS or that for 34 type 1 infants diagnosed clinically was better than that for 26 type 1 children diagnosed clinically ($P < 0.001$ and $P = 0.005$).

clinically ($P = 0.014$) or for children with type 2 tumors diagnosed clinically ($P < 0.001$) (Fig. 3). There was no significant difference in the OS between type 2 infants and type 2 children both diagnosed clinically. The OS was unfavorable for all three clinical groups of patients with type 3 tumors, and there was no significant difference in the OS between any two of the three clinical groups of patients with type 3 tumors (Fig. 4).

Correlation of the Number of Chromosome 1 Determined by FISH With the DNA Index Determined by Flow Cytometry

Of the 134 tumors whose DNA index (DI) was determined, 23 tumors with disomy 1 had a median DI of 1.0 ranging from 1.0 to 1.26, 5 tumors with teterasomy 1 had a median DI of 1.9 ranging from 1.8 to 2.4, 87 tumors with trisomy 1 had a median DI of 1.44 ranging from 1.17 to 1.71, and 19 tumors with a mixed population of cells with trisomy 1 and cells with tetrasomy 1 with or without cells having pentasomy 1 had a median DI of 1.91 ranging from 1.72 to 2.46. Thus, tumors with disomy 1, trisomy 1, and tetrasomy 1 corresponded with those with DI of 1–1.17, 1.18–1.71, and 1.80–2.40, respectively. Only 2 of the 134 tumors (1.5%), one with disomy 1 and a DI of 1.26 and the other with trisomy 1 and a DI of 1.17, showed a DI that fell outside of the above correlation. Tumors with tetrasomy 1 and those with a mixed population of the various cells occupying 4% and 14% of the 134 tumors, respectively, showed overlapped DI ranges, and those 2 groups of tumors could be separated by FISH, but not by flow cytometry.

DISCUSSION

Epidemiological studies on the reduction of mortality by MS have recently been reported in Quebec, Germany

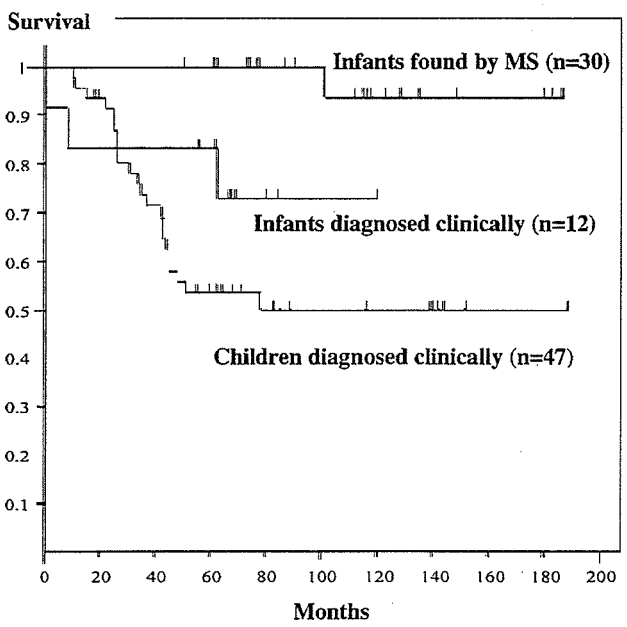

Fig. 3. The OS curve for 30 type 2 infants found by MS was better than 12 type 2 infants diagnosed clinically ($P = 0.014$), or 47 type 2 children diagnosed clinically ($P < 0.001$).

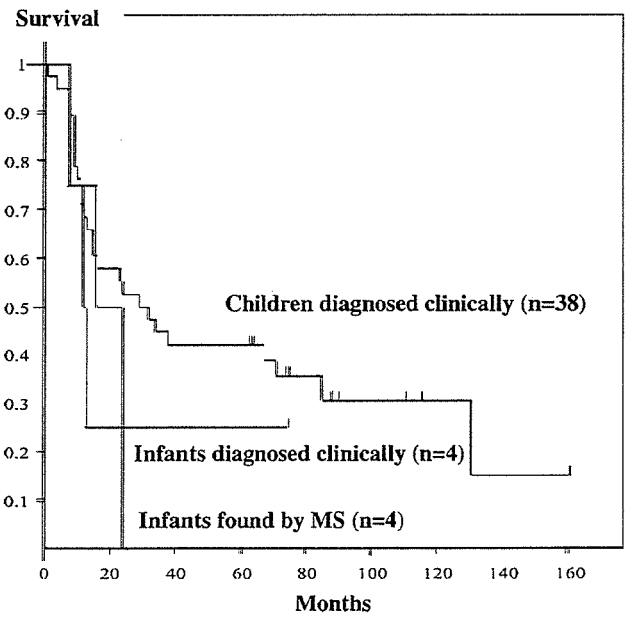

Fig. 4. OS curves for four type 3 infants found by MS, four type 3 infants diagnosed clinically, and 38 type 3 children diagnosed clinically. There were no significant differences between any two of the three groups.

and Japan [3–6]. All three studies showed an increased incidence of neuroblastoma in the first year of life, and strongly suggested that the screening may have detected a large number of neuroblastomas that would have regressed spontaneously [3–5]. While the Quebec and German studies denied the reduction of mortality, the Japanese studies showed a modest decrease in mortality. Because it is clear that harm has been caused by excessive treatment of some neuroblastomas that would have regressed spontaneously, and the effectiveness of the program has been controversial, the program in Japan was discontinued on March 31, 2004. Although the efficacy of the screening will be determined by epidemiological methods, biological studies on neuroblastomas detected clinically or by MS may explain the background of the epidemiological data, and clarify the incidence of neuroblastomas with unfavorable features that may be a target for MS.

The present study classified neuroblastomas into three biological types on the basis of the number of chromosome 1, *MYCN* status and clinical outcome, namely type 1, type 2 and type 3. We also examined the incidence ratio of the three biological types in each of three clinical groups of patients (infants found by MS, infants diagnosed clinically and children diagnosed clinically). Type 1, type 2 and type 3 tumors were found in 88.2%, 10.5%, and 1.4% of infants found by MS, in 68.0%, 24.0%, and 8.0% of infants diagnosed clinically and in 23.4%, 42.3%, and 34.2% of children diagnosed clinically ($P < 0.001$). The distinct differences in the incidence of the 3 types of tumors between infants found by MS, infants diagnosed clinically and children diagnosed clinically are closely correlated with the favorable or unfavorable outcome of each clinical group of patients (Table II).

While infants with type 1 tumors found by MS and those with type 1 tumors diagnosed clinically had similarly excellent prognoses, children with type 1 tumors diagnosed clinically had intermediate prognoses (Fig. 2). Children with type 1 tumors diagnosed clinically who had an unfavorable outcome were at the advanced stages, and had frequent 1p deletion (Tables I and II). While the incidence of type 1 tumors was very high and most of the type 1 tumors were at the early stages in infants found by MS, the incidence of type 1 tumors was low and one half of the type 1 tumors were at the advanced stages in children diagnosed clinically. These findings suggest that some type 1 tumors with the accumulation of genomic alterations in older children may have originated from subclinical type 1 tumors in infants although the incidence of such tumors may be quite low.

While the children with type 2 tumors diagnosed clinically had advanced stages and dismal prognoses, the infants with type 2 tumors found by MS had early stages and good prognoses. The infants with type 2 tumors diagnosed clinically had a stage distribution and prognoses between the infants with type 2 tumors found by MS and the children with type 2 tumors diagnosed clinically (Table II and Fig. 3). The worse outcome for the infants with type 2 tumors diagnosed clinically than for the infants with type 2 tumors found by MS may be related to the fact that the type 2 tumors diagnosed clinically were at more advanced stages than those found by MS, although the small number of infants diagnosed clinically may have affected the worse survival curve. Thus, type 2 tumors may be a suitable target for MS. In contrast, all three clinical groups of patients with type 3 tumors had advanced stages and dismal prognosis, suggesting that the type 3 tumors may not be a suitable target for MS. The type 2 tumors comprise about 10% of MS-positive tumors, and adding the small portion of MS-positive type 1 tumors to MS-positive type 2 tumors results in 10%–15% of MS-positive tumors that may be a target for MS.

