TABLE 2 Induction Chemotherapy and Preconditioning Regimens

Patient	Conventional protocol	Chemotherapy (no. of courses)	Time to HDC from onset (days)	Time of stem cell harvest (course)	Stem cell source	Conditioning regimen	
						Thiotepa (mg/m^2)	Melphalan (mg/m^2)
1	new A1[a]	3	120	3	Auto-BM	1000	300
2	new A1	5	130	2	Auto-PB	800	280
3	new A1[a]/ CPT-11	6		N.D.	N.D.		
4	new A1[a]	5	167	3	Auto-BM	1000	280
5	98A3 / CPT-11	6	185	N.D	u-CB	26[c]	6[b,c]
6	98A3 / CPT-11	5	165	N.D	u-CB	760[b]	210[b]
7	98A3→98A3/ CPT-11	4	139	2 (PB) and 4 (BM)	Auto-PB, BM	800	280
8	98A3	5	167	5	Auto-BM	800	280
9	98A3	4	167	2	Auto-PB	720[d]	252[d]
10	98A3	4	132	1	Auto-PB	720[d]	252[d]
11	98A3	4	148	3 (PB) and 4 (BM)	Auto-PB, BM	570[d]	200[d]

Note. Auto-BM, autologous bone marrow; Auto-PB, autologous peripheral blood; CBDCA, carboplatin; CDDP, cisplatin; CPT-11, irinotecan N.D., not done; u-CB, unrelated cord blood.

[a]CBDCA was administered instead of CDDP.

[b]Drug dose was reduced because of transplantation from allogeneic donors.

[c]Drug was administered in terms of body weight (mg/kg).

[d]Drug dose was reduced to 70–90% of the prescribed dose according to renal function.

patients aged ≥2 years, dosage was adjusted according to the following formula: given dose (mg/m^2) = (Ccr/100) × 800 mg/m^2 (thiotepa) or 280 mg/m^2 (melphalan). In the case of allogeneic transplantation, doses of these drugs were reduced, because of severe gastrointestinal toxicity due to these alkylating agents. Peripheral blood stem cells (PBSCs) and bone marrow cells were used as salvage stem cells in 4 and 3 patients, respectively. Because PBSC count was insufficient for stem cell rescue in 2 patients, bone marrow cells were also transfused with PBSCs. Autologous bone marrow was used in the patients in whom PBSCs could not be harvested: this was performed at the end of induction chemotherapy. PBSCs were harvested after the 1st to 4th course of induction chemotherapy, after morphologic disappearance of tumor cells from bone marrow. In the 2 patients in whom disappearance of tumor cells from bone marrow was delayed, unrelated umbilical cord blood was used (Table 2).

Local Therapy

After all courses of chemotherapy including HDC, radical surgery was finally applied to remove tumor tissue in local lesions when bone marrow function was acceptably recovered for surgery. Total resection for primary tumor as well as lymph node metastases was attempted. All lesions where the primary tumor and local lymph node metastases existed in onset of the disease were explored and if any suspected tumor tissue was existed, then resection was performed.

CT scan was performed after surgery to confirm no residual tumor in local lesions in all cases. Irradiation was not applied to any local lesions.

RESULTS

Response to Induction Chemotherapy

In 1 patient (#3), tumors did not respond to induction chemotherapy and he showed progressive disease. He died from progression of pulmonary metastatic tumors 6 months after diagnosis before HDC. After 3–6 courses (median, 5 courses) of induction chemotherapy, 10 patients received HDC. Time from initial diagnosis to HDC was 4–6 months (median, 5 months). With respect to metastases at initial diagnosis in patients who received HDC, these were detected in bone (n = 8), bone marrow (n = 7), lymph node (n = 9), and liver (n = 1)and evaluated by computed tomography, technetium-99 bone scan, bilateral bone marrow aspiration, and iodine-123 metaiodobenzyl-guanidine scan. After induction chemotherapy, the bone marrow metastases disappeared in all patients, but liver and bone metastases each remained in 1 patient, respectively. Primary tumors and regional lymph node metastases remained in all patients. Tumor marker levels were

normalized in all patients. At HDC, 7 patients attained PR and 2 VGPR according to International Neuroblastoma Response Criteria.

Response to High-Dose Chemotherapy

Nine patients received HDC at PR or VGPR. The size of one primary tumor did not change. After HDC, residual bone metastases disappeared in 1 patient. Liver metastases persisted in 1 patient. Five primary tumors that decreased to below 50% after conventional chemotherapy decreased to below 10% and the sizes of primary tumors did not change dramatically, but metastatic lymph nodes disappeared in 2 patients. With respect to adverse reactions observed during HDC, fungal osteomyelitis was observed in 1 patient who received allograft. In addition, gastrointestinal tract mucositis with bloody stools was observed in 1 patient and NCI-CTC grade III mucositis was noted in all patients.

Surgery and Pathological Evaluation of Tumors

Radical surgery was performed in each patient, resecting all recognizable lesions, including the primary tumor and affected lymphatic tissues. The timing of surgery was 2 months after the initiation of HDC in most patients who received autologous stem cell transplantation, and it was prolonged to 4 months in the patients who underwent allogeneic transplantation and/or had HDC-related complications (Table 3).

We evaluated the effect of chemotherapy including HDC by comparing tumor specimens resected at outset and second-look surgery in 6 patients, according to the histologic criteria for the effects of anticancer therapy for pediatric solid malignant tumors in Japan (Table 4) [13]. We were not able to evaluate the remaining 4 patients as insufficient amounts of pretreatment specimen were available. Necrotic or fibrous lesions were seen in one-third to two-thirds of the area of tumor tissues (Ef1b) of 1 patient. In the 4 cases, prominent necrosis and loss of tumor cells were observed in more than two-thirds of the tumor area and was associated with fibrosis and calcification (Ef2). On histological examination, the specimens from almost all, except one (#11), resected primary tumors had some degree of residual tumor tissue and in occasional cases viable tumor tissue was recognized in concurrently resected lymph nodes. However, residual tumor tissue consisted of scattered nests of neuroblastic cells in degenerative fibrous tissue, occasionally associated with Schwannian cell proliferation. Neuroblastic cells were more differentiating with abundant neutrophil formation as compared to pretreatment tumors. The preoperative induction chemotherapy and HDC produced remarkable cytotoxic effects and induced differentiation toward ganglionic cells. Examples of the histopathologic changes resulting from treatment are shown in Figures 1 and 2.

TABLE 3 Response to Treatment and Outcome

Patient	Response to induction chemotherapy			Response to induction chemotherapy and HDC			Time to surgery		Post therapy histology classification[a]	Outcome from diagnosis (mo.)
	Response	Residual site	VMA,HVA (mg/mg_{Cr})[c]	Response	Residual site	VMA, HVA (mg/mg_{Cr})[c]	From HDC (days)	From onset (days)		
1	PR	P/LN	≦20, ≦20	PR	P/LN	≦20, ≦20	96	216	N.E.	EFS (171)
2	PR	P/LN	N.E.[d]	VGPR	P	N.E.[d]	79	209	N.E.	EFS (104)
3	PD	–		–	–		––	––	–	PD (3[b])
4	PR	P/LN/B	N.E.[d]	VGPR	P	N.E.[d]	68	235	Ef1b	EFS (159)
5	PR	L/LN	≦20, ≦20	PR	L/LN	≦20, ≦20	106	291	Ef2	EFS (73)
6	PR	P/LN	N.E.,[d] ≦20	VGPR	P	N.E.[d] ≦20	99	264	N.A	EFS (57)
7	PR	P/LN	N.E.[d], ≦20	VGPR	P	N.E.[d], ≦20	52	191	Ef2	Relapse in LN (24)
8	VGPR	P	≦20, ≦20	VGPR	P	≦20, ≦20	83	250	N.E.	EFS (38)
9	VGPR	P	≦20, ≦20	VGPR	P	≦20, ≦20	85	252	Ef2	Relapse in multiple sites (20)
10	PR	P	≦20, ≦20	VGPR	P	≦20, ≦20	50	182	Ef2	Relapse in LN (18)
11	PR	P	≦20, ≦20	PR	P	≦20, ≦20	55	203	Ef3	EFS (21)

Note. B, bone; CR, complete response; EFS, event-free survival; HDC, high-dose chemotherapy; L, liver; LN, lymph node; NR, no response; P, primary; PD, progressive disease; PR, partial response; VGPR, very good partial response. HVA, urine homovanillic acid; VMA, urin vanillylmandelic acid.

