TABLE 1. Brock Criteria and Chang Criteria

Brock Criteria[9]		Chang Criteria[17]	
Grade 0	< 40 dB at all frequencies	Grade 0	≤20 dB at 1, 2, and 4 kHz
Grade 1	≥ 40 dB at 8 kHz	Grade 1a	≥ 40 dB at any frequency 6-12 kHz
		1b	> 20 and < 40 dB at 4 kHz
Grade 2	≥ 40 dB at 4-8 kHz	Grade 2a	≥ 40 dB at 4 kHz and above
		2b	> 20 and < 40 dB at any frequency below 4 kHz
Grade 3	≥ 40 dB at 2 kHz	Grade 3	≥ 40 dB at 2 or 3 kHz and above
Grade 4	≥ 40 dB at 1-8 kHz	Grade 4	≥ 40 dB at 1 kHz and above

The timing of audiometric assessment was not clearly defined and was at the discretion of the primary doctor.

The criteria for classification of hearing loss were based on the results of audiography. Severity of hearing loss was graded according to the Brock criteria that are designed and validated specifically for cisplatin-induced hearing loss, with a focus on high frequencies[9] (Table 1). The numerical grade assigned to patients with asymmetric hearing loss corresponded to audiometric results for the ear with better hearing capacity. To evaluate the utility of the Brock grading system, audiograms were also assigned Chang grades, which are minor modifications of the Brock grades developed to incorporate functional deficits caused by hearing loss of < 40 dB that are not covered by the Brock criteria[17] (Table 1). Apart from hearing loss at high frequencies, a threshold hearing loss of > 40 dB at low frequencies (125, 250, 500 Hz) was also defined to be abnormal in this study.

RESULTS

Patients

Patient characteristics are summarized in Table 2. Six (11%) patients experienced disease relapse. Forty-three (78%) patients were alive at the last follow-up. The median follow-up from diagnosis to the most recent audiometry analysis was 2.2 years (range, 0.2 to 19.6 y).

TABLE 2. Patient Characteristics

Characteristics	No. Patients
Total, n	55
Median age at diagnosis (n [range]) (y)	2 (0-14)
Males:females, n	31:24
Diagnosis, n	
Neuroblastoma	29
Hepatoblastoma	16
Central nervous system tumors	9
Nasopharyngeal carcinoma	1
Cumulative dose of cisplatin (mg/m^2)	200-1500 (median: 400)
Carboplatin, n	30
Myeloablative carboplatin	10
Cumulative dose of carboplatin (mg/m^2)	200-5850 (median: 1600)
Radiotherapy, n (Gy)	16
≥ 50	8
30-50	3
< 30	5
Status at last follow-up [n (%)]	
Alive	43 (78)
Dead	12 (22)

Treatment

All patients were treated with cisplatin-based chemotherapy. Twenty-two patients received both cisplatin and carboplatin; 8 patients received cisplatin and radiotherapy; and 8 patients received cisplatin, carboplatin, and radiotherapy. The total cumulative dose of cisplatin at the end of treatment ranged from 200 to 1500 mg/m^2 (median, 400 mg/m^2). The total cumulative dose of carboplatin ranged from 200 to 5850 mg/m^2 (median, 1600 mg/m^2). Cisplatin was changed to carboplatin because of ototoxicity in 2 patients and nephrotoxicity in 2 patients. Seven patients diagnosed with a brain tumor that was treated by radiation therapy received craniospinal irradiation at a dose of 18 to 30 Gy, which was boosted to a total dose of 24.4 to 50 Gy during irradiation of the tumor bed. Six patients diagnosed with neuroblastoma received cranial irradiation at a dose of 12 to 20 Gy, whereas another 2 patients diagnosed with neuroblastoma that metastasized to the brain received focal irradiation at a dose of 36 Gy. The remaining patients diagnosed with nasopharyngeal carcinoma received irradiation of 60 Gy to the neck lesion.

Ototoxicity

The characteristics of patients with or without hearing loss are shown in Table 3. Thirty-five (64%) patients developed ≥ grade 1 hearing loss according to the Brock criteria. Two patients with hearing loss experienced tinnitus, whereas 1 patient experienced dizziness. The cumulative dose of cisplatin at the time of hearing loss diagnosis ranged from 200 to 1500 mg/m^2 (median, 400 mg/m^2). Ten (56%) of the 18 patients who received a cumulative dose of

TABLE 3. Profile of Children With Hearing Loss Versus Those Without Hearing Loss After Cisplatin Treatment

	Hearing Loss (Brock ≥ 1)	No Hearing Loss (Brock 0)	P
Patients (n [%])	35 (64)	20 (36)	
Median age at diagnosis (y)	0-14 (median: 2)	0-13 (median: 1)	0.43†
Males:females, n	22:13	9:11	0.26‡
Cumulative dose of cisplatin (mg/m^2)	200-1500* (median: 400)	200-1170 (median: 380)	0.74†
Carboplatin exposure, n	22	8	0.16‡
Myeloablative carboplatin	8	2	0.30‡
Radiotherapy, n	12	4	0.36‡

*The dose at the time of diagnosis of hearing loss, not at the end of treatment.
†Student *t* test.
‡Fisher exact test.

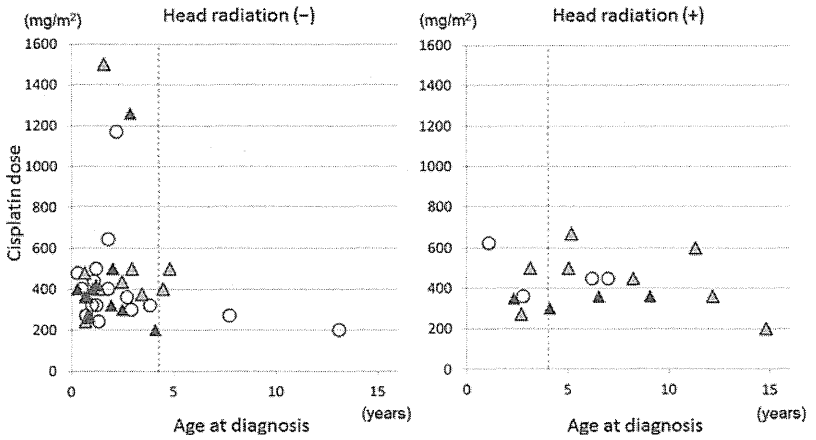

FIGURE 1. Role of cisplatin dose and age at diagnosis. ○, normal hearing; △, with hearing loss (Brock grade 1 and 2); ▲, with hearing loss (Brock grade 3 and 4); ----, 4 years of age.

cisplatin of $<360\,mg/m^2$ developed hearing loss, whereas 25 (68%) of the 37 patients who received $\geq 360\,mg/m^2$ developed hearing loss (Fig. 1). Seven (88%) of the 8 patients treated with a combination of cisplatin, carboplatin, and radiotherapy, and 8 (47%) of the 17 patients treated with cisplatin only, developed hearing loss. Twenty-three (59%) of the 39 patients who were treated before the age of 4 years and 12 (75%) of the 16 patients who were treated after the age of 4 years developed hearing loss. Nine (75%) of the 12 patients in the older group with hearing loss had received radiotherapy (Fig. 1).

The median time to the onset of hearing loss after the last dose of cisplatin was 71 days (range, −110 to 3446 d). Hearing loss developed after a year in 4 patients (11%) and at or after 2 years in 6 patients (17%; Fig. 2). Their total cumulative dose of cisplatin ranged from 240 to 1500 mg/m^2 (median, 410 mg/m^2). Only 1 patient received radiotherapy and 8 patients were treated before the age of 4 years. Nine (75%) of the 12 patients who received radiation therapy developed hearing loss within 6 months from the end of cisplatin administration.

The severity of hearing loss graded by the Brock criteria and Chang criteria is shown in Table 4. Of the 35 patients who developed hearing loss, severe hearing loss was identified in 16 (46%) patients according to the Brock criteria (grade 3 and 4) and 28 (80%) patients according to the Chang criteria (grade 2b to 4). Severe hearing loss according to the Brock criteria (grade 3 and 4) was observed in 12 of the 23 (52%) younger patients (under 4 y) and 4 of the 12 (33%) older patients (4 y or older). In contrast, severe hearing loss according to the Chang criteria (grade 2b to 4) was observed in 18 of the 23 (78%) younger patients and 5 of the 12 (42%) older patients. Four patients with Brock grades 2 to 4 and Chang grades 2b to 4 required hearing aids. Their total cisplatin doses were 260, 264, 360, and 450 mg/m^2, respectively, and 2 patients had received radiotherapy. Fifteen (43%) of the 35 patients with hearing loss exhibited hearing loss even at low frequencies; 4 patients showed persistent hearing failure, whereas 11 patients showed transient failure. Six patients experienced progressive hearing loss with time (Table 5); progression to hearing loss took ≥ 2 years in 5 of them.