Biological features of neuroblastomas found by the Austrian mass-screening project showed unfavorable features including *MYCN* amplification, 1p deletion/imbalance, and di-/tetraploidy in 17 (37%) of 45 tumors [14]. When we used the similar criteria, unfavorable features were found in 60 (21%) of 287 tumors in the present study. The Austrian program also emphasized the presence of intratumoral heterogeneity of *MYCN* amplification and 1p deletions in some neuroblastomas, especially in triploid tumors detected by MS [14,15]. As we used Southern blot but not FISH to evaluate the *MYCN* copy number, we could not assess the heterogeneity of the foci of cells with or without *MYCN* amplification in the same tumors. Intratumoral heterogeneity of 1p deletion reported by Ambros et al. was not found in the present study [15]. The different times for the screening and the different sample sizes might have reflected the different incidences of tumors with unfavorable features or with intratumoral heterogeneity.

Ploidy, 1p deletion, and *MYCN* copy number are important biological factors predicting outcome of neuroblastoma patients. The present study showed that 1p deletion was a poor prognostic factor for tumors with disomy 1 but not for those with trisomy 1. The reason why 1p deletion has different prognostic significance between diploid and triploid neuroblastomas was explained elsewhere [16]. The present study also showed that there was no significant difference in OS between children diagnosed clinically having disomy 1 with 1p deletion and no *MYCN* amplification and those having disomy 1 with 1p deletion and *MYCN* amplification. The similar findings were also reported by other investigators [12]. In addition, multivariate analyses in neuroblastoma showed that 1p deletion had stronger predictive power than *MYCN* amplification [8,17]. These findings reflected that most tumors with *MYCN* amplification had disomy 1 with 1p deletion, and most children having tumors with disomy 1

with 1p deletion and no *MYCN* amplification had unfavorable prognosis.

We determined the number of chromosome 1 s that correlated with the ploidy number by interphase FISH [16]. In addition, the present simultaneous FISH and flow cytometric analysis showed that the number of chromosome 1 s is correlated with the specific ranges of DI, and that FISH but not flow cytometry can detect the Tris1 tumors with a mixed population of cells with trisomy 1, tetrasomy 1, with or without pentasomy 1. The tumors with mixed populations were included in the Tris1 tumors because of the presence of triploid cells, frequent occurrence in infants, and favorable clinical outcome, although cells with tetrasomy 1 and/or cells with pentasomy 1 sometimes predominated over cells with trisomy 1 in the tumor. Tumors with mixed populations of cells accounted for 22% of the 453 tumors that were examined by FISH. Because the DI range of tumors with mixed populations overlapped with that of the tumors with simple tetrasomy 1, it is important to keep in mind that tetraploid tumors in infants determined by flow cytometry may belong to the Tris1 tumors if FISH analysis is performed.

The Pediatric Oncology Group (POG) studied the OS of infants with unresectable or metastatic neuroblastomas, and found that patients with hyperdiploid tumors (DI, >1) including tetraploid tumors had a better OS than those with diploid tumors (DI = 1) [18]. In contrast, Ladenstein et al. studied the OS of infants and children with localized or disseminated neuroblastomas, and found that patients with triploid tumors had a better OS than those with diploidy/tetraploidy [19]. While tumors with tetrasomy 1 (6/338, 2%) were far less frequent than those with a mixed population of cells with trisomy 1 and cells with tetrasomy 1 in infants, tumors with simple tetrasomy 1 were more frequent than those with mixed populations in older children. Thus, the present simultaneous FISH and flow cytometric study suggested that a classification of unresectable neuroblastomas using diploidy and hyperdiploidy may discriminate infants with a favorable outcome from those with unfavorable ones, and another classification using diploidy/tetraploidy and triploidy may predict the outcome of infants and children at all stages. Furthermore, the present classification using interphase FISH on chromosome 1 may also predict the outcome of patients of any age and at all stages.

The present study suggests that 10%–15% of MS-positive tumors may be the target for MS. The low incidence of tumors with unfavorable biology in MS-positive population suggests that the decrease in mortality may be small if present, and the marginal decrease may have caused the discrepancy between the Quebec or German studies and Japanese one although the inferior statistical design of the Japanese study may have played a major role in the inconsistency.

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A cytogenetic analysis in two cases of malignant peripheral nerve sheath tumor showing hypodiploid karyotype

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Abstract. In this study, we report cytogenetic findings in two cases of malignant peripheral nerve sheath tumor (MPNST) with hypodiploid karyotypes. A G-band technique, multicolor fluorescence *in situ* hybridization (m-FISH) and comparative genomic hybridization (CGH) were used and compared in this investigation. In both tumors, the G-band and m-FISH analysis demonstrated multiple rearrangements on chromosomes 1-5, 8-12, 15-17, 20 and 21, whereas CGH exhibited gains at 8q and 4q. Both of the structural aberrations and the genomic imbalances of the chromosomes may play an important role in the pathogenesis and development of MPNST. No cytogenetic abnormalities specific for MPNST were found in the present cases or in other previously reported cases. This may reflect the diversity or heterogeneity of MPNST that exhibit various clinical and histological features. However, there are few cases described in detail on a morphologic pattern of MPNST, a correlation between the cytogenetic aberrations and the histologic patterns are still uncertain.

Introduction

Malignant peripheral nerve sheath tumor (MPNST) is a rare neoplasm arising from peripheral nerves and can occur sporadically or in association with neurofibromatosis 1 (NF1; von Recklinghausen's disease) (1). Although it clearly originates from peripheral nerves, MPNST is one of the most difficult diagnoses in pathology owing to its morphologic diversity. In only 50% of MPNST, immunohistochemical examination shows a positive reaction to the neural marker S-100 protein (2).

Cytogenetic analyses of MPNST have been reported in more than 100 cases. Their chromosome ploidy distributes between hypodiploid and tetraploid, some tumors have been described as hypodiploid (3-8). Regardless of ploidy range, however, karyotypes of MPNST exhibit complex abnormalities with numerical and structural changes (3-13). Cytogenetic aberrations on chromosomes 1, 2, 5, 7-9, 11-14, 17, 18 and 22 were found to be the most frequent, although no consistent karyotypic patterns have been detected (3-18).

In the present study, we examined the cytogenetic details of two tumors of myxoid MPNST with hypodiploid karyotypes by combining conventional G-band, multicolor fluorescence *in situ* hybridization (m-FISH) and comparative genomic hybridization (CGH) techniques, and compared the results with existing cytogenetic information of MPNST to consider its relevance to carcinogenesis.

Materials and methods

Clinical data

Case 1. A 46-year-old man noticed a painless mass on the left side of his neck. The mass was excised and diagnosed histologically as MPNST; the patient showed no clinical signs of neurofibromatosis. Three years later, a recurrent tumor developed at the same site and was surgically removed. One year later, the tumor recurred for a third time at the same site and was again excised. A sample was collected for cytogenetic analysis from the initial recurrent tumor.

Case 2. A 70-year-old man had low back pain for a period of 4 years. He felt pain in his left leg for one month and it became difficult to walk. A computer tomography (CT) evaluation revealed a tumor in his left buttock. The tumor was surgically removed and diagnosed as MPNST; no clinical signs of neurofibromatosis were present.

