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神経芽細胞腫のスクリーニングの展望と問題点

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はじめに

神経芽細胞腫は，その予後が腫瘍の生物学的悪性度に大きく依存し，分化・退縮する予後良好な腫瘍と集学的治療にもかかわらず進行が速く悪性度の高い腫瘍が存在する。前者は乳幼児に多く，後者は1歳以上に多いことから，1980年頃から生後6カ月児を対象として神経芽細胞腫マススクリーニング（以下神経芽マス）が開始され，1985年に全国的な神経芽細胞腫検査事業として開始された。その結果，この事業が施行後，神経芽細胞腫の発生率が2倍以上に上昇し，予後良好な腫瘍の発見が増加した。通常，検診などを開始すると一次的に診断される数が増加するが，その後減少して元の発症率に復帰するはずである。しかし，神経芽細胞腫の罹患数は減少せず，予後良好な腫瘍の絶対数が増加し，明らかに過剰診療であることが指摘され，実際に予後不良な神経芽細胞腫が神経芽マスによって減少したか否かの明らかなエビデンスが少ないことから，平成15年度に休止が決定した。

神経芽マスの有効性，施行の是非に関しては未だに賛否両論があり，結論に至っていないのが現状と言わざるを得ない。ドイツや北米のケベックなどで，神経芽マスの有効性に関するスタディが行われた[1,2]が，評価に値する十分なスタディとは言いがたい面も残されている。世界に類をみない大規模な検査事業を行ってきた本邦は，神経芽マスに対して，正しい効果判定と有効性，問題点を評価する義務があると考えている。

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神経芽細胞腫マススクリーニングの効果について

神経芽マスを全国的に展開する以前に，この有効性と蓋然性についてパイロットスタディを行うことが必要であったことは否めないが，この検査事業を行ったことでこの腫瘍の本態がかなり明らかになったことも事実であり，この議論は結果論であることも否めないところもある。

現在までに，本邦では，さまざまな施設や機関からマススクリーニングの効果に関する報告がなされてきている（表）[1~9]。受検者と非受験者の比較をしたものと施行前後の比較をしたものに大きく分けられる。前者では有意に死亡率が低下したとの報告が多く，後者では有意であったとの報告と，ないとの報告に分けられる。前者の研究のなかで，規模が大きな報告としては，コホート研究として厚生労働研究として行われた久繁班の研究と，林らによる前向きコホート研究があり，両者とも有意に死亡率が低下したと報告している。神経芽マスがある程度有効性があることを示唆しているといえるが，コストベネフィットの面でどの程度有効性があるのかを示すまでに至っていない。

一方，日本では施行地域と非施行地域が同時期にデータを得ることができなかったために，カナダのケベックとドイツで行われたスタディがよく引用され，対照地域の選択，施行時期の問題などもあるが，有意な死亡率が得られていないことが

表　神経芽細胞腫マススクリーニング（MS）の効果に関する主な報告

報告地域（報告者、年度）	観察対象月齢（施行時期）	対照群	検査法	罹患率（受診群/対照群）10万対	罹患率の比（95%信頼区間等）	死亡率（受診群/対照群）	死亡率の比（95%信頼区間等）
ドイツ（Shilling, 2002）	12～60（12）	未実施地域	定量	14.3/7.3	1.94	1.4/1.2	1.17
ケベック（Woods, 2002）	0～71（3週と6）	未実施地域	定性	不明	2.85（1.79～2.57）	4.78/3.33～5.29	0.90～1.40
25道府県（久繁，2001）	6～84（6）	未受検者	定量	20.02/11.63	1.72	1.855/3.403	0.55
全国（林，2005）	6～72（6）	未受検者	定量	—	—	1.54/4.31	0.281
九州（Suita, 2002）	0～60（6）	未受検者	定量	16.9/11.8	1.43	1.4/0	—
7県（Yamamoto, 2002）	0～59（6）	実施前	定性	14.18/9.82	1.44（p=0.01）	2.98/4.21	0.71（p=0.21）
			定量	25.98/9.82	2.65（p<0.0001）	2.18/4.21	0.52（p=0.07）
札幌市（Nishi, 1997）	0～48（6）	実施前	定性	24.41/11.97	2.04	4.88/6.21	0.79（p>0.5）
			定量	19.98/11.97	1.66	1.05/6.21	0.17（p<0.5）
新潟県（Asami, 1995）	0～60（6）	実施前	定性	18.6/10.5	1.77	4.5/5.9	0.76
			定量	22.1/10.5	2.10	0/5.9	0
7府県市（Ajiki, 1998）	1～48（6）	実施前	定性	12.0/8.20	1.5（1.2～1.7）	2.6/3.5	0.8（0.5～1.0）
			定量	20.8./8.20	2.5（2.3～2.8）	1.4/3.5	0.4（0.3～0.6）
九州（Suita, 2002）	0～60（6）	実施前	定量	16.9/6.1	2.77	1.4/2.1	0.67（p=0.419）
大阪府（Honjo, 2003）	0～168（6）	実施前	定性	15.2/7.4	2.05（p<0.05）	5.4/4.3	1.26
			定量	23.5～26.8./7.4	3.18～3.62	4.0～4.1/4.3	0.93～0.95（p>0.05）
大阪（Ajiki, 1998）	0～180（6）	実施前	定性/定量	20.5/7.5	2.8（1.3～5.7）	4.3/4.3	1.0（0.3～2.9）

報告され[1,2]，今回のこの事業の休止の大きな根拠になったことは否めない。これらのスタディが正しく計画され，実施されたものでないことから，この結果の評価も一定せず，果たして神経芽マスが有効であるのかどうかを多くの人に納得させるエビデンスが乏しいというのが現実である。

神経芽細胞腫マススクリーニングの特殊性と問題点

新生児マススクリーニングにおいて発見される代謝異常の患者は，無症状あるいは症状が軽い時期に早期発見して治療を開始することで通常の発達が期待され，発見例の受ける利益は大きなものがあり，通常1回のスクリーニングで相応の効果

