ン・ドキソルビシンなどから2-4剤を選択し，それらを組み合わせて併用する種々の化学療法計画が考案され実施されている．また大量化学療法としては，現在主として行われているのはメルファラン＋エトポシド＋カルボプラチンの組み合わせによるMEC療法(CEM療法)やブスルファン＋メルファランの組み合わせによるBU/Mel療法などである．TBIについては議論の多い部分であるが，近年ではTBIによる腎障害，間質性肺炎，肝中心静脈閉塞症などの急性毒性と，成長障害，内分泌障害，白内障，二次がんなどの晩期毒性の重大性から，生存者の生活の質も重視したnon-TBIによる治療開発が世界的に進行している[1)]．

これらの高リスク神経芽腫に対する世界各国における標準的治療戦略による臨床試験成績はほぼ類似している．米国のCCG-A 3891臨床試験の大量化学療法群のデータでは，3年無増悪生存割合は34±4%で，非大量化学療法群では22±4%である[11)]．またドイツ及びスイスのNB97臨床試験の大量化学療法群のデータでは，3年無増悪生存割合は47%(95% CI38-55)で，非大量化学療法群では31%(95% CI 23-39)である[12)]．一方，日本から報告される3年無増悪生存割合は大体30～50%の範囲にある[13)]．

神経芽腫を含め発症数の少ない稀少疾患である小児固形腫瘍はいずれも未だ治療法開発段階であり，常に整備された前方視的な多施設共同臨床研究に従い治療が行われることが望ましいと考えられる．小児固形腫瘍は症例数が少なく，診断が不確実になることも多いため中央病理診断の必要性がある．各施設での個々の経験に基づいた治療では症例数が少なすぎて確かなデータが得られず，しかもデータ管理が不十分となりやすい．治療成績の向上には多施設臨床研究が不可欠である．

進行神経芽腫の再発例では再発後の予後はさらに悪い．現在でも非常に難治であり，再発後の長期生存例は少ない．新規薬剤や新規治療戦略の開発が望まれている．

現在世界的に期待されている新規薬剤・治療法には Topotecan, Irinotecan, Fenretinide, 131I-MIBG＋ASCT, Tandem Auto SCT, Allo SCT, Anti-GD2 monoclonal antibody 3F8＋GM－CSF, Demethylating agent(Decitabine)などがあげられる[1)]．

XI　治療終了後の長期的な問題点

治療終了後の長期的な問題点も最近クローズアップされるようになり，重大な課題として再認識されている．これらには副鼻腔炎，低身長，やせ，肥満，歯牙の崩出障害，甲状腺機能低下症，性腺機能障害，不妊症，毛髪，難聴，白内障，腎機能障害，心機能障害，呼吸器系の障害，学校に関連する問題，精神神経的問題，二次がんなどの種々の問題点がある[1)]．

XII　結　　語

本稿では，神経芽腫についての基礎的知識の概略と，進行神経芽腫に対する化学療法についての知見を紹介した．

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SPECIAL ARTICLE

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The International Neuroblastoma Risk Group (INRG) Classification System: An INRG Task Force Report

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ABSTRACT

Purpose

Because current approaches to risk classification and treatment stratification for children with neuroblastoma (NB) vary greatly throughout the world, it is difficult to directly compare risk-based clinical trials. The International Neuroblastoma Risk Group (INRG) classification system was developed to establish a consensus approach for pretreatment risk stratification.

Patients and Methods

The statistical and clinical significance of 13 potential prognostic factors were analyzed in a cohort of 8,800 children diagnosed with NB between 1990 and 2002 from North America and Australia (Children's Oncology Group), Europe (International Society of Pediatric Oncology Europe Neuroblastoma Group and German Pediatric Oncology and Hematology Group), and Japan. Survival tree regression analyses using event-free survival (EFS) as the primary end point were performed to test the prognostic significance of the 13 factors.

Results

Stage, age, histologic category, grade of tumor differentiation, the status of the *MYCN* oncogene, chromosome 11q status, and DNA ploidy were the most highly statistically significant and clinically relevant factors. A new staging system (INRG Staging System) based on clinical criteria and tumor imaging was developed for the INRG Classification System. The optimal age cutoff was determined to be between 15 and 19 months, and 18 months was selected for the classification system. Sixteen pretreatment groups were defined on the basis of clinical criteria and statistically significantly different EFS of the cohort stratified by the INRG criteria. Patients with 5-year EFS more than 85%, more than 75% to ≤ 85%, ≥ 50% to ≤ 75%, or less than 50% were classified as very low risk, low risk, intermediate risk, or high risk, respectively.

Conclusion

By defining homogenous pretreatment patient cohorts, the INRG classification system will greatly facilitate the comparison of risk-based clinical trials conducted in different regions of the world and the development of international collaborative studies.

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INTRODUCTION

Neuroblastoma (NB) is remarkable for its broad spectrum of clinical behavior, with some tumors regressing or maturing, whereas others progress despite intensive multimodality treatment.[1,2] This diversity in behavior correlates closely with a number of clinical and biologic features,[2] and combinations of prognostic variables are used for risk-group assignment and treatment stratification. However, the factors selected by various cooperative groups to define risk are not uniform. For example, the International Society of Pediatric Oncology Europe Neuroblastoma Group (SIOPEN) uses age, surgical risk factors defined by imaging, and *MYCN* status for risk-group assignment of locoregional tumors, whereas the Children's Oncology Group (COG) uses age, postsurgical staging, *MYCN* amplification, histology, and DNA ploidy.[3,4] Furthermore, the increasing number of genetic features included in more recently developed clinical trials to guide therapy decisions[5-7] further complicates comparisons.

To facilitate comparison of clinical trials performed throughout the world, the William Guy Forbeck Research Foundation sponsored an international conference more than 20 years ago. The outcome of the conference was published as the International Neuroblastoma Staging System (INSS).[8,9] During the last two decades, there have been major advances in understanding the genetics

of NB. Although the unfavorable prognostic factor *MYCN* amplification[10] is used by all cooperative groups for risk-group stratification and therapeutic decisions, other prognostically significant genetic features[5-7,11] have not been consistently incorporated into risk classification schemas. Furthermore, only some cooperative groups include tumor histology for risk-group assessment.[12,13]

To develop a consensus approach to pretreatment risk stratification, a task force of investigators with expertise in NB from the major pediatric cooperative groups around the world was established in 2004. A new International Neuroblastoma Risk Group (INRG) Staging System (INRGSS) was designed to stratify patients at the time of diagnosis before any treatment, as detailed in the companion article by Monclair et al.[14] In the INRGSS, extent of locoregional disease is determined by the absence or presence of image-defined risk factors (L1 and L2, respectively). Stage M will be used for widely disseminated disease, and MS describes metastatic NB limited to skin, liver, and bone marrow without cortical bone involvement in children age 0 to 18 months with L1 or L2 primary tumors. In addition, the Task Force's recommendations for defined standard operating procedures for molecular diagnostic testing of NB tumor tissue, criteria for the evaluation of bone marrow metastatic disease by immunocytochemistry and RT-PCR and for the assessment of metastatic disease by MIBG will be described in future reports.

PATIENTS AND METHODS

INRG Task Force Members

In 2004, investigators from the major cooperative groups, COG (North America and Australia), the German Pediatric Oncology and Hematology Group (GPOH), the Japanese Advanced Neuroblastoma Study Group (JANB), the Japanese Infantile Neuroblastoma Co-operative Study Group (JINCS), SIOPEN and China with expertise in NB were contacted by ADJP and SLC and invited to participate in an initiative to establish the INRG classification system. The major goal of the Task Force was to develop a consensus approach for pretreatment risk stratification of NB, based on statistical analyses of prognostic factors.

The leaders of the cooperative groups were asked to nominate six individuals with expertise in one or more of the following categories: clinical trials related to NB, chemotherapy, surgery, pathology, biology, radiology, nuclear medicine and statistics. In addition, young investigators were invited, and 52 investigators were identified. Four committees were formed: Surgery, Chair—Tom Monclair; Statistics, Chair—Wendy B. London; Biology, Chair—Peter F. Ambros; and Metastatic Disease, Chair—Katherine K. Matthay. The four chairs of the committees and the co-chairs of the INRG Task Force (A.D.J.P. and S.L.C.) comprised the INRG Executive Committee. Four international conferences were held: June 2004 in Genoa, Italy; September 2005 in Whistler, Vancouver, Canada sponsored by the William Guy Forbeck Research Foundation; May 2006 in Los Angeles, CA; and September 2006 in Geneva, Switzerland.

Table 1. Number of Patients in the International Neuroblastoma Risk Group Analytic Cohort by Country or Cooperative Group of Origin

Country or Cooperative Group	No.	%
COG	4,235	48.1
SIOPEN: Previous European Neuroblastoma Study Group (ENSG)	917	10.4
SIOPEN: Italy	304	3.5
SIOPEN: Spain	410	4.7
SIOPEN: LNESG1 trial	526	6.0
Germany	1,938	22.0
Japan	470	5.3
Total	8,800	100

Abbreviations: COG, Children's Oncology Group; SIOPEN, International Society of Pediatric Oncology Europe Neuroblastoma Group.

Patient Cohort

Data were collected on patients enrolled on COG, GPOH, JANB, JINCS, or SIOPEN trials. Enrollment cutoff of 2002 was chosen to allow at least 2 years of follow-up at the 2004 data freeze. Eligibility for inclusion in the INRG cohort included (1) confirmed diagnosis of NB, ganglioneuroblastoma (GNB), or ganglioneuroma (GN) maturing; (2) age no older than 21 years; (3) diagnosis between 1990 and 2002; and (4) informed consent. In addition to date of diagnosis and follow-up data, information on 35 potential risk factors were requested: age, INSS stage, Evans stage, Shimada classification, Shimada histologic category, Shimada grade, Shimada mitosis-karyorrhexis index (MKI), International Neuroblastoma Pathology Classification (INPC), INPC histologic category, INPC grade of tumor differentiation, INPC MKI, *MYCN* status, DNA ploidy (defined as DNA index ≤ 1.0 $v > 1.0$), 11q loss of heterozygosity (LOH), 11q aberration, unbalanced 11q LOH, 1p LOH, 1p aberration, 17q gain, serum ferritin, serum lactate dehydrogenase (LDH), six primary tumor sites, and eight metastatic sites. Analyses were performed on 8,800 unique patients.

Statistical Considerations

Objective, inferential criteria formed the initial basis for definition of the risk groups. However, because there were too few patients who had known values for all the factors and challenges of reaching international agreement, the final decision regarding the delineation of pretreatment risk groups was made by consensus on the basis of treatment strategies and overall survival (OS), in addition to event-free survival (EFS) results.

