Results

FISH analysis

8 of all 47 cases analyzed using FISH showed *MYCN* amplification without *MYCN* gain. 7 of 47 cases showed *MYCN*-gain cells without *MYCN* amplification. The other 32 of 47 cases showed no *MYCN* amplification and they showed neither *MYCN*-gain cells nor *MYCN* amplified cells (Fig. 1).

The comparison between the results determined by FISH and the results determined by an SNP array

The status of chromosome 2p was determined with an SNP array in all 47 samples. All 47 cases were classified into 4 groups; 6 cases which had no distal 2p gain with amplification of *MYCN* gene region were defined as Group 1. Two cases which had a distal 2p gain with amplification of *MYCN* gene region were defined as Group 2. Seven cases which had a distal 2p gain without the amplification of the *MYCN* gene region was defined as Group 3. The other 32 cases which had neither any amplification of the *MYCN* gene region nor distal 2p gain were defined as Group 4.

The correlation between the results of the FISH examination and the results of the SNP array are shown in Table 1. Of the 8 cases that showed *MYCN* amplification with FISH, 6 were identified as Group 1 and 2 were identified as Group 2 by the SNP array. All 7 cases that showed *MYCN* gain with FISH were identified as Group 3 by the SNP array. All 32 cases that showed no *MYCN* amplification with FISH were identified as Group 4 by the SNP array.

The clinical and biological features of the seven cases with MYCN gain based on the FISH assay

The clinical and biological features were examined in all seven *MYCN*-gain cases (Table 2). The age at the initial diagnosis ranged from 8 to 60 months, and the median age was 36 months. One patient (case 3) was identified through mass screening at 6 months of age. Six of the seven cases were patients with stage 4 NB and the other case was a patient with stage 3. The ratio of *MYCN* signals to the centrosome of chromosome 2 signals in the *MYCN*-gain cells varied in each of the cases. All 7 cases showed distal 2p gain and the incidence of 1p loss, 3p loss, 11q loss, 17q gain, that are poor prognostic factors in NB, in *MYCN*-gain cases ($n = 7$) was significantly higher than that of each genetic anomaly in no *MYCN* amplification cases ($n = 32$) as determined by FISH, respectively (1p loss, 43 vs. 6%, $p = 0.008$; 3p loss, 57 vs. 13%, $p = 0.008$; 11q loss, 71 vs. 22%, $p = 0.01$; 17q gain, 71 vs. 28%, $p = 0.03$). All seven cases showed a single copy of *MYCN* gene by SB. Q-PCR showed that three of seven cases had a slight increase in the *MYCN* gene dosage and no case showed *MYCN* amplification.

The outcome of the patients with MYCN gain based on FISH

Figure 2 shows the survival curves of the patients of *MYCN* amplification, *MYCN* gain and no *MYCN* amplification determined by FISH. The 5-year overall survival (OS) rate of patients with *MYCN* gain ($n = 7$, $71.4 \pm 17.1\%$) was not significantly different from that ($n = 32$, $90.6 \pm 5.2\%$) of patients in no *MYCN* amplification ($p = 0.11$).

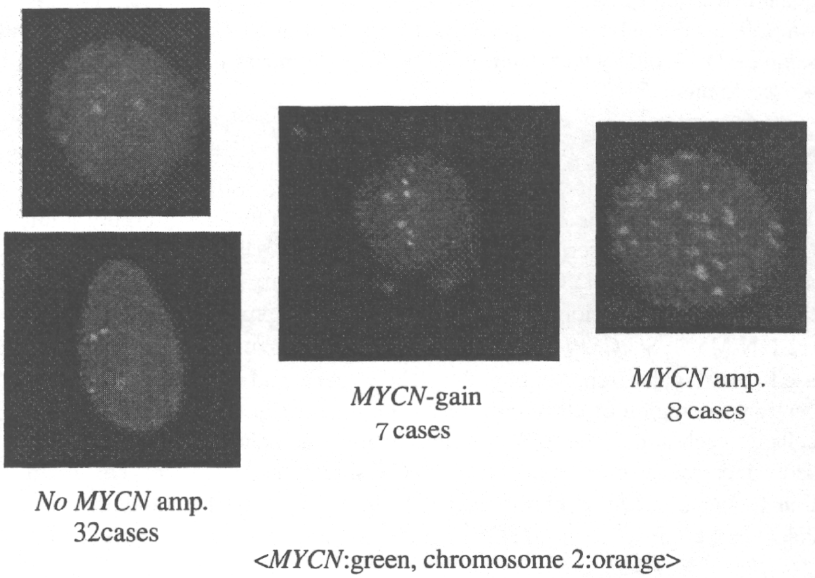

Fig. 1 The result of the FISH assay. 8 of the 47 cases analyzed using FISH showed *MYCN* amplification. Seven cases showed *MYCN* gain. Thirty-two cases showed no *MYCN* amplification

Table 1 The correlation between the result of FISH examinations and the result of SNP array

The status of Chr. 2p by SNP array	Group 1 2p gain (−) *MYCN* (+)	Group 2 2p gain (+) *MYCN* (+)	Group 3 2p gain (+) *MYCN* (−)	Group 4 2p gain (−) *MYCN* (−)
The status of *MYCN* by FISH				
MYCN amp.	6	2	0	0
MYCN gain	0	0	7	0
No *MYCN* amp.	0	0	0	32

Table 2 The clinical and biological characteristics of the seven patients in *MYCN* gain assessed by FISH

Case	Age (months)	Mass	Stage	Fish	*MYCN*: 2CEP	SB (copy)	Q-PCR	Ploidy	1p loss	2p gain	3p loss	11q loss	17q gain	Outcome
1	48	(−)	4	Gain	9:3	1	3.25	D	+	+	−	−	+	Dead
2	60	(−)	4	Gain	3:2	1	0.65	ND	−	+	+	+	+	Alive
3	8	(+)	3	Gain	5:3	1	2.63	Tri	−	+	−	−	+	Alive
4	36	(−)	4	Gain	3:2	1	1.57	D	+	+	+	+	+	Dead
5	8	(−)	4	Gain	5–6:4	1	1.41	Tetra	−	+	+	+	−	Alive
6	42	(−)	4	Gain	3:2	1	1.18	D	−	+	−	+	+	Alive
7	13	(−)	4	Gain	5:2	1	3.67	D	+	+	+	+	−	Alive

Mass mass screening, *SB* southern blotting method, *Q-PCR* quantitative PCR, *gain MYCN* gain, *ND* not determined, *MYCN:CEP2* MYCN signals: cetromeric region of chromosome 2 signals, *D* diploidy, *tri* triploidy, *tetra* tetraploidy

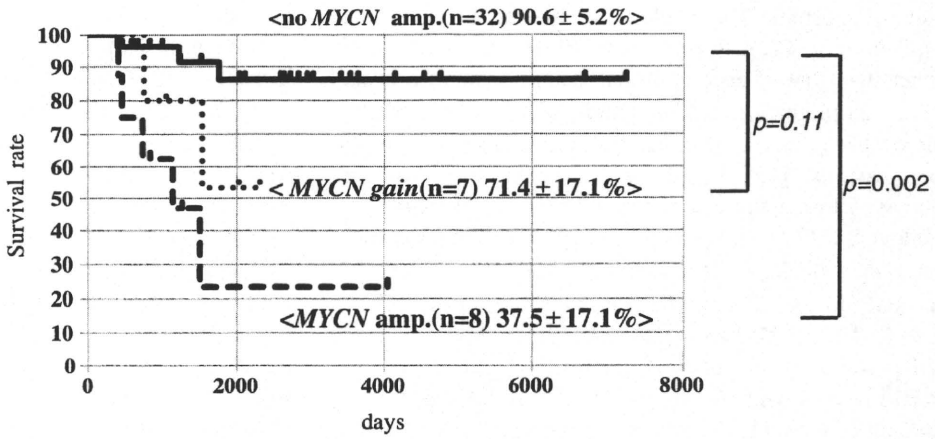

Fig. 2 Kaplan–Meier survival curves in three groups (*MYCN* amplification, *MYCN* gain and no *MYCN* amplification determined by FISH)

Discussion

The present study evaluated the correlation between *MYCN* signals using FISH and chromosomal 2p status using an SNP array. There were 7 cases that showed *MYCN* gain by FISH and all these 7 cases showed distal 2p gain without any amplification of *MYCN* gene regions in the SNP array. This result suggested that *MYCN*-gain using FISH represents the chromosomal status of distal 2p gain. The samples with *MYCN* amplification by FISH showed amplification of *MYCN* region with or without 2p gain by an SNP array. Of course, the number of samples in this study is small; however, we can think about one of the hypothesis that the progression from the tumor without *MYCN* amplification to the tumor with *MYCN* amplification in NB detected by FISH (Fig. 3). In this study, only 2 of 8 cases with *MYCN* amplification by FISH showed distal 2p gain with the amplification of the *MYCN* region using the SNP array. These results suggested the majority of *MYCN*-gain cases do not pass through a state of *MYCN* gain.

Spitz et al. [7] reported that the 3-year overall survival of the patients with *MYCN* gain is not significantly different in comparison to the patients with *MYCN* single copy. In addition, Schleiermacher et al. [13] reported that 2p gain was not an independent prognostic factor. To assess whether *MYCN* gain affects the prognosis of the

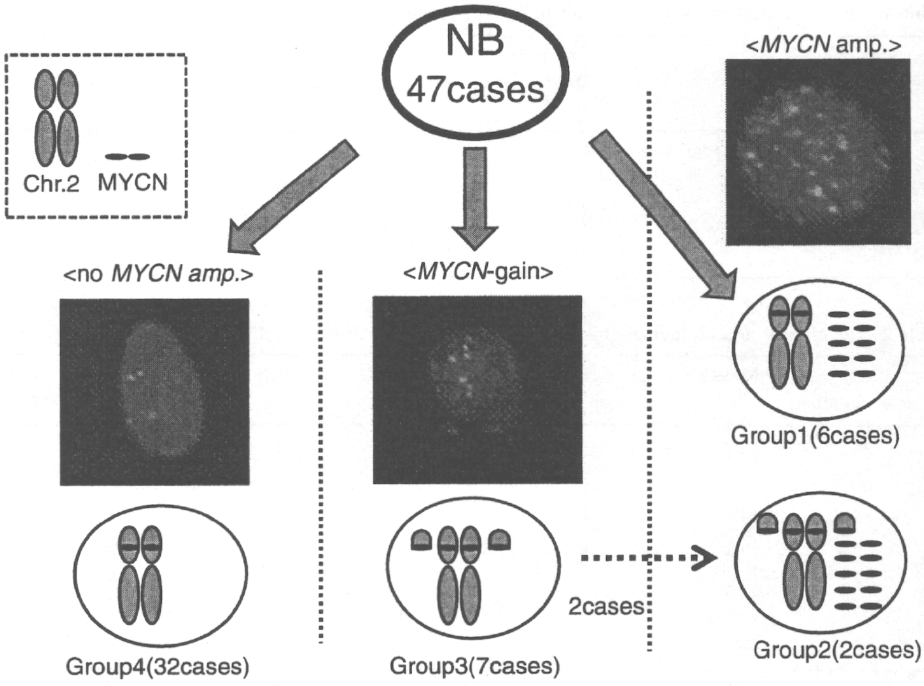

Fig. 3 A hypothesis of the progression from no *MYCN* amplification tumor to amplified tumor in neuroblastoma