Cytogenetic analysis. We received tissue samples from the recurrent tumor in case 1 and from the primary gluteal tumor in case 2. Tumor tissues from each patient were disaggregated with collagenase, cultured and harvested as previously described (19). The trypsin-Giemsa banding technique was performed for the karyotypic analysis. The karyotype was expressed according to the detailed system of the International System for Human Cytogenetic Nomenclature (ISCN 1995) (20). A multicolor painting was employed with the 24XCyte mFISH probe kit according to the recommendations of the manufacturer (Meta systems GmbH, Germany). Slides were

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Key words: malignant peripheral nerve sheath tumor, neurofibromatosis, sarcoma, CGH, mFISH, karyotype, chromosome

Table I. Antibodies and immunohistochemical conditions.

Antibodies	Dilution	Source	Pretreatment
Vimentin	1:100	Dako	+
S-100	Prediluted	Nishirei, Japan	-
NSE	1:200	Dako	+
Leu7 (CD 57)	1:50	BK	+
CD34 (My10)	1:40	BK	+
EMA	1:100	Dako	+
CK AE1/AE3	1:50	Dako	+
CK 7	1:50	Dako	+
CAM5.2	Prediluted	BK	+
CD56	1:25	Dako	+
Desmin	1:100	Dako	+
HHF35	1:50	Enzo, NY, USA	-
α-SMA	1:100	Dako	-
HMB45	1:50	Dako	-
MyoD1	1:100	Dako	-
p53 (DO7)	1:100	Novocastra, UK	+

EMA, epithelial membrane antigen; NSE, neuronspecfic enolase; Dako, Dakopatts, Denmark; BK, Becton-Dickinson.

observed with a Zeiss Axioplan 2 microscope (Carl Zeiss Jena GmbH, Jena, Germany). Images were captured with a Sensys CCD camera and analyzed by using the automated mFISH analysis software (MetaSystems, Altlusheim, Germany) based on the digital image analysis system (Isis, Carl Zeiss Vision, Oberkochen, Germany).

Comparative genomic hybridization. DNA was extracted from fresh frozen tissues and labeled directly with fluorescein-12-dUTP (Roche Diagnostics, Mannheim, Germany) by nick translation (Vysis, Downers Grove, IL). SpectrumRed (Vysis) was used as the reference DNA and the hybridization procedure was carried out as described by Koga *et al* (18). Three-color CGH signals (red for reference DNA, green for tumor DNA and blue for counterstaining) were captured using a Zeiss Axioplan 2 microscope (Carl Zeiss Jena GmbH) and analyzed with Isis/CGH software (Metasystems, Altlussheim, Germany). At least 10 representative images were analyzed to detect increases and decreases of DNA sequence copy numbers on the chromosome. The chromosomal regions with a green/red ratio above 1.20 were considered to have gained DNA sequence copy numbers, whereas regions with a ratio below 0.80 were considered to have lost DNA copy numbers. These cut-off values were based on measurements from a series of normal mail/femail control. Heterochromatic, centromeric and telomeric regions, the short arm of the acrocentric chromosomes and Y chromosome were excluded from evaluation, because these are known to be areas in which hybridization is unreliable (21).

Pathologic evaluations. Formalin-fixed, paraffin-embedded tissue samples were used for light microscopicy studies.

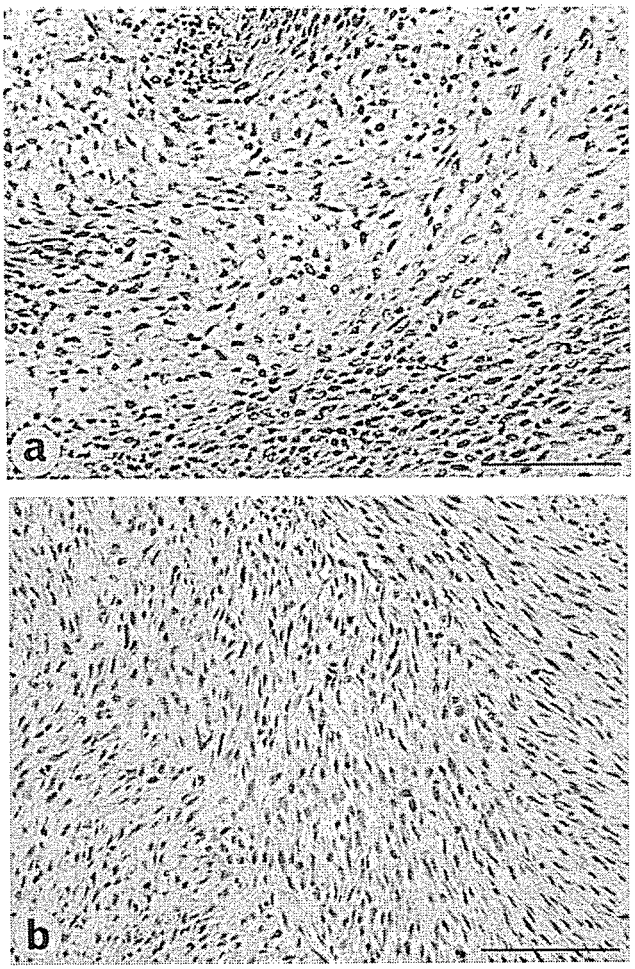


Figure 1. (a), The tumor is composed of a mixture of spindle cells, polyfonal cells and a few bizarre giant cells. A prominent myxoid feature is seen (H&E). Scale bar, 100 μm. (b), Section shows spindle-shaped cells with wavy nuclei in a myxomatous background (H&E). Scale bar, 100 μm.

Serial 3-μm thick sections were prepared on glass slides and stained with hematoxylin and eosin (H&E). For immunocytochemistry of paraffin-embedded sections, antigen retrieval was performed for EMA, CytokeratinAE1/AE3, NSE, CD34, CD56, CD57, vimentin and p53 (clone DO-7) by using microwave pretreatment in standard citrate buffer (0.01 M, pH 6.0). The summary of immunohistochemical conditions is listed in Table I. The samples were reacted with each of the primary antibodies for 1 h at room temperature. The bound antibodies were visualized using a labeled streptavidin-biotin system and an alkaline phosphatase technique.

Results

Pathologic findings. In case 1, the tumor showed a mixed proliferation of atypical spindle cells and round or stellate cells in a delicate fibrillar matrix. The tumor exhibited wide myxoid areas alternating with dense hypercellular foci and increased perivascular cellularity (Fig. 1a). Mitotic figures including abnormal forms were found among the tumor cells.

In case 2, the tumor showed proliferation of spindle-shaped cells with plump or wavy nuclei and fine, eosinophillic cytoplasm in a prominent myxomatous background (Fig. 1b).