Survival Analyses

The primary analytic end point was EFS. Time to event was defined as time from diagnosis until time of first occurrence of relapse, progression, secondary malignancy, or death, or until time of last contact if none of these occurred. EFS was selected as the primary end point because the majority of patients with non–high-risk disease who have an event successfully achieve treatment salvage, and it is difficult to discriminate subsets using OS because of fewer events (deaths) in the lower-risk cohorts, resulting in lower power. Univariate analyses using a log-rank test, at a 5% significance level and without adjustment for multiple testing, were performed to identify factors statistically significantly predictive of EFS to be carried forward into the survival-tree regression. Kaplan-Meier curves were examined for each factor (data not shown).[15] Cox proportional hazards regression models were used to identify the most highly statistically significant variable to create a given split or "branch" in the survival tree.[16-19] The survival tree methodology, rather than attempting to develop a prognostic index, was used to develop the classification because the consensus of the clinical and scientific participants involved was that the survival tree approach was more intuitive, reflected the customary format for risk-group presentation in this disease, and could be used more easily internationally. The assumption of proportional hazards was tested. For practical reasons, all factors were analyzed as binary variables. All EFS and OS values are reported at the 5-year time point $\pm$ the SE.

Methods to Dichotomize Age, LDH, and Ferritin

Age was dichotomized using methods previously described by London et al ($n = 3,666$ COG patients from the INRG database).[20] Excluding these 3,666 patients, the analysis to identify an optimal age cutoff was repeated (data not shown). For LDH and ferritin respectively, the median value was used to dichotomize the cohort, and two binary variables were created for the survival-tree analysis.

Justification for Utilizing Underlying Components of Histologic Classification

The INPC and Shimada histology systems use age at diagnosis and histologic features of the tumor to categorize tumors as favorable versus unfavorable. This results in a duplication of the prognostic contribution

("confounding") of age when histology is used in a risk-group schema that includes age. To determine which histologic features were independently associated with outcome, tumor grade (differentiating *v* poorly differentiated or undifferentiated), MKI (low or intermediate *v* high), histologic category (GN-maturing or GNB-intermixed *v* GNB-nodular or NB), and age (< 547 *v* ≥ 547 days) were analyzed with EFS tree regression.[17-19,21]

Methods to Reduce the Number of Prognostic Variables

The 35 potentially prognostic factors were consolidated to 13 for analysis. Only factors where data were available for more than 5% of the 8,800 patients were included. Because Shimada and INPC are similar, histology data were consolidated into a single system. INPC was the default, but Shimada diagnosis, grade of tumor differentiation, or MKI were used if the corresponding INPC value was unknown. INSS was selected as the staging criteria. In situations where INSS and Evans definitions were the same (ie, INSS stage 1 = Evans stage I), Evans stage was used if INSS was unknown. Unbalanced 11q LOH and 11q aberrations data were combined into a single variable: "11q aberration." Similarly, 1p LOH and 1p aberrations were combined into the variable "1p aberration." 17q gain data were available for less than 5% of the patients, so 17q was not further analyzed. Using univariate analyses, six primary tumor sites were consolidated into one binary variable (adrenal *v* nonadrenal), as were eight metastatic sites (presence of metastases *v* no metastases).

The INRG database included a crude categoric variable for initial treatment. However, no statistical adjustment for treatment was performed. Because treatment has been assigned for many years using prognostic factors, treatment group is confounded with the prognostic factors,

Table 2. Clinical Characteristics of the International Neuroblastoma Risk Group Analytic Cohort (N = 8,800)

Factor	EFS		Patients		5-Year EFS (%)			5-Year OS (%)		
	Hazard Ratio	95% CI	No.	%	Rate	SE	Log-Rank *P*	Rate	SE	Log-Rank *P*
Age, days										
< 365	3.6	3.3 to 4.0	3,734	42	84	1		91	1	
≥ 365			5,066	58	49	1	< .0001	55	1	< .0001
Age, days										
< 547	3.7	3.4 to 4.0	4,773	54	82	1		88	1	
≥ 547			4,027	46	42	1	< .0001	49	1	< .0001
Year of enrollment/diagnosis										
≥ 1996	1.4	1.2 to 1.4	4,493	51	69	1		76	1	
< 1996			4,307	49	59	1	< .0001	66	1	< .0001
Initial treatment										
Observation, surgery, or standard chemotherapy	4.1	3.8 to 4.4	4,515	68	79	1		86	1	
Intensive multimodality			2,170	32	34	1	< .0001	41	1	< .0001
INSS stage										
1, 2, 3, 4S	5.2	4.8 to 5.7	5,131	60	83	1		91	1	
4			3,425	40	35	1	< .0001	42	1	< .0001
Evans stage										
I, II, III, IVS	6.6	5.8 to 7.6	2,022	63	86	1		91	1	
IV			1,177	37	31	2	< .0001	36	2	< .0001
Serum ferritin (ng/mL)										
< 92	3.6	3.2 to 4.0	2,170	50	81	1		87	1	
≥ 92			2,175	50	46	1	< .0001	52	1	< .0001
LDH (U/L)										
< 587	2.4	2.2 to 2.7	2,586	50	77	1		85	1	
≥ 587			2,592	50	53	1	< .0001	58	1	< .0001
Histologic classification (INPC, Shimada if INPC missing)										
Favorable	6.6	5.7 to 7.5	2,724	64	89	1		95	1	
Unfavorable			1,536	36	40	2	< .0001	49	2	< .0001
Diagnostic category (INPC, Shimada if INPC missing)										
1 = NB, stroma-poor			3,657	90	64	1		71 ±	1	
2 = GNB, intermixed, stroma-rich			144	3	95	3		96	2	
3 = GNB, well diff., stroma-rich			38	1	80	9	< .0001	79	9	< .0001
4 = GNB, nodular (composite)			232	6	53	5		68	5	
(2 and 3) *v* (1 and 4)	4.7	2.8 to 7.8								
Grade of NB differentiation (INPC, Shimada if INPC missing)										
Differentiating	2.5	2.0 to 3.3	518	16	83	2		89	2	
Undifferentiated			2,759	84	63	1	< .0001	72	1	< .0001
MKI (INPC, Shimada if INPC missing)										
Low, intermediate	3.2	2.8 to 3.8	2,690	87	74	1		82	1	
High			393	13	37	4	< .0001	44	4	< .0001

NOTE. Hazard ratios denote increased risk of an event for the second row within a given category compared with the first row.
Abbreviations: INPC, International Neuroblastoma Pathology Classification; EFS, event-free survival; OS, overall survival; NB, Neuroblastoma; GNB, Ganglioneuroblastoma; MKI, Mitosis Karyorrhexis Index.

resulting in reduced ability to detect the effect of a prognostic factor if adjustment for treatment is made. Therefore, instead of statistically adjusting for treatment, post hoc interpretation and the delineation of pretreatment groups were based on knowledge of how groups of patients had been treated historically.

Methods to Identify Prognostically Distinct Subgroups

The methodologic goal was to identify subgroups that were both statistically and clinically significantly different from one another, such that resulting subgroups of patients would be as homogenous as possible in terms of biology and outcome. The prognostic significance of the 13 factors was tested in the overall cohort, and the one with the highest χ^2 value was retained to create two subgroups or "nodes." The remaining factors were then tested within each node. This process was repeated within each node until the sample size was too small to proceed, or until no further statistically significant variables were found. In some nodes, the number of patients with known values for all factors being tested became too small for multivariate analysis. In this situation, factors were tested in a pairwise fashion in the model. The winner for each comparison was recorded, and the factor with the most "wins" was selected to create the next branch. Although not optimal, this approach was deemed necessary to overcome the problem of missing data.

RESULTS

INRG Cohort

The proportion of patients in the INRG analytic cohort of 8,800 was fairly evenly distributed between North America (48%) and Europe (47%), plus patients from Japan (5%) (Table 1). Tables 2 and 3 and Appendix Table A2 (online only) summarize the clinical and biologic characteristics of the cohort. The overall 5-year EFS and OS rates were 63% ± 1% and 70% ± 1%, respectively, with median follow-up of 5.2 years in 5,819 patients alive without an event. The assumption of proportional hazards was not violated for either EFS or OS except for 17q gain and skin metastases which were of no consequence because they were not among the final 13 risk factors evaluated. Also, at each split of the survival regression tree, the assumption of proportional hazards was upheld for EFS and OS.

Stage

The EFS tree regression analysis was performed on the basis of INSS stage. As described in Monclair et al,[14] an analysis of SIOPEN data (n = 474) found both INSS stage and INRGSS highly prognostic of EFS, and validated the German study.[22] This retrospective analysis supports the translation of EFS tree regression results (in terms of INSS stage) into the INRG Classification system (in terms of INRGSS): INSS 1 → INRGSS L1; INSS 2, 3 → INRGSS L2; INSS 4 → INRGSS M; and INSS 4S → INRGSS MS.

Age

The predictive ability of age was shown to be continuous in nature in the analysis of COG patients (n = 3,666) and within the balance of INRG patients. As recognized by the Task Force, it would be optimal to evaluate age as a continuous variable for risk stratification because outcome gradually worsens with increasing age. However, using two age groups was considered more feasible for these analyses. The analysis of non-COG patients within the INRG cohort confirmed the findings of London et al,[20] with support for an optimal "cutoff" between 15 and 19 months. For practical reasons, the Task Force's consensus was an age cutoff of 18 months (547 days) for the INRG classification system. Although the cutoff could be anywhere in this range, once selected, this age cutoff must be consistently applied as the exact number of days. However, for patients with diploid, stage M, *MYCN* nonamplified tumors, the Task Force elected to use the more conservative age cutoff of 12 months (365 days).

LDH and Ferritin

The median value to dichotomize LDH was 587 U/L, and for ferritin was 92 ng/mL.

Tumor Histology

In the EFS tree analysis testing histologic category, grade of tumor differentiation, MKI, and age, we found evidence of independent prognostic ability of each factor. This was tested in half the patients

Table 3. Genetic Characteristics of the International Neuroblastoma Risk Group Analytic Cohort (N = 8,800)

Factor	EFS		Patients		5-Year EFS (%)			5-Year OS (%)		
	Hazard Ratio	95% CI	No.	%	Rate	SE	Log-Rank *P*	Rate	SE	Log-Rank *P*
MYCN status										
Not amplified	4.1	3.8 to 4.5	5,947	84	74	1		82	1	
Amplified			1,155	16	29	2	< .0001	34	2	< .0001
Ploidy										
> 1 (hyperdiploid)	2.3	2.0 to 2.6	2,611	71	76	1		82	1	
≤ 1 (diploid, hypodiploid)			1,086	29	55	2	< .0001	60	2	< .0001
11q										
Normal	2.3	1.9 to 2.9	844	79	68	3		79	2	
Aberration			220	21	35	5	< .0001	57	5	< .0001
1p										
Normal	3.2	2.8 to 3.8	1,659	77	74	2		83	1	
Aberration			493	23	38	3	< .0001	48	3	< .0001
17q gain										
No gain	1.7	1.3 to 2.3	187	52	63	4		74	4	
Gain			175	48	41	5	.0006	55	5	.0009

NOTE. Hazard ratios denote increased risk of an event for the second row within a given category compared with the first row.
Abbreviations: INPC, International Neuroblastoma Pathology Classification; EFS, event-free survival; OS, overall survival; LOH, loss of heterozygosity.