patients without *MYCN* amplification, other genetic anomalies were assessed in the 47 NB cases using the SNP array in this study. The incidence of 1p loss, 11q loss, and 17q gain in *MYCN* gain detected by FISH was significantly higher than that of each of those genetic anomalies in no *MYCN* amplification determined by FISH, respectively. These aberrations are among the unfavorable factors in neuroblastoma [13]. In this study, the prognosis of the patients with genetic anomalies, such as 1p loss, 3p loss, 11q loss and 17q gain is significantly poor in comparison to that of the patients without them in the patients with *MYCN* gain and no *MYCN* amplification by FISH [with 1p loss ($n = 5$: $40.0 \pm 22.0\%$) versus without 1p loss ($n = 34$: $94.1 \pm 4.0\%$), $p = 0.0004$; with 3p loss ($n = 8$: $62.5 \pm 17.1\%$) versus without 3p loss ($n = 31$: $93.5 \pm 4.4\%$), $p = 0.008$; with 11q loss ($n = 13$: $69.2 \pm 12.8\%$) versus without 11q loss ($n = 26$: $96.2 \pm 3.7\%$), $p = 0.007$; with 17q gain ($n = 15$: $66.7 \pm 12.2\%$) versus without 17q gain ($n = 24$: $100 \pm 0\%$), $p = 0.001$]. However, the prognosis of patients with *MYCN* gain (=2p gain) was not significantly different from that of patients in no *MYCN* amplification. We suggest that *MYCN* gain may not be an independent prognostic factor and the prognosis of *MYCN* gain may be affected by multiple factors associated with genetic aberrations.

We reported that a slight increase in the gene dosage of *MYCN* detected by Q-PCR may indicate that the NB tissue contains a small number of cells with either *MYCN* amplification or a large number of cells with *MYCN* gain using the microdissection system and FISH method [18]. However, 3 of 7 cases with *MYCN* gain showed a slight increase of the *MYCN* gene by Q-PCR. The other 4 cases with *MYCN* gain did not show a slight increase in *MYCN* gene quantified by Q-PCR. This result suggests that it is difficult to detect the all *MYCN*-gain cases accurately by the Q-PCR method, because the degree of the gain of the *MYCN* gene and the ratio of the tumor cells and the normal cells, such as lymphocytes is different in each *MYCN*-gain case. Therefore, the FISH method might thus make it possible to evaluate the *MYCN* gene status more accurately in comparison to the Q-PCR method.

In conclusion, the present study suggested that the *MYCN* gain detected by FISH represents the 2p gain, and the *MYCN* gain is not considered to represent the pre-status of *MYCN* amplification. In addition, *MYCN* gain may not be an independent prognostic factor in NB.

Acknowledgments The authors thank Dr. Ken Yamamoto for helpful discussions and thank Mr. Brian Quinn for reading and editing the manuscript.

References

1. Brodeur GM, Maris JM, Yamashiro DJ, Hogarty MD, White PS (1997) Biology and genetics of human neuroblastomas. J Pediatr Haematol Oncol 19:93–101
2. Brodeur GM, Seeger RC, Schwab M, Varmus HE, Bishop JM (1984) Amplification of N-myc in untreated human neuroblastoma correlates with advanced disease stage. Science 224: 1121–1124
3. Seeger RC, Brodeur GM, Sather H, Dalton A, Siegel SE, Wong KY, Hammond D (1985) Association of multiple copies of the

Nmyc oncogene with rapid progression of neuroblastomas. N Engl J Med 313:1111–1116
4. Tanaka S, Tajiri T, Noguchi S, Shono K, Ihara K, Hara T, Suita S (2004) Clinical significance of a highly sensitive analysis for gene dosage and the expression level of MYCN in neuroblastoma. J Pediatr Surg 39:63–68
5. Tajiri T, Shono K, Fujii Y, Noguchi S, Kinoshita Y, Tsuneyoshi M, Suita S (1999) Highly sensitive analysis for N-myc amplification in neuroblastoma based on fluorescence in situ hybridization. J Pediatr Surg 34:1615–1619
6. Tajiri T, Tanaka S, Shono K, Kinoshita Y, Fujii Y, Suita S, Ihara K, Hara T (2001) Quick quantitative analysis of gene dosages associated with prognosis in neuroblastoma. Cancer Lett 166: 89–94
7. Spitz R, Hero B, Skowron M, Ernestus K, Berthold F (2004) MYCN-status neuroblastoma: characteristics of tumors showing amplification, gain, and non-amplification. Eur J Cancer 40:2753–2759
8. Ambros PF, Ambros IM (2001) SIOP Europe neuroblastoma pathology, biology, and bone marrow group: pathology and biology guidelines for resectable and unresectable neuroblastic tumors and bone marrow examination guidelines. Med Pediatr Oncol 37:492–504
9. Guo C, White PS, Weiss MJ, Hogarty MD, Thompson PM, Stram DO, Gerbing R, Matthay KK, Seeger RC, Brodeur GM, Maris JM (1999) Allelic deletion at 11q23 is common in MYCN single copy neuroblastomas. Oncogene 18:4948–4957
10. Plantaz D, Mohapatra G, Matthay KK, Pellarin M, Seeger RC, Feuerstein BG (1997) Gain of chromosome 17 is the most frequent abnormality detected in neuroblastoma by comparative genomic hybridization. Am J Pathol 150:81–89
11. Łastowska M, Viprey V, Santibanez-Koref M, Wappler I, Peters H, Cullinane C, Roberts P, Hall AG, Tweddle DA, Pearson AD, Lewis I, Burchill SA (2007) Identification of candidate genes involved in neuroblastoma progression by combining genomic and expression microarray with survival data. Oncogene 26: 7432–7444
12. Carén H, Erichsen J, Olsson L, Enerbäck C, Sjöberg RM, Abrahamsson J, Kogner P, Martinsson T (2008) High-resolution array copy number analyses for detection of deletion, gain, amplification and copy-neutral LOH in primary neuroblastoma tumors: four cases of homozygous deletions of the CDKN2A gene. BMC Genomics 9:353
13. Schleiermacher G, Michon J, Huon I, d'Enghien CD, Klijanienko J, Brisse H, Ribeiro A, Mosseri V, Rubie H, Munzer C, Thomas C, Valteau-Couanet D, Auvrignon A, Plantaz D, Delattre O, Couturier J, Société Française des Cancers de l'Enfant (SFCE) (2007) Chromosomal CGH identifies patients with a higher risk of relapse in neuroblastoma without MYCN amplification. Br J Cancer 97:238–246
14. Van Roy N, Van Limbergen H, Vandesompele J, Van Gele M, Poppe B, Laureys G, De Paepe A, Speleman F (2000) Chromosome 2 short arm translocations revealed by M-FISH analysis of neuroblastoma cell lines. Med Pediatr Oncol 35:538–540
15. Corvi R, Sevelyeva L, Schwab M (1995) Duplication of N-MYC at its resident site may be a mechanism of activation alternative to amplification in human neuroblastoma cells. Cancer Res 55:3471–3474
16. Tsuda T, Obara M, Hirano H (1987) Analysis of N-myc amplification in relation to disease stage and histologic types in human neuroblastomas. Cancer 60:820–826
17. Taylor SR, Roedere M, Murphy RF (1987) Flow cytometric DNA analysis of adenocortical tumors in children. Cancer 59:2059–2063
18. Souzaki R, Tajiri T, Higashi M, Kinoshita Y, Tanaka S, Kohashi K, Tsuneyoshi M, Taguchi T (2008) Clinical implications of a slight increase in the gene dosage of MYCN in neuroblastoma determined using quantitative PCR. Pediatr Surg Int 24:1095–1100
19. Gotoh T, Hosoi H, Iehara T, Kuwahara Y, Osone S, Tsuchiya K, Ohira M, Nakagawara A, Kuroda H, Sugimoto T (2005) Prediction of MYCN amplification in neuroblastoma using serum DNA and real-time quantitative polymerase chain reaction. J Clin Oncol 23:5205–5210

Journal of Pediatric Surgery (2010) 45, 2299–2304

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Hedgehog signaling pathway in neuroblastoma differentiation

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Received 27 July 2010; accepted 12 August 2010

Key words:
Neuroblastoma;
Hedgehog signal;
GLI1

Abstract

Purpose: The hedgehog (Hh) signaling pathway is activated in some adult cancers. On the other hand, the Hh signaling pathway plays an important role in the development of the neural crest in embryos. The aim of this study is to show the activation of Hh signaling pathway in neuroblastoma (NB), a pediatric malignancy arising from neural crest cells, and to reveal the meaning of the Hh signaling pathway in NB development.

Methods: This study analyzed the expression of Sonic hedgehog (Shh), GLI1, and Patched 1 (Ptch1), transactivators of Hh signaling pathway, by immunohistochemistry in 82 NB and 10 ganglioneuroblastoma cases. All 92 cases were evaluated for the status of *MYCN* amplification.

Results: Of the 92 cases, 67 (73%) were positive for Shh, 62 cases (67%) were positive for GLI1, and 73 cases (79%) were positive for Ptch1. Only 2 (10%) of the 20 cases with *MYCN* amplification were positive for Shh and GLI1, and 4 cases (20%) were positive for Ptch1 (*MYCN* amplification vs no *MYCN* amplification, $P \leqq .01$). The percentage of GLI1-positive cells in the cases with INSS stage 1 without *MYCN* amplification was significantly higher than that with INSS stage 4. Of 72 cases without *MYCN* amplification, 60 were GLI1-positive. Twelve cases were GLI1-negative, and the prognosis of the GLI1-positive cases was significantly better than that of the GLI1-negative cases ($P = .015$).

Conclusions: Most of NBs without *MYCN* amplification were positive for Shh, GLI1, and Ptch1. In the cases without *MYCN* amplification, the high expression of GLI1 was significantly associated with early clinical stage and a good prognosis of the patients. In contrast to adult cancers, the activation of the Hh signaling pathway in NB may be associated with the differentiation of the NB.

The hedgehog (Hh) signaling pathway plays an important role in the growth and patterning in a variety of tissues during embryonic development [1,2]. In addition, Hh signaling is associated with neural crest cell development [3,4]. On the other hand, the activation of the Hh signal pathway because of mutations or amplification of the pathway is associated with tumorigenesis of glioma [5], medulloblastoma [6], and basal cell carcinoma [7]. Furthermore, the hedgehog ligand-dependent stimulation of the Hh pathway is associated with

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0022-3468/$ – see front matter © 2010 Elsevier Inc. All rights reserved.
doi:10.1016/j.jpedsurg.2010.08.020

tumor development in other cancers such as gastric cancer [8], pancreas cancer [9], and breast cancer [10].

