the main types of childhood cancer. A total of 33 059 childhood cancer deaths were reported in Japan during 1970–2006, of which 353 cancer deaths occurred in 2006. For all cancers combined, the mortality rate during 2000–2006 was 2.20 per 100 000 population for boys and 1.89 for girls. Leukemia was the most common diagnosis. Death rates from leukemia were 0.84 for boys and 0.68 for girls. Mortality from childhood CNS tumors was 0.43 for boys and 0.42 for girls. Geographic variations were observed. The rates of childhood CNS tumor and malignant kidney tumor were lower for both genders in Japan than in other countries.

### temporal changes in mortality

Trends of age-standardized mortality from childhood cancer are shown in Figures 1 and 2 and Tables 3 and 4. Mortality for all cancers combined decreased since 1970s in Japan. For boys, a declining trend of 1.58% per year ($P < 0.05$) was observed during 1970–1979, followed by an accelerated decline of 3.78% per year ($P < 0.05$) during 1979–2006. For girls, mortality was high in the 1970s and remained stable in 1996–2006 at a low level, after two significant periods of decline (1972–1995 and 1995–1999). The average annual per cent change (AAPC) in recent 10 years was −3.8% ($P < 0.05\%$) for boys and −1.9% ($P < 0.05$) for girls. In recent 5 years, declining trend only occurred in boys. The average annual per cent change during 2002–2006 was −3.8% ($P < 0.05\%$) for boys, and for girls a nonsignificant decline was observed from 2002 (AAPC = −0.6%, $P > 0.05$) for girls.

The mortality rate from leukemia in boys remained stable during 1970–1976 (APC = −1.10, $P > 0.05$) and then declined by 4.77% per year ($P < 0.05$) during 1976–2006. For girls, mortality decreased by 4.53% per year ($P < 0.05$) throughout the whole period. The average annual change in recent 10 years was −4.8% ($P < 0.05\%$) for boys and −4.5% ($P < 0.05\%$) for girls. Similar decline trends were also observed in Canada, the United States, Italy, UK (girls) and New Zealand.

In contrast with the dramatic decline in mortality for childhood leukemia, mortality rates from childhood CNS tumor in Japan remained stable at a low level for both genders during 1980–2006. The average annual change in recent 10 years was 0.5% ($P > 0.05$) for boys and 0.0% ($P > 0.05$) for girls. On the contrary, Canada, the United States, UK and New Zealand (girls) showed significant declining trends in the whole period.

With reference to the pattern of mortality for lymphomas, death rates for boys were stable during 1970–1985 and declined significant thereafter by 8.56% per year. The trend for girls leveled off during 1970–1991 and showed a declining trend of 11.85% per year during 1991–2006; however, except for New Zealand females, the death rates in other countries for both genders significantly declined throughout the whole period.

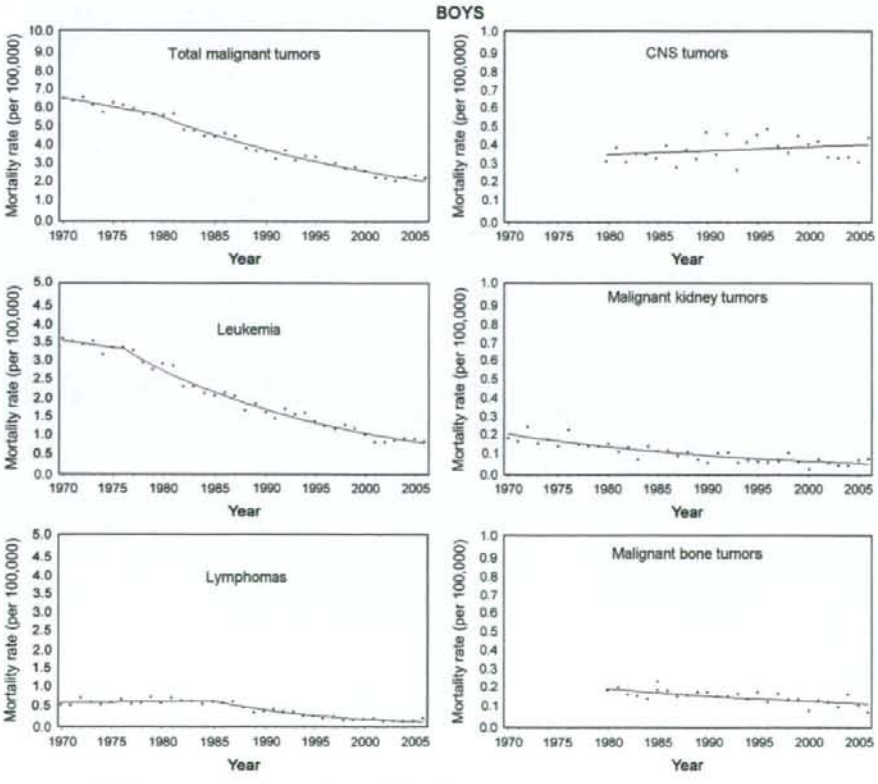


**Figure 1.** Mortality rates of childhood cancer deaths, boys, Japan, 1970–2006.

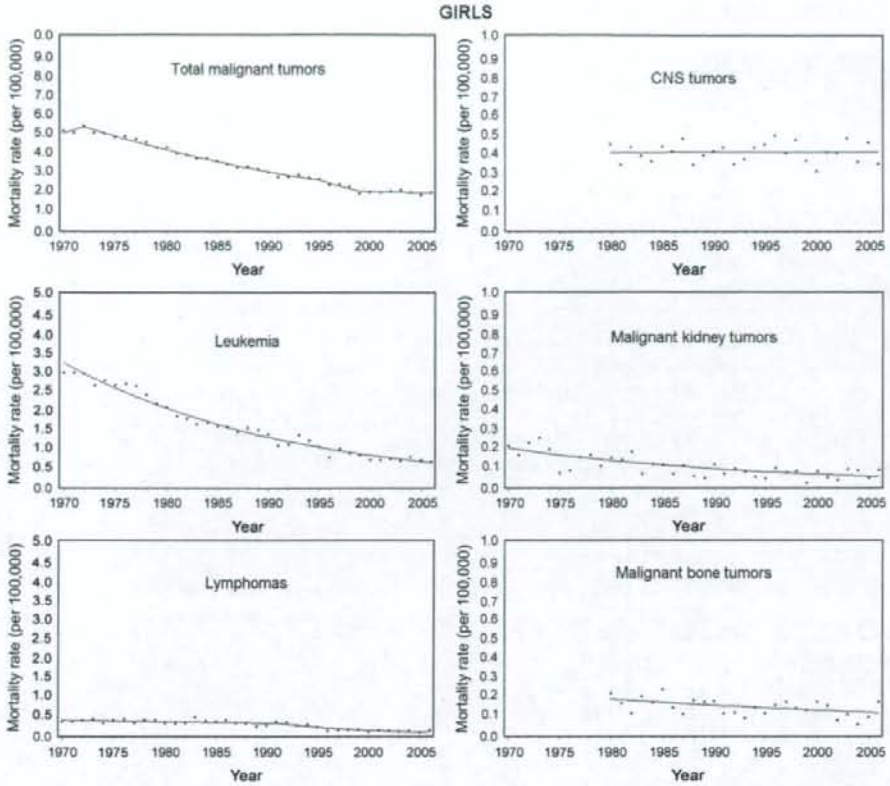


**Figure 2.** Mortality rates of childhood cancer deaths, girls, Japan, 1970–2006.

Regression analysis also revealed that the death rate for malignant kidney tumors declined by 4.12% per year for boys and 3.98% per year for girls during 1970–2006. Similar trends were observed for malignant bone tumor. Mortality decreased by 2.03% per year for boys and 1.79% per year for girls throughout the whole period.

Mortality rates varied from prefecture to prefecture in Japan. A map of SMR by gender is shown in Figure 3. The SMR was significantly highest among children in Kochi prefecture for boys and Tokushima and Kagoshima prefectures for girls.

## discussion

In this study, we quantified the childhood cancer burden in Japan, focusing on mortality, and compared these figures with other developed countries. The results indicated that mortality from childhood cancer in Japan is substantial, while the number of deaths is small. There were 33 059 cases of childhood cancer death over the period 1970–2006 in Japan. Approximately 400 deaths each year were attributed to cancer in children aged 0–14 years. Mortality from all cancers combined in Japan is comparable to that in the European, North American and Oceanic countries included in this study for contrast.

The joinpoint regression method was used in our research to evaluate the trend in childhood cancer deaths. This method has allowed a detailed and accurate description of the pattern of childhood cancer mortality since it identifies the calendar years in which statistically significant changes in trends occurred. This offers a clearer picture of actual trends in mortality over long periods of time rather than using only one trend statistics. We also reported the average annual percentage change in this study. The AAPC can be used to characterize a short segment based on a joinpoint model fit over a much longer series. This is especially advantageous for situations when the data are sparse (e.g. a rare cancer or data from a small geographic area) [7]. Our results showed a declining cancer mortality rate for boys in the whole period and a stable trend for girls in recent 5 years. It is unlikely that the observed time trends in the mortality rate are due to variations in the completeness and accuracy of the population data because the analyzed data were provided by official sources and based on the population census. The significant time trend observed for most tumor types is congruent with improvements in diagnosis, therapy and supportive care.

The dramatic decrease in mortality observed for childhood leukemia, which accounts for ~50% of all childhood cancer

**Table 3.** The APC of childhood cancer mortality rates (boys)

| Country | Trend 1 | | Trend 2 | | Trend 3 | | Trend 4 | | AAPC | |
|---|---|---|---|---|---|---|---|---|---|---|
| | Years | APC | Years | APC | Years | APC | Years | APC | Last 10 observations | Last 5 observations |
| Total malignant tumors | | | | | | | | | | |
| Japan | 1970–1979 | −1.58* | 1979–2006 | −3.78* | | | | | −3.8* | −3.8* |
| Canada | 1970–2004 | −3.64* | | | | | | | −3.6* | −3.6* |
| United States | 1970–1998 | −3.22* | 1998–2005 | −0.26 | | | | | −0.3 | −0.9 |
| Italy | 1970–1985 | −2.32* | 1985–1989 | −8.69 | 1989–1993 | 6.96 | 1993–2003 | −5.89* | −5.9* | −5.9* |
| UK | 1970–2005 | −2.93* | | | | | | | −2.9* | −2.9* |
| New Zealand | 1970–2004 | −2.50* | | | | | | | −2.5* | −2.5* |
| Leukemia | | | | | | | | | | |
| Japan | 1970–1976 | −1.10 | 1976–2006 | −4.77* | | | | | −4.8* | −4.8* |
| Canada | 1970–2004 | −5.00* | | | | | | | −5.0* | −5.0* |
| United States | 1970–1984 | −4.95* | 1984–2005 | −3.39* | | | | | −3.4* | −3.4* |
| Italy | 1970–2003 | −3.69* | | | | | | | −3.7* | −3.7* |
| UK | 1970–2005 | −3.74* | 2003–2005 | −27.41 | | | | | −9.6 | −16.4 |
| New Zealand | 1970–1997 | −2.12* | 1997–2004 | −18.03* | | | | | −14.7* | −18.0* |
| Lymphomas | | | | | | | | | | |
| Japan | 1970–1985 | 0.39 | 1985–2006 | −8.56* | | | | | −8.6* | −8.6* |
| Canada | 1970–2004 | −6.10* | | | | | | | −6.1* | −6.1* |
| United States | 1980–2005 | −5.63* | | | | | | | −5.6* | −5.6* |
| Italy | 1970–2003 | −4.46* | | | | | | | −4.5* | −4.5* |
| UK | 1970–2005 | −4.56* | | | | | | | −4.6* | −4.6* |
| New Zealand | 1970–2004 | −2.57* | | | | | | | −2.6* | −2.6* |
| Central nervous system tumors | | | | | | | | | | |
| Japan | 1980–2006 | 0.48 | | | | | | | 0.5 | 0.5 |
| Canada | 1980–2004 | −2.13* | | | | | | | −2.1* | −2.1* |
| United States | 1980–2005 | −1.07* | | | | | | | −1.1* | −1.1* |
| Italy | 1980–2003 | −2.19* | | | | | | | −2.2* | −2.2* |
| UK | 1980–2005 | −1.25* | | | | | | | −1.2* | −1.2* |
| New Zealand | 1980–2004 | −0.86 | | | | | | | −0.9 | −0.9 |
| Malignant kidney tumors | | | | | | | | | | |
| Japan | 1970–2006 | −4.12* | | | | | | | −4.1* | −4.1* |
| Canada | 1970–1996 | −7.91* | 1996–2004 | 17.70* | | | | | 14.5* | 17.7* |
| United States | 1970–1987 | −5.46* | 1987–2005 | −1.73* | | | | | −1.7* | −1.7* |
| Italy | 1970–2003 | −4.91* | | | | | | | −4.9* | −4.9* |
| UK | 1970–2005 | −3.64* | | | | | | | −3.6* | −3.6* |
| New Zealand | 1970–2004 | −1.99 | | | | | | | −2.0* | −2.0* |
| Malignant bone tumors | | | | | | | | | | |
| Japan | 1980–2006 | −2.03* | | | | | | | −2.0* | −2.0* |
| Canada | 1980–2004 | −2.32* | | | | | | | −2.3* | −2.3* |
| United States | 1980–1990 | −4.41* | 1990–2005 | 1.31 | | | | | 1.3 | 1.3 |
| Italy | 1980–2003 | −4.43* | | | | | | | −4.4* | −4.4* |
| UK | 1980–2005 | −2.93* | | | | | | | −2.9* | −2.9* |
| New Zealand | 1980–2004 | −0.23 | | | | | | | −0.2 | −0.2 |