(randomly selected) and the results confirmed in the other half. Excellent outcome was seen for patients with GN-maturing and GNB-intermixed tumors. For patients with GNB-nodular and NB tumors, age (younger than 18 $v \geq$ 18 months) was the most statistically significant factor. Within patients younger than 18 months with GNB-nodular and NB tumors, high MKI was associated with significantly lower EFS than low/intermediate MKI. Within patients 18 months of age or older with GNB-nodular and NB tumors, undifferentiated or poorly differentiated grade was associated with significantly lower EFS than differentiating grade. To prevent confounding of the effect of age, we analyzed histologic features (histologic category, MKI, and grade of differentiation) in lieu of the INPC.

Primary Site and Metastases

Adrenal primary tumor site had statistically significantly worse EFS than all other primary sites combined. For metastases, the most significant split was the presence versus absence of metastases.

EFS Tree Regression Analyses

The presence of classic metastases was the most significant prognostic factor in the EFS tree regression analysis of the overall cohort. The EFS and OS of INSS non–stage 4 (including 4S) patients were 83% ± 1% and 91% ± 1%, respectively, and 35% ± 1% and 42% ± 1% for children with stage 4 disease (Fig 1A).

Subclassification of Non–Stage 4 Patients

Within the patients with non–stage 4 disease (INSS stage 1, 2, 3, and 4S), histologic category (ie, GN-maturing and GNB-intermixed versus GNB-nodular and NB) was the most powerful prognostic factor (EFS: 97% ± 2% and 83% ±1, respectively). Of the 162 non–stage 4 INSS stage patients with GN-maturing or GNB-intermixed, only two had *MYCN* amplification, and both were alive without event at the time of this analysis. Because these tumors have a distinct clinical nature, the cohort of GN-maturing and GNB-intermixed was regarded as a terminal node. Within non–stage 4 GNB-nodular and NB patients, *MYCN* status was the most powerful prognostic factor (Fig 1A). Patients with *MYCN*-nonamplified tumors had EFS of 87% ± 1% and OS of 95% ± 1%, and 46% ± 4% and 53% ± 4% for patients with *MYCN*-amplified tumors. Within the *MYCN*-nonamplified cohort, patients with stage 1 disease had significantly better outcome than those with stages 2,3,4S (EFS: 93% ± 1% v 82% ± 1%; OS: 98% ± 1% v 92% ± 1%; Fig 1B). EFS for stage 1 patients with normal chromosome 1p was statistically better compared with those with 1p aberration (94% ± 2% v 78% ± 10%). However, OS was excellent regardless of the status of chromosome 1p (normal 1p: 99% ± 1%; 1p aberration: 100%). Therefore, 1p status was not included as a criterion in the INRG classification system and stage 1 was a terminal node.

Although survival rates for patients with stages 2, 3 disease (EFS: 82% ± 1%; OS: 92% ± 1%) and stage 4S patients (EFS: 82% ± 2%; OS: 91% ± 2%) were not statistically significantly different, treatment intensity differed. Because there are different treatment approaches in this group (4S disease is commonly observed whereas treatment for stage 2 and 3 tumors is surgery with or without chemotherapy), stage 2, 3 patients were split from stage 4S patients for further survival tree analyses. Within stage 2, 3 patients, those younger than 18 months old had statistically higher EFS than those 18 months of age or older (88% ± 1% v 69% ± 3%). In *MYCN* nonamplified stage 2, 3 patients younger than 18 months old, 11q aberration was the most highly prognostic of the biomarkers evaluated, with lower EFS (60% ± 20%) and OS (84% ± 14%) than normal 11q (EFS: 83% ± 5%; OS: 98% ± 2%; Fig 1B).

In patients with *MYCN*-nonamplified stage 2, 3 tumors who were 18 months of age or older, 11q aberration was the most statistically significant factor, but grade of tumor differentiation was also highly significant and identified additional poor-prognosis patients without evidence of 11q aberration (Fig 1B). The Task Force therefore decided to combine 11q aberration with grade into a single prognostic factor, categorizing patients who had either 11q aberration and/or undifferentiated (or poorly differentiated) histology (EFS: 61% ± 11%; OS: 73% ± 11%) versus those who did not have either one of the poor-outcome features (EFS: 80% ± 16%; OS: 100%).

Within the patients with *MYCN*-nonamplified stage 4S tumors, 11q aberration was the most highly prognostic factor (11q aberration—EFS: 38% ± 30%, OS: 63% ± 38%; normal 11q—EFS: 87% ± 7%, OS: 97% ± 4%). The number of patients within this cohort is small, and additional evaluation will be needed to further evaluate the impact of 11q aberration in this subset of patients.

MYCN-amplification was detected in only 8% of patients with stage 1 to stage 4S disease (Fig 1C). Although EFS rates for stage 1 patients were not statistically significant different from those of stage 2, 3, and 4S patients, less intensive treatment was administered to patients with *MYCN*-amplified stage 1 tumors. Because of the difference in treatment strategies, further survival tree analyses were performed separately in stage 1 patients versus stage 2, 3, and 4S patients. LDH was most highly prognostic for patients with *MYCN*-amplified stage 1 tumors (< 587 U/L—EFS: 55% ± 15%, OS: 85% ± 10% $v \geq$ 587 U/L—EFS: 40% ± 22%, OS: 58% ± 22%) and within the stage 2, 3, and 4S subset (< 587 U/L—EFS: 67% ± 9%, OS: 72% ± 8% $v \geq$ 587 U/L—EFS: 43% ± 5%, OS: 47% ± 5%). LDH is known to reflect tumor burden, and of the 169 *MYCN*-amplified stage 2, 3, and 4S patients with elevated LDH, 72% were stage 3. In view of the small number of patients in this cohort and the nonspecific nature of LDH, the Task Force decided not to include LDH in the classification system.

Subclassification of Patients With Stage 4 Disease

Age was the most powerful prognostic variable within 3,425 patients with stage 4 disease (Fig 1D). Children younger than 18 months had EFS and OS rates of 63% ± 2% and 68% ± 2%, respectively. Children 18 months of age or older had EFS and OS rates of 23% ± 1% and 31% ± 1%, respectively. Although serum ferritin ($< v \geq$ 92 ng/mL) was shown to be prognostic in the cohort of patients age 18 months and older by the EFS tree regression, outcome was poor in both cohorts, with EFS rates of 43% ± 4% and 20% ± 2%, respectively. Further statistically significant splits for *MYCN* status were identified within both ferritin cohorts ($< v \geq$ 92 ng/mL), but EFS and OS were poor in all of these subsets. Thus, serum ferritin did not add clinically relevant information in this cohort of patients with poor prognosis and was not included in the INRG classification schema. Within patients younger than 18 months with stage 4 disease, *MYCN* status was the most powerful prognostic factor. EFS was 83% ± 2% for children younger than 18 months with stage 4 disease lacking *MYCN* amplification versus 26% ± 4% for those with *MYCN*-amplified tumors. Within *MYCN*-nonamplified patients younger than 18 months with stage 4 disease, ploidy had prognostic significance. Patients with a DNA index greater than 1.0 had EFS of 85% ±

