Table 3. Expression of hTERT and N-Myc mRNA in 16 neuroblastomas

No.	Age/sex	type	stage	status	Shimada class	hTERT (nested)	hTERT (real-time)	N-Myc
1	8 m/M	NB	II		favorable	+	146	4.61
2	2 y/F	GNB	III		favorable	+	76	0.37
3	11 m/M	GNB	II		favorable	+	0	0.56
4	7 m/F	NB	II		favorable	–	0	3.77
5	8 m/M	NB	I		favorable	–	28	5.8
6	10 m/F	NB	II		favorable	+	957	1.45
7	1 y/M	GNB	II		unfavorable	+	223	2.56
8	8 m/M	NB	III	progressive	favorable	+	55	3.59
9	1 y/F	NB	IVa	progressive	unfavorable	+	768	66.51
10	1 y/F	NB	IVa	progressive	unfavorable	+	241	36.57
11	1 y/M	NB	IVa	death	unfavorable	+	1305	26.77
12	3 y/F	NB	IVa	death	unfavorable	+	99	3.88
13	4 y/F	NB	IVa	death	unfavorable	+	714	49.33
14	2 y/F	NB	IVb	progressive	unfavorable	+	164	0.29
15	1 y/M	NB	IVa	progressive	unfavorable	+	539	5.51
16	3 y/F	NB	IVa	progressive	unfavorable	+	589	44.95

GNB = ganglioneuroblastoma; NB = neuroblastoma

The three suspected malignant cases, 6, 7, and 8, have as yet no malignant clinical manifestation. The hTERT mRNA positive cases in the benign samples may develop metastasis in the future.

Kinosita *et al.* and Kubota *et al.* [9,10] reported that telomerase activity was a potential marker for malignancy, but Bamberger *et al.* [11] noted that telomerase activity was not a suitable marker for malignancy in the adrenal gland. A potential reason for the different results may be that telomerase activity easily decreases in storage and may vary in different regions of a sample. hTERT mRNA seems to be more stable than the telomerase activity, but also varies in different regions of the sample. We could not rule out the possibility of false positive samples, since lymphocytes always contain hTERT mRNA [15] and may contaminate the samples.

A number of markers have been examined as possible predictors of malignancy, for example, MIB-1, a monoclonal antibody against the Ki-67 protein. It was recently reported that the MIB-1 proliferative index score may be a useful indicator for diagnosing malignant pheochromocytoma [16], and according to Prof. H Sasano (Tohoku Univ, Japan, personal communication), the score correlates with telomerase activity. In this study, the MIB-1 index was a useful indicator for discriminating the malignancy and correlated with hTERT mRNA in the 4 malignant samples. Elder *et al.* [17] also reported that in all three patients with malignant tumors, the tumors were positive for either hTERT expression or Ki-67/MIB-1 immunoreactivity, and concluded that combined use of Ki-67 and hTERT was a very useful tool to identify malignant pheochromocytomas.

In neuroblastomas, the expression of hTERT mRNA correlated with the N-myc mRNA expression, a prognostic marker. Hiyama *et al.* [12] reported that in neuroblastomas high telomerase activity correlated with poor outcome, while the tumors with low activity of this enzyme showed favorable outcome. Krams *et al.* [17] reported that the presence of full-length hTERT transcripts detected by RT-PCR analysis of neuroblastic tumor samples might help in appraising the malignant potential in individual cases.

However, we could not discriminate progressive neuroblastomas from benign ones by the real-time PCR for the full-length type. The basal expression of hTERT mRNA seems to be higher in neuroblastomas than in pheochromocytomas, and that causes the low sensitivity in discriminating progressive neuroblastomas. We previously examined hTERT mRNA expression in other tumors: All of 12 mammary gland tumors, including 2 malignant ones, were positive for hTERT mRNA, whereas all of 8 parathyroid gland tumors, including 7 cancers, were negative (data not shown). Thus basal expression of hTERT mRNA may vary in these tumors due to their tissue origin, and this variation may reduce its utility as a prognostic marker.

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Usefulness of Tyrosine Hydroxylase mRNA for Diagnosis and Detection of Minimal Residual Disease in Neuroblastoma

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Neuroblastoma (NB) is the most common malignant solid tumor in childhood and, among all childhood malignancies, is second only to leukemia. NB originates before birth in the neural crest, which develops into the adrenal medullae and sympathetic ganglia. In the adrenal medulla, tyrosine hydroxylase (TH) is the first enzyme in the pathway of catecholamine synthesis. We used reverse transcription polymerase chain reaction (RT-PCR) to examine the expression of TH mRNA in NB and Ewing's sarcoma cell lines, small round cell tumors (SRCTs) containing NB, and other clinical tumor samples (osteosarcoma, osteochondroma, and Wilms' tumor). In total, we analyzed 33 clinical tumor samples. TH mRNA was expressed in all three NB cell lines examined, but not in two ES cell lines or in a breast cancer cell line. We detected TH mRNA in 23 of 25 NB tumor samples (92%), but in none of the SRCTs or other clinical tumor samples. This RT-PCR technique showed a sensitivity for TH mRNA of one NB cell per 10^5 negative cells. Based on these results, the detection of TH mRNA is very useful both as a tumor marker for NB and for detecting minimal residual disease. Therefore, we can use this method to detect tumor cell contamination before hematopoietic stem cell transplantation.

Key words neuroblastoma; tyrosine hydroxylase; hematopoietic stem cell transplantation; minimal residual disease; reverse transcription polymerase chain reaction

Neuroblastoma (NB) is a very common malignant solid tumor in childhood. NB belongs to the small round cell tumors (SRCTs), which include other solid tumors such as Ewing's sarcoma (ES), rhabdomyosarcoma, and malignant lymphoma.[1] SRCTs are histologically ambiguous, so it is necessary to analyze adequate tumor markers for an accurate diagnosis. Patients who are over the age of 1 year at diagnosis usually have poor prognoses.[2]

NB is also characterized by elevated levels of catecholamine production. Tyrosine hydroxylase (TH) is very important as the first and rate-limiting step in the synthesis of catecholamines.[3—6] Therefore, we used reverse transcription polymerase chain reaction (RT-PCR) to examine the expression of TH mRNA in some cell lines, SRCTs, and other clinical tumor samples, to assess whether we can use it as a tumor marker and detect cell contamination in hematopoietic stem cells. Specific TH mRNA could be detected in NB cell lines and clinical NB tumor samples, but not in other cell lines and tumor samples. Moreover, the technique had a high sensitivity of $1/10^5$.

We think that this method should be used for detecting minimal residual disease because the prognoses of patients in NBs depend on being positive or negative for TH mRNA in bone marrow (BM) samples within 4 months after chemotherapy.[7] Moreover, the risk of relapse after autologous peripheral blood stem cell (PBSC) or BM transplantation is high if there is NB cell contamination.[8,9]

Here, we describe a very useful method for detecting minimal residual disease. The method can also be used as a tumor marker.

MATERIALS AND METHODS

Cell Lines and Tumor Samples Three NB cell lines (IMR-32,[10,12] SK-N-SH,[11,12] and NB-39,[12] two ES cell lines (NCR-EW2,[13] SCMC-ES1[14]), and one breast cancer cell line (MCF-7[15]) were examined. They were cultured in RPMI 1640 medium supplemented with 10% fetal bovine serum, 100 U/ml penicillin, 0.1 mg/ml streptomycin, and 2 g/l sodium bicarbonate under 5% CO_2 at 37 °C. Thirty-three clinical tumor samples (25 NBs, 1 ES, 1 osteosarcoma, 1 osteochondroma, 1 Wilms' tumor, 1 malignant lymphoma, and 3 rhabdomyosarcoma) were examined. One of the three rhabdomyosarcoma samples was from a recurrent tumor. These tissues had been stored at −80 °C since collection. The clinical diagnoses for these patients were made by histopathology. Informed consent was obtained from all patients before they entered this study.

