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

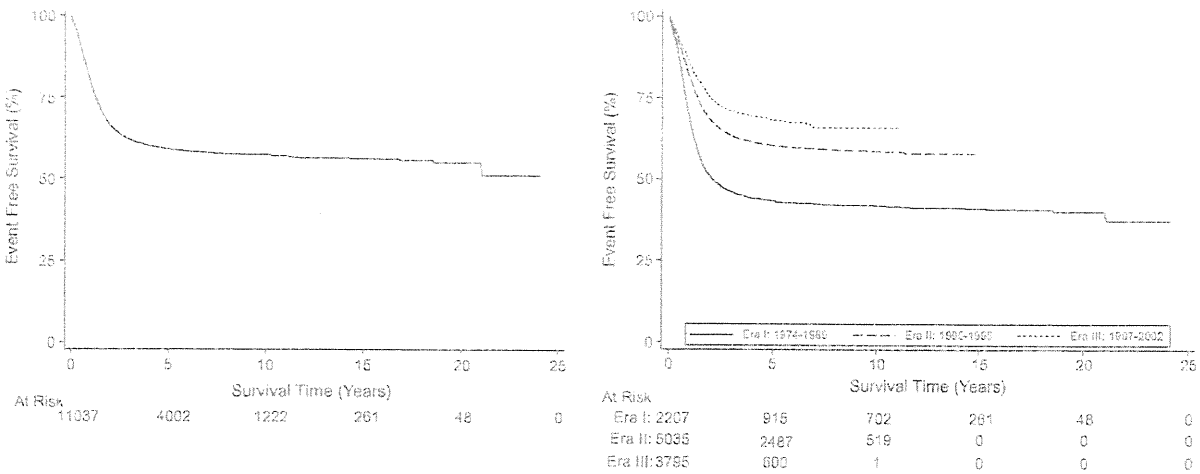


**Fig. 1 – EFS from date of diagnosis of all 11,037 patients and by their respective Era of diagnosis (1974–1989, 1990–1996 and 1997–2002).**

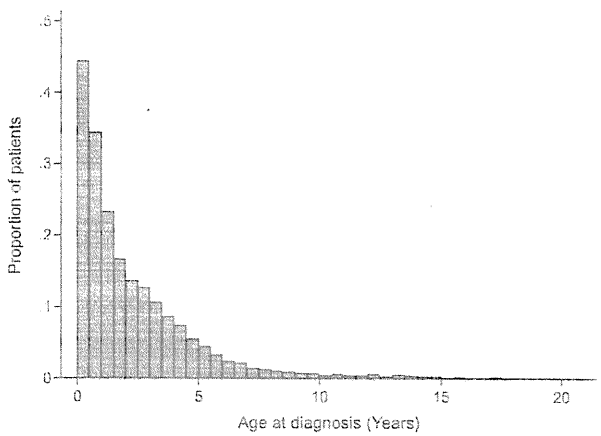


**Fig. 2 – Distribution of age-at-diagnosis in 11,037 cases of neuroblastoma under 21 years of age.**

(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 | – | | |

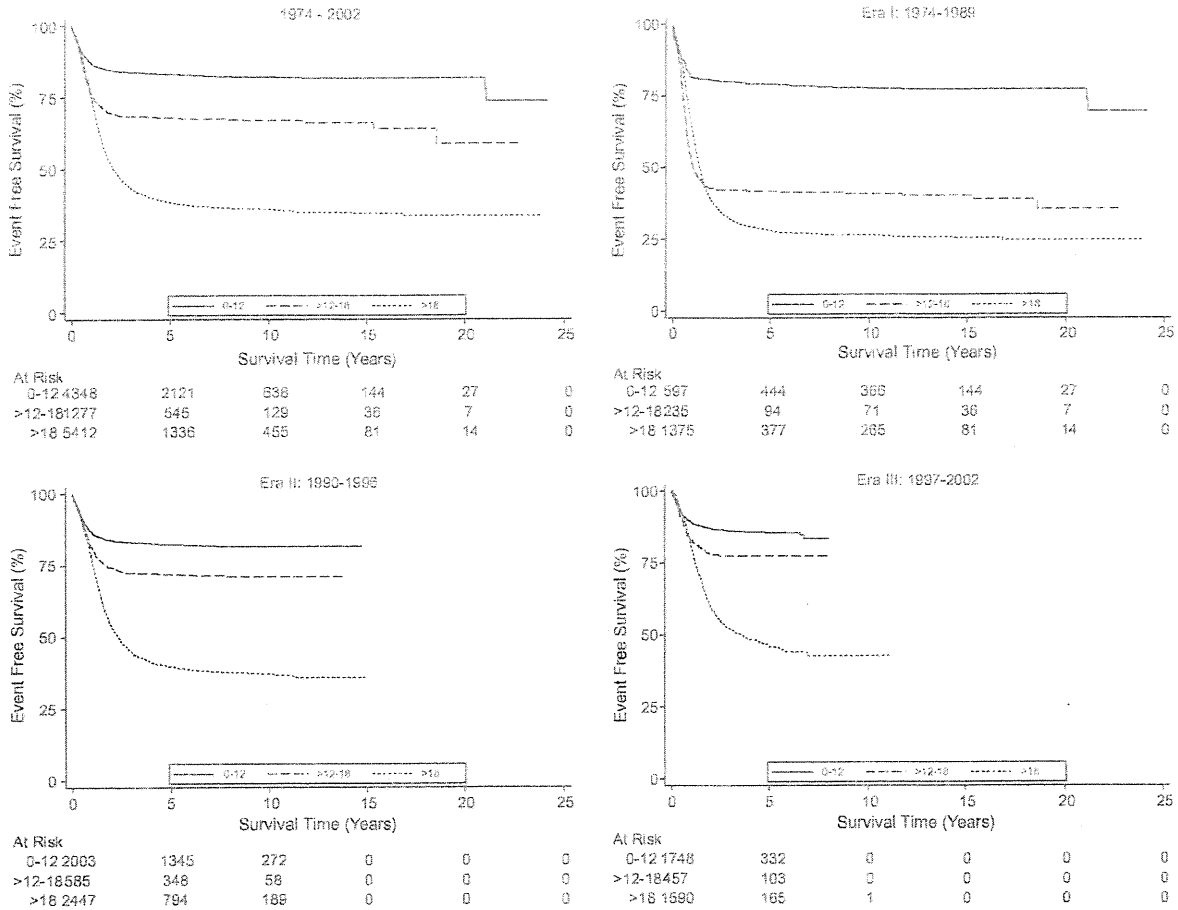


**Fig. 3 – EFS for all 11,037 patients, and within each Era, for the age categories 0–12, 13–18 and 19 or more months.**

in a decrease in the risk associated with MYCN amplification compared to the risk if MYCN is considered alone. However, amplification remains adversely prognostic in Era III (HR 2.62: 2.35–3.04, $p < 0.001$).

An alternative approach to assessing outcome is to assign a weight of −1, 0, or 1 to each risk factor (age-at-diagnosis, BM metastases, MYCN status), where the weight corresponds to the low-, intermediate- and high-risk categories. Using the weights, analyses determined that each risk factor exerted approximately the same level of prognostic influence on outcome (results not shown). On this basis, all possible combinations of low-, intermediate-, or high-risk of age, BM metastases and MYCN status create different groups. For each patient, the weights are summed to a score and those with the same score are combined into groups. For example, the weights for patients age-at-diagnosis 0–12 (weight: −1), unknown BM status (0) and amplified MYCN (1) sum to 0 (zero). These patients would be combined with others who have a sum of 0, for example, those aged 19+ months (weight: 1), with no BM metastases (−1), and unknown MYCN (0). This process results in seven groups with scores ranging from −3 (all factors indicating low-risk) to +3 (high-risk) but then coded 0–6 for convenience. The corresponding EFS curves indicate a worsening prognosis with increasing score, within each era as well as over the entire period (Fig. 6). On this basis, visual inspection of the EFS curves of Era III suggests a possibility of four major risk categories with scores (0, 1), (2), (3), and (4, 5, 6), respectively. This underlines the fact that, despite the considerable improvement in the overall prognosis of young patients with NB, major outcome differences remain.

## 4. Discussion

The International Neuroblastoma Risk Group (INRG) Task Force collated, after extensive consultation and international collaboration, data from 11,037 patients with neuroblastoma (NB) in those less than 21 years of age-at-diagnosis and recruited to clinical studies between 1974 and 2002. With these data INRG have proposed a revised classification system based on the 8800 patients diagnosed between 1990 and 2002.[9,13] The objective of the revised classification was to identify risk groups, ranging from very-good to very-poor prognosis, to enable focus for devising better therapeutic strategies. It is hoped that this will accelerate the improvement in outcome achieved over previous decades for all patients of whatever risk category. A key consideration for this revised classification was whether the age-at-diagnosis cut-off of greater than 12 months should be revised as it had been suggested, from an analysis of 3666 patients, that this might be raised to those who are >18 months.[15] The further data

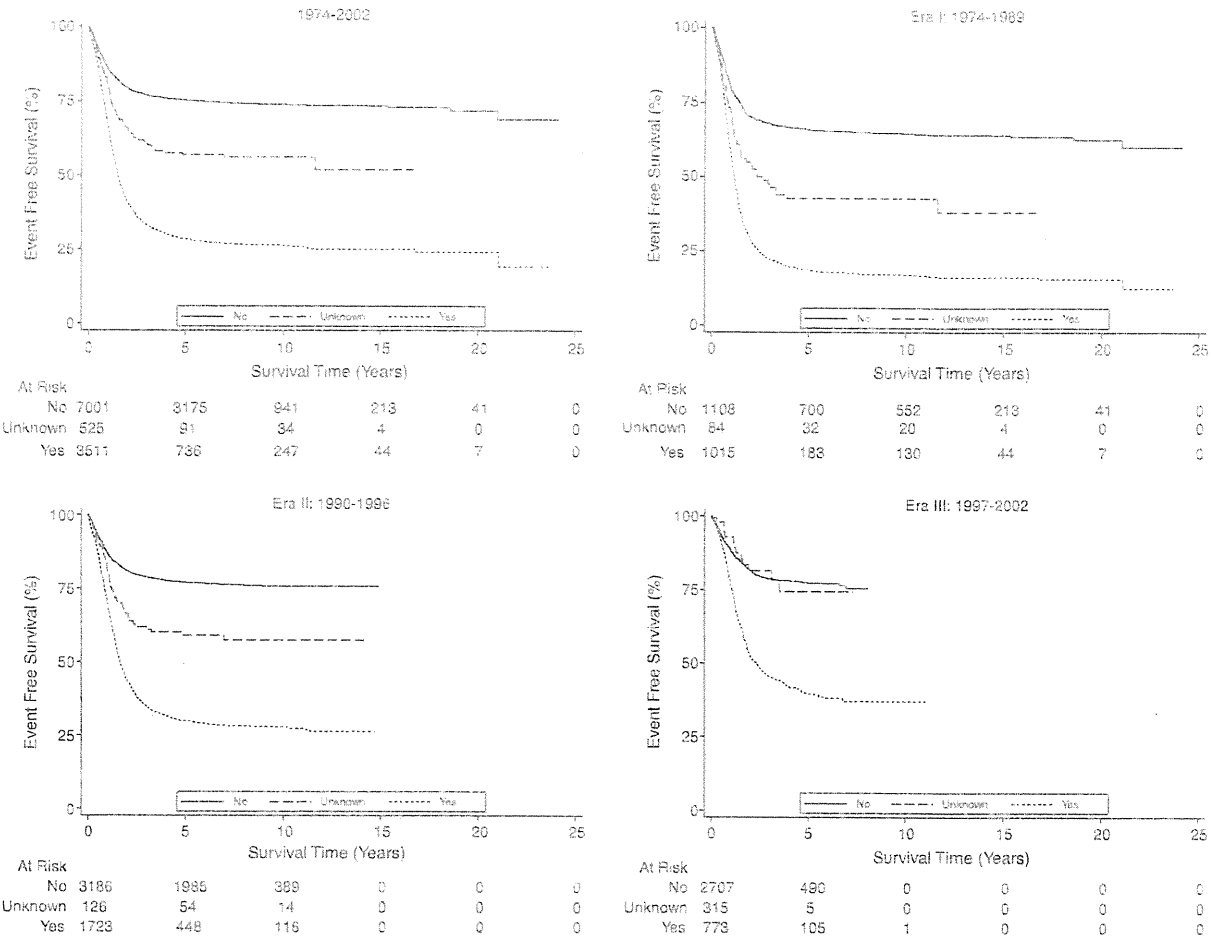


**Fig. 4 – EFS for all 11,037 patients, and within each Era, for the three BM involvement groups.**

available from 1990 to 2002 subset of the INRG database have demonstrated statistical and biological confirmation for the revision. Thus the INRG classification system regards those with age-at-diagnosis >18 months as at higher risk although this is only one of the seven criteria used to determine risk.