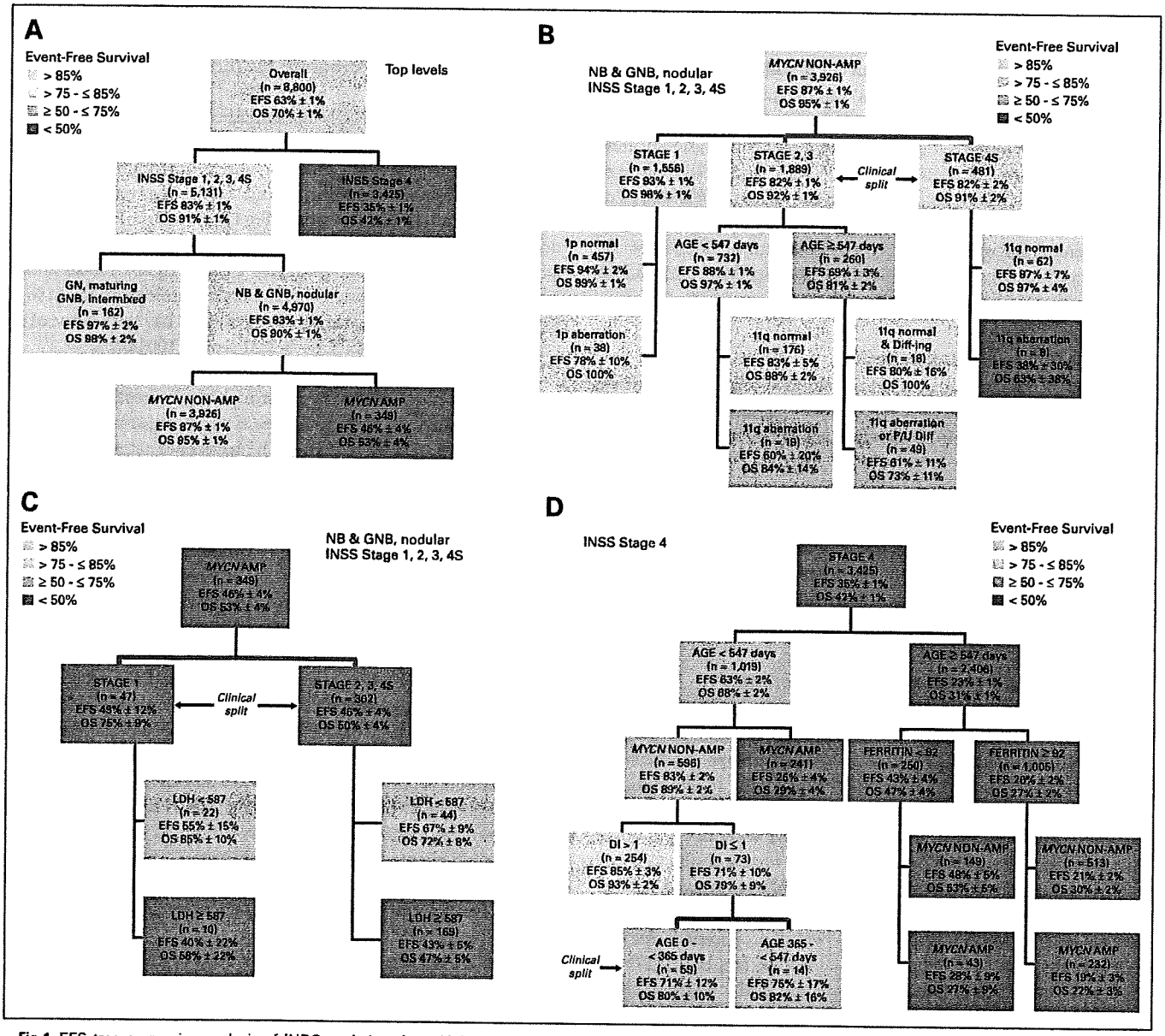

Fig 1. EFS tree regression analysis of INRG analytic cohort. Unless otherwise noted, a split or branch occurs for the most highly statistically significant factor as identified using a Cox proportional hazards regression model. (A) Top levels of the overall tree. (B) Subtree for NB and GNB-nodular, non–stage 4 *MYCN* NON-AMP patients. The split of stage 2, 3 from stage 4S patients was a clinical decision and not the result of statistical significance. (C) Subtree for NB and GNB-nodular, non–stage 4 *MYCN* AMP patients. The split of stage 1 from stage 2, 3, 4S patients was a clinical decision and not the result of statistical significance. (D) Subtree for INSS stage 4 patients. EFS, event-free survival; OS, overall survival; DI, DNA index; AMP, amplified; NON-AMP, nonamplified; INRG, International Neuroblastoma Risk Group; NB, neuroblastoma; GNB, ganglioneuroblastoma; GN, ganglioneuroma; INSS, International Neuroblastoma Staging System; LDH, lactate dehydrogenase.

3%, whereas EFS was 71% ± 10% for DNA index 1.0 or less. Although EFS for patients with stage 4 tumors younger than 12 months were not statistically significantly different from those 12 months or older to younger than 18 months, substantially higher-intensity treatment regimens were administered to patients who were 12 to younger than 18 months of age. On the basis of ploidy data and the excellent outcome of young children with stage 4 disease with favorable biologic features, several cooperative groups have developed clinical trials testing reduction in treatment for this cohort. In patients with diploid, *MYCN*-nonamplified stage 4 tumors, clinical justification was used to split patients younger than 12 months from 12 months and older to younger than 18 months of age, as the international consensus is that the intensity of therapy should not be reduced in this later group.

INRG Classification System

In summary, the consensus INRG classification schema includes the criteria INRG stage, age, histologic category, grade of tumor differentiation, *MYCN* status, presence/absence of 11q aberrations, and tumor cell ploidy. Sixteen statistically and/or clinically different pretreatment groups of patients (lettered A through R) were identified using these criteria (Fig 2). The proportion of patients grouped using EFS cut points for 5-year EFS of more than

INRG Stage	Age (months)	Histologic Category	Grade of Tumor Differentiation	*MYCN*	11q Aberration	Ploidy	Pretreatment Risk Group
L1/L2		GN maturing; GNB intermixed					A Very low
L1		Any, except GN maturing or GNB intermixed		NA			B Very low
				Amp			K High
L2	< 18	Any, except GN maturing or GNB intermixed		NA	No		D Low
					Yes		G Intermediate
	≥ 18	GNB nodular; neuroblastoma	Differentiating	NA	No		E Low
					Yes		H Intermediate
			Poorly differentiated or undifferentiated	NA			
				Amp			N High
M	< 18			NA		Hyperdiploid	F Low
	< 12			NA		Diploid	I Intermediate
	12 to < 18			NA		Diploid	J Intermediate
	< 18			Amp			O High
	≥ 18						P High
MS	< 18			NA	No		C Very low
					Yes		Q High
				Amp			R High

Fig 2. International Neuroblastoma Risk Group (INRG) Consensus Pretreatment Classification schema. Pretreatment risk group H has two entries. 12 months = 365 days; 18 months = 547 days; blank field = "any"; diploid (DNA index ≤ 1.0); hyperdiploid (DNA index > 1.0 and includes near-triploid and near-tetraploid tumors); very low risk (5-year EFS > 85%); low risk (5-year EFS > 75% to ≤ 85%); intermediate risk (5-year EFS ≥ 50% to ≤ 75%); high risk (5-year EFS < 50%). GN, ganglioneuroma; GNB, ganglioneuroblastoma; Amp, amplified; NA, not amplified; L1, localized tumor confined to one body compartment and with absence of image-defined risk factors (IDRFs); L2, locoregional tumor with presence of one or more IDRFs; M, distant metastatic disease (except stage MS); MS, metastatic disease confined to skin, liver and/or bone marrow in children < 18 months of age (for staging details see text and Monclair et al[14]); EFS, event-free survival.

85%, more than 75% to ≤ 85%, ≥ 50% to ≤ 75%, or less than 50%, were 28.2%, 26.8%, 9.0%, and 36.1%, respectively (Table 4). The categories were designated as very low (A, B, C), low (D, E, F), intermediate (G, H, I, J), or high (K, N, O, P, Q, R) pretreatment risk subsets.

DISCUSSION

In recent years, the need to develop an international consensus for pretreatment risk stratification for children with NB has become increasingly apparent. To achieve this goal, an international task force established the INRG classification system. The prognostic effect of 13 variables in an 8,800-patient cohort was analyzed, with EFS, not OS, as the primary end point for the reasons identified earlier in this article. The INRG classification system includes the seven factors that were highly statistically significant and also considered clinically relevant. Similar to other series, patients with widely disseminated stage 4 disease had significantly worse outcome than those with locoregional disease or stage 4S NB.[9,23] As described in the article by Monclair et al,[14] a new pretreatment staging system was designed for the INRG classification system. In the INRGSS, extent of locoregional disease is determined by the absence or presence of image-defined risk factors (L1 and L2, respectively). Stage M will be used for disseminated disease, analogous to INSS stage 4. Similar to INSS stage 4S tumors, metastases are limited to skin, liver, and bone marrow without cortical bone involvement in INRGSS MS disease. However, the definition of MS has been expanded to include toddlers age 12 to younger than 18 months and large "unresectable" primary tumors (L1 or L2). As discussed in the companion article by Monclair et al,[14] the inclusion of L2 tumors is based on the excellent outcome of all 30 children enrolled on the SIOPEN 99.2 trial who met the criteria for INSS stage 4S disease and, in addition, had midline infiltration of the primary tumor, after treatment with a few cycles of chemotherapy or observation alone (B. De Bernardi, personal communication, February 2008). Although there is some concordance of patients between the INRGSS and the INSS staging systems, the two systems differ in the sense that the INSS staging system contains inherent confounding of surgical treatment versus extent of tumor, whereas INRGSS removes that confounding because it is assigned before surgery. The important similarity of the two systems is that INRGSS retains the prognostic value of staging that has been well documented for INSS staging, with statistically significantly higher EFS for L1 compared with L2. There is statistical justification for use of INRG staging for assigning patients to pretreatment groups, although prospective evaluation of the risk grouping based on the INRGSS staging system will be mandatory.

Table 4. Proportion of Patients When Arbitrary EFS Cut Points Are Applied to Cluster Rows of the International Neuroblastoma Risk Group Consensus Stratification (for illustrative purposes)

Pretreatment Risk Group	% 5-Year EFS	% Proportion of Patients
Very low	> 85	28.2
Low	> 75 to ≤ 85	26.8
Intermediate	≥ 50 to ≤ 75	9.0
High	< 50	36.1

Abbreviation: EFS, event-free survival.

The analysis of the INRG data confirmed that the predictive ability of age is continuous in nature for NB. By convention, virtually all cooperative groups have used the 12-month cutoff to determine risk.[1] Similar to a previous study of COG patients,[20] our analysis of the INRG cohort indicated that the optimal age cutoff is between 15 and 19 months. Children age 12 to younger than 18 months with hyperdiploid stage 4 disease who lack *MYCN* amplification have excellent outcome when treated with intensive therapy on high-risk clinical trials.[24,25] These results suggest that therapy may be reduced safely in a subset of young children with stage 4 disease, and clinical trials testing this question have recently been developed. An age cutoff of 18 months (547 days) was, therefore, selected for the INRG classification system for all children except those with diploid, stage M tumors

without amplification of *MYCN* for whom the more conservative, 12-month cutoff will be maintained.

Tumor histology is another well established prognostic variable in NB.[12,13] To avoid confounding of age and INPC, we tested histologic category, MKI, grade of tumor differentiation, and age in the EFS tree regression analyses in lieu of INPC. We found that histologic category and tumor differentiation were statistically significantly associated with EFS. Consistent with the inferior prognosis that has been reported in patients with Shimada unfavorable histology INSS stage 3 tumors that lack *MYCN* amplification,[26] we found that outcome was worse for patients age 18 months and older with *MYCN*-nonamplified stage 2, 3 poorly differentiated or undifferentiated tumors compared with those with differentiating tumors.

To accurately stratify patients with locoregional tumors using the INRG classification system, sufficient samples of tumor tissue will be required for genetic/expression studies and for histologic category determination. In addition, there is a need for wide-scale education of pediatric pathologists to ensure that different histopathologic grades are uniformly and reproducibly recognized. The challenges of distinguishing GNB-intermixed from GNB-nodular are significant when the entire tumor is not resected. Surgical biopsy needs to be guided by the radiological appearances of the tumor, with any heterogeneous areas targeted. Adequate tissue samples are mandatory to evaluate histologic grade of differentiation in locoregional NBs that lack *MYCN* amplification in children 18 months of age or older. In most cases, multiple "true-cut" cores will yield sufficient tissue to determine tumor grade of differentiation, but fine-needle aspirates are not likely to provide adequate quantities of tissue for histologic analysis and are not appropriate. In metastatic tumors, fine-needle aspirates may provide adequate information for genetic analysis.

A number of genetic aberrations have been identified in NB tumors that are strongly associated with outcome. Our analysis confirmed the unfavorable prognostic significance of *MYCN* amplification, and in the INRG classification system, *MYCN* status is used to stratify patients into different pretreatment risk groups. We also found that 11q aberration was associated with worse outcome in patients with L2 or MS tumors that lack *MYCN* amplification. Similar to previous studies,[25,27-29] the prognostic value of DNA ploidy was demonstrated in children younger than 18 months of age with stage 4 disease and normal *MYCN* copy number. Recommendations regarding standardized methods for evaluating *MYCN* copy number, tumor cell ploidy, and other genetic aberrations in NB tumors will be reported in a future article.