The Hh proteins, Sonic hedgehog (Shh), Desert hedgehog, and Indian hedgehog, act as ligands for the receptor Patched 1 protein (Ptch1) that is located on the cell membrane [11]. Hedgehog signal transduction is initiated by the binding Hh proteins to Ptch1. Ptch1 inhibits the activity of a transmembrane protein (smoothened; SMO) that activates factors downstream of Hh signaling pathway when those ligands are not bound to Ptch1. SMO stimulates a signaling cascade that results in the activation of the transcription factors Gli proteins (GLI1, GLI2, and GLI3) [12] when ligands are bound to Ptch1. GLI1 is amplified in glioma [5] and is a strong positive activator of downstream target genes in the nuclear, and GLI1 is a transcriptional activator of Hh signaling itself [13]. Therefore, GLI1 staining in the nucleus by immunohistochemistry is a marker of activation for Hh signaling [8,10].

Neuroblastoma (NB) is the most common solid malignant tumor in children arising from neural crest cells and usually occurs in the adrenal medulla. Neuroblastomas showed various clinical courses, and many studies have found both clinical and biological markers associated with the prognosis. *MYCN* gene amplification occurs in approximately 25% of primary NBs, and this factor is one of the most unfavorable prognostic factors in NB [14-16].

Hh signaling activation is associated with the development of neural crest cell, but there has been no evidence of a role in NB development. The aim of this study is to determine whether Hh signaling activation is associated with differentiation or tumorigenesis in NB.

1. Materials and methods

1.1. Clinical data of patients and biological data of NB samples

Patients were diagnosed with NB between April 1988 and March 2008. Eighty-two NB samples and 10 ganglioneuroblastoma samples were obtained. The tumor was staged according to the International Neuroblastoma Staging System (INSS). All of the parents of the patients provided informed consent for tumor preservation and the biological analysis before surgery. This study was performed according to ethical guidelines for the clinical studies by Ministry of Health, Labour, and Welfare of Japan in July 30, 2003. The patients included 56 males and 36 females; and 33 were INSS stage 1, 9 were stage 2, 16 were stage 3, 29 were stage 4, and 5 were stage 4S. Fifty-eight had been diagnosed when they were younger than 12 months. Forty-six patients were identified by a mass screening program in Japan at 6 months of age. Immunohistochemical analyses were performed in all 92 cases, and evaluated for *MYCN* gene amplification using Southern blotting or quantitative polymerase chain reaction as described previously [17,18]. *MYCN* amplification was defined as an *MYCN* gene copy of 2 or more in Southern blotting and a corrected *MYCN* gene dosage of more than 4.00 in quantitative polymerase chain reaction.

1.2. Immunohistochemistry

The immunohistochemical study was performed using the streptavidin-biotin-peroxidase method (Histofine; Nichirei, Tokyo, Japan). Samples were fixed in 10% formalin and embedded in paraffin. The primary antibodies used in this study were anti-SHH (1:100, N-19, sc-1194, Santa Cruz Biotechnology, Santa Cruz, Calif), anti-GLI1 (1:100, N-16, sc-6153, Santa Cruz Biotechnology), and anti-PTCH1 (1:100, H-267, sc-9016, Santa Cruz Biotechnology). All primary antibodies were incubated 2 hours at room temperature. Secondary antibodies were applied for 1 hour at room temperature. The results were visualized with diaminobenzidine. Slides were counterstained by hematoxylin. A number of cytoplasmic-positive cells or cell membrane–positive cells in neuroblasts were counted. The staining was judged as negative if the intensity of staining was similar to that of background staining. Three hundred cells were counted, and the percentage of positive cells was calculated for each section. The immunoreactivities were classified into 3 categories: −, 0% to 50% tumor cell positive; +, 50% to 90%; ++, 90% to 100%. The sample was judged to be "positive" if more than 50% of the tumor cells were positive. We determined the percentage of neuroblasts showing nuclear staining of GLI1 strongly in relation to the total number of neuroblasts. The NB component was used to judge the cases of ganglioneuroblastoma nodular. The specimens were determined by an independent pathologist who knew neither staging nor the status of *MYCN*.

1.3. Statistical analysis

Mann-Whitney U test and χ^2 test were used for statistical analysis. The survival curve was estimated using the Kaplan-Meier procedure and then it was statistically evaluated by the log-rank test. Results were considered to be significantly different when $P < .05$.

2. Results

2.1. Association of expression of Shh, GLI1, and Ptch1 proteins and the status of MYCN gene

The Shh and Ptch1 staining was observed in the cell membrane strongly, and GLI1 was localized in the cytoplasm in neuroblasts with ganglionic differentiation in GNB samples (Fig. 1A). The Shh, GLI1, and Ptch1 staining intensity of GNBs was higher than that of NBs, and most

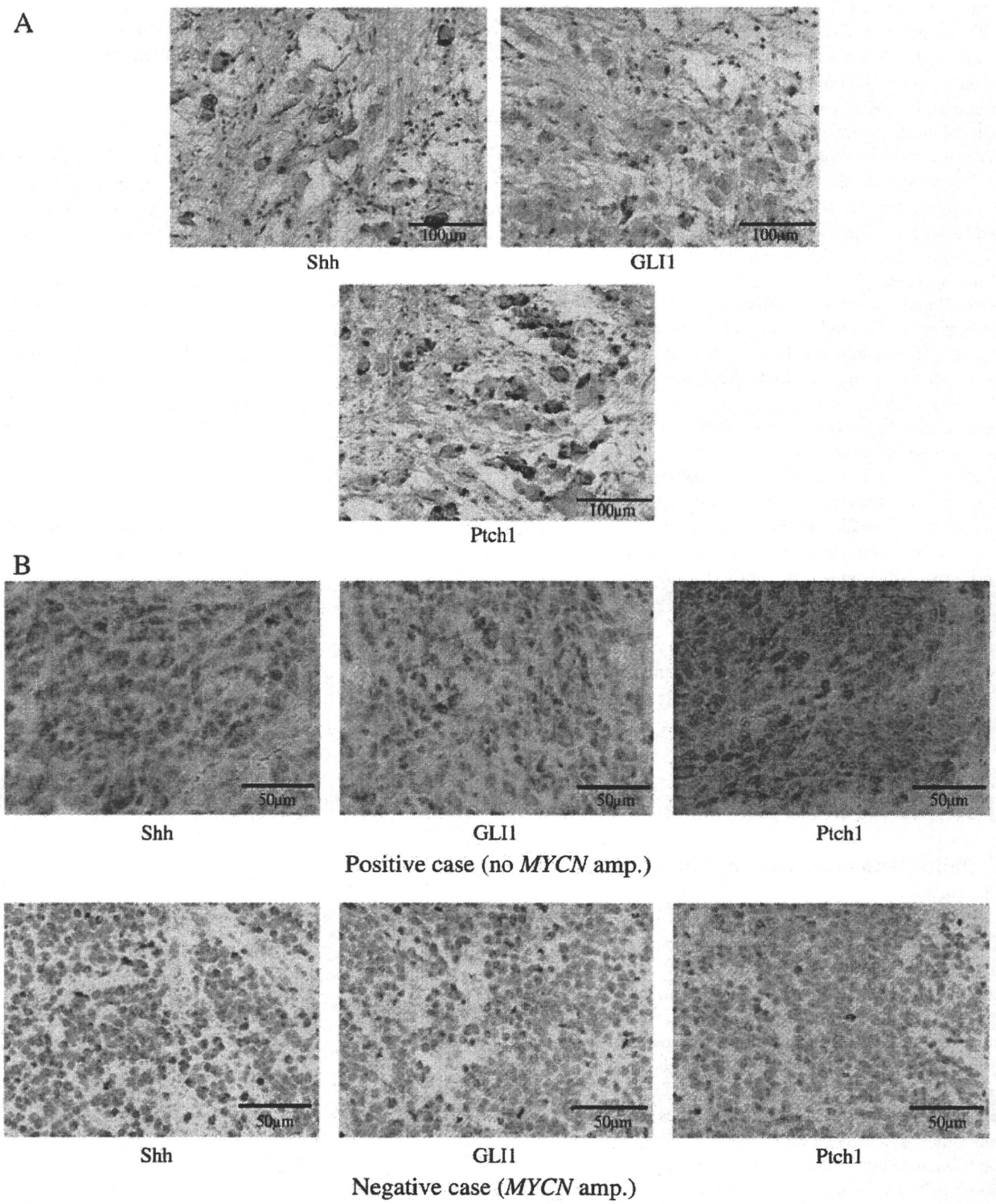

Fig. 1 A, Shh and Ptch1 staining was observed in the cell membrane strongly and GLI1 was localized in the cytoplasm of neuroblast with differentiation in ganglioneuroblastoma. No cells showed GLI1 nuclear stain (original magnification ×200). The cells demonstrating a positive expression cells are brown in color. The nuclei were stained with hematoxylin (purple). B, Shh-, GLI1-, and Ptch1-positive cases in NB without *MYCN* amplification and negative cases with *MYCN* amplification. No cells showed GLI1 nuclear stain in GLI1-positive case (top center panel) (original magnification ×400). The cells demonstrating a positive expression are brown in color. The nuclei were stained with hematoxylin (purple).

Table 1 Expression of Hh signal proteins (Shh, GLI1, Ptch1) in 92 samples

	Positive		Negative
	++	+	−
Shh	64	3	25 (27%)
	67(73%)		
GLI1	53	9	30 (33%)
	62(67%)		
Ptch1	70	3	19 (21%)
	73(79%)		

Schwann cells were negative for Shh, GLI1, and Ptch1 (Fig. 1A, B).

The result of immunohistochemistry of 92 samples is shown in Table 1. Sixty-seven of 92 samples (73%) were positive for Shh, 62 (67%) for GLI1, and 73 (79%) for Ptch1. Of 46 samples identified by mass screening system in Japan, 43 samples (93%) were positive for Shh; 39 (85%), for GLI1; and 45 (98%), for Ptch1. As shown in Table 2, only 2 (10%) of 20 samples with *MYCN* amplification were positive for Shh and GLI1, respectively, and 4 samples (20%) were positive for Ptch1. On the other hand, 65 (90%) of 72 samples without *MYCN* amplification were positive for Shh, 60 samples (83%) for GLI1, and 69 samples (96%) for Ptch1. There was a significant association between Hh signal proteins (Shh, GLI1, and Ptch1) and the status of *MYCN* gene ($P \leqq .01$).

2.2. Correlation of the expression of GLI1 and clinical stage (INSS) in the 72 NBs without MYCN amplification

Fig. 2 shows the correlation of the percentage of GLI1-positive cells and clinical stage (INSS) in the 72 cases without *MYCN* amplification. GLI1 is located downstream of the Hh signaling pathway, and it is a strong positive activator of target genes. The percentage of GLI1-positive cells in the cases with stage 1 was significantly higher than that with stage 4 (92.5 ± 21.3% vs 69.6 ± 38.8%; $P = .04$). However, there were no significant difference between stage 1 and stage 2 ($P = .23$), stage 1 and stage 3 ($P = .15$), or stage 1 and stage 4S ($P = .57$), respectively.