*$P < 0.05$.

APC is the annual per cent change; AAPC is average annual per cent change.

deaths, is consistent with improvements in survival, particularly for patients with acute lymphoblastic leukemia. This increase in survival is due to more effective antileukemic therapy, such as multidrug chemotherapy protocols, with a reduction in the number of relapses and resistant disease, but also due to improvements in supportive care, such as antibiotics, antifungal treatment, blood banking, transplant procedures and pediatric intensive care. In fact, the 5-year survival rate of acute lymphoblastic leukemia increased from 20% to 30% in the 1960s to 60% to 75% in the 1980s in developed countries. Current survival rates are ~80% for acute lymphoblastic leukemia (ALL) [8] and 50%–70% for acute myelogenous leukemia. In Japan, a population-based study in Osaka prefecture indicated that the 5-year survival rate of childhood leukemia increased from 32.4% in 1975–1984 to 60.4 in 1985–1994 [1]. National incidence trends could not be

**Table 4.** The APC of childhood cancer mortality rates (girls)

| Country | Trend 1 | | Trend 2 | | Trend 3 | | Trend 4 | | AAPC | |
|---|---|---|---|---|---|---|---|---|---|---|
| | Years | APC | Years | APC | Years | APC | Years | APC | Last 10 observations | Last 5 observations |
| Total malignant tumors | | | | | | | | | | |
| Japan | 1970–1972 | 3.24 | 1972–1995 | −3.21* | 1995–1999 | −6.46* | 1999–2006 | −0.57 | −1.9* | −0.6 |
| Canada | 1970–2004 | −3.42* | | | | | | | −3.4* | −3.4* |
| United States | 1970–1977 | −4.46* | 1977–1995 | −2.72* | 1995–2005 | −1.07* | | | −1.1* | −1.1* |
| Italy | 1970–2003 | −2.80* | | | | | | | −2.8* | −2.8* |
| UK | 1970–2005 | −2.73* | | | | | | | −2.7* | −2.7* |
| New Zealand | 1970–2004 | −2.57* | | | | | | | −2.6* | −2.6* |
| Leukemia | | | | | | | | | | |
| Japan | 1970–2006 | −4.53* | | | | | | | −4.5* | −4.5* |
| Canada | 1970–2004 | −5.28* | | | | | | | −5.3* | −5.3* |
| United States | 1970–1980 | −6.09* | 1980–2005 | −3.14* | | | | | −3.1* | −3.1* |
| Italy | 1970–2003 | −4.33* | | | | | | | −4.3* | −4.3* |
| UK | 1970–2005 | −3.88* | | | | | | | −3.9* | −3.9* |
| New Zealand | 1970–2004 | −3.17* | | | | | | | −3.2* | −3.2* |
| Lymphomas | | | | | | | | | | |
| Japan | 1970–1991 | −1.13 | 1991–2006 | −11.85* | | | | | −11.8* | −11.8** |
| Canada | 1970–2004 | −4.55* | | | | | | | −4.6* | −4.6* |
| United States | 1980–2005 | −4.39* | | | | | | | −4.4* | −4.4* |
| Italy | 1970–2003 | −3.93* | | | | | | | −3.9* | −3.9* |
| UK | 1970–2005 | −4.56* | | | | | | | −4.6* | −4.6* |
| New Zealand | 1970–2004 | −0.35 | | | | | | | −0.4 | −0.4 |
| Central nervous system tumors | | | | | | | | | | |
| Japan | 1980–2006 | 0.03 | | | | | | | 0.0 | 0.0 |
| Canada | 1980–2004 | −1.50* | | | | | | | −1.5* | −1.5* |
| United States | 1980–2005 | −0.87* | | | | | | | −0.9* | −0.9* |
| Italy | 1980–2003 | −2.28* | | | | | | | −2.3* | −2.3* |
| UK | 1980–2005 | −1.68* | | | | | | | −1.7* | −1.7* |
| New Zealand | 1980–2004 | −2.32* | | | | | | | −2.3* | −2.3* |
| Malignant kidney tumors | | | | | | | | | | |
| Japan | 1976–2006 | −3.98* | | | | | | | −4.0* | −4.0* |
| Canada | 1970–2004 | −2.90* | | | | | | | −2.9* | −2.9* |
| United States | 1970–1991 | −4.60* | 1991–2005 | 0.16 | | | | | 0.2 | 0.2 |
| Italy | 1970–2003 | −4.62* | | | | | | | −4.6* | −4.6* |
| UK | 1970–2005 | −3.49* | | | | | | | −3.5* | −3.5* |
| New Zealand | 1970–2004 | −2.91* | | | | | | | −2.9* | −2.9* |
| Malignant bone tumors | | | | | | | | | | |
| Japan | 1980–2006 | −1.79* | | | | | | | −1.8* | −1.8* |
| Canada | 1980–2004 | −0.24 | | | | | | | −0.2 | −0.2 |
| United States | 1980–2005 | −1.59* | | | | | | | −1.6* | −1.6* |
| Italy | 1980–2003 | −3.52* | | | | | | | −3.5* | −3.5* |
| UK | 1980–2005 | −2.22* | | | | | | | −2.2* | −2.2* |
| New Zealand | 1980–2004 | 1.52 | | | | | | | 1.5 | 1.5 |

*$P < 0.05$.

APC is the annual per cent change; AAPC is average annual per cent change.

obtained in the current study. Research in Great Britain [9, 10], Italy [11] and Sweden [12] showed increased trends in childhood leukemia. A report from Britain indicated that small peaks in the incidence of ALL in 1976 and 1990 coincided with the years immediately following influenza epidemics [13]. Other explanations of the increased trend were characteristics of the environment, such as population mixing, although the etiology of cancer remains complicated and largely unknown.

The stable trend in mortality for childhood CNS tumor implied a modest increase trend in the incidence rate in Japan because of the survival improvement reported in childhood CNS tumors in developed countries in recent decades, while progress in therapy for brain tumors has not been as great as for leukemia. For CNS tumors, computed tomography, which was introduced in the 1970s, and magnetic resonance imaging, which has been used widely since the 1980s, has become

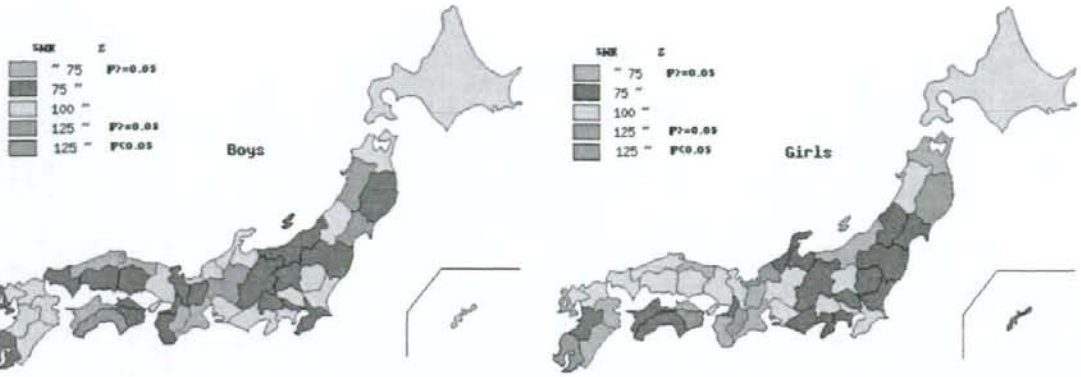


**Figure 3.** Standardized mortality ratios for childhood cancer in Japan, 2000–2006 by prefecture.

a standard tool for CNS tumor diagnosis and evaluation [14]. Furthermore, improvements in neurosurgical techniques have occurred during the past two decades, including stereotactic surgery, Cavitron Ultrasonic Surgical Aspirator and so on. Childhood cancer survival research from Osaka prefecture in Japan reported a slight increase in 5-year survival [1]. Incidence trends were not evaluated in this study. Data from the population-based cancer registry of Hokkaido prefecture in Japan indicated that the incidence of childhood brain tumors has been increasing, though the cause is unknown [15]. Other studies conducted in developed countries reported a significant increase in childhood CNS tumor incidence [10, 12, 16–21]. This has been explained by changes in detection and/or reports of childhood CNS tumors [22]. Because magnetic resonance imaging became ubiquitous at tertiary pediatric centers in the mid-1980s, it is likely to have increased the rate of detection; however, in the current study, the mortality rate of childhood CNS tumors in Japan was low and constant since the 1980s, and no significant increase in the number of deaths occurred in the middle of the 1980s to support the suggestion that the incidence increase was due to improved diagnostic techniques, if this increase really exists in Japan, and it seems unlikely to explain the long-term continued leveling off of mortality. The etiology of childhood CNS tumors remains largely unknown. Environmental factors are suggested to have a relationship with brain tumors. Further investigation in this field is needed to identify the incidence trends and reasonable explanations for these trends in Japan.

A previous childhood cancer mortality study in Japan presented data up to 1998. Furthermore, trend analysis was according to the correlation coefficient between the mortality rate and death year. Our analysis provides an updated mortality rate and reliable time trend analysis. In general, the mortality trends observed in other developed countries were compatible with Japan, although some differences were apparent. For example, a decrease in mortality during 0–14 years was observed in leukemia in the United States, Canada, Italy, New Zealand and Japan; however, the mortality rate from CNS tumors has decreased in the United States, Canada, UK and Italy in recent two decades. No evidence of decline appeared during 1980-2006 in Japan. For lymphoma, the decline occurred relatively late in Japan, compared with a significant decline without a leveling off period in the United States, Canada, Italy and UK. There is no simple explanation for these trend disparities. It is possible that the distribution of the histology pattern is markedly different among different countries, even in the same diagnostic group. The possible causes for these disparities in the childhood cancer death rate (e.g. late diagnosis, poor treatment quality, lack of health insurance and difficulty in accessing health care) need to be studied further.