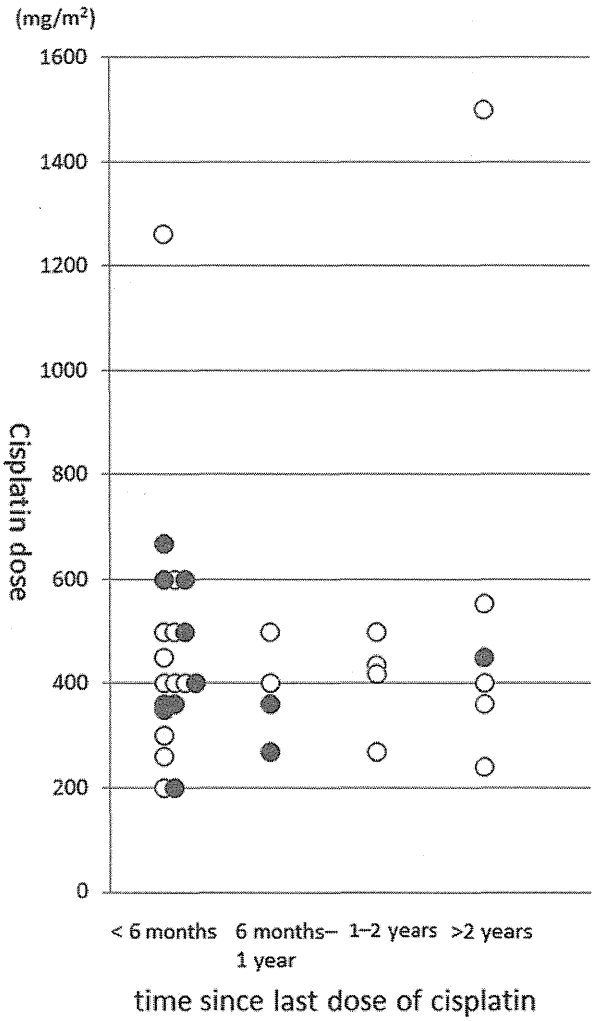

FIGURE 2. Time to onset of hearing loss since cisplatin treatment. ○, Patients without radiation therapy; ●, Patients with radiation therapy.

TABLE 4. Severity of Hearing Loss

	Brock Criteria		
	1-2	3-4	Total
Chang criteria			
1a-2a	12	0	12
2b-4	7 (1)	16 (3)	23
Total	19	16	35

( ) hearing aids.

DISCUSSION

This study evaluated hearing loss in 55 pediatric patients who had received cisplatin as anticancer therapy. We included all patients who were treated with cisplatin with or without carboplatin and/or radiotherapy. In total, 64% patients treated with cisplatin developed hearing loss. In agreement with previous studies, a cumulative dose of cisplatin > 360 to 400 mg/m^2 is directly related to the onset of ototoxicity. In our cohort, 56% patients who had received < 360 mg/m^2 at least 200 mg/m^2 of cisplatin and 68% patients who had received ≥ 360 mg/m^2 of cisplatin developed hearing loss. Contrary to the previous findings, we found that the cumulative dose of cisplatin did not correlate exactly with the onset of hearing loss. In contrast, patients treated with cisplatin, carboplatin, and radiotherapy developed ototoxicity at a high rate (88%). We found that the risk of hearing loss was associated with cisplatin-based multimodal therapy that included carboplatin and/or radiotherapy rather than a cumulative dose of cisplatin alone. Therefore, we could not predict by total dose of cisplatin whether they develop hearing loss or not. It is very important for all pediatric patients treated with cisplatin to undergo screening for hearing loss from the early treatment and posttreatment phases in addition to the regular follow-up evaluations, irrespective of the cumulative dose of cisplatin. Another issue is deciding between discontinuation of cisplatin to preserve residual hearing or continuation of the drug to maximize survival. Despite the current recommended monitoring for hearing loss during cisplatin therapy and existing guidelines for cisplatin dose modifications, the ultimate effects of these dose modifications on hearing outcome has not been thoroughly evaluated. Prospective studies are warranted to clarify this.

The time to onset of hearing loss from the last dose of cisplatin was variable in this study. Previous studies suggested that a possible delay in the onset of hearing impairment ranged from 1 to 50 months after therapy.[7,18] Our study demonstrated that it took > 2 years to progress to hearing loss in 17% patients. Results of most reviews suggest that hearing loss is permanent and stable after the completion of therapy. Bertolini and colleagues observed progressive hearing loss after as long as 136 months from the end of therapy with platinum compounds for pediatric solid tumors. Radiation therapy also increases the risk of ototoxicity, and survivors > 5 years after diagnosis remain at risk for the development of ototoxicity.[12,19] We found that 6 patients had a 5-year progression of hearing loss, and 4 of them had received radiation for head lesions. Forty-three percent patients with hearing loss (≥ Brock grade1) also exhibited hearing loss at low frequencies that were not defined in the Brock criteria. As previously reported, hearing loss at low frequencies is sometimes reversible, and some of our patients experienced transient low-frequency hearing loss. In most cases, the cause was edema or inflammation. However, 4 patients exhibited persistent low-frequency hearing loss for reasons unknown. Even if audiograms are normal at the end of cisplatin-based therapy, it is important to maintain long-term observations for ototoxicity. On the basis of our results, we suggest that patients treated with cisplatin should receive audiologic management for > 5 to 10 years after the end of treatment. With the recent advances in technology, online hearing tests or hearing check lists are easily accessible at home, making prolonged assessment possible and cost-effective.

Younger age at the time of treatment has been found to increase the risk of hearing loss.[9,10,11] Children younger than 4 to 5 years of age at the time of treatment were more likely to acquire high-frequency hearing loss compared with older children. However, in our study, 59% patients who were younger (4 y or below) and 75% patients who were older (above 4 y) at diagnosis developed hearing loss. This may be because more patients in the older (above 4 y) group received radiation therapy.

In most studies, age was directly correlated with the severity of hearing loss. This is because of the immaturity of the cochlea cells or the age-related pharmacokinetics of cisplatin.[20] In our study, the younger group (under 4 y) comprised a higher proportion of patients with Brock grades 3 and 4 compared with the older group (above 4 y). The severity of hearing loss as graded by the Brock criteria did not correlate with the adaptation of hearing aids. In this report, the severity in 4 patients with hearing aids widely ranged from grade 2 to 4. In contrast, it is recommended that patients with Chang grades of ≥ 2b should use hearing aids. Of our patients, 4 with Chang grades 2b to 4 required hearing aids and accounted for 17% patients with grades 2b to 4. Brock and Chang criteria did not always predict audiologic intervention in our study. The Brock criteria focus on high-frequency hearing loss, where cisplatin-induced hearing loss manifests first. As mentioned previously, some patients presented with hearing loss at low frequencies. Although speech frequencies are considered to be 500 to 2000 Hz, they differ with language. For example, Japanese words comprise lower

TABLE 5. Patients With Progressive Hearing Loss

Cases	Age at Diagnosis (y)	Radiotherapy (Gy)	Time From Onset of Hearing Loss to Progression (y)	Severity (Grade)	
				Brock Criteria	Chang Criteria
1	9	+ (50)	3.9	1-4	1-4
2	14	+ (60)	5.1	1-2	2b-2b
3	3	+ (18)	3.5	1-2	1a-2b
4	3	—	3.3	3-4	2a-4
5	4	—	0.2	2-3	2b-3
6	4	+ (12)	4.2	1-3	2b-3

frequencies compared with English words. Therefore, it is important for the grading system to predict the need for hearing support more reliably by including lower frequencies. Another problem with hearing aids is that they require advanced techniques to adjust the hearing level, particularly in patients with high-frequency hearing loss, and therefore, cochlear implants are sometimes required in these patients. A system to evaluate how hearing loss affects communication is also required.