[a]Pathological classification according to the Committee on Histological Classification of Childhood Tumors, Japanese Society of Pathology (see Table 4).

[b]Decreased.

[c]The level of VMA and HVA are revised by urin creatinin. Normal levels of VMA and HVA are below under 20 mg/mg Cr in our institute for every age.

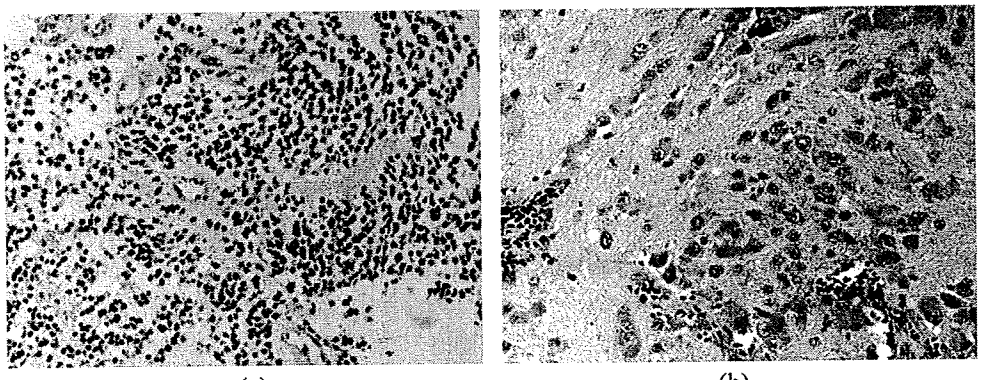
[d]Normal levels of cathecholamine at onset.

TABLE 4 Pathological Classification of Treatment Effect According to the Committee on Histological Classification of Childhood Tumors, Japanese Society of Pathology

Ef0	No effect
Ef1a	Necrosis of tumor cells in less than one-third of tumor area
Ef1b	Necrosis of tumor cells in less than two-thirds and in more than one-third of tumor area
Ef2	Necrosis and disappearance of tumor cells plus calcification and fibrosis in more than two-thirds of tumor area
Ef3	All tumors are affected by obvious necrotic tissue and no tumor cells are seen

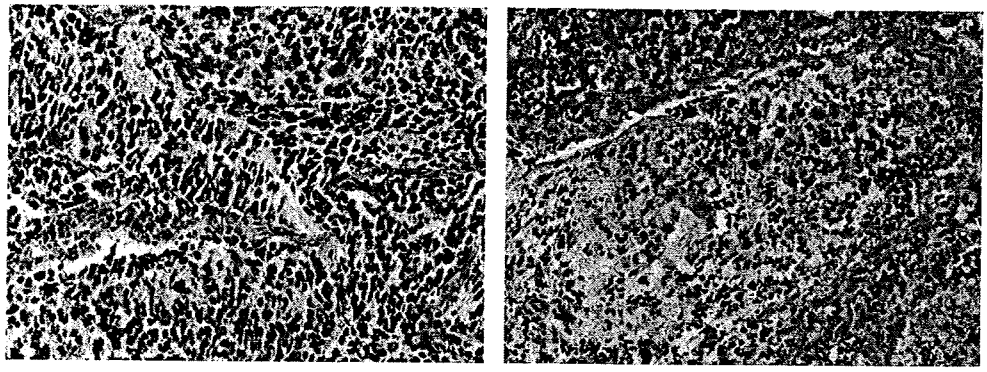
Outcome

Altogether, 10 of 11 patients received HDC and 7 patients have remained in remission for 21–171 months (median, 73 months). In 2 patients (#7 and #10), the tumor relapsed in regional abdominal and thoracic lymph nodes after 24 and 18 months, respectively, from initial diagnosis. In patient #9, relapse was observed in multiple sites including bone, bone marrow, and lymph nodes 20 after from diagnosis. After gross total resection of the tumors, patient #7 received salvage chemotherapy, consisting of irinotecan and topotecan, and local irradiation. Finally, allogeneic stem cell transplantation preconditioned with fludarabine and busulfan was performed. She has been in remission for 21 months after relapse. The other 2 patients are currently undergoing treatment.



(a) (b)

FIGURE 1 Histological findings for primary tumor is from patient 4: (a) before treatment—poorly differentiated subtype with low mitosis karyorrhexis index (MKI); and (b) after HDC—residual tumor nests of differentiating neuroblastic cells.



(a) (b)

FIGURE 2 Histological findings for lymph node metastasis from patient 5: (a) before treatment—poorly differentiated subtype with low mitosis karyorrhexis index (MKI); and (b) after HDC—extensive necrosis with residual differentiating neuroblastic cell nests.

DISCUSSION

Primary surgery is generally and traditionally performed between induction chemotherapy and HDC. It might be possible that tumor cells become more sensitive to chemotherapy after mass reduction, but the rationale of the timing of local therapy is unclear. In this case series, we performed primary surgery after completion of induction chemotherapy and HDC based on the hypothesis that consecutive conventional and high-dose chemotherapies without interruption by local therapy can eradicate systemically spread tumor cells before acquisition of resistance to cytotoxic drugs and clonal evolution of resistant clones. The disadvantage of this treatment strategy is the increased risk for metastasis of tumor cells residing in the local tumors and emergence of resistant clones in these. Among 11 consecutive high-risk patients with stage 4 neuroblastoma, except 1 patient whose tumors were primarily refractory to induction chemotherapy, none displayed progressive disease before local surgery; 7 patients remain in event-free survival; and systemic relapse was observed in only 1 patient.

The disadvantage of performing surgery during chemotherapy appears to be related to the interruption of systemic therapy. Furthermore, when intraoperative/postoperative complications occur, discontinuation of systemic chemotherapy may be prolonged and this may cause systemic relapse. In performing surgery after all courses of chemotherapy, the timing of surgery can be selected under conditions of sufficient tumor control. Surgery was safely performed after recovery of hematopoiesis in this series.

In this treatment strategy, HDC plays a key role, since less potent HDC may allow progression of the local tumor. For HDC, we employed a double-conditioning regimen consisting of thiotepa and melphalan, as previously reported [12]. These agents were chosen for the treatment of neuroblastoma, as they show efficacy as high-dose, single-agent therapy for

neuroblastoma and are not used for conventional chemotherapy prior to HDC [4, 14, 15]. This HDC regimen consisted of 2 cycles of administration of thiotepa and melphalan with a 1-week interval: this interval facilitated combination therapy at the maximum tolerated dose of a single agent without severe complications. The major adverse effect of this regimen is gastrointestinal mucositis and narcotic drugs are frequently required. However, life-threatening complications such as veno-occlusive disease and renal insufficiency are not observed.

In this case series, the effect of chemotherapy was pathologically validated in primary tumors. No residual tumor cells were observed except in one patient. Scattered viable tumor cells were detected in other resected tumor specimens, though the number of these cells was small and they were embedded in the connective tissue. Similar findings were observed in regional lymph nodes. These scattered cells are tightly embedded in fibrous tissue and might possibly proliferate, contributing to relapse. Thus, it was shown that even HDC rarely totally eradicates tumor cells of the primary tumors.

Concerning local therapy, gross total resection was eventually performed after completion of all systemic chemotherapies. No conclusion has been drawn concerning the role of gross total resection. With our treatment strategy, systemic disease seemed to be controlled sufficiently, and under such conditions the significance of the local therapy may increase. Radiation therapy was not performed in this case series and local recurrence was observed in 2 patients without recurrence in other sites. This might suggest that radiotherapy is beneficial to selected patients who undergo gross total resection. To identify these patients, histopathological evaluation of chemotherapy outcome may provide useful information, in addition to the extent of local disease at initial diagnosis and the extent of surgery. Although the relationship between histopathological findings and clinical outcome was unclear in this series, recruitment of additional patients may assist in drawing some conclusions.

This novel treatment strategy consisting of the postponement of local surgery until the end of chemotherapy combined with intensive induction and consolidation chemotherapy seems feasible. A multicenter phase II study is being planned in Japan to confirm the utility of this strategy.