RNA Extraction Total RNA was extracted from the six cell lines using the acid-guanidium-phenol chloroform method after treatment with Catrimox-14™. Total RNA from the 33 clinical tumor samples was extracted by TRIZOL reagent (GIBCO BAL) based on the acid-guanidium-phenol chloroform extraction method.[16]

Reverse Transcription Polymerase Chain Reaction Total RNA (1 μg) was reverse-transcribed in a 10 μl reaction mixture with a first strand cDNA synthesis kit (Rever Tra-α-TM, TOYOBO). RT was performed with Oligo-dT. The mixture was annealing at 42 °C for 20 min, followed by incubated at 99 °C for 5 min, and then held at 4 °C. PCR amplification was carried out in 10× reaction mixture containing 1.2 pmol of the respective primers. We used a HOT START PCR kit from KOD-Plus- (TOYOBO).[17—19] The PCR conditions were one cycle of template denaturing at 94 °C for

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Table 1. PCR Primers

Gene	Sequence	Location
TH sense	5′-TGT CAG AGC TGG ACA AGT GT-3′	Exon 8
TH anti-sense	5′-GAT ATT GTC TTC CCG GTA GC-3′	Exon 9
GAPDH sense	5′-TCC TCT GAC TTC AAC AGC GAC ACC-3′	Exon 5
GAPDH anti-sense	5′-TCT CTC TTC CTC TTG TGC TCT TGG-3′	Exon 8

2 min, followed by 28 cycles of denaturing at 94 °C for 15 s, annealing at 60 °C for 90 s, extension at 68 °C for 20 s, and then holding at 4 °C. We used glyceraldehyde-3-phosphate dehydrogenase (GAPDH) as an internal marker and IMR-32, NB-39, and SK-N-SH neuroblastoma cell lines as positive controls. NCR-EW2, SCMC-ES1, and MCF-7 were examined as negative controls. The primer sequences are listed in Table 1.[20] The primers for TH are located in exons that are not affected by alternative splicing. To avoid contamination with genomic DNA, the reverse primers for both TH and GAPDH were located in successive exons.

Analyses of PCR Products The PCR products were electrophoresed through 2.0% agarose gel, stained with ethidium bromide (WAKO), and visualized under a UV lamp. We used a Bioanalyzer (Agilent Technologies) to accurately determine band sizes.

Detection for Sensitivity A NB cell line (IMR-32) was examined as a positive control and an ES cell line (NCR-EW2) as a negative control. We counted the number of each cell type and diluted them to make suspensions containing one NB cell per 10 ES cells, one per 10^2, one per 10^3, one per 10^4, one per 10^5, and one per 10^6. We investigated the sensitivity of this RT-PCR technique for detecting TH mRNA.

RESULTS AND DISCUSSION

We analyzed six cell lines for detecting a specific TH mRNA with the RT-PCR technique. The electrophoresis and Bioanalyzer results are presented in Figs. 1A and 1B. The band size for TH was 299 bp, and the internal marker GAPDH was 209 bp. TH mRNA was detected in three NB cell lines (IMR-32, NB-39, and SK-N-SH), but it was not detected in the ES cell lines (NCR-EW2, SCMC-ES1) or the breast cancer cell line (MCF-7).

We investigated 33 clinical tumor samples by the same method as that used for the cell lines. The electrophoresis and Bioanalyzer results are presented in Tables 2 and 3. In 23 of 25 NB tumor samples (92%), TH mRNA could be detected, but it was not detected in the T1 and T2 samples (Table 2). TH mRNA was not detected in the osteosarcoma (T26), osteochondroma (T27), Wilms' tumor (T28), and SRCT (T29—T33) samples (Table 3). The rhabdomyosarcoma from a recurrent tumor (T33) also did not express TH mRNA.

We examined the sensitivity for detecting minimal residual disease by this RT-PCR technique. We used a NB cell line (IMR-32) as a positive control and an ES cell line (NCR-EW2) as a negative control. The electrophoresis and Bioanalyzer results are presented in Figs. 2A and 2B. On the electrophoresis, a TH mRNA band could be seen in samples with concentrations down to one NB cell per 10^5 ES cells, but not at a concentration of one NB cell per 10^6 ES cells (Fig. 2A).

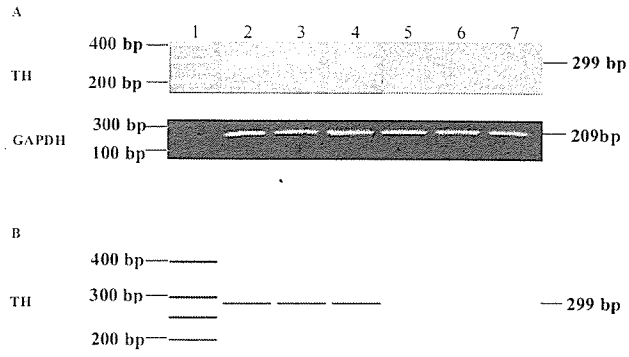

Fig. 1. RT-PCR Products Detected in Cell Lines

(A) Bands detected by electrophoresis. Lane 1, marker; lane 2, IMR-32; lane 3, NB-39; lane 4, SK-N-SH; lane 5, NCR-EW2; lane 6, SCMC-ES1; lane 7, MCF-7. (B) Bands detected by Bioanalyzer. Lanes are as for electrophoresis. A 299 bp RT-PCR product for TH was seen by both electrophoresis and Bioanalyzer, and a 209 bp product for GAPDH was also seen.

Table 2. NB Patients' Clinical Profile and TH mRNA Bands Detected by Electrophoresis and Bioanalyzer

Tissue	Sex	Age	Diagnosis	TH mRNA
T1	F	1 y	NB	−
T2	F	10 m	NB	−
T3	M	10 m	NB	+
T4	M	10 m	NB	+
T5	M	9 m	NB	+
T6	M	3 y	NB	+
T7	M	7 m	NB	+
T8	M	4 m	NB	+
T9	F	10 m	NB	+
T10	F	1 y	NB	+
T11	F	6 y	NB	+
T12	F	1 y	NB	+
T13	F	8 m	NB	+
T14	F	6 m	NB	+
T15	M	4 y	NB	+
T16	F	3 y	NB	+
T17	M	4 y	NB	+
T18	F	10 y	NB	+
T19	M	9 y	NB	+
T20	F	7 m	NB	+
T21	F	9 m	NB	+
T22	F	10 m	NB	+
T23	M	3 y	NB	+
T24	M	5 y	NB	+
T25	F	5 y	NB	+

NB: neuroblastoma.

Similarly, using the Bioanalyzer, TH mRNA could be detected at one NB cell per 10^5 ES cells but not at one NB cell per 10^6 ES cells (Fig. 2B).

Abnormal amplification and expression are well known for *MYCN*,[21—23] *trk-A*,[24—27] and protein gene product 9.5 (PGP9.5)[28] in NB. However, these markers are not very useful for diagnosis. Recently, by using monoclonal antibodies

Table 3. Other Tumor Patients' Clinical Profile and TH mRNA Bands Detected by Electrophoresis and Bioanalyzer

Tissue	Sex	Age	Diagnosis	TH mRNA
T26	M	13 y	OS	–
T27	F	12 y	OC	–
T28	M	8 m	WT	–
T29	F	6 m	ML	–
T30	M	3 y	ES	–
T31	M	11 y	RMS	–
T32	F	3 y	RMS	–
T33	M	4 y	RMS	–

OS: osteosarcoma, OC: osteochondroma, WT: Wilms' tumor, ML: malignant lymphoma, ES: Ewing's sarcoma, RMS: rhabdomyosarcoma.

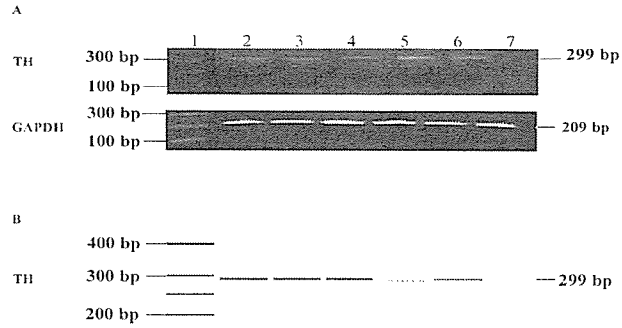

Fig. 2. Sensitivity of RT-PCR Technique for TH mRNA

Lane 1, marker; lane 2, one NB cell per 10 ES cells; lane 3, one NB cell per 10^2 ES cells; lane 4, one NB cell per 10^3 ES cells; lane 5, one NB cell per 10^4 ES cells; lane 6, one NB cell per 10^5 ES cells; lane 7, one NB cell per 10^6 ES cells. (A) Bands detected by electrophoresis. (B) Bands detected by Bioanalyzer. The 299 bp RT-PCR product for TH was detected by both electrophoresis and Bioanalyzer, and a 209 bp product for GAPDH was also seen.

that react selectively to cells of neuroectodermal origin, the diagnostic usefulness has been improved. However, the monoclonal antibodies often produce false positive results,[29,30] and the clinical importance of detecting positive cells as evidence of infiltration is still debated.[31] As NB has a specific catecholamine metabolism, it may be more useful to detect TH, which is the first and rate-limiting enzyme of catecholamine synthesis. We found that TH mRNA was expressed in all NB cell lines (100%) and in 23 of 25 (92%) clinical NB tumor samples. On the other hand, it was not expressed in any of the other cell lines and clinical tumor samples.