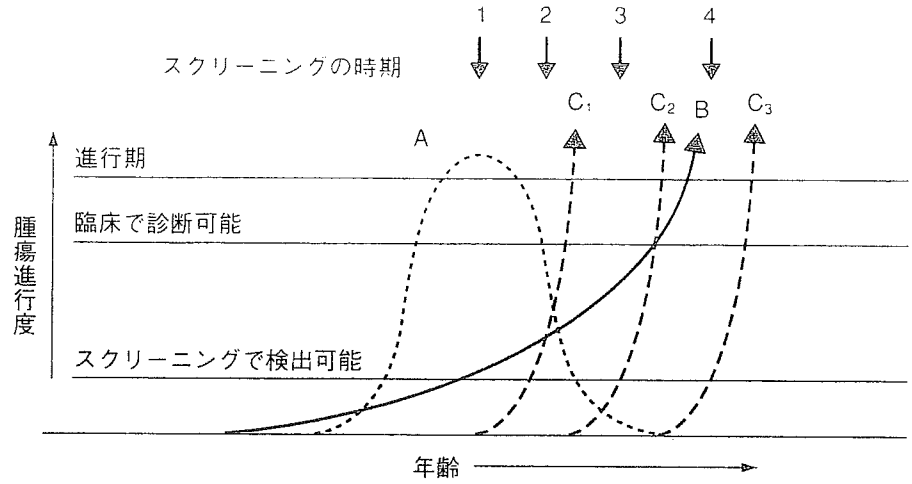

図　神経芽細胞腫スクリーニングの時期

A：自然退縮・分化する腫瘍，B：年齢に従い進行する腫瘍，$C_{1\sim3}$：急速に増大する腫瘍

スクリーニングを1の時期に行うと大部分がAの腫瘍で過剰診断となる。2の時期であれば，半数以上は将来進行する腫瘍が発見されるが，過剰診断の症例もかなりある。3の時期に行うと過剰診断はほとんどなく，B，C_2の腫瘍を早期発見できる。4の時期であれば過剰診断はないが，C_3を除いては早期発見できず，すでに進行している。神経芽細胞腫の多くが$C_{1\sim3}$のような発症形式であれば，1回のスクリーニングで早期発見することは困難であり，複数回の検査が必要となる。

が期待される。一方，腫瘍である神経芽マスは，確かに無症状あるいは症状が軽い早期に発見して治療を開始することで予後が改善し，恩恵を受ける児は確かに存在するのであろうが，この腫瘍の発生形態が明確でないことから，図のごとく，神経芽マスを行う時期によって効果は異なり，また，1回の検査で十分な効果が得られるという科学的根拠もなく，効果を上げるためには複数回行うことも必要かもしれない[2]。従来のスクリーニングは，科学的根拠なしに施行時期が決定されたことから，効果もそれなりのものであった可能性もある。

もう一つの大きな問題は，過剰診断と過剰治療である。生後6カ月で行われた神経芽細胞腫検査事業の成果ともいうべきかもしれないが，従来，1歳未満発見のこの腫瘍が予後良好であったとの臨床経験は，その多くが自然退縮あるいは分化する腫瘍であったことによる。すなわち，神経芽マスによって罹患率が2倍以上に上昇したことから，従来，発見されずに退縮あるいは分化していた腫瘍が乳児期に数多く存在していたことが明らかになったのである[10,11]。従来，自然に退縮あるいは分化する腫瘍が神経芽細胞腫の中に存在することは知られた事実であった[12]が，神経芽細胞腫検査事業はこの腫瘍の自然歴解明に大きなエビデンスを与えたことにちがいはない。さらに，実際に自然に退縮あるいは分化していることが明らかになった頃，第2の過ちを犯してしまったのである。再び何も明らかなスタディがなく，倫理性も十分討議されないまま，経験論から，各施設の判定基準も異なった形で無治療経過観察を始めたのである[10,11]。自然歴が明らかでないまま，大きさ，部位，年齢などの因子から判定し，無治療経過観察となった患者の長期経過観察，家族への負担，医療費などのコストベネフィット，分化した腫瘍は増大しないのかなどの諸問題を棚上げし，いきなり無治療経過観察が開始されたのである。代謝性疾患の擬陽性とは，全く異なることで，再検査により健常と診断されれば健常児であるが，この場合は，実際に腫瘍が体内に存在している。軽症で治療対象にならないものと考えることもできるが，これらの保証はなく，転移したり，進行例に

なってから治療することは果たして倫理的にも正しいのかどうかの議論も必要であった。無治療経過観察が間違った治療法であるといっているのではなく，この治療方針の選択の方法に疑問を投げかけているのである。神経芽マスが始まる前は，こうした患者は臨床上発見されれば無論のこと治療されてきたわけであり，これらは過剰診断，過剰治療といわれてはいない。マススクリーニングが休止になれば，こうした患者は出てこないのかというと，そんなはずはなく，欧米でも incidental neuroblastoma として報告されている[13]ように，臨床上発見される。マススクリーニングでないから治療してよいのか？　マススクリーニングの休止後であるから，無治療経過観察はしないのか？　無治療経過観察の選択肢を患者に提示してきた医療側の選択基準を今こそ明確にすべきかもしれない。そのためにも，神経芽マス施行中のこれらの腫瘍の特性を正確かつ緻密に解析し，自然歴を把握した後に，無治療経過観察が治療選択の一つとして成り立つ根拠を提示し，患者側にその選択肢を示すことが必要ではないのかと考えている。

測定上の問題点

神経芽マスの問題点として，測定値の精度管理もあげられる。尿中の VMA, HVA を高速液体クロマトグラフィー（HPLC）で測定し，その値をクレアチニン（Cr）で除した値を用いてきたが，施設間のばらつきも大きく，その原因として，技術不足，器械の老朽化，測定条件などがあげられる。新生児マススクリーニングで行われているような外部精度管理の導入がなければ，陽性者を正常と判定したり，その逆も起きうる可能性が高く，今後の神経芽細胞腫をスクリーニングするのであれば，外部精度管理の導入は必至と考えられる[14]。最近，タンデムマスで VMA, HVA を測定する方法が報告されてきており，新たな方法の導入も考慮すべきかもしれない[15]。