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Centrosome amplification is correlated with ploidy divergence, but not with *MYCN* amplification, in neuroblastoma tumors

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Abstract Ploidy is an important biologic feature defining heterogeneous neuroblastoma. To clarify whether centrosome amplification is correlated with ploidy status or *MYCN* amplification, we examined centrosomes by immunostaining, and ploidy and *MYCN* copy numbers by fluorescence in situ hybridization in 27 neuroblastomas. There were 8 infant triploid, 9 infant diploid, and 10 childhood diploid tumors. Ploidy divergence, defined as a mixed population of cells with trisomy 1, cells with tetrasomy 1, and/or cells with pentasomy 1 in diploid tumors and that of cells with tetrasomy 1 and cells with pentasomy 1 in triploid tumors, each occupying more than 5% of cells, was found in 78% of infant diploid tumors, but not in triploid and childhood diploid tumors ($P<0.0001$). Childhood and infant diploid tumors had higher incidences of centrosome amplification than infant triploid tumors ($P=0.0001$ and 0.07, respectively). While both infant and childhood diploid tumors share a high incidence of centrosome amplification, only infant diploid tumors showed ploidy divergence, implying the presence of cytokinesis failure. These findings suggest that centrosome amplification found in cells of infant diploid tumors and that found in cells of childhood diploid tumors may be generated by different mechanisms. *MYCN* amplification was not correlated with centrosome amplification in sporadic neuroblastomas.

1. Introduction

Neuroblastoma is one of the most common childhood tumors and has a broad spectrum of clinical behavior [1,2]. Neuroblastoma patients are clinically classified into two groups: one has a good prognosis with minimal therapy and the other has a very poor prognosis despite aggressive therapy. Various molecular and cytogenetic markers predict favorable and unfavorable prognoses of neuroblastoma; these include *MYCN* copy number, *TRKA* expression, 1p and/or 11q deletion, 17q gain, chromosomal ploidy, and hypermethylation of tumor suppressor genes [1–3]. Prognosis is closely correlated with chromosomal features: most stage 1, 2, and 4S tumors are triploid, whereas the majority of stage 4 tumors are diploid or tetraploid [4–10]. Correlation between prognosis and ploidy has been puzzling, and we previously offered a hypothesis that explains how the ploidy state of the tumor plays a fundamental role in heterogeneity and why various factors are correlated [11].

The centrosome, a small nonmembranous organelle, normally localized at the periphery of the nucleus, regulates the nucleation of microtubules, cell polarity, cell cycle, and chromosomal stability [12,13]. Analysis of human tumors revealed a strong positive correlation between centrosome abnormalities and the number of chromosome aberrations [14]. The presence of more than two centrosomes (centrosome amplification) severely disturbs the mitotic process and cytokinesis via the formation of more than two spindle poles, resulting in an increased frequency of chromosome segregation errors (chromosome instability), although recent studies showed that centrosome amplification does not necessarily result in multipolar spindles [12,15]. More

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recently, a significant correlation was found between centrosome amplification and *MYCN* amplification in neuroblastoma tumors [16]. Other oncogenes and tumor suppressor genes also affect centrosome duplication and/or amplification in various cancers and their cell lines [12,13,17–19].

To clarify the relationship between centrosome amplification and tumor cell ploidy or *MYCN* copy numbers, we examined 27 neuroblastoma tumors by immunofluorescence staining and fluorescence in situ hybridization (FISH) analyses.

2. Materials and methods

2.1. Patients and samples

Tumor samples were obtained from 27 Japanese infants or children with neuroblastoma who underwent biopsy or surgery between June 2003 and January 2008 (Table 1). In this study, 17 patients who were 18 months old or younger and 10 patients who were at least 18 months old were classified as infants and children, respectively, in this study. Of the 17 infants, 12 had undergone a mass-screening program; 10 were diagnosed as having neuroblastoma, and 2 had screened negative but later were diagnosed clinically. Five infants did not undergo screening, but later were diagnosed clinically. All 10 children who were older than 18 months were negative for screening and were found clinically later. All 17 infants, except for 1, were alive with no evidence of disease; 1 infant with a stage 4 and *MYCN*-amplified tumor was undergoing chemotherapy, 2 children died of the disease, and the other 8 children were alive with or without disease at the last follow-up (January 31, 2008).

The tumor samples were minced with scissors and cultured in plastic flasks containing RPMI 1640 medium (GIBCO-BRL, Grand Island, NY) with 15% calf serum in 5% CO_2 atmosphere for 5–7 days and then harvested. One colon cancer cell line, HCT-116, was also cultured under the same conditions, and their chromosomes were studied.

2.2. FISH analysis

To detect the copy number of chromosome 1 and the presence or absence of 1p deletion, we used repetitive DNA, D1Z1 (pUC 1.77) specific for the pericentromeric region (1q12), and D1Z2 (p1-79) specific for the subtelomeric region (1p36.33) as probes [6]. D1Z1 was labeled with digoxigenin 11-dUTP, and D1Z2 with biotin 16-dUTP (Roche Laboratories, Indianapolis, IN) by nick translation. Two-color FISH was performed by modification of the standard FISH procedure. Hybridized probes were detected with fluorescein isothiocyanate (FITC)-labeled avidin and antidigoxigenin rhodamine (Roche). Chromosomes were counterstained with 4',6-diamidino-2-phenylindole dihydrochloride hydrate (DAPI) maintained in Vectashield (Vector Laboratories Inc., Burlingame, CA). In each tumor, 100 interphase nuclei were counted.

MYCN copy numbers were examined using commercially available DNA probes, D2Z1 specific for the pericentromeric region of chromosome 2 labeled with SpectrumGreen-dUTP, and *MYCN* (2p24.1) labeled with SpectrumRed-dUTP (Qbiogene, Inc.). Hybridization was performed under the conditions recommended by the suppliers.

2.3. Flow cytometry

Of the 27 tumors examined by FISH, 5 were also examined by flow cytometry. The DNA index was analyzed on a Becton-Dickinson FACScan flow cytometer by DNA cell cycle analysis software, version C. The tumor samples were considered to have a diploid population when a G0/G1 peak was located on the same channel as the G0/G1 peak of the normal lymphocyte sample.

2.4. Immunofluorescence staining

Tumor cells from 27 neuroblastoma tumors and 1 colon cancer cell line were cultured on coated coverslips for a few days, and examined by the immunostaining method using the antibodies γ-tubulin and β-tubulin (T-3559 and T-2657; Sigma, St. Louis, MO) against centrosomes and spindles, respectively. A colon cancer cell line, HCT-116, served as controls that show the proper immunostaining of cultured tumor cells (Fig. 4a). The tumor cells were fixed in −20°C acetone for 10 minutes, permeabilized in 0.2% Tween-20 for 5 minutes, and blocked in phosphate-buffered saline containing 1% bovine serum albumin and 1% human immunoglobulin for 1 hour, followed by standard indirect immunohistochemistry. The antibody–antigen complexes were detected by TRITC (T-2402, Sigma) or FITC-conjugated secondary antibodies. Nuclear DNA was counterstained with DAPI in Vectashield (Vector Laboratories). In each tumor, centrosome numbers from 100 cells were counted.

2.5. Statistical analysis

Student's *t*-test with or without Welch's correction compared the incidences of centrosome amplification between any two of the three groups of tumors classified by age and ploidy (i.e., infant diploid tumors, infant triploid tumors and childhood diploid tumors). The incidences of centrosome amplification were also compared by the same test between infant diploid tumors with ploidy divergence and infant triploid tumors with no ploidy divergence, and between tumors with a single *MYCN* copy and those with *MYCN* amplification. Differences in the incidence of ploidy divergence among the three groups of tumors classified by age and ploidy were examined by the chi-square test using a 2×3 table. Statistical analysis was performed using Prism software for Mac, version 5.0 (Graphpad Software, La Jolla, CA).