Many modifications in treatment and supportive care have occured over time: the three Era (1974–89; 1990–96 and 1997–2002) serve as a surrogate variable encapsulating all factors that have changed. The main focus of this study is to investigate whether the general prognosis for children with NB has improved over time and, in particular, to establish whether age-at-diagnosis still plays a major role in subsequent outcome. It is well established that other features recorded at diagnosis will also be important determinants but to investigate change over the full 28-year period we are constrained to variables that were recorded in the earlier times. This precluded, for example, detailed study of chromosome 11q status, and DNA ploidy but permitted investigation of *MYCN* status and the presence of metastatic disease in the bone marrow. Although stage is a well established clinical prognostic factor in NB, as age is used within the INSS procedure itself to define 4s disease, it could not be considered as a prognostic variable for the purposes of our analysis.

It is recognised that definitions, investigative methods, laboratory techniques and standards of recording are very likely to differ widely over the different international groups each collating information from many individual participating centres on the 11,037 patients concerned. It is also recognised that some subjectivity was utilised in choosing, for example, BM involvement rather than bone metastases for our model although each appeared to be similarly influential on outcome. The former was chosen on statistical grounds as having the fewer missing values, a more even spread in those with and without involvement, and a larger hazard ratio. Further, we were not attempting to develop a full prognostic model for use with future patients (this would need to consider the most recent items now available) but merely to give a broad description of the changes over the era and the influence of age-at-diagnosis using simple statistical models. In full recognition that a substantial part of the data (Era II and III) had been used in developing the INRG classification, we used an entirely different statistical approach, the proportional hazards regression model,[10] to substantiate or otherwise the age-at-diagnosis classification.

We show that the 3-year EFS increased from 46% for patients diagnosed from 1974–1989 to 63% for 1990–1996, to 71% for 1997–2002.

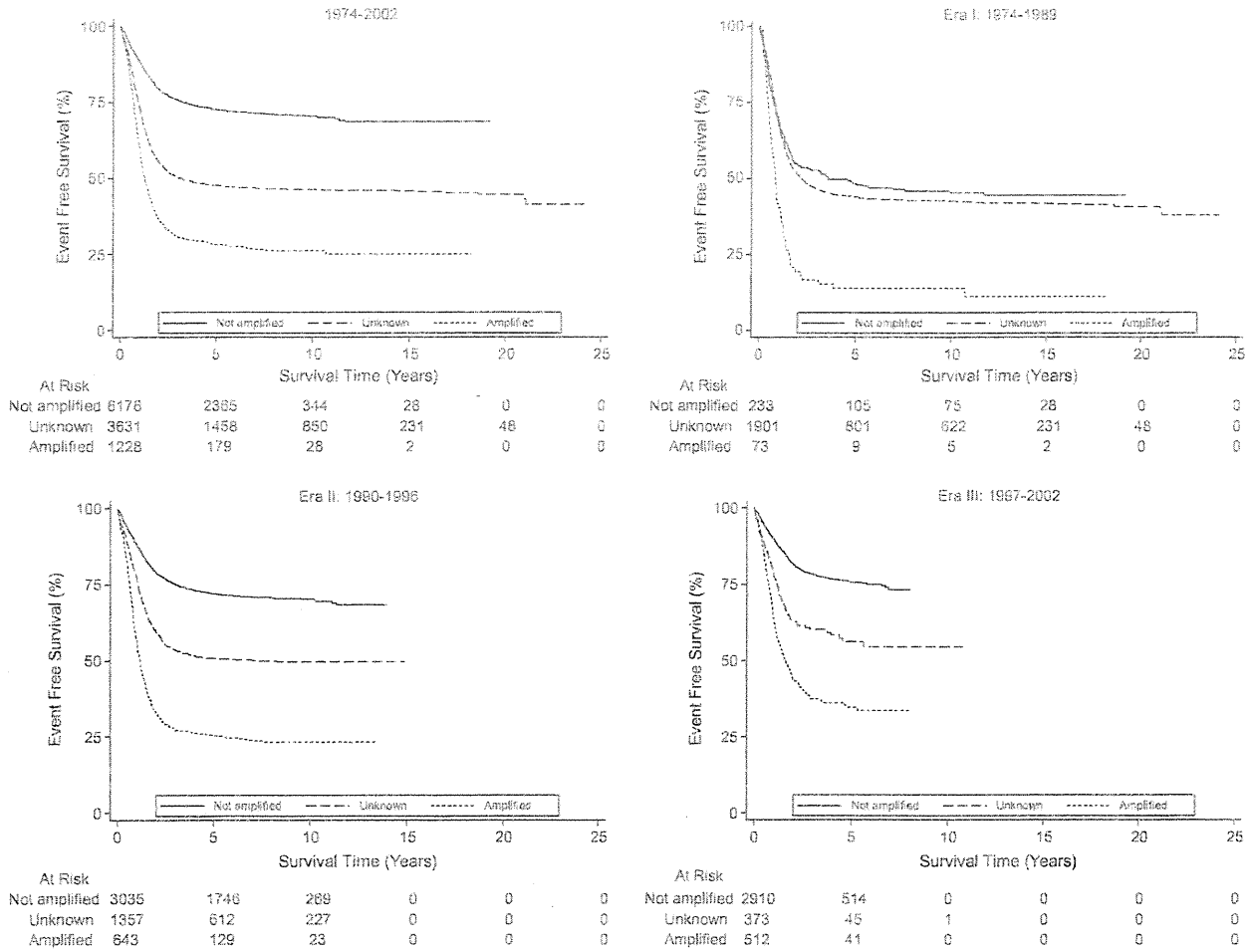


**Fig. 5 – EFS for all 11,037 patients, and within each Era, for the *MYCN* amplification groups.**

**Table 3 – Multivariable estimates of HRs for EFS by age in 6 categories and evidence of BM disease and *MYCN* amplification for each of three era and all times. (Univariate HRs are indicated in parenthesis).**

| | | *n* (%) | All events | Hazard ratio (HR) | | | | EFS (%) | |
|---|---|---|---|---|---|---|---|---|---|
| | | | | Era I 1974–1989 | Era II 1990–1996 | Era III 1997–2002 | All era | 3-year | 5-year |
| Age (*m*) | –6 | 2441 (22) | 384 | 1 | 1 | 1 | 1 | 84.11 | 83.51 |
| | 7–12 | 1907(17) | 327 | 1.22 | 0.98 | 0.95 | 1.02 | 83.46 | 82.46 |
| | 13–15 | 703 (6) | 187 | 3.03 | 1.00 | 1.42 | 1.47 | 72.43 | 72.24 |
| | 16–18 | 574 (5) | 210 | 4.52 | 1.97 | 1.51 | 2.34 | 63.38 | 62.65 |
| | 19–24 | 925 (8) | 412 | 3.29 | 2.10 | 2.09 | 2.39 | 53.92 | 52.06 |
| | 25+ | 4487 (41) | 2746 | 3.57 | 2.68 | 2.83 | **2.95** | 41.16 | 35.62 |
| | Univariate HR of 25+ group | | | (5.52) | (4.65) | (4.04) | **(4.83)** | | |
| Bone marrow metastases | No | 7001(63) | 1665 | 1 | 1 | 1 | 1 | 77.00 | 75.36 |
| | Unknown | 525 (5) | 109 | 1.64 | 1.65 | 1.05 | 1.25 | 60.97 | 56.99 |
| | Yes | 3511 (32) | 2492 | 2.81 | 2.86 | 2.14 | 2.68 | 33.34 | 28.62 |
| | Univariate HR of Yes group | | | (3.66) | (4.25) | (3.16) | (4.00) | | |
| *MYCN* amplified | No | 6178 (56) | 1555 | 1 | 1 | 1 | 1 | 75.60 | 72.52 |
| | Unknown | 3631 (33) | 1880 | 0.98 | 1.47 | 1.62 | 1.64 | 50.13 | 47.52 |
| | Yes | 1228 (11) | 831 | 1.88 | 2.77 | 2.62 | 2.58 | 30.80 | 28.28 |
| | Univariate HR of Yes group | | | (2.50) | (4.24) | (3.86) | (3.97) | | |
| | Total | 11037 | 4266 | 2207 | 5035 | 3795 | | | |

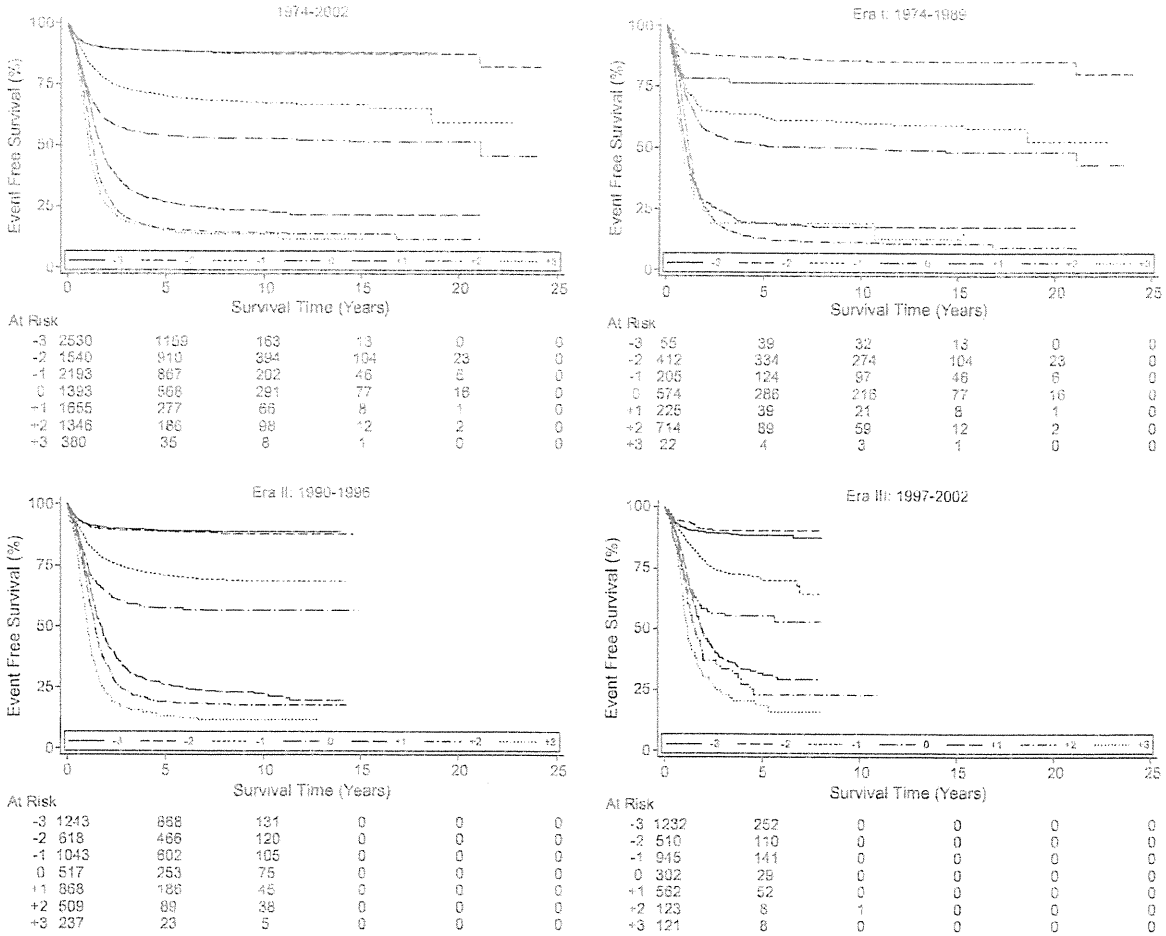


**Fig. 6 – EFS for the seven risk groups defined by age-at-diagnosis (3 groups), together with the presence or absence of metastatic BM involvement and *MYCN* amplification.**