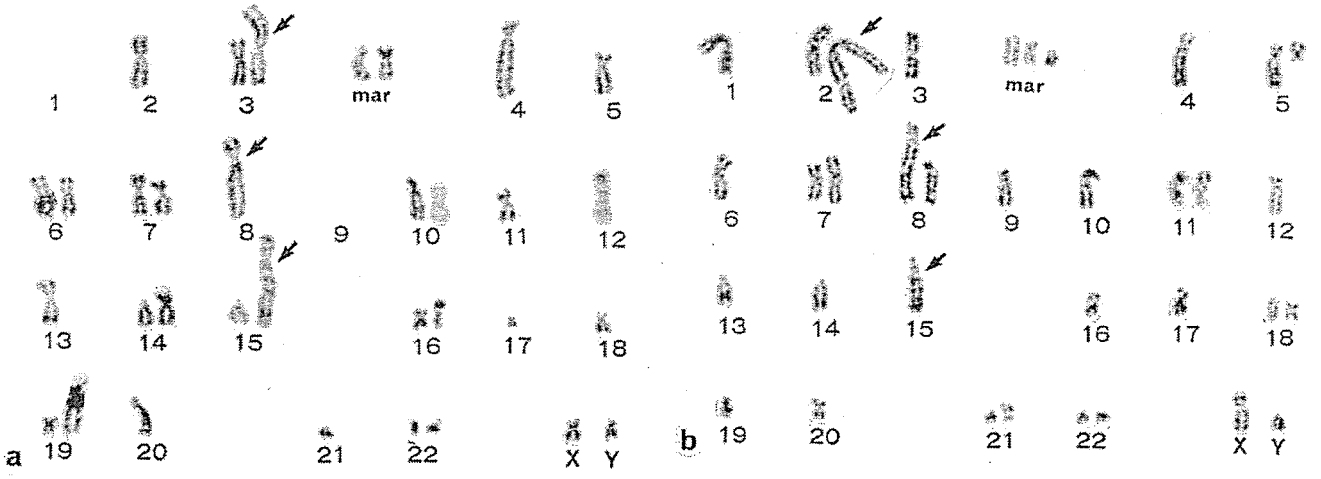


Figure 2. Representative G-banded karyotype revealed by m-FISH analysis [(a), case 1; (b), case 2]. Arrows point to unclassified marker chromosomes detected by G-band analysis alone.

Table II. Cytogenetic findings in two MPNST.

Case 1	34,Y,der(X)(:p21→q22:),-1,-2,der(3)(17?→17?::3p11→3qter),-4,der(4)(4pter→4q35::22?→22?::8?→8?),-5,-8,der(8)(8qter→8q10::8q10→8qter::9?→9?::3?→3?::19?→19?),-9,-9[3], der(10)(10pter→10q26::Xq23→Xqter),-11,-12[4],der(12)(4?→4?:: 12p13→12qter),-13,der(13)(3?→3?::13p11→13qter),der(14)(4?→4?::22?→22?::14q10→14qter),-15,der(15)(X?→X?::12?→12?::X?→X?::20?→20?::15p10→15qter::21?→21?::9?→9?:: 1q32→1qter),der(16)(11qter→11q10::16p11→16qter),der(17)(:p12→q21:)der(19)(19pter→19q13::3?→3?::2q11→2qter),-20,der(20)(20pter→20q12::8q12→8qter),-21,-22,der(?)(19?→19?::8?→8?::22?→22?::10q10→10qter),+2mar [cp6][a]
Case 2	33-35,XY,-1,der(2)(4?→4?::6?→6?::1?→1?::8?→8?::20?→20?::13?→13?::2p14→2q37::7?→7?::1q22::1qter),-3,der(4)(4pter→4q31::3q12→3qter),del(4)(pter→q11:),del(5)(pter→q11:),-6,der(7)(7pter→q11::12q11→12q15::15q11→15qter),der(8)(8pter→8q24.3::17?→17?::12?→12?::17?→17?::8?→8?::21?→21?::20?→20?::5?→5?),der(8)(p21→q10::4q11→4qter),-9,-10,der(10)(17?→17?::10p15→10qter),der(11)(11pter→11q22::13?→13?),-12,-13,-14,-15,der(15)(15pter→15q24::14q11→14ater),-16,der(17)(17pter→17q11.1::9q13→9q21),-19,-20,der(21)(5?→5?::21p11.2→21qter),+mar [cp5]

[a]8 cells showed normal karyotype.

Moderate nuclear atypia, scattered mitotic figures and focal necrosis were observed.

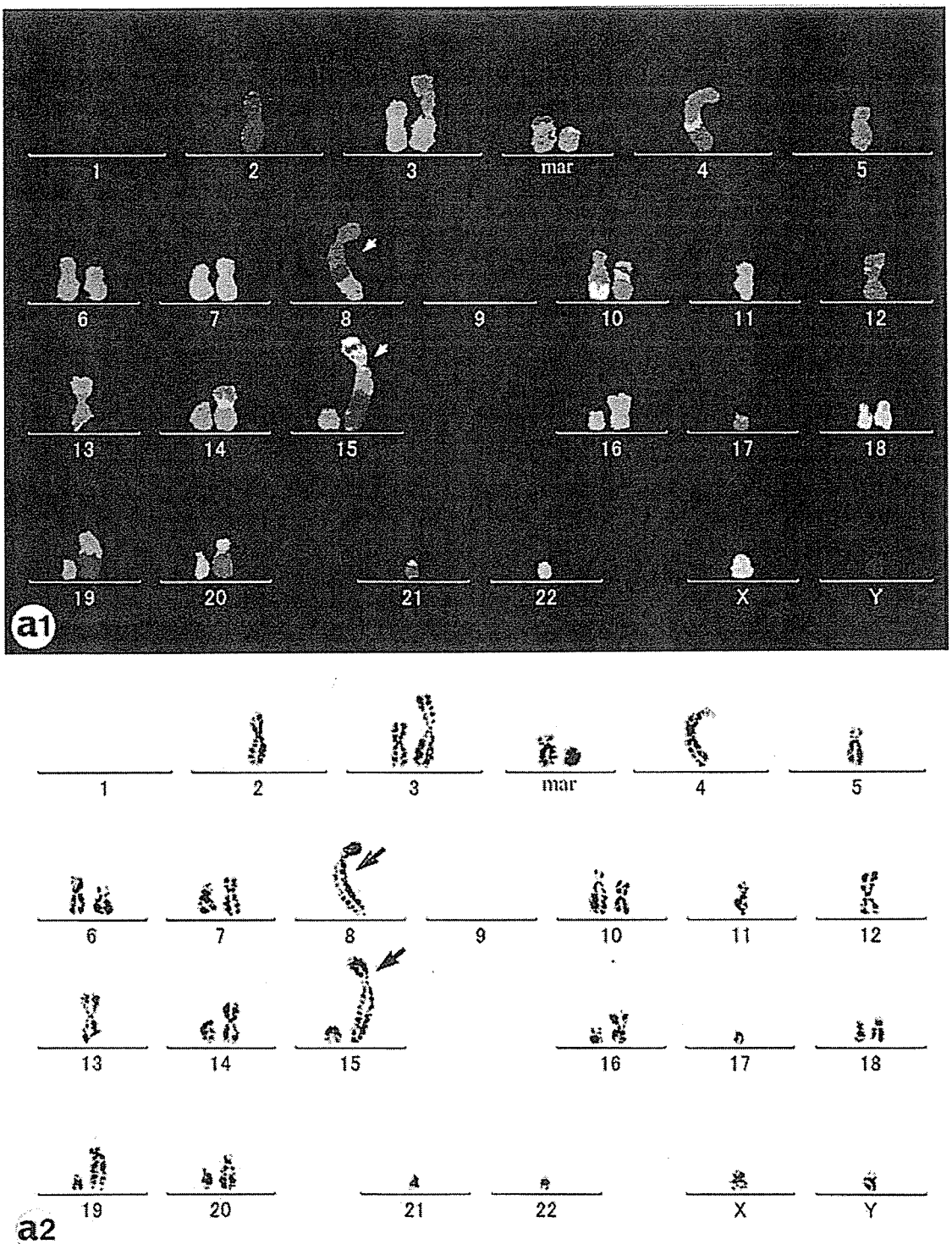
In both cases the tumor cells showed positive reactions for NSE, CD56 and vimentin. S-100 protein and CD57 were also positive in some cells. The tumor cells were uniformly negative for cytokeratin7, cytokeratin AE1/AE3, CAM5.2, HMB45, MyoD1, HHF35, desmin, EMA and CD34. Positive immunoreactions for p53 were observed in more than 60% of the tumor cell nuclei in case 1 and more than 10% of the tumor cell nuclei in case 2.

