HEN2. COS7 cells were cotransfected with the expression plasmids for HA-LMO3 and FLAG-HEN2 and double stained with anti-HA and anti-FLAG antibodies. As shown in Fig. 3*B*, LMO3 as well as HEN2 appear exclusively nuclear. On closer inspection by merging two images, these two proteins colocalized in the nucleus. Consistent with the previous reports (35), HA-LMO3 was coimmunoprecipitated with FLAG-HEN1 under our experimental conditions (Fig. 3*C*).

Overexpression of LMO3 accelerates growth of SH-SY5Y neuroblastoma cells. We addressed the question whether LMO3 could induce cell growth of neuroblastoma. To this end, we transfected the expression plasmid for FLAG-LMO3-A or the empty plasmid into SH-SY5Y neuroblastoma cells and established two stable transfectants overexpressing FLAG-LMO3-A (named as LMO3-15 and LMO3-20). As shown in Fig. 4*A*, the expression levels of FLAG-LMO3-A were higher in LMO3-15 and LMO3-20 cells than in the parental SH-SY5Y and the control transfectants (V-2 and V-12). LMO3-15 expressed FLAG-LMO3-A at the level comparable with that in LMO3-20. Similar results were also obtained by RT-PCR analysis (Fig. 4*B*). No obvious morphologic changes could be observed in LMO3-15 and LMO3-20 cells (data not shown). As shown in Fig. 4*C*, LMO3-15 and LMO3-20 cells proliferated at a much faster rate than the control transfectants and SH-SY5Y cells in culture medium containing 10% serum. More importantly, LMO3-15 and LMO3-20 cells continued to grow exponentially even in the low serum culture medium, whereas the growth of the vector-transfected cells as well as SH-SY5Y cells was significantly suppressed under this condition.

To examine whether the LMO3-A-overexpressing cells have an ability to grow in soft agar medium, each transfectants were cultured in soft agar medium for 3 weeks. The numbers of colonies with diameters >300 μm formed by each transfectants in soft agar were scored. LMO3-15 and LMO3-20 cells formed large distinct colonies and showed a statistically significant increase in the number of colonies compared with the vector-transfected cells and SH-SY5Y cells (Fig. 4*D*). These results strongly suggest that overexpression of LMO3 is sufficient to induce malignant transformation in neuroblastoma cells. We also tried to obtain the cells stably transfected with HEN2 but never been successful with unknown reason.