A high mortality rate was observed in Kochi prefecture in boys and Tokushima and Kagoshima prefectures in girls. As mentioned above, the geographic disparity might be due to differences in cancer incidence and survival in different regions. Studies of the relationship between social class and childhood cancer have not been consistent. Research from Brazil suggested that higher decreases in the mortality rate were observed in more developed regions, possibly reflecting better health care [23]. We did not perform a similar ecologic study here, because of the small number of death, and we could not even calculate mortality by subtype by prefecture. Further detailed individual-level study is needed to identify a more reasonable explanation for the mortality disparities in childhood cancer.

A few points should be borne in mind when interpreting these findings. Some stable trends in the present study, such as mortality in lymphoma, and malignant bone tumors in New Zealand are more difficult to explain because of the small absolute number and substantial random variation. Other limitations included the wide time span and changes in diagnostic capabilities during the study period, and we were not able to collect any information on social status, employment of individuals and other genetic, environmental factors that would have allowed us to analysis etiological hypotheses.

Despite these limitations, when considering the absence of a national cancer registry system in Japan, estimates of incidence may have their own limitations (for example, they may be significantly influenced by errors in diagnosis and classification); evaluation of death may be an alternate effective method to identify more population-based point estimates of

mortality from childhood cancer under these circumstances. Furthermore, the results presented here are based on 100% national coverage and provide an important baseline for monitoring the further progress against childhood cancer in Japan. Analysis of trends in national mortality rates over several decades may provide additional insight into the burden and impact of childhood cancer and suggest more targeted avenues for interventions that further delineate and ultimately reduce mortality from childhood cancer.

## conclusions

The present study provides updated figures and trends in childhood cancer mortality in Japan and other developed countries. This will help to estimate care needs and to plan interventions and the quantity of appropriate childhood cancer treatment. Comprehensive efforts designed to identify risk factors for childhood cancer, promote early detection and reduce morbidity and mortality are warranted.

## funding

Grant-in-Aid from the Ministry of Health, Labor, and Welfare of Japan (H19-GANRINSHO-IPPAN-012).

## acknowledgements

Author contributions: LY designed and carried out analyses and drafted the paper; DQ prepared data and made the figure; JF, and NS edited the paper and commented on the interpretation of the result. All authors read and approved the final draft of the paper. Competing interests: The authors have no competing interests.

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# Neuroblastoma Presenting With Dilated Cardiomyopathy

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We report an infant with neuroblastoma who presented with dilated cardiomyopathy. A 3-month-old girl presented with dilated cardiomyopathy diagnosed as stage III neuroblastoma. Since total resection was impossible, chemotherapy was started. Cardiomyopathy was normalized by improvement of neuroblastoma. The prompt improvement of cardiac function following treatment of neuroblastoma suggested that cardiomyopathy in this patient was caused by the increase in catecholamines secreted by neuroblastoma and that reduction in catecholamines by treatment of neuroblastoma led to improvement in the cardiomyopathy. Pediatr Blood Cancer 2008;50:391–392. © 2006 Wiley-Liss, Inc.

**Key words:** cardiomyopathy; infant; neuroblastoma

## INTRODUCTION

Neuroblastoma is a neurogenic tumor and one of the most common solid tumors in childhood. It produces catecholamines or its metabolites. Catecholamine-induced cardiomyopathy is known to occur with pheochromocytoma, but is very rarely associated with neuroblastoma. Here we report an infant with neuroblastoma who presented with dilated cardiomyopathy.

## CASE HISTORY

A 3-month-old female presented with a 3-day history of fever. Chest X-ray (Fig. 1) showed enlarged heart (cardiothoracic ratio: 0.60). She was persistently hypertensive (systolic 130 mm Hg, diastolic 80 mm Hg). Echocardiography demonstrated dilated left ventricle, with poor contractility (ejection fraction: 0.25), suggesting dilated cardiomyopathy. During echocardiography, an abdominal mass was detected. Abdominal computed tomographic (CT) scan showed a large retroperitoneal mass. CT scan also showed that the bilateral kidneys were well enhanced and there was no apparent obstruction of the renal vasculature. Laboratory data on admission showed increased urinary vanillylmandelic acid (VMA) and homovanillic acid (HVA). There were no metastatic regions detected on 3-meta-iodo benzylguanidine (MIBG) scintigraphy. Stage III neuroblastoma was suspected based on the criteria proposed by Evans et al. [1], but total resection was thought to be impossible because the mass involved the bilateral renal arteries and the celiac artery. Therefore, we started digoxin, diuretics, captopril, and amrinon (phosphodiesterase inhibitor) to control hypertension. After hypertension was controlled (106/78 mm Hg), biopsy was performed. Pathological examination suggested poorly differentiated type neuroblastoma, intermediate MKI. N-MYC was not amplified, the DNA index was 2.42, and the trk A expression was high.

After the biopsy, we started carvedilol for cardiomyopathy and chemotherapy. For the first course, we gave 20 mg/kg of cyclophosphamide (CPA) on day 1, 0.65 mg/kg of pirarubicin (THP) on day 3, and 7.5 mg/kg of carboplatin (CBDCA) on day 5. To avoid severe toxicity, the dose of first course was reduced by 50% from we usually gave for infant with neuroblastoma. After the first course of chemotherapy, the blood pressure improved to 90/64 mm Hg. So amrinon was tapered off. The cardiomyopathy was also improved following chemotherapy (ejection fraction improved to 50%, after first course of chemotherapy). For the second course, 75% dose of the ordinary regimen (30 mg/kg of CPA on day 1, 1 mg/kg of THP on day 3, and 11.25 mg/kg of CBDCA on day 5) was given. After the second course, the full dose of chemotherapy (40 mg/kg of CPA, 1.33 mg/kg of THP, 15 mg/kg of CBDCA) was administered; there were no adverse effects greater than grade 2 of common terminology criteria for adverse events (CTCAE) except for hematological toxicity. After five courses of chemotherapy, VMA and HVA normalized, hypertension resolved and cardiac function also normalized (ejection fraction 0.71, and there was no sign of cardiomegaly on chest X-ray). Atrial natriuretic peptide (ANP) and brain natriuretic peptide (BNP) had been elevated before treatment, but these values also normalized after chemotherapy (Table I). Total cumulative dose of pirarubicin was 6.98 mg/kg.

## DISCUSSION

The signs and symptoms of neuroblastoma are heterogeneous: fever, bone pain, diarrhea, and anemia. Catecholamine secretion by neuroblastoma is well known, but hypertension due to excess catecholamines from neuroblastoma is relatively rare, possibly because the storage mechanism of neuroblastoma is less efficient than pheochromocytoma, and it leads to increased intracellular breakdown, therefore resulting in releasing less active cathecolamine in circulation [2,3]. Cardiomyopathy caused by pheochromocytoma is a well-known complication. Excess catecholamine secreted from pheochromocytoma causes cardiac muscle damage and downregulation of adrenergic receptor, which result in cardiac failure. However, there have only been five reported cases of neuroblastoma (or ganglioneuroma) presenting with dilated cardiomyopathy [4–8]. All patients were <3 years of age, and four of these patients were hypertensive. Blood catecholamines were elevated in three cases and the levels of catecholamines were not indicated in two other reports. In all cases, cardiomyopathy was improved by treatment of the neuroblastoma and favorable outcome was achieved, similar to the present case.

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Received 17 April 2006; Accepted 28 August 2006


DOI 10.1002/pbc.21065
Published online 25 October 2006 in Wiley InterScience (www.interscience.wiley.com)

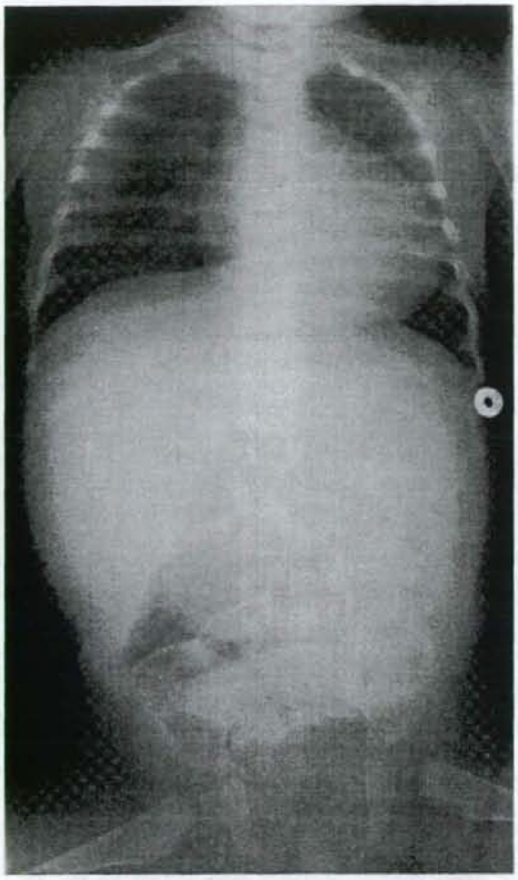

Fig. 1. Chest X-ray at admission showed an enlarged heart.

Dilated cardiomyopathy is a disease characterized by impairment of the systolic function with dilated ventricle. In most cases, the etiology of this disease is unknown and metabolic, infectious and immunological factors have been suggested. It has also been reported that not only hypertension due to excess catecholamine but also high catecholamine levels could cause direct cardiac muscle damage, resulting in cardiomyopathy. Since we could not rule out viral infection as a cause of cardiomyopathy, the association of cardiomyopathy with neuroblastoma in our case might be an unrelated association. However, the prompt improvement of cardiac function following treatment of neuroblastoma strongly suggests that cardiomyopathy in this patient was caused by neuroblastoma.

TABLE I. Laboratory Data Before and After Treatment

| | At admission | After 3rd course of chemothrapy | After 6th course of chemothrapy |
|---|---|---|---|
| VMA | 371 | 45.6 | 12.4 |
| HVA | 159 | 41.2 | 21.1 |
| ANP | 1093 | 72.1 | 93.9 |
| BNP | 1052 | 0.0 | 0.0 |
| EF | 25 | 51 | 71 |

VMA, vanillylmandelic acid (microgram/mgCre); HVA, homovanillic acid (microgram/mgCre); ANP, atrial natriuretic peptide (pg/ml); BNP, brain natriuretic peptide (pg/ml); EF, ejection fraction (%).

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# Effectiveness of screening for neuroblastoma at 6 months of age: a retrospective population-based cohort study

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## Summary

**Background** In Japan, a nationwide programme between 1984 and 2003 screened all infants for urinary catecholamine metabolites as a marker for neuroblastoma. Before 1989, this was done by qualitative spot tests for vanillylmandelic acid in urine, and subsequently by quantitative assay with high-performance liquid chromatography (HPLC). However, the Japanese government stopped the mass-screening programme in 2003, after reports that it did not reduce mortality due to neuroblastoma. We aimed to assess the effectiveness of the programme, by comparing the rates of incidence and mortality from neuroblastomas diagnosed before 6 years of age in three cohorts.

**Methods** We did a retrospective population-based cohort study on all children born between 1980 and 1998, except for a 2-year period from 1984. We divided these 22 289 695 children into three cohorts: children born before screening in 1980–83 (n=6 130 423); those born during qualitative screening in 1986–89 (n=5 290 412); and those born during quantitative screening 1990–98 (n=10 868 860). We used databases from hospitals, screening centres, and national cancer registries. Cases of neuroblastoma were followed up for a mean of 78·7 months.