Thus, we found that TH mRNA is expressed specifically in NB, and this specific expression can be use to distinguish NB from SRCTs. TH mRNA can also be used as a tumor marker for the accurate diagnosis of NB.

The expression of TH mRNA did not correlate with the patient's age or sex, and it might not be a prognostic factor for NB patients.

Another important problem is determining whether tumor cell contamination exists when a patient's PBSC or BM is used for autologous transplantation. Our method can detect contamination of one cell in 10^5.

In future, our method may be very useful for diagnosing NB patients and detecting minimal residual disease in clinical samples.

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神経芽腫マス・スクリーニング ―過去と現在
―日本のマス・スクリーニングが示したもの―

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はじめに

1971年，日本で小児悪性固形腫瘍への関心が乏しかったころ，米国ではEvansらCCSGA (Children's Cancer Study Group A) により小児がんの中で最も多く，予後不良な神経芽腫(neuroblastoma：NB) 246例の2年生存率は年齢と病期に強く依存していると報告された[1]。全例の2年生存率は32%（79/246例）で，1歳未満の生存例は74%（51/69），1～2歳26%（12/47），2歳以後12%（16/130）であった。1歳未満の発見例が全体の25%にすぎず，2歳以後に発見される例が53%（130例）もあった。病期ではI，II，IVsは良好，III，VIは不良であった。年齢が増し，病期が進行すると予後不良となった。早期発見の必要性が示された。一方，1966年には『Pediatr Clin North Am』の総説で，NBはカテコラミン産生腫瘍で尿中のカテコラミン（CA）の代謝産物であるVMA（vanillylmandelic acid）やHVA（homovanillic acid）の測定が診断に有用であると述べられていた。当時の日本とは雲泥の差があった。CCSGAでは多数例で統一プロトコールによる治療など，羨ましいことであった。しかし，「早期発見で治る」を目標に診断からスクリーニングへと進行した。

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I．マス・スクリーニングへの道[2]

骨痛，貧血，持続発熱，脾腫の幼児の骨髄から異常細胞がみつかり，臨床的にNBを疑った例の剖検で脾腫は腫瘍で，組織診断で確定した例に出会った（1965年）。この例を契機に尿中CAや代謝産物の測定に取りかかった。Gitlowらがpheochrmocytomaの診断に用いたVMA比色判定量法の検討から，尿中VMAだけでもNBを疑うことができることがわかった[3]。さらに，CAや代謝産物を測定し，尿でNBの診断ができたが，測定は煩雑で時間がかかり，早期発見にはつながらなかった。比色半定量法を改良し，数分でVMAの増加を濾紙上で肉眼で判定できるVMA spot testを開発した。この方法では診察しなくても尿だけで疑うことができ，早期発見に利用できた。

II．VMA spot testによるスクリーニング開始

1972年，京都市内保健所での3歳児検診受診時に試行し，1例を発見したが遅かった。1974年，6か月乳児を対象に尿添加濾紙郵送法でスクリーニングを開始した。1/1.5～2万人の頻度で5例の早期例を発見し良好な予後が期待された。「このスクリーニングシステムでNBを発見できる」ことがわかった[4]。その後，いくつかの地域でスクリーニ

ングが開始された。

III. 6か月の選択理由

1）臨床NB例の年齢分布には1歳未満の大きなピークと3歳ごろの小さいピークがあった（米国，オランダ，デンマークでも同じ）。

2）*in situ* neuroblastoma（1963）が乳児期早期には高い頻度であり，大部分（？）が消失するので，その発見を避けるため。

3）1歳までに治療を開始したい。

4）6か月にスクリーニングを受けNBを疑い，確定診断に至ると1歳近くになると予想した。

以上のことから6か月を選択した。代謝異常スクリーニングで有名な故ガスリー教授が「興味をもっている」のあと，「なぜ，6か月か？」が最初の質問であった。

IV. 国レベルの実施へ

1983年，NBマス・スクリーニングの評価のために厚生省助成研究班ができ，その結果から1985年にVMA spot testによる6か月乳児のスクリーニングが全国実施となった。乳児の家庭，保健所，検査機関と病院の連携でマス・スクリーニングが行われた（詳細は省略）。

V. HPLCによるVMA/HVA定量スクリーニング法への変更[5)]

VMA spot testの発見感度（sensitivity）は77.2%であるために約23%のNBが見落とされることから，精度向上のために1988年から高速液体クロマトグラフィー（HPLC：high performance liquid chromatography）によるVMA，HVA定量法へと徐々に変わった。その感度はVMA 84.2%，HVA 88.2%で，VMA+HVA併用で96.1%と良好であった。HPLCスクリーニング実施から16年を経過し，6か月スクリーニング発見例は増加し（1/5000～6000人），予後良好であることをはじめ，分子生物学的特徴や組織像と予後の関連など，多くのことが明瞭となった。

この間の海外の関心も高く，カナダ・ケベック-米国・ミネソタ，英国・ニューカッスル，ドイツ・

表1　神経芽腫スクリーニング受診者・年度別発生数および頻度

	対象者数（出生数）	受診者数	受診年	NB例	頻度
1976-1983	—	—	—	27	—
1984	1,469,923	124,870	8.5%	24	1/5,203
1985	1,425,043	834,536	58.6%	56	1/14,902
1986	1,374,666	997,643	72.6%	66	1/15,116
1987	1,332,491	1,024,841	76.9%	79	1/12,973
1988	1,314,006	1,036,740	78.9%	112	1/9,257
1989	1,246,802	1,033,535	82.9%	105	1/9,843
1990	1,214,600	1,023,005	84.2%	158《653》	1/6,475
1991	1,223,245	1,026,741	83.9%	132（189）	1/7,778
1992	1,208,989	1,049,905	86.8%	136（194）	1/7,720
1993	1,206,219	1,042,578	86.4%	173（202）	1/6,026
1994	1,238,328	1,046,953	84.5%	173（208）	1/6,052
1995	1,187,067	1,043,490	87.9%	172（194）	1/6,067
1996	1,206,253	1,030,179	85.4%	191（232）	1/5,394
1997	1,191,359	1,037,043	87.0%	173（252）	1/5,994
1998	1,202,858	1,042,238	86.6%	170（206）	1/6,130
1999	1,177,421	1,038,040	88.2%	143（202）	1/5,139
2000	1,190,337	1,020,815	85.8%	166（201）	1/5,079
合計	20,219,270	16,453,152	81.37%	2,257	

1985：VMA spot testで全国的にはじまる。　（　）は厚生省発表
1988：HPLCに変更開始。1991：全国的にHPLCとなる。

表2　年齢別神経芽腫

	小児数（15歳以下）	<1	1≦ <2	2≦ <4	4≦ <7	7≦	?
1980	27,507,078	35	31	29	19	12	8
1981	27,603,000	25	26	29	17	12	13
1982	27,254,000	37	22	33	28	13	3
1983	26,907,000	48	29	38	25	11	1
1984	26,504,000	55	27	27	18	11	
1985	26,033,218	58	23	31	21	12	
1986	25,434,000	74	19	36	23	13	
1987	24,753,000	85	12	24	16	16	
1988	23,985,000	108	29	30	26	5	1
1989	23,201,000	108	19	25	21	5	
1990	22,486,000	114	10	18	17	12	
1991	21,904,000	163	36	28	31	11	
1992	21,364,000	145	29	25	22	9	

シュツットガルト，フランスの一部でも開始され，1989年以後NBマス・スクリーニング国際シンポジウムがすでに7回開催された。

VI. 日本のマス・スクリーニングの結果（表1）

スクリーニング開始から2000年までに2257例を発見した[6]。HPLCの導入で年間160～170例が発見されている。日本の小児慢性特定疾患申請書からみた新発生例が年間320例くらいであることから，約半数のNBがマス・スクリーニングで発見されている。病期は大部分Ⅰ，Ⅱ，Ⅳsで，Ⅲが18%，Ⅳが5%で進行例は少ない。しかし，約半数に転移があり，多くは局所リンパ節であったが，骨転移例もみられた。報告時と5年後の調査で生存率98%，97%であった。今日，予後因子として重要なN-myc増幅は検査された1737例中33例（1.9%）で，10コピー以上は0.6%であった。「スクリーニング発見例の多くは早期で98%が治る。Ⅲ，Ⅳ期でも治る」，「予後不良例は少なく，強力な治療は不要である」と結論できた。