Recent studies suggest that low-risk tumors may be best defined by the absence of *MYCN* gene amplification and any structural genetic abnormalities, (including either 11q and/or 1p aberrations and/or 17q gain).[30,31] The Task Force agreed that it would be optimal to evaluate genetic aberrations in NB tumors using genome-wide methods. However, because this type of analysis is not routinely performed by the large cooperative groups, incorporation of more global genetic data in the current INRG was not considered feasible at the present time. The immediate challenges are (1) to ensure that adequate tumor material is available for prospective "comprehensive" genetic investigations on every patient and (2) to identify technologies that are not cost prohibitive and will yield rapid and reproducible results. It is anticipated that the future INRG classification system will rely on the genetic profile of NB tumors, rather than the presence or absence of individual genetic abnormalities.

A limitation of this analysis is that there was no statistical adjustment for treatment, and therefore, patients in any of the 16 lettered rows may have received very different therapy. It is intended to extend the INRG database prospectively, and it will be critical to collect data on details of therapy.

In conclusion, the INRG classification system will ensure that children diagnosed with NB in any country are stratified into homogenous pretreatment groups. We strongly recommend that cooperative groups begin using this risk schema now. The very low-, low-, intermediate-, and high-risk categories were defined according to EFS cutoffs. These four categories were included in the classification schema to assist treating physicians in evaluating the prognostic impact of the combination of factors in each of the 16 lettered rows in the INRG classification system. Although these risk categories could have been defined differently, we selected EFS cutoff values that are commonly used for treatment stratification at the present time. For example, at most centers around the world, patients with features that are associated with estimated EFS rates of less than 50% are treated with intensive, multimodality strategies, whereas those predicted to have more than 85% EFS receive minimal therapy. We anticipate that risk group stratification will be further refined as treatment for high-risk disease improves and genome-wide DNA and expression analysis of tumors becomes more routine. It must be emphasized that we are not recommending that treatment be assigned according to these four broad risk-group categories. Rather, the key to reaping the benefits of this system will be the assignment of patients in one of the 16 pretreatment lettered designations in the INRG classification system to a single treatment group without splitting that row in different treatment subgroups. We anticipate that eligibility criteria for treatment protocols will likely include several of the 16 INRG pretreatment designations, and that the combinations of the 16 pretreatment groups that will be included in clinical trials studies conducted by each of the cooperative groups may be different. Therefore, it will be critical to individually report the outcome of patients assigned to each of the 16 pretreatment designations. This approach will greatly facilitate the comparison of risk-based clinical trials conducted in different regions of the world, provide a platform to ask randomized surgical questions, and lead to the development of international collaborative studies.

AUTHORS' DISCLOSURES OF POTENTIAL CONFLICTS OF INTEREST

The author(s) indicated no potential conflicts of interest.

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RASSF1A hypermethylation in pretreatment serum DNA of neuroblastoma patients: a prognostic marker

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The tumour suppressor gene *RASSF1A* is known to be frequently silenced by promoter hypermethylation in neuroblastoma tumours. Here we explored the possible prognostic significance of aberrant promoter hypermethylation of *RASSF1A* in serum DNA samples of patients with neuroblastoma as a surrogate marker for circulating tumour cells. We analysed the methylation status of the *RASSF1A* gene in matched tumour and pretreatment serum DNA obtained from 68 neuroblastoma patients. Hypermethylation of *RASSF1A* in tumour samples was found in 64 patients (94%). In contrast, serum methylation of *RASSF1A* was observed in 17 patients (25%). Serum methylation of *RASSF1A* was found to be statistically associated with age $\geqslant$ 12 months at diagnosis ($P=0.002$), stage 4 ($P<0.001$) and *MYCN* amplification ($P<0.001$). The influence of serum *RASSF1A* methylation on prognosis was found to be comparable with that of the currently most reliable marker, *MYCN* amplification on univariate analysis (hazard ratio, 9.2; 95% confidence interval (CI), 2.8–30.1; $P<0.001$). In multivariate analysis of survival, methylation of *RASSF1A* in serum had a hazard ratio of 2.4 (95% CI, 0.6–9.2), although this association did not reach statistical significance ($P=0.194$). These findings show that the methylation status of *RASSF1A* in the serum of patients with neuroblastoma has the potential to become a prognostic predictor of outcome.

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Neuroblastoma is the most common extracranial solid tumour in children and is characterised by a wide range of clinical behaviours, from spontaneous regression to rapid progression with a fatal outcome (Maris *et al*, 2007). The clinical outcome is associated with disease stage, age at diagnosis, *MYCN* amplification and histological classification. Although numerous genetic abnormalities, including *MYCN* amplification, are associated with tumour progression and poor outcome, the molecular mechanisms responsible for the pathogenesis of aggressive neuroblastoma remain unclear. Identifying such molecular changes may contribute to improved clinical management and outcome prediction of newly diagnosed neuroblastomas.

In recent years, changes in the status of DNA methylation, known as epigenetic alterations, have turned out to be one of the most common molecular alterations in human neoplasia including neuroblastoma (Misawa *et al*, 2005; Sugino *et al*, 2007). Several potential tumour-suppressor genes have been described as frequently silenced by hypermethylation in neuroblastomas. Methylation of promoter CpG islands is known to inhibit transcriptional initiation and cause permanent silencing of downstream genes. Loss of heterozygosity of chromosome 3p21.3 is one of the most frequent alterations in solid tumours. Located within this 3p21.3 locus, the RAS-association domain family 1, isoform A gene (*RASSF1A*) encodes a RAS effector that has been identified as a tumour suppressor of many different cancer types (Dammann *et al*, 2000). *RASSF1A* falls into the category of genes frequently inactivated by methylation rather than mutational events. This gene is silenced and inactivated by promoter region hypermethylation in many adult and childhood cancers, including neuroblastoma (Astuti *et al*, 2001; Harada *et al*, 2002; Wong *et al*, 2004; Yang *et al*, 2004; Banelli *et al*, 2005; Lazcoz *et al*, 2006; Michalowski *et al*, 2008). *RASSF1A* has been shown to play important roles in cell cycle regulation, apoptosis and microtubule stability as a tumour suppressor gene (Agathanggelou *et al*, 2005).

It is well known that DNA fragments are frequently and abundantly found in the serum of cancer patients, with significantly higher levels in patients with metastasis (Hesson *et al*, 2007). A number of studies have evaluated the potential of circulating tumour-related methylated DNA in serum for the molecular diagnosis and prognosis of various types of cancer (Müller *et al*, 2003; Ibanez de Caceres *et al*, 2004; Mori *et al*, 2005). Methylation-specific PCR assay is a sensitive and specific assay for tumour-related DNA methylation in serum. Several studies have investigated the prospect of using DNA methylation as a surrogate marker for circulating tumour cells in serum samples from breast cancer or melanoma patients (Fiegl *et al*, 2005; Koyanagi *et al*, 2006). However, no studies of neuroblastoma have assayed serum samples for aberrant DNA methylation. Therefore, this study

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investigated whether it is possible to detect *RASSF1A* epigenetic alterations in the serum of neuroblastoma patients, and aberrant *RASSF1A* methylation in patient pretherapeutic serum is of prognostic significance in neuroblastoma using a series of matched neuroblastoma tumour and serum DNA.

MATERIALS AND METHODS

Patients and sample collection

Clinical data were collected retrospectively by reviewing the medical database at the Hospital of Kyoto Prefectural University of Medicine for the period between 1985 and 2004. After approval by the Institutional Review Board, 68 neuroblastoma patients were identified on the basis of histological examination of tumour specimens that met the following criteria: the patient had an available tumour specimen; a serum specimen was available; and the patient either died or had >1 year of follow-up time. The clinical data included information regarding tumour stage, age at diagnosis, sex, *MYCN* gene status and outcome. Staging was evaluated according to the criteria of the International Neuroblastoma Staging System (Maris *et al*, 2007). Patients of any age who had stage 1 or 2 disease and those younger than 12 months with stage 3 or 4S disease were given either surgery or surgery with chemotherapy (Matsumura and Michon, 2000). Patients aged 12 months or older with stage 3 and any patients with stage 4 disease were treated according to the protocol by the Japanese Neuroblastoma Study Group (Sawaguchi *et al*, 1990; Tsuchida and Kaneko, 2000; Kaneko *et al*, 2002; Suita *et al*, 2007). The patients with stage 4 disease underwent high-dose chemotherapy with autologous stem-cell rescue after the initial chemotherapy. Instead of pre-specified sample size determination, power analysis was conducted after collecting clinical data to guarantee statistical power and to evaluate whether *RASSF1A* methylation is a prognostic marker for survival. In a realistic scenario, a study of 68 patients had power of 96% to detect a single marker with hazard ratio larger than 5.

Tumour samples at the time of diagnosis and before the administration of chemotherapy were frozen immediately and stored at −80°C until DNA extraction. In addition, match-paired serum samples were assessed. Peripheral blood was obtained before any therapy or surgery. To avoid contamination of serum DNA by the DNA from WBCs, serum was prepared exclusively from the liquid fraction of clotted blood after centrifugation at 1000 × g for 10 min and stored it at −20°C until DNA extraction. For the extraction of free DNA, we used 200 μl of stored serum, which supplemented with 1 μg salmon testes DNA (Sigma, St Louis, MO, USA) as a carrier DNA. DNA was extracted from tumour samples using a QIAmp DNA Mini Kit and from serum samples using a QIAmp DNA Blood Mini Kit (Qiagen, Hilden, Germany) according to the manufacturer's instructions.