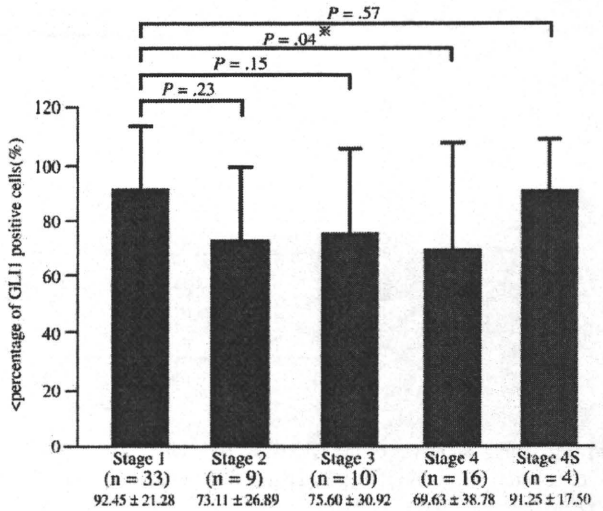

Fig. 2 Correlation between the percentage of GLI1-positive cells and clinical stage (INSS) in the 72 cases without *MYCN* amplification. The percentage of GLI1-positive cells in stage 1 cases was significantly higher than that in stage 4 (92.5 ± 21.3% vs 69.6 ± 38.8%; $P = .04$).

2.3. Association of the outcome of patients and the expression of GLI1 protein

Fig. 3 shows the survival curves of the patients with GLI1-positive cases (n = 60) and GLI1-negative cases (n = 12) in 72 NBs without *MYCN* amplification. The 5-year overall survival rate (OS) of GLI1-positive patients (93.7 ± 3.5%) was significantly higher in comparison to that of GLI1-negative patients (70.0 ± 14.5%; $P = .015$). On the other hand, 5-year OS of the patients with *MYCN*

Table 2 Associations of the expression of Hh signal proteins and the status of *MYCN* amplification in NBs

		Positive		Negative		
		++	+	−		
Shh	*MYCN* amplification	2	0	18	20	$P \leqq .01$
	No *MYCN* amplification	62	3	7	72	
		64	3	25		
GLI1	*MYCN* amplification	2	0	18	20	$P \leqq .01$
	No *MYCN* amplification	51	9	12	72	
		53	9	30		
Ptch1	*MYCN* amplification	4	0	16	20	$P \leqq .01$
	No *MYCN* amplification	66	3	3	72	
		70	3	19		

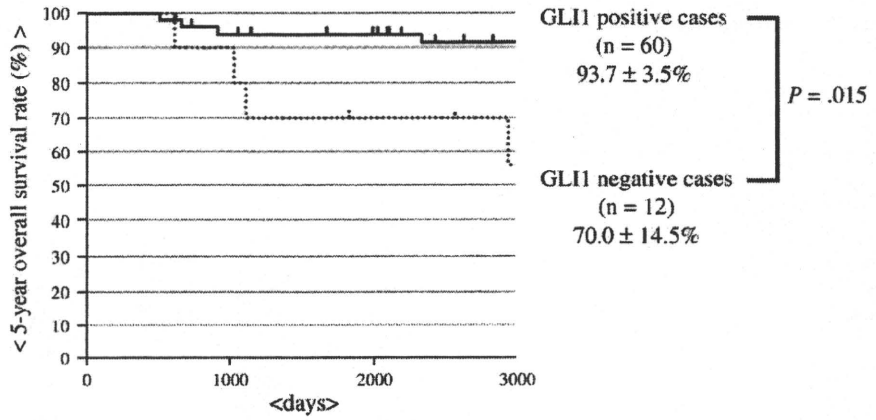

Fig. 3 Survival curves of the GLI1-positive patients (n = 60) and GLI1-negative cases (n = 12) in 72 NBs without *MYCN* amplification. The 5-year overall survival rate of GLI1-positive patients (n = 60, 93.7 ± 3.5%) was significantly higher in comparison to that of GLI1-negative patients (n = 12, 70.0 ± 14.5%).

amplification (n = 20) was 36.1 ± 11.2% and the 2 GLI1-positive cases with *MYCN* amplification are alive without disease after treatment.

2.4. GLI1 nuclear staining in NBs

The percentage of GLI1 nuclear staining was very low in all 92 cases (1.98% ± 3.83%, 0%-15%; Fig. 1A, B) and only 2 of 92 samples were higher than 10%.

3. Discussion

The current study showed that Hh signaling pathway–associated proteins such as Shh, GLI1, and Ptch1 were expressed in most NB cases, especially cases without *MYCN* amplification. Moreover, a number of positive cases show "++", whereas "+" cases are rare. As a result, most of the positive cases tended to be easy to distinguish as positive cases. The percentage of GLI1-positive cells in early-stage samples was higher than that with advanced stage. The 5-year OS rate of GLI1-positive cases without *MYCN* amplification is significantly higher than that in GLI1-negative cases. Only 2 of all 92 samples showed more than 10% of GLI1 nuclear staining.

The proteins of Hh signaling pathway such as Shh, Ptch1, and GLI1 are highly expressed in various pediatric malignant tumors such as NB [19,20], rhabdomyosarcoma [21], and clear cell sarcoma [22]. This study is the largest series evaluated for the Hh signal activation of the primary NB samples.

Mao et al [20] reported that 48% to 70% of primary NBs were positive for Hh signal–associated proteins and the ligand-dependent Hh pathway was activated in NB cell lines. These results are consistent similar with the current results. The current study found that 67% to 79% of NB samples showed expression of Shh, GLI1, and Ptch1. Only 2 of 92 cases were GLI1-positive, Shh-negative, and Ptch1-negative (data not shown). Therefore, the Hh signal activation of NB may be via the ligand-dependent pathway, and may not be a mutation or amplification of the transactivator on the Hh signal pathway as observed in medulloblastoma [6] and basal cell carcinoma [7]. Oue et al [19] reported that early-stage NBs highly express GLI1 in comparison to advanced-stage NBs. Our data also show that not only early-stage samples but also samples without *MYCN* amplification tended to be positive for Hh signal–associated proteins.

Some studies have suggested an association between Hh signaling activation and NB in vitro, although these results remain controversial. GLI1 transduction of an NB cell line inhibits proliferation and it induces a pattern of gene expression that resembles the gene expression of ganglioneuroma and the transcriptional response of treatment with the retinoic acid [23]. Therefore, Hh signal activation may be associated with the differentiation of NB. On the contrary, the inhibition of Hh signaling of NB cell line by cyclopamine, inhibitor of SMO, induces apoptosis and the Hh signal stimulates the tumorigenicity of NB cells [20]. Further examination will be necessary for NB primary samples to reveal the association between NBs development and Hh signaling.

The GLI1 nuclear staining with the immunochemistry examination has been reported to be observed in gastric cancer [8] and breast cancer [10], strongly suggesting the activation of Hh signaling. Although a large number of NBs and GNBs cases were positive for GLI1, there were very few cases with nuclear staining of GLI1 in the current study. This result might be associated with the good prognosis of the GLI1-positive cases in NB in contrast to that in adult cancers.

In conclusion, most of NBs without *MYCN* amplification were positive for Shh, GLI1, and Ptch1. In contrast, most of NBs with *MYCN* amplification were negative for Shh, GLI1, and Ptch1. In the cases without *MYCN* amplification, the high expression of GLI1 was significantly associated with early clinical stage and a good prognosis of the patients. In contrast to adult cancers, these findings may show that the activation

of the Hh signaling pathway in NB is associated with the differentiation of the NB and a good prognosis of the patients.

References

[1] Ramalho-Santos M, Melton DA, McMahon AP. Hedgehog signals regulate multiple aspects of gastrointestinal development. Development 2000;127(12):2763-72.

[2] Hebrok M. Hedgehog signaling in pancreas development. Mech Dev 2003;120(1):45-57.

[3] Ahlgren SC, Bronner-Fraser M. Inhibition of sonic hedgehog signaling in vivo results in craniofacial neural crest cell death. Curr Biol 1999;9: 1304-14.

[4] Williams Z, Tse V, Hou L, et al. Sonic hedgehog promotes proliferation and tyrosine hydroxylase induction of postnatal sympathetic cells in vitro. Neuroreport 2000;11:3315-9.

[5] Kinzler KW, Bigner SH, Bigner DD, et al. Identification of an ampliied, highly expressed gene in a human glioma. Science 1987;236:70-3.

[6] Pietsch T, Waha A, Koch A, et al. Medulloblastomas of the desmoplastic variant carry mutations of the human homologue of Drosophila patched. Cancer Res 1997;57:2085-8.

[7] Toftgard R. Hedgehog signalling in cancer. Cell Mol Life Sci 2000;57: 1720-31.

[8] Yanai K, Nagai S, Wada J, et al. Hedgehog signaling pathway is a possible therapeutic target for gastric cancer. J.Surg. Oncol 2007;95 (1):55-62.

[9] Thayer SP, di Magliano MP, Heiser PW, et al. Hedgehog is an early and late mediator of pancreatic cancer tumorigenesis. Nature 2003;425 (6960):851-6.

[10] Kubo M, Nakamura M, Tasaki A, et al. Hedgehog signaling pathway is a new therapeutic target for patients with breast cancer. Cancer Res 2004;64(17):6071-4.

[11] Hooper JE, Scott MP. Communicating with Hedgehogs. Nat Rev Mol Cell Biol 2005;6:306-17.

[12] Paces-Fessy M, Boucher D, Petit E, et al. The negative regulator of Gli, Suppressor of fused (Sufu), interacts with SAP18, Galectin3 and other nuclear proteins. Biochem J 2004;378:353-62.

[13] Lee J, Platt KA, Censullo P, et al. Gli1 is a target of Sonic hedgehog that induces ventral neural tube development. Development 1997;124: 2537-52.

[14] Brodeur GM, Maris JM, Yamashiro DJ, et al. Biology and genetics of human neuroblastomas. J Pediatr Hematolo Oncol 1997;19:93-101.

[15] Brodeur GM, Seeger RC, Schwab M, et al. Amplification of N-myc in untreated human neuroblastoma correlates with advanced disease stage. Science 1984;224:1121-4.

[16] Seeger RC, Brodeur GM, Sather H, et al. Association of multiple copies of the Nmyc oncogene with rapid progression of neuroblastomas. N Engl J Med 1985;313:1111-6.

[17] Souzaki R, Tajiri T, Higashi M, et al. Clinical implications of a slight increase in the gene dosage of MYCN in neuroblastoma determined using quantitative PCR. Pediatr Surg Int 2008;24:1095-100.

[18] Tsuda T, Obara M, Hirano H. Analysis of N-myc amplification in relation to disease stage and histologic types in human neuroblastomas. Cancer 1987;60:820-6.

[19] Oue T, Yoneda A, Uehara S, et al. Increased expression of the hedgehog signaling pathway in pediatric solid malignancies. J Pediatr Surg 2010 Feb;45(2):387-92.

[20] Mao L, Xia YP, Zhou YN, et al. A critical role of Sonic Hedgehog signaling in maintaining the tumorigenicity of neuroblastoma cells. Cancer Sci 2009 Oct;100(10):1848-55.