Ignoring all other considerations, age-at-diagnosis appears to have retained an important role over the 28-year period. However, this influence is gradually waning over calendar time with, for example, those of >18 months having a 3-year EFS increasing from 25% in those diagnosed 1974–1989 to 45% in those from 1997–2002 (Fig. 3). Adjusting treatment in those over 12 months to compensate for their relatively poorer prognosis may have contributed to this. It also remains apparent (Table 2) that the risk of relapse increases steadily with age so that no single cut is likely to divide good and poor prognosis unambiguously for all. Thus, there remains some suggestion that those diagnosed between 13 and 18 months remain at higher risk than the youngest children, and that those >18 months are at an even greater risk of relapse. A pragmatic cut beyond 18 months for prognostic purposes for future patients does not seem unreasonable. It is important to note that wherever a cut is made, those close to this (convenient) boundary are at very similar risk. The increasing age risk remains the case, although diminished in magnitude, whether or not the presence or otherwise of metastases in the BM or *MYCN* oncogene status is taken into account. Although only future data can substantiate this, it is likely that strength of increasing age-at-diagnosis as an adverse prognostic variable will continue to decline.

For patients with high-risk disease, treatment strategies have been increasingly intensified over time. Further, the use of myeloablative therapy and stem cell transplants increased following the Children's Cancer Group randomized trial[16] and this too may have contributed to both the improved EFS and the decreasing influence of age-at-diagnosis.

Although older age, *MYCN* amplification and the presence of BM metastases were independently predictive of poor outcome, the unfavourable prognostic influence of either one of these is offset by the absence of one or more of the remaining unfavourable factors (Fig. 6).

It has been suggested that age-at-diagnosis may be a proxy for as undescribed tumour-related factor. However, it may be that some current prognostic features, such as *MYCN*-status, should be reconsidered but on a continuous scale rather than categorised (amplified, not amplified) as when considered in this way they may mimic more closely the situation that is apparent with age.

There is strong evidence that the strategy of increasing therapeutic intensity has improved the outcome for children with NB over the 1974–2002 period and that there is a declining influence of the prognostic effect of age-at-diagnosis. Nevertheless the strength of the three most powerful factors, *MYCN* status, BM metastases and age, remains high.

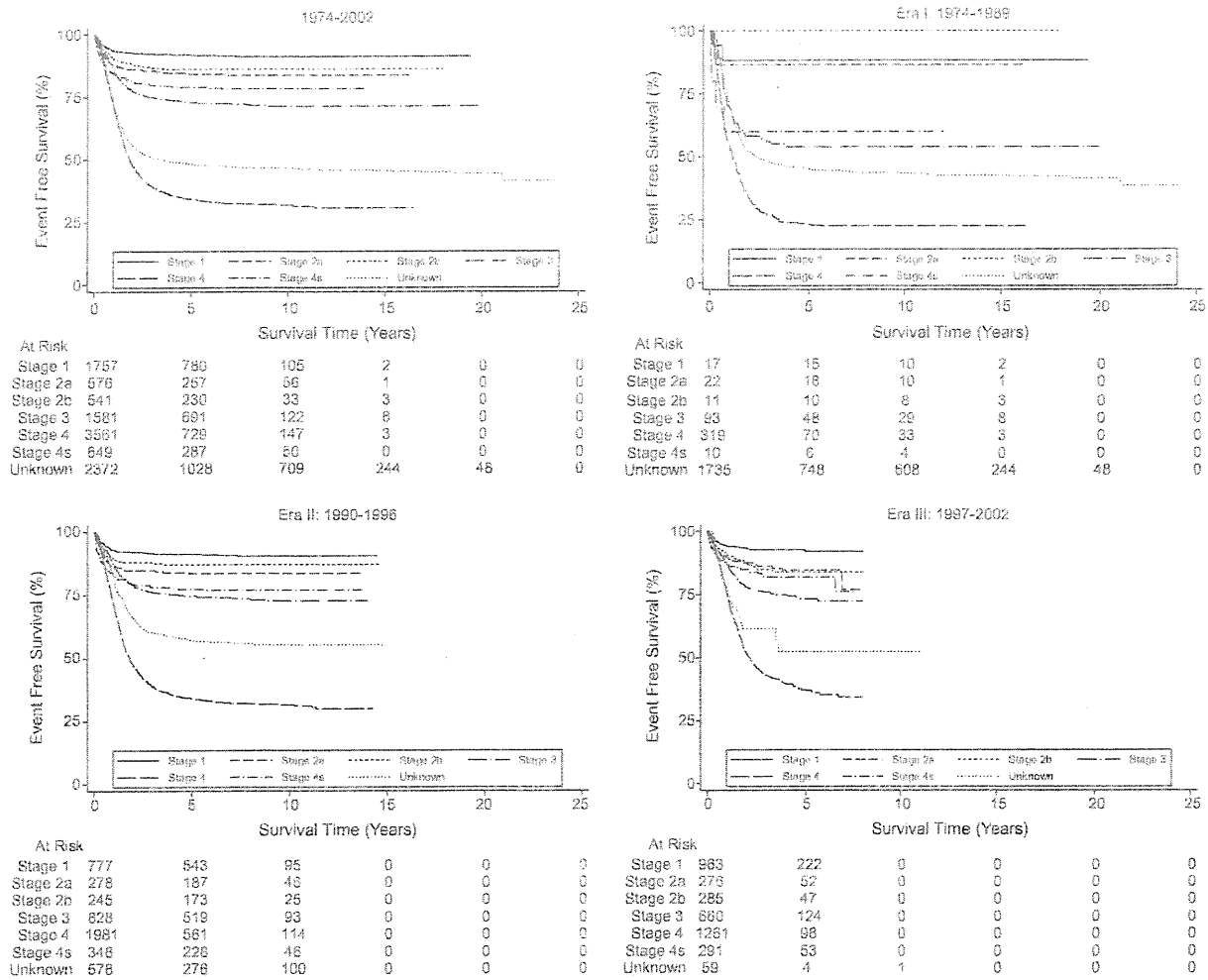


**Fig. A1 – EFS for all 11,037 patients, and within each Era, for each INSS stage.**

## Conflict of interest statement

None declared.

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## Appendix A

See Fig. A1.

## REFERENCES

1. Breslow N, McCann B. Statistical estimation of prognosis for children with neuroblastoma. *Cancer Res* 1971;31:2098–103.
2. Evans AE. Staging and treatment of neuroblastoma. *Cancer* 1980;45:1799–802.
3. Brodeur GM. Neuroblastoma: biological insights into a clinical enigma. *Nat Rev Cancer* 2003;3:203–16.
4. Cecchetto G, Mosseri V, De Bernardi B, et al. Surgical risk factors in primary surgery for localized neuroblastoma: the LNESG1 study of the European International Society of Pediatric Oncology Neuroblastoma Group. *J Clin Oncol* 2005;23:8483–9.
5. Kushner BH, Cohn SL. Intermediate-risk Neuroblastoma. In: Cheung N-KV, Cohn SL, editors. *Neuroblastoma*. Heidelberg: Springer-Verlag; 2005. p. 131–7.

6. Attiyeh EF, London WB, Mosse YP, et al. Chromosome 1p and 11q deletions and outcome in neuroblastoma. *N Engl J Med* 2005;**353**:2243–53.
7. Caron H, van Sluis P, de Kraker J, et al. Allelic loss of chromosome 1p as a predictor of unfavorable outcome in patients with neuroblastoma. *N Engl J Med* 1996;**334**: 225–30.
8. Bown N, Cotterill S, Lastowska M, et al. Gain of chromosome arm 17q and adverse outcome in patients with neuroblastoma. *N Engl J Med* 1999;**340**:1954–61.
9. Cohn SL, Pearson ADJ, London WB, et al. The International Neuroblastoma Risk Group (INRG) Classification System: An INRG Task Force Report. *J Clin Oncol* 2009;**27**:289–97.
10. Cox DR. Regression models and life-tables. *J Royal Stat Soc (B)* 1972;**34**:187–220.
11. Machin D, Cheung Y-B, Parmar MKB. *Survival analysis: a practical approach*. Chichester: John Wiley; 2006.
12. Brodeur GM, Pritchard J, Berthold F, et al. Revisions of the international criteria for neuroblastoma diagnosis, staging, and response to treatment. *J Clin Oncol* 1993;**11**:1466–77.
13. Monclair T, Brodeur GM, Ambros PF, et al. The International Neuroblastoma Risk Group (INRG) Staging System. *J Clin Oncol* 2009;**27**:298–303.
14. StataCorp. Stata Statistical Software: Release 10.0. College Station. Texas: Stata Press; 2007.
15. London WB, Castleberry RP, Matthay KK, et al. Evidence for an age cutoff greater than 365 days for neuroblastoma risk group stratification in the Children's Oncology Group. *J Clin Oncol* 2005;**27**:6459–65.
16. Matthay KK, Villablanca JG, Seeger RC, et al. Treatment of high-risk neuroblastoma with intensive chemotherapy, radiotherapy, autologous BM transplantation, and 13-cis-retinoic acid. Children's Cancer Group. *N Engl J Med* 1999;**341**:1165–73.

Pediatr Blood Cancer 2011;56:865–867

BRIEF REPORT

# Detection of *MYCN* DNA in the Cerebrospinal Fluid for Diagnosing Isolated Central Nervous System Relapse in Neuroblastoma

**Tomiko Kimoto, MD,[1]* Masami Inoue, MD,[1] Sadao Tokimasa, MD, PhD,[1] Shigeki Yagyu, MD, PhD,[2] Tomoko Iehara, MD, PhD,[2] Hajime Hosoi, MD, PhD,[2] and Keisei Kawa, MD, PhD[1]**

We present the case of a 1-year-old female with stage-4 neuroblastoma with *MYCN* amplification; she was treated with five chemotherapy courses, resulting in normalization of elevated serum levels of tumor markers. Complete remission was achieved after allogeneic hematopoietic stem-cell transplantation with reduced-intensity conditioning. Nine months later, however, the tumor relapsed in the central nervous system (CNS). The serum and cerebrospinal fluid (CSF) levels of the tumor markers were normal, but the *MYCN* copy number was high only in the CSF DNA, suggesting an isolated CNS recurrence. The *MYCN* copy number in the CSF DNA was reflective of response to treatment. Pediatr Blood Cancer 2011;56:865–867.