新たなマススクリーニングにむけて

6 カ月時に施行された神経芽細胞腫検査事業は，死亡率低下があったもののその過剰診断の多さから，その施行時期を変更して行うことが現在検討されている。すでに，札幌市では生後 14 カ月，大阪府，京都府では生後 18 カ月での神経芽マスが開始され，陽性で本症と診断された児も少しずつ報告されてきている。今，求められているのはこうした時期に行う神経芽マスが果たして有効であるかどうかを正確な臨床研究として行うことである。倫理的問題から，まず神経芽マスを受ける児の親に正しい説明と理解を求め，臨床研究であることのインフォームドコンセントを得ておくことが前提となる。そして，どの時期に行うことが最も有効であるかを綿密に検討し，さらに有意な結果が得られる人口を対象として，神経芽マスの前向き研究を行うことが必要である。この結果を評価し，神経芽マスを施行すべきか否かを判定すべきである。現在，上記の地方公共団体を中心に，これと同等の発生率の地域を対照として，前向き研究を行うことが計画されつつある。施行時期についても，多くの意見があるが，過剰診断を最小にすることと予後不良の神経芽細胞腫の発生状況から，生後 16〜18 カ月が適切であることが示唆されている。過剰診断を最小にする時期の根拠は，無治療経過観察例の尿中 VMA・HVA の経過からその多くの症例が正常化する時期があげられる[11]。病理の嶋田分類においても，18 カ月が予後を層別化する年齢であり[16]，今後生後 18 カ月以降と以前でリスク分類しようとする動きもあり，治療を一定にする意味においても生後 18 カ月でまず検討し，その結果が有効であれば，より有効な時期をさらに詳細に検討するとともに，新たなスクリーニング事業のあり方を行政とともに検討し，構築することが必要になると考えている。

おわりに

神経芽マスは平成 15 年にいったん休止され，その後のこの疾患の動向は極めて興味深いところである。死亡率が有意に低下したとの事実もあり，過剰診断を最小にしたスクリーニング事業にむけた検討が必要な時期であると考えられ，解決すべき問題点も数多く存在し，今後の慎重かつ迅速な

対応が求められている領域である。

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お知らせ（6）

第 21 回日本糖尿病・妊娠学会

日　時：2005 年 11 月 25 日（金），26 日（土）
会　場：岡山コンベンションセンター
岡山市駅元町 14-1　TEL：086-214-1000
会　長：平松祐司（岡山大学産科・婦人科学教授）
演題締切：平成 17 年 7 月 31 日
特別講演：インスリン抵抗性に関する最近の知見
門脇　孝教授　東京大学
招請講演：Recent Progress in Understanding the Molecular Causes of Diabetic Embryopathy
Mary R. Loeken 博士（Joslin Diabetes Center, Harvard Medical School）
シンポジウム：糖尿病合併妊婦の分娩前後の取り扱い
ワークショップ，一般演題，ランチョンセミナー
学会事務局：第 21 回日本糖尿病・妊娠学会事務局
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詳細は学会ホームページ（http：//202.238.86.111/jsdp）または事務局までお問い合せください。

Pediatr Blood Cancer 2006;46:285–291

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

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

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

INTRODUCTION

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

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

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

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

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

PATIENTS AND METHODS

Patients and Specimens

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

Interphase FISH and MYCN Copy Number Analyses

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

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

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

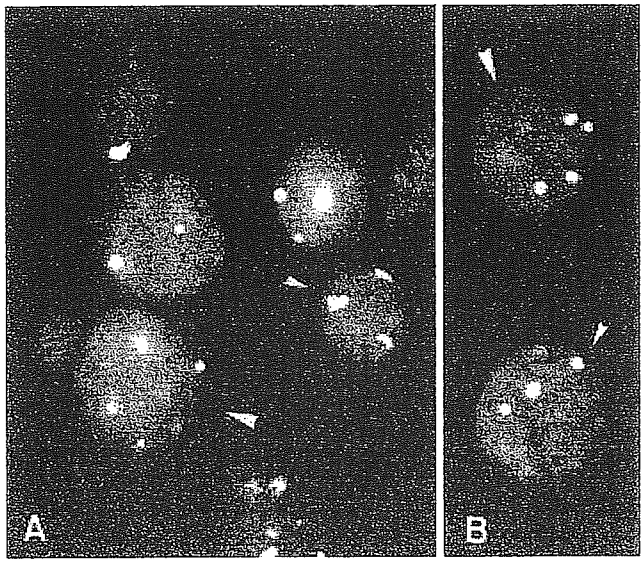

Fig. 1. Interphase FISH analysis using a chromosome 1 probe (D1Z1) on Tris1 tumors with a mixed population of cells with trisomy 1 and tetrasomy 1, with or without pentasomy 1. Cells in A and those in B were derived from 2 different tumors. Large and small arrowheads show cells with tetrasomy 1 and cells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

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

Flow Cytometry

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

Statistical Analysis

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

RESULTS

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

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

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

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

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

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

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

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

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

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

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

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

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

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

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

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

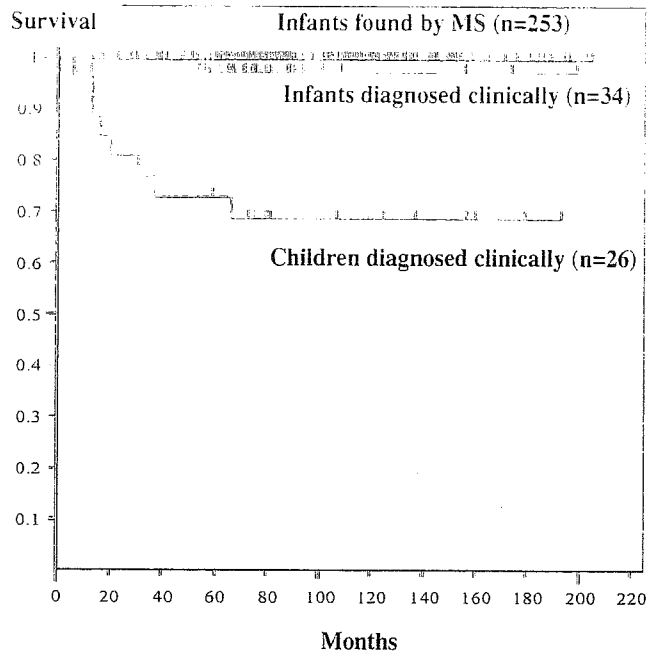

Fig. 2. The OS curve for 253 type 1 infants found by MS or that for 34 type 1 infants diagnosed clinically was better than that for 26 type 1 children diagnosed clinically ($P < 0.001$ and $P = 0.005$).