Cytogenetic analysis. In case 1, 26 days of culture produced adequate metaphases whereas cultures sustained for 2, 12 and 23 days produced no metaphases. Twenty-three metaphases were analyzed and the modal chromosomal number was 34. The karyotype based on G-band (Fig. 2a) in combination with mFISH (Fig. 3a) showed hypodiploidy with complex abnormalities (Table II).

In case 2, 18 adequate metaphases were obtained at 53 days of culture and the cells passaged twice during cultivation. Forty-five metaphases were analyzed and the modal chromosomal number was 34. The karyotype based on G-band (Fig. 2b) in combination with mFISH (Fig. 3b) showed hypodiploidy with complex abnormalities (Table II).

Comparative genomic hybridization. In case 1, the gains of DNA sequences were observed for 4q28→qter, 8q, 17q22→qter and the losses of DNA sequences were observed for 4p14→pter (Fig. 4a).

In case 2 the gains of DNA sequences were observed for 1q21.3→q24, 2p22→pter, 2q12→q14, 2q24→q32, 4p14→pter, 4q26→qter, 5p14→p15.2, 5q32→qter, 7p13→pter, 8q12→q21.1,



12q13→q21, 15q14→q24, 21q11.1→q22.1; no DNA sequence losses were observed in case 2 (Fig. 4b).

Discussion

Histologically, malignant peripheral nerve sheath tumor (MPNST) is composed chiefly of spindle-shaped cells mixed with round or stellate cells. A differential diagnosis of malignancy therefore includes various spindle cell malignancies such as monophasic fibrous synovial sarcoma, fibrosarcoma, leiomyosarcoma, hemangiopericytoma and clear cell sarcoma (1). Both cases lacked signs of neurofibrmatosis 1 (NF1) and it was difficult to make a precise diagnosis based solely on histological findings; we therefore carried out immunohistochemical staining of the tumors to establish the diagnosis. The tumor cells showed positive immunoreactivities for vimentin and neural makers including NSE, S-100 protein, CD56 and CD57 and they were uniformly negative for myogenic, epithelial and melanocytic markers. On the basis of these results, the diagnosis of MPNST was established in both cases.

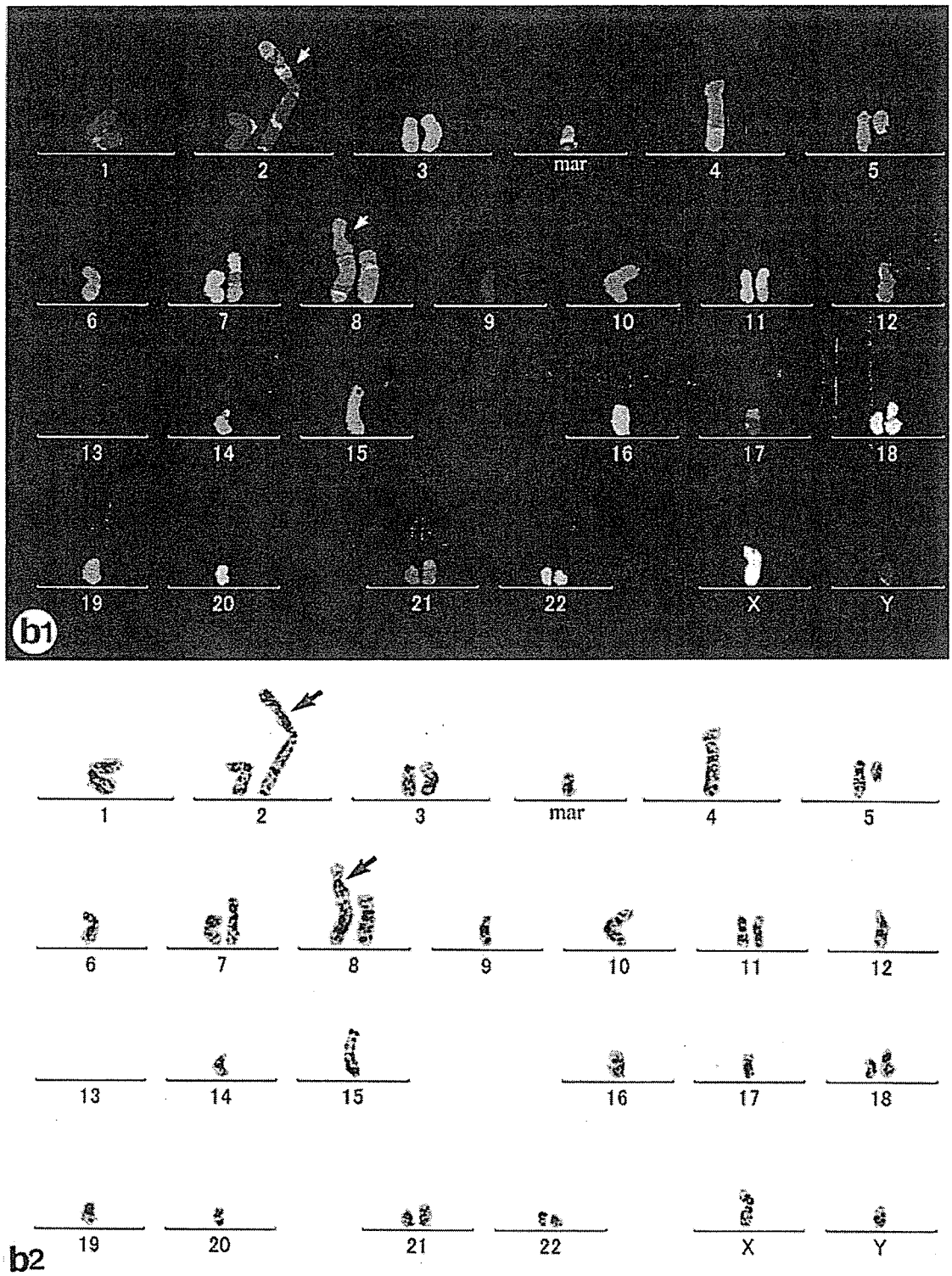


Figure 3. Complete m-FISH karyotypes in pseudo colors [(a1), case 1; (b1), case 2], and reverse DAPI banding pattern form the metaphase [(a2), case 1; (b2), case 2]. Some unclassified marker chromosomes detected by G-banding are composed of various portions of the affected chromosomes (arrows).

In recent years, many cytogenetic analyses of MPNST have been carried out utilizing the conventional G-band procedure and comparative genomic hybridization (CGH). As a result of these efforts, more than 100 cases of cytogenetic studies in which approximately 80 cases of chromosomal aberrations have been reported (3-18). The majority of them displayed highly complex karyotypes with many numerical and structural alterations. Their chromosome numbers varied with tetraploidy from hypodiploid range. Many chromosomal imbalances per sample were also detected by CGH.