VII. 乳児期スクリーニングが示したこと

1）乳児マス・スクリーニング発見例の予後は良好で，これらの例に対して全国統一乳児NB治療プロトコールを作成し治療の軽減化ができた（治療プロトコール#9405，#9805，現在改変中）。進行例でも高率に治癒する。

2）生物学的な予後因子（N-myc，Trkなど）の不良例はまれである（本号次項参照）。

3）119例の組織の検討でfavorable histologyが95%で，unfavorable histologyが5%にすぎなかった。Schwann細胞様細胞や線維組織の増生，腫瘍細胞の部分的消失像や腫瘍細胞の分化，線維化傾向がみられた。退縮傾向が多くにみられた[7]。

4）HPLCマス・スクリーニング実施後，日本小児外科学会悪性腫瘍登録委員会の報告では，スクリーニング発見例を含む1歳未満例は激増したが，1歳以後の例の有意な減少はなかった（表2）。また，病期も同様で早期の例の増加に対応する進行例の有意な減少はなかった。スクリーニングによる発見頻度がVMA spot testで1/1.5～2万人からHPLCでの1/0.5～0.6万人で約3倍増で自然治癒例を含んでいる可能性があった。自然治癒についてはすでに成書にも記載されているが，その機序は不明であるが，幼若乳児にみられる。

5）自然治癒する例に手術や化学療法などの治療に伴う副作用の出現は，無用なだけでなく，害を及ぼしている。

6）乳児期マス・スクリーニングの有効性は1998年リヨンでのNBマス・スクリーニング国際シンポジウムで陪審員制度で検討され，6か月マス・スクリーニングに有効性がみられず，死亡例の減少を評価の対象とすべきだと結論された。疫学的に

デザインされたスタディが必要との指摘があった。わが国のスクリーニングは診断から始まり，一斉に全国へ広がった経過から疫学的に有効性を比較・評価することができなかった。平成10年(1998)度に厚生科学研究「神経芽細胞スクリーニングの評価」，続いて「神経芽腫のマス・スクリーニングプログラムの疫学的評価」の研究が行われた。全国乳児コホートを対象にHPLCによるスクリーニングの受検群と未受検群での神経芽腫死亡を比較した研究では，生後6か月以降の神経芽腫死亡率は受検群で0.234/10万人年，未受検群で0.831/10万人年であり，群間の累積死亡の差は100万人年当たり20〜30人で3年間で100人近くが救命されたことになる[8]。この数字が有効か，否かが話題となった。

VIII. 海外からの報告—スクリーニングに対する批判

2002年の「N Engl J Med」のカナダ・ケベック-米国・ミネソタ，ドイツ・シュツットガルトの2つの論文[9,10]で乳児期マス・スクリーニングでは死亡例の減少が認められないと結論した[11,12]。population-based registryをもとに，前者は薄層クロマトグラフィー法で感度は77%？，発見頻度1/10200，後者はHPLC法でそれぞれ68.8%，1/9095人で，日本の96.0%，1/5000〜6000に比べると感度が悪く，発見頻度の悪いシステムとの比較には問題があると感じているが，原因はわからい。日本のデザイン（研究のつもりで実施したのでなかったが）の無さのために反論ができていない。

IX. 厚生労働省の対応と現在

上記2論文とわが国の報告をもとに，平成15年5月，厚生労働省が疫学・統計学，外科，病理，行政，マスコミ，医師会などのメンバーによる4回の「神経芽細胞腫マススクリーニング検査のあり方に関する検討会」で文献的検討が行われ，同年7月に「現行の生後6か月時に実施する神経芽細胞腫検査事業は事業による死亡率減少効果の有無が明確でない一方，自然退縮する例に対して手術などの治療を行うなどの負担をかけており，このまま継続することは難しいと判断される」，と結論されたが，① 神経芽細胞腫の罹患と死亡の正確な把握，② NBマス・スクリーニングの実施時期の変更など，新たな検査法の検討，評価，③ NBによる死亡の減少を目指した，臨床診断と治療に向上のための研究と実施体制の確立，を条件として，いったん休止することが適切で，行政が適切に対応することを望むという報告書を出した[11]。そして，平成16年度「NBマススクリーニングの効果判定と医療体制の確立」研究班を立ち上げ，その中の一つに「前向き介入研究—NBの実施時期変更の検討と評価」分担研究班ができ，検討が開始された。

おわりに

1985年からの6か月乳児を対象として全国的に実施されていたNBマス・スクリーニングが，いったん休止となった。乳児期NBの特徴が明瞭になるなど学問的にはある程度の役割りを果たしたと思っている。しかし，期待した治癒率の増加は自然治癒例の増加によって評価ができず，対象年齢を変えて，より効率的なマス・スクリーニングができるか，考える必要がある。① NBマス・スクリーニングを行うべきか，否か，② 実施するとすれば，いつか？　その根拠は？　を明瞭にする必要がある。一方，研究的には③ 自然消褪の機序や自然暦は，④ 乳児期NBから幼児期NBへ移行するのか？　別のNBか？　などが今後の課題であり，行政的には，⑤ NBに関する疫学的調査ができるシステムの確立が必須である。

近い将来に，これらが明らかになることを期待している。

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Clinical Significance of a Highly Sensitive Analysis for Gene Dosage and the Expression Level of *MYCN* in Neuroblastoma

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Background: The amplification of the *MYCN* gene is one of the most powerful adverse prognosis factors in neuroblastoma, but the clinical significance of an enhanced expression of *MYCN* remains controversial. To reassess the clinical implications of *MYCN* amplification and expression in neuroblastoma, the status of amplification and the expression level of the *MYCN* gene of primary neuroblastoma samples were analyzed using highly sensitive analyses.

Methods: Using a quantitative polymerase chain reaction (PCR) method (TaqMan), the gene dosages (*MYCN/p53*) of 66 primary neuroblastoma samples were determined. In all 66 samples, the status of *MYCN* amplification has been determined previously by the Southern blotting method. Of the 54 samples with a single copy of *MYCN* based on the Southern blotting method, 23 samples were analyzed for *MYCN* amplification using the fluorescence in situ hybridization (FISH) method. The expression levels (*MYCN/GAPDH*) of 56 samples were determined by a quantitative reverse transcriptase (RT)-PCR method.

Results: Of the 54 samples with a single copy of *MYCN* based on the Southern blotting method, 46 samples showed *MYCN* gene dosages of less than 2.0, whereas the remaining 8 samples with dosages of more than 2.0 were tumors from patients with advanced-stage disease. The results of FISH supported the fact that these 8 samples contained a small number of *MYCN*-amplified cells. The cases of *MYCN* gene dosages of more than 2.0 were significantly associated with all other unfavorable prognostic factors (an age of >1 year at diagnosis [$P < .0001$], nonmass screening [$P = .0003$], advanced stage [$P < .0001$], diploid or tetraploid [$P < .0001$], and a Shimada unfavorable histology [$P < .0001$]). *MYCN* gene dosages of more than 2.0 were significantly associated with a high expression of *MYCN* ($P = .0459$). However, the expression level of *MYCN* was not significantly associated with any other prognostic factors.

Conclusions: Quantitative PCR may thus be a useful modality for performing a highly sensitive and accurate assessment of the amplification and expression levels of the *MYCN* gene. In particular, the combination of the quantitative PCR system and the FISH method is considered to be a highly effective method for evaluating the status of *MYCN* amplification. In this highly sensitive analysis, *MYCN* amplification (*MYCN/p53* $\geq$ 2.0) was reconfirmed to be a strongly unfavorable factor, whereas the expression level of *MYCN* does not appear to be an independently significant prognosis factor. *J Pediatr Surg 39:63-68.*

INDEX WORDS: Neuroblastoma, *MYCN*, quantitative polymerase chain reaction, FISH.