There is evidence that platinum ototoxicity shows significant interindividual genetic variability and racial variability.[15] In a study, genetic variations in 2 specific genes, namely, thiopurine *S*-methyltransferase (*TPMT*) and catechol-*O*-methyltransferase (*COMT*), were identified as having a strong association with cisplatin-induced ototoxicity in pediatric patients. *TPMT* and *COMT* variants were found to be associated with severe ototoxicity, and patients who carried at least 3 of the 4 risk alleles exhibited a rapid decline in their hearing, often after the first dose of cisplatin. Of late, Japanese patients have been reported to be more susceptible to cisplatin-induced hearing loss.[20] Ethnic differences in the prevalence of one or more of these gene polymorphisms may be the underlying cause of the higher susceptibility of Japanese patients to cisplatin. Study on cisplatin-induced hearing loss targeting westerners have been reported a lot; however, a study on other races including the Japanese is quite limited.

Our study faces many limitations inherent to any retrospective analysis, particularly those imposed by the variability in timing and quality of audiometry data available for patients at different time points. In addition, there were no clear criteria for conducting hearing tests in our cohort, and patients treated with lower doses of cisplatin did not undergo hearing evaluation. Audiologic evaluation was more difficult in the younger patients, whose hearing level may have been evaluated to be worse than it actually was. Our study are largely influenced by many factors because of the variety of diagnoses. This study contains patients with vastly different exposures in terms of type of chemotherapy, dose, age at treatment, and exposure of radiation. Because of the small sample size, we could not include the use of statistical analysis. We emphasize the result that low dose of cisplatin could develop hearing loss and some of them also develop late hearing loss or hearing loss at lower frequencies (125 to 500 Hz). We would not be able to predict by the total dose of cisplatin whether they develop hearing loss or not and only close follow-up evaluation would do so. Our results suggest that all patients who received cisplatin-based chemotherapy would undergo audiometry at baseline, after each cisplatin or carboplatin dose, at the end of therapy, and every several months or years after the end of therapy. Further prospective studies with analysis of genetic polymorphisms were required to confirm our observations.

In conclusion, our study did not validate the risk factors demonstrated by earlier studies: the cumulative dose of cisplatin and/or younger age, both associated with the onset of hearing loss. A lower dose ($< 360\ mg/m^2$) of cisplatin was found to cause hearing loss not only in younger (under 4 y) patients but also in older (4 y or older) patients. The onset of hearing loss may be associated with many factors, including genetic background and combination with ototoxic therapy. Future prospective studies are therefore required to predict the risk factors associated with hearing loss, including genetic background. Ultimately, a long-term follow-up system that helps in identifying individuals who will require hearing support is crucial for providing the appropriate intervention.

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Long-Term Follow-Up Results of the Observation Program for Neuroblastoma Detected at 6-Month Mass Screening

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We conducted an observation program of neuroblastoma in infants, detected by mass screening at 6 months of age; we followed up with them for 15 years. No recurrence was observed after disappearance of tumors, and persistent tumors showed no malignant transformation or metastasis. Histology of the resected tumors showed age-related differentiation. *(J Pediatr 2014; ■: ■-■).*

In 1985, nationwide mass screening (MS) for neuroblastoma (NBL) in 6-month-old infants began in Japan. During this period, no clear decrease in the incidence of unfavorable NBL at an older age was observed.[1] Similar results of large-scale interventional studies in Canada[2] and Germany[3] were reported around the same time, and MS in Japan was discontinued in March 2004.

In 1994, we initiated an observation program for localized NBL cases detected by MS without any therapy and reported the benign characteristics of these tumors after a 2-year observation period.[4] Although our short-term observation, such as several similar ones,[5-8] confirmed the tendency of NBL to spontaneous regression, the natural course of NBL over longer periods remains unclear. Therefore, we have continued follow-up of these patients for up to 15 years (median follow-up period of 81 months) and report here the observation results for 45 tumors.

Methods

Among 132 patients with NBL who were identified by MS and diagnosed at Saitama Children's Medical Center between April 1994 and March 2004, 45 patients met the following criteria and were enrolled in the observation program: (1) Evans stage I or II; (2) tumor diameter of <5 cm; (3) no intraspinal canal or great vessel invasion; (4) urinary vanillylmandelic acid (VMA) and homovanillic acid (HVA) levels of <50 μg/mg creatinine; and (5) informed consent obtained from guardians. All 12 cases reported in our short-term observations were further noted and included in our analysis.[4] Our workup included confirmation of elevated urine catecholamine metabolites, ultrasonography, computed tomography/magnetic resonance imaging (MRI), bone scintigraphy, metaiodobenzylguanidine scan, and bone marrow aspiration from at least one site. The cut-off values for VMA and HVA were 16.4 and 29.9 μg/mg creatinine, respectively.

To avoid invasive diagnostic procedures for patients with these potentially benign tumors, we diagnosed NBL without biopsies if they fulfilled the following 2 typical NBL clinical characteristics: tumor consistent with NBL radiologically showing metaiodobenzylguanidine uptake and elevated urine VMA and/or HVA levels. One patient with a stage III tumor was enrolled because the parents had declined surgery or chemotherapy for religious reasons. This patient's adrenal tumor disappeared at the age of 67 months. At follow-up, we measured VMA/HVA levels and used ultrasonography to evaluate the maximal diameter of the tumor's horizontal section. In patients with posterior mediastinal tumors, we used MRI to evaluate tumor volume. We used computed tomography/MRI to confirm tumor disappearance. During follow-up, tumor resection was performed in the following cases: tumor growth >5 cm in maximal diameter; continuous VMA/HVA elevation >50 μg/mg creatinine; physician's decision for surgery because of intensive tumor growth; or guardians' request. Patients were classified into the following 3 groups: group A, patients whose tumors disappeared during follow-up; group B, patients whose tumors remained detectable during follow-up; and group C, patients whose tumors were resected for any reason during follow-up.

Results

Patient characteristics are shown in the Table. The primary tumor site was the adrenal gland in 32 patients (71%), the retroperitoneum in 9 patients (20%), and the posterior mediastinum in 4 patients (9%). Overall, 17 cases were in group A (37%), 19 cases were in group B (42%), and 9 cases were in group C (20%). No significant differences

HVA	Homovanillic acid
MRI	Magnetic resonance imaging
MS	Mass screening
NBL	Neuroblastoma
VMA	Vanillylmandelic acid

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Table. Clinical findings and results of observation program of 45 infants with NBL detected through MS

	Group A, observed/ disappeared (n = 17)	Group B, observed/ detectable (n = 19)	Group C, resected (n = 9)		All patients (n = 45)
Patient characteristics (%)				*P**	
Sex				**.049**	
Male	7 (41)	10 (53)	8 (89)		25 (56)
Female	10 (59)	9 (47)	1 (11)		20 (44)
Tumor site				.458	
Adrenal gland	14 (82)	11 (58)	6 (67)		31 (69)
Retroperitoneum	2 (12)	5 (26)	3 (33)		10 (22)
Posterior mediastinum	1 (6)	3 (16)	0		4 (9)
Clinical variables (range)				*P*†	
Median age at presentation (mo)	7.2 (6.3-9.5)	7.5 (6.7-9.7)	7.4 (6.2-8.7)	.549	7.2 (6.2-9.7)
Median observation period (mo)	101 (35-139)	81 (4-184)	73 (56-126)	.274	81 (4-184)
Median tumor diameter at diagnosis (mm)	32 (19-49)	32 (19-51)	30 (24-46)	.408	30 (19-51)
Median VMA value at diagnosis (μg/mg Cre)	26.1 (15.7-39.5)	27.4 (11.5-55.8)	24.9 (17.2-48.2)	.529	26.1 (11.5-55.8)
Median HVA value at diagnosis (μg/mg Cre)	28.9 (20.0-45.0)	28.5 (17.5-59.7)	30.7 (18.5-40.8)	.878	28.9 (17.5-59.7)
Median age at VMA normalization (mo)	10 (8-15)	15 (7-39)	18 (10-39)	**.004**	12 (8-39)

Statistically significant values ($P < .05$) are in bold.
*Fisher exact test.
†Kruskal-Wallis test.

among the 3 groups were observed in terms of age, tumor diameter, or initial VMA/HVA levels (Table).

Among the 17 group A cases, the median ages of VMA normalization and tumor disappearance were 10 months (range, 8-15 months) and 64 months (range, 16-134 months), respectively. In group A, urinary VMA levels normalized earlier than in groups B or C (Table). After tumor disappearance, no relapse or VMA/HVA re-elevation was observed during the median follow-up period of 26 months (range, 0-108 months) (Figure 1, A).