In the present cases, both tumors displayed the hypodiploid karyotype with complicated aberrations and some unclassified marker chromosomes. With the combination of G-band and m-FISH analyses, some unclassified marker

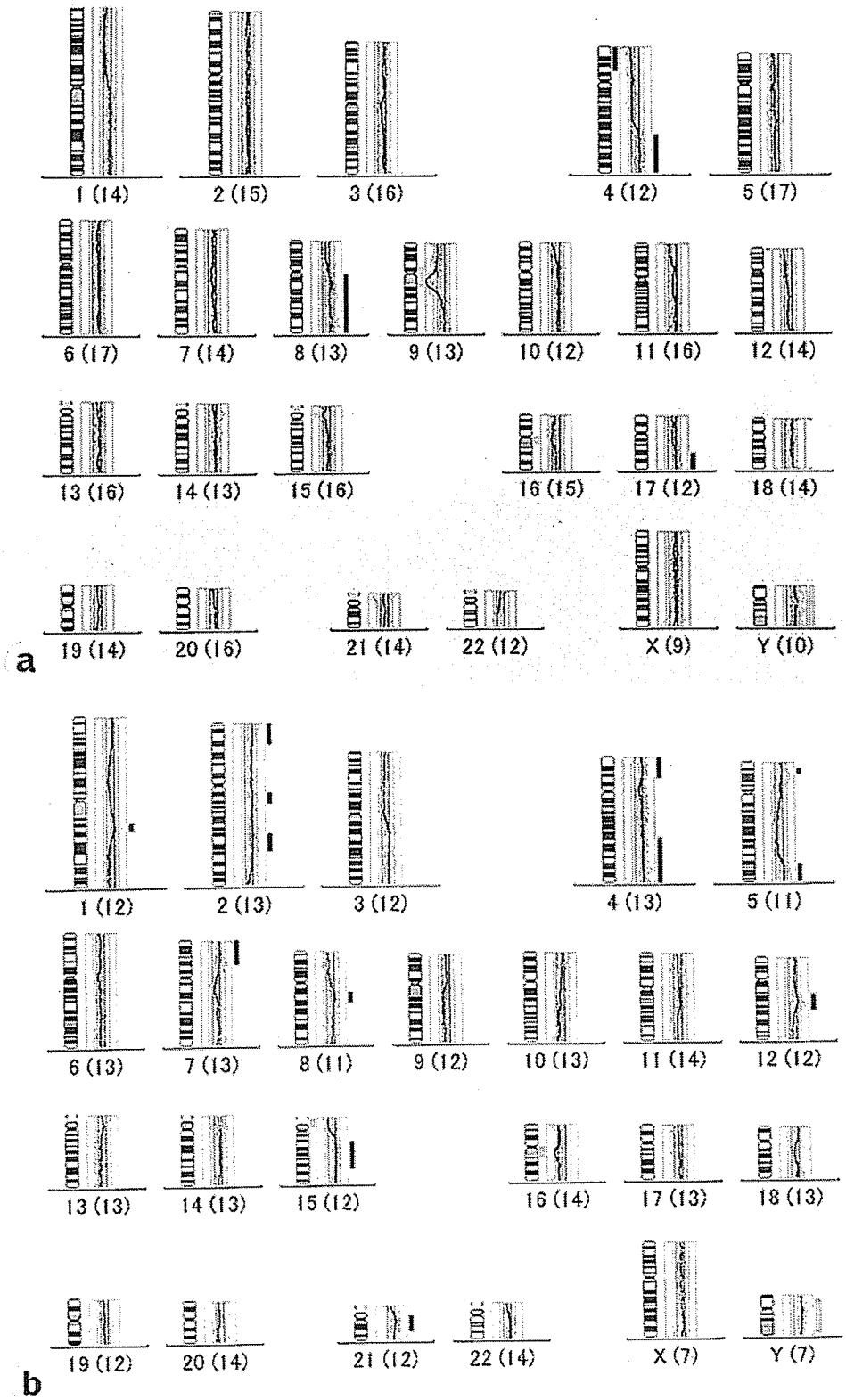


Figure 4. CGH single profiles of test to reference fluorescent intensity for each chromosome. Gains are shown on the right side of the profiles and the losses on the left by black bars [(a), case 1; (b), case 2]. Heterochromatic, centromeric and telomeric regions, the short arm of the acrocentric chromosomes and Y chromosome were excluded from the analysis and shown by the gray bars.

chromosomes could be identified (Fig. 2). A number of these chromosomes were proved to be composed of various portions of the affected chromosomes (Fig. 3). These results confirm the usefulness of m-FISH in the diagnosis of difficult cases of chromosomal aberration. As the structural aberrations were very complex and involved the frequent insertion or deletion of a single band, we were not able to determine even the chromosome number in some marker chromosomes. In a few

instances, many rearrangements were distributed randomly throughout the entire length of the chromosome.

Recently published results highlight a recurrent pattern of chromosomal imbalance by CGH. The recurrent gains of chromosome 1q, 5p, 6p, 7, 8q, 12, 13q, 15 and 17q the most frequent losses of chromosomes 1p, 9p, 11p, 17p and 17q have been reported (3-18). Loss of chromosome 9p is the most common feature in MPNST. *CDNK2A* has been mapped to 9p21 as a candidate tumor suppressor gene. The *CDNK2A* encodes p16 and $p14^{ARF}$ and inactivation of this gene impairs both RB1 and TP53 tumor suppressor pathways, respectively (22,23). Tumor suppressor genes *p53* and *NF1* are located on 17p12 and 17q11.2, respectively. Functional inactivation of these genes may play an important role in MPNST progression and development (9,24,25). Putative tumor suppressor genes located on chromosomes 1p and 11q are still unknown. In the present cases, we detected the gain of chromosome 17q by CGH. Whereas we were unable to identify genomic imbalances using conventional banding analysis because of complicated aberrations of karyotype, however, structural aberration of chromosomes 1, 9, 11 and 17 were found. These chromosomal aberrations may cause disruption of the tumor suppressor genes and possibly inactivate their suppressor function. Immunohistochemical analysis detected overexpression of the *p53* gene product in both our tumors. The half-life of the wild-type *p53* gene product is much shorter than that of mutated *p53* gene products; this may explain the overexpression of *p53* immunoreactivity (24,25).

CGH analysis of our tumors demonstrated that gains were more frequent than losses. This result supports the hypothesis that a proto-oncogene is predominant during tumor progression (16). In case 1, CGH analysis demonstrated that the recurrent tumor had the gain of 17q22-qter. This agrees with a previous report that suggested that the gain of 17q is associated with poor clinical outcome in MPNST (15,17). In case 2, CGH analysis found gains on chromosomes 5p, 7, 8q, 12 and 15. Gains in these chromosomes have been detected in several tumor types (26,27).

In our two cases, the commonly affected chromosomes detected by G-band, m-FISH and CGH analysis were chromosomes 4 and 8. Gains of 4q28-qter and 8q12-21 were detected by CGH analysis. The gain in chromosome 4q detected by CGH analysis has been reported previously in peripheral nerve sheath tumors (18); however, the relationship between the aberration of 4q and pathogenesis in MPNST is not clarified. Although the gain in 8q occurs frequently in sporadic and NF1-associated MPNST, it also occurs in various kinds of malignant solid tumors (10,12,17,26,27). The gain of 8q might therefore be associated with malignant tumor progression rather than the pathogenesis of MPNST.

These findings agree with a previous report that suggests MPNST develop by multi-step process with several genetic events (16). Several tumor suppressor genes and proto-oncogenes may contribute to tumorigenesis, development and progression in MPNST. This may explain the fact that in spite of many reports of numerical and structural cytogenetic aberrations in MPNST, an aberration common to all MPNST has not been found yet. Incidentally various combinations of genetic changes may reveal various histological patterns in MPNST. MPNST is one of the most difficult diagnoses in pathology due to the heterogeneity of their constituent cells and the morphologic diversity. However, there are not sufficient cases described in detail to suggest a correlation between phenotypic and genotypic features in MPNST. It is therefore necessary to investigate the possibility of a relationship between the histological patterns and the cytogenetic changes in a large series of MPNST.