NEUROBLASTOMA is a tumor derived from neural crest origin, which arises in the adrenal medulla or paraspinal sympathetic ganglia. The prognosis in neuroblastoma tends to vary greatly, and many studies have found both clinical and biological factors to be closely correlated with the outcome.[1,2] To select the optimal treatment according to the degree of malignancy of neuroblastoma, it is essential to accurately and rapidly identify any genetic abnormalities associated with the prognosis using several molecular biological methods. The amplification of the *MYCN* gene is strongly associated with rapid tumor progression.[3,4] An amplification of the *MYCN* gene occurs in approximately 25% of primary tumors, and this factor is known to be one of the most unfavorable prognostic factors in neuroblastoma.[3,4] We assume that an enhanced expression consequent to the gene amplification of proto-oncogene contributes to tumorigenesis. Regarding the *MYCN* gene, it is easy to consider that the amplification of *MYCN* gene results in an enhanced expression of *MYCN*, which activates the transcription of genes associated with the cell proliferation.[5,6] However, the clinical significance of *MYCN* expression in children with neuroblastoma remains controversial.[7-11]

The amplification of the *MYCN* status has been mainly analyzed for the whole tumor using the Southern blotting method, but this method is not able to detect intratumor heterogeneity. We previously reported our findings for a

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highly sensitive analysis for *MYCN* amplification in neuroblastoma based on the FISH (fluorescence in situ hybridization) method.[12] Furthermore, we reported preliminarily the quantitative polymerase chain reaction (PCR) method (TaqMan) to be useful as a quick and accurate modality for evaluating for the status of *MYCN* amplification in 25 neuroblastoma samples.[13]

In the current study, the status of *MYCN* amplification in more neuroblastoma samples was evaluated using the quantitative PCR method and the FISH method to reassess the clinical implication of *MYCN* amplification in neuroblastoma. Furthermore, the expression level of *MYCN* was analyzed also by the quantitative PCR method to elucidate the correlation between *MYCN* expression and the biology of neuroblastoma.

MATERIALS AND METHODS

Clinical Data of Patients and Biological Data of Neuroblastoma Samples

Neuroblastoma in patients evaluated at the Department of Pediatric Surgery, Kyushu University was diagnosed and staged according to the International Neuroblastoma Staging System (INSS).[14] Sixty-six frozen tumor samples were obtained from untreated neuroblastoma patients. The characteristics of the patients were shown to be as follows: the sex of the patients was 39 boys and 27 girls, and the age at diagnosis ranged from 19 days after birth to 11 years of age. Of the 66 cases, 25 cases were diagnosed in patients greater than 1 year of age, whereas the remaining 41 cases were diagnosed in patients younger than 1 year of age. Thirty-six patients were identified by a neuroblastoma mass screening system. Of the 66 samples, 39 were tumors that were stage 1, 2, or 4S, whereas 9 were stage 3, and 18 were stage 4. Fifty-three patients are still alive, of whom, 5 patients are still under treatment, whereas 13 patients have died of the disease. The follow-up period after treatment ranged from 1 month to 12 years. In all 66 tumors, the *MYCN* amplification status was analyzed by the quantitative PCR system, and the expression level of *MYCN* was analyzed by the quantitative RT-PCR system in the 56 tumors. In all 66 samples, the status of *MYCN* amplification was also previously determined by the Southern blotting method.[15] In 23 of 54 cases with a single copy identified by Southern blotting, the *MYCN* amplification status was examined using the FISH methods. DNA ploidy was examined previously using flow cytometry[16] in 46 cases. Thirty-one cases were triploid, whereas 15 cases were diploid or tetraploid. Regarding the histologic findings, 64 cases were classified based on the Shimada classification.[17] Forty-five cases showed a favorable histology, whereas the remaining 19 cases showed an unfavorable histology.

DNA or RNA Extraction and cDNA Synthesis

DNA was extracted from the frozen tumor samples using proteinase K and phenol. Isogen LS (Nippon Gene, Osaka, Japan) was used to extract total RNA, and reverse transcription (RT) was performed with a First-strand cDNA synthesis kit (Amersham Pharmacia, Uppsala, Sweden) using random hexanucleotide primers.

Quantitative PCR (TaqMan)

As described previously, the *p53* gene was used as an internal control gene to obtain the gene dosage (*MYCN/p53*).[13] The *p53* gene is a tumor suppresser gene in which mutations or deletions are found in a variety of malignant tumors. However, no aberration of the *p53* gene in neuroblastoma has ever been found, and the gene status in neuroblastoma is known to be stable.[18] The corrected gene dosage of the *MYCN* gene was obtained based on the assumption that the mean gene dosage of 20 normal individual lymphocytes was 1.00. The mean ± 2 SD of gene dosage of 20 normal individual lymphocytes was 1.00 ± 0.58. In this study, we evaluated that the *MYCN*-amplified cells apparently present in the samples with a corrected gene dosage (*MYCN/p53*) of more than 2.0. The information on the *MYCN* gene and the *p53* gene sequences was obtained from a GenBank database search. The primers and TaqMan probes for the *MYCN* gene and the *p53* gene were designed to be located on intron 2 of *MYCN* and exon 1 of the *p53* gene using the application-based primer design software Primer Express (Applied Biosystems, Foster City, CA). The sequences of the PCR primers and TaqMan probes were as follows: *MYCN:* forward primer 5′-CCC AGC GTG GTA GTC AAT GA-3′, reverse primer 5′-TTA ATG ACA AAG CCA TAA TCC ACA G-3′, TaqMan probe 5′-AGA ATG CGC ACA TGA TGC TAC ACG TTT CT-3′; *p53:* forward primer 5′-GCC CTT ACT TGT CAT GGC GA-3′, reverse primer 5′-ATC CCA CAA CCC CTG CG-3′, TaqMan probe 5′-TGT CCA GCT TTG TGC CAG GAG CC-3′. Quantitative PCR was performed in a final volume of 25 μL, and each sample was analyzed in duplicate. Each reaction mixture contained 0.1 pmol/μL TaqMan probe, 0.2 pmol/μL each primer, 1x TaqMan PCR master mix, and 10 to 50 ng DNA. Thermal cycling was started with a 2 minute incubation at 50°C, followed by a first denaturation step of 10 minutes at 95°C, and then 40 cycles of 2-step PCR consisting of 95°C for 5 seconds and 60°C for 1 minute. The quantification of the *MYCN* gene was achieved by means of the ABI Prism 7700 Sequence Detection System (Applied Biosystems). Genomic DNA from one neuroblastoma with 90 copies of *MYCN* by Southern blotting method was serially diluted to establish the calibration curve.

Quantitative RT-PCR (TaqMan)

The primer and TaqMan probe for *MYCN* mRNA were designed to be located on exon 2-3, thereby avoiding the amplification contaminating genomic DNA. *GAPDH* was used as an internal control gene to analyze the *MYCN* gene expression (*MYC/GAPDH*). The sequences of the PCR primers and TaqMan probe were as follows: *MYCN:* forward primer 5′-GAC CAC AAG GCC CTC AGT ACC-3′, reverse primer 5′-TGA CCA CGT CGA TTT CTT CCT-3′, TaqMan probe 5′-CCG GAG AGG ACA CCC TGA GCG A-3′. PCR primer and TaqMan probe for *GAPDH* were purchased from ABI as a kit of TaqMan GAPDH Control Regent and Predeveloped TaqMan Assay Regents Control Kit. The measurement of *MYCN* cDNA was based on 2 reporter dyes, namely, FAM for *MYCN* and VIC for *GAPDH*. The quantitative RT-PCR system was performed in the same manner as that for the quantitative PCR. The corrected expression level of *MYCN* (*MYCN/GAPDH*) was obtained based on the assumption that the value of one primary tumor sample (12 copies of *MYCN* by Southern blotting) was 1.00.

FISH Analysis of the MYCN Gene

Fresh tumor single cells were suspended in potassium chloride. The nuclei were denatured immediately before hybridization in 70% formamide and 2 times standard saline citrate (SSC) at 75°C for 2 minutes and then were dehydrated through ethanol. The hybridization buffer, which contained 10 ng aliqour of the *MYCN* probe was denatured for 5 minutes at 75°C, chilled on ice, and then applied to the slide. Hybridization was performed overnight at 37°C. After the slide was washed by formamide, SSC, and Triton, the nuclei were counterstained with 30 μL of DAPI containing 5 μL of antifade solution. The signals representing the *MYCN* gene were countered for 100 cells on one slide.

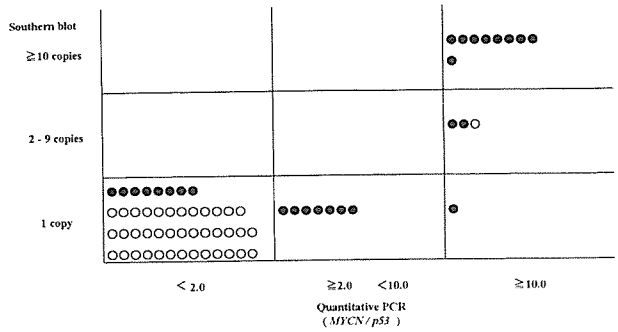

Fig 1. The comparison between the quantitative PCR method and Southern blotting method for the status of *MYCN* amplification in 66 primary neuroblastomas. ●, stage 3, 4; ○, stage 1, 2, 4S.