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

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

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

DISCUSSION

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

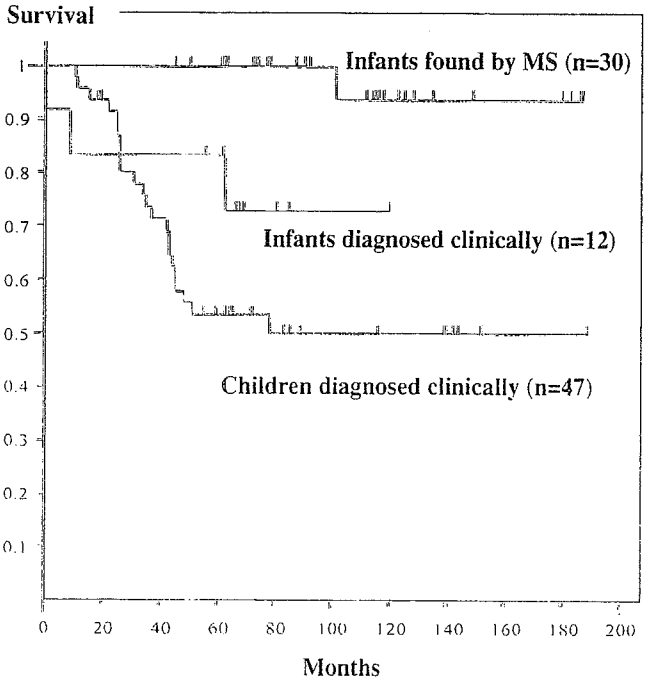

Fig. 3. The OS curve for 30 type 2 infants found by MS was better than 12 type 2 infants diagnosed clinically ($P = 0.014$), or 47 type 2 children diagnosed clinically ($P < 0.001$).

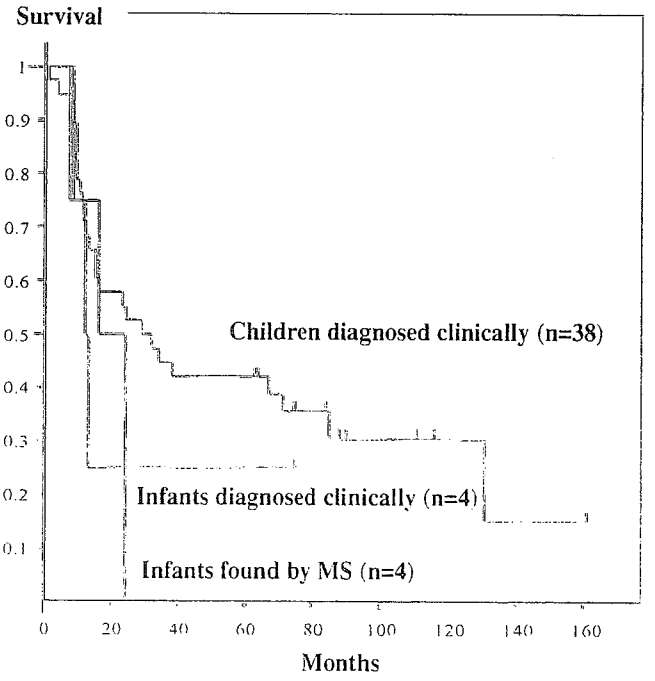

Fig. 4. OS curves for four type 3 infants found by MS, four type 3 infants diagnosed clinically, and 38 type 3 children diagnosed clinically. There were no significant differences between any two of the three groups.

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

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

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

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

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

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

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

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

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

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

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Clinical significance of serum NM23-H1 protein in neuroblastoma

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We have previously reported that *NM23* genes are overexpressed in various hematological malignancies and that serum NM23-H1 protein levels are useful for predicting patient outcomes. In this study we assessed the clinical implications of serum NM23-H1 protein on neuroblastoma. We examined serum NM23-H1 protein levels in 217 patients with neuroblastoma, including 131 found by mass-screening and 86 found clinically by an enzyme-linked immunosorbent assay, and determined the association between levels of this protein, and known prognostic factors or the clinical outcome. The serum NM23-H1 protein level was higher in neuroblastoma patients than in control children ($P < 0.0001$). Patients with *MYCN* amplification had higher serum NM23-H1 levels than those with a single copy of *MYCN*. Overall survival was assessed in the 86 patients found clinically, and was found to be worse in patients with higher serum MN23-H1 levels (≥ 250 ng/mL) than in those with lower levels (< 250 ng/mL; $P = 0.034$). The higher level of NM23-H1 was correlated with a worse outcome in patients with a single *MYCN* copy, or in those younger than 12 months of age. Serum NM23-H1 protein levels may contribute to predictions of clinical outcome in patients with neuroblastoma. (*Cancer Sci* 2005; 96: 653–660)

The *NM23* gene was identified by differential hybridization of a cDNA library with total RNA extracted from slightly and highly metastatic melanoma cell lines.[1] The *NM23* gene has been identified as a family of genes encoding different isoforms of nucleoside diphosphate kinase (NDPK).[2] *NM23* genes play critical roles in cellular proliferation, differentiation, oncogenesis, and tumor metastasis.[1,3] The mechanisms for these pleiotropic effects are not well understood. Eight isotypes of the human *NM23* gene (*NM23-H1*, *NM23-H2*, *NM23-H3/DR-NM23*, *NM23-H4*, *NM23-H5*, *NM23-H6*, *NM23-H7*, and *NM23-H8*) have been identified.[2] Among these, only *NM23-H1* and *NM23-H2* have been studied extensively in human cancers.