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＜特集「分子標的医療研究の新展開」＞

EGFR阻害剤（gefitinib/イレッサ®）の小児固形腫瘍における臨床応用への可能性

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The possibility of gefitinib, epidermal growth factor receptor inhibitor, for pediatric solid tumors

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抄　録

小児固形腫瘍の予後は治療の進歩により改善してきた．しかし，難治性の腫瘍に関しては分子標的療法など新規の治療の開発が望まれている．上皮性増殖因子受容体（EGFR）は小児固形腫瘍においても確認されている．また，EGFRチロシンキナーゼ阻害剤のゲフィチニブは腫瘍細胞の増殖を抑制し，非小細胞性肺がんの臨床試験でも有効性が確認されている．有効性の予測因子の結論は出ていないが，EGFRの遺伝子変異，遺伝子の増幅また下流のシグナルのAKTのリン酸化などが報告・議論されている．

我々は，小児固形腫瘍のなかでも特に難治性である悪性横紋筋肉腫様腫瘍（MRT）におけるEGFRの発現とゲフィチニブの効果を*in vitro*と*in vivo*で検討し，MRTにおいても*in vitro*と*in vivo*ともに抗腫瘍効果があることを明らかにした．ゲフィチニブはMRTの治療に有効な新規治療薬である可能性を示した．

最近，治療抵抗性の小児固形腫瘍の患児に対するゲフィチニブの第Ⅰ相試験の結果が報告され，その耐用性が示された．ゲフィチニブは小児固形腫瘍の治療において大きな可能性を持つ薬剤であると考えられる．そのためには，腫瘍別に分子標的を明確にし，基礎研究の成果を臨床へと繋ぐ，トランスレーショナルリサーチの基盤整備が必要である．

キーワード：小児固形腫瘍，EGFR，ゲフィチニブ，悪性横紋筋肉腫様腫瘍．

Abstract

The prognoses of pediatric solid tumors (PST) have improved according to progression of therapies. However, current treatments have had only limited success. Then innovative therapies, such as molecular target therapy, are needed. Epidermal growth factor receptor (EGFR) was found to be expressed on some PST. Gefitinib is an oral EGFR-tyrosine kinase inhibitor and has been demonstrated to be effective in inhibiting the proliferation of cancer cells *in vivo* as well as in clinical trials. The effective

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molecular predictors for gefitinib are reported that mutation or gene amplification of EGFR and AKT phosphorylation, however this predictors are controversial.

Among PST, malignant rhabdoid tumor (MRT) is a rare and highly aggressive neoplasm in young children. EGFR was found to be expressed on MRT cell lines and tumor tissues. This encouraged us to examine the antitumor effects of gefitinib on MRT cells *in vitro* and *in vivo*. Gefitinib inhibited EGFR-phosphorylation and *in vitro* cell growth, and a high concentration of gefitinib (20 μM) induced apoptosis *in vitro*. Furthermore, gefitinib had a cytostatic effect on established MRT xenografts. Our results demonstrate that gefitinib has antitumor effects in MRT cells *in vitro* and *in vivo*, and has promise as a novel and therapeutic strategy for MRT.

Recently, the result of Phase I study of gefitinib in children with refractory solid tumor demonstrated that gefitinib is well tolerated. The possibility that gefitinib has the efficacy for PST was demonstrated, however, further clinical studies are needed for the establishment of treatments with gefitinib for PST.

Key words: Pediatric solid tumor, EGFR, Gefitinib, MRT.

はじめに

化学療法，外科治療，放射線治療によるがん治療の進歩により，小児がんの治療成績は目覚しく向上してきた．特に骨髄移植を併用した超大量化学療法や支持療法の進歩は，小児固形腫瘍の予後改善に大きな役割を果たした．しかし，化学療法に用いられてきた従来の抗腫瘍薬は，正常細胞と腫瘍細胞間での選択性が低く，副作用が大きくなるという欠点があった．効果が期待でき，副作用の少ないことが治療薬の理想であり，作用分子の明らかな分子標的薬の開発が進んだ．

がん治療で分子標的薬が注目されるようになった背景には，(1)腫瘍細胞の増殖，浸潤，転移などの悪性化のメカニズムが明らかになり，腫瘍細胞と正常細胞との差異が分子のレベルで明確になったこと，(2)腫瘍細胞周囲の微小環境の研究が進み腫瘍細胞と細胞外器質の関係や転移，浸潤のメカニズムの解明が進んだことがある．これらにより，腫瘍細胞に対する治療の標的が明確になってきた．分子標的療法は，難治性腫瘍に対する，新たな治療法の確立だけではなく，個々の腫瘍の性質，遺伝情報に基づいた個別化した治療（テーラーメイド治療）の可能性を大きくすると期待される．今回，小児悪性固形腫瘍における分子標的療法の現状と今後の可能性について，特にEGFR（epidermal growth factor receptor）チロシンキナーゼ阻害剤であるゲフィチニブ（gefitinib，イレッサ®）を中心に述べる．

EGFRとゲフィチニブ

EGFRは細胞膜を貫通する受容体型チロシンキナーゼで，erbB-1によりコードされる170kDaの糖タンパクである．リガンドとしてEGF, TGF-α, Amphiregulin, β-cellulin, heparin binding-EGF, Epiregulinが知られている．これらのリガンドが受容体に結合すると，二量体を形成し細胞膜内のチロシンキナーゼ部位へのATPの結合が促進し，チロシンキナーゼが活性化する．活性化したチロシンキナーゼからさまざまなシグナル伝達系が活性化され，細胞増殖，生存，浸潤など腫瘍細胞の特性に関与している(図1)．EGFR以外にHER2, HER3，HER4と相同性を有する受容体ファミリーがあることが明らかになり，erbBレセプターファミリーと呼称されるようになり，さらに，erbBレセプターファミリーは同じレセプター同士のhomodimerだけでなく，EGFR/HER2, HER2/HER3といったheterodimerを形成することが知られている[1)2)]．

一方，ゲフィチニブはEGFRチロシンキナーゼ阻害剤として開発された，低分子量の合成アニリノキナゾリンである．チロシンキナーゼが阻害されることで下流のシグナル伝達が阻害され，腫瘍細胞の細胞増殖，生存，浸潤などを抑