Analysis of DNA methylation

Treatment of tumour DNA and serum DNA with sodium bisulphite was performed with an EZ DNA methylation kit (Zymo Research, Orange, CA, USA) following the protocol of the manufacturer. Methylation-specific PCR was performed with primers specific for either methylated or unmethylated DNA spanning the region within the *RASSF1A* gene (Figure 1). The primers used were methylation-specific RAM-1 (5′-GTG TTAACGCGTTGCGTATC-3′) and RAM-2 (5′-AACCCCGCGAACT AAAAACGA-3′) and unmethylation-specific RAU-1 (5′-TTTGGT TGGAGTGTGTTAATG-3′) and RAU-2 (5′-CAAACCCCACAAACT AAAAACAA-3′), as described earlier (Lo *et al*, 2001). PCR conditions consisted of an initial incubation for 10 min at 95°C followed by 35 cycles for tumour samples or 40 cycles for serum

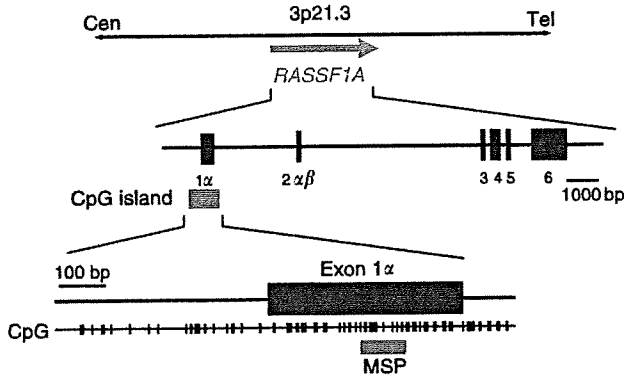

Figure 1 Genomic structure of the *RASSF1A* gene. Vertical tick marks, CpG sites; blue boxes, exons; green box, CpG island in the promoter; red box, region analysed by methylation-specific PCR.

samples of denaturation at 95°C for 45 s, annealing at 60°C for 45 s and extension at 72°C for 60 s, followed by a final extension step of 72°C for 10 min. Lymphocyte DNA and *in vitro* methylated (using *Sss*I CpG methylase; New England Biolabs, Beverly, MA, USA) lymphocyte DNA were used as unmethylated and methylated controls, respectively. The PCR products obtained were analysed by electrophoresis in 2% agarose gels and stained with ethidium bromide. Samples were scored as methylation positive when methylated alleles were visualised as bands in the methylated DNA lane and as methylation negative when bands were seen only in the unmethylated DNA lane. The analysis of the samples in this study was performed by an analyst blinded to the clinical and biological information.

Statistical analysis

The primary end point was overall survival defined by the period from diagnosis of the primary tumour to any cause of death. The relationship between clinicopathological variables and methylation status of the *RASSF1A* gene was shown initially using contingency tables and χ^2 test. Survival curves for *RASSF1A* methylation were derived by the Kaplan–Meier method. Univariate analysis was conducted using Cox's proportional hazard models and log-rank test. Performance of *RASSF1A* methylation as a prognostic marker was also analysed after adjustment for known prognostic factors by (i) subset analysis of stage 3 patients using contingency tables and Fisher's exact test and (ii) multivariate Cox's proportional hazard models including age, sex and tumour stage. Two-sided *P*-values <0.05 were considered as significant. SAS 9.13 (SAS Institute Inc., Cary, NC, USA) was used for statistical analyses.

RESULTS

A total of 124 patients with histologically confirmed neuroblastoma or ganglioneuroblastoma were treated at the Hospital of Kyoto Prefectural University of Medicine between January 1985 and May 2004. Sixty-eight patients met the criteria of this retrospective study. The detailed patient disposition is shown in Figure 2 and the baseline characteristics of patients are presented in Table 1. Of the 68 patients, 24 were classified as stage 1, 11 as stage 2, 11 as stage 3, 18 as stage 4 and 4 as stage 4S. At the time of diagnosis, 42 patients (62%) were younger than 12 months, and 26 (38%) were older. We found no significant differences between included and excluded patients for age or stage statistically. Twelve patients (18%) had tumours with *MYCN* amplification, and *MYCN* amplification was not detected in the tumours from 56 (82%) patients by southern

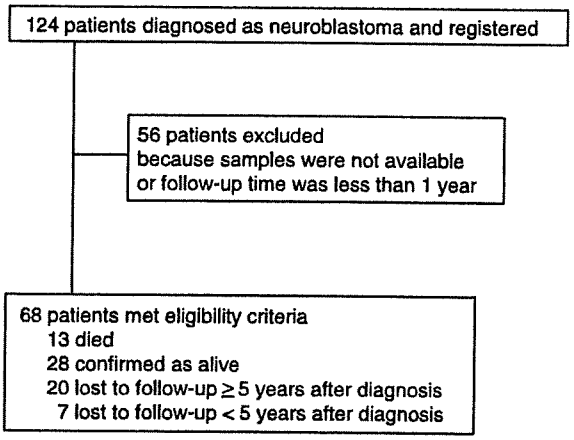

Figure 2 Patient disposition.

Table 1 Characteristics of patients

Characteristic	No. of patients (%)
Sex	
Male	27 (39.7)
Female	41 (60.3)
Age at diagnosis	
< 12 months	42 (61.8)
⩾ 12 months	26 (38.2)
Stage	
1	24 (35.3)
2	11 (16.2)
3	11 (16.2)
4	18 (26.5)
4S	4 (5.9)
MYCN	
Non-amplified	56 (82.4)
Amplified	12 (17.6)
Diagnosis	
GNB	7 (10.3)
NB	61 (89.7)
Serum RASSF1A	
Unmethylated	51 (75.0)
Methylated	17 (25.0)

blot analysis or fluorescence *in situ* hybridisation. The median follow-up time was 72 months, with a range from 9 to 248 months.

Detection of *RASSF1A* promoter methylation in tumours

This study initially investigated the hypermethylation status of the *RASSF1A* tumour suppressor genes in 68 neuroblastoma tumours. Only four (one each at stage 1, 2, 4S and 3) tumours showed no methylation of *RASSF1A* (Supplementary Table). All other neuroblastoma tumours (64 of 68; 94%) showed methylated *RASSF1A*. Hypermethylation in tumours was observed very frequently in all of the stages of neuroblastoma examined, including stage 1, 2 and 4S tumours (Supplementary Table) and no correlation between *RASSF1A* methylation and known prognostic factors including stage, age and *MYCN* amplification was detected. No relationship between *RASSF1A* methylation in tumours and outcome was also observed. *RASSF1A* methylation was not observed in any of the three benign ganglioneuromas.

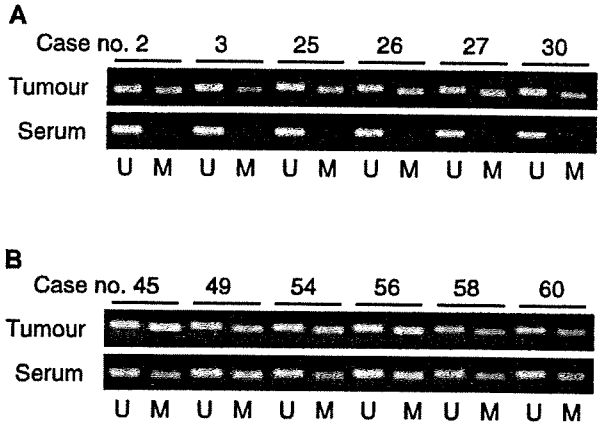

Figure 3 *RASSF1A* methylation status of tumour and serum DNA in neuroblastoma patients. M, methylated; U, unmethylated. The sizes of the PCR products for methylated and unmethylated primers are 93 and 105 bp, respectively. (**A**) Cases stage 1 and 2 with good prognosis tumour DNAs are methylated but absent in the serum DNAs. (**B**) In contrast, in stage 3 and 4 *MYCN*-amplified cases, methylated DNAs are detected in both tumour and serum samples.

Table 2 Associations between clinical factors and serum *RASSF1A* methylation status

Characteristic	Methylated no.	Unmethylated no.	Total no.	*P*-value
Age at diagnosis				0.002
< 12 months	5	37	42	
⩾ 12 months	12	14	26	
Stage				<0.001
1/2/4S	3	36	39	
3	3	8	11	
4	11	7	18	
MYCN				<0.001
Non-amplified	5	51	56	
Amplified	12	0	12	

Detection of *RASSF1A* promoter methylation in serum

The hypermethylation status of *RASSF1A* in the matched serum DNA samples was then determined and compared with the pattern of hypermethylation found in the corresponding tumour DNA samples (Figure 3). *RASSF1A* hypermethylation was detected in 17 of 68 (25%) matched serum DNA samples (Table 1). The detailed overview is shown in Supplementary Table.

Correlation of serum *RASSF1A* methylation status with clinical factors

The methylation status of *RASSF1A* in the pretherapeutic serum of the 68 patients was analysed for association with known prognostic factors (Table 2). Serum *RASSF1A* methylation showed a significant statistical association with age ⩾12 months ($P=0.002$). *RASSF1A* methylation in serum was detected more frequently in disseminated stage 4 tumours than local-regional (stage 1, 2 and 3) and 4S tumours ($P<0.001$). Furthermore, serum-methylated *RASSF1A* was significantly correlated with *MYCN* amplification ($P<0.001$). Notably, all cases with *MYCN* amplification showed *RASSF1A* methylation of serum DNA (Supplementary Table).

Table 3 Univariate analysis of survival

Characteristic	Hazard ratio	95% CI	P-value
Age at diagnosis			
<12 months	Reference		
⩾12 months	23.6	3.1–181.9	0.002
Sex			
Male	Reference		
Female	1.0	0.3–3.0	0.983
Stage			
1/2/3/4S	Reference		
4	19.8	4.4–89.5	<0.001
MYCN			
Non-amplified	Reference		
Amplified	8.2	2.7–24.7	<0.001
Serum RASSF1A			
Unmethylated	Reference		
Methylated	9.2	2.8–30.1	<0.001

CI = confidence interval.

Analysis of prognostic significance of *RASSF1A* methylation in serum

The association of pretherapeutic serum methylation status of *RASSF1A* with clinical outcome was analysed in 68 patients with known follow-ups. Univariate analyses revealed prognostic significance for age at diagnosis ⩾12 months, stage 4 and *MYCN* amplification ($P=0.002$, $P<0.001$ and $P<0.001$, respectively; Table 3) in this cohort, as expected. Patients with serum-methylated *RASSF1A* had significantly worse overall survival than patients with serum-unmethylated *RASSF1A* ($P<0.001$, log-rank test; Figure 4A). The 5-year survival was more than 90% in patients without serum methylation of *RASSF1A*, whereas lower than 50% in patients with serum methylation of *RASSF1A* (hazard ratio, 9.2; 95% confidence interval (95% CI), 2.8–30.1; $P<0.001$); Table 3). *RASSF1A* methylation in serum and the known prognostic factors were also correlated with relapse-free survival as well as with overall survival ($P<0.001$; Table 4; Figure 4B). Furthermore, a subset analysis revealed that stage 3 patients also had a trend towards poorer prognosis when *RASSF1A* was methylated in serum. When limited to cases in stage 3, two of the three patients with serum-methylated *RASSF1A* died, whereas all eight patients with serum-unmethylated *RASSF1A* are alive ($P=0.055$, Fisher's exact test). In a multivariate analysis including age, sex and tumour stage, serum *RASSF1A* methylation was still associated with poor outcome with a hazard ratio of 2.4 (95% CI, 0.6–9.2), although this did not reach statistical significance ($P=0.194$; Table 5).