The images were photographed using a fluorescence microscope (Olympus, BX60, Tokyo, Japan) and Provia 400 (Fuji, ISO 400, Tokyo, Japan).

Statistical Analysis

Fisher' Exact test was used to test the association between *MYCN* amplification (*MYCN/p53* ≧ 2.0) or no amplification (*MYCN/p53* < 2.0) and other prognostic factors. The expression levels of *MYCN* (*MYCN/p53*) in the subgroups were represented by Percentile (50% [25%, 75%]). A comparison of the gene dosage and expression in relation to clinical and genetic parameters was made using Mann-Whitney U test.

RESULTS

The Gene Dosages of MYCN by the Quantitative PCR Method

Regarding the status of *MYCN* amplification, the findings of a comparison between the quantitative PCR method and the Southern blotting method are shown in Fig 1.

Of the 54 samples with a single copy of *MYCN* based on the Southern blotting method, 46 samples showed the corrected gene dosage (*MYCN/p53*) to be less than 2.0, whereas the remaining 8 samples with more than 2.0 had tumors from patients with an advanced stage of disease (stages 3, 4). Of the 8 samples with a dosage of more than 2.0, 3 patients died of the disease.

In 23 of 54 cases with a single copy of *MYCN* based on the Southern blotting method, the status of *MYCN* amplification was also examined by the FISH method (Fig 2). Eighteen of the samples were tumors from the patients with a nonadvanced stage, whereas the remaining 5 samples were those from patients with a advanced stage. The patients with a gene dosage of more than 2.0 (*MYCN/p53* ≧ 2.0) and a single copy of *MYCN* by the Southern blotting method, only 2 patients had an advanced stage. Furthermore, the FISH analysis showed that these 2 cases (case 1 and 2) contained a small number of *MYCN* amplified cells at rates of 15% and

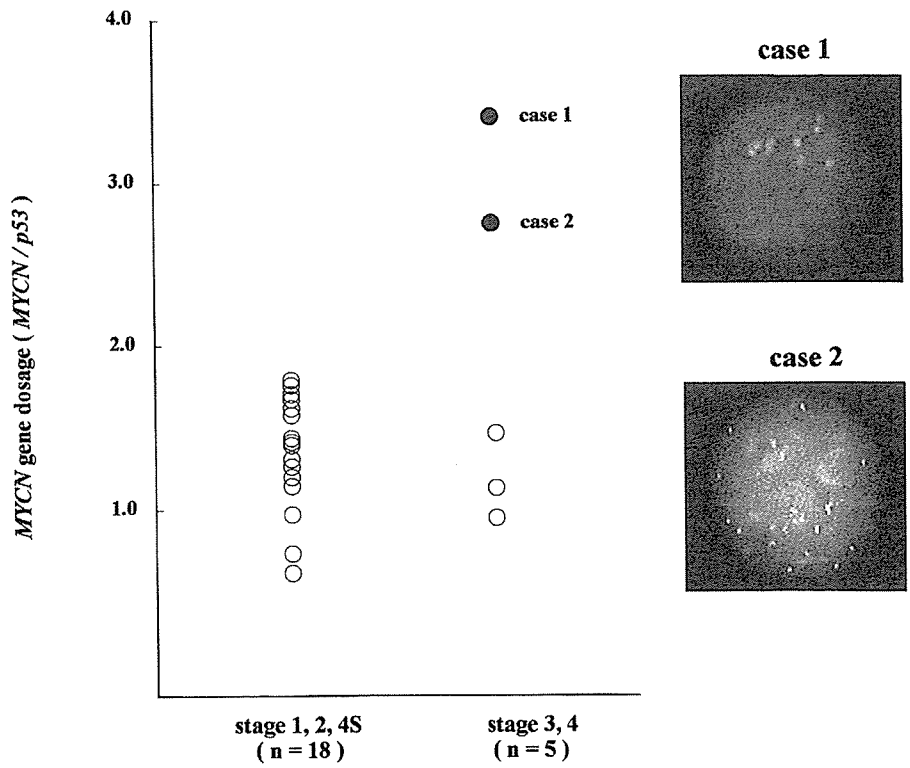

Fig 2. The gene dosage of *MYCN* based on the quantitative PCR and the status of *MYCN* amplification by the FISH method in 23 neuroblastomas with a single copy of *MYCN* based on the Southern blotting method. The 2 cases (cases 1 and 2) contained a small number of *MYCN*-amplified cells at rates of 15% and 29% of the cells with a nuclei, respectively. ●, *MYCN*-amplified cell (+) by FISH; ○, *MYCN*-amplified cell (−) by FISH.

Table 1. Gene Dosage of *MYCN* in Relation to Clinical and Biological Prognostic Factors

Category	Number	Gene Dosage of *MYCN* (*MYCN/p53*) <2.0	Gene Dosage of *MYCN* (*MYCN/p53*) ≥2.0	*P* Value*
Age				
<1 year of age	41	37 (90.2%)	4 (9.8 %)	<.0001
≥1 year of age	25	9 (36.0%)	16 (64.0%)	
Mass screening				
Mass positive	36	32(88.9%)	4 (11.1%)	.0003
Sporadic	30	14 (46.7%)	16 (53.3%)	
Stage				
Stage 1,2,4S	39	38 (97.4%)	1 (2.6 %)	<.0001
Stage 3,4	27	8 (29.6%)	19 (70.4%)	
DNA ploidy				
Aneuploid	31	30 (96.6%)	1 (3.4 %)	<.0001
Diploid or tetraploid	15	4 (26.7%)	11 (73.3%)	
Shimada				
Favorable	45	41 (91.1%)	4 (8.9 %)	<.0001
Unfavorable	19	5 (26.3%)	14 (73.7%)	

*P value was determinated by Fisher's Exact test.

29% of cells with a nuclei, respectively. These findings suggested that these samples showed intratumor heterogeneity with respect to *MYCN* amplification.

In 9 cases of more than 2 copies of *MYCN* based on the Southern blotting method, the corrected *MYCN* gene dosages by the quantitative PCR were all more than 10.0 (Fig 1). In the majority of these cases, the analytic value based on the quantitative PCR was shown to be a higher than that based on a Southern blotting analysis.

The relationship between the *MYCN* gene dosage and the known prognostic factors (age, mass screening, clinical stage, DNA ploidy, Shimada classification) is shown in Table 1. The cases of a gene dosage of more than 2.0 were strongly associated with an age of more than 1 year at diagnosis ($P < .0001$), nonmass screening ($P = .0003$), advanced stage ($P < .0001$), diploid or tetraploid ($P < .0001$), and a Shimada unfavorable histology ($P < .0001$), which are all unfavorable factors.

The Expression Level of MYCN by the Quantitative RT-PCR Method

The dot graph of gene dosages and expression level of *MYCN* gene in 56 neuroblastomas is shown in Fig 3. As for the expression level of *MYCN* (*MYCN/ GAPDH*), the median value was 0.295. Of 14 cases of *MYCN* amplification (*MYCN/p53* ≥ 2.0), 10 cases showed to be more than the median value (0.295). On the other hand, the cases of no amplification (*MYCN/p53* < 2.0) showed a variety of expression levels of the *MYCN* gene.

The relationship between the *MYCN* gene expression level and prognostic factors are shown in Table 2. The level of *MYCN* expression in cases of *MYCN* amplification (*MYCN/p53* ≥ 2.0) was significantly higher than that of cases of no *MYCN* amplification (*MYCN/p53* < 2.0; $P = .0459$). However, the expression level of *MYCN* was not significantly associated with any other prognostic factors (age, mass screening, clinical stage, DNA ploidy, Shimada classification).

Figure 4 shows the relationship between the *MYCN* gene expression level and age at diagnosis in subsets of tumors with or without *MYCN* amplification. In the cases of no *MYCN* amplification (*MYCN/p53* < 2.0), the *MYCN* expression levels in the samples from patients less than 1 year of age (n = 35, 0.28 [0.06, 1.24]) were significantly higher than those from patients older than 1 year of age (n = 7, 0.02 [0.02, 0.15]; $P = 0.014$). In the cases of no *MYCN* amplification (*MYCN/p53* < 2.0), the majority of patients less than 1 year of age showed an early clinical stage and a good prognosis in spite of an expression level of the *MYCN* gene, whereas the majority of patients older than 1 year of age showed an advanced clinical stage even if the level of *MYCN* expression was very low.