**Key words:** CNS relapse; *MYCN* DNA in the CSF; neuroblastoma,

## INTRODUCTION

Neuroblastoma is the most common extracranial tumor of childhood. Metastatic disease is commonly found at its diagnosis. Although the frequency of spread of neuroblastoma to bone, bone marrow, and liver is high both at the initial diagnosis and at recurrence, metastasis to the central nervous system (CNS) is rare in terms of both brain parenchymal and leptomeningeal involvement. A recent study by the Children's Cancer Group (CCG) on metastatic sites in 567 patients with stage-4 neuroblastoma diagnosed between 1989 and 1996 revealed that only 0.7% and 2.2% of the patients had CNS involvement at diagnosis and at recurrence, respectively [1]. However, the improvement in the survival of children with metastatic neuroblastoma owing to recent advances in treatment has been accompanied by an increase in the frequency of CNS involvement [2,3].

*MYCN* amplification is strongly associated with rapid tumor progression and a poor outcome, independent of the stage of the tumor [4]. In addition, *MYCN* amplification is a significant risk factor for CNS relapse [3]. Reports have suggested that early recognition and aggressive treatment of this complication helps prevent tumor growth. Since the radiological features vary in each the patient [5,6], it is sometimes difficult to detect CNS relapse of neuroblastoma.

We report a case in which *MYCN* copy numbers in both serum and cerebrospinal fluid (CSF) samples were correlated with CNS neuroblastoma.

## CASE REPORT

A 1-year-old female presented with proptosis and a left-sided abdominal mass. Computed tomography (CT) demonstrated a left adrenal mass (diameter, 4 cm; primary tumor) and cervical and celiac lymphadenopathy. Brain CT scan demonstrated soft tissue masses involving the skull. No areas of abnormal parenchymal or meningeal enhancements were visible, which indicated the absence of brain invasion. A lumber puncture was not performed at diagnosis. A primary tumor and generalized bone uptake were observed on meta-iodobenzylguanidine (MIBG) scanning; accordingly, stage-4 neuroblastoma was diagnosed. The tumor exhibited the unfavorable Shimada histology, with more than 40 *MYCN* copies, as evident on polymerase chain reaction (PCR) analysis. Tumor markers were abnormally elevated: the levels of urinary homovanillic acid (HVA) and serum neuron-specific enolase (NSE) were 145.24 μg/mg creatinine and 1,678 ng/ml, respectively. Complete remission was achieved after five courses of combination chemotherapy (regimen A1, comprising cyclophosphamide, vincristine, pirarubicin hydrochloride, and cisplatin) and resection of the primary tumor. The patient received allogeneic stem-cell transplantation from her human-leukocyte-antigen haplo-identical father after reduced-intensity conditioning, with melphalan and fludarabine, which was aimed at eliciting the graft-versus-tumor (GVT) response [7]. For prophylaxis against the graft-versus-host disease (GVHD), tacrolimus, and methotrexate were administered. The CD34 cell count was $4.4 \times 10^6$/kg and the nucleated cell count was $6.3 \times 10^8$/kg. Engraftment was confirmed by fluorescent in-situ hybridization on day 12. The patient developed GVHD grade III (skin, stage 3; gut, stage 3), which was controlled well by steroid administration. To induce a GVT effect, we attempted to wean this patient off the immunosuppressive drugs in a month. Four months after the stem-cell transplantation, the patient was admitted with altered consciousness [Glasgow coma scale: 3] and seizure associated with hydrocephalus. She also presented with paralysis of the lower extremities and a neuropathic bladder. A T1-weighted magnetic resonance (MR) image obtained at this time point showed extensive intraspinal pachymeningeal thickening and nodularity (Fig. 1). Tumor masses disturbed the flow of CSF. On the same day, ventriculo-peritoneal (V-P) shunt was implanted to decrease the pressure.

The new lesions were negative for MIBG uptake. Abdominal and pelvic CT did not reveal any other abnormalities. Diagnostic biopsy was not performed because of surgical risk. CSF was removed from the cranial end of the shunting system. The results of cytological analysis of the CSF were negative. Tumor markers were within the normal range: the levels of urinary HVA, serum NSE, CSF HVA, and

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Conflict of interest: The authors declare no competing financial interests.

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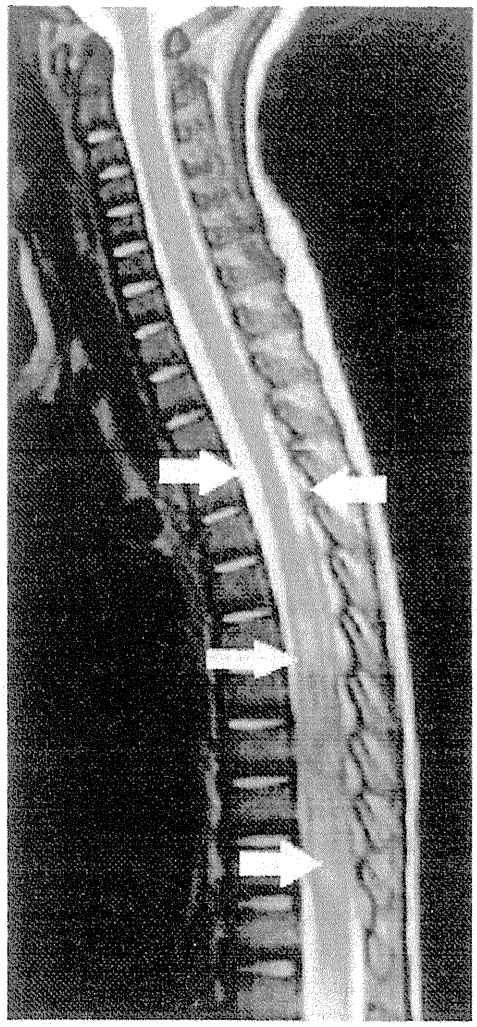

**Fig. 1.** Contrast-enhanced T1-weighted magnetic resonance image obtained during the first relapse shows an extensive intraspinal pachymeningeal thickening.

CSF NSE were 19.33 μg/ng creatinine, 14.4 ng/ml, 357 ng/ml (normal range: 322–520 ng/ml), and 111 ng/ml (indicating hemolysis), respectively. We measured the level of circulating *MYCN* DNA both in the CSF and serum, by using DNA-based real-time quantitative PCR with the *N*-acetylglucosamine kinase gene (*NAGK*, 2p12). The *MYCN* copy number was evaluated as previously described [8]. The *MYCN/NAGK* (M/N) ratio in the CSF sample obtained was 142.77, whereas that in the serum was 1.79, which was within the expected range. The clinical symptoms showed gradual improvement with repeated A1. Although, the contrast-enhancing masses of the spine remained, the CSF M/N ratio decreased to that in the serum (from 142.77 to 2.41). Seven months after the relapse, the patient complained of headache. CT scan revealed multiple masses in the brain, and she died from cerebral hemorrhage. The family did not permit post-mortem examination. The CSF M/N ratio increased from 2.41 to 12.09, while the serum M/N ratio and the other tumor markers were within the normal range (Fig. 2).

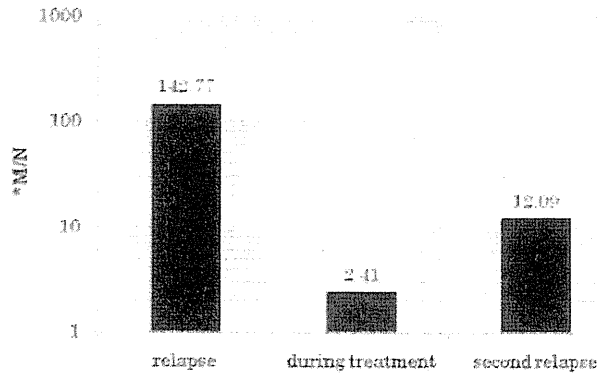


**Fig. 2.** The *MYCN/NAGK* (M/N) ratio in the CSF sample.

## DISCUSSION

The prognosis of neuroblastoma with *MYCN* amplification is extremely poor. The long-term overall survival of patients with this tumor remains unsatisfactory (<40%) despite the improvement in the outcome by the use of intensive multimodal therapy with autologous hematopoietic stem-cell rescue. *MYCN* amplification is strongly associated with a poor prognosis, irrespective of age [9].

Molecular techniques such as PCR have enabled the detection of small amounts of free DNA in the serum and plasma of several cancer patients, and the level of free DNA is known to decrease when the patients respond to radiotherapy; this suggests that the level of free DNA can be considered as a valuable marker for cancer detection [10,11].

Combaret et al.[12] reported that high levels of *MYCN* DNA were present in the peripheral blood of patients with *MYCN*-amplified neuroblastoma (MNA).

However, the results of the assay for this DNA could be influenced by the quality of the template DNA or a numerical change in chromosome 2. Gotoh et al. [8] used DNA-based real time quantitative PCR and a single copy reference gene (*NAGK*) located on 2p12 for assessment of *MYCN*. *NAGK* is located on chromosome 2 but is distant from the region usually affected by changes in the number of *MYCN* copies of chromosome 2; therefore, the number of *MYCN* copies to the number of reference gene copies gives the *MYCN* copy number, even if there is a change in the number of copies of chromosome 2. Both the sensitivity and specificity of the serum M/N ratio as a diagnostic test were high in the above-mentioned study. In addition, Gotoh et al. reported that the serum M/N ratio was elevated even in patients having MNA with localized tumors. A quantitative method using serum DNA is not only a diagnostic tool for assessing MNA status, but serum DNA is also being considered an early marker in disease progression for monitoring patients with neuroblastoma [8,13].

The CNS is a rare site of neuroblastoma recurrence; however, with the increase in the survival of patients, CNS recurrence of tumors is being increasingly diagnosed. The CNS can serve as a sanctuary site for cancer cells, because the blood–brain barrier impedes the penetration of most chemotherapy agents. CNS involvement can be clinically occult and carries a poor prognosis. Predictive features at the time of diagnosis for CNS recurrence are patient age of 2–3 years, tumor *MYCN* amplification, and positive findings of CSF cytology [3]. High-risk patients with neuroblastoma should be monitored with appropriate modalities such as contrast-enhanced MRI of the brain

and spine to enable timely detection of CNS metastasis. Current treatments for CNS relapse are still inadequate. The MSKCC groups reported the potential to increase survival rates for patients with CNS neuroblastoma treated with compartmental intrathecal antibody-based radioimmunotherapy targeting minimal residual disease following surgery, craniospinal irradiation, and chemotherapy [14].

Our patient developed an isolated CNS relapse of neuroblastoma after allogeneic stem-cell transplantation. Although most of the conventional diagnostic techniques were only slightly useful for the detection of neuroblastoma in this case, the high level of *MYCN* copies in the CSF helped us confirm the relapse. When the patient developed a relapse of the tumor, the CSF M/N ratio was elevated, while the serum M/N ratio remained normal. These data suggest that tumors localized in the CNS could be overlooked if diagnostic assays based on serum DNA are used. The tumors in CNS may not release a significant amount of DNA into the systemic circulation. Our report suggests that the CSF M/N ratio may be an early tumor marker even if the tumor is isolated to the CNS. The finding seems to be important in the face of an increase in the number of deaths due to CNS recurrence of neuroblastoma. In similar cases, it will be imperative to confirm observation via tissue biology examination as well as our findings about the detection of *MYCN DNA*.