Table 1
Clinical and cytogenetic characteristics of neuroblastoma tumors classified by chromosome 1 and *MYCN* copy numbers

				FISH analyses												
				D1Z2/D1Z1					*MYCN* /D2Z1							
Patient	age	Tumor site	Stage	1/2	2/2	3/3	4/4	5/5	dmin/2	2/2	4/2	3/3	4/4	5/5	MS	Abnormal centro. (%)
Infant diploid tumors (n=9)																
1	9m	Retroperitoneum	3	0	96	4	0	0		92		2	6		+	3
2[a]	9m	Mediastinum	2	0	91	7	2	0		100					+	30
3[a]	9m	Adrenal gland	1	0	90	6	3	1		21		75	3	1	+	9
4[a]	1y6m	Retroperitoneum	3	0	86	2	12	0		100					NU	0
5[a]	8m	Abd. sym. gangl.	3	0	85	10	4	1		91		4	5		+	3
6[a]	1y3m	Sacral region	2	0	78	11	8	3		26			70	4	−	30
7[a]	1y0m	Mediastinum	2	0	77	11	12	0		83		4	12	1	+	21
8[a]	7m	Adrenal gland	4	0	55	21	22	2		37		15	47	1	+	43
9	8m	Adrenal gland	4	96	4	0	0	0	99		1				NU	2
Infant triploid tumors (n=8)																
10	9m	Adrenal gland	1	0	17	82	0	1		16		78	6		+	2
11	5m	Retroperitoneum	3	0	19	80	1	0		8		90	2		NU	2
12	9m	Mediastinum	1	0	24	76	0	0		14		74	9	3	+	0
13	17d	Retroperitoneum	3	0	25	73	0	2		21		47	32		NU	2
14	8m	Mediastinum	3	0	33	65	2	0		29		67	3	1	NU	2
15	8m	Retroperitoneum	1	0	46	54	0	0		40		60			+	21
16	7m	Retroperitoneum	3	0	63	37	0	0		47		5	46	2	+	3
17	1y0m	Retroperitoneum	3	0	64	36	0	0		51		46	3		−	0
Childhood diploid tumors without *MYCN* amplification (n=5)																
18	4y	Mediastinum	4	0	98	1	1	0		98		1	1		−	54
19	3y	Adrenal gland	4	0	96	2	2	0		100					−	19
20	3y	Adrenal gland	4	0	96	4	0	0		75	19	3	3		−	50
21	2y	Mediastinum	4	4	90	4	2	0		10	89		1		−	68
22	4y	Pelvis	4	5	87	8	0	0		67	28		5		−	58
Childhood diploid tumors with *MYCN* amplification (n=5)																
23	6y	Adrenal gland	4	90	9	0	1	0	98	2					−	26
24	5y	Adrenal gland	4	93	7	0	0	0	100						−	26
25	5y	Adrenal gland	4	33	59	8	0	0	88	12					−	18
26	2y	Adrenal gland	4	95	1	4	0	0	79 (21)[b]						−	27
27	4y	Adrenal gland	4	94	1	2	3	0	98	2					−	33

Abbreviations: d, days; m, months; y, years; MS, mass screening; centro., centrosome; dmin, double minutes; Abd. sym. gangl., abdominal sympathetic ganglion; (mass screening) +, found by mass screening; -, negative by mass screening and found clinically; NU, not undergone mass screening.

[a] Tumors had ploidy divergence: a mixed population of cells was underlined (see the definition in Results 3.1).

[b] Twenty-one cells had dim/4 D2Z1 signals.

3. Results

3.1. Constitution of chromosome 1 examined by FISH in neuroblastoma tumors

D1Z1 (red) and D1Z2 (green) signals were located in pericentromeric and subtelomeric regions, respectively, of normal chromosome 1 (Fig. 1a). In interphase nuclei, two (Fig. 1b) and three (Fig. 1c) pairs of D1Z1 and D1Z2 signals, indicating disomy 1 and trisomy 1, respectively, were demonstrated. Our study showed that neuroblastoma tumors could be classified as tumors with a disomy 1 or trisomy 1 modal clone. These clones served as surrogates for diploidy and triploidy on the basis of correlation of two and three D1Z1 signals with near-diploid (44–57) and near-triploid (58–80) chromosome numbers, respectively, or DNA indexes of 1.0–1.26 and 1.18–1.71, respectively, by flow-cytometry [6,11]. In a recent study on neuroblastoma, Spitz et al. [20] confirmed the validity of using disomy 1 and trisomy 1 as surrogates for diploidy and triploidy, respectively. Thus, tumors were classified into diploid and triploid groups on the basis of the constitution of chromosome 1. When a tumor had a population of cells with trisomy 1 occupying more than 30% of 100 cells examined, it was defined as a triploid tumor; normal diploid cells usually coexist with aneuplid tumor cells. If the tumor had a population of cells with trisomy 1 occupying less than 30% of 100 cells and another population with disomy 1 in more than 50% of 100 cells, it was defined as a diploid tumor. In addition, we used the D2Z1 signal number in the present study, and found that the numbers of chromosome 1 detected by the D1Z1 signal and those of chromosome 2 detected by the D2Z1 signal were generally consistent, with two exceptions: a diploid tumor (no. 3) had 90 cells with disomy 1 and 75 cells with trisomy 2, and a triploid tumor (no. 16) had 37 cells with trisomy 1 and 46 cells with tetrasomy 2 (Table 1).

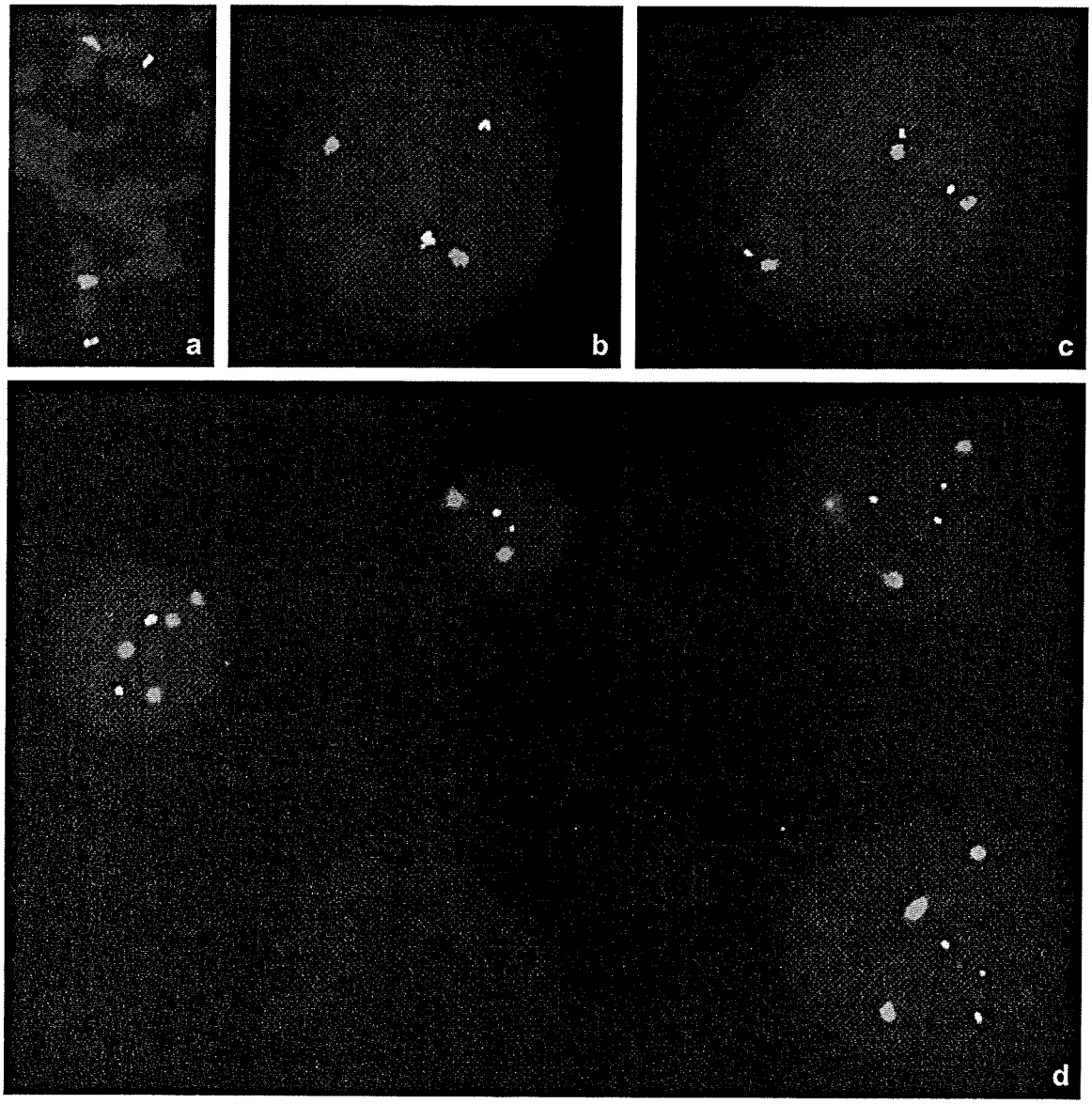

Fig. 1. Evaluation of ploidy by FISH. (a) Normal human chromosomes 1. Red signals indicate pericentromeric D1Z1 (1q12), and green signals indicate subtelomeric D1Z2 (1p36.33). (b) Interphase nucleus of an infant diploid tumor (no. 3). Two pairs of D1Z1 and D1Z2 signals are observed. (c) Interphase nucleus of an infant triploid tumor (no. 16). Three pairs of D1Z1 and D1Z2 are observed. (d) Interphase nuclei of an infant diploid tumor (no. 8). A mixed population of cells with trisomy 1 and cells with tetrasomy 1, in addition to the diploid clone of the cells, are seen.