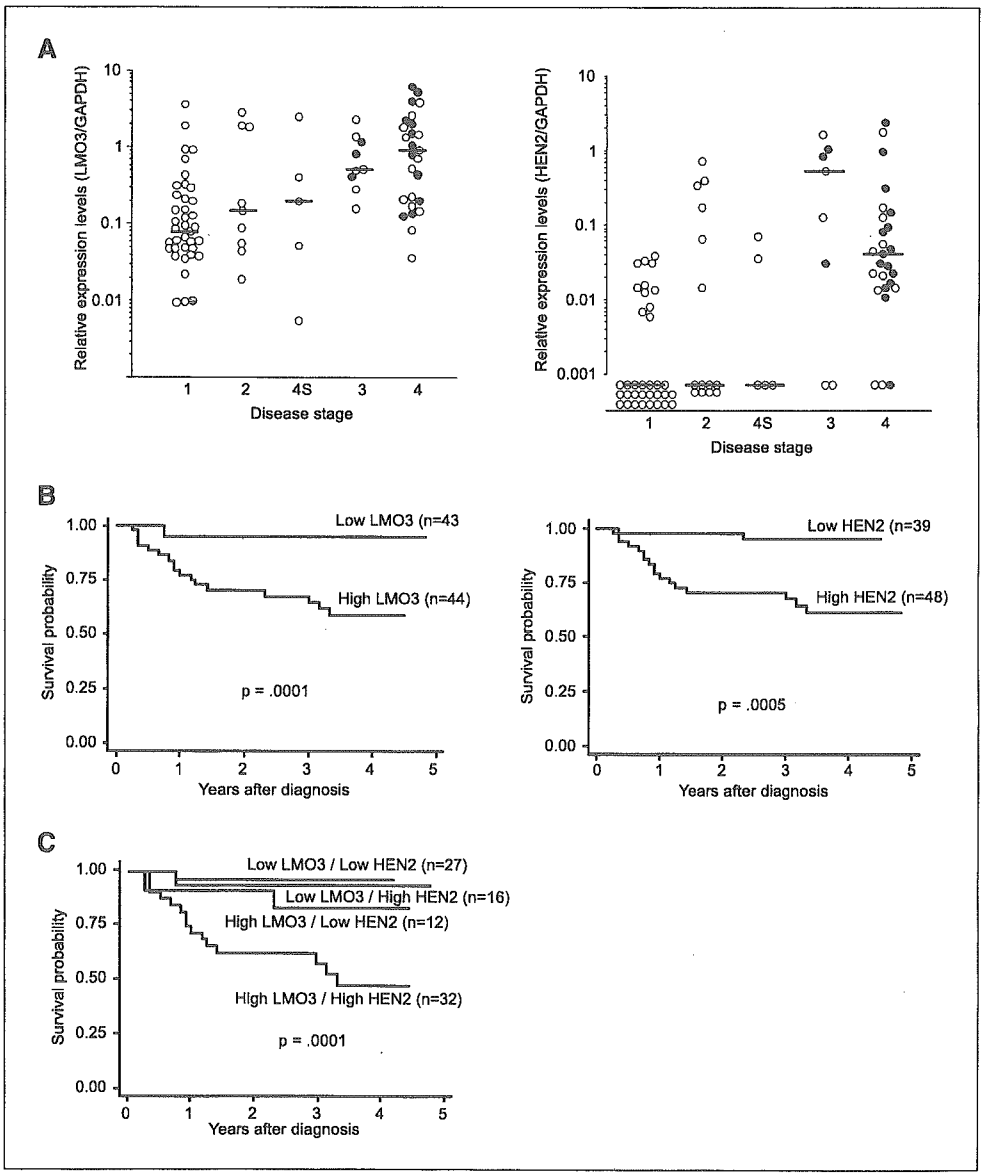

Figure 6. Expression of *LMO3* and *HEN2* mRNA in 87 primary neuroblastomas. *A*, expression levels of *LMO3* (*left*) and *HEN2* (*right*) transcripts in 87 primary neuroblastoma samples categorized by the patient's clinical stage were examined by a quantitative real-time RT-PCR. Relative expression levels of *LMO3* or *HEN2* mRNA were determined by calculating the ratio between *GAPDH* and *LMO3* or *HEN2*. *Bars*, median levels of *LMO3* or *HEN2* expression in each stage; *open* and *closed circles*, samples from patients who are alive and dead, respectively. *B* and *C*, Kaplan-Meier survival curves of patients with neuroblastomas based on high or low expression of *LMO3*, *HEN2* (*B*), or *LMO3* and *HEN2* (*C*).

LMO3 induces marked tumor growth in nude mice. SH-SY5Y cells with a single copy of *MYCN* form tumors in nude mice, although the growth rate is slow compared with that of the other neuroblastoma cell lines with *MYCN* amplification (38). To examine whether overexpression of LMO3 in SH-SY5Y cells could affect the tumor growth *in vivo*, we injected the each transfectants into the left flank of athymic nude mice, and the tumor volumes were measured weekly. V-2 and V-12 cells slowly formed tumors with similar kinetics and of similar sizes 35 to 42 days after injection (Fig. 5*A*). In contrast, the tumors grew rapidly in nude mice implanted with LMO3-15 or LMO3-20 cells. The sizes of the excised tumors from the LMO3-15-implanted mice on day 49 were >10-fold larger than those of control mice (Fig. 5*B*) and showed histologically undifferentiated neuroblastoma with small round cell shapes and small amounts of stromal components (Fig. 5*C*).