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## REFERENCES

1. DuBois SG, Kalika Y, Lukens JN, et al. Metastatic sites in stage IV and IVS neuroblastoma correlate with age, tumor biology, and survival. Pediatr Hematol Oncol 1999;21:181–189.
2. Kramer K, Kushner B, Heller G, Cheung NK. Neuroblastoma metastatic to the central nervous system. The Memorial Sloan-Kettering Cancer Center experience and a literature review. Cancer 2001;91:1510–1519.
3. Matthay KK, Brisse H, Couanet D, et al. Central nervous system metastases in neuroblastoma: Radiologic, clinical, and biologic features in 23 patients. Cancer 2003;98:155–165.
4. Seeger RC, Brodeur GM, Sather H, et al. Association of multiple copies of the N-myc oncogene with rapid progression of neuroblastomas. N Engl J Med 1985;313:1111–1116.
5. Palasis S, Egelhoff JC, Morris JD, et al. Central nervous system relapse of treated stage IV neuroblastoma. Pediatr Radiol 1998;28:990–994.
6. Porto L, Kieslich M, Yan B, et al. Isolated CNS relapse in neuroblastoma. Neuropediatrics 2005;36:112–116.
7. Inoue M, Nakano T, Yoneda A, et al. Graft-versus-tumor effect in a patient with advanced neuroblastoma who received HLA haploidentical bone marrow transplantation. Bone Marrow Transplant 2003;32:103–106.
8. Gotoh T, Hosoi H, Iehara T, et al. Prediction of MYCN amplification in neuroblastoma using serum DNA and real-time quantitative polymerase chain reaction. J Clin Oncol 2005;23:5205–5210.
9. Matthay KK, Villablanca JG, Seeger RC, et al. Treatment of high-risk neuroblastoma with intensive chemotherapy, radiotherapy, autologous bone marrow transplantation, and 13-cis-retinoic acid. Children's Cancer Group. N Engl J Med 1999;341:1165–1173.
10. Leon SA, Shapiro B, Sklaroff DM, Yaros MJ. Free DNA in the serum of cancer patients and the effect of therapy. Cancer Res 1977;37:646–650.
11. Silva JM, Gemma Dominguez, Garcia JM, et al. Presence of tumor DNA in plasma of breast cancer patients clinicopathological correlations. Cancer Res 1999;59:3251–3256.
12. Combaret V, Audoynaud C, Iacono I, et al. Circulating MYCN DNA as a tumor-specific marker in neuroblastoma patients. Cancer Res 2002;62:3646–3648.
13. Combaret V, Hogarty MD, London WB, et al. Influence of neuroblastoma stage on serum-based detection of MYCN amplification. Pediatr Blood Cancer 2009;53:329–331.
14. Kramer K, Kushner BH, Modak S, et al. Compartmental intrathecal radioimmunotherapy: Results for treatment for metastatic CNS neuroblastoma. J Neurooncol 2010;97:409–418.

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# Identification of therapy-sensitive and therapy-resistant neuroblastoma subtypes in stages III, IVs and IV

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ABSTRACT

The aim was to present a new model of risk stratification with high predictive sensitivity for non-localized neuroblastomas (NBs). "*MYCN* amplification", "unfavorable histology of the International Neuroblastoma Pathology Classification (INPC) system" and "low Ha-ras/trk A expression" could be defined as an independent predictor for high-risk NBs. A risk stratification flow chart was applied to 103 advanced NBs in which all three factors were examined and 69 were grouped as high-risk NBs of which 38 patients died. The predictive sensitivity for poor patient outcome was 86%, which included 38 of the 44 total deaths in this analysis. Using the number of the three independent risk factors in each tumor, the 69 high-risk NBs were classified into three subgroups. NBs with the three risk factors (triple risk) represented the most aggressive character and survival of the affected patients was only 10% (*"therapy-resistant NBs"*). Survivals of the patients with NBs possessed the two (double) risk factors or the one (single) risk factor were 29% and 66%, respectively. This stratification also elucidated a subgroup in which patient survival was 90% (*"therapy-sensitive"*). There were 21 NBs with "high Ha-ras/trk A expression", "favorable INPC histology" and "unamplified *MYCN*" (no risk NBs). Among the four subgroups without a risk factor, with a single risk factor, with double risk and with triple risk, Kaplan–Meier analysis showed a significant difference in NB patient outcome ($p < 0.0001$). Risk stratification might improve the therapeutic efficacy for the high-risk NBs and might decrease therapy-related sequelae in the lower risk NBs.



## 1. Introduction

Neuroblastoma (NB) is the most common extracranial solid malignant tumor to occur during childhood. It originates from primordial neural crest cells that develop into the sympathetic nervous system, including the adrenal medulla. A majority of patients with localized NB have a good clinical outcome with minimum medical intervention. However, many of the advanced NBs have been resistant to chemotherapy and thereby have been associated with a poor clinical outcome [1]. Advances of highly intensive treatment, such as myeloablative-therapy with hematopoietic stem cell transplantation (HSCT), have been able to save some patients with NBs [2,3]. The more intense the therapies are, the more risk of therapeutic sequela the

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patients are charged with. It is well known clinically that NBs have heterogeneous clinical behavior. In addition to highly rapid tumor progression, spontaneous differentiation and regression of the tumors are also observed in infants and young children.

Over the last two decades advances in NB research have provided valuable insight into the biology of this type of tumor. Many predictors for patient prognosis have been reported including chromosomal aberrations, gene alterations and expressions [4,5]. *MYCN* gene amplification [6], International Neuroblastoma Pathology Classification (INPC) [7], trk A and Ha-ras gene expression [8] have been reported to correlate significantly with the clinical behaviors of NBs. These studies dealing with NB biology have been focused on factors in association (specificity) with patient outcome. However, the respective predictor could only detect ~50% of patients with poor clinical outcome [9–11]. More information with high sensitivity to patient outcome is needed for determining their therapeutic intensity shortly after diagnosis. It is critical that therapeutic intensity should be in accordance with the risk for poor clinical outcome to optimize patient survival and to avoid sequela from therapies that are too intense.

This study presents a model of risk stratification that identifies subgroups of "therapy sensitive" NBs as well as "therapy resistant" NBs in non-localized stages III, IVs and IV.

## 2. Methods

### 2.1. Patients

Between 1975 and June 2007, 264 patients with NB were clinically diagnosed and referred for estimating the clinical significance of biological markers. No cases with NB detected by a Japanese mass-screening program were included in this study because of the therapeutic bias of early intervention on clinical outcome. Tumor samples were submitted to our laboratory from more than 50 medical institutes in association with the Japanese Infantile Neuroblastoma Cooperative Study [12] and the Japanese Advanced Neuroblastoma Cooperative Study [13] for examining Ha-ras and trk A expression. The clinical outcome of the patient was confirmed by using a questionnaire completed every 2-years through June 2008. Informed consent to join the cooperative studies was obtained from all parents or guardians in the respective medical institutes, which included an all-inclusive permission of data availability for biologic study of tumors on the condition of patient anonymity. All data from NB cases diagnosed before the 1990s were dealt with under the anonymous condition. Data extracted from the database for this study were limited to "era at the diagnosis", "gender and age of the patients at diagnosis", "stage (Evans classification)" [14], "*MYCN* status", "INPC finding", "Ha-ras/trk A expression", "treatment", "period of event-free survival (EFS) and over-all survival (OS)" and "clinical outcome". No personal information, such as name, birthday or hospital where they received therapies, was available for this study. Consideration to guard patient privacy was approved by ethics committee of the National Hospital Organization Hiroshimanishi Medical center.

A total of 196 patients with NB were evaluated in this study, 111 male and 85 female patients. The cohort consisted of 56 stage III, 117 stage IV and 23 stage IVs patients. The reason why 23 stage IVs were enrolled into this study was that clinical behavior of stage IVs NBs was heterogeneous and the survival of patients was very close to that of stage III (Fig. 1). The mean patient age at diagnosis was 25.5 months (range: 0 month–20 years). There were 38 infants among 117 cases in stage IV. The EFS of infants was 68% (26/38) and was much better than 29% (23/79) of the patients older than 12 months. This finding was also compatible with previous reports [1]. Eighty-four patients were deceased and the mean OS period was 1.43 years (range: 0 month–5.63 years). Of the 112 surviving patients, the mean follow-up period was 6.41 years (range: 0 month–21.1 years). The events experienced by patients during their clinical course were defined as relapse and/or progression of tumor.

### 2.2. Therapies and event-free survival

Due to the escalation of multimodal therapeutic intensity over the past 30 years, the number of long-term survivors from NB has increased. To examine the effects of advances in the treatment of NB, patients were classified into three groups based on the time of their diagnosis. In the first group, patients were diagnosed between 1975 and 1984 ($n = 66$; 26 in stage III, 8 in stage IVs and 32 in stage IV) and were typically treated with several anti-tumor agents including vincristin, adriamycin and cyclophosphamide [15,16]. In the second group, patients were diagnosed between 1985 and 1994 ($n = 49$; 12 in stage III, 3 in stage IVs and 34 in stage IV) and were typically treated with a combination of multiple drugs and high-dose chemotherapy. For patients enrolled from the *Japanese Advanced Neuroblastoma Cooperative Study*, they had been administered vincristin, THP-adriamycin, cyclophosphamide, etoposide and cisplatin [17,18]. The third group included patients diagnosed between 1995 and 2007 ($n = 81$; 18 in stage III, 12 in stage IVs and 51 in stage IV).

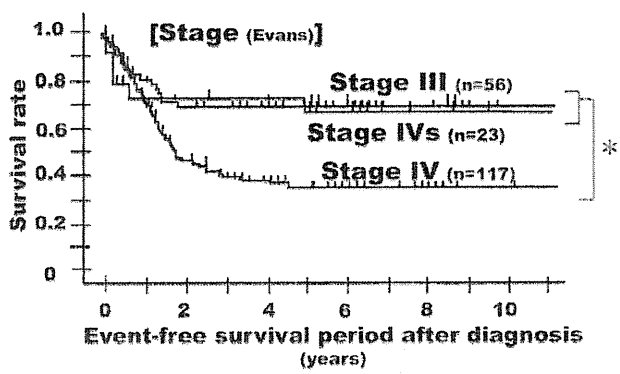


**Fig. 1.** Stage at diagnosis and event-free survival of patients with advanced neuroblastoma. Log-rank test (Mantel–Cox): $\chi^2_0 = 13.197$, df = 2, $p = 0.0014$ ($n = 196$) 'Breslow–Graham–Wilcoxon test: $\chi^2_0 = 8.013$, df = 1, $p = 0.0046$ staging was based on Evans classification.

For these patients, in addition to a modified regimen described for the previous decade, some patients received myeloablative-therapy followed by HSCT [12,13,19]. The ratio of patients in stage IV showed 15–20% increases in last two decades. The difference in EFS for these three groups is shown in Fig. 2. While the survival in the first and second decades did not show any significant differences, the survival in the third decade was improved relative to the previous two decades ($p = 0.0405$).

This retrospective study was performed based on a therapeutic bias in which therapy in the third decade might partially overcome the risk that the NBs possessed originally.