[21] Tostar U, Malm CJ, Meis-Kindblom J, et al. Deregulation of the hedgehog signaling pathway: a possible role for the PTCH and SUFU genes in human rhabdomyoma and rhabdomyosarcoma development. J Pathol 2006;208:17-25.

[22] Cutcliffe C, Kersey D, Huang CC, et al. Clear cell sarcoma of the kidney: up-regulation of neural markers with activation of the sonic hedgehog and Akt pathways. Clin Cancer Res 2005;11:7986-94.

[23] Gershon TR, Shirazi A, Qin LX, et al. Enteric neural crest differentiation in ganglioneuromas implicates Hedgehog signaling in peripheral neuroblastic tumor pathogenesis. PLoS One 2009;4 (10):e7491.

Journal of Pediatric Surgery (2010) 45, 2312–2316

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Journal of Pediatric Surgery

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Concordance for neuroblastoma in monozygotic twins: case report and review of the literature

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Received 27 July 2010; accepted 12 August 2010

Key words:
Neuroblastoma;
Monozygotic twin;
Twin-to-twin metastasis

Abstract The patients were infant male twins born by cesarean delivery following a healthy pregnancy at 36 weeks' gestation to unrelated parents. At 4 months of age, twin 2 presented with hepatomegaly and a right suprarenal mass. Resection of an adrenal tumor and a liver tumor biopsy were performed. Twin 1 had no symptoms at 4 months of age. Screening by abdominal ultrasonography showed multiple masses in the liver but no adrenal mass. Metaiodobenzylguanidine scintigraphy showed positive findings in multiple liver masses. A laparoscopic biopsy for a liver tumor was performed. All primary tumor and liver tumor specimens from twin 2 and the liver tumor of twin 1 had the same histologic classification of neuroblastoma and nearly identical genetic aberrations, including a chromosome gain or loss using array–comparative genomic hybridization. From these clinical and pathologic findings and genetic analyses, we strongly demonstrate the transplacental metastatic spread from twin 2 to twin 1. In the literature, 9 pairs of concordant twin neuroblastomas, including the current twin, have been presented; and the clinical findings of 5 twin pairs may represent placental metastases from one twin with congenital neuroblastoma to the other twin. This study is the first report presenting the possibility of twin-to-twin metastasis in monozygotic twins with neuroblastoma based on an analysis of the clinical features and genetic aberrations.

Neuroblastoma is the most common neonatal solid abdominal tumor [1]. The tumor originates from the neural crest cells of the adrenal medulla or sympathetic ganglia. The neuroblastoma concordance in monozygotic twins, that is, when both twins 1 and 2 have neuroblastoma and present either simultaneously or at different times, has not yet been described in detail. Furthermore, neuroblastoma in monozygotic twins is of interest because of the resulting insights regarding tumorigenesis and/or metastasis. In the present study, we reported simultaneous-onset neuroblastoma in monozygotic twins. Furthermore, we assessed the

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0022-3468/$ – see front matter © 2010 Elsevier Inc. All rights reserved.
doi:10.1016/j.jpedsurg.2010.08.025

mechanism of this shared pathology using a genetic analysis and a review of the pertinent literature.

1. Case report

1.1. Clinical features and outcomes

The patients were infant male twins born by cesarean delivery following a healthy pregnancy at 36 weeks' gestation to unrelated parents. During the gestational course, the twins did not undergo exposure to extrinsic factors that might affect neuroblastomas. At 4 months of age, twin 2 presented with hepatomegaly and a right suprarenal mass (Fig. 1A-D). The patient's urinary vanillylmandelic acid (339 μg/mg Cr) and homovanillic acid (447 μg/mg Cr) were markedly elevated. Metaiodobenzylguanidine (MIBG) scintigraphy showed positive findings in the patient's right adrenal tumor and multiple liver masses (Fig. 1B). An adrenal tumor resection and liver tumor biopsy were performed. Twin 1 had no symptoms at 4 months of age. A screening by abdominal ultrasonography and a computed tomographic scan showed multiple masses in the patient's liver, but no adrenal mass (Fig. 1C). The patient's urinary vanillylmandelic acid (15.9 μg/mg Cr) and homovanillic acid (23.4 μg/mg Cr) were not elevated. Metaiodobenzylguanidine scintigraphy showed positive findings in multiple liver masses (Fig. 1D). A laparoscopic-assisted biopsy was performed for the liver tumor. All specimens of the primary adrenal tumor and liver tumor of twin 2 and the liver tumor of twin 1 exhibited favorable neuroblastoma regarding the Shimada classification [2] (Fig. 2A, B, C) and no MYCN amplification. According to these histology and biology of

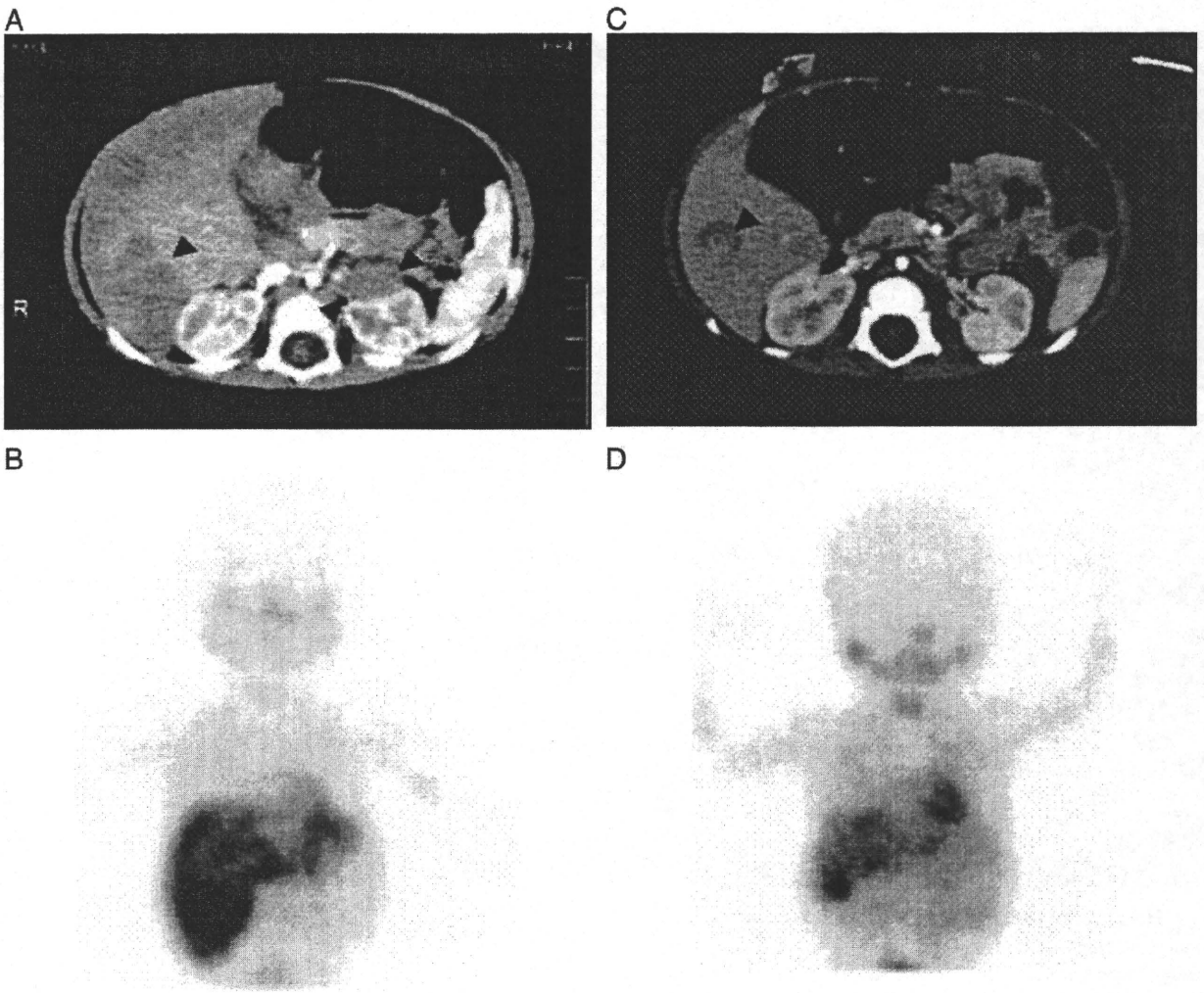

Fig. 1 Computed tomographic scan and MIBG scintigraphy of twin. A, Hepatomegaly and a right suprarenal mass in twin 2. B, Positive findings at right adrenal tumor and multiple liver masses in twin 2 as analyzed by MIBG scintigraphy. C, Multiple masses in liver, but no adrenal masses in twin 1. D, Positive finding at multiple liver masses in twin 1 using MIBG scintigraphy.

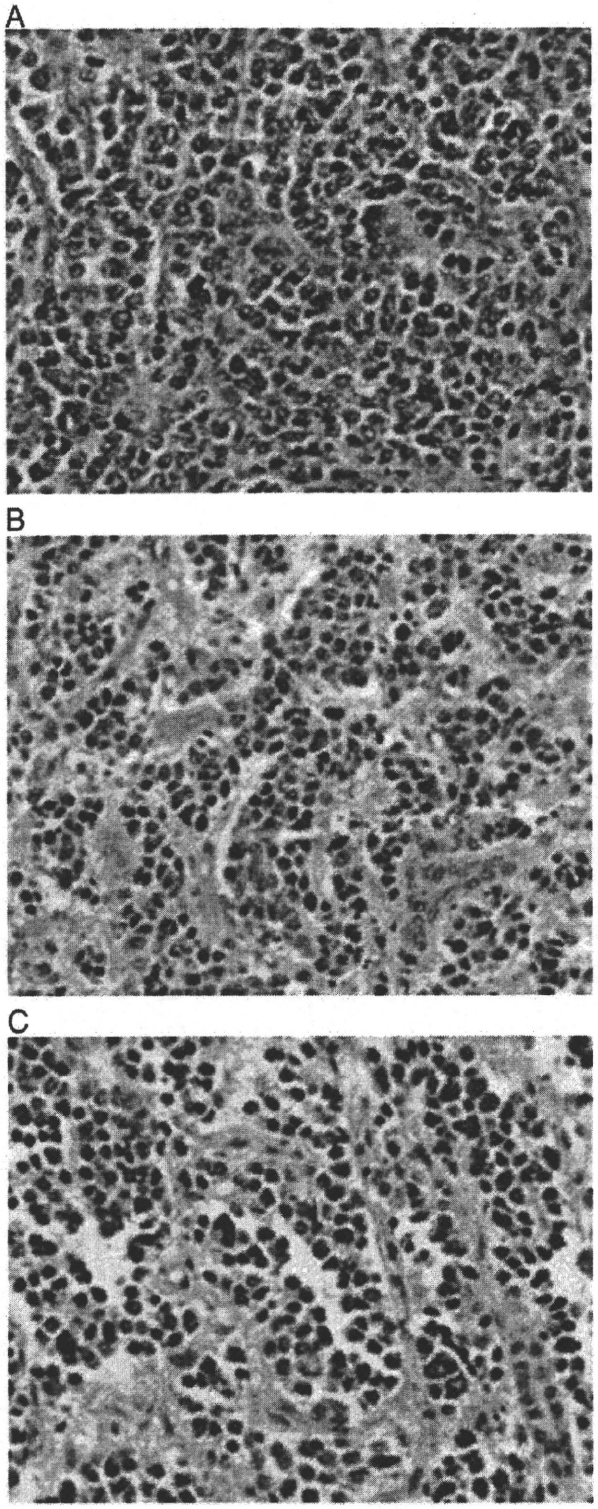

Fig. 2 Histology of the primary adrenal and liver tumors of twin 2 and the liver tumor of twin 1. A, Primary adrenal tumor of twin 2 (hematoxylin and eosin [H&E] stain). B, Liver tumor of twin 2 (H&E stain). C, Liver tumor of twin 1 (H&E stain). All sections show a proliferation of rounded cells with hyperchromatic nuclei and scant cytoplasm in a neuropil background, with histopathologic similarities at 3 sites.

tumors, mild chemotherapy including low dose of cyclophosphamide and vincristine was administered to twin 2 for a period of 6 months and patient observation is continuing for twin 1 for a period of 6 months after surgical intervention in both cases, respectively.