Among the 19 group B cases, VMA levels normalized at the median age of 15 months (range, 7-39 months) despite continued tumor presence. Patients were followed to the median age of 80.5 months (range, 13-191 months), and no sign of metastasis or VMA/HVA re-elevation was observed (Figure 1, B). Despite the continuous presence of tumors, tumor-related complications or symptoms were not observed. The median size of the remaining tumors at last follow-up was 19 mm (range, 7-47 mm).

Among the 9 group C cases, the median age at resection was 21 months (range, 10-54 months). Resection was performed in 3 cases because of rapid tumor growth (Figure 1, C): 1 tumor grew beyond a diameter of 5 cm, and in the other 2 cases, the physician opted for resection because of rapid tumor growth. For the other 6 cases, the guardians requested resection, although the patients still

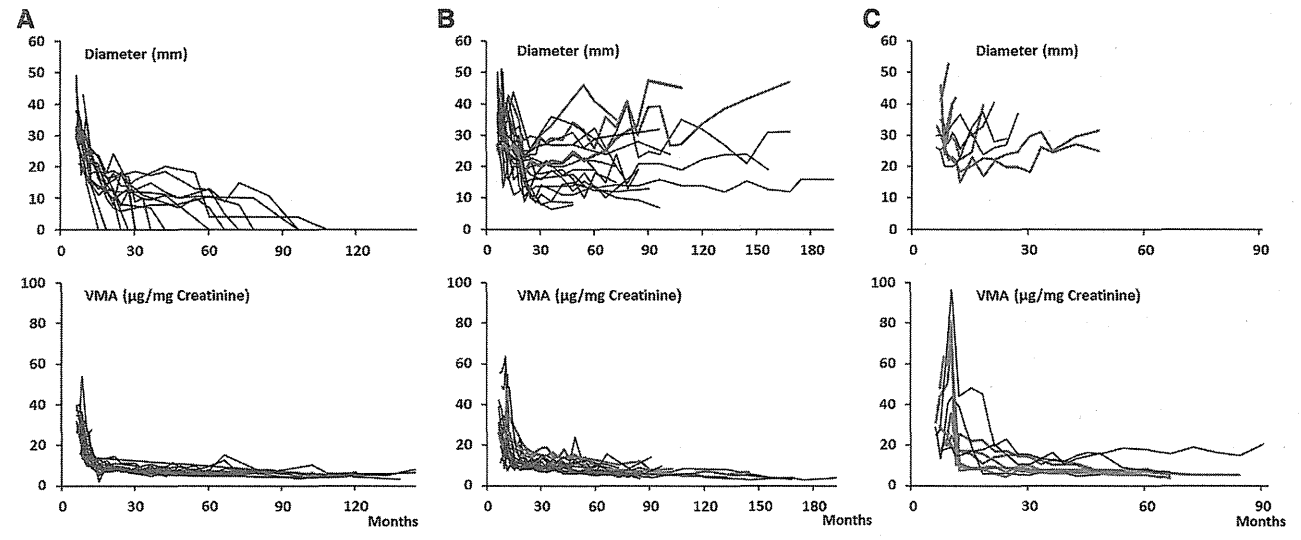

Figure 1. Maximal tumor diameter and VMA value trends in **A,** group A, **B,** group B, and **C,** group C. Tumors that gradually grew after temporal regression are shown with a *blue line* and tumors that were resected because of rapid tumor growth are shown with a *red line*.

fulfilled the criteria for observation only. Tumor histology showed a tendency to differentiation that correlated with the patients' age at resection (**Figure 2**; available at www.jpeds.com). Some tumors in groups B and C showed a gradual increase in size after temporal regression (**Figure 1**).

Discussion

Our follow-up results reaffirmed the benign characteristics of the NBLs detected by MS and indicated that these tumors are unlikely to undergo malignant transformation after regression. In our analysis, 2 different types of responses were observed: regressing and remaining tumors and tumors demonstrating an increase in size after temporal regression. Marachelian et al reported that regressing and maturing tumors presented different histologic characteristics.[9] In this report, cytoplasmic enlargement and proliferation of Schwann stroma were observed in some maturing tumors, which could explain tumor regrowth. Banelli et al showed that benign ganglioneuroma had notably different patterns of gene methylation compared with malignant NBL cells.[10] Thus, these 2 groups presumably have different biological characteristics, although we cannot draw conclusions because of the lack of genetic and histologic analysis in our patients.

Among the tumors resected, we observed histologic differentiation proportional to longer observation period and increasing age of the patients, which is consistent with previous reports.[5,7,11,12] Although many of these tumors had to be resected upon guardians' requests, we believe that the characteristics of these tumors were not substantially different from benign tumors in groups A and B (except 3 tumors showing rapid growth). Thus, this differentiation reflects the regression/maturation process and the benign characteristics of these tumors and strongly supports our initial hypothesis that majority of these tumors could have been safely observed without intervention.

Our long-term follow-up, together with other reports of observation programs,[5-8] supports the hypothesis that most NBLs identified during MS are not related to the progressive and malignant NBL tumors detected at older ages. Thus, MS at 6 months of age could not decrease the population-based incidence of advanced-stage disease or NBL-associated mortality, and cessation of MS as a national health project was appropriate.

It is highly plausible that clinically detected NBLs in infants include a considerable proportion of benign tumors that regress without treatment, and some researchers have reported their prospective study results that did not involve any treatment for selected infant patients with NBL.[12,13] To establish an observation-only strategy in these patients, more attempts should be explored in a prospective trial. ■

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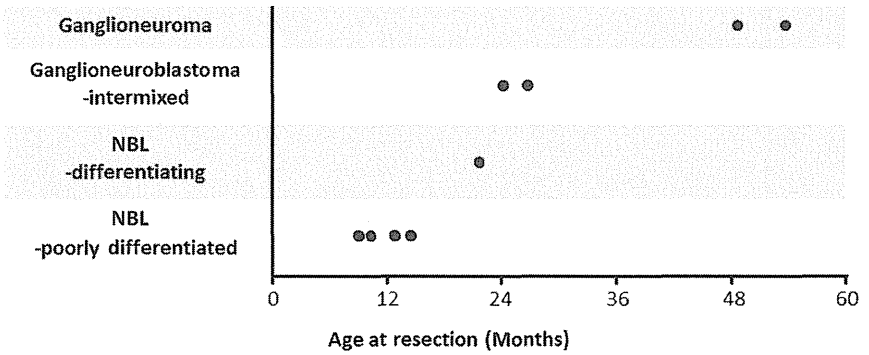

Figure 2. Correlation between histology of the resected tumors and age at surgery.

臨床試験デザイン

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京都府立医科大学大学院医学研究科生物統計学

Clinical Trial Design

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抄 録

臨床試験はデザイン・計画の段階から始まり，試験実施・データ管理・モニタリングを経て，データ解析・報告書作成に至る．この各ステップが統計的方法を必要としている．再現性によって結果を保証することが可能な基礎実験と異なり，同じデザインで繰り返すことが困難な臨床試験においては，デザインと手続きの妥当性から結果を保証するしかない．計画段階から生物統計学の専門家が参画していれば，質の高い臨床試験を実施できる可能性は高い．近年，臨床試験にベイズ流の方法が有用であるという報告は着実に増えている．効率的かつ倫理的な試験デザインの開発は，資源を有効に活用するという観点から今後ますます重要になるであろう．本稿では，ランダム化対照試験の標準的方法，および探索的臨床試験のデザインとして有用であるベイズ流の方法について述べる．

キーワード：技術評価，統計的仮説，ランダム化，統計的考察，ベイズ流統計学．

Abstract

Every clinical trial starts from the design and planning stage, moves to trial conduct, data management and monitoring, and finally to the data analysis and conclusions. Each step along the way calls for statistical methods. While basic research which can be guaranteed a result by reproducibility, clinical trials which cannot be repeated with the same design must be guaranteed by the validity of design and procedure. If a biostatistician take part in the planning stage, we can do the high quality of clinical trials. In recent years, reports insisting on the usefulness of Bayesian statistics in clinical studies have steadily increased. The development of efficient and ethical design will become important in the future, from the viewpoint of the best use of resources. In this paper, I will describe the standard methods for randomized clinical trials, and Bayesian methods which could be useful for the design of exploratory clinical trials.