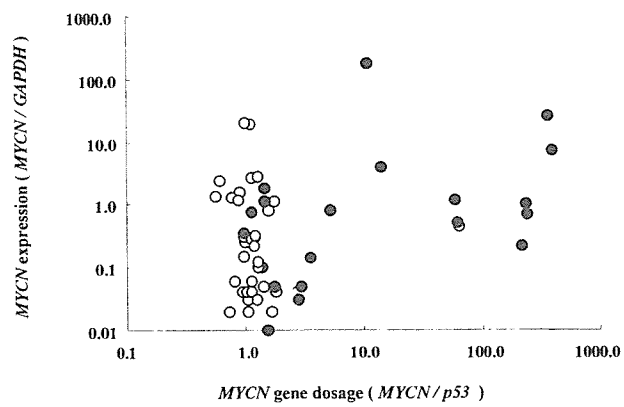

Fig 3. A dot graph of the gene dosages and the expression level of the *MYCN* gene in 56 neuroblastomas. ●, stage 3, 4; ○, stage 1, 2, 4S.

Table 2. *MYCN* Expression in Relation to Clinical and Biological Prognostic Factors

Category	No.	*MYCN/GAPDH* % TILE 50% [25%, 75%]	*P* Value*
Age			
<1 year of age	39	0.31 [0.08,1.24]	.4983
≥1 year of age	17	0.25 [0.03,1.00]	
Mass screening			
Mass positive	34	0.31 [0.10,1.26]	.3739
Sporadic	22	0.24 [0.04,0.95]	
Stage			
Stage1,2,4S	37	0.15 [0.04,1.12]	.0805
Stage 3,4	19	0.78 [0.18,1.53]	
DNA ploidy			
Aneuploid	28	0.22 [0.06,1.14]	.3667
Diploid or tetraploid	9	0.10 [0.03,0.45]	
Shimada			
Favorable	43	0.25 [0.06,1.16]	.4727
Unfavorable	13	0.52 [0.05,1.19]	
MYCN/p53			
<2.0	42	0.19 [0.04,1.12]	.0459
≥2.0	14	0.75 [0.28,3.19]	

**P* value was determinated by Mann-Whitney U test.

DISCUSSION

The amplification of the *MYCN* gene is the most powerful unfavorable prognostic factor in neuroblastoma.[3,4] In the Study Group of Japan for Advanced Neuroblastoma (JANB), 2 chemotherapeutic regimens for advanced neuroblastoma have been designed based on the *MYCN* amplification status (more than 10 copies of *MYCN* or less than 10 copies of *MYCN*) since 1991.[19] As a result, it is essential for the treatment of neuroblastoma to accurately and rapidly identify the status of *MYCN* amplification. The quantitative analysis of the *MYCN* gene has so far been mainly analyzed for whole tumors by the Southern blotting method, but this method is not able to detect intratumor heterogeneity. The FISH method is able to evaluate the status of *MYCN* amplification in individual neuroblastoma cells; however, it is difficult to determine the copy number of the *MYCN* oncogene using the FISH method. The quantitative PCR system is a new technique,[20] which is able to overcome the demerits of both the FISH and the Southern blot methods.

In the current study, the combined analysis of the quantitative PCR and the FISH method suggested that approximately 15% (8 of 54) of all cases with a single copy by the Southern blotting method might have a small number of *MYCN* amplified cells in those tumors. The quantitative PCR system may be able to detect a small number of *MYCN* amplified cells, which could not be found by the Southern blotting method. The combination of the quantitative PCR system and the FISH method is considered to be highly effective for evaluating the amplification of the *MYCN* status in neuroblastoma. However, it remains unclear as to which treatment should be selected in tumors with a small number of *MYCN*-amplified cells. Further studies based on this combined analysis should lead to the development of new therapeutic strategies. In addition, the quantitative PCR system is also available for small amounts of samples. Therefore, the quantitative PCR system combined with the microdisection technique[21] is considered to be more effective for evaluating the status of *MYCN* amplification.

As mentioned previously, the clinical significance of *MYCN* expression in children with neuroblastoma remains controversial. Bordow et al[7] reported that in patients older than 1 year of age with *MYCN* nonamplified tumors, high levels of *MYCN* expression correlated with poor outcome. On the other hand, a poor survival rate has been observed in patients older than 1 year with advanced-stage tumors that lack *MYCN* amplification, even if the *MYCN* was expressed at low levels.[22] In addition, several researchers reported that the *MYCN* expression was not predictive of a poor prognosis.[8,10] In the current study, *MYCN* gene dosages of more than 2.0 were significantly associated with a high expression of *MYCN*. However, the patients with no amplification (*MYCN/p53* < 2.0) showed various expression levels of *MYCN* gene. In addition, no statistically significant difference between the *MYCN* expression and other prognostic factors (age, mass screening, DNA ploidy, Shimada classification) could be found. These findings suggest that the only gene dosage of *MYCN* does not always

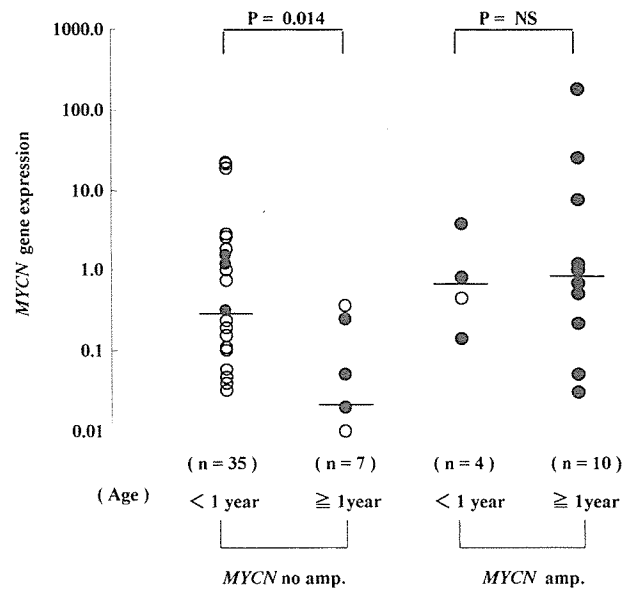

Fig 4. The relationship between the *MYCN* gene expression level and age at diagnosis in the subsets of tumors with or without *MYCN* amplification. *MYCN* amp, *MYCN/p53* ≧ 2.0; *MYCN* no amp, *MYCN/p53* < 2.0. ●, stage 3, 4; ○, stage 1, 2, 4S; bar, 50%tile; NS, not significant.

contribute to the level of *MYCN* expression in neuroblastoma. Furthermore, moderately high levels of *MYCN* expression in patients less than 1 year of age were shown to not be a poor prognostic factor, whereas the low levels of *MYCN* expression in patients greater than 1 year of age were not shown to be a favorable prognostic factor. These results are consistent with the results previously reported by Matsunaga et al[23] who showed the enhanced expression of *MYCN* in cases detected by mass screening.[23] Taken together, the expression level of *MYCN* does not seem to be an independently significant prognostic factor in this highly sensitive analysis.

An enhanced expression of *MYC* has been shown to confer growth potential to cells in vitro as well as in vivo: however, the *MYC* family of oncogenes are also strong inducers of apoptosis in conditions in which their expression is deregulated from mitogenic signaling pathways.[24] Several investigators have reported a correlation between *MYCN* and apoptosis in neuroblastoma in vitro.[25] *MYCN* may thus be considered to play an important role in both the cellular proliferation and apoptosis of neuroblastoma in vivo, and *MYCN* coordinately induces cellular proliferation and apoptosis through different pathways. Further studies on *MYCN*-related proteins should provide an explanation for the mechanism of cellular proliferation and spontaneous regression in neuroblastoma.