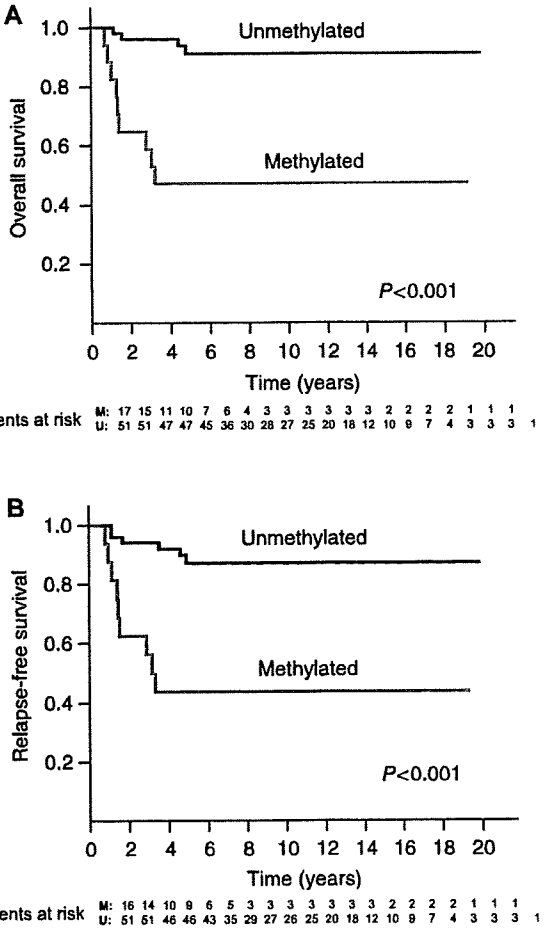

Figure 4 Kaplan–Meier survival curves of 68 neuroblastoma patients: correlation of pretherapeutic serum *RASSF1A* methylation status with overall survival (**A**) and relapse-free survival (**B**). M, methylated: patients with serum methylation of *RASSF1A*. U, unmethylated: patients without serum methylation of *RASSF1A*. The 5-year overall survival was more than 90% in patients without methylation, whereas lower than 50% in patients with methylation ($P<0.001$).

DISCUSSION

In patients with malignancies, aberrant methylation of serum DNA has been reported (Müller *et al*, 2003; Ibanez de Caceres *et al*, 2004; Fiegl *et al*, 2005; Mori *et al*, 2005; Koyanagi *et al*, 2006). We have detected cell-free tumour DNA in serum of neuroblastoma patients (Gotoh *et al*, 2005). Prognosis in stage 4 neuroblastoma patients with metastases is poor despite intensive chemotherapy (Maris *et al*, 2007). Therefore, this study aimed to explore the possible prognostic significance of aberrant promoter hypermethylation of *RASSF1A*, which has been found frequently in neuroblastoma tumours, using pretherapeutic serum of neuroblastoma patients as a surrogate marker for circulating tumour cells.

We first investigated the *RASSF1A* methylation status in 68 neuroblastoma tumour DNA samples in comparison with matched serum DNA samples. The methylation of *RASSF1A* was observed in this study in 94% of primary tumours. Our results show that promoter hypermethylation of *RASSF1A* occurs at a high frequency in primary neuroblastoma tumours and no correlation between *RASSF1A* methylation and known prognostic factors including stage, age and *MYCN* amplification, or outcome was seen. The high proportion of *RASSF1A* promoter methylation in tumours agrees with earlier reports in the literature, which have found *RASSF1A* to be hypermethylated in 52–94% of tumour DNA samples (Astuti *et al*, 2001; Harada *et al*, 2002; Wong *et al*, 2004; Yang *et al*, 2004, 2007; Banelli *et al*, 2005; Lazcoz *et al*, 2006; Michalowski *et al*, 2008). Several earlier studies with one exception (Yang *et al*, 2004) failed to find a statistical correlation between *RASSF1A* methylation in tumours and poor outcome (Astuti *et al*, 2001; Harada *et al*, 2002; Banelli *et al*, 2005; Michalowski *et al*, 2008). We also did not observe any relationship between *RASSF1A* methylation in tumours and prognosis. *RASSF1A* hypermethylation in tumours can be a relatively early event in neuroblastoma tumorigenesis as it is detectable in non-advanced early-stage tumours with high frequency. Although the prognostic significance of epigenetic changes of single genes in neuroblastoma tumour DNA remain controversial, a few studies have indicated that poor prognosis is associated with the CpG island methylator phenotype

Table 4 Univariate analysis of relapse

Characteristic	Hazard ratio	95% CI	*P*-value
Age at diagnosis			
<12 months	Reference		
⩾12 months	12.5	2.8–55.3	<0.001
Sex			
Male	Reference		
Female	1.0	0.4–2.9	0.972
Stage			
1/2/3/4S	Reference		
4	14.3	4.0–51.0	<0.001
MYCN			
Non-amplified	Reference		
Amplified	7.2	2.6–20.0	<0.001
Serum RASSF1A			
Unmethylated	Reference		
Methylated	6.8	2.4–19.1	<0.001

CI = confidence interval.

Table 5 Multivariate analysis of survival

Characteristic	Hazard ratio	95% CI	*P*-value
Age at diagnosis			
<12 months	Reference		
⩾12 months	1.2	1.0–1.5	0.066
Sex			
Male	Reference		
Female	0.6	0.1–2.5	0.452
Stage			
1/2/3/4S	Reference		
4	8.4	1.5–46.4	0.014
Serum RASSF1A			
Unmethylated	Reference		
Methylated	2.4	0.6–9.2	0.194

CI = confidence interval.

(Abe *et al*, 2005; Banelli *et al*, 2005; Yang *et al*, 2007), suggesting that aberrant methylation of multiple genes is likely to contribute to neuroblastoma pathogenesis.

As a next step, we analysed *RASSF1A* methylation status in 68 paired serum DNA samples. In contrast to tumours, *RASSF1A* methylation was detected in neuroblastoma patient serum from only 25% (17 out of 68). To investigate the clinical significance of the serum *RASSF1A* methylation, associations with established prognostic factors and outcome were evaluated. *RASSF1A* methylation in serum was found to be statistically associated with established prognostic factors. Serum *RASSF1A* methylation was more frequently detected in neuroblastoma patients with age ⩾12 months at diagnosis ($P=0.002$), stage 4 ($P<0.001$) and *MYCN* amplification ($P<0.001$). Furthermore, the presence of methylation of *RASSF1A* in serum was associated with poorer outcome. The influence of serum *RASSF1A* methylation on prognosis was found to be comparable with that of the currently most reliable marker, *MYCN* amplification in univariate analysis. A subset analysis of stage 3 patients showed a trend associating poor survival with serum *RASSF1A* methylation ($P=0.055$), although the data were limited due to the small number of patients in the subgroup. In multivariate analysis of survival, methylation of *RASSF1A* in serum had a hazard ratio of 2.42, but this association did not reach statistical significance ($P=0.194$). Further validation studies using a larger set of patients are necessary to confirm our findings.

The presence of tumour-derived DNA within the blood stream has been identified earlier (Müller *et al*, 2003; Fiegl *et al*, 2005; Mori *et al*, 2005). Recently, one study showed that the detection of circulating tumour cells was correlated with tumour-related methylated DNA in patients with melanoma (Koyanagi *et al*, 2006), suggesting that circulating tumour cells are a potential source of circulating methylated DNA. Our study suggests that methylated *RASSF1A* DNA in serum is a surrogate marker for circulating neuroblastoma cells. Another recently published study showed that *RASSF1A* methylation was also detectable in ovarian cancer patient's serum at a high frequency from methylated tumour cases including several stage I tumours (Ibanez de Caceres *et al*, 2004). In the earlier study, there was no statistical association between tumour stage and positive detection in serum. However, some other studies have shown limited detection of *RASSF1A* methylation in the serum of patients with other neoplasms (Murray *et al*, 2004; Hesson *et al*, 2007). These differing results may suggest that free neoplastic DNA from ovarian cancer can access the blood stream more readily than that from other neoplasms including neuroblastoma.

In conclusion, this is the first study to examine epigenetic changes in a tumour suppressor gene, *RASSF1A*, the promoter of which is hypermethylated at a high frequency in neuroblastoma tumours, using serum DNA in a cohort of neuroblastoma patients. This study demonstrates the utility of detecting circulating methylated *RASSF1A*, which can be measured in serum, as a potentially predictive marker of neuroblastoma outcome. *RASSF1A* methylation in serum could have useful clinical applications in neuroblastoma management, if our results are confirmed in larger studies. However, we should not forget the limitation when attempting to translate our findings into the clinical fields as highly sensitive methylation analysis could be tricky because of incomplete bisulphite conversion by inexperienced analysts.

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CASE REPORT

Unusual fatty metamorphosis observed in diffuse liver metastases of stage 4S neuroblastoma

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Abstract We report a case of stage 4S neuroblastoma in which CT showed diffuse liver metastases containing a geographical fatty area in the periportal region. MRI showed this abnormality to correspond to an area with an unusual pattern of fatty change. ^{123}I-metaiodobenzylguanidine (MIBG) scintigraphy demonstrated increased accumulation throughout the liver, except for the region showing fatty change. To the best of our knowledge, this is the first report of liver metastases from neuroblastoma with geographical fatty infiltration.

Keywords Infantile neuroblastoma · Liver · Metastasis · ^{123}I-MIBG · MRI · Child

Introduction

Neuroblastoma (NB) is the most common malignant solid tumour of infancy and childhood. Age and initial stage are the important prognostic factors for this disease [1]. In the International NB Staging System, stage 4 encompasses all patients with distant disease, except a special category that is labelled stage 4S [2]. Stage 4S is defined as infants who have a small resectable primary tumour, with distant metastases restricted to the liver, bone marrow, or skin.

^{123}I-metaiodobenzylguanidine (MIBG) has high affinity for tumours derived from neural crest tissue, and is thought to be indispensable for staging and evaluation of response to therapy of NB [3]. Here, we report a case of stage 4S NB with diffuse liver metastases containing an atypical fatty area. In this case, MRI and ^{123}I-MIBG scintigraphy were helpful in the evaluation of the unusual liver finding demonstrated by CT.

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Case report

A 3-month-old girl with abdominal distension was admitted to our hospital. She was born at 39 weeks' gestation and delivery and early postnatal period were uncomplicated. Her parents first noticed her abdominal distension at 1 month of age that gradually increased over time. Physical examination revealed tachycardia, tachypnoea and hepatomegaly. Her laboratory findings were: white blood cell count 14.8×10^9/l, haemoglobin 10.9 g/dl, platelets 45.7×10^4/μl, AST 103 IU/l, LDH 348 IU/l. Some tumour markers were elevated; serum neuron specific enolase (NSE) 110 ng/ml, urine vanillylmandelic acid (VMA)/creatinine (Cr) 1,938 mg/g Cr, urine homovanillic acid (HVA)/Cr 1,058 mg/g Cr, α-fetoprotein (AFP) 12,435 ng/ml. Bone marrow studies showed a clump of atypical tumour cells (5.8%) and revealed normal karyotype with no rearrangement or gene deletion or N-myc gene amplification.