Key Words: Technology assessment, Statistical hypothesis, Randomization, Statistical consideration, Bayesian statistics.

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はじめに

臨床試験はデザイン・計画の段階から始まり，試験実施・データ管理・モニタリングを経て，データ解析・報告書作成に至る．この各ステップが統計的方法を必要としている．臨床試験は，良いデザインおよび正しい遂行がなければ，台無し（例えば，結論の出ないまたは誤った知見を導く）になり，さらには大惨事（例えば，不必要に多数の患者が毒性または死を被ることを引き起こす）になる可能性さえある．臨床試験は効率的かつ倫理的であるべきで，資源を節約し，より多くの患者に恩恵を与え，より迅速に正しい結論を引き出し，結果として不必要な毒性をより少なくすべきである[1]．本稿では，ランダム化対照試験の標準的方法，および探索的臨床試験のデザインとして有用であるベイズ流（Bayesian）の方法について述べる．

医学・医療と技術評価

医学は普遍性のある真実を追求する科学の一分野である一方，医療は多様性のある個人に対して最適な技術を選択して適用することが要求される場である．技術評価は，主に統計学に基づく科学的方法を駆使して医療技術を相対的に評価し，医学から医療への橋渡しを行う（図1）．

統計学を医学・医療の領域に導入する際には，2つの大きなギャップを認識しておく必要がある．1つは，「決定論」と「非決定論（確率論）」のギャップである．1800年代半ばにクロード・ベルナールが「統計学に立脚している限り，医学は永久に推測科学に止まるであろう」と決定論的な考え方を主張して以来，医学の世界では決定論的な思想が支配的である．もう1つのギャップは，意思決定の主体に関わる問題であり，「対集団の確率」と「対個人の確率」とのギャップである．たとえば，ある医薬品を承認すべきかどうかという判断は，その国の人々という集団に対するベネフィットとリスクのバランスで決定される．その決定は「対集団の確率」に基づく一方，医療の場で診断や治療を行う際には，個人に対するベネフィットとリスクを評価しなければならない．たとえば，胎児診断を行って，医師が「胎児に異常がある確率は80%」と言ったとき，その80%は集団での頻度に基づいたものである．しかしながら，それを聞いた母親の「子供には異常があるか（100%），ないか（0%）のどちらか」という感覚で，この確率を解釈することはそれほど容易ではない．これは「確率とは何か」という哲学的課題につながっている．

臨床試験は，20世紀を代表する英国の統計学

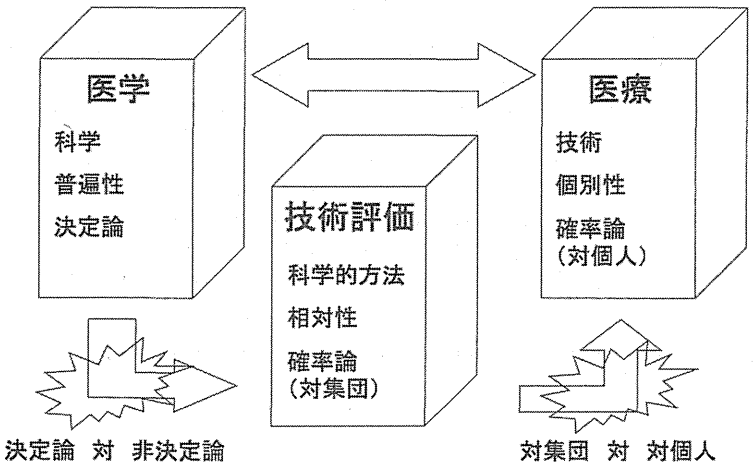

図1 医学・医療と技術評価

者R.A.フィッシャー（1890～1962）が創始した統計的実験（技術的実験とも呼ばれる）の方法論を基礎としている．科学的実験は，人工的に作り出された純粋な条件のもとでの因果関係を確定しようとするのに対して，統計的実験は以下の特徴を有する[2]．

・実験の場は，現実の応用の場に近い状況に設定される
・結果の分析には誤差の存在を前提にしなければならない
・いくつかの因子を同時に変化させて結果を見る必要があることがある
・目的は，何らかの基準によって現実の場において最も良い結果が得られるような条件を求めることである

様々な種類の誤差を伴うデータを扱うためには統計的方法が不可欠である．また，再現性によって結果を保証することが可能な基礎研究と異なり，同じデザインで繰り返すことが困難な臨床試験においては，デザインと手続きの妥当性から結果を保証するしかない．

探索的試験と検証的試験

臨床試験の性格は検証的試験と探索的試験に大別される．検証的試験は有効性または安全性の確固たる証拠を提示するための試験と位置付けられる．ただし，個々のいかなる試験も検証的側面と探索的側面の両方を持つ．試験実施計画書（以下，プロトコル）には，各試験について検証的な証明として用いられる側面と，探索的解析のためにデータを提供する側面とを，明確に区別しておくべきである[3]．探索的解析から得られた結果は仮説に過ぎず，その仮説は検証的試験によって確認しなければならない．

統計的仮説

統計的仮説の代表的なものは，優越性仮説と非劣性仮説である．優越性仮説を証明しようとする試験（優越性試験）とは，試験治療の効果が対照治療（活性対照またはプラセボ対照）よりも「臨床的に優れること」を示すことが目的の試験である．一方，非劣性仮説を証明しようとする試験（非劣性試験）とは，試験治療の効果が対照治療よりも「臨床的に劣らないこと」を示すことが目的の試験である．多くの場合は優越性仮説が設定されるが，対照治療（通常，活性対照）に比べて安全性あるいは経済性に優れていることが見込まれる場合に，このような非劣性仮説が許容されることがある．

非劣性試験を計画する際には，非劣性マージン（臨床的に意味のある最小の差：Δ）の決定，データの質などについて十分な注意が必要である．ハザード比を治療効果の尺度とした臨床試験の場合，優越性試験では，ハザード比の95%信頼区間の上限が1より小さければ，有意水準5%で試験治療が優れると判断される．一方，非劣性試験では，その95%信頼区間の上限が$1+\Delta$よりも小さければ，有意水準5%で試験治療は対照治療にΔ以上は劣らない，と判断される（図2）．

ランダム化

ランダム化対照試験では，登録の際にランダム化という操作が必要になる（図3）．ランダム化を行うことにより，試験群と対照群の比較可能性（内的妥当性とも呼ばれる）が保証される．ランダム化は「実験計画法」を確立したフィッシャーの偉大な発明の一つである．実験に伴う誤差には以下の2種類がある．ちなみに，フィッシャーは臨床試験ではなく，農事実験に従事していた．

・偶然誤差…測定誤差のようにある確率分布に従うと想定できる誤差であり，繰り返し測定を行えばその大きさについて推定可能である
・系統誤差またはバイアス…圃場の肥沃度や日当たりの不均一性のように確率変動と見なせない誤差であり，繰り返しには関係なく結果を歪める原因となる