**Findings** 21·56 cases of neuroblastoma per 100 000 births over 72 months were identified in the qualitatively screened group (relative risk [RR] 1·87, 95% CI 1·66–2·10), and 29·80 cases per 100 000 births over 72 months in the quantitatively screened group (RR 2·58, 2·33–2·86). The cumulative incidence of neuroblastoma in the prescreening cohort (11·56 cases per 100 000 births over 72 months) was lower than that in other cohorts ($p<0·0001$ for all comparisons), but more neuroblastomas were diagnosed after 24 months of age in this cohort ($p=0·0002$ for qualitative screening *vs* prescreening, $p<0·0001$ for quantitative screening *vs* prescreening). Cumulative mortality was lower in the qualititative screening (3·90 cases per 100 000 livebirths over 72 months) and quantitative screening cohorts (2·83 cases) than in the prescreening cohort (5·38 cases). Compared with the prescreening cohort, the relative risk of mortality was 0·73 (95% CI 0·58–0·90) for qualitative screening, and 0·53 (0·42–0·63) for quantitative screening. Mortality rates for both the qualitative and quantitative screening groups were lower than were those for the prescreening cohort ($p=0·0041$ for prescreening *vs* qualitative screening, $p<0·0001$ for prescreening *vs* quantitative screening).

**Interpretation** More infantile neuroblastomas were recorded in children who were screened for neuroblastoma at 6 months of age than in those who were not. The mortality rate from neuroblastoma in children who were screened at 6 months was lower than that in the prescreening cohort, especially in children screened by quantitative HPLC. Any new screening programme should aim to decrease mortality, but also to minimise overdiagnosis of tumours with favourable prognoses (eg, by screening children at 18 months).

**Funding** Japanese Ministry of Health, Labour, and Welfare.

*Lancet* 2008; 371: 1173–80

See Comment page 1142

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## Introduction

Neuroblastoma is one of the most common extracranial solid tumours in childhood. It has been shown to affect one in 7000 children in a non-screening period and to account for about 15% of cancer mortality in children.[1,2] Its prognosis depends on the age and stage of disease at diagnosis. Localised tumours that are diagnosed before 1 year of age have a favourable prognosis, whereas cure of disseminated tumours in children older than 1 year remains difficult despite recent advances in treatment. Since most neuroblastomas produce catecholamines, their metabolites—vanillylmandelic acid and homovanillic acid—can be measured in urine samples to allow early detection of preclinical tumours in infancy.[3,4] Therefore, mass-screening tests for neuroblastoma at 6 months of age were initiated in Kyoto in 1973, and launched nationwide across Japan in 1984–85.[5] Mass screening was also introduced in other countries.[6–10] Japan's programme initially used a qualitative test for vanillylmandelic acid in urine, but in 1990 this test was replaced by quantitative measurements with high-performance liquid chromatography (HPLC).[4,11,12]

However, the Japanese mass-screening programme tended to overdiagnose localised tumours with favourable prognoses, including occult tumours that spontaneously regressed or matured without ever becoming clinically overt.[13–16] In 2003, after two reports that screening of infants did not reduce mortality due to neuroblastoma,[17,18] the Japanese government decided to halt screening, on the condition that rates of incidence and mortality should be assessed. We aimed to assess the effectiveness of the Japanese 6-month screening programme, by investigating

| | Livebirths | Infants screened | Compliance rate | Livebirths in cohort | Screened in cohort |
|---|---|---|---|---|---|
| Prescreening cohort | | | | 6130423 | 0 |
| 1980 | 1576889 | .. | .. | | |
| 1981 | 1529455 | .. | .. | | |
| 1982 | 1515392 | .. | .. | | |
| 1983 | 1508687 | .. | .. | | |
| No cohort | | | | | |
| 1984 | 1489780 | 124870 | 8·4% | | |
| 1985 | 1431577 | 834536 | 58·3% | | |
| Qualitative screening cohort | | | | 5290412 | 4092759 (77·4%) |
| 1986 | 1382946 | 997643 | 72·1% | | |
| 1987 | 1346658 | 1024841 | 76·1% | | |
| 1988 | 1314006 | 1036740 | 78·9% | | |
| 1989 | 1246802 | 1033535 | 82·9% | | |
| Quantitative screening cohort | | | | 10868860 | 9342132 (86·0%) |
| 1990 | 1221585 | 1023005 | 83·7% | | |
| 1991 | 1223245 | 1026741 | 83·9% | | |
| 1992 | 1208989 | 1049905 | 86·8% | | |
| 1993 | 1188282 | 1042578 | 87·7% | | |
| 1994 | 1238328 | 1046953 | 84·5% | | |
| 1995 | 1187064 | 1043490 | 87·9% | | |
| 1996 | 1206555 | 1030179 | 85·4% | | |
| 1997 | 1191665 | 1037043 | 87·0% | | |
| 1998 | 1203147 | 1042238 | 86·6% | | |

*Table 1:* Number of livebirths and compliance with screening objectives throughout Japan (1980–98)

*Panel:* **International Neuroblastoma Staging System**

**Stage 1**
Localised tumour with complete gross excision, with or without microscopic residual disease

**Stage 2A**
Localised tumour with incomplete gross resection

**Stage 2B**
Localised tumour with or without complete gross excision, with ipsilateral lymph nodes positive for tumour (enlarged contralateral lymph nodes must be negative microscopically)

**Stage 3**
Unresectable unilateral tumour infiltrating across the midline, with or without regional lymph node involvement; localised unilateral tumour with contralateral regional lymph node involvement; or midline tumour with bilateral extension by infiltration (unresectable) or lymph node involvement

**Stage 4**
Any primary tumour with dissemination to distant lymph nodes, bone, bone marrow, liver, and other organs

**Stage 4S**
Localised primary tumour (as defined for stage 1, 2A, or 2B) in a patient younger than 1 year, with dissemination limited to skin, liver, or bone marrow (marrow involvement should be <10%)

the incidence of neuroblastoma diagnosed in children younger than 6 years of age, and the associated mortality rates.

## Methods

### Study participants

We analysed all livebirths between 1980 and 1998 in Japan, excluding those in 1984–85 when compliance with neuroblastoma screening was still low (table 1). We divided these children into three cohorts: children born between 1980 and 1983, when few infants were screened (n=6 130 423); children born between 1986 and 1989, during nationwide qualitative screening (n=5 290 412); and children born between 1990 and 1998, after introduction of the quantitative HPLC screening method (n=10 868 860). The total population included in this study was 22 289 695.

At the social health check-up received by all infants in Japan at the age of 3 months, public-health nurses explained the neuroblastoma screening programme to the parents. Parents were asked to collect urine samples at 6 months from their infants on filter paper or in small bottles and post them to screening centres. From 1984 until 1989, qualitative tests (spot or dip) for vanillylmandelic acid in urine were used. Quantitative HPLC analysis of urinary vanillylmandelic acid and homovanillic acid, with normalisation to urinary creatinine concentrations, was introduced around 1990 across most of Japan, in 67 screening centres, with a single quality control centre.

The positive criterion for neuroblastoma was that vanillylmandelic acid or homovanillic acid was more than three standard deviations above the mean concentration, in μg/mg of creatinine. Infants with positive screening results were reassessed, and urinary tests were repeated. A thorough examination was required for any child who showed positive a second time.

The designated hospitals in each prefecture or region examined these cases for uniform neuroblastoma diagnosis, staging, treatment, and follow-up. For detection of neuroblastoma, the treating hospitals used diagnostic imaging with echography, chest radiography, and CT scan or MRI.

Patients were classified according to the Evans staging system or International Neuroblastoma Staging System (INSS; panel).[19,20] Patients of any age who had stage 1 or 2 disease, and those younger than 12 months with stage 3 or 4S disease were given either surgery or surgery with chemotherapy. Patients aged 12 months or older with stage 3 and any patients with stage 4 disease were treated according to the protocol by the Japanese Neuroblastoma Study Group.[21] The proportion of patients with stage 4 disease who underwent bone marrow transplantation after myeloablative chemotherapy increased gradually after 1985.

### Data gathering

Data for patients who were born between 1980 and 1998 and in whom neuroblastoma was diagnosed were obtained

from the cancer registries of the Japanese Society of Paediatric Surgeons and the Japanese Society of Paediatric Oncology. Both of these registries were launched before 1980, and enrolled children with neuroblastoma directly from the hospitals where they had been treated, independently of the Japanese mass-screening programme. In both registries, all cases were followed up for at least 5 years, and were resurveyed 5 years after enrolment. Moreover, we cross-referenced the database against that of the Japanese Infantile Neuroblastoma Cooperative Study Group. Use of these databases for this study was approved by our consortium, by the ethics committees of each of the societies, and by Hiroshima University. We assembled the new database as a single registry for each case, with careful checks for duplicates. For the prescreening and qualitative screening cohorts (1980–89), we asked major hospitals nationwide to supply clinical data, under informed consent; we obtained such data for 1203 cases.

In Japan, residency and death registration is required by law, and the registries are complete. Information about the cause of each death was supplemented from death certificate files provided by the Ministry of Health, Labour, and Welfare. We matched the cases of death in our database to those in the government death certificate files according to birthdate, sex, address, date of death, cause of death, underlying diseases, and day of surgery or autopsy. Matching showed that 527 of the 843 deaths due to neuroblastoma (62·5%) were registered in our database; we used this calculated registration rate to adjust data for our population-based analysis. Between 1980 and 1983, our database had 206/329 deaths; between 1986 and 1989 it had 129/201 deaths; and between 1990 and 1998 it had 192/313 deaths. The feasibility of this ratio was supported by matching cases diagnosed between 1998 and 2000 with those in the database of the Research Project for Children's Specific Chronic Diseases, which started from 1998.

To calculate the specificity and sensitivity of the Japanese HPLC mass-screening programme, screening results were collected from the databases of 50 of the 67 screening centres in the Japanese Society for Mass Screening, which examined 2718259 (65·5%) of 4152950 infants screened between 1995 and 1998.[12]

Since we used a historical cohort as a control, we assessed differences in treatment between the three eras by comparing the numbers of cases who underwent current protocols including myeloablative therapy and the overall survival curves of cases classified as INSS 4 cases who were diagnosed at later than 1 year old (panel).

### Statistical analysis

We estimated the cumulative incidence of neuroblastoma and associated mortality rates per 100000 births diagnosed at younger than 72 months of age by use of the calculated registration rate (62·5%). We compared these rates after applying variance stabilisation with arcsine transformation to assess significance for a large number of comparisons. We did not adjust for multiple comparisons because these incidence rates and mortality rates were mainly compared with the prescreening cohort as control. We used two-sided tests and log-rank tests as appropriate. Two-sided p values of less than 0·05 were regarded as significant.

| | Cases | Cumulative incidence* | Relative risk (95% CI) | p† |
|---|---|---|---|---|
| **Prescreening cohort** | | | | |
| Total neuroblastoma | 443 | 11·56 | 1·00 | – |
| Cases diagnosed at 1–5 months | 60 | 1·57 | 1·00 | – |
| Cases diagnosed at 6–72 months | 383 | 10·00 | 1·00 | – |
| **Qualitative screening cohort** | | | | |
| Total neuroblastoma | 713 | 21·56 | 1·87 (1·66–2·10) | <0·0001 |
| Cases diagnosed at 1–5 months | 71 | 1·37 | 1·37 (0·97–1·94) | 0·071 |
| Cases diagnosed at 6–72 months | 642 | 19·42 | 1·94 (1·71–2·21) | <0·0001 |
| Cases in screened children | 554‡ | 21·66 | 2·17 (1·90–2·47) | <0·0001 |
| Cases in unscreened children | 88 | 11·76 | 1·18 (0·92–1·47) | 0·14 |
| **Quantitative screening cohort** | | | | |
| Total neuroblastoma | 2025 | 29·80 | 2·58 (2·33–2·86) | <0·0001 |
| Cases diagnosed at 1–5 months | 135 | 1·99 | 1·27 (0·94–1·74) | 0·13 |
| Cases diagnosed at 6–72 months | 1890 | 27·82 | 2·78 (2·50–3·11) | <0·0001 |
| Cases in screened children | 1781§ | 30·50 | 3·05 (2·74–3·41) | <0·0001 |
| Cases in unscreened children | 109 | 11·42 | 1·14 (0·91–1·40) | 0·21 |

Data are number of cases, or cumulative incidence per 100 000 births until 72 months of age, unless otherwise specified. *Rates calculated from the registration rate of the database. †Arcsine transformation was used to assess p values for difference against the prescreening cohort. ‡430 cases of neuroblastoma were detected by positive results from qualitative (VMA spot) screening. 124 were false negative results. §1537 cases were detected by positive results from quantitative (HPLC) screening. 244 were false negative results.