All 8 triploid tumors occurred in infants younger than 18 months (infant triploid tumors), 9 diploid tumors occurred in infants younger than 18 months (infant diploid tumors), and 10 diploid tumors developed in children who were older than 18 months (childhood diploid tumors). The presence of a mixed population of cells with trisomy 1, cells with tetrasomy 1, and/or cells with pentasomy 1 in diploid tumors, and that of a mixed population of cells with tetrasomy 1 and cells with pentasomy 1 in triploid tumors, each population occupying more than 5% of cells examined, was defined as ploidy divergence (Fig. 1d). Divergence was found in 7/9 infant diploid tumors, but not in 8 infant triploid and 10 childhood diploid tumors ($P < 0.0001$ by the chi-square test; Table 1).

3.2. Correlation of the number of chromosome 1 determined by FISH with DNA index determined by flow cytometry

One infant diploid tumor (no. 5) had a main diploid peak and a second peak with DNA content twice that of the main peak, indicating DNA index of 1.0 (Fig. 2a). Another infant diploid tumor (no. 8) had a main diploid peak and a small second peak with a DNA index of 1.56 (Fig. 2b). Both tumors showed ploidy divergence (Fig. 1d, Table 1). Three infant triploid tumors had a main diploid peak and a second peak, with DNA indexes ranging from 1.50 to 1.58, and showed no ploidy divergence (Fig. 2c, Table 1).

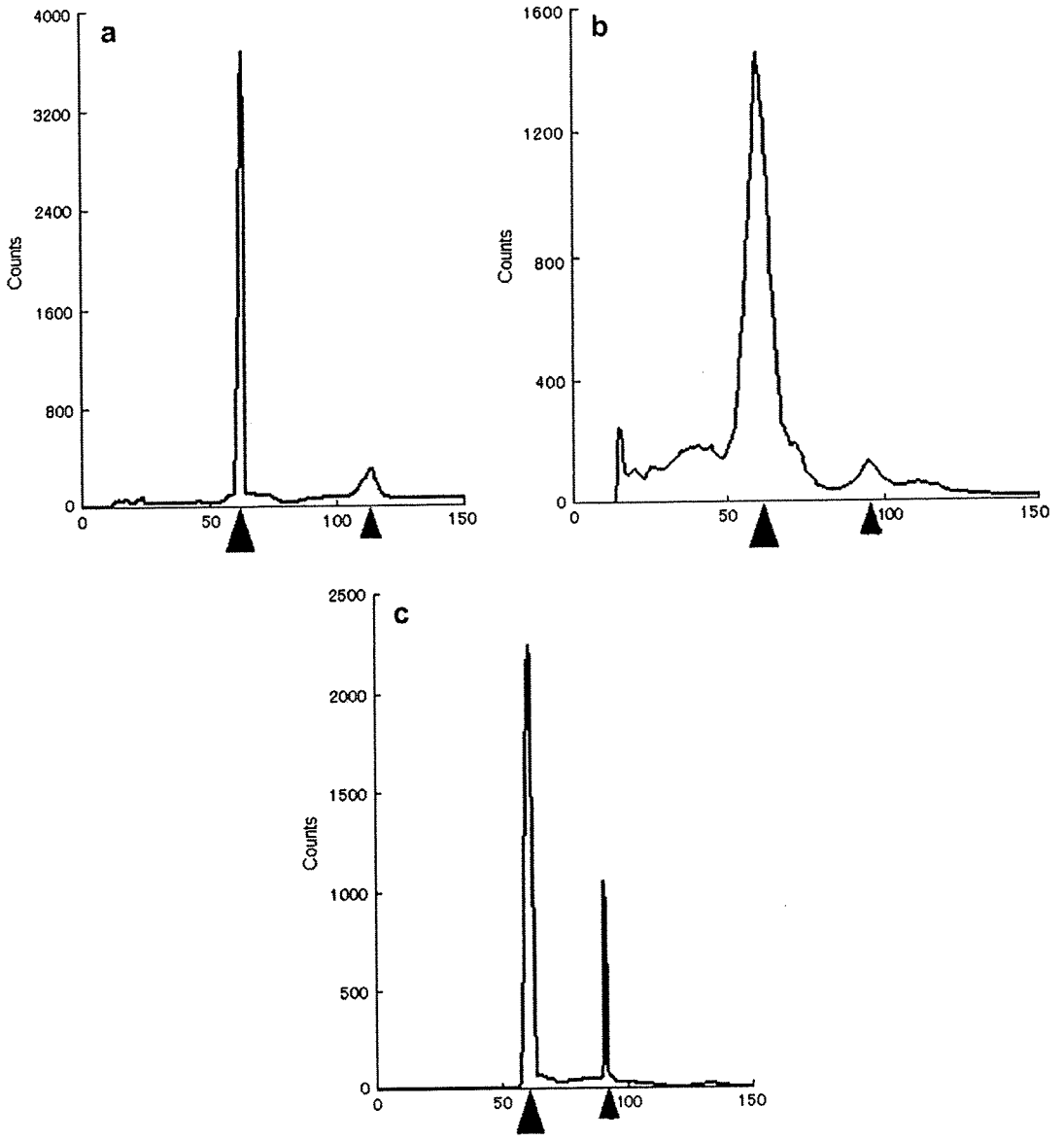

Fig. 2. Examination of DNA index by flow cytometry. Large arrowheads indicate a main diploid peak of G0/G1 cells from tumor tissues that was located on the same channel as the G0/G1 peak of the normal lymphocyte sample. A small arrowhead indicates the G2/M peak of the tumor tissue in a, and the aneuploid peak in b and c. (a) An infant diploid tumor (no. 5) with DNA index of 1.0. (b) Another infant diploid tumor (no. 8) with DNA indexes of 1.0 and 1.56. (c) An infant triploid tumor (no. 11) with DNA index of 1.50. Ploidy divergence was seen in nos. 5 and 8, but not in no. 11 (Table 1).

3.3. Analysis of MYCN copy number in neuroblastoma tumors

MYCN copy numbers were examined by FISH using D2Z1 and *MYCN* probes. Eight of nine infant diploid tumors and all triploid tumors had two or three pairs of D2Z1 (green) and *MYCN* (red) signals in interphase nuclei, indicating a single copy of *MYCN* per haploid genome (Fig. 3, a and b). One infant diploid tumor had numerous copies of *MYCN*, consistent with double minutes. In contrast, of 10 childhood diploid tumors, 3 had a single copy, 3 had 2 copies (Fig. 3c), and 5 had numerous copies of *MYCN* (Fig. 3d). All 6 tumors with *MYCN* amplification showed 2 D1Z1 and 1 D1Z2 signals, indicating 1p deletion (Table 1).

3.4. Analysis of centrosomes in neuroblastoma tumors

Centrosomes and spindles were stained with anti–γ-tubulin and anti–β-tubulin antibodies, respectively, for microscopic examination (Fig. 4a). We defined one or two centrosomes in a cell as normal (Fig. 4, b and c), and three or more centrosomes in a cell as amplification (Fig. 4, d and e, the cell on the right). Extra centrosomes