ランダム化の目的は，一言で言うと「系統誤差を偶然誤差に転化すること」である．臨床試験におけるその意義は，

・予後因子が既知か未知かにかかわらず，予後因子の分布が類似したグループを作る

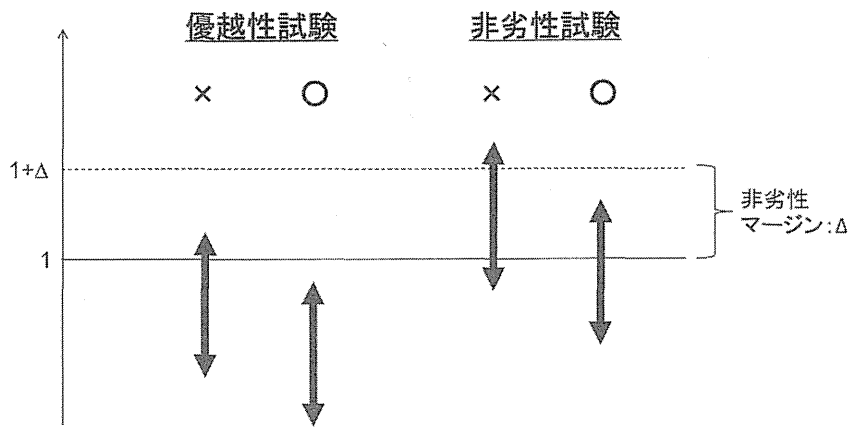

図2 優越性試験と非劣性試験の判断規準

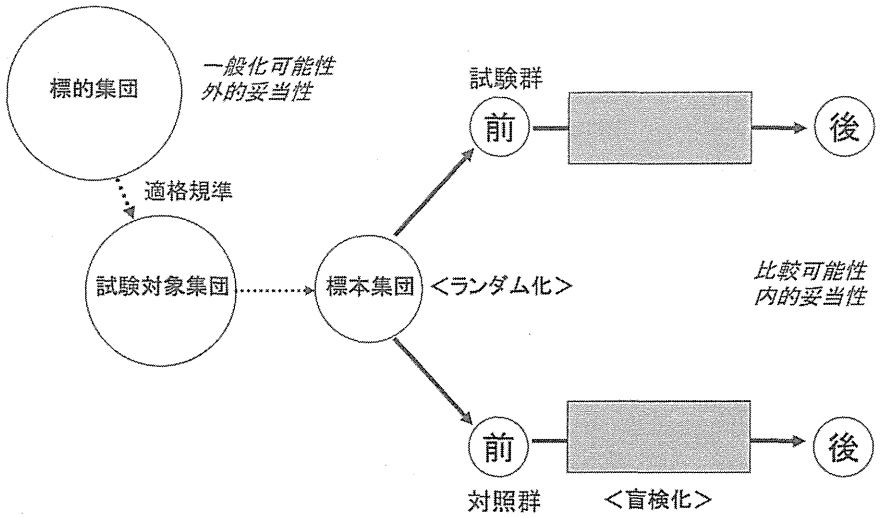

図3 ランダム化対照試験

・データ解析の際に治療効果の定量的な評価のための正しい統計的根拠を与えることである[3].

臨床試験で利用されるランダム化の方法は，固定的割付（単純ランダム化，並べ替えブロックランダム化など）と適応的割付（偏コインデザイン，最小化法など）とに分類される．重要な予後因子の分布を治療群間で均等にするためには，それらの因子で層を作り，その層ごとにランダム化（層別ランダム化）を行う必要がある．ランダム化の方法には多くの選択肢があり，試験統計家がそれぞれの利点と欠点を考慮して決定すべきである．

統 計 的 考 察

試験統計家がプロトコルに記載すべき統計的事項として，「標本サイズの設定根拠」，「解析対象集団」，「解析項目・方法」，「中間モニタリン

グ」がある．

1．標本サイズの設定根拠

臨床試験に参加していただく対象数（標本サイズ）は科学性，倫理性，実施可能性のバランスを考慮して決める必要があり，多すぎても少なすぎてもいけない．標本サイズは主要評価項目に関する情報に基づいて計算される．その時点における情報を最大限利用するものの，一時的な仮定に基づく概算であることに注意が必要である．例えば，ある癌の補助化学療法の臨床試験において，標準治療を受ける患者の5年生存率を予測する場合，利用できる情報にはかなり大きなばらつきがある．これらの前提条件を慎重に検討した上で，通常は仮説検定に基づく決定方式に従って，帰無仮説，対立仮説，検定統計量，有意水準，第Ⅰ種の過誤，第Ⅱ種の過誤または検出力などが試験の性格（探索的または検証的）を考慮して決定される．

2．解析対象集団

解析対象集団は，ITT（intention-to-treat）の原則に従って定義すべきである．これは，「被験者が実際に受けた治療ではなく，被験者を治療しようとした意図（intention to treat）に基づいて評価する」という原則である[3]．従って，登録されたすべての被験者を解析対象とすることが原則であるが，登録後に判明した不適格例，試験治療を全く受けなかった例を対象から除くことは一般的に許容される．いずれにしても，有効性および安全性に関する主要な解析対象集団の定義をプロトコルに明記し，報告時には解析対象から除外した対象数とその理由を明記する必要がある．

全体集団のある一部のグループを対象とした解析をサブグループ解析（サブセット解析，部分集団解析）と呼ぶ．通常，患者特性やベースライン情報に基づいてサブグループ化を行う．「試験治療を完遂した集団」のように介入後の情報に基づいて対象を選択する場合には，比較可能性など別の問題が生じるため，サブグループ解析とは区別しておく必要がある．ランダム化の際に層別に用いた因子によるサブグループ解析は，ランダム化に基づく比較可能性の条件を満たしている．そうでない場合は比較可能性の条件を満たさない．ただ，その条件を無視したとしても，多数の検定の実施により第1種の過誤確率が上昇すること，一方ではサブグループ内の標本サイズ不足により検出力が低下することが問題となる．対応策としては，1）関心のある少数のサブグループを事前にプロトコルに記載する，および2）交互作用の検定が有意な場合にのみサブグループでの検定を行うことが推奨されている．交互作用には，量的交互作用と質的交互作用の2種類があり，交互作用が検出されたときは有意差の有無だけでなく，医学的な意義と解釈について十分な吟味が必要である．いずれにしても，サブグループ解析は，探索的解析の代表的なものであり，その目的は仮説の生成である．

3．解析項目・方法

臨床試験で利用される標準的な統計解析手法について表1に示す．ランダム化対照試験では，ランダム化によって比較可能性が保証されているので，通常は観察研究のように複雑な回帰モデルを用いて交絡因子を調整する必要はない．

4．中間モニタリング

中間モニタリング（中間解析，中間評価とも呼ばれる）の目的は，

- 試験治療の優越性が疑いなく立証された場合
- 適切な試験治療の差を示す見込みがないことが判明した場合
- 許容できない有害事象が明らかになった場合

に試験を早期に中止することである[3]．検定の多重性を考慮した中止規則の設定には多くの方法（グループ逐次法など）が開発されてきているが，実際に臨床試験を中止すべきかどうかという判断は純粋に統計的な問題ではなく，臨床的のみならず社会的な影響も考慮する必要がある．そのような判断を公正に行う場として，中間モニタリングを実施する際には，当該臨床試験に関与しない第三者からなるデータモニタリング委員会（効果安全性評価委員会とも呼ばれ

表1 変数の型別の標準的な統計解析手法

目的	連続変数	分類変数	時間-イベント変数
分布の記述	ヒストグラム、箱ヒゲ図、散布図	ヒストグラム、分割表	生存曲線（Kaplan-Meier法）
要約統計量	平均、分散、中央値、パーセント点、相関係数	頻度、一致度、相関係数	x年生存率、中央生存期間
検定（単純）	t検定、分散分析、Wilcoxon検定	χ^2検定、Fisher正確検定	ログランク検定
検定（層別）	共分散分析	Mantel-Haenszel検定	層別ログランク検定
回帰モデル	重回帰分析	ロジスティック回帰分析	Cox回帰分析

る）を設置しなければならない.

探索的臨床試験のデザイン —ベイズ流の方法

1950年頃に臨床試験の方法論がほぼ確立して以来，統計的評価の方法として，フィッシャーあるいはネイマン・ピアソンによる頻度流（frequentist）の仮説検定・推定が主に用いられてきた．データ解析へのベイズ流統計学の適用は，物理学をはじめとする多くの自然科学分野および社会科学分野で広く行われており，医学・生物学分野においても，ベイズ流の統計モデルを適用したデータ解析の事例は多く存在する．しかしながら，臨床試験のデザインにベイズ流の方法を適用した事例としては，抗がん剤の第Ⅰ相試験（最大耐用量を決定するための試験）でのCRM（continual reassessment method），ランダム化試験の中間解析でのベイズ流予測確率の利用などがあるが，未だそれほど多くない.

大学等の研究機関が主体となって実施するトランスレーショナルリサーチおよび臨床試験の対象疾患は，難治性かつ重篤であり，そのうえ患者数が限られているという特徴がある．このような状況では，基礎研究で認められたコンセプトを実証するためのPOC（proof of concept）試験と呼ばれる探索的試験の実施が主であり，疾患の重篤性などを考えると同時対照を設定すること自体困難な場合が多い．また，被験者のリスクを最小にするために臨床試験の途中で結果をモニタリングしながら意思決定を行うというような柔軟な対応も必要である．さらに，被験者数を最小にするために，過去に得られた証拠や情報（事前情報）を十分に生かすことも重要となる．これらを鑑みると，予期しない事態が発生して試験途中でデザイン（標本サイズや中間モニタリングの時期・方法など）の変更を行う場合などに，頻度流接近法に基づく方法は柔軟性の観点から不十分であり，新しい方法の開発が必要となる．近年，ベイズ流接近法は柔軟性と効率性の面から有望と考えられている．臨床試験におけるベイズ流接近法の主な特長は以下の通りである.