*Table 2:* **Cases of neuroblastoma diagnosed at younger than 72 months of age and rates of incidence for the three cohorts**

### Role of the funding source

The sponsor of the study had no role in study design, data collection, data analysis, data interpretation, or writing of the report. The corresponding author had full access to all the data in the study and had final responsibility for the decision to submit for publication.

## Results

In the three cohorts, with 22289695 children, 3181 children with cases of neuroblastoma that were diagnosed at younger than 72 months of age were registered (table 2). Of these cases, 266 were diagnosed at younger than 6 months and 2915 were diagnosed between 6 and 72 months of age. Compliance rates for quantitative screening were higher than were those for qualitative screening (table 1). 430 neuroblastomas were detected and registered by positive results from qualitative screening, and 1537 by quantitative screening (table 2). Nearly all tumours in both screened cohorts were detected between 6 and 16 months of age (median 8 months). The exceptions were two of the 430 cases in the qualitative screening cohort (which were detected at 19 and 28 months of age) and 29 of the 1537 cases in the quantitative screening cohort. False negative results from screening were registered between 7 and 72 months of age (median

| | Cases | Cumulative incidence* | Relative risk (95% CI) | p† |
|---|---|---|---|---|
| **Prescreening** | | | | |
| Total neuroblastoma | 383 | 10·00 | 1·00 | |
| Stages 1–3 | 153 | 3·99 | 1·00 | |
| Stage 4 | 200 | 5·21 | 1·00 | |
| Stage 4S | 8 | 0·21 | 1·00 | |
| **Qualitative screening** | | | | |
| Total neuroblastoma | 642 | 19·42 | 1·52 (1·35–1·71) | <0·0001 |
| Screened children | 554 [430]‡ | 21·66 | 1·70 (1·50–1·92) | <0·0001 |
| Unscreened children | 88 | 11·76 | 1·18 (0·92–1·42) | 0·14 |
| Stages 1–3 | 439 | 13·28 | 3·32 (2·78–4·01) | <0·0001 |
| Screened children | 393 [355]‡ | 15·36 | 3·85 (3·20–4·66) | <0·0001 |
| Unscreened children | 46 | 6·14 | 1·54 (1·69–1·38) | 0·0098 |
| Stage 4 | 155 | 4·69 | 0·90 (0·73–1·11) | 0·31 |
| Screened children | 116 [35]‡ | 3·51 | 0·87 (0·69–1·09) | 0·21 |
| Unscreened children | 39 | 5·21 | 1·00 (0·69–1·38) | 0·99 |
| Stage 4S | 26 | 0·78 | 3·77 (1·89–9·36) | 0·00042 |
| Screened children | 25 [24]‡ | 4·68 | 4·68 (2·24—11·7) | <0·0001 |
| Unscreened children | 1 | 0·13 | 0·64 (0·00–3·17) | 0·67 |
| Unknown | 22 | | | |
| **Quantitative screening** | | | | |
| Total neuroblastoma | 1890 | 27·82 | 2·18 (1·98–2·41) | <0·0001 |
| Screened children | 1781 [1537]‡ | 30·50 | 3·05 (2·74–3·41) | <0·0001 |
| Unscreened children | 109 | 11·42 | 1·14 (0·91–1·40) | 0·21 |
| Stages 1–3 | 1478 | 21·76 | 5·44 (4·63–6·47) | <0·0001 |
| Screened children | 1434 [1346]‡ | 24·56 | 6·12 (5·21–7·28) | <0·0001 |
| Unscreened children | 44 | 4·61 | 1·15 (0·81–1·58) | 0·42 |
| Stage 4 | 290 | 4·27 | 0·82 (0·69–0·98) | 0·028 |
| Screened children | 230 [76]‡ | 3·94 | 0·75 (0·62–0·91) | 0·0030 |
| Unscreened children | 60 | 6·29 | 1·20 (0·89–1·58) | 0·22 |
| Stage 4S | 101 | 1·49 | 7·11 (3·79–16·9) | <0·0001 |
| Screened children | 97 [97]‡ | 1·66 | 7·92 (4·21–18·9) | <0·0001 |
| Unscreened children | 4 | 0·42 | 1·99 (0·48–6·29) | 0·25 |
| Unknown | 21 | | | |

Data are number of cases or cumulative incidence per 100 000 births, unless otherwise specified. *Cumulative incidence between 6 and 72 months of age. †Arcsine transformation was used to assess significance. p values were calculated against the prescreening cohort with the observed data. ‡Among the neuroblastomas in screened children, case numbers were detected by positive screening results.

*Table 3:* **Stages of neuroblastoma diagnosed between 6 and 72 months of age in the three cohorts**

28·5 months with qualitative screening and 30 months with quantitative screening).

Table 2 shows that in the three cohorts, 443 neuroblastomas were diagnosed before 72 months in the prescreening cohort, and 713 and 2025 were detected in the quantitatively and qualitatively screened cohorts, respectively. The 227 survivors in the prescreening cohort were followed up for a mean duration of 89·3 (SD 32·4, median 79) months; 548 survivors in the qualitative screening cohort were followed up for 88·0 (22·1, 85) months, and 1833 in the quantitative screening cohort for 69·4 (22·2, 72) months. The overall mean follow-up was 78·7 (20·4, 73) months. The 206 children in the prescreening cohort who died, survived for a mean of 16·3 (14·5, 13) months, the 129 in the qualitative screening group survived for 24·5 (20·3, 17) months, and the 192 in the quantitative screening group survived for 23·5 (20·5, 19) months. Since 517 of the 527 children who died (98%) did so before 69 months after diagnosis, these durations of follow-up were regarded as sufficient.

The rates of neuroblastoma detected in children in the screened cohorts were higher than were those detected in the prescreening cohort (p<0·0001, table 2). In the prescreening cohort, 11·56 cases per 100 000 births were detected before 72 months of age, compared with 21·56 cases in the qualitatively screened and 29·80 cases in the quantitatively screened cohorts. Furthermore, more cases per 100 000 livebirths were recorded in the quantitatively screened cohort than in the qualitatively screened cohort (p<0·0001).

Similar rates of cases were diagnosed at younger than 6 months of age in the three cohorts (table 2). The cumulative incidence of tumours diagnosed between 6 and 72 months of age was 10·00 cases per 100 000 births in the prescreening cohort, which was lower than both 19·42 in the qualitative screening cohort (p<0·0001) and 27·82 in the quantitative screening (p<0·0001). Incidence rates of clinically diagnosed tumours between 6 and 72 months in children in the two screened cohorts who were not screened were similar to those in the prescreening cohort.

To estimate the specificity and sensitivity of the quantitative HPLC screening, data from 50 representative centres were analysed against the report from the Japanese Society for Mass Screening.[22] 3302 of 2718 258 screened infants had two positive results on screening, and received a thorough examination; of those, 698 cases of neuroblastoma were detected by screening. We estimated from the incidence data (30·50 cases per 100 000 livebirths) that 829 cases of neuroblastoma would be diagnosed between 6 and 72 months old (table 2) in HPLC-screened children, with 131 false negative cases. Thus, sensitivity of this type of screening was 84·2%; specificity was 99·9%; and the positive predictive value, including possible overdiagnosis of non-progressive tumours was 21·1%.

Localised tumours, classified as stages 1–3 and 4S, were recorded more often in both the screened cohorts than in the prescreening cohort (p<0·0001 for all comparisons, table 3). Fewer disseminated stage 4 tumours were found in the screened subset of the quantitatively screened cohort than in the prescreening cohort (p=0·003). The rates of tumours in the unscreened subsets of the two screened cohorts did not differ from the rate in the prescreening cohort.

Table 4 shows numbers of neuroblastoma patients and incidences according to stage and age at diagnosis for the three cohorts. In infants younger than 6 months of age, the incidence of total tumours for the three cohorts did not differ. Between 6 and 12 months of age, the incidence was higher in both screened cohorts than in the prescreening cohort (p<0·0001 for both

comparisons). Incidences of all tumours diagnosed between 12 and 23 months of age were similar in the three cohorts, except that more localised tumours were detected at 12–17 months of age in the quantitatively screened cohort than in the prescreening cohort ($p<0.0001$). Some children who had positive results after quantitative screening were diagnosed at older than 1 year because consultation was delayed or diagnosis was difficult.

More tumours were diagnosed between 24 and 72 months in the prescreening cohort (5·72 cases per 100 000 births) than in the qualitatively screened (3·78, $p=0.00020$) or the quantitatively screened cohort (2·81, $p<0.0001$). Moreover, fewer neuroblastomas were diagnosed in the quantitatively screened cohort than in the qualitatively screened cohort at this age ($p=0.0096$). In particular, screening diagnosed fewer stage 4 tumours between 24 and 72 months than those detected in the prescreening cohort (table 4, $p=0.012$ for prescreening *vs* qualitatively screened and $p<0.0001$ for prescreening *vs* quantitatively screened, $p=0.17$ for qualitatively *vs* quantitatively screened).

On the other hand, more stage 4S tumours were diagnosed at 6–11 months of age in the screened cohorts than in the prescreening cohort ($p<0.0001$ in both). As a result, in all neuroblastomas diagnosed between 6 and 72 months of age, stage 4 tumours did not differ in the qualitatively screened cohort, but were lower in the quantitatively screened cohort (table 3, $p=0.21$ for prescreening *vs* qualitatively screened and $p=0.028$ for prescreening *vs* quantitatively screened).

Of the 3181 cases whose tumours were diagnosed before 72 months old in the three cohorts, 527 patients died. Cumulative mortality rates are summarised in table 5. Mortality rates were 3·90 in the qualitatively screened and 2·83 in the quantitatively screened cohorts; the rate in the prescreening cohort was higher (5·38) ($p=0.0041$ and $p<0.0001$, respectively). Moreover, the mortality rate in the quantitatively screened cohort was lower than that in the qualitatively screened cohort ($p=0.0043$).