The level of *NM23-H1* expression is inversely correlated with the tumor's metastatic potential in experimental rodent cells and in human tumors such as breast, ovarian, cervical and gastric cancer, hepatocellular carcinoma, and melanomas.[4] Therefore, *NM23-H1* is implicated in the regulation of metastasis in a variety of human cancers. However, overexpression of the *NM23-H1* gene has been reported in various neoplasms including neuroblastoma, hematological malignancies, and pancreatic, lung, ovarian and gastric cancers.[5–8] Overexpression of *NM23-H1* is indicative of a poor patient prognosis for patients with neuroblastoma, acute myelogenous leukemia (AML), or non-Hodgkin's lymphoma (NHL).[8–10]

In neuroblastoma, a gain of 17q is the most frequent genetic abnormality, followed by 1p deletion and *MYCN* amplification, both of which correlate closely with 17q gain. The three genetic events are strong predictors of unfavorable prognosis.[11,12] The *NM23* genes are located at the edge of the common chromosomal region of 17q gain. Godfrid *et al.* identified genes that are activated in the *MYCN* downstream pathway using SAGE libraries of *MYCN*-transfected and control neuroblastoma cell lines.[13] The *NM23-H1* and *NM23-H2* genes are strongly induced in *MYCN*-expressing cells. Neuroblastoma tumor and cell line panels reveal a striking correlation between *MYCN* amplification and mRNA or protein expression of both *NM23* genes. These findings suggest that *NM23-H1* and *NM23-H2* expression may be increased by 17q gain in neuroblastoma, and can be further upregulated by *MYCN* overexpression. These observations suggest a role of *NM23-H1* and *NM23-H2* in the tumorigenesis of an unfavorable type of neuroblastoma.

We previously established an enzyme-linked immunosorbent assay (ELISA) technique for determining the serum level of NM23-H1 protein.[14] Serum levels of NM23-H1 in patients with NHL and AML are significantly higher than those in controls, and elevated NM23-H1 levels correlate with poor prognosis in these patients.[10,15] It has been strongly suggested that serum NM23-H1 protein is produced directly by tumor cells and its level depends on the total mass of malignant cells overexpressing *NM23-H1*.[14,16] These results indicate that the serum level of NM23-H1 protein may be clinically useful as a prognostic factor in NHL and AML. The present study assessed the clinical implications of serum NM23-H1 protein levels in patients with neuroblastoma, in whom tumor samples were used to determine the biological prognostic factors.

Materials and Methods

Patients and controls

Serum NM23-H1 protein was measured in 217 untreated neuroblastoma patients who were admitted to various institutions in Japan and underwent biopsy or surgery between 2000 and 2002. The 217 patients included 131 who were found by

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a mass-screening (MS) program for infants at 6 months of age by measuring urinary catecholamine metabolites and 86 who were found clinically.[17] Of the 86 patients, 29 who were younger than 12 months old were mostly found before MS, and 57 who were 12 months old or older underwent MS with a negative result, or did not undergo MS. Patients were staged according to the International Neuroblastoma Staging System (INSS).[18] Patients of any age with stage 1 or 2 disease, and those younger than 12 months of age with stage 3 disease were treated by surgery or surgery and chemotherapy consisting of cyclophosphamide and vincristine; patients 12 months or older with stage 3 or stage 4 disease and those younger than 12 months of age with stage 4 disease were treated according to the protocol published by the Japanese Neuroblastoma Study Group.[19] Serum samples from 23 children consisting of 22 with inguinal hernias and one with an edematous scrotum before surgery were analyzed for comparison. The median age of the children was 23 months (range: 3–49 months). Informed consent was obtained from patients and/or their parents, and the ethics committee of Saitama Cancer Center approved the study design.

Venous blood samples

Peripheral venous blood samples were collected in sterile test tubes with heparin and placed on ice. The samples were centrifuged at 2000*g* for 15 min at 4°C, and stored at −20°C. As a marker of hemolysis, free serum hemoglobin (Hb) was determined according to the method of Testa *et al.*[20]

ELISA for human NM23-H1 protein

NM23-H1 protein levels in serum were determined using a sandwich ELISA assay, as described previously.[14,15] Recombinant NM23-H1-GST protein was used as a standard.

Examination of *MYCN* copy number, TRKA expression and ploidy

DNA preparation, digestion, and Southern blot analysis using the *MYCN* probe were carried out as described previously.[12] The presence of more than three copies of the *MYCN* gene per haploid genome was considered to indicate amplification.[21] *TRKA* expression was examined by northern blotting as reported previously.[22] DNA index was analyzed on a Becton-Dickinson FACScan flow cytometer by DNA cell-cycle analysis software (version C).

Statistical analysis

The significance of differences in various clinical and biological aspects of the disease among the patient groups was examined by using the Mann–Whitney *U* or Kruskal–Wallis test (non-parametric analysis). Spearman's correlation coefficient (rs) by ranks was used to evaluate the correlation between paired values. Survival analysis was performed according to the Kaplan–Meier method, and the significance of differences in survival was determined by using the generalized Wilcoxon's and log-rank tests. A multivariate analysis of prognostic factors was performed using Cox's proportional-hazards regression model. All statistical analyses were performed with Excell Statcel and Stat Flex software (version 5.0, Artech Co. Ltd, Osaka, Japan), and $P < 0.05$ was taken to indicate significance.

Results

Examination of serum NM23-H1 protein levels in neuroblastoma patients and control children

The serum level of NM23-H1 was examined in 217 neuroblastoma patients and 23 control children. The serum levels of NM23-H1 were significantly higher in patients with neuroblastoma ($n = 217$, mean ± SD 176 ± 280 ng/mL) than in the control children ($n = 23$, 27 ± 41 ng/mL, $P < 0.0001$; Fig. 1a). The serum NM23-H1 levels of the control children were higher than those of the healthy adults (data not shown). The serum NM23-H1 levels in patients with neuroblastoma were significantly higher than those in patients with various hematological malignancies (data not shown). Next, the relationship between serum levels of NM23-H1 and Hb was examined in 217 neuroblastoma patients and 23 control children, because the NM23-H1 protein leaked from red blood cells by hemolysis may have elevated the serum NM23-H1 levels.[23] The results showed a weak correlation (rs = 0.3958, $P = 7.5356 \times 10^{-10}$, Spearman's correlation coefficient by ranks), although some patients had a higher Hb level but a lower NM23-H1 level, or a lower Hb level but a higher NM23-H1 level (Fig. 1b). When we chose samples from 156 patients