① 解釈が容易な「確率」だけを用いて整合性のある推測と意思決定を行うことができる
② 標本サイズに関わらず事前分布を事後分布に更新して推測ができる
③ 予測分布を用いて試験結果を予測することができる

また，ベイズ流デザインの動作特性が頻度流に評価できることも1つの利点である.

ベイズ流デザインの例

すべての被験者に同一の試験治療を行う単群臨床試験は，探索的な臨床試験の大部分を占めている．その主目的は，治療効果に対する確定的な証拠を得ることではなく，さらに研究を継続すべき有望な治療をスクリーニングすることである．単群臨床試験デザインの多くは，効果が認められない場合は試験を早期中止することが望ましい致死的な疾患の領域で開発されてきた．抗がん剤の第Ⅱ相単群臨床試験については，1960年代から頻度流の方法が開発され，1990年代以降ベイズ流接近法を用いたデザインがいくつか提案されている．その中には，効用／損失関数を明示的に用いるベイズ流決定理論に基づく手法も含まれる．

ここで，被験者20名にある試験治療を行い，14名に「効果あり（成功）」，6名に「効果なし（失敗）」という結果が得られた，二値（成功または失敗）評価項目の単群臨床試験という単純な事例を用いてベイズ流の方法を概説する．まず，事前情報が存在しないと仮定し，事前分布を一様分布（Beta(1,1)と表現されるベータ分布）と定める．次に，ベイズの定理を用いて，事前分布と観察データ（実際には尤度［ゆうど］と呼ばれる形に変換されたもの）を結合し，事後分布Beta(15,7)（＝Beta (1＋14, 1＋6)）を得る（図4）．このように更新された事後分布から，この治療の成功確率の平均は0.68(＝15/(15＋7))，成功確率が0.5以下の確率は分布下面積から0.039と得られる．この結果から，引き続いて5名の被験者に同じ治療を行ったときに何名の被験者に成功が観察されるかという予測分布を得ることもできる（図5）．

お　わ　り　に

フィッシャーは1938年に次のように述べている：「同じだけの時間と労力をかけたとしてもデータ収集の過程，または実験計画を厳密に検討しているか否かによって，得られる収穫は10倍から12倍にもなる．実験終了後に統計学者に相談を持ちかけるのは，統計学者に，単に死後診察を行って下さいと頼むようなものである．統計学者はおそらく何が原因で実験が失敗したかという実験の死因について意見を述べてくれるだけであろう」[4]．

臨床試験に統計的方法は必須であり，計画段階から生物統計学の専門家が参画していれば，

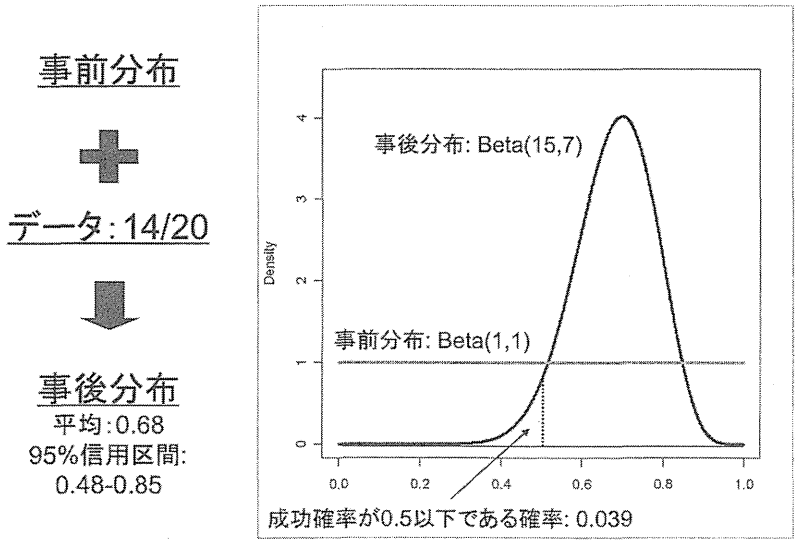

図4　事前分布から事後分布へ

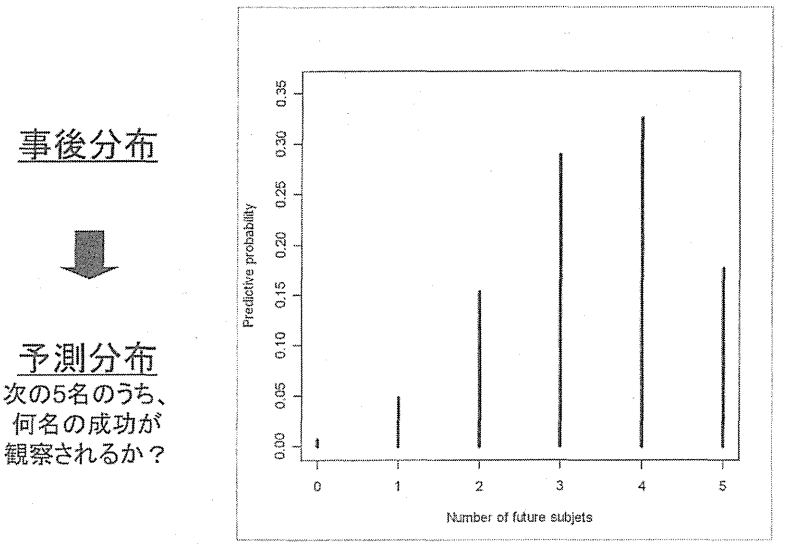

図5 事後分布から予測分布へ

質の高い臨床試験を実施できる可能性は高い．近年，臨床試験にベイズ流の方法が有用であるという報告は着実に増えている．効率的かつ倫理的な試験デザインの開発は，資源を有効に活用するという観点からも今後ますます重要になるであろう．

開示すべき潜在的利益相反状態はない．

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RASSF1A methylation may have two biological roles in neuroblastoma tumorigenesis depending on the ploidy status and age of patients ☆

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ABSTRACT

RASSF1A methylation was frequent in neuroblastomas found in infants by mass-screening or infants and children diagnosed clinically, whereas *CASP8* and *DCR2* methylation was only frequent in tumors in children. When classified according to the ploidy status, *RASSF1A* and *PCDHB* methylation was only associated with *MYCN* amplification and poor outcomes in infants with a clinically diagnosed diploid, not triploid tumor. *RASSF1A* and *PCDHB* methylation was associated with poor outcomes in children with triploid and diploid tumors, respectively, and with *MYCN* amplification in children with diploid tumor. *RASSF1A* methylation may have two biological roles based on the ploidy status and patient's age.

1. Introduction

Neuroblastoma is the most common solid tumor in children, and accounts for 8–10% of childhood cancers and 15% of childhood cancer deaths [1]. While localized neuroblastomas in infants regress spontaneously or mature, disseminated tumors in children resist intensive multimodal treatment [2,3]. A mass-screening program has been conducted on infants in Japan and other countries, based on the assumption that the early detection of tumors in infants could improve overall outcomes [4–6]. Because it is clear that damage has been caused by excessive treatment of some neuroblastomas that would have regressed spontaneously, and the effectiveness of the program has been questioned [5–7], this program was discontinued in Japan.

RASSF1A functions as a tumor suppressor gene that plays an important role in cell cycle arrest, apoptosis, genomic stability, microtubule stabilization, and cell motility [8–10]. The mRNA expression levels of *RASSF1A* are controlled by DNA methylation in the promoter region, and it is a representative gene that shows hypermethylation in various primary tumors [8,11–24]. Caspase 8 encoded by *CASP8* is a family member of cysteine proteases that play essential roles in apoptosis, and silencing of *CASP8* by methylation has frequently been found in neuroblastomas, especially those with *MYCN* amplification [14–19,21,22,24–28]. DCR2 has been shown to prevent binding of TNF-related apoptosis-inducing ligand (TRAIL) to the death receptors, DR4 and DR5 as a decoy receptor, and exhibits antiapoptotic activity. The down-regulation of *DCR2* by promoter methylation was reported in various types of cancer including neuroblastoma [29,30]. In addition, the CpG island methylator phenotype (CIMP) was shown to have stronger prognostic power than methylation of individual genes in neuroblastomas; CIMP was detected by methylation analysis of the *PCDHB* CGIs [31].