Stratifying by age, mortality rates in cases diagnosed at younger than 6 months of age did not differ in the three cohorts (0·37 in the prescreening cohort, 0·33 for qualitative screening, and 0·26 for quantitative screening). In cases diagnosed between 6 and 72 months of age, mortality rates in the qualitative and quantitative screening cohorts were lower than that in the prescreening cohort ($p=0.0034$ and $p<0.0001$, respectively). The mortality rate in the quantitative screening cohort was also lower than that of the qualitative screening cohort ($p=0.0051$). Mortality rates in patients with tumours diagnosed at 6–17 months of age were similar in the three groups (0·86 for 33 patients in the prescreening cohort, 0·97 for 32 patients who had qualitative screening, and 0·90 for the 61 patients who had quantitative screening). Moreover, mortality rates in patients with tumours diagnosed between 6 and 72 months of age in the prescreening cohort (5·01) were similar to those in the unscreened subgroups in the qualitative screening and quantitative screening cohorts (4·81 and 4·79, respectively), indicating that more recent treatment regimen did not improve

| | Prescreening | | | Qualitative screening | | | | Quantitative screening | | | |
|---|---|---|---|---|---|---|---|---|---|---|---|
| | Cases | Cumulative incidence* | Relative risk (reference) | Cases | Cumulative incidence* | Relative risk (95% CI) | p† | Cases | Cumulative incidence* | Relative risk (95% CI) | p† |
| 0–5 months at diagnosis | 60 | 1·57 | 1·00 | 71 | 2·15 | 1·37 (0·97–1·94) | 0·071 | 135 | 1·99 | 1·27 (0·94–1·74) | 0·13 |
| Stages 1–3 | 24 | 0·63 | 1·00 | 35 | 1·06 | 1·69 (1·01–2·90) | 0·085 | 78 | 1·15 | 1·83 (1·19–2·99) | 0·0085 |
| Stage 4 | 16 | 0·42 | 1·00 | 14 | 0·42 | 1·01 (0·48–2·09) | 0·97 | 21 | 0·31 | 0·74 (0·39–1·46) | 0·36 |
| Stage 4S | 19 | 0·50 | 1·00 | 17 | 0·51 | 1·04 (0·53–2·01) | 0·91 | 40 | 0·59 | 1·19 (0·70–2·12) | 0·54 |
| 6–11 months at diagnosis | 66 | 1·72 | 1·00 | 442 | 13·37 | 7·76 (6·06–10·19) | <0·0001 | 1460 | 21·49 | 12·47 (9·87–16·20) | <0·0001 |
| Stages 1–3 | 35 | 0·91 | 1·00 | 357 | 10·80 | 11·82 (8·54–17·20) | <0·0001 | 1254 | 18·46 | 20·05 (14·67–28·90) | <0·0001 |
| Stage 4 | 20 | 0·52 | 1·00 | 44 | 1·33 | 2·49 (1·49–4·38) | 0·00032 | 86 | 1·26 | 2·42 (1·54–4·12) | 0·00023 |
| Stage 4S | 8 | 0·21 | 1·00 | 26 | 0·79 | 3·77 (1·81–9·36) | 0·00042 | 101 | 1·49 | 7·11 (3·79–16·93) | <0·0001 |
| 12–17 months at diagnosis | 48 | 1·25 | 1·00 | 43 | 1·30 | 1·04 (0·68–1·57) | 0·86 | 165 | 2·43 | 1·93 (1·42–2·71) | <0·0001 |
| Stages 1–3 | 20 | 0·52 | 1·00 | 28 | 0·85 | 1·62 (0·92–2·95) | 0·095 | 121 | 1·78 | 2·20 (3·41–5·74) | <0·0001 |
| Stage 4 | 27 | 0·70 | 1·00 | 14 | 0·42 | 0·60 (0·30–1·12) | 0·12 | 43 | 0·63 | 0·90 (0·56–1·48) | 0·66 |
| 18–23 months at diagnosis | 50 | 1·30 | 1·00 | 32 | 0·97 | 0·74 (0·47–1·15) | 0·19 | 74 | 1·09 | 0·83 (0·59–1·20) | 0·32 |
| Stages 1–3 | 22 | 0·57 | 1·00 | 9 | 0·27 | 0·47 (0·20–0·99) | 0·054 | 38 | 0·56 | 0·97 (0·58–1·69) | 0·92 |
| Stage 4 | 28 | 0·73 | 1·00 | 22 | 0·67 | 0·91 (0·51–1·59) | 0·74 | 35 | 0·51 | 0·70 (0·43–1·17) | 0·16 |
| 24–72 months at diagnosis | 219 | 5·72 | 1·00 | 125 | 3·78 | 0·66 (0·53–0·82) | 0·00020 | 191 | 2·81 | 0·49 (0·40–0·60) | <0·0001 |
| Stages 1–3 | 76 | 1·98 | 1·00 | 45 | 1·36 | 0·69 (0·47–0·99) | 0·094 | 65 | 0·96 | 0·33 (0·23–0·48) | <0·0001 |
| Stage 4 | 125 | 3·26 | 1·00 | 75 | 2·27 | 0·70 (0·52–0·92) | 0·012 | 126 | 1·87 | 0·57 (0·44–0·73) | <0·0001 |
| Total | 443 | 11·56 | | 713 | 21·56 | 1·87 (1·66–2·10) | <0·0001 | 2025 | 29·80 | 2·58 (2·33–2·86) | <0·0001 |

Data are number of cases or cumulative incidence per 100 000 births, unless otherwise specified. *Cumulative incidence rate until 72 months of age per 100 000 births was adjusted with the registration rate of the database. †Arcsine transformation was used to assess significance. p values were calculated against the prescreening cohort with observed data.

*Table 4:* **Distribution of cases of neuroblastoma diagnosed at younger than 72 months of age according to age at diagnosis and stage in the three cohorts**

mortality rates in those who had not undergone the screening. These findings suggested that the rate of death was not affected by a lead time effect on tumours with unfavourable prognosis due to screening. Moreover, in the screened subgroups of the qualitatively and quantitatively screened cohorts, mortality rates of cases diagnosed between 6 and 72 months of age were 3·21 and 2·19 (p=0·00056 and p<0·0001, respectively).

In the stage 4 cases that were diagnosed at later than 1 year, 24/199 patients in the prescreening cohort, 27/117 in the qualititatively screened cohort, and 113/199 in the quantitatively screened cohorts were treated with current protocols, which included myeloablative therapy (p=0·010 for prescreening *vs* qualitative screening, p<0·0001 for both comparisons with quantitative screening). However, the overall survival curves of these patients showed that survival rates 48 months after diagnosis were similar, whereas survival rates between 24 and 48 months differed (figure).

## Discussion

According to two well-known reports[17,18] screening did not reduce mortality from neuroblastoma, and was associated with a high incidence of early-stage disease. Previous reports from Japan had noted that the incidence of early-stage neuroblastoma was higher in screened infants, but that mortality was lower.[11,23] We analysed a nationwide Japanese cohort, with more than 13 million screened infants, and showed that mortality was lower in those who were screened than in those born before the screening programme was introduced.

Divergence in other reports could derive from several factors, including age at screening, screening methodologies, compliance rates, and diagnostic activity or ability. In a study in Quebec, Canada, 476 654 children who were born between 1989 and 1994 were assessed by qualitative thin-layer chromatography, which might be less effective than the quantitative HPLC method.[6,11] This programme recorded 118 neuroblastoma cases, with 22 deaths.[17] These numbers might be too small to adequately assess a reduction in mortality rate. In a German study, 1475 773 children (61·2% of those who were born between 1994 and 1999) underwent screening at 1 year of age by HPLC.[7] Although this programme was larger than that in Quebec, the results did not support the usefulness of general screening for neuroblastoma.[18] In that study,[7] the incidence of neuroblastoma in the control cohort between 12 and 60 months of age was low, at 7·3 (compared with a generally reported incidence of about 10),[1,2] which would have affected the assessment of effectiveness.

In 1998, an international consensus conference concluded that screening in children younger than 7 months was not recommended, and emphasised the necessity for retrospective analysis of the Japanese screening programme to investigate the clinical importance of neuroblastoma screening.[24] Our whole-population data included more than 500 deaths. Therefore, the specificity and positive predictive value were higher for our quantitatively screened cohort than for those of the screening cohorts reported in other studies.[18,25,26] Although the screening meant that many overdiagnosed tumours were identified, cumulative mortality rates in children who were diagnosed at younger than 72 months of age were lower in both of the screened cohorts than in the prescreening cohort. According to several studies of the adoption of wait-and-see strategies[27,28] urinary vanillylmandelic acid and homovanillic acid concentrations fell to within the normal ranges by 18 months of age in almost all tumours that regressed. In a pilot study that assessed screening for 14-month-old children, many overdiagnosed cases were thought to have been detected.[29] To decrease overdiagnosis without sacrificing effectiveness, a pilot programme of neuroblastoma screening for 18-month-old children has been proposed and launched by several prefectural committees in Japan.

Since a historical cohort was used as a control, any decreases in mortality in the screened cohorts could potentially have been due to advances in treatment, espcially the widespread initiation of intensive treatment regimens with myeloablative therapy, rather than a direct effect of the screening programme. Although the

| | Prescreening cohort | | | Qualitative screening | | | | Quantitative screening | | | |
|---|---|---|---|---|---|---|---|---|---|---|---|
| | Cases | Mortality rate* | Relative risk (ref) | Cases | Mortality rate* | Relative risk (95% CI) | p† | Cases | Mortality rate* | Relative risk (95% CI) | p† |
| Total deceased cases | 206 | 5·38 | 1·00 | 129 | 3·90 | 0·73 (0·58–0·90) | 0·0041 | 192 | 2·83 | 0·53 (0·42–0·63) | <0·0001 |
| Deceased cases by neuroblastoma diagnosed at 0–5 months | 14 | 0·37 | 1·00 | 11 | 0·33 | 0·91 (0·40–2·01) | 0·82 | 18 | 0·26 | 0·73 (0·36–1·50) | 0·36 |
| Deceased cases by neuroblastoma diagnosed at 6–72 months | 192 | 5·01 | 1·00 | 118 | 3·57 | 0·71 (0·56–0·89) | 0·0034 | 174 | 2·56 | 0·51 (0·42–0·63) | <0·0001 |
| Deceased cases in screened children | – | – | – | 82‡ | 3·21 | 0·64 (0·49–0·82) | 0·00056 | 128‡ | 2·19 | 0·44 (0·35–0·54) | <0·0001 |
| Deceased cases in unscreened children | 192 | 5·01 | 1·00 | 36 | 4·81 | 0·96 (0·6–1·34) | 0·86 | 46 | 4·79 | 0·96 (0·68–1·30) | 0·82 |

Data are number of cases or cumulative mortality per 100 000 births, unless otherwise specified. *Cumulative mortality rate in children with neuroblastoma until 72 months of age per 100 000 births was adjusted with the registration rate of the database. †Arcsine transformation was used to assess significance. p values were calculated against prescreening cohort using the observed data. ‡Among the deceased cases in screened children, 12 children died of tumours diagnosed by positive results of qualitative (VMA spot) screening, and 20 of tumours diagnosed by quantitative (HPLC) screening.

*Table 5:* Mortality rates of neuroblastoma diagnosed at younger than 72 months of age for the three cohorts

proportion of stage 4 cases who underwent this intensive treatment was greater in the later cohorts, the long-term prognosis in stage 4 cases did not differ between the three cohorts, indicating that improvements in treatment did not contribute to the improved survival in the screened cohorts. The estimated incidence rates without screening in the present study were similar to those reported previously in Japanese restricted areas[15,16] and in Europe,[30] indicating that our database contains generally comparable data. Moreover, mortality rates were similar in the prescreening cohort and unscreened subgroups in the two screened cohorts.

Socioeconomic factors, such as poorer access to health care in unscreened groups, could also correlate with mortality rates in general. However, since Japanese mass-screening tests had been done free of charge and almost all costs of neuroblastoma treatment were covered by Japanese official insurance and subvention, socioeconomic factors were effectively controlled for in our study. A reduction in the underlying incidence of progressive disease over time could potentially have affected the mortality rate; however, the incidence rates of total cases and stage 4 cases in unscreened groups in the two screened cohorts did not decrease compared with the prescreening cohort. Thus, no obvious differences between the three cohorts—other than the intervention of screening—would affect mortality.

The cumulative mortality rate in the quantitatively screened cohort was lower than that in the qualititatively screened cohort (p=0·0043), suggesting that the method of quantitative measurement of urinary vanillylmandelic acid and homovanillic acid, corrected against creatinine, can identify cases of neuroblastoma more accurately than can the qualitative method.[12] Thus, the effect of screening for neuroblastoma should have been mostly heavily weighted in quantitatively screened cohort. In fact, the largest reduction in the incidence of advanced (stage 4) neuroblastoma detected at older than 2 years of age was in the quantitative screening cohort, which might have contributed to the reduction of mortality. Recently, Hisashige and co-workers[31] and Hayashi and co-workers[32] also reported low mortality rates in those screened with HPLC in a prospective cohort study.[31,32] The relative risk of death due to neuroblastoma in the screened cohort was 0·55 and the difference in cumulative mortality rate until 6 years of age between screened and unscreened cohorts was 27·7 per million.[31,32] These data are in agreement with our findings: relative risk was 0·53 (95% CI 0·42–0·63), and the difference in cumulative mortality rates was 26·0 per million in our study.