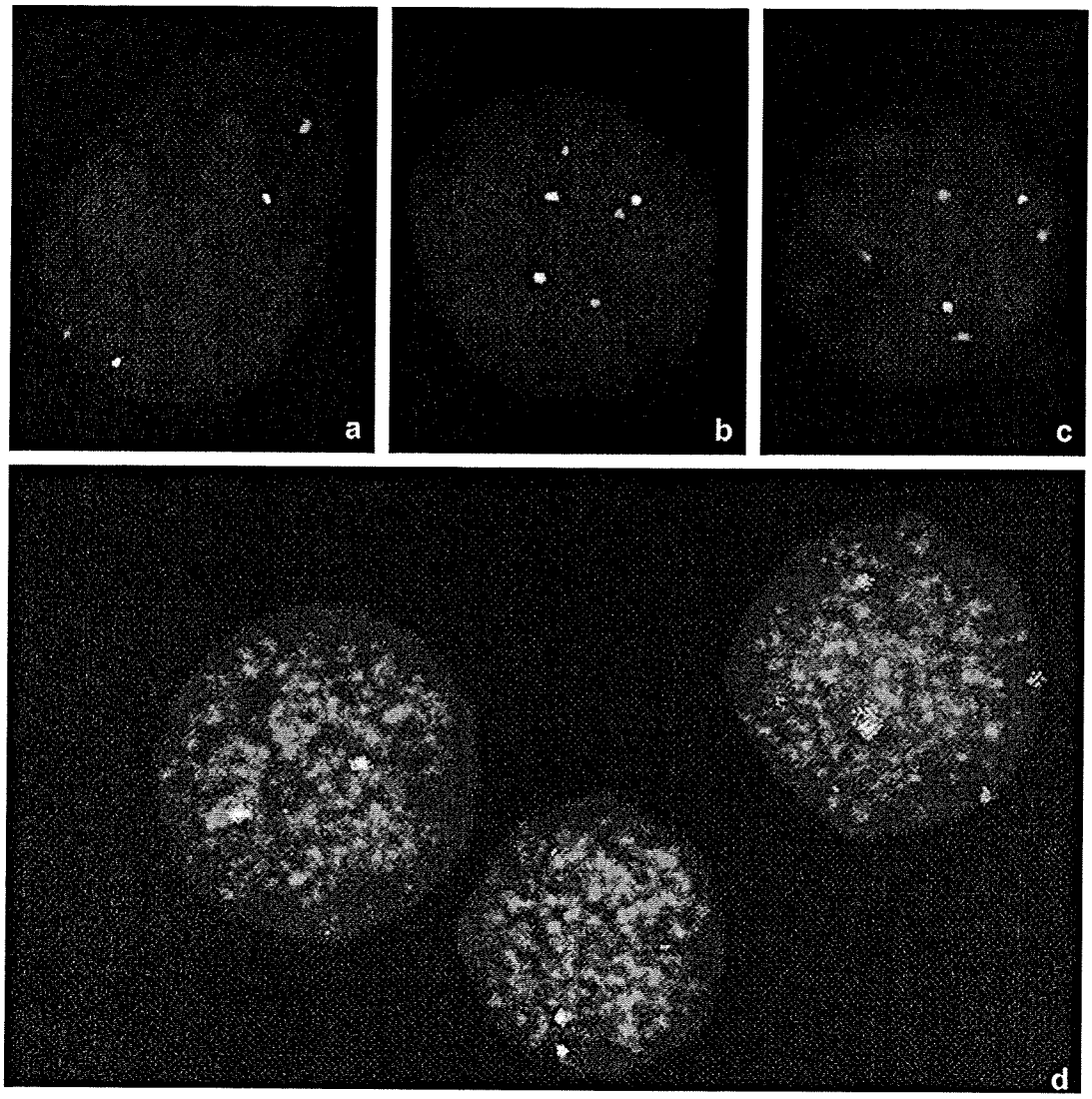

Fig. 3. Evaluation of the *MYCN* copy number by FISH using the *MYCN* (red) and D2Z1 (green) probes located at 2p24 and pericentromeric region of chromosome 2, respectively. (a) Interphase nucleus of a diploid tumor with single-copy *MYCN* (No. 1). Two pairs of D2Z1 and *MYCN* signals are observed. (b) Interphase nucleus of a triploid tumor with single-copy *MYCN* (No. 14). Three pairs of D2Z1 and *MYCN* signals are observed. (c) Interphase nucleus of a diploid tumor that has two copies of *MYCN* (no. 21). Four *MYCN* and two D2Z1 signals are seen. (d) Interphase nucleus of a diploid tumor that has multiple copies of *MYCN* (no. 26). Numerous *MYCN* signals and two D2Z1 signals in a nucleus are observed, indicating *MYCN* amplification.

were often located in the cytoplasm rather than in the nucleus or perinuclear region (Fig. 4, d and e).

Centrosome amplification was detectable in 15.7% of infant diploid tumors, 4.0% of infant triploid tumors, and 37.9% of childhood diploid tumors (Fig. 5a). Childhood diploid tumors had a higher incidence of centrosome amplification than infant triploid tumors ($P=0.0001$) or infant diploid tumors ($P=0.01$), and infant diploid tumors tended to have a higher incidence of centrosome amplification than infant triploid tumors ($P=0.07$; Fig. 5a). Of 9 infant diploid tumors, 7 had ploidy divergence, and these 7 tumors had a higher incidence of centrosome amplification than 8 infant triploid tumors ($P=0.05$), all of which had no ploidy divergence (Fig. 5b). Of 27 tumors, 17 were found clinically, and 10 were found by mass screening. There was no difference in the incidences of centrosome amplification between 11 sporadic tumors with a single copy of *MYCN* and 6 sporadic tumors with *MYCN* amplification ($P=0.74$; Fig. 5c).

4. Discussion

Our study of ploidy in neuroblastoma revealed that all triploid tumors occurred in infants, whereas diploid tumors occurred in both infants and children. When we compared clinical characteristics, including the age of patients, the tumor site, and the mass-screening status, there were no differences between infant diploid and triploid tumors. We

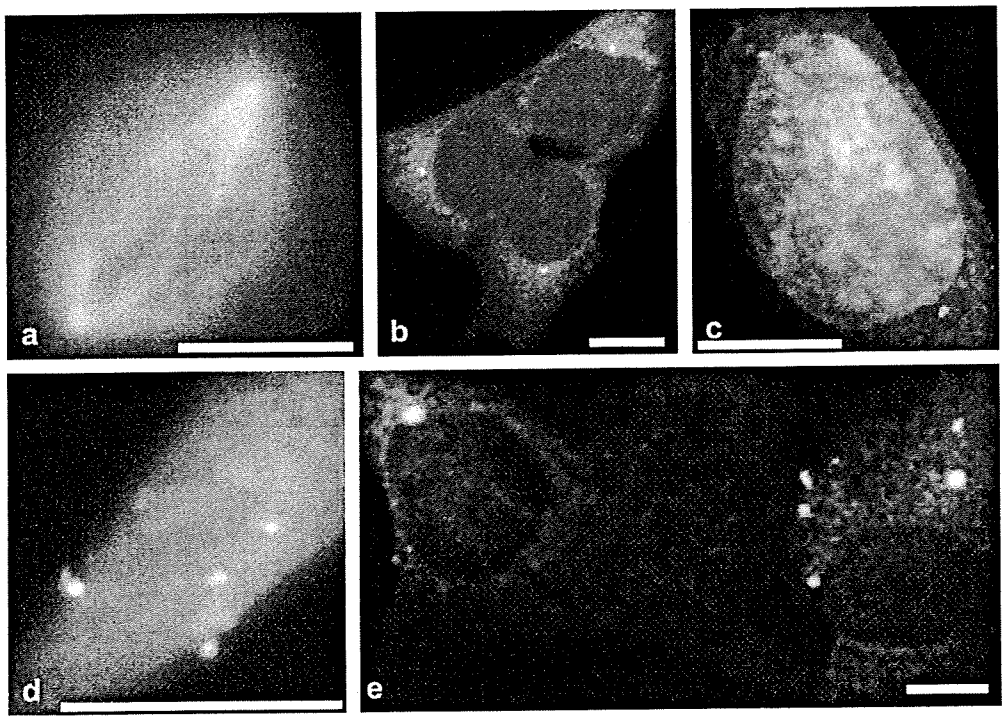

Fig. 4. Examination of centrosome numbers by immunofluorescence staining with anti–γ-tubulin (orange) and anti–β-tubulin (green) antibodies. The scale bars represent 10 μm. (a) The antibodies detected centrosomes (orange) and spindles (green) in a metaphase cell from a control cell line, HCT-116. (b) One centrosome signal is shown in each of three cells from an infant diploid tumor with *MYCN* amplification (no. 9). (c) Two centrosome signals are shown in a cell from an infant triploid tumor (no. 12). (d) Four centrosome signals are shown in a cell from a childhood diploid tumor with a single *MYCN* copy (no. 22). Note that the excess centrosomes are located in the cytoplasm. (e) Five centrosome signals in one cell (right) and one centrosome signal in the other cell (left) are shown in a childhood diploid tumors with *MYCN* amplification (no. 24). All 100 cells from this tumor showed *MYCN* amplification that was detected by FISH.

found, however, that ploidy divergence was very frequent in infant diploid tumors but not in infant triploid tumors. Furthermore, the ploidy divergence was correlated with centrosome amplification in the infant tumors. The centrosome amplification was also found in the majority of childhood diploid tumors, but none of these showed ploidy divergence.