1.2. Histologic and genetic analysis for specimens of the primary tumor and the liver tumor of twin 2 and the liver tumor of twin 1

All specimens of primary adrenal tumor and liver tumor of twin 2 and liver tumor of twin 1 were indicative of a poorly differentiated neuroblastoma and also demonstrated a favorable histology regarding the Shimada classification. A histologic examination of several blocks of placenta revealed no metastatic tumor thrombi in villous stem vessels. No specimens had MYCN amplification, and all specimens were triploid.

To evaluate whole-genome aberrations, a microarray-based comparative genomic hybridization (array-CGH) was performed according to the methods described in a previous report by Tomioka et al [3] (244K Human CGH Oligo Microarray, Agilent Technology). For all specimens, the CGH type revealed entire chromosomal gains and losses type [3], which is associated with a favorable prognosis. Regarding the whole genome aberrations such as chromosome gains or losses, the only difference between the primary adrenal tumor and the liver tumor of twin 2 was chromosome 2; and the only difference between the primary adrenal tumor of twin 2 and the liver tumor of twin 1 was chromosome 14. Regarding chromosome 2, the primary adrenal tumor of twin 2 had a 2p whole gain and a 2q whole gain, whereas the liver tumor of twin 2 had a 2p partial gain and a 2q partial gain. Chromosome 14 was unchanged in the primary adrenal tumor of twin 2, but there was a 14q whole loss in the liver tumor of twin 1.

The consents of patients' parents for tumor preservation and the biological analysis were obtained before surgery. This study was performed according to the Ethical Guidelines for Clinical Research published by the Ministry of Health, Labor, and Welfare of Japan on July 30, 2003.

2. Discussion

Neuroblastomas can be multifocal and can be concordant or discordant (only one of the twins has the tumor) among monozygotic twins. To the best of our knowledge, as shown in Table 1, only 9 sets of monozygotic twins concordant for neuroblastoma, including the current cases, have been described; and no concordant neuroblastoma cases have been described for dizygotic twins [4-11].

Neuroblastomas can present in multiple ways. The most frequent presentation occurs in a child with a primary tumor (abdominothoracic) with or without metastases. In 1972,

Table 1 Review of literature of monozygotic twins concordant for neuroblastoma

References	Case	Sex	Age	Primary	Metastasis	Outcome	Zygosity
Lee, 1953	1	M	4 mo	L adrenal	Liver, bone, brain, testis	DOD 8.5 mo	Monozygous
	2	M	4 mo	R adrenal	Liver, bone, brain, testis	AWD 20 mo	
Cochran, 1963	3	F	–	R sympathetic	Liver	DOD 9.5 mo	Monozygous
	4	F	–	R sympathetic	Liver	DOD 11 mo	
Barrett and	5	–	Infancy	–	–	–	Identical twin
Toye, 1963	6	–	Infancy	–	–	–	
Miller, 1971	7	M	–	–	–	DOD 13 d	Twins, male
	8	M	–	–	–	DOD 16 mo	
Mancini, 1982	9	M	Birth	L adrenal	Liver, r adrenal medulla	Stillbirth	Identical twin and
	10	M	2 mo	None found	Liver	DOD 13 mo	single placenta
Boyd, 1995	11	M	1 wk	Retroperitoneum	Liver	DOD 1wk	*Diamnionic
	12	M	2 wk	None found	Liver, bone, skin, bil adrenal medulla	DOD 1 mo	monochorionic placenta
J Anderson, 2001	13	F	3 wk	L adrenal	Liver, bone marrow	NED 18 mo	Monozygous
	14	F	6 mo	R adrenal	Liver, r adrenal medulla	NED 18 mo	
I Adaletli, 2006	15	F	2 mo	L adrenal	Liver	NED 16 mo	Monozygous
	16	F	2 mo	None found	Liver	NED 16 mo	
Tajiri,	17	M	4 mo	None found	Liver	Ongoing mild	Monozygous
(current cases)						chemotherapy 6 mo	
	18	M	4 mo	L adrenal	Liver	Ongoing observation 6 mo	

DOD indicates died of disease; AWD, alive with disease; NED, no evidence of disease.

* Diamnionic monochorionic placenta: Histopathologic examination of metastatic placental tumors revealed tumor thrombi in the villous stem vessels and terminal villi without gross macroscopic placental lesions.

Knudson and Strong [12] suggested 2 hypotheses that are the most widely accepted etiologic model for childhood neuroblastoma. The hypotheses invoke both inherited and acquired genetic defects as the basis for tumor development. In familial (hereditary) cases of neuroblastoma, the first defect is an inherited germ line mutation present in all cells of the body. A second defect or hit occurs postzygotically in only somatic target cells, the neuroblast. In sporadic (nonhereditary) cases, mutations are postzygotic events in the same neuroblast. The pathogenesis of multifocal neuroblastoma appears to occur through the multicentric growth of neuroblastoma nodules or neuroblastoma in situ with a potential for regression or maturation [13].

The cause of the shared pathology of concordance for neuroblastoma in monozygotic twins has not been well established. The question remains whether the disease is a simultaneous onset of malignancy in both twins or is because of metastatic spread via placental vascular anastomoses in utero from one twin with congenital disease to the second twin. Only monochorionic placentas share fetoplacental circulation and thus are a potential mechanism for metastasis. In cases 11 and 12 in Table 1, the histopathologic examination of metastatic placental tumors revealed tumor thrombi in villous stem vessels and terminal villi without gross macroscopic placental lesions [9]. The massive hepatic metastases could have arisen as a first-pass effect through the entrance of blood through the umbilical vein. Systemic metastases could also occur if an umbilical venous flow with tumor cells enters the right atrium. Fetoplacental metastases are favored in cases in which one twin within a twin pair manifests a readily identifiable primary tumor and twin 2 manifests the disease either simultaneously or later without a recognizable primary site. When both twins of a given twin pair display obvious primary tumors with similar disease extents, a diagnosis of simultaneous primary neuroblastoma is favored. In view of these clinical features, 5 pairs (cases 9, 10; cases 11, 12; cases 13, 14; cases 15, 16; and cases 17, 18) of 9 pairs of concordant twin neuroblastoma, as shown in Table 1, are considered to be placental metastases from one twin with congenital neuroblastoma to the other.

With the introduction of molecular and genetic markers such as MYCN and DNA ploidy, clear-cut biological markers are currently available to aid in differentiating simultaneous primary tumors from the metastatic spread between twins. However, the detailed whole genome analysis of tumor samples from monozygotic twins had never been done in the previous reports. When the primary neuroblastoma generates in one of a twin, a variety of chromosomal changes occur in the primary tumor. We have identified a variety of chromosomal changes in primary neuroblastomas using array-CGH or SNP array [3]. Afterward, a little chromosomal change may occur in the metastatic tumors, when neuroblastoma cells spread to other organs. In the present study, the adrenal primary adrenal tumor of twin 2 had a variety of genetic aberrations

including the gain or loss of chromosomes as analyzed by array-CGH, whereas the liver tumor of twin 2 and the liver tumor of twin 1 had only one genetic change from primary adrenal tumor of twin 2 using array-CGH, respectively. This result of genetic analysis suggests that placental metastases from one twin with congenital neuroblastoma to the other has occurred in the present cases.

In summary, in the current cases, twin 1 presents the disease simultaneously without a recognizable primary site; and all primary tumor and liver tumor specimens from twin 2 and the liver tumor of twin 1 had the same histologic classification of neuroblastoma and nearly identical genetic aberrations, including a chromosome gain or loss using array-CGH. From these clinical and pathologic findings and genetic analyses, we herein strongly demonstrate the transplacental metastatic spread from twin 2 to twin 1 as the mechanism of shared neuroblastoma pathology. This article is the first report presenting the possibility of twin-to-twin metastasis in monozygotic twins with neuroblastoma by the analysis of clinical features and genetic aberrations.

Acknowledgments

We thank Dr Tomoko Iehara, Pediatrics, Kyoto Prefectural University of Medicine, Kyoto, Japan, for the personal communication. The English used in this manuscript was reviewed by Brian Quinn (Editor-in-Chief, Japan Medical Communication).

References

[1] Crist WM, Kun LE. Common solid tumors of childhood. N Engl J Med 1991;324:461-71.
[2] Shimada H, Chatten J, Newton Jr WA, et al. Histopathologic prognostic factors in neuroblastic tumors: definition of subtypes of ganglioneuroblastoma and an age-linked classification of neuroblastomas. J Natl Cancer Inst 1984;73:405-16.
[3] Tomioka N, Oba S, Ohira M, et al. Novel risk stratification of patients with neuroblastoma by genomic signature, which is independent of molecular signature. Oncogene 2008;17(27):441-9.
[4] Lee M. The surgical significance of tumors in identical twins: a short review of the literature and a report of sympathicoblastoma occurring in monozygotic twins. Am Surg 1953;19:803-11.
[5] Cochran W. Neuroblastoma (sympathicoblastoma) in Northern Ireland: a review over a ten year period. Ulster Med J 1963;32:82-98.
[6] Barrett AF, Toye DKM. Sympathicoblastoma: radiological findings in forty-three cases. Clin Radiol 1963;14:33-42.
[7] Miller RW, Fraumeni JF, Hill JA. Neuroblastoma: epidemiologic approach to its origin. Am J Dis Child 1968;115:253-61.
[8] Mancini Af, Rosito P, Faldella G, et al. Neuroblastoma in a pair of identical twins. Med Pediatr Oncol 1982;10:45-51.
[9] Boyd KT, Schofield ED. Monozygotic twins concordant for congenital neuroblastoma: case report and review of the literature. Pediatr Pathool Lab Med 1995;15:931-40.
[10] Anderson J, Kempski H, Hill L, et al. Neuroblastoma in monozygotic twins. A case of probable twin-to-twin metastasis. Br J Cancer 2001; 85:493-6.
[11] Adaletli I, Kurugoglu S, Aki H, et al. Simultaneous presentation of congenital neuroblastoma in monozygotic twin: a case of possible twin-to-twin metastasis. AJR Am J Roentgenol 2006;186:1172-5.
[12] Knudson Jr AG, Strong LC. Mutation and cancer: neuroblastoma and pheochromocytoma. Am J Hum Genet 1972;24:514-32.
[13] Hiyama K, et al. Multifocal neuroblastoma: biologic behavior and surgical aspects. Cancer 2000;88:1955-63.