Since the natural incidence of neuroblastoma diagnosed at younger than 15 years old was estimated to be 10–18 per 100 000 children,[33,34] several reports have discussed overdiagnosis of clinically insignificant neuroblastoma because of screening efforts.[9,13–15,18,30] In this study, although we cannot distinguish cases of overdiagnosis from progressive diseases among the tumours detected by

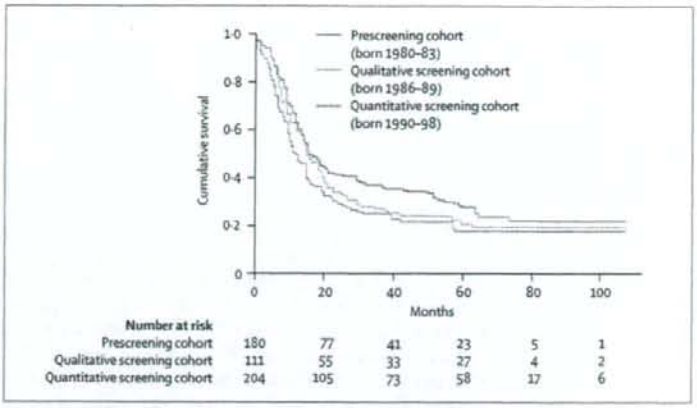


*Figure:* **Kaplan-Meier cumulative survival curves for patients with stage 4 neuroblastoma diagnosed at older than 1 year**
The overall survival rates of the patients in the quantitative screening cohort were higher in the 24–48 year range (p=0·027) but survival rate did not differ in the long term.

screening, comparison of incidences between screened and unscreened groups showed that 9·9 cases per 100 000 livebirths might have been overdiagnosed in the qualitatively screened cohort, compared with 19·1 cases in the quantitatively screened cohort. For tumours diagnosed between 6 and 72 months of age, more localised and stage 4S tumours were diagnosed in the two screened cohorts, whereas the incidence of stage 4 tumours did not differ in the qualitatively screened cohort, and were similar to those reported in the Quebec cohort.[4] On the other hand, in the quantitatively screened cohort, the incidence rate of stage 4 tumours decreased, despite the increase of infant stage 4 tumours, which usually have favourable outcomes. In this cohort, the decrease of these tumours diagnosed between 24 and 72 months of age could be related to mortality reduction.

The mortality rates of unscreened subgroups in older patients did not decrease during the two decades of the study, indicating that neuroblastoma treatment has not improved for cases of disseminated tumours in older patients. Thus, early diagnosis and prevention of dissemination for tumours with unfavourable prognosis, including screening, might be the most efficient means for improvement of the outcome of neuroblastoma. The usefulness of mass screening as a public-health service should be assessed not only by decrease in mortality, but also by factors such as potential overdiagnosis, the cost of screening, and the potential psychological burden on children and families.

In Japan, a sample for HPLC analysis costs about US$5 (700 yen). US$35 000 would be needed to detect one case by screening 7000 asymptomatic children. However, if this case were diagnosed at an early stage, the overall treatment cost might be less than US$10 000, whereas that for advanced neuroblastoma would be closer to US$50 000; therefore, one can argue for the cost-effectiveness of this

prevention programme. Thus, effective screening to detect tumours with unfavourable prognoses at early stages might reduce the cost of screening to within an acceptable range. The recommendation of mass screening for neuroblastoma as a public-health policy should not be made until several key issues are resolved. However, our results show that adequate treatments, including wait-and-see strategies for early stage neuroblastoma (eg, incidental or infant neuroblastomas), are necessary to improve the outcomes and help in understanding whether neuroblastomas with unfavourable prognoses arise from unfavourable tumours or not.

#### Contributors

EH, TI, TS, MF, KA, and MO participated in designing the study and statistical analysis. EH, TI, TS, MF, YH, FS, MS, SK, AY, HY, and TT also treated patients and obtained samples. EH, TI, TS, MF, and AY contributed to the conclusions of the paper. All authors have seen and approved the final version of this manuscript.

#### Conflict of interest statement

We declare that we have no conflict of interest.

#### Acknowledgments

This study was supported by Grants-in-Aid from the Ministry of Health, Labour, and Welfare of Japan (H16-KODOMO-IPPAN-012). We thank Minoru Hamasaki who reviewed the pathology of neuroblastoma tissue specimens; all contributing physicians, paediatric oncologists, and paediatric surgeons; the Committees on Tumour Registrations in the Japanese Society of Paediatric Surgeons and Japanese Society of Paediatric Oncology for the use of their neuroblastoma database; the Ministry of Internal Affairs and Communications and the Ministry of Health, Labour, and Welfare for approving the use of the death registration files; and Tada-aki Kato (scientific research by Grants-in-Aid from the Ministry of Health, Labour and Welfare of Japan) for providing us with the database of neuroblastoma in the registry of the Research Project for Children's Specific Chronic Diseases.

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Pediatr Surg Int (2008) 24:1095–1100
DOI 10.1007/s00383-008-2228-3

ORIGINAL ARTICLE

# Clinical implications of a slight increase in the gene dosage of *MYCN* in neuroblastoma determined using quantitative PCR

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Published online: 23 August 2008


**Abstract**
*Introduction* Recently, determining the *MYCN* status in neuroblastoma (NB) using the quantitative PCR (Q-PCR) and FISH instead of the Southern blotting (SB) has been recommended. In order to assess the implications of the gene dosage of *MYCN* in NB, the *MYCN* status was evaluated using Q-PCR on DNA extracted from small areas of NB specimens obtained using laser capture microdissection (LCM).
*Materials and methods* *MYCN* gene dosages (*MYCN/NAGK*) were determined in 63 primary NB block samples, as well as in 243 microdissected tissues from 63 samples using Q-PCR. In 23 of 63 cases, the *MYCN* gene status was evaluated using FISH.
*Results* Nine block samples with the amplification of *MYCN* based on SB showed a remarkable increase of the *MYCN* gene dosage using Q-PCR. Twelve of 54 block samples with no amplification of *MYCN* based on SB showed a slight increase of the *MYCN* gene dosage ($3.56 \geqq MYCN/NAGK > 1.84$), and 8 of these 12 cases were in the advanced stage. Among these 12 cases, 1 case had several LCM areas with a high copy number of *MYCN* and several LCM areas which showed no increase of *MYCN* gene. Another case showed a slight increase in the *MYCN* gene dosage ($3.65 \leqq MYCN/NAGK \leqq 4.82$) in all LCM areas. In addition, a large number of cells with the *MYCN* gain were found using FISH in the block sample. In 2 other cases of 12 cases, although no LCM areas showed an increased gene dosage of *MYCN*, a small number of cells with *MYCN* amplification were found using FISH were found in the block sample.
*Conclusion* 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 the *MYCN* amplification or a large number of cells with the *MYCN* gain, which are associated with the aggressive progression of NB.

**Keywords** Neuroblastoma · *MYCN* · Laser capture microdissection

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## Introduction

Neuroblastoma (NB) is the most common type of solid tumor in children. Amplification of the *MYCN* gene has been reported to be strongly associated with rapid tumor progression [1, 2]. An amplification of the *MYCN* gene occurs in approximately 25% of primary tumors, and it is known to be one of the most unfavorable prognostic factors in NB [3]. Therefore, for the treatment of NB, it is important to accurately identify the status of *MYCN* amplification. *MYCN* amplification of the whole tumor has been mainly analyzed by Southern blotting (SB) [4, 5], but this method is unable to detect intratumor heterogeneity. We previously reported that our findings for a highly sensitive analysis of *MYCN* amplification in NB based on the FISH and the quantitative PCR (Q-PCR) [6–8]. This combination is useful as a quick and accurate modality for evaluating for the status of *MYCN* amplification in NB. The

international NB risk group (INRG) committee of 2006 recommended using the Q-PCR and dual color FISH to determine *MYCN* status instead of SB. With FISH, individual cells with *MYCN* amplification are detected with the *MYCN* probe signals, but it is difficult to determine the copy number throughout the whole tumor with *MYCN* amplification. Q-PCR can accurately determine the copy number of the whole tumor. However, the significance of a slight increase in the gene dosage of *MYCN* detected by Q-PCR in NB is still unclear. Therefore, the implications of a slight increase of *MYCN* determined by Q-PCR were assessed by evaluating the *MYCN* status using Q-PCR on DNA extracted from small areas of NB specimens obtained using laser capture microdissection system (LCM).

## Materials and methods

### Clinical data of patients and biologic data of neuroblastoma samples

Patients with NB, evaluated at the Department of Pediatric Surgery Kyushu University, Fukuoka, Japan, were diagnosed and the tumor was staged according to the International Neuroblastoma staging system (INSS). Sixty-three NB frozen and paraffin-embedded samples were obtained from untreated patients with neuroblastoma. The patients included 39 males and 24 females, and 24 were stage 1 as INSS, 9 were stage 2, 9 were stage 3, 18 were stage 4, and 3 were stage 4S. Of the 54 patients with single copy MYCN detected by SB, 24 were stage 1, 9 were stage 2, 6 were stage 3, 12 were stage 4, and 3 were stage 4S. Of the 63 NB patients, 40 were diagnosed at younger than 12 months, 5 were between 12 and 18 months, and 18 were older than 18 months. Thirty-six patients were identified by the mass screening system in Japan at 6 months old. The *MYCN* gene amplification in these NB block samples were quantified using SB and Q-PCR. SB showed that 9 of 63 NB samples had more than 2 copies of the *MYCN* gene and 54 had a single copy of *MYCN* gene. In all 63 samples, LCM was used to obtain specimens from the paraffin-embedded tissue, and the *MYCN* gene dosages were quantified using the Q-PCR. In 23 of 63 cases, the *MYCN* gene status was evaluated using FISH.

### DNA extraction form the frozen block samples

Total DNA was extracted from frozen block samples of tumors by using proteinase K and phenol.

### Southern Blot analysis

The number of copies of the *MYCN* gene was determined by Souther Blot analysis as described previously [9]. Human placental DNA was used as the single copy number control tissue.

### DNA extraction method from small areas obtained by LCM from the paraffin-embedded samples

The tumor cells were identified microscopically and small areas in the tumor were laser captured from 7–10 μm paraffin sections mounted onto membrane slides using a laser captured microdissection system (LMD 6000, Leica). Two or more small areas were captured from each slide and a total 243 small areas were captured. DNA was extracted from samples using a PicoPure™ DNA Extraction Kit (Arcturus Engineering).