### 2.3. Biological factors

*MYCN* status was determined from Southern blot analysis and *MYCN* amplification was defined as greater than 10 copies of the gene present [6]. Expression of Ha-ras and trk A were determined from immunohistochemical studies and have a relationship with a patient's favorable outcome as previously reported [8,20–22]. When expression of both Ha-ras and trk A were high or low, the expressions were defined as "High" or "Low", respectively. When Ha-ras and trk A were expressed inversely, the expression was defined as "Intermediate". Histopathological prediction was determined according to the INPC criteria reported in 1999 [23,24] and 2000 [25], and NBs diagnosed before 1999 were all re-evaluated according to the criteria as well.

### 2.4. Statistical analysis

SPSS (Version 11.5, SPSS Inc., Chicago, IL, USA) was used for statistical analyses. Survival curves were plotted using the Kaplan–Meier method and analyzed using the log-rank test for comparison among more than three groups and the Breslow–Gehan–Wilcoxon test was used for pairwise comparisons. To assess the independence of the variables associated with the clinical outcome, multivariate analysis using the Cox proportional hazard model was performed. Significant statements refer to $p$ values less than 0.05, which were considered statistically significant.

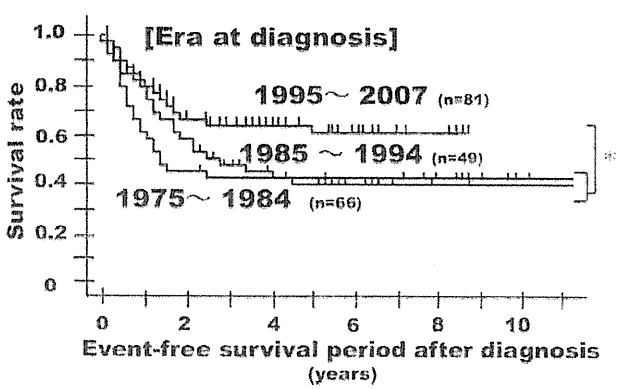


**Fig. 2.** Era at diagnosis and event-free survival of patients with advanced neuroblastoma. Log-rank test (Mantel–Cox): $\chi^2_0 = 6.945$, df = 2, $p = 0.0310$ ($n = 196$) Breslow–Graham–Wilcoxon test: $\chi^2_0 = 4.197$, df = 1, $p = 0.0405$.

## 3. Results

### 3.1. Statistical significances of three predictors for clinical outcome of the patients

*MYCN* status was examined in 135 advanced NBs and the amplification was detected in the 34 NBs (Table 1). *MYCN* amplification was found to have a highly significant association with the patient outcome in cases of advanced NB (Breslow–Graham–Wilcoxon test: $\chi^2_0 = 26.373$, degree of freedom (df) = 1, $n = 135$, $p < 0.0001$) and was associated with aggressive tumor biology. For 26 (76%) out of 34 patients identified with *MYCN* amplification, events such as progression, relapse or death were observed within 2 years after diagnosis. However, the sensitivity for poor outcome of patients represent only 42% of the 62 patients who experienced the clinical events.

Both unfavorable histology according to INPC criteria and low Ha-ras/trk A expression were also significantly associated with poor clinical outcome of the patients. The Breslow–Graham–Wilcoxon test was used for the former: ($\chi^2_0 = 12.479$, df = 1, $n = 170$, $p = 0.0004$), and the log-rank test was used for the latter: ($chi^2_0 = 19.278$, df = 2, $n = 164$, $p < 0.0001$). The predictive specificity and the detective sensitivity of INPC unfavorable histology were 70% (42/60) and 51% (42/83), respectively. Similarly, the specificity and sensitivity of low Ha-ras/trk A expression were 66% (41/62) and 53% (41/77), respectively (Table 1). Although each of the three factors was found to be specific for predicting high-risk NBs, ~50% of the high-risk NBs were not correctly identified when NBs were evaluated using only one predicting factor.

Multivariate analysis using the Cox proportional hazard model was performed to evaluate the independence of the three factors used to predict the clinical outcome for patients with NB. *MYCN* amplification, INPC unfavorable histology and low Ha-ras/ trk A expression were independently associated with poor clinical outcome (Table 2).

### 3.2. Stratification using a flow chart of the three independent risk factors

Based on the independence of the risk factors, *MYCN* amplification, INPC unfavorable histology and low expression of Ha-ras/trk A, 103 NBs in whom all three factors were examined were stratified (Fig. 3). Of these cases, 22 were in stage III disease, 12 were in stage IVs disease and 69 patients were in stage IV disease. Fifty-nine patients were male and 44 patients were female and the mean age of the patients at diagnosis was 30.8 months (range; 0 month–20 years). Forty-four patients died and their mean OS period was 1.68 years (range; 0 month–5.63 years). Fifty-nine patients were event-free survivors and their mean follow-up period was 5.70 years (range; 2.32 years–16.7 years). These surviving patients had a period without disease for more than 2 years after diagnosis or a state of remission achieved following a certain event during their clinical courses.

Initially, 28 NBs identified with *MYCN* amplification were classified as high-risk NBs, and 20 of these patients died with an OS period of 453 days after diagnosis. The 75 NBs without *MYCN* amplification were subsequently evaluated based on their INPC histology. Then, the 24 NBs with INPC unfavorable histology were stratified as high-risk NBs, and 14 of these patients died with a mean OS period of 610 days. For the remaining 51 NBs without MYCN amplification and with INPC favorable histology, 17 NBs with low Ha-ras/trk A expression were categorized as high-risk NBs. Four of these patients died and were associated with a mean OS period of 656 days. In total, 69 NBs were classified as high-risk NBs using these three-step stratification criteria and the 38 patients with these high-risk NBs died from disease. These deaths represent 86% of the total 44 deceased patients included in this study. Using the proposed stratification process, high-risk NBs could be successfully predicted with high sensitivity at the time of diagnosis.

On the other hand, a favorable outcome of the patients was associated closely with 21 NBs with high expression of both Ha-ras/trk A, an absence of amplified *MYCN* and INPC favorable histology. Nineteen (90%) of 21 patients were disease-free survivors. Of these surviving patients, 9 were in stage III, 5 were in stage IVs and 7 were in stage IV. Among the 21 patients 12 received a conventional chemotherapy for advanced tumors and all patients were disease-free survivors. The remaining 9 patients received mega-dose chemotherapy and HSCT in addition to the conventional chemotherapy and 7 were disease-free survivors. Only 2 patients died, which represented 4.5% of the total 44 deceased patients in this study. Therefore the NBs with the above characteristics could be considered

**Table 1**
Predictive specificity and sensitivity for clinical outcome of patients with neuroblastoma.

| Predictive factors | Cases with factor/cases examined | Number of cases with clinical events[a] | Predictive intensity for poor outcome | | |
|---|---|---|---|---|---|
| | | | Specificity[b] | & | Sensitivity[c] |
| MYCN amplification | 34/135 | 26[a] | 76% (26/34) | | 42% (26/62) |
| INPC unfavorable | 60/170 | 42[a] | 70% (42/60) | | 51% (42/83) |
| Ha-ras/trk A low expression | 62/164 | 41[a] | 66% (41/62) | | 53% (41/77) |

[a] Cases with clinical events: relapse, progression, death, etc.
[b] Specificity: ratio of cases with clinical events relative to those with the predictive factor.
[c] Sensitivity: ratio of cases with clinical events among cases predicted relative to all cases with clinical events in this study.

**Table 2**
Independence of predictive factors in neuroblastomas.

| Variables | df[a] | Parameter estimates | Standard error | $\chi_0^2$ | *p* Value | Hazard ratio | 95% CI[b] |
|---|---|---|---|---|---|---|---|
| *MYCN amplification* | | | | | | | |
| "Amplified" vs. "unamplified" | 1 | 0.819 | 0.295 | 7.726 | 0.005 | 2.267 | 1.273, 4.039 |
| *INPC histology* | | | | | | | |
| "Favorable" vs. "unfavorable" | 1 | 0.948 | 0.297 | 10.203 | 0.001 | 2.581 | 1.443, 4.618 |
| *Ha-ras/trk A expression* | | | | | | | |
| "High and intermediate" vs. "low" | 1 | −0.808 | 0.347 | 5.424 | 0.02 | 0.446 | 0.226, 0.88 |
| *Ha-ras/trk A expression* | | | | | | | |
| "High" vs. "intermediate & low" | 1 | 0.281 | 0.433 | 0.423 | 0.516 | 1.325 | 0.568, 3.09 |

[a] Degree of freedom.
[b] 95% Confidence intervals of hazard ratio.

*"therapy-sensitive NBs"*. Of 13 NBs with low expression of either Ha-ras / trk A (intermediate), 4 patients died from disease, which represented 9% of the total deceased patients in this study.

### 3.3. Biological stratification of high-risk NBs compared to therapy-sensitive NBs

We supposed that number of the independent risk factors might affect the patient survival in the 69 high-risk NBs. The ten patients associated with all three (triple) risks were three patients in stage III and seven patients in stage IV and there was only one survivor (10%). Moreover, tumor aggressiveness was consistent with the clinical course. For example, of the nine deceased patients, the mean EFS period from diagnosis was 292 days, which indicated that their relapse and/or progression had begun during or shortly after their initial treatment. Furthermore, this mean EFS period was shorter than 469 days for the 17 deceased patients with double risk NBs and 456 days for the 12 deceased patients with single risk NBs, respectively. The survival rates for the 24 patients with double risk NB and the 35 patients with single risk NB were also 29% and 66%, respectively. When the three subgroups of high-risk NBs combined with the therapy-sensitive subgroup, significant differences in their clinical outcome were confirmed by using Kaplan–Meier analyses (Fig. 4). Under intragroup comparison, differences in their clinical outcome of patients were also statistically significant, except for the triple vs. double risk groups (Table 3). Therefore, NBs with triple risk could be defined as *"therapy-resistant"*.

## 4. Discussion

Since 1971 when Evans et al. proposed a staging system for NBs [14], many clinical markers associated with patient prognosis have been reported [26]. As results from advances of pediatric surgery and chemotherapy, the favorable outcome of patients with localized NB has been

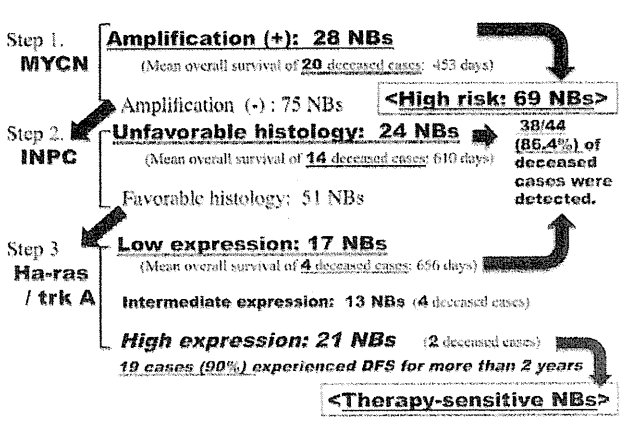


**Fig. 3.** Risk stratification flow chart of 103 advanced neuroblastomas. *Abbreviation*: NB, neuroblastoma.

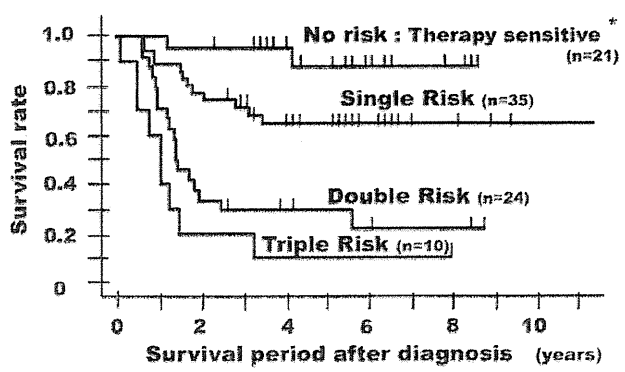


**Fig. 4.** Risk factors and survival of patients with advanced neuroblastoma. Log-rank test (Mantel–Cox): $\chi_0^2 = 40.908$, df = 3, $p < 0.0001$ ($n = 90$). *Ha-ras/trk A expressions were "high"/"high", in addition to INPC "favorable" and "unamplified" MYCN.