Tumor cell ploidy is one of the biomarkers that predicts outcomes of patients with neuroblastoma. The majority of tumors found by mass-screening have been characterized by triploidy [32]. The International Neuroblastoma Risk Group (INRG) classification system used ploidy (DNA index) to classify tumors with distant metastasis and less than 18 months of age [33].

Many studies have examined the methylation status of tumor suppressor genes in neuroblastomas; however, none have clarified the association between methylation of the genes and the subtypes of tumors classified by age, method for tumor detection, or the ploidy status, although the disease is well-known for its biological heterogeneity [14–28]. We found that *RASSF1A*, *CASP8*, *DCR2*, and *PCDHB* family were significantly methylated, and associated with clinical and *MYCN* status. When tumors were classified according

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to the ploidy status, *RASSF1A* and *DCR2* methylation was associated with poor outcomes in infants with a diploid, not triploid tumor, and in children with a triploid, not diploid tumor, suggesting age-dependent heterogeneity in triploid tumors.

2. Material and methods

2.1. Patients and samples

Tumors were obtained from 259 Japanese infants or children with neuroblastoma who underwent biopsy or surgery between January 1985 and December 1998. One-hundred and twenty-three patients were found by mass-screening to have neuroblastomas at 6 months of age (group A1) and 64 patients at 18 months of age or less (group A2), and 72 children over 18 months (group B) were diagnosed clinically.

2.2. Ploidy determined by interphase FISH and flow cytometry

Pathologists in each institution verified that each sample contained 70% or more tumor cells. To detect the copy number of chromosome 1s and the status of 1p, two-color FISH was performed using the two probes, D1Z1 and D1Z2, as described previously [34]. Disomy 1, trisomy 1, tetrasomy 1, or pentasomy 1 was determined based on the number of D1Z1 signals. The DNA index of tumor tissues was analyzed on the Becton–Dickinson FACScan flow cytometer by DNA cell-cycle analysis software-version C. Tumors were classified into 2 types (diploid, 2n and triploid, 3n) based on the numbers of chromosome 1 or the DNA index obtained by flow cytometry. Triploidy included triploidy and hyperdiploidy determined by flow cytometry, and tumors with a combination of cells with 3, 4, and 5 chromosomes 1 examined by FISH were classified as triploid (3n) tumors.

2.3. Sodium bisulfite modification and conventional methylation-specific PCR (MSP) analysis

Bisulfite treatment was performed as previously described [11,35]. The genes examined were *RASSF1A*, *CASP8*, *DCR2*, *HOXA9*, *RUNX3*, *NORE1A*, *p16INK4A*, *p14ARF*, *RASSF2A*, *SOCS1*, *RIZ1*, and *HOXB5*. Primer sequences and PCR conditions were described in a previous study [11]. PCR products were run on 2% agarose gels and visualized after staining with ethidium bromide.

2.4. Quantitative MSP analyses of RASSF1A, DCR2, and PCDHB family

Bisulfite-modified DNA was used as a template for TaqMan- or SYBR green I-based real-time PCR using a LightCycler (Roche Diagnostics), as described previously [11]. Primers and probes used to specifically amplify bisulfite-converted DNA for the internal reference gene (*ACTB*) and target genes (*RASSF1A*, *DCR2*, and *PCDHB family*) were described in Supplementary Table 1 [11,31]. Each amplification reaction included positive and negative controls for the methylation status of target genes, and tumor DNA samples with the bisulfite treatment. *ACTB* was used as a reference gene to determine the relative level of methylated DNA for one of the target genes in each sample.

We failed to detect quantitative PCR products using PCR primers and a probe for the exon 4 region of *CASP8*, from which PCR primer sequences for conventional PCR were obtained, probably because of low CpG contents of the region.

2.5. MYCN amplification analysis

DNA preparation, digestion, and Southern blot analysis using the *MYCN* probe were performed as described previously [34]. More than 3 copies of the *MYCN* gene per haploid genome were considered to indicate amplification.

2.6. Statistical analysis

The significance of 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 Student's *t* test with or without Welch's correction compared the mean percentages of *RASSF1A*, *DCR2*, or *PCDHB* methylation between two types of tumors with or without *MYCN* amplification or any two ploidy groups classified by the age of patients and the method of tumor detection. The overall survival 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. The influence of various biological and clinical factors on overall survival was estimated using the Cox proportional-hazards model calculated with Stat Flex software for Windows, version 6.0.

3. Results

3.1. Conventional and quantitative MSP analysis

The methylation status of *RASSF1A*, *CASP8*, *DCR2*, *HOXA9*, *RUNX3*, *NORE1A*, *p16INK4A*, *p14ARF*, *RASSF2A*, *SOCS1*, *RIZ1*, and *HOXB5* was examined using conventional MSP. Conventional MSP analysis of the *RASSF1A*, *CASP8*, and *DCR2* genes was performed in 259 neuroblastomas [123 found by mass-screening (group A1) and 136 found clinically (groups A2 + B)], and of the other 9 genes in 45 tumors (25 found by mass-screening and 20 diagnosed clinically). *RASSF1A*, *CASP8*, and *DCR2* were methylated in 57.7%, 3.3%, and 3.3% of 123 neuroblastomas found by mass-screening, in 51.6%, 10.9%, and 1.6% of 64 tumors diagnosed clinically (<18 months), and in 70.8%, 40.3%, and 38.9% of 72 tumors diagnosed clinically (>18 months) (Supplementary Tables 2–4). None of the 9 other genes (*HOXA9*, *RUNX3*, *NORE1A*, *p16INK4A*, *p14ARF*, *RASSF2A*, *SOCS1*, *RIZ1*, and *HOXB5*) were methylated in the 45 tumors.

Quantitative MSP analysis of *RASSF1A*, *DCR2*, and *PCDHB* methylation was carried out in 221 (85.3%), 116 (44.8%), and 116 (44.8%), respectively, of 259 neuroblastomas. Group A1 was included in *RASSF1A* analysis, and excluded from *DCR2* and *PCDHB* analysis. We performed ROC analysis, and determined cut-off values of 26%, 7%, and 18% of *RASSF1A*, *DCR2*, and *PCDHB* methylation (Fig. 1, A, B, and C). We then examined the dose–response relationship between percentages of *RASSF1A*, *DCR2* and *PCDHB* methylation (10%, 20%, 30%, 40%, 50%, 60%, 70% and 80%) and overall survival, and adopted the cut-off value of 40%, 70%, and 60%, respectively, which gave the highest HR (Fig. 1D–F). Although cut-off values were determined based on data of overall survival, they were also used for association analysis between gene methylation and clinical and *MYCN* features.

3.2. Correlation between RASSF1A, CASP8, DCR2, and PCDHB methylation and stage of the disease

We found no significant difference in stage distribution between *RASSF1A*-, *CASP8*-, and *DCR2*-methylated tumors and *RASSF1A*-, *CASP8*-, and *DCR2*-unmethylated tumors, respectively, determined by conventional MSP and found by mass-screening with an exception of *RASSF1A*-methylated diploid tumors (Table 1). *RASSF1A*-methylated tumors were at more advanced stages than *RASSF1A*-unmethylated tumors in infants and children diagnosed clinically ($P = 0.018$ and $P = 5.49E{-}05$). Quantitative MSP analysis confirmed the association. *CASP8*- and *DCR2*-methylated tumors were at a more advanced stage than *CASP8*- and *DCR2*-unmethylated tumors in children ($P = 0.026$ and $P = 5.06E{-}05$), however, such an association was not detected in tumors in infants diagnosed clinically. Quantitative MSP analysis of *DCR2* confirmed the association between the methylation and an advanced stage in children, but not in infants. The similar association was also found between *PCDHB*-methylated and –unmethylated tumors in children.

When tumors were further classified according to the ploidy status, *RASSF1A*-methylated diploid tumors were or were more likely to be a more advanced stage than *RASSF1A*-unmethylated diploid tumors in infants found by mass-screening ($P = 0.029$) or clinically diagnosed (<18 months) ($P = 0.052$) and in children (>18 months) ($P = 0.006$) (Table 1). *RASSF1A*-methylated triploid tumors in children were at a more advanced stage than *RASSF1A*-unmethylated triploid tumors in children ($P = 3.12E{-}03$), but not in infants found by mass-screening or clinically diagnosed. Quantitative MSP could not confirm the association in children.

Children with *CASP8*-methylated tumors were at a more advanced stage than those with *CASP8* unmethylated tumors in children ($P = 0.026$). *DCR2*-methylated diploid and triploid tumors in

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