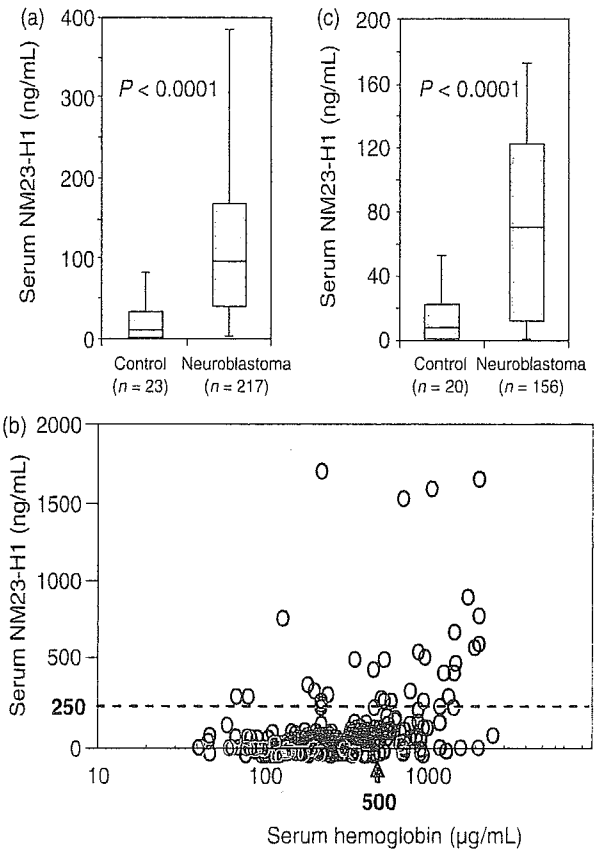

Fig. 1. Serum NM23-H1 levels in patients with neuroblastoma and in control children. (a) Box plots of NM23-H1 serum levels for 217 patients with neuroblastoma and 23 control children with any serum hemoglobin levels. (b) Relationship between the serum levels of NM23-H1 and hemoglobin in all samples examined (black circles, neuroblastoma patients [$n = 217$]; red circles, control children [$n = 23$]). (c) Box plots of NM23-H1 serum levels for 156 patients with neuroblastoma and 20 control children with serum hemoglobin levels less than 500 μg/mL.

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Table 1. Relationship between serum NM23-H1 protein levels and clinicopathological findings in 217 patients with neuroblastoma and 23 control children

Clinicopathological findings	Number of patients (mean ± SD)	Serum NM23-H1 (ng/mL)	*P*-value (analysis)
Control children	23	27 ± 41	
All patients	217	176 ± 280	< 0.0001 (MW)
Method of detection			
Mass-screening	131	135 ± 206	
Found clinically	86[†]	239 ± 357	0.0595 (MW)
Age of patients			
< 12 months	134	168 ± 292	
≥ 12 months	83	190 ± 260	0.2427 (MW)
Stage of the disease			
1 + 2 + 4s	122	136 ± 159	
3 + 4	95	227 ± 378	0.8088 (MW)
Primary site			
Mediastinum	31	145 ± 212	
Adrenal	101	187 ± 290	
Abdomen	78	184 ± 302	0.3393 (KW)
Others	7	74 ± 82	
MYCN copy number			
1	186	143 ± 204	
> 3	31	378 ± 519	0.0006 (MW)
TRKA expression	173		
Medium or high	125	150 ± 209	
None or low	48	238 ± 373	0.4629 (MW)
Ploidy	168		
Diploid	69	188 ± 273	
Hyperdiploid	99	185 ± 284	0.9012 (MW)
Others	7	112 ± 126	

MW, Mann–Whitney *U*-test; KW, Kruskal–Wallis test. [†]Table 2.

and 20 control children with serum Hb less than 500 μg/mL, the correlation between serum NM23-H1 and Hb levels was negligible (rs = 0.2351, $P = 0.0035$). Even in these patients, the serum levels of NM23-H1 were significantly higher (n = 156, 113 ± 184 ng/mL) than in the control children (n = 20, 20 ± 35 ng/mL, $P < 0.0001$; Fig. 1c).

Relationship between serum NM23-H1 protein levels and clinicopathological features in neuroblastoma

The relationship between serum NM23-H1 levels and various clinical and biological features in the 217 patients is shown in Table 1. The serum NM23-H1 levels tended to be higher in patients found clinically than in those found by MS ($P = 0.0595$), and were significantly higher in patients with amplified *MYCN* copies than in those with a single *MYCN* copy ($P = 0.0006$; Table 1). There was a correlation between *MYCN* amplification and the elevated serum NM23-H1 level (≥ 250 ng/mL) in all 217 patients (rs = 0.6970, $P = 0.0005$). However, serum Hb concentrations did not correlate with *MYCN* amplification ($P = 0.6320$), or other factors (data not shown). There was no significant difference in the serum NM23-H1 levels between two groups of patients classified by age of the patients, stage of the disease, expression levels of *TRKA*, or tumor cell ploidy (Table 1).

Serum NM23-H1 levels and overall survival

Of the 217 patients, the 86 patients who were found clinically were included and the 131 patients found by MS were excluded from survival analysis, because all the 131 patients were alive at the last follow-up (18–51 months), and the clinical and biological features are different for the patients found by MS and those found clinically.[12] The relationship between serum NM23-H1 levels and various clinical and biological features in the 86 patients was similar to that found for all 217 patients (Tables 1, 2). The 86 patients were divided into two groups according to various cut-off points over 100 ng/mL, which was the upper limit in control serum (mean + 2 × SD = 20 + 2 × 35 = 90). The cut-off points used here were 100 ng/mL (< 100, n = 39, *vs* ≥ 100, n = 47), 150 ng/mL (< 150, n = 54, *vs* ≥ 150, n = 32), 200 ng/mL (< 200, n = 60, *vs* ≥ 200, n = 26) and 250 ng/mL (< 250, n = 64, *vs* ≥ 250, n = 22). The cut-off value of greater than 250 ng/mL showed the most significant prognostic effects with generalized Wilcoxon's and log-rank test analysis (data not shown). Therefore, we used 250 ng/mL of serum NM23-H1 as a cut-off value. As shown in Figure 2a, patients with the higher serum NM23-H1 levels had worse overall survival than those with the lower levels ($P = 0.0219$ according to the generalized Wilcoxon test, $P = 0.0340$ according to the log-rank test). Overall survival was significantly worse for patients who were 12 months or older than for those younger than 12 months of age ($P = 0.0364$ and $P = 0.0158$), for patients at stages 3 and 4 than for those at stages 1, 2 and 4S ($P = 0.0157$ and $P = 0.0082$), and for patients with *MYCN* amplification than for those with a single copy of *MYCN* ($P = 0.0195$ and $P = 0.0054$; Fig. 2b,c,d). These results