**Table 3**
Intragroup comparisons for the number of risk factors and the clinical outcome of patients with neuroblastoma.

| Comparison between two groups | Breslow–Gehan–Wilcoxon test | | |
|---|---|---|---|
| | df[a] | $\chi^2_0$ | *p* Value |
| Triple risk ($n$ = 10) vs. double risk ($n$ = 24) | 1 | 3.158 | 0.756 |
| Triple risk ($n$ = 10) vs. single risk ($n$ = 35) | 1 | 19.037 | <0.0001 |
| Triple risk ($n$ = 10) vs. no risk[b] ($n$ = 21) | 1 | 26.557 | <0.0001 |
| Double risk ($n$ = 24) vs. single risk ($n$ = 35) | 1 | 10.963 | 0.0009 |
| Double risk ($n$ = 24) vs. no risk[b] ($n$ = 21) | 1 | 18.368 | <0.0001 |
| Single risk ($n$ = 35) vs. no risk[b] ($n$ = 21) | 1 | 4.573 | 0.0325 |

[a] Degree of freedom.
[b] Expression of both Ha-ras and trk A was "high", MYCN amplification was "absent" and INPC was "favorable histology".

recognized and the therapeutic intensity has decreased. In contrast, despite elevating intensity of chemotherapies for patients with non-localized NBs, especially in stage IV, the survival rates have not improved accordingly. Recently, the International Neuroblastoma Risk Group reported a large cohort study for risk classification for prospective comparison in a risk-based clinical trial [27]. Our study focused on non-localized NBs and examined both the specificity and sensitivity of the prediction. The clinical relevance of each *MYCN* status and INPC finding linked with patient age was compatible with the results from the cohort study. We presented here a model of prognostic prediction with high sensitivity to patient outcome. The current study also elucidated a subtype sensitive to chemotherapy currently available for advanced NBs and another subtype resistant to current intense regimen (Fig. 4).

Stage IVs has been well-known as a subgroup of disseminated NBs with a favorable natural outcome and the treatment modalities are milder. However, some of the stage IVs patients have unfavorable outcomes. In our study, 30% of stage IVs patients experienced tumor progression, relapse, and/or death and the EFS rate was similar to that of stage III patients (Fig. 1). These findings were consistent with a report by van Noesel et al. [28] that 33/119 (28%) stage IVs patients died and 45% of the 33 deceased patients progressed to stage IV. They mentioned that the progression from stage IVs to stage IV was strongly related to the presence of unfavorable biological markers. Stage IVs NBs were heterogeneous; therefore, they were enrolled into the analysis of this study.

First, the current study evaluated the predictive sensitivity of a newly proposed risk assessment system, which could be performed in conventional medical laboratories. Each of "*MYCN* amplification", "INPC unfavorable histology" and "low expression of Ha-ras/trk A" has statistical specificity for poor clinical outcome of patients, however, ~50% of patients with poor clinical outcome were not correctly identified when the evaluation was performed by using a single predictor (Table 1).

It might be reasonable that different kinds of intracellular dysfunctions attribute to the diversity of NBs. Each of the *MYCN* [29], trk A [30] and Ha-ras [31–33] plays different role in cellular signaling pathways. *MYCN* might control the proliferating activity and the amplification might promote tumor aggressiveness. However, both trk A and Ha-ras have been shown to contribute to the development of fetal and postnatal neuronal tissues, respectively [30,34,35]. The histopathology of NBs can also range from immature NBs to mature ganglioneuroma, as well as the associated cellular indicators such as the mitosis-karyorrhexis index. Shimada's classification was proposed in 1984 [36], which evolved into the INPC (Shimada system) in 1999 [23] as a histopathological prognostic factor linked with patient age. If these three factors independently contribute to the cellular biology of NB, an evaluation using their combination might improve the predictive sensitivity regarding clinical outcome of patients with NB. Therefore, this study was designed to determine whether *MYCN* status, INPC histology and Ha-ras/trk A expression could be independent predictors for the patient's EFS (Table 2) and the results of this study show that 86% (38/44) of all deceased patients were successfully identified using the proposed 3-step risk assessment criteria (Fig. 3). The sensitivity of prognostic prediction might be enough to accept as a model of risk stratification.

This 3-step risk assessment could also elucidate a therapy-sensitive subgroup. There were 21 NBs with unamplified *MYCN*, INPC favorable histology and high Ha-ras/trk A expression. Their EFS rate was 90% (19/21). They were treated with conventional multi-modal therapies for advances NBs; 12 patients were treated with conventional chemotherapy and were disease-free for more than 2 years. The remaining 9 patients received HSCT treatment in addition to the conventional chemotherapy, of which 7 remained disease-free. Their disease-free survival rate was equal to that for patients with stage I or II disease. Thus, they could be defined as a subgroup of "*therapy-sensitive NBs*". It is suggested that patients in this subgroup could achieve disease-free survival with standard therapeutic regimens for advanced NBs.

This study also verified a hypothesis that heterogeneous advanced NBs could be stratified using numbers of the independent factors (Fig. 4). Over-all, 23/35 (66%) patients with single risk NBs and 7/24 (29%) patients with double risk NBs achieved disease-free survival. While it has been well known that *MYCN* amplification is a strong predictor for poor prognosis, results of this study suggest that NB biology with amplified *MYCN* might also be modified with the remaining two factors. For example, four patients with *MYCN* amplified NB identified as a single risk factor were treated with intensive chemotherapy followed by HSCT and all of them achieved disease-free survival. It is possible that current chemotherapy with high intensity and followed by HSCT may improve the salvage of these patients, especially when only *MYCN* amplification is a risk factor. On the other hand, the survival was only 3/14 (21%) *MYCN* amplified NB patients associated with either INPC unfavorable histology or low expression of Ha-ras/trk A (double risk). There were three patients with the double risk NB including *MYCN* amplification and they received high intensive chemotherapy with HSCT. The survival for more than 2 years could be achieved in 2/3 (67%) patients. The remaining eleven patients were treated with intensive chemotherapy for advanced NB without HSCT, however, their survival rate was 1/11(9%). Although this study was a retrospective and the therapies were not controlled, 23/30

(77%) patients with single or double risk factors and received HSCT were also disease-free survivors. Based on these results, HSCT regimen following conventional chemotherapy appears to be effective for NBs with the single or double risk factors. The improved survivals of patients with non-localized NBs was compatible with findings from a Italian cohort study reported by Haupt et al. [37]. For example, our study showed that EFS of the patients in stage IV was 23.5% in era of 1974–1984 and improved to 66.6% in era of 1995–2007. The effectiveness was provided by intensifying chemotherapies including HSCT. This study elucidated another subtype which is still resist to the current intensifying therapies.

Consistent with the risk stratification criteria proposed in this study, the most striking tumor aggressiveness was exhibited by the 10 NBs with triple risk factors, *MYCN* amplification, INPC unfavorable histology and low expression of Ha-ras/trk A. Only one (10%) patient survived. For the nine deceased patients, the mean EFS period (292 days) was much shorter than that of the other two groups (469 days for double risk NBs and 456 days for single risk NBs, respectively). Moreover, a majority of these patients experienced tumor progression during their initial chemotherapies, and as a result, it was too early to prepare an ordinary schedule for HSCT. These patients were referred to as having *therapy-resistant* NBs, and further treatment strategies for them should be modified to a different regimen with more steeply escalating initial therapeutic intensity with early tandem HSCT or other adjuvant immunotherapies or radiotherapies [38,39].

There are still many questions to be answered regarding the pathogenesis of heterogeneous NBs. Recent advances in gene technology may provide much more information and the means to address these questions, such as DNA or RNA micro arrays and genomic or molecular signatures [40,41]. However, these methods are still expensive and require special circumstances to handle properly. We can expect to be realized soon that the newly developing technologies promote to elucidate tumor biology more precisely and make the cost suitable for clinical performance. While, the much more subject genes we can analyze, the more complex algorithm we need for the interpretation. Our study is a preceding model to stratify heterogeneous NBs by using conventional methods such as Southern blotting/FISH, histopathology, and immunohistochemistry. A point of view in our study could be adaptable to the further evaluation using newly developing technology. The more multi-sided information about tumor biology can be available, the better selection of therapeutic intensity can be achieved adequately for patients with advanced NB. It is important to note that there is no regimen without any risk caused by the therapy itself [42–44]. When the intensity of treatment administered is matched with the tumor targeted, therapeutic efficacy can be maximized and therapy-related sequelae can be minimized.

## 5. Conclusion

This study provides a clinically applicable stratification model for the identification of high-risk NBs in advanced stages. In addition to the highly predictive sensitivity and specificity for patient outcome, the stratification model elucidates subgroups, such as *"therapy-sensitive NBs"* and *"therapy-resistant NBs"*. The number of independent risk factors was used to classify heterogeneous NBs into four subgroups and showed significant differences in patient survival. Based on this stratification, the appropriate selection of therapeutic intensity should maximize the treatment efficacy of high-risk NBs while minimizing therapy-related sequelae for therapy-sensitive NBs.

## Conflicts of interest

None declared.

## Acknowledgments

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## References

[1] G.M. Brodeur, R.P. Castleberry, Neuroblastoma, in: P.A. Pizzo, D.G. Poplack, (Eds.), Principles and Practice of Pediatric Oncology, 3rd ed. J.B. Lippincott-Raven, Philaelphia, 1997, pp. 761–797.
[2] K.K. Matthay, J.G. Villablanca, R.C. Seeger, D.O. Stram, R.E. Harris, N.K. Ramsay, P. Swift, H. Shimada, C.T. Black, G.M. Brodeur, R.B. Gerbing, C.P. Reynolds, Treatment of high-risk neuroblastoma with intensive chemotherapy, radiotherapy, autologous bone marrow transplantation and 13-cis-retinoic acid. Children's Cancer Group, New Engl. J. Med. 341 (1999) 1165–1173.
[3] J.R. Park, J.G. Villablanca, W.B. London, R.B. Gerbing, D. Haas-Kogan, E.S. Adkins, E.F. Attiyeh, J.M. Maris, R.C. Seeger, C.P. Reynolds, K.K. Matthay, Outcome of high-risk stage 3 neuroblastoma with myeloablative therapy and 13-cis-retinoic acid: a report from the Children's Oncology Group, Pediatr. Blood Cancer 52 (2009) 44–50.
[4] J.M. Maris, K.K. Matthay, Molecular biology of neuroblastoma, J. Clin. Oncol. 17 (1999) 2264–2279.
[5] N. Bown, Neuroblastoma tumor genetics: clinical and biological aspects, J. Clin. Pathol. 54 (2001) 897–910.
[6] R.C. Seeger, G.M. Brodeur, H. Sather, A. Dalton, S.E. Siegel, K.Y. Wong, D. Hammond, Association of multiple copies of the N-myc oncogene with rapid progression of neuroblastomas, New Engl. J. Med. 313 (1985) 1111–1116.
[7] H. Shimada, S. Umehara, Y. Monobe, Y. Hachitanda, A. Nakagawa, S. Goto, R.B. Gerbing, D.O. Stram, J.N. Lukens, K.K. Matthay, International neuroblastoma pathology classification for prognostic evaluation of patients with peripheral neuroblastic tumors. A report from the Children's Cancer Group, Cancer 92 (2001) 2451–2461.
[8] T. Tanaka, T. Sugimoto, T. Sawada, Prognostic discrimination among neuroblastomas according to Ha-ras/trk a gene expression, Cancer 83 (1998) 1626–1633.
[9] T. Tanaka, T. Iehara, T. Sugimoto, M. Hamasaki, S. Teramukai, Y. Tsuchida, M. Kaneko, T. Sawada, Diversity in neuroblastomas and discrimination of the risk to progress, Cancer Lett. 228 (2005) 267–270.
[10] S. Bilke, Q. Chen, J. Khan, Whole chromosome alterations predict survival in high-risk neuroblastoma without MYCN amplification, Clin. Cancer Res. 14 (2008) 5540–5547.
[11] A. Oberthuer, P. Warnat, Y. Kahlert, F. Westermann, R. Spitz, B. Brors, B. Hero, R. Eils, M. Schwab, F. Berthold, M. Fischer, Classification of neuroblastoma patients by published gene-expression markers