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Changes over three decades in outcome and the prognostic influence of age-at-diagnosis in young patients with neuroblastoma: A report from the International Neuroblastoma Risk Group Project

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ARTICLE INFO

Article history:
Received 28 July 2010
Received in revised form 21 October 2010
Accepted 27 October 2010
Available online 26 November 2010

Keywords:
Neuroblastoma
Outcome

ABSTRACT

Purpose: Increasing age has been an adverse risk factor in children with neuroblastoma (NB) since the 1970's, with a 12-month age-at-diagnosis cut-off for treatment stratification. Over the last 30 years, treatment intensity for children >12 months with advanced-stage disease has increased; to investigate if this strategy has improved outcome and/or reduced the prognostic influence of age, we analysed the International Neuroblastoma Risk Group (INRG) database.

Patients and methods: Data from 11,037 children with NB (1974–2002) from Australia, Europe, Japan, North America. Cox modelling of event-free survival (EFS) tested if the era and prognostic significance of age-of-diagnosis, adjusted for bone marrow (BM) metastases and *MYCN* status, effects on outcome had changed.

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0959-8049/$ - see front matter
doi:10.1016/j.ejca.2010.10.022

Prognosis
Age-at-diagnosis
INRG

Results: Outcome improved over time: 3-year EFS 46% (1974–1989) and 71% (1997–2002). The risk for those >18 months against ⩽12 decreased: hazard ratio (HR); 4.61 and 3.94. For age 13–18 months, EFS increased from 42% to 77%. Outcome was worse if: >18 months (HR 4.47); BM metastases (HR 4.00); and *MYCN* amplified (HR 3.97). For 1997–2002, the EFS for >18 months with BM involvement and *MYCN* amplification was 18%, but 89% for 0–12 months with neither BM involvement nor *MYCN* amplification.
Conclusions: There is clear evidence for improving outcomes for children with NB over calendar time. The adverse influence of increasing age-at-diagnosis has declined but it remains a powerful indicator of unfavourable prognosis. These results support the age-of-diagnosis cut-off of greater than 18 months as a risk criterion in the INRG classification system.

1. Introduction

Numerous prognostic clinical and biological factors have been identified in neuroblastoma (NB). Age >12 months and widely disseminated disease were shown to be associated with poor outcome more than 40 years ago.[1,2] Amplification of the *MYCN* oncogene, genetic aberrations of chromosomes 1p, 11q, and 17q, and specific histologic features of the tumour are also associated with poor outcome.[3–8] Combinations of prognostic variables are now routinely used for risk-group assignment and for treatment stratification with significantly intensified regimens for those with high risk disease. If this is an effective strategy, outcome for children with NB should have increased over time while the prognostic influence of variables ascertained at diagnosis should decrease.

To evaluate the influence of these variables on outcome, the International Neuroblastoma Risk Group (INRG) established a database of 11,037 children with NB diagnosed between 1974 and 2002 by Australian, European, Japanese, and North American groups in order to develop a consensus approach to pre-treatment risk stratification. Because treatment regimens have changed substantially over the years, the resulting INRG classification system[9] was based on data from the more recent 8800 patients diagnosed between 1990 and 2002. The analysis identified sixteen risk groups, and age-at-diagnosis played an important role in group identification. The groups were amalgamated into very-low-, low-, intermediate- and high-risk categories based on projected event-free survival rates.

This analysis goes beyond the creation of the INRG classification system[9] as here we examine the changing influence of important prognostic indicators over the whole three decades. The purpose of this paper is to establish the magnitude of any changes and to examine in greater detail the influence of age-at-diagnosis; as a dichotomy at >18 months plays a pivotal role in determining greater risk in the INRG classification.

2. Patients and methods

2.1. *INRG database*

Data were collected on patients enrolled on the Children's Oncology Group, German Gesellschaft für Pädiatrische Onkologie und Hämatologie, Japanese Advanced Neuroblastoma Study Group, Japanese Infantile Neuroblastoma Co-operative Study Group, and International Society of Paediatric Oncology Europe Neuroblastoma Group trials. Enrollment cut-off of 2002 was chosen to allow at least two years follow-up at the 2004 data freeze. Eligibility included: confirmed diagnosis of NB or ganglioneuroblastoma (GNB); age ⩽21 years; diagnosis 1974–2002; informed consent. In addition to outcome data, information on 35 potential risk factors was requested although only age-at-diagnosis, the presence of bone marrow (BM) metastases and *MYCN* status are considered in detail here.

2.2. *Era*

In recognition of treatment changes that occurred from 1974 to 2002, the data have been divided into three analytic periods: those diagnosed between 1974–1989, 1990–1996 and 1997–2002. The years defining the era were selected based on major changes in therapeutic strategy. During 1974–1989, multi-agent chemotherapy regimens were introduced, though surgery and radiation therapy remained key modalities. During 1990–1996, the use of risk-based regimens became widespread, and therapy was intensified in patients at greater risk of relapse. Some patients with high-risk disease received stem cell transplants. After 1996, almost all high-risk patients underwent stem cell transplantation. In addition, the use of 13-*cis*-retinoic acid following transplant became widely accepted, and reductions of chemotherapy took place for low- and intermediate-risk patients.

2.3. *Statistical methods*

Event-free survival (EFS) is defined as the period from diagnosis to the first event: relapse, progression, secondary malignancy or death. Patients who experienced no event are censored at the date of last follow-up. Overall survival (OS) is calculated from diagnosis to death, while patients still alive are censored. EFS and OS curves were calculated using the Kaplan–Meier technique. The Cox proportional hazards model was used to estimate the hazard ratios (HRs) and 95% confidence intervals (CI).[10,11] The HR quantifies the increased risk of an event for one group of patients in comparison to another.

2.4. *Modelling strategy*

Univariate Cox regression models, for example for age-at-diagnosis alone, and multivariable models, for example for age and *MYCN* amplification status, have been constructed and compared. The magnitude of the effect of age on outcome is modified by the influence of other variables, particularly the era of initial diagnosis and the therapeutic approach at that time, so different modelling strategies were adopted. Some strategies are dictated by the way age was used 'clinically' to define risk groups.

2.5. *Justification for modelling with the presence of bone marrow metastases instead of INSS stage*

To test the effect of age in a multivariable model adjusting for the extent of disease, one would typically use INSS stage[12,13] for the adjustment. However, the INSS system uses age in the definition of 4s disease; thus, INSS stage and age are confounded. Results below support the use of BM metastases as more highly prognostic than any other site of metastases. As a consequence, the extent of disease is characterised by the presence of BM metastases instead of INSS stage.

2.6. *Missing values*

Age-at-diagnosis was available on all patients; however, serum ferritin, for example, was available on only 1.8% of patients from 1974 to 1989. Because we wish to examine trends over the whole calendar period, we considered only variables that are available in Era I and are of major prognostic importance. In addition an 'Unknown' category (a mixture of patients with and without the attribute) was created for each variable. This enabled, for example, the inclusion *MYCN* status in our models as the 'Unknown' survival curve group in each Era take a central position between the amplified and not amplified groups. This approach allows the modelling process to retain the same numbers of patients irrespective of which variables are included in the Cox model.

2.7. *Calculations*

Calculations were made using Stata version 10 (Stata Corp., 2007).[14]

3. Results

3.1. *Era*

From 11,037 patients, 4266 (39%) experienced an event, and 3627 (33%) died (Table 1). Although we focus on era, age, BM metastases and *MYCN* status on outcome, the characterisation of patients by INSS stage, other sites of metastases and initial treatment is presented. The overall 3-year EFS and OS rates were 62% and 70% (Fig. 1). The long-term EFS curve plateaus beyond 5 years at approximately 60%. EFS improved from 43% (Era I: 1974–1989) to 60% (Era II), to 68% (Era III: 1997–2002). Those diagnosed in Era III had one third of the event rate compared to those diagnosed in Era I (HR = 0.35; 95% CI: 0.32–0.38) (Fig. 1, Table 1). There was considerable variation in outcome within each Era, highly dependent on patient and tumour features.

3.2. *Age-at-diagnosis*

The age-at-diagnosis distribution (Fig. 2) is markedly skewed to the younger age groups. Therefore, for the first 2 years of life, EFS and OS analyses were performed on cohorts of patients divided into 3-month (91-day) intervals, whereas 6-month (182-day) intervals were used thereafter. The percentage of patients with an event increased from 15% in the youngest to over 60% in the oldest (Table 2). Further when compared to infants less than 3 months, HRs increase with age up to 911 days (30 months) and then plateau at approximately 5.0. In addition, the corresponding age-specific HRs (fitted by separate Cox models within each era) consistently decrease over time. For example, within Era I, the risk is 2.28 times greater for children with age-of-diagnosis 274–364 days compared to those of 0–91 days. For children of 274–364 days, the risk decreases over time (HRs: 2.28, 1.36, and 0.98 by era). Nevertheless, even in Era III, age remains a strong predictor of adverse outcome in patients >22 months, with HRs close to 4. Despite a gradually increasing risk with increasing age-at-diagnosis beyond 22 months, there is no obvious cut-point for categorisation into low- and high-risk groups.

Using the three broader age categories beyond 12 and 18 months, which correspond to the previous and newly recommended categories for higher risk children suggested by INSS and INRG classification system, respectively (Table 2), shows an increasing HR with age but now more smoothly because of the merged categories. The EFS (Fig. 3) shows a clear decline. There remains a suggestion of a weakening effect of age over the Era with, in general, HRs closer to unity but there remain a clear indication of declining 3-year EFS from 83.82%, through 68.35% to 43.28% as patient age-at-diagnosis increases. The outcome for those of 13–18 months of age has got progressively closer to that of the youngest patients over calendar time.

3.3. *Bone marrow metastases*

In single variable Cox models for EFS, the presence of BM metastases is more highly predictive of an event than any other metastatic site, with a HR = 1.89 for the unknown and 4.00 (CI: 3.76–4.26, $p < 0.001$) for those with involvement. The corresponding HRs for OS are 2.53 and 5.19 (Table 1). The long-term EFS is approximately 75% without BM involvement, 50% when the marrow status is unknown, and only 25% in those with confirmed BM involvement (Fig. 4). There is little relative change in the adverse prognosis associated with BM involvement over the three era, with successive HRs of 3.66, 4.25 and 3.16. Nevertheless, even in patients with BM metastases, the 3-year EFS improved from 22% to 35%, to 45%.

3.4. *MYCN*

MYCN status is also highly predictive of outcome with the HRs for amplified tumours 3.97 (CI: 3.64–4.32, $p < 0.001$) for EFS and 5.31 (4.84–5.82, $p < 0.001$) for OS (Table 1). Those unknown

Table 1 – Variables by Era of diagnosis for NB patients ≤21 years of age.