Table 2. Relationship between serum NM23-H1 protein levels and clinicopathological findings in 86 patients with neuroblastoma found clinically

Characteristics	No. of patients (mean ± SD)	Serum NM23-H1 (ng/mL)	*P*-value (analysis)
All patients	86	239 ± 357	
Age			
< 12 months	27	282 ± 471	
≥ 12 months	59	219 ± 294	0.7694 (MW)
Stage			
1 + 2 + 4s	21	154 ± 187	
3 + 4	65	266 ± 394	0.3900 (MW)
Primary site			
Mediastinal	11	124 ± 207	
Adrenal	46	285 ± 383	
Abdominal	26	220 ± 375	0.0982 (KW)
Others	3		
MYCN copy number			
1	59	157 ± 193	
> 3	27	418 ± 534	0.0028 (MW)
TrkA expression	63		
Medium + high	28	154 ± 189	
0 + low	35	296 ± 422	0.1865 (MW)
Ploidy	66		
Diploid	37	255 ± 436	
Hyperdiploid	27	234 ± 352	0.4304 (MW)

MW, Mann–Whitney *U*-test; KW, Kruskal–Wallis test.

indicate that the serum NM23-H1 level serves as a useful prognostic factor for neuroblastoma, as well as the other well-known prognostic factors.

Subsequently, we classified the 86 patients into two groups according to the age of the patients, stage of the disease, or copy numbers of *MYCN*, and evaluated the influence of the serum NM23-H1 levels on the overall survival in each one of the six groups (Fig. 3). Of the 29 patients younger than 12 months of age, the seven patients with higher levels of NM23-H1 had a worse outcome than the 22 patients with the lower levels ($P = 0.0401$ according to the generalized Wilcoxon test and $P = 0.0273$ according to the log-rank test; Fig. 3a). The seven patients with higher levels of NM23-H1 had the following attributes: stage 1 + 2 + 4S ($n = 3$); stage 3 + 4 ($n = 4$); with non-amplified *MYCN* ($n = 4$); with more than three *MYCN* ($n = 3$). Likewise, of the 19 patients with a stage 3 tumor, four patients with higher levels had a worse outcome than the 15 patients with lower levels ($P = 0.0005$ and $P < 0.0001$; Fig. 3c). The four patients with higher levels of NM23-H1 had the following attributes: < 12 months of age ($n = 0$); > 12 months of age ($n = 4$); with non-amplified *MYCN* ($n = 1$); with more than three *MYCN* ($n = 3$). Of the 59 patients with a single copy of *MYCN*, the 11 patients with higher levels had a worse outcome than the 48 patients with lower levels of serum NM23-H1 ($P = 0.0301$ and $P < 0.0366$; Fig. 3e). The 11 patients with higher levels of NM23-H1 had the following attributes: < 12 months of age ($n = 4$); > 12 months of age ($n = 7$); stage 1 + 2 + 4S ($n = 2$); stage 3 + 4 ($n = 9$). In contrast, a higher serum NM23-H1 level did not influence overall survival in the 57 patients 12 months old or older, in the 46 patients with stage 4 disease, or in the 27 patients with *MYCN* amplification (Fig. 3b,d,f).

Four prognostic factors, including the age of the patients, stage of the disease, *MYCN* copy number, and the serum NM23-H1 level, were available for multivariate analysis in the 217 patients (Table 3a) and 86 patients (Table 3b). According to multivariate analysis, the serum NM23-H1 level provided no significant influence on overall survival in either group of patients (Table 3).

Discussion

The *NM23-H1* gene is overexpressed in various hematological malignancies and other neoplasms including neuroblastoma. Overexpression of *NM23-H1* mRNA is indicative of a poor prognosis in patients with neuroblastoma, and mutations and increased copy numbers of *NM23-H1* have been reported in advanced neuroblastoma.[6,24] In the present study, we found that the serum NM23-H1 level was significantly higher in patients with neuroblastoma than in the control children (Fig. 1), and that the serum NM23-H1 level predicted a poor outcome for patients with tumors (Fig. 2a). Furthermore, the higher level of NM23-H1 was correlated with a worse outcome in patients younger than 12 months of age, in those with stage 3 disease, or in those with a single *MYCN* copy (Fig. 3). In contrast, a higher serum NM23-H1 level did not influence overall survival in patients who were 12 months old or older, in those with stage 4 disease, or in those with *MYCN*

Table 3. Univariate and multivariate analysis for predictors of survival in neuroblastoma

Prognostic factors	Univariate (χ^2, log-rank)	*P*-value	Multivariate (relative risk & 95% CI)	*P*-value
Patients found by mass-screening or clinically ($n = 217$)				
Serum NM23-H1(< 250/> 250 ng/mL)	11.211	0.0008	1.7294 (0.7997–3.7398)	0.1639
Age (< 12/≥ 12 months)	32.353	< 0.00001	3.8979 (1.3818–10.996)	0.0101
Stage (1, 2, 4s/3, 4)	33.142	< 0.00001	8.2514 (1.8173–37.466)	0.0063
NMYC amplification (–/+)	43.997	< 0.00001	2.3253 (1.0541–5.1297)	0.0366
Patients found clinically ($n = 86$)				
Serum NM23-H1(< 250/> 250 ng/mL)	4.493	0.0340	1.6143 (0.7386–3.5282)	0.2299
Age (< 12/≥ 12 months)	5.825	0.0158	1.4742 (0.4877–4.4563)	0.4916
Stage (1, 2, 4s/3, 4)	6.994	0.0082	3.5721 (0.7158–17.826)	0.1206
NMYC amplification (–/+)	7.749	0.0054	1.9682 (0.9016–4.2967)	0.0892

CI, confidence interval.