reveals a low sensitivity for unfavorable courses of *MYCN* non-amplified disease, Cancer Lett. 250 (2007) 250–267.
[12] T. Matsumura, J. Michon, Treatment of localized neuroblastoma, in: G.M. Brodeur, T. Sawada, Y. Tsuchida, P.A. Voute (Eds.), Neroblastoma, Elsevier Science, BV, Amsterdam, 2000, pp. 403–415.
[13] Y. Tsuchida, M. Kaneko, Treatment of advanced neuroblastoma: the Japanease experience, in: G.M. Brodeur, T. Sawada, Y. Tsuchida, P.A. Voute (Eds.), Neroblastoma, Elsevier Science BV, Amsterdam, 2000, pp. 453–469.
[14] A.E. Evans, G.J. D'Angio, J.A. Randolph, A proposed staging for children with neuroblastoma, Cancer 27 (1971) 347–378.
[15] D. Pinkel, C. Pratt, C. Holton, D. James, E. Wrenn, O. Hustu, Survival of children with neuroblastoma treated with combination chemotherapy, J. Pediatr. 73 (1968) 928–931.
[16] T.E. Williams, M.H. Donaldson, Neuroblastoma in: W.W. Sutow, T.J. Vietti, D.J. Fernbach (Eds.), Clinical Pediatric Oncology, The C.V. Mosby Company, Saint Louis, 1973, pp. 384–410.
[17] S. Sawaguchi, M. Kaneko, J. Uchino, T. Takeda, M. Iwafuchi, S. Matsuyama, H. Takahashi, T. Nakajo, Y. Hoshi, I. Okabe, J. Yokoyama, H. Nishihira, S. Sakurai, T. Sawada, N. Nagahara, Y. Tsuchida, Treatment of advanced neuroblastoma with emphasis on intensive induction chemotherapy: a report from the study group of Japan, Cancer 66 (1990) 1879–1887.
[18] M. Iwafuchi, J. Utsumi, Y. Tsuchida, M. Kaneko, S. Matsuyama, N. Ohnuma, N. Taguchi, H. Mugishima, J. Yokoyama, S. Sasaki, A. Yoshioka, N. Nagahara, M. Fukuzawa, S. Suita, Evaluation of the patients with advance neuroblastoma more than 5 years after initiation of intensive Japanese protocol: a report of the study group of Japan for advanced neuroblastoma, Med. Pedatr. Oncol. 27 (1996) 515–520.
[19] M. Kaneko, Y. Tuchida, H. Mugishima, N. Ohnuma, K. Yamamoto, K. Kawa, M. Iwafuchi, T. Sawada, S. Suita, Intensified chemotherapy increases the survival rates in patients with stage 4 neuroblastoma with *MYCN* amplification, J. Pediatr. Hematol./Oncol. 24 (2002) 613–621.
[20] T. Tanaka, D.J. Slamon, H. Shimoda, C. Waki, Y. Kawaguchi, Y. Tanaka, N. Ida, Expression of Ha-ras oncogene products in human neuroblastomas and the significance correlation with a patient's prognosis, Cancer Res. 48 (1988) 1030–1034.
[21] T. Tanaka, D.J. Slamon, H. Shimada, H. Shimoda, T. Fujisawa, N. Ida, R.C. Seege, A significant association of Ha-ras p21 in neuroblatoma cells with patient prognosis, Cancer 68 (1991) 1296–1302.
[22] T. Tanaka, E. Hiyama, T. Sugimoto, T. Sawada, M. Tanabe, N. Ida, Trk A gene expression in neuroblastoma. The clinical significance of an immuno- histochemical study, Cancer 76 (1995) 1086–1095.
[23] H. Shimada, I.M. Ambros, L.P. Dehner, J. Hata, V.V. Joshi, B. Roald, Terminology and morphologic criteria of neuroblastic tumors. Recommendations by the International Neuroblastoma Pathology Committee, Cancer 86 (1999) 349–363.
[24] H. Shimada, I.M. Ambros, L.P. Dehner, J. Hata, V.V. Joshi, B. Roald, D.O. Stram, R.B. Gerbing, J.N. Lukens, K.K. Matthay, R.P. Castleberry, The international neuroblastoma pathology classification (the Shimada system), Cancer 86 (1999) 364–372.
[25] S. Umehara, A. Nakagawa, K.K. Matthay, J.N. Lukens, R.C. Seeger, D.O. Stram, R.B. Gerbing, H. Shimada, Histopathology defines prognostic subsets of ganglioneuroblastoma nodular, Cancer 89 (2000) 1150–1161.
[26] G.M. Brodeur, P.F. Ambros, Genetic and biological markers of prognosis in neuroblastoma, in: G.M. Brodeur, T. Sawada, Y. Tsuchida, P.A. Voute (Eds.), Neroblastoma, Elsevier Science BV, Amsterdam, 2000, pp. 355–369.
[27] L.S. Cohn, A.D.J. Peason, W.B. London, T. Monclair, P.F. Ambros, G.M. Brodeur, A. Faldum, B. Hero, T. Iehara, D. Machin, V. Mosseri, T. Simon, A. Garaventa, V. Castel, K.K. Matthay, The International Neuroblastoma Risk Group (INRG) classification system: an INRG task force report, J. Clin. Oncol. 10 (2009) 289–297.
[28] M.M. van Noesel, K. Hahlen, F.G.A.J. Hakvoort-Cammel, R.M. Egeler, Neuroblastoma 4s A heterogeneous disease with variable risk factors and treatment strategies, Cancer 80 (1997) 834–843.
[29] M. Schwab, *MYCN* in neuronal tumors, Cancer Lett. 204 (2004) 179–187.
[30] A. Nakagawara, M. Arima-Nakagawara, N.J. Scavarda, C.G. Azar, A.B. Cantor, G.M. Brodeur, Association between high levels of expression of the trk gene and favorable outcome in human neuroblastoma, New Engl. J. Med. 328 (1993) 847–854.
[31] C. Kitanaka, K. Kato, R. Ijiri, K. Sakurada, A. Tomiyama, K. Noguchi, Y. Nagashima, A. Nakagawara, T. Momoi, Y. Toyoda, H. Kigasawa, T. Nishi, M. Shirouzu, S. Yokoyama, Y. Tanaka, Y. Kuchino, Increased Ras expression and caspase-independent neuroblastoma cell death: possible mechanism of spontaneous neuroblastoma regression, J. Natl. Cancer. Inst. 94 (2002) 358–368.
[32] C.P. Reynolds (Ed.), Ras and seppuku in neuroblastoma, J. Natl. Cancer. Inst. 94 (2002) 319–321.
[33] A. Agathanggelou, W.N. Cooper, F. Latif, Role of the Ras-association domain family 1 tumor suppressor gene in human cancers, Cancer Res. 65 (2005) 3497–3508.
[34] T. Tanaka, N. Ida, H. Shimoda, C. Waki, D.J. Slamon, M.J. Cline, Organ specific expression of ras oncoproteins during growth and development of the rat, Mol. Cell. Biochem. 70 (1986) 97–104.
[35] T. Tanaka, N. Ida, C. Waki, H. Shimoda, D.J. Slamon, M.J. Cline, Cell-type specific expression of c-ras gene products in the normal rat, Mol. Cell. Biochem. 75 (1987) 23–32.
[36] H. Shimada, J. Chatten, W.A. Newton Jr., A.B. Hamoudi, T. Chiba, H.B. Marsten, K. Misugi, Histopathologic prognostic factors in neuroblastic tumors: definition of subtypes of ganglioneuroblastoma and an age-linked classification of neuroblastomas, J. Natl. Cancer Inst. 73 (1984) 405–416.
[37] R. Haupt, A. Garaventa, C. Gambini, S. Parodi, G. Cangemi, F. Casale, E. Viscardi, M. Bianchi, A. Prete, A. Jenkner, R. Luksch, A. Di Cataldo, C. Favre, P. D'Angelo, G.A. Zanazzo, G. Arcamone, G.C. Izzi, A.R. Gigliotti, G. Pastore, B. De Bernardi, Improved survival of children with neuroblastoma between 1979 and 2005: a report of the Italian Neuroblastoma Registry, J. Clin. Oncol. 28 (2010) 2331–2338.
[38] K.K. Matthay, A. Quach, J. Huberty, B.L. Franc, R.A. Hawkins, H. Jackson, S. Groshen, S. Shusterman, G. Yanik, J. Veatch, P. Brophy, J.G. Villablanca, J.M. Maris, Iodine-131-metaiodobenzylguanidine double infusion with autologous stem-cell rescue for neuroblastoma: a new approaches to nerublastoma therapy phase I study, J. Clin. Oncol. 27 (2009) 1020–1025.
[39] K.K. Matthay, C.P. Reynolds, R.C. Seeger, H. Shimada, E.S. Adkins, D. Haas-Kogan, R.B. Gerbing, W.B. London, J.G. Villablanca, Long-term results for children with high–risk neuroblastoma treated on a randomized trial of myeloablative therapy followed by 13-cis-retioic acid: a Children's Oncology group study, J. Clin. Oncol. 27 (2009) 1007–1013.
[40] M. Ohira, S. Oba, Y. Nakamura, T. Hirata, S. Ishii, A. Nakagawara, A review of DNA microarry analysis of human neuroblastomas, Cancer Lett. 228 (2005) 5–11.
[41] A. Schramm, I. Mierswa, L. Kaderali, K. Morik, A. Eggert, J.H. Schulte, Reanalysis of neuroblastoma expression profiling data using improved methodology and extended follow-up increases validity of outcome prediction, Cancer Lett. 282 (2009) 55–62.
[42] M. Barrera, A.K. Shaw, K.N. Speechley, E. Maunsell, L. Pogany, Educational and social late effects of childhood cancer and related clinical, personal, and familial characteristics, Cancer 104 (2005) 1751–1760.
[43] G.T. Armstrong, Q. Liu, Y. Yasui, W. Leisenring, L.L. Robison, A.C. Mertens, Late mortality among 5-year survivors of childhood cancer: a summary from the Childhood Cancer Survivor Study, J. Clin. Oncol. 27 (2009) 2328–2338.
[44] A.T. Meadows, D.L. Friedman, J.P. Neglia, A.C. Mertens, S.S. Konaldson, M. Stovall, S. Hammond, Y. Yasui, P.D. Inskip, Second neoplasm's in survivors of childhood cancer: findings from the Childhood Cancer Survivor Study Cohort, J. Clin. Oncol. 27 (2009) 2356–2362.