		Era I (%) 1974–1989	Era II (%) 1990–1996	Era III (%) 1997–2002	All era (%)		
Number	*n*	2207	5035	3795	11037		
Age (y)	Median	2.14	1.41	1.16	1.44		
	Range	0–20.0	0–19.5	0–20.9	0–20.9		
Age (*m*)	0–12	597 (27)	2003 (40)	1748 (46)	4348 (39)		
	13–18	235 (11)	585 (12)	457 (12)	1277 (12)		
	19–24	218 (10)	411 (8)	296 (8)	925 (8)		
	25+	1157 (52)	2036 (40)	1294 (34)	4487 (41)		
INSS	I	17 (1)	777 (15)	963 (25)	1757 (16)		
	IIa	22 (1)	278 (6)	276 (7)	576 (5)		
	IIb	11 (0)	245 (5)	285 (8)	541 (4)		
	III	93 (4)	828 (16)	660 (17)	1581 (15)		
	IV	319 (14)	1981 (39)	1261 (33)	3561 (32)		
	IVs	10 (0)	348 (7)	291 (8)	649 (6)		
	Unknown	1735 (79)	578 (11)	59 (2)	2372 (22)		
Metastases						Hazard ratio (HR)	
						EFS	OS
Bone marrow	No	1108 (50)	3186 (63)	2707 (71)	7001 (64)	1	1
	Unknown	84 (4)	126 (3)	315 (8)	525 (4)	1.89	2.53
	Yes	1015 (46)	1723 (34)	773 (20)	3511 (32)	4.00	5.19
Bone	No	1276 (58)	3587 (71)	2911 (77)	7774 (71)	1	1
	Unknown	109 (5)	141 (3)	314 (8)	564 (5)	2.14	2.80
	Yes	822 (37)	1307 (26)	570 (15)	2699 (24)	3.70	4.66
Distant lymph nodes	No	1481 (67)	3943 (78)	3181 (84)	8605 (78)	1	1
	Unknown	128 (6)	142 (3)	345 (9)	615 (5)	1.33	1.54
	Yes	598 (27)	950 (19)	269 (7)	1817 (17)	2.11	2.48
Liver	No	1728 (78)	4216 (84)	3166 (83)	9110 (83)	1	1
	Unknown	129 (6)	130 (3)	307 (8)	566 (5)	1.49	1.76
	Yes	350 (3)	689 (14)	322 (8)	1361 (12)	1.34	1.43
Skin	No	1134 (16)	4361 (87)	3325 (88)	8820 (80)	1	1
	Unknown	1049 (48)	578 (11)	410 (11)	2037 (18)	1.90	2.36
	Yes	24 (1)	96 (2)	60 (2)	180 (2)	0.93	0.83
Lung	No	1144 (52)	4271 (85)	3273 (86)	8688 (79)	1	1
	Unknown	1049 (48)	702 (14)	480 (13)	2231 (20)	1.77	2.18
	Yes	14 (1)	62 (1)	42 (1)	118 (1)	3.05	3.58
CNS	No	1124 (51)	4279 (85)	3281 (86)	8684 (79)	1	1
	Unknown	1049 (48)	702 (14)	480 (13)	2231 (20)	1.77	2.18
	Yes	34 (2)	54 (1)	34 (1)	122 (1)	2.65	3.12
Other	No	1590 (72)	4149 (82)	3223 (85)	8962 (81)	1	1
	Unknown	157 (7)	136 (3)	357 (9)	650 (6)	1.68	2.00
	Yes	460 (21)	750 (15)	215 (6)	1425 (13)	2.46	2.81
MYCN	Not amplified	233 (11)	3035 (60)	2910 (77)	6178 (56)	1	1
	Unknown	1901 (86)	1357 (27)	373 (10)	3631 (32)	2.33	3.12
	Amplified	73 (3)	643 (13)	512 (13)	1228 (12)	3.97	5.31
Therapy	Observation	24 (1)	261 (5)	80 (0)	365 (3)		
	Surgery alone	150 (7)	736 (15)	1206 (32)	2092 (19)		
	Chemo + surgery	653 (30)	1612 (32)	631 (17)	2896 (26)		
	HDT no stem cell	189 (9)	364 (7)	187 (5)	740 (7)		
	HD unknown Stem cell	112 (5)	500 (10)	293 (8)	905 (8)		
	HDT with stem cell	57 (3)	399 (8)	446 (12)	902 (9)		
	Unknown	1022 (46)	1163 (23)	952 (25)	3137 (28)		
Event		1285	2013	968	4266		
EFS (%)	3-year	46.3	63.3	71.0	62.0		
	5-year	43.3	60.4	68.3	59.1		
	HR	1	0.60	0.45			
Dead		1224	1725	678	3627		
OS (%)	3-year	52.0	71.6	80.3	70.0		
	5-year	47.1	66.8	76.6	65.3		
	HR	1	0.53	0.35			

MYCN status had HRs of 2.33 and 3.12 for EFS and OS. The long-term EFS for children without *MYCN* amplification is approximately 75%, 50% for unknown status and 25% with *MYCN*-amplified tumours (Fig. 5). Little relative change in the adverse prognosis of *MYCN* amplification across era is seen (Table 3), with successive HRs of 2.50 during 1974–1989

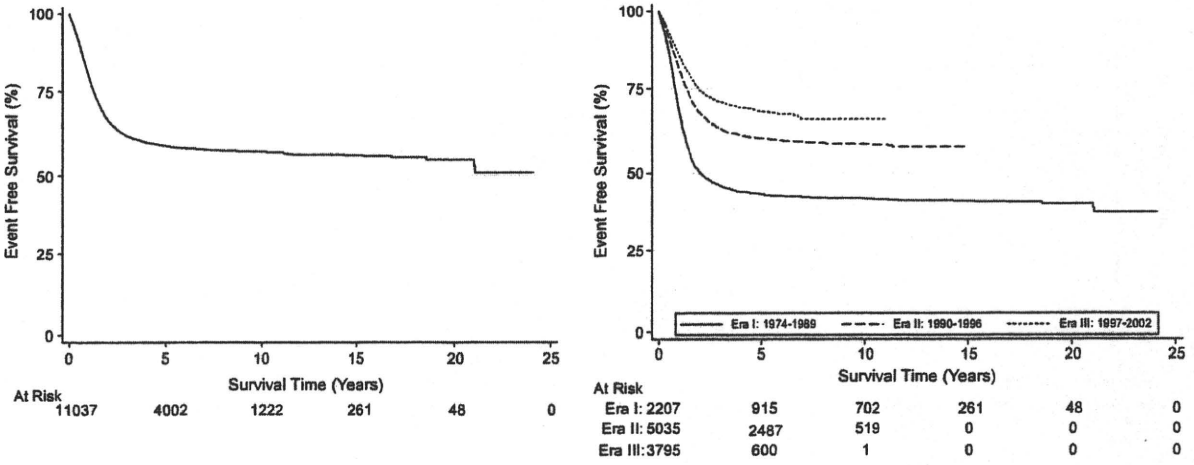

Fig. 1 – EFS from date of diagnosis of all 11,037 patients and by their respective Era of diagnosis (1974–1989, 1990–1996 and 1997–2002).

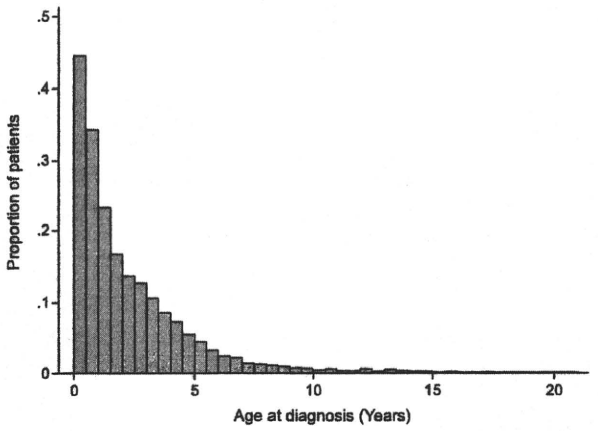

Fig. 2 – Distribution of age-at-diagnosis in 11,037 cases of neuroblastoma under 21 years of age.

(an era of many of unknown *MYCN* status), 4.24 and 3.86. Nevertheless, even in patients with *MYCN*-amplification, the 3-year EFS improved from 17% to 28%, to 37% over the decades.

3.5. *Age-of-diagnosis adjusted for the presence of bone marrow metastases and MYCN amplification*

Inclusion of BM involvement and *MYCN* status in a multivariable EFS model with age-at-diagnosis weakens the influence of age. For example, the risk for an event for age 25+ months falls from 4.83 in the model with age alone (Table 3, highlighted entries), to 2.95 when adjusted for BM metastases and *MYCN* status. Nevertheless, increasing age is still predictive of adverse prognosis. Adjustment for age and *MYCN* status results in a decrease in the risk associated with the presence of BM metastases, but it remains prognostic of poor outcome in Era III (HR 2.14: 1.87–2.44, $p < 0.001$). Similarly, adjustment for age and the presence of BM metastases results

Table 2 – Hazard ratios (HRs) for EFS for each Era, and by age (13 and 3 categories) within each Era and all patients combined.

Age (*d*)	Age (*m*)	*n*	All events (%)	Hazard ratio (HR)				EFS (%)	
				Era I 1974–1989	Era II 1990–1996	Era III 1997–2002	All	3-year	5-year
0–91	0–3	1540	237 (15)	1	1	1	1	84.33	83.95
92–182	4–6	901	147 (16)	1.55	1.03	0.88	1.06	83.75	82.76
183–273	7–9	1170	178 (15)	1.16	0.91	0.90	0.94	85.76	84.91
274–364	10–12	728	149 (20)	2.28	1.36	0.98	1.36	79.47	78.20
365–455	13–15	703	183 (26)	3.98	1.39	1.34	1.78	73.03	72.84
456–546	16–18	575	210 (37)	7.12	2.26	1.73	2.74	63.43	62.70
547–637	19–21	520	206 (40)	4.61	2.72	2.27	3.04	58.96	57.85
638–728	22–24	402	206 (51)	5.38	4.19	3.99	4.41	46.96	44.11
729–911	25–30	750	418 (56)	6.66	4.31	3.49	4.61	44.92	40.82
912–1093	31–36	697	434 (62)	7.07	4.58	4.47	5.14	39.49	34.78
1094–1275	37–42	585	362 (62)	7.47	4.42	4.10	5.12	38.96	35.22
1276–1456	43–48	480	305 (64)	6.40	5.08	4.12	5.11	39.77	34.10
1457+	49+	1986	1231 (62)	5.96	4.91	3.65	4.87	41.48	34.66
	0–12	4348	711 (16)	1	1	1	1	83.82	83.04
	13–18	1277	397 (31)	3.94	1.70	1.63	2.11	68.35	67.92
	19+	5412	3158 (58)	4.61	4.29	3.94	4.47	43.28	38.32
Total		11,037	4266 (39)	2207	5035	3795	–		