Contrast-enhanced CT scan (Fig. 1) demonstrated marked hepatomegaly and a low-attenuation area in the periportal region with a geographically widespread distribution. Intrahepatic vessels seemed to pass through this area normally. A

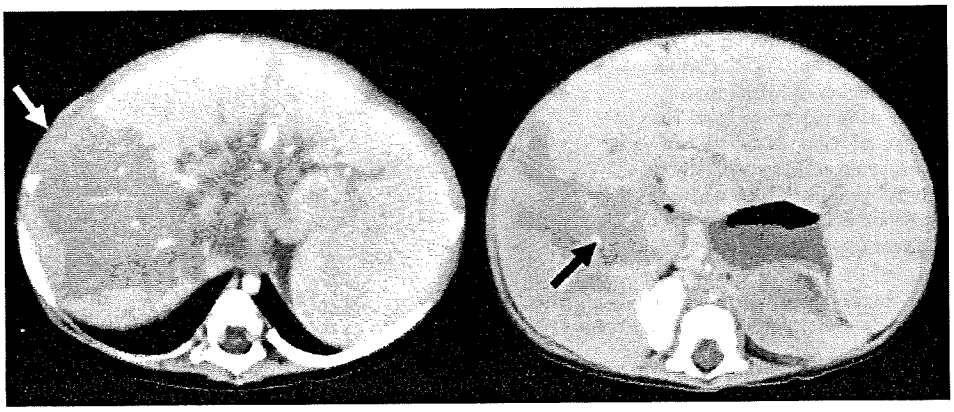

Fig. 1 Axial contrast-enhanced CT shows a geographically hypodense area in the periportal region (*white arrow*), in which the vascular architecture can be identified (*black arrow*)

heterogeneous mass was seen in the left adrenal grand. No enlarged lymph nodes were observed.

Abdominal MRI (Fig. 2) was performed for further evaluation. The area corresponding to the low-attenuation region on CT was hyperintense on T1-weighted (T1-W) in-phase images with decreasing signal on out-of-phase imaging and hypointense on superparamagnetic iron oxide (SPIO)-enhanced imaging. SPIO-enhanced imaging demonstrated hyperintense signal spread widely throughout the liver with sparing of the periportal region. Inhomogeneous nodular high signal was visualized on T2-weighted (T2-W) imaging. These findings indicate that the low-attenuation area represents fatty change with normal Kupffer cell function, while abnormal cells had infiltrated the remainder of the liver. The left adrenal mass had inhomogeneous high signal intensity on T2-W images with very intense signal in its centre suggesting cystic change.

^{123}I-MIBG scintigraphy (Fig. 3) was performed to confirm the diagnosis of NB and for staging. Intense accumulation was seen in the markedly enlarged liver. The periportal area with SPIO accumulation was photopenic on single photon emission CT (SPECT). Areas of

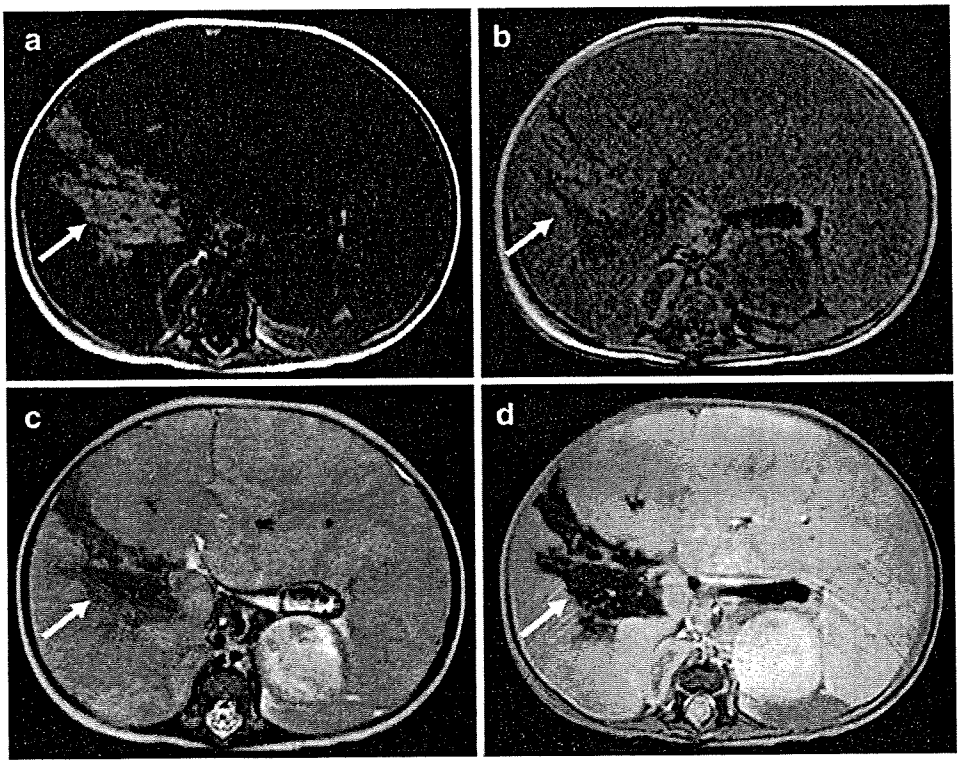

Fig. 2 Axial MRI. The periportal area is demonstrated as a fat-containing area by high signal on **a** T1-W in-phase and decreased signal on **b** T1-W out-of-phase images. Normal Kupffer cell function is shown by hypointense signal on **c** T2* SPIO-enhanced and **d** proton density-weighted SPIO images with surrounding diffusely abnormal areas without SPIO concentration (*arrows*)

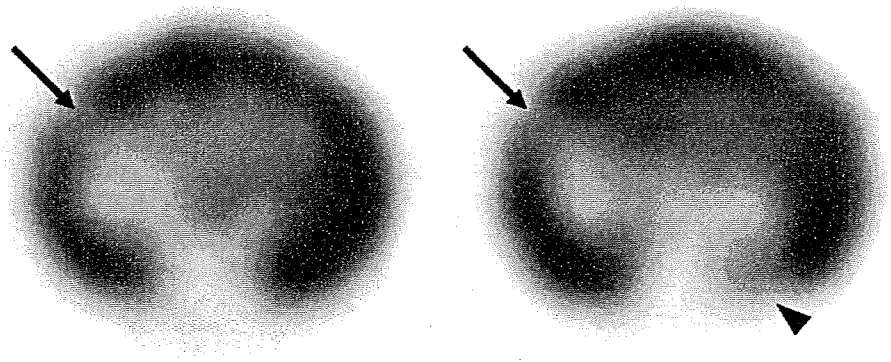

Fig. 3 ^{131}I-MIBG SPECT. Images shown correspond to the CT levels of Fig. 1. Intense accumulation of MIBG is seen in the periphery of the liver. The periportal region is shown as a photopenic area (*arrow*). Abnormal ring-like MIBG accumulation is seen in the cystic left adrenal mass (*arrowhead*)

doughnut-like abnormal accumulation were also seen in the cystic left adrenal gland mass. Bone marrow biopsy revealed infiltration of NB cells, and so the girl was diagnosed with left adrenal NB with liver and bone marrow metastases (stage 4S).

Since she had severe dyspnoea caused by the prominent hepatomegaly, radiotherapy was performed. When the dyspnoea had improved, chemotherapy was commenced. Four months later, the fatty area without MIBG accumulation increased in size, and the hyperintense area on T2-W images that showed MIBG accumulation became restricted to the periphery of the liver (Fig. 4). Following radiotherapy, six courses of chemotherapy and resection of the primary tumour, she is under observation.

Discussion

Approximately 80% of cases of stage 4S NB have liver metastases [4]. In previous reports, the morphological characteristics of the liver metastases were divided into two patterns; multiple nodular and diffuse infiltrative that spread throughout the liver [5]. Liver enlargement is frequently observed.

In our case, characteristic geographical fatty infiltration surrounding the porta hepatis was seen associated with diffuse tumour throughout the liver. SPIO-MRI and ^{123}I-MIBG scintigraphy were useful for the diagnosis of NB liver metastases, and revealed that the area of fatty change was spared from the surrounding diffuse tumour infiltration.

Stage 4S NB often disappears without treatment. The Ras gene is considered to work as a mediator of the programmed cell death system [6]. When tumour growth is more rapid than the natural death of tumour cells, marked liver enlargement causes dyspnoea and renal dysfunction resulting in early patient demise. However, some tumours vanish with no treatment (when natural death of the tumour cells is faster than tumour growth) and patients in this group have a good prognosis.

In the liver, SPIO particles are sequestered by reticuloendothelial Kupffer cells. Their superparamagnetic properties cause a shortening of the T2 time and subsequent decrease in the signal intensity of liver containing normal Kupffer cells. In this case, SPIO-MRI was useful to distinguish the fatty area from tumour infiltration. The periportal fatty area, with SPIO concentration and without high MIBG accumulation, surrounded by active NB is a unique and interesting finding in this case. However, we could not obtain pathological proof of this. The expansion of this area after treatment raises the possibility of it reflecting the natural regressive course of 4S tumour.

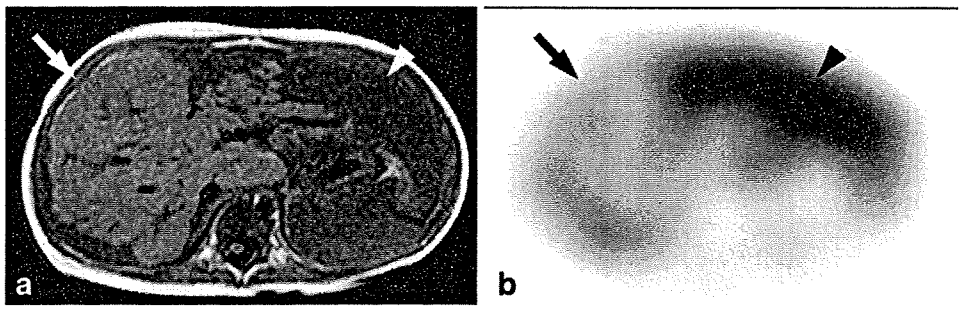

Fig. 4 Follow-up imaging 4 months after chemotherapy. **a** T1-W in-phase axial MRI and **b** MIBG SPECT. The liver has decreased in size and the fatty area without MIBG accumulation has expanded (*arrow*). The hypointense area with MIBG accumulation is restricted to the periphery of the liver (*arrowhead*)