Expression of *LMO3* and *HEN2* is associated with a poor outcome of neuroblastoma. To verify whether a significant relationship could be observed between the expression of *LMO3* and/or *HEN2* in primary neuroblastomas and the patients' survival, we quantitatively measured the expression levels of *LMO3* and *HEN2* mRNA in 87 primary tumors by using a quantitative real-time RT-PCR. The values of the levels of *LMO3* and *HEN2* expression were normalized to that of *GAPDH* expression [relative expression values (REV)]. The high level of *LMO3* expression was significantly associated with high expression of *HEN2* (Student's *t* tests, mean ± SE: 1.43 ± 0.27 REV, n = 48 versus 0.54 ± 0.17 REV, n = 39; P = 0.001), older age (≥1-year-old: 1.37 ± 0.29, n = 32 versus <1-year-old: 0.84 ± 0.21, n = 55; P = 0.008), advanced disease stages (stages III + IV: 1.83 ± 0.35, n = 34 versus stages I + II + IVS: 0.52 ± 0.14; P < 0.00005; Fig. 6*A*), low levels of *TrkA* expression (low TrkA: 1.63 ± 0.34, n = 37 versus high *TrkA*: 0.59 ± 0.15, n = 50; P = 0.0003), *MYCN* amplification (amplification: 1.91 ± 0.44, n = 27 versus single copy: 0.64 ± 0.13, n = 60; P = 0.0002), and sporadic cases of neuroblastoma (sporadic: 1.68 ± 0.32, n = 39 versus mass screening: 0.51 ± 0.14, n = 48; P < 0.00005). The high level of *HEN2* expression was also significantly correlated with high expression of *LMO3* (χ^2 tests: P = 0.001), older age (P < 0.0005), advanced stages (P < 0.0005; Fig. 6*B*), low *TrkA* expression (P < 0.0005), *MYCN* amplification (P < 0.0005), and sporadic cases of neuroblastoma (P < 0.0005). Thus, high expression of *LMO3* and *HEN2* was well associated with conventional markers indicating the poor prognosis of neuroblastoma.

We next tested if expression levels of *LMO3* and *HEN2* could have prognostic significance in primary neuroblastomas. The results for log-rank tests showed that high expression of *LMO3* or *HEN2* was significantly associated with poor survival (P = 0.0002 and 0.0005, respectively; Fig. 6*C* and *D*). Remarkably, the combination of high expression of both *LMO3* and *HEN2* showed the significantly worse prognosis compared with the other combinations of *LMO3* and *HEN2* expression levels as shown in Fig. 6*E*. As expected, older patients and the patients with advanced tumors, low expression of *TrkA*, amplified *MYCN*, and the tumors found by mass screening were associated with short time to survival (P < 0.00005). However, the adrenal origin of the tumor was not associated with the outcome (P = 0.19; data not shown).

The univariate analysis suggested that *LMO3* expression (P < 0.0005), *HEN2* expression (P = 0.004), age (P < 0.0005), *MYCN* amplification (P < 0.0005), and mass screening (P = 0.001) were of prognostic importance, supporting the results of the log-rank test (Table 1). Furthermore, the multivariate analysis showed that

Table 1. Simple Cox regression models using LMO3 expression and dichotomous factors of HEN2 expression, age, MYCN amplification, mass screening, and origin (n = 87)

Model	Factor	P	Hazard ratio (95% confidence interval)
A	LMO3 expression (high vs low)	<0.0005	1.80 (1.32-2.47)
B	HEN2 expression (high vs low)	0.004	8.69 (2.00-37.7)
C	Age (≥1 vs <1 y)	<0.0005	8.75 (2.87-26.7)
D	MYCN amplification (1 copy vs >1 copy)	<0.0005	0.049 (0.014-0.171)
E	Mass screening (+ vs −)	0.001	0.032 (0.004-0.237)
F	Origin (adrenal gland vs others)	0.20	2.06 (0.684-6.23)

NOTE: All variables with two categories, except *LMO3* expression (log). Hazard ratio shows the relative risk of death of first category relative to the second. Because all patients with advanced tumor stages and low expression of *TrkA* had died of the tumor, a Cox regression model with the tumor stage or *TrkA* expression was not fitted.