Centrosome amplification found in cells of most infant diploid tumors may be generated by cytokinesis failure because such diploid cells had ploidy divergence accompanying tetraploid cells. In contrast, the centrosome amplification found in cells of childhood diploid tumors may be generated by multiple centrosome duplications in a single cell cycle, as reported in mouse or human cancer cell lines with mutation in *TP53* or other tumor suppressor genes or oncogenes [17–19,21,22]. The occurrence of the multiple centrosome amplification in childhood diploid tumors is consistent with the finding that childhood diploid tumors have more frequent cytogenetic changes than infant diploid tumors [6,23] (Fig. 6). Recent studies showed that supernumerary centrosomes are forced to coalesce into two spindle poles, and cells with coalesced centrosomes underwent bipolar division in certain experimental conditions or mouse neuroblastoma cell lines [12,15]. Thus, the presence of centrosome amplification in the majority of childhood diploid tumors may not be surprising if we presume that the centrosome coalescing takes place in these tumor cells. Although infant diploid and childhood diploid tumors share the trait of centrosome amplification, the mechanisms for amplification may be different, and different mechanisms may be closely correlated with the clinical behavior of the tumors (Fig. 6).

It is tempting to speculate that both diploid and triploid cells in infant tumors originate through tripolar or tetrapolar division of tetraploid cells with centrosome amplification caused by cytokinesis failure [11] (Fig. 6). The resulting diploid clone of cells coexists with small clones of divergent ploidies and retains centrosome amplification, whereas the resulting triploid cells form a triploid tumor when the triploid chromosome constitution has a proliferative and survival advantage, so the triploid tumors may proliferate by bipolar division. Therefore, there is no residual chromosome instability, thus explaining the benign nature of the triploid tumors. These phenomena may be explained by genetic divergence and convergence proposed on the basis of cytogenetic studies of leukemias and solid tumors or the continuous observation of centrosomes in tumor cell lines [21,24].

We previously reported that infant diploid tumors were classified into favorable and unfavorable types on the basis

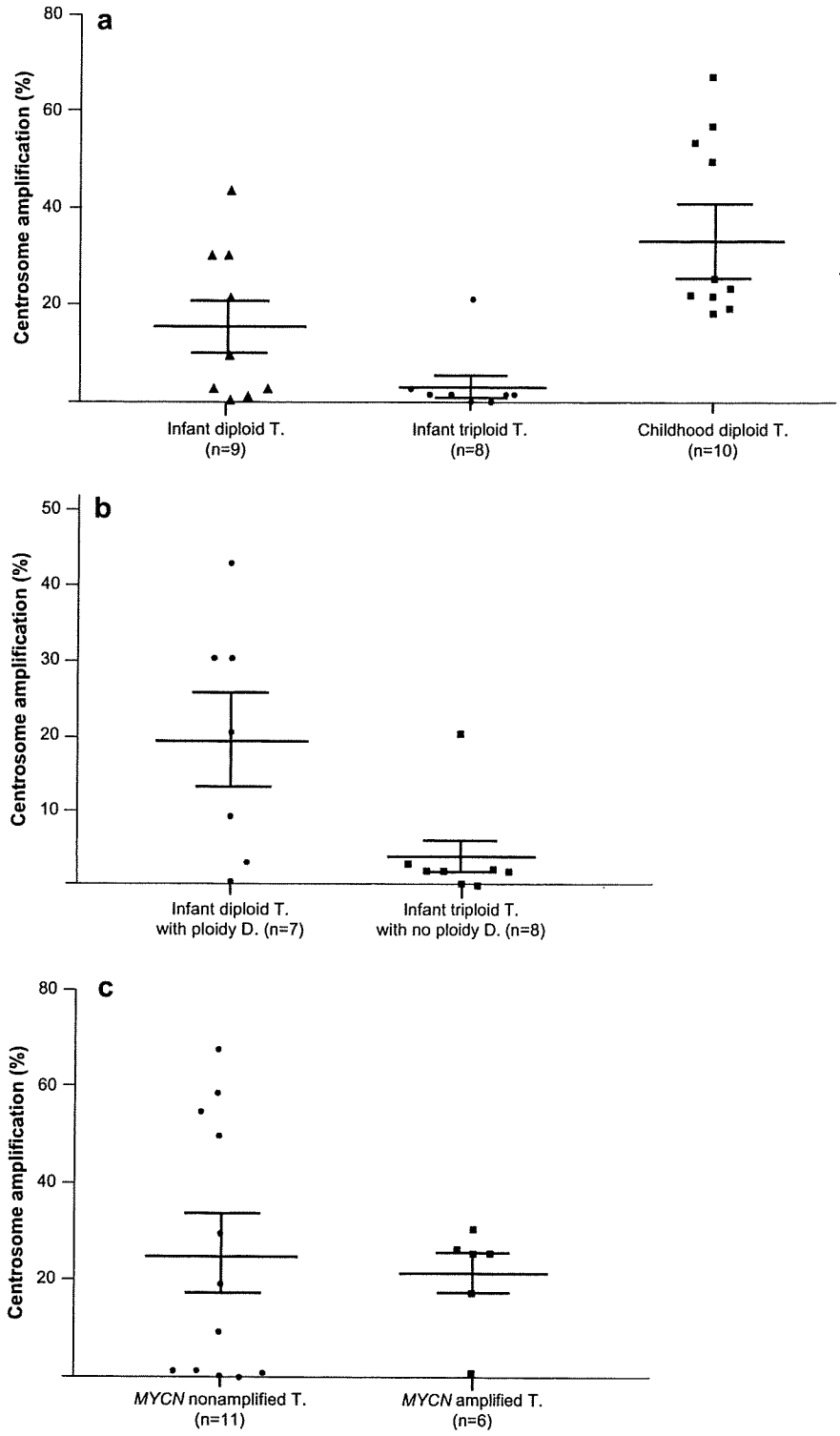

Fig. 5. Percentage of centrosome amplification in tumor cells from 27 neuroblastoma tumors. The large and small horizontal bars indicate the mean percentage of centrosome amplification and the mean percentage ± standard error, respectively. (a) Childhood diploid tumors had a higher incidence of centrosome amplification than infant diploid ($P=0.01$) or infant triploid ($P=0.0001$) tumors. Infant diploid tumors had a higher incidence of centrosome amplification than infant triploid tumors ($P=0.07$). (b) Infant diploid tumors with ploidy divergence had a higher incidence of centrosome amplification than infant triploid tumors with no ploidy divergence ($P=0.05$). (c) There was no significant difference in the incidence of centrosome amplification between sporadic tumors with a single *MYCN* copy and those with *MYCN* amplification ($P=0.74$). T, tumors; D, divergence.

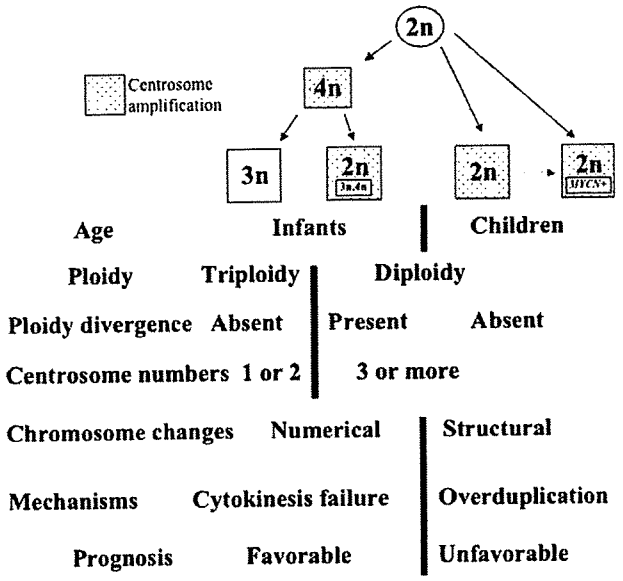

Fig. 6. Model of the mechanism by which infant and childhood diploid tumors may arise. Oval and rectangle indicate a neuroblastoma precursor cell and a neuroblastoma tumor, respectively. Dots in rectangles indicate that the tumors had centrosome amplification. The rectangle with a large 2n and a small 3n and 4n indicates an infant diploid tumor with ploidy divergence. The rectangle with 2n and *MYCN*+ indicates a childhood diploid tumor with *MYCN* amplification.

of the presence or absence of 1p deletion [6], and other investigators also reported the favorable outcome of most infant diploid tumors [25]. The present study examined mostly favorable diploid tumors in infants, and explains why many infants with a diploid tumor had a favorable outcome; i.e., most infant diploid tumors and infant triploid tumors may be derived from the common precursor tetraploid cells with cytokinesis failure (Fig. 6). Only one infant diploid tumor in the present study was at stage 4 and had *MYCN* amplification and 1p deletion; such a tumor shares an unfavorable outcome with childhood diploid tumors with *MYCN* amplification and/ or 1p and/or 11q loss, and/ or 17q gain [5,6,23].