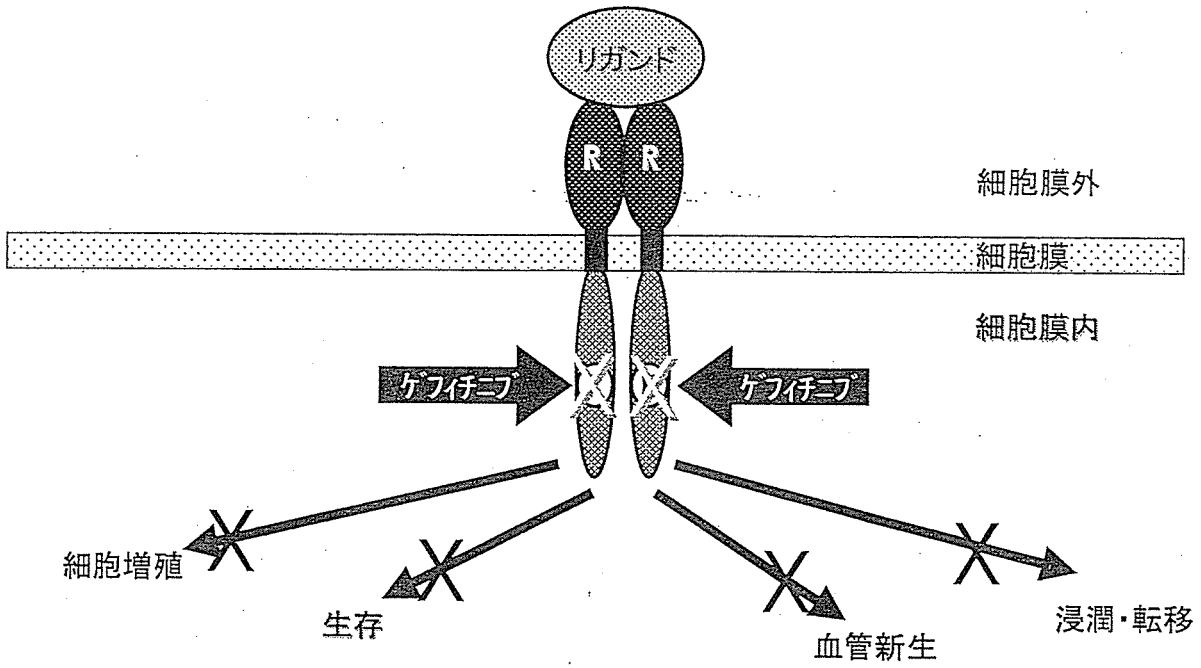

図1　EGFRとゲフィチニブの作用

制し効果を発揮する（図1）．分子標的薬剤では，腫瘍での標的の発現をみれば，治療効果が得られると考えられていた．しかし，ゲフィチニブでは標的となるEGFRの発現量と臨床効果が一致しない結果が得られている[3]．さらに，近年ゲフィチニブの感受性に関して多くの議論がなされている．非小細胞肺がんに発現するEGFRのチロシンキナーゼドメインに変異のある患者での，ゲフィチニブの有効性が[4]報告され，EGFR遺伝子変異が効果の予測に有効である可能性が示された．さらに，2005年にはゲフィチニブの反応性にEGFRの遺伝子のコピー数が重要であること[5]，EGFRの下流に存在するAKTのリン酸化の状態の重要性が指摘されている[6]．また，分子標的治療薬は，がんの原因となった分子を標的にするため，既存の抗がん剤よりも副作用が少ないと考えられていた．しかし，ゲフィチニブでの間質性肺炎など重篤な有害事象の報告から，やはり，リスクとベネフィットを慎重に評価し投与されるべき薬剤である．また，EGFRに変異があっても化学療法とゲフィチニブとの併用療法では生存率に差はなく有効性は証明されなかった[7]．このように，臨床試験が進行していくにつれ，あらたな知見が集積され，次々と問題が提起されているのが現状である．したがって，基礎研究と臨床研究が密接に連携し，分子標的治療の開発を行うことが非常に重要である．

小児固形腫瘍の分子標的と分子標的薬

小児に好発する固形腫瘍である脳腫瘍のなかでもgliomaではEGFRが[8]，神経芽腫にはTrk受容体[9]が，Wilms腫瘍ではHER（human epidermal growth factor receptor）-2[10]が，そしてmedulloblastomaにはHER2とHER4が発現し[11]，予後や悪性化に関与していることが報告されてきた．

したがって，小児固形腫瘍においても，腫瘍細胞の増殖に関与する各種の増殖因子とその受容体で標的分子となりうる．代表的なものとしてTrk，EGFR，HER2，VEGFR（vascular endothelial growth factor receptor），PDGFR（platelet-derived growth factor receptor）あるいはc-Kitなどを挙げることができ，臨床応用できる可能性がある．一方正常細胞には発現していないキメラ遺伝子も標的になる．慢性骨髄性白血病（CML）の*BCR-Abl*遺伝子と急性前骨髄性白血病（APL）のPML/RARαキメラ遺伝子は典型的な分子標的であり，CMLに対してはBCR-

Ablチロシンキナーゼ阻害剤としてメシル酸イマチニブ（imatinib mesylate，グリベック®），またAPLに対して，全トランス型レチノイン酸（ATRA）はすでに臨床応用されている．固形腫瘍では横紋筋肉腫に見られるPAX3/FKHRやEwing肉腫のEWS/FLI1などのキメラ遺伝子も標的分子としての可能性がある．

従来の抗がん剤では，腫瘍の縮小効果が，早期臨床試験の効果判定として重要であった．しかし，分子標的薬剤の場合，腫瘍の増殖抑制効果や患者のQOL向上も含めて有効性を評価するようになってきた．臨床的有効性を評価するには，薬剤が標的分子に作用していることの証明（proof of target：POT），標的分子に作用することで*in vitro*と*in vivo*ともに，増殖抑制などの効果を示すことの証明（proof of principle：POP），さらに分子標的薬が客観的に臨床症状やQOLの改善などベネフィットにつながることの客観的な証明（proof of efficacy：POE）が最終的に必要となる．

悪性横紋筋肉腫様腫瘍へのゲフィチニブの有用性の検討

我々は，悪性横紋筋肉腫様腫瘍（malignant rhabdoid tumor; MRT）に対するチロシンキナーゼを阻害するゲフィチニブ（gefitinib，イレッサ®）の効果を検討したので紹介する[12]．

MRTは，乳幼児に好発する非常に予後の悪い腫瘍である．組織学的には好酸性封入体の存在が特徴であり，分子生物学的には*hSNF5INI1*遺伝子の異常が認められる．化学療法など治療法が進歩した現在においてもその予後は悪く，4年生存率は非進行例では41.8%であるが，進行例では15.9%である．特に6か月未満発症例の4年生存率は8.8%以下と悲劇的であり[13]，新規治療法の開発が急務である．そこで，MRTに対する分子標的療法の可能性を検討した．

最初にMRTに対する標的分子を検索した．MRT腫瘍組織でのEGFRの発現を確認した報告はなかったが，MRT細胞株にEGFRが発現しているという報告はあった．そこで，我々は，経験した2症例のMRT組織と，それらより樹立したMRT細胞株でのEGFRの発現を検討した．

使用したMRT腫瘍組織は組織学的にMRTと診断された，肝原発のMRT-ANと腎原発のMRT-NSを用いた．また細胞株はこれら2例の腫瘍より樹立されたMP-MRT-AN（以下AN株）とKP-MRT-NS（以下NS株）を使用した．これら細胞株では，*INI1*遺伝子の欠損がみられ，ともに分子生物学的にもMRT細胞株であることが確認された．腫瘍組織におけるEGFRの発現は免疫組織化学法で，細胞株におけるEGFRの発現は間接蛍光抗体法とWestern blot法で検討した．腫瘍組織ではANで瀰漫性にNSでは結節性にEGFRの発現を認め，細胞株ではAN株の90%とNS株の40%にEGFR陽性細胞を認めた（図2）．また，Western blot法でもEGFR蛋白の発現を確認した．これらの結果から，MRTにおいてEGFRが分子標的療法の標的分子となりうることが期待できた．そこで，ゲフィチニブの抗腫瘍効果を*in vitro*と*iv vivo*で評価し，MRTの治療へのゲフィチニブの有用性を検討した．

次に，MRTのEGFRがリン酸化し，ゲフィチニブがEGFRのリン酸化を抑制することが可能かを検討した．リン酸化EGFRの検出は抗EGFR抗体を用いた免疫沈降法を用いて，EGFR蛋白を抽出し，抗チロシンリン酸化抗体を用いたWestern blot法で評価した．無血清下で，MRT細胞株ではリン酸化EGFRは検出できなかったが，EGFを添加すると強くリン酸化された．しかし，1 μMのゲフィチニブ添加によりEGFRのリン酸化は抑制され，ゲフィチニブはMRT細胞株に存在するEGFRのリン酸化を抑制することが確認された（POT）（図3）．

続いて，ゲフィチニブがMRT細胞株の増殖を抑制するかどうか検討した．まず，*in vitro*での細胞増殖は，培養細胞にゲフィチニブを添加し，24時間ごとに細胞数を計測した．MRT細胞株にゲフィチニブを0.1～100 μMで添加すると，48時間後から濃度依存性に増殖が抑制され，IC_{50}値はAN株が10 μMでNS株が12 μMであった．また，MRT細胞に20 μMのゲフィチニブを添加した場合，細胞は小球形に変形し浮