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総会記録

第19回日本小児がん学会

教育講座

神経芽腫新国際分類　ＩＮＰＣについて

—従来の分類との関連を含めて—

秦　順一

はじめに

近年，神経芽腫の病期の統一，治療法の改善，生物学的特異性の解明を多面的な領域を，国際レベルで行うInternational Neuroblastoma Risk Group(INRG)が結成された．その中で本腫瘍の国際病理組織分類の策定を目的とするInternational Neuroblastoma Pathology Committeeが6名のメンバーで組織された．筆者もそのメンバーの一人として加わってきた．4年ほどの討議の後1999年にInternational Neuroblastoma Pathology Classification (INPC)が発表されるに至った．本組織分類は神経芽腫の亜型を病理学的に明確に定義し，分類の再現性(reproducibility)，予後を含む本腫瘍の生物学的特異性(biological relevance)，分類が容易であることを目指している(user friendly)．神経芽腫の腫瘍細胞は年齢とともに分化・成熟を果たすという事実が知られており，本分類ではShimada分類に準じて発症年齢と組織型との対応によって予後判定基準(age-linked histological classification and prognosis system)を取り入れた．この分類はすでに米国，カナダ，オーストラリアで使用が開始され，EU諸国でもその導入が考慮されている．わが国においても，腫瘍の病態，生物学的特性の解明ならびに治療法の開発を進める点からも，国際的な共通言語（組織分類）を取り入れる必然性が共通認識となりつつある．本年3月に改訂，出版された日本病理学会小児腫瘍アトラス「神経芽腫群腫瘍」では全面的にこの分類を取り入れている．本稿ではINPCを紹介するとともに従来の分類との相違点を述べる．

国立成育医療センター研究所

1. INPC分類とその方針について

分類の概要は以下に記載した．国際分類であり，和訳による誤解を避けるためオリジナルの診断名で統一した．

1）Neuroblastoma/subgroup
  a) undifferentiated
  b) poorly differentiated
  c) differentiating
2）Ganglioneuroblastoma, intermixed
3）Ganglioneuroma/subgroup
  a) maturing
  b) mature
4）Ganglioneuroblastoma, nodular

A）INP組織学的分類の方針

神経芽腫の組織像は，基本的には腫瘍細胞である神経冠由来の神経芽腫細胞（腫瘍性，以下同様）と反応性に増生すると考えられるシュワン様細胞から成る間質によって構成される．このような観点から本組織学的分類は，1）シュワン様細胞の量，腫瘍細胞である神経芽細胞の増殖巣との関連，2）増殖している神経芽細胞の分化・成熟度，3）腫

瘍細胞の増殖性の目安となる Mitosis-Karyorrhexis-Index(MKI) を取り入れる，4) 神経芽腫の腫瘍細胞の分化・成熟度は年令と極めて高い相関があるので，発生年令と組織像によって腫瘍の予後を推定する．すなわち，この分類では単なる組織像の記載にとどまらず，予後の推定をも行うことを特徴としている（表 1，2）[1-4]．

B) INP の説明

1. Neuroblastoma(NB, Schwannian stroma-poor tumor)

神経芽細胞の増殖が主体で間質であるシュワン細胞は認められないか，わずかに腫瘍細胞増殖巣を取り囲むように存在する血管結合織周囲にみられる．以下の亜群が存在する．

a) Undifferentiated sub type

小型ないし中型の未熟な神経芽細胞からなり，胞体に乏しい．腫瘍細胞間に光顕上明らかな神経細線維 (neuropil) がみられない．この亜型に診断されると年令に関連なく予後不良群にはいるので，診断は慎重にする必要がある．

b) Poorly differentiated sub type

神経細線維が増殖する腫瘍細胞間に明らかに介在する腫瘍をいう．腫瘍細胞は小型，裸核状である．時には好酸性の胞体と核小体が明瞭な交感神経節細胞への分化を窺わせる細胞が混じるが，これらの細胞は腫瘍組織全体として５％以下に止まる．なお，交感神経節細胞への分化傾向を示す細胞の形態的学的基準として，①細胞の腫大（核面積の２倍以上），②胞体の好酸性の増加，③核が腫大すると同時に核小体が明瞭化する，と定められている．

c) Differentiating neuroblastoma sub type

腫瘍細胞の多くは，なお未熟な神経芽細胞であるが前述した交感神経節細胞へ分化傾向を示す細胞が腫瘍全体で５％を超える腫瘍をいう．

d) Mitosis karyorrhexis-Index(MKI)

Neuroblastoma では腫瘍細胞の増殖性を示し，予後とも密接に関連する組織学的所見として MKI の程度を必ず付記する．MKI はその出現頻度に従って高度(high, ＞ 4%：200個/5000細胞)，中等度 (intermediate, 2-4%：100-200個/5000細胞)，軽度 (low, ＜ 2%：100個/5000細胞) に分類される，神経芽腫の組織学的予後判定に重要な指標となるので，正確なカウントが必要である．

2. Ganglioneuroblastoma(GNB), intermixed type

成熟したシュワン様細胞が腫瘍組織の 50% 以

表 1　INPC の基本的考え方

1，全ての神経芽腫群腫瘍に適応できる
2，組織分類の定義を明確にする組織分類の基準 ・神経芽細胞の分化度 ・間質であるシュワン細胞の発達 ・Mitosis-Karyorrhexis-Index（←細胞増殖の指標）
3，予後との相関（予後を推定しうる）を重視する組織分類とする Age-linked Morphologic Classification ←Shimada system

表 3　日本病理学会分類との相違

1，腫瘍の間質であるシュワン細胞の量を考慮している (vs　神経芽細胞の分化度を重視)
2，MKI を採用←腫瘍細胞の増殖指標
3，発生年齢を加味した組織分類 予後を推定し得る組織分類

表 2　INPC 腫瘍亜分類とシュワン細胞の発達との関連

Neuroblastoma(NB) Undifferentiated(UD) Poorly differentiated(PD) Differentiating(D)	➡	50％未満
Ganglioneuroblastoma(GNB) Intermixed		50％以上
Ganglioneuroma(GN) Maturing GN Mature GN	➡	シュワン細胞が主体
Ganglioneuroblastoma(GNB) Nodular		Composite Stroma poor/rich

上を占め，その中に神経節細胞様の大型な腫瘍細胞や小型で未熟な神経芽細胞の増殖巣がする．

3. Ganglioneuroma (GN, Schwannian stroma-rich)

シュワン様細胞からなる間質が腫瘍の大部分を占めるもので，その他の構成細胞は分化したまたは分化途上にある交感神経節様細胞である．分化した神経節様細胞には外套細胞を伴う．以下の亜群が存在する．

a) Ganglioneuroma, maturing sub type

腫瘍のほとんどが神経節腫様組織から成るが，一部に神経細線維を伴った神経芽細胞が散在している腫瘍をいう．

b) Ganglioneuroma, mature sub type

成熟したシュワン様細胞から成る間質が腫瘍組織の殆どを占め，神経突起を伴った成熟した神経節細胞が散在性に存在する．未熟な神経芽細胞は認められない．

4. Ganglioneuroblastoma, nodular type (GNB, nodular)

肉眼的に神経節腫様組織から成る腫瘍で，その中に出血を伴う未熟神経芽細胞の増殖巣が結節状にみとめられる腫瘍をいう．すなわち，前者は後者とは明らかに異なるクローンから成ると考えられる．組織学的には未熟神経芽細胞の増殖巣（結節）と成熟した神経節腫様組織との間には明瞭な境界が存在する．また，予後の判定のため未熟神経芽細胞から成る胞巣の所見はneuroblastomaのそれに準じて評価する．

C) INPCによる組織分類の手順

i) 大項目としてまずNB，GNB，GNに分類する．

ii) それぞれの腫瘍の亜型(subtype)を決定する

iii) NBについては腫瘍細胞の分化度とともにMKIの程度を決定する．

iv) NBでは組織像が一様でなく，しばしば組織型が混在する場合が少なくない．その際には優勢度で診断するのが原則であるが，判断が困難な場合はその成分を所見として記載する．

v) 転移先から採取された腫瘍の組織分類

INPCでは未治療で原発巣の摘出腫瘍に対して腫瘍分類を付すことを原則としている．しかしながら神経芽腫の場合，腫瘍が進展し原発腫瘍が採取不可能で，転移巣を生検する場合が少なくない．この場合，転移腫瘍であっても未治療の腫瘍であれば充分に予後を推定できる情報が得られるので，原発巣に準じた組織分類をすることができる．

D) 発生年齢と組織分類から判断（推定）される神経芽腫の予後

表4　ＩＮＰＣ／従来の分類との比較

INPC/Category and Subtype	日本病理学会 小児腫瘍分類委員会	Shimada Classification
Neuroblastoma(NB)	神経芽腫	NB(Stroma-poor)
undifferentiated	円形細胞＋花冠細線維形成型	undifferentiated
poorly differentiated	花冠細線維形成型	
differentiating	花冠＋神経節芽腫・低分化型	differentiating
Ganglioneuroblastoma intermixed/Ganglioneuroma	神経節芽腫／神経節腫 (GN)	GNB/GN (Stroma-rich)
GNB	神経節芽腫	
intermixed	分化型	GNB intermixed
GN		
maturing	神経節芽腫分化型＋神経節腫	GNB, well differentiated
mature	神経節腫	GN
Ganglioneuroblastoma, nodular	神経節芽腫・混成型	GNB, nodular