MYCN amplification is a hallmark of neuroblastoma with aggressive clinical behavior [1,2], and centrosome amplification was reported in various invasive carcinomas and neuroblastoma tumors with *MYCN* amplification [14,16]. We tried to clarify the relationship between the two types of amplification in neuroblastoma tumors, and found that there was no difference in the incidence of centrosome amplification between sporadic tumors with a single *MYCN* copy and those with *MYCN* amplification. The present findings seem to contradict the previous report of a higher incidence of centrosome abnormality in neuroblastoma tumors with *MYCN* amplification than in tumors without *MYCN* amplification [16]. This discrepancy may be resolved when a large number of neuroblastoma tumors with and without *MYCN* amplification are examined. Sugihara et al. [26] showed that ectopic expression of *MYCN* alone in a neuroblastoma cell line did not cause centrosome amplification, but centrosome amplification and micronucleus formation occurred in these cells after DNA damage. These findings suggest that *MYCN* amplification per se may not induce centrosome amplification.

Acknowledgments

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Risks and benefits of ending of mass screening for neuroblastoma at 6 months of age in Japan

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Abstract

Purpose: The mass screening (MS) for neuroblastoma (NB) at 6 months of age in Japan was discontinued in 2004. This study assessed the risks and benefits of MS based on an analysis of NB detected before or after discontinuation of MS in Japan.

Methods: The clinical features and Brodeur's genetic type based on *MYCN*, DNA ploidy, and other genetic aberrations were assessed in 113 NB patients (20 cases after and 93 cases [55 MS cases] before the discontinuation of MS) older than 6 months treated at one institution since 1985.

Results: The 20 patients with NBs detected after MS was discontinued ranged in age from 7 to 67 months, 12 patients were stage 4, and 11 patients would have been detected at 6 months of age if they had undergone MS. The Brodeur's genetic type of these 20 patients showed that 30% (6/20) were type 1 (low risk), 55% (11/20) were type 2A (intermediate risk), and 15% (3/20) were type 2B (high risk). Of 93 patients with NB detected before MS was discontinued, 60% (56/93) were type 1, 18% (17/93) were type 2A, and 22% (20/93) were type 2B. Among the type 2A patients, 82% (9/11) of the patients detected after MS was discontinued showed stage 4, whereas only 50% (9/18) of those diagnosed before MS was discontinued were stage 4. The genetic analysis using single nucleotide polymorphism (SNP) array for type 2A showed that the pattern of genetic aberration was equivalent in those detected either before or after MS was discontinued.

Conclusions: There was a decrease of type 1 and an increase of type 2A NB in patients after MS was discontinued in Japan. These results suggest that most of the type 1 detected by MS has regressed, and most of the type 2A detected by MS has appeared sporadically as advanced NB in patients older than 1 year.

Beginning in 1985, a nationwide mass screening program (MS) for neuroblastoma (NB) was conducted for 6-month-old infants throughout Japan [1,2]. More than 2000 Japanese children have been diagnosed with NB based on the MS at 6 months of age. The outcome of these patients has been excellent, and more than 97% of all such patients are alive [3,4]. The number of patients with NB has increased since the initiation of MS started. Likewise, the number of patients younger than 1 year as well as the number of cases with early stage NB has also increased. However, the number of advanced-stage NB patients older than 1 year has not

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substantially changed in several reports [5,6]. These findings imply that a number of tumors in this age group (6 months of age) have the capacity to either spontaneously regress or mature, and thus they may not be detected clinically.

Mass screening for NB at 6 months of age in Japan was discontinued in 2004. Most of the NBs detected by MS show a good prognosis [7], whereas only a few cases demonstrate an unfavorable outcome [8]. This study aims to assess the risk and benefit of MS based on an analysis of NB detected before or after the discontinuation of MS in Japan.

1. Materials and methods

The details of the MS protocol in Japan have been published elsewhere [9]. Briefly, the presence of urinary vanillylmandelic acid (VMA) and homovanillic acid (HVA) is measured by high-performance liquid chromatography.

Between 1985 and 2008, 113 NB patients older than 6 months were treated at our institution. Fifty-five of the 93 NBs detected before the discontinuation of MS (MS-positive cases) were detected through MS at 6 months of age, 27 cases (MS-negative cases) were detected in children older than 6 months sporadically in the cohort of children who underwent MS at 6 months of age, and 11 cases (nonscreened cases) were detected in children older than 6 months sporadically in the cohort of children who did not undergo MS at 6 months of age. Twenty cases in children older than 6 months were detected in the cohort of children who did not undergo MS after the discontinuation of MS.

The following clinical features were analyzed: age, initial symptom, the clinical stage (International Neuroblatoma Staging System), the site of the primary tumor, urinary VMA and HVA, the treatment, and the outcome of the patients.

The following biological features were also examined: MYCN amplification; DNA ploidy; other genetic aberrations such as 1p loss, 2p gain, 3p loss, 11q loss, and 17q gain; and the Shimada histology [10]. Consent was obtained from the patient's parents for tumor preservation and biological analysis before surgery. The *MYCN* amplification status was examined using the fluorescence in situ hybridization (FISH) method, quantitative polymerase chain reaction (PCR), and Southern blotting [11]. The 1p loss was determined by 2-color FISH method or SNP array. Other genetic aberrations such as the 3p loss, 11q loss, and the 17q gain were evaluated by SNP array. DNA ploidy was assessed using flow cytometry. The protocols used for FISH and quantitative PCR have been described in detail in previous reports [11-13]. SNP array experiments were done using Human CMV370-Duo (Illumina, San Diego, CA) according to the manufacturer's protocol. Genomic profiles were created using the Illumina Genome Viewer (IGV) and Chromosome Browser (ICV) of Illumina's BeadStudio2.0 software program. Based on these genetic features, all NBs were classified into 3 groups (type 1, type 2A, type 2B) using Brodeur's genetic type (BGT) [14]. Type 1 is triploid and does not have any MYCN amplification, 1p loss, 3p loss, 11q loss, or 17q gain. Type 2A is diploid, and at least one of other genetic aberration such 1p loss, 3p loss, 11q loss, and 17q gain. Type 2B is diploid and MYCN amplification. The statistical test of difference in each genetic

Table 1 The clinical features of NB detected after the discontinuation of MS

Case	Age (mo)	Stage (INSS)	Symptom	Primary site	Urinary VMA or HVA	Treatment	Outcome	Time from diagnosis (mo)
1	13	2A	Free	Ad	Increase	Biopsy-chemo	CR	58
2	10	1	Free	Ad	Increase	Ope	CR	47
3	25	4	Fever, anemia	Ad	Increase	Biopsy-chemo-ope-SCT	CR	39
4	8	4	Free	Med	Increase	Biopsy-chemo-ope-chemo	CR	37
5	18	1	Stridor	Med	Increase	Ope-chemo	CR	33
6	9	4	Fever	Ad	Increase	Biopsy-chemo-SCT-ope	CR	32
7	9	2B	Diarrhea	Ad	Increase	Biopsy-chemo-ope-chemo	CR	25
8	7	3	Free	Ad	Increase	Biopsy-chemo	CR	21
9	42	4	Fever, leg pain	Ad	Increase	Biopsy-chemo	PD	20
10	13	4	Exophthalmos	Ad	Increase	Biopsy-chemo	Undergoing	18
11	49	4	Abdominal mass	Ad	Increase	Ope-chemo	Undergoing	17
12	45	4	Leg pain	Ret	Increase	Biopsy-chemo	Undergoing	16
13	67	4	Cervical mass	Ad	Increase	Biopsy-chemo-ope-SCT	CR	13
14	13	1	Abdominal mass	Ad	Increase	Ope	CR	11
15	8	1	Free	Ad	Increase	Ope	CR	10
16	57	4	Abdominal mass	Ret	Increase	Biopsy-chemo	Undergoing	8
17	14	3	Opsoclonus-myoclonus	med	Increase	Ope-chemo	Undergoing	8
18	21	4	Fever, leg pain	Ad	Increase	Biopsy-chemo	Undergoing	4
19	28	4	Abdominal mass	Ad	Increase	Biopsy-chemo	Undergoing	1
20	10	4	Exophthalmos	Ad	Increase	Biopsy-chemo	Undergoing	1

Ad indicates adrenal gland; med, mediastinum; ret, retroperitoneum; chemo, chemotherapy; ope, tumor extirpation; SCT, stem cell transplantation; CR, complete response; PD, progressive disease; INSS, International Neuroblatoma Staging System.