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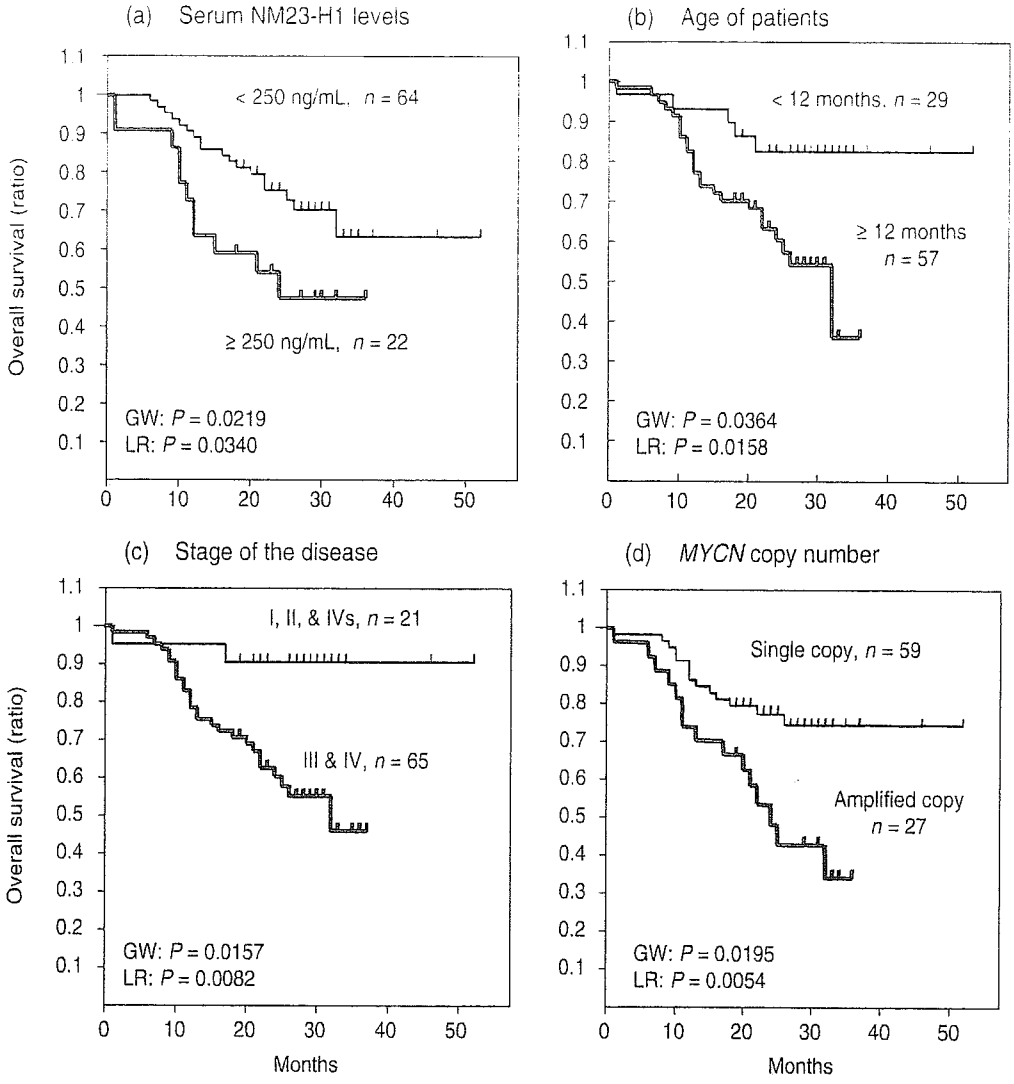

Fig. 2. Overall survival curves for 86 patients with neuroblastoma who were found clinically. (a) Overall survival curves for 22 patients with a serum NM23-H1 level ≥ 250 ng/mL, and for 64 patients with a level < 250 ng/mL. (b) Overall survival curves for 57 patients 12 months of age or older, and for 29 patients younger than 12 months. (c) Overall survival curves for 65 patients at stages 3 and 4 of the disease, and for 21 patients at stages 1, 2 and 4s. (d) Overall survival curves for 27 patients with *MYCN* amplification, and for 59 patients with a single copy of *MYCN*. GW, generalized Wilcoxon's test; LR, log-rank test.

amplification (Fig. 3). These findings suggest that the NM23-H1 level may be an important factor for predicting the outcome of patients in these low or intermediate risk groups (i.e. patients younger than 12 months of age, with stage 3 disease, or with a single copy of *MYCN*). In addition, the serum NM23-H1 level may be a clinically useful prognostic factor, because the measurement of serum NM23-H1 protein is easily and quickly carried out prior to treatment.

According to multivariate analysis, the serum NM23-H1 level provided no significant influence on overall survival in either group of patients shown in Table 3. These results might be due to the short observation time, the small number of cases, or the strong correlation between *MYCN* amplification and the elevated serum NM23-H1 level.

Although all the 131 patients found by MS were alive at the last follow-up (18–51 months) and were excluded from survival analysis, they contained 15 patients (the last follow-up: 19–37 months) with higher levels than 250 ng/mL of serum NM23-H1. It might be interesting to follow up these patients to clarify the clinical significance of serum NM23-H1 in the MS group.

Prognostic factors in neuroblastoma have been thoroughly investigated and include *MYCN* copy number, *TRKA* expression level, chromosomal ploidy, 1p loss, and 17q gain in tumor cells. Laborious and time-consuming work is required to examine these biological factors in tumor tissues. Therefore, serum markers that are easily measurable and can predict a clinical outcome are desired. Serum levels of lactate dehydrogenase (LDH) and ferritin are high in advanced stage neuroblastomas, but both may reflect a rapid cellular turnover or a large tumor burden.[25,26] Neuron-specific enolase (NSE) is a cytoplasmic protein that is associated with neural cells,