### Quantitative PCR (TaqMan)

The *N-acetylglucosamine kinase gene* (*NAGK*) was used as an internal control gene to determin the gene dosage [10]. The *NAGK* gene is located at 2p13 and it is located on the chromosome 2 as is the *MYCN* gene. *NAGK* gene is well separated from the *MYCN* amplicom [11]. The mean gene dosage of *MYCN* of 10 normal individual lymphocytes was $1.00 \pm 0.42$. In this study, a corrected *MYCN* gene dosage (*MYCN/NAGK*) of more than the mean + 2SD (1.84) was defined as an increase in the gene dosage of *MYCN*. The primers and the TaqMan probe for *MYCN* gene were: *MYCN* Forward, 5′-GTGCTCTCAATTCTCGCCT-3′; *MYCN* reverse, 5′-GATGGCCTAGAGGAGGGCT-3′; *MYCN* Probe, 5′-FAM-CACTAAAGTTCCTTCCACCCT CTCCT-TAMRA-3′. The primers and TaqMan probe for *NAGK* gene were: *NAGK* forward, 5′-TGGGCAGACA-CATCGTAGCA-3′; *NAGK* reverse, 5′-CACCTTCA CTCCCACCTCAAC-3′; and *NAGK* probe, 5′-VIC-TGTTGCCCGAGATTGACCCGGT-TAMRA-3′. Q-PCR was performed in a final volume of 30 μL, and each sample was evaluated in duplicate. Each reaction mixture contained 0.1 pmol/μL TaqMan probe, 0.2 pmol/μL each primer, 1× TaqMan PCR master mix, and extracted DNA. PCR amplification was started a 2 min incubation at 50°C, followed by a denaturation step of 10 min at 95°C, and then 40 cycles at 95°C for 15 s and 60°C for 1 min. The genes quantified using the ABI Prism 7700 Sequence Detection System (Applied Biosystems). Genomic DNA from normal lymphocyte cells from healthy donors was serially diluted to establish the calibration curve.

### FISH method

Fresh tumor samples were homogenized and suspended in potassium chloride. The sample was fixed in Carnoy's fixative (3:1 methanol/glacial acetic acid). The cells were denatured standard saline citrate (SSC) at 37°C for 30 min

and then were dehydrated in an ethanol series. The hybridization buffer, containing 10 ng aliquot of the MYCN gene probe (LSI N-MYC SO, Vysis) or MYCN gene and the alpha satellite region of human chromosome 2 probes (LSI N-MYC SG/CEP 2 SO DNA probe, Vysis) were denatured for 5 min at 75°C, chilled on ice, and then applied to a slide. Hybridization was performed overnight at 37°C. After the slides were washed in SSC and Triton, the nuclei were counterstained with 10 μL of DAPI containing 5 μL of antifade solution. The signals representing the *MYCN* gene and the alpha satellite region of chromosome 2 were counted in 100 cells on each slide. The images were photographed using a fluorescence microscope (Olympus, BX60, Tokyo, Japan) and Provia 400 (Fuji, ISO 400, Tokyo, Japan). The amplification of the *MYCN* gene was defined as a more than the 4-fold increase of *MYCN* signals in relation to the number of chromosomes 2 in a dual color probe, or a more than 8 MYCN signals in single color probe. Additional copies up to the 4-fold in the dual color probe, or 5 to 8 *MYCN* signals in single color probe was defined as *MYCN* gain cells. [12].

## Results

### Analysis of *MYCN* gene dosage using Q-PCR with DNA extracted the block samples

Figure 1 shows the correlation between *MYCN* gene dosage of 64 block samples based on SB and Q-PCR. All of 8 samples with more than 10 copies of the *MYCN* gene based on the SB showed a remarkable increase of the *MYCN* gene dosages ($MYCN/NAGK \geqq 10$) with Q-PCR. One sample with six copies of *MYCN* gene based on SB showed a *MYCN* gene dosage of 6.63 with Q-PCR. Of 54 samples with a single copy of *MYCN* detected by SB, 12 samples showed a slight increase in the gene dosage of *MYCN* ($3.56 \geqq MYCN/NAGK > 1.84$).

### Analysis of *MYCN* gene dosage using Q-PCR in specimens isolated by LCM

All eight samples with more than 10 copies of the *MYCN* gene detected by SB showed that the *MYCN* gene dosage of more than 10 (minimum value: 15.00) in small areas captured by LCM and there was no evidence of heterogeneity. One sample with six copies of *MYCN* detected by SB showed a range of *MYCN* gene dosage from 5.50 to 24.59. In this case, no analysis by FISH was available. Figure 2 shows the maximum and the minimum values of the *MYCN* gene dosage based on Q-PCR in several small areas obtained by LCM from 54 cases with a single copy of *MYCN* detected by SB in the block sample. All 42 cases

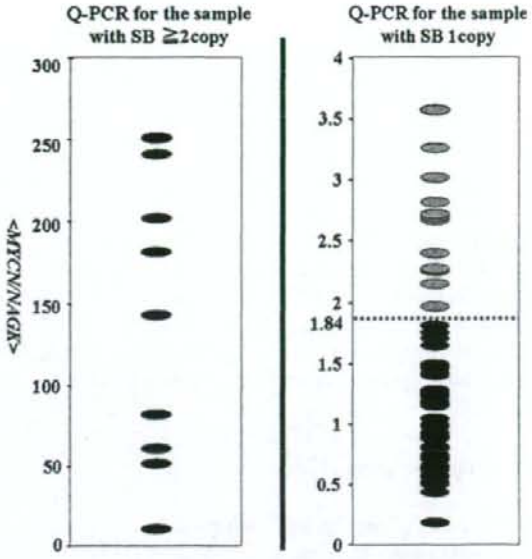


**Fig. 1** The correlation between Southern blotting and quantitative PCR in the block sample. Samples with a slight increase in the gene dosage of *MYCN* using Q-PCR are indicated as *gray points*. Twelve cases with a single copy of *MYCN* based on SB showed a slight increase in the gene dosase of *MYCN* ($3.56 \geqq MYCN/NAGK > 1.84$). *SB* Southern blotting method, *Q-PCR* quantitative PCR

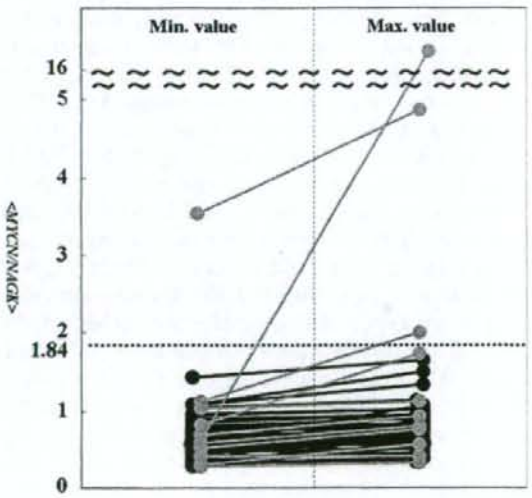


**Fig. 2** The maximum value and minimum value of *MYCN* gene dosage in several small areas obtained by LCM from 54 cases with a single copy of *MYCN* in the block sample based on SB. The *gray points* represent the values of *MYCN* gene dosage in the areas obtained by LCM from 12 block samples with a slight increase of *MYCN* gene dosage ($3.56 \geq MYCN/NAGK > 1.84$) based on Q-PCR

with an *MYCN* gene dosage of less than 1.84 detected in the block samples by Q-PCR, the gene dosage of *MYCN* in all specimens obtained by LCM areas was less than 1.84. In

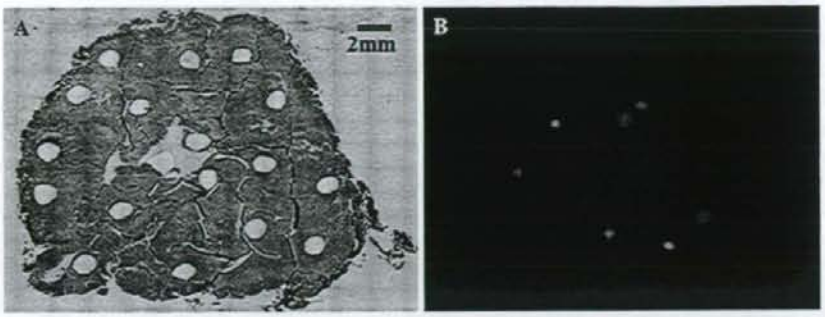


**Fig. 3** **a** The small areas microdissected by LCM in case 10. All microdissected small areas showed a slight increase for the gene dosage of *MYCN*. **b** The cell with the *MYCN* gain detected by FISH (*green MYCN* signal, *orange* the alpha satellite region of chromosome 2) in case 10. The majority of cells of the sample were detected as *MYCN* gain cells

12 cases with a slight increase in the gene dosage of *MYCN* ($3.56 \geqq MYCN/NAGK > 1.84$) in the block sample, 1 case (=case 1) had several LCM areas with a remarkable increase in the *MYCN* gene dosage (the maximal value: 16.40) and others with a single copy number of *MYCN* ($1.84 \geqq MYCN/NAGK$). The tumor of case 1 had an intratumoral necrosis. The central necrotic area of the tumor showed a single copy number of the *MYCN* gene and the variable tumor cells area around the necrosis showed a remarkable increase in the dosage of the *MYCN* gene. The *MYCN* gene status was not determined by FISH in this case. Another case (case 10) of the 12 samples showed a slight increase in *MYCN* gene dosage ($3.65 \leqq MYCN/NAGK \leqq 4.82$) in all LCM areas (Fig. 3a). Furthermore, there were a large number of cells with *MYCN* gain were found using FISH method (Fig. 3b). In two other cases (cases 2 and 12), almost all of the LCM areas showed a gene dosage of *MYCN* of less than 1.84. (Only one small area from case 2 showed an *MYCN* gene dosage as high as 2.00). However, these two samples showed *MYCN* amplification in 15 and 29% of the cells with nuclei by the FISH method.

## Summary of *MYCN* gene status and the clinical characteristics in 12 cases with a slight increase in the *MYCN* gene dosage detected by Q-PCR in the block sample

Table 1 shows the clinical characteristics of the patients in the 12 cases with a slight increase in the *MYCN* gene dosage detected by Q-PCR in the block sample. Eight (67%) of these 12 cases showed advanced stage (INSS stage 3 and 4) and 6 cases (50%) died of the disease. As a result, 4 cases (cases 1, 2, 10 and 12) with a slight increase in the *MYCN* gene dosage based on Q-PCR for block sample had a large number of *MYCN* gain cells or a small number of *MYCN* amplified cells in the tumor tissue.

**Table 1** The characteristics of 12 cases with a slight increase of the gene dosage of *MYCN* by Q-PCR from the block sample

| Case | Age | INSS | Mass | *MYCN* copy (SB) | *MYCN* copy (Q-PCR) | LCM-PCR (max) | LCM-PCR (min) | FISH | Outcome |
|---|---|---|---|---|---|---|---|---|---|
| 1 | 3 month | 4s | − | 1 | 2.70 | 16.40 | 0.54 | ND | Dead |
| 2 | 4 year | 4 | − | 1 | 3.25 | 2.00 | 1.12 | Amp. | Dead |
| 3 | 7 month | 3 | + | 1 | 2.80 | 0.43 | 0.42 | ND | CR |
| 4 | 7 month | 3 | + | 1 | 1.96 | 0.94 | 0.69 | ND | CR |
| 5 | 6 month | 2B | + | 1 | 2.16 | 1.73 | 0.83 | Not Amp. | CR |
| 6 | 4 year | 4 | − | 1 | 2.27 | 0.76 | 0.52 | ND | Dead |
| 7 | 1 year | 4 | − | 1 | 2.28 | 1.13 | 1.03 | ND | Dead |
| 8 | 9 year | 4 | − | 1 | 2.40 | 0.58 | 0.32 | ND | Dead |
| 9 | 6 month | 1 | + | 1 | 3.04 | 0.33 | 0.29 | Not Amp. | CR |
| 10 | 4 year | 4 | − | 1 | 3.56 | 4.82 | 3.65 | Gain | PR |
| 11 | 4 month | 4s | − | 1 | 2.70 | 0.80 | 0.60 | ND | CR |
| 12 | 3 year | 4 | − | 1 | 2.65 | 0.99 | 0.87 | Amp. | Dead |

*Mass* mass screening, *SB* southern blotting method, *Q-PCR* quantitative PCR, *LCM-PCR* the gene dosage of *MYCN* for the samples captured by laser captuered microdissection, *ND* not determined, *CR* complete remission, *PR* partial remission