Table 2. Multiple Cox regression models using LMO3 expression and dichotomous factors of HEN2 expression, age, MYCN amplification, mass screening, and origin (n = 87)

Model	Factor	P	Hazard ratio (95% confidence interval)
A	LMO3 expression (high vs low)	0.005	1.61 (1.16-2.23)
	HEN2 expression (high vs low)	0.029	5.32 (1.19-23.9)
B	LMO3 expression (high vs low)	0.005	1.62 (1.15-2.28)
	Age (>1 vs <1 y)	0.002	5.79 (1.86-18.1)
C	LMO3 expression (high vs low)	0.066	1.36 (0.98-1.89)
	MYCN amplification (1 copy vs >1 copy)	<0.0005	0.075 (.02-.282)
D	LMO3 expression (high vs low)	0.044	1.42 (1.01-2.01)
	Mass screening (+ vs −)	0.005	0.051 (0.007-0.404)
E	LMO3 expression (high vs low)	<0.0005	1.78 (1.31-2.41)
	Origin (adrenal gland vs others)	0.21	2.02 (0.666-6.12)

NOTE: All variables with two categories, except *LMO3* expression (log). Hazard ratio shows the relative risk of death of first category relative to the second.

LMO3 expression was significantly associated with survival after controlling *HEN2* expression ($P = 0.005$), age ($P = 0.005$), mass screening ($P = 0.044$), and origin ($P < 0.0005$), suggesting that *LMO3* expression was an independent prognostic factor from the other factors (Table 2). *LMO3* expression was marginally associated with survival after controlling *MYCN* amplification ($P = 0.066$). On the other hand, because *HEN2* expression was highly associated with age, *MYCN* amplification, and mass screening, it was not significantly associated with survival after controlling age, *MYCN* amplification, and mass screening in the corresponding multiple Cox regression models (data not shown).

Discussion

In the present study, we have identified that both *LMO3* and *HEN2* are expressed at higher levels in aggressive neuroblastomas especially with *MYCN* amplification than those with favorable prognosis. Coexpression of *LMO3* and *HEN2* has been observed almost exclusively in neuroblastoma cell lines, not the other lines, suggesting that their expression and function are neuronal specific. Furthermore, LMO3 physically interacted with HEN2 in mammalian cells. The functional significance of LMO3 expression was shown by a stable transfection into SH-SY5Y neuroblastoma cells, colony formation in soft agar, and tumor growth in nude mice, all of which have suggested that LMO3, probably by interacting with endogenous HEN2, markedly promotes the tumor growth. Indeed, the tumors with high expression of both *LMO3* and *HEN2* have shown the worst prognosis in the analysis of 87 primary neuroblastomas. Thus, our results suggested that, in concert with HEN2, the neuronal specifically expressed LMO3 plays an important role in the tumorigenesis of neuroblastoma. Our observation is strikingly intriguing because that *LMO1* or *LMO2* is already known to be the oncogene in T-cell acute lymphoblastic leukemia and that *LMO4* has recently been implicated in the genesis of breast cancer (4, 9).

We have identified a *Nbla3267/LMO3* clone from the screening of differentially expressed genes between favorable and unfavorable subsets of neuroblastoma. *LMO3* was one of the genes expressed at higher levels in the latter than the former (24), like *MYCN* oncogene and *DDX1*, a DEAD box gene coamplified with *MYCN* in aggressive neuroblastomas. In the development of hematopoietic system, LMO1 and LMO2 form a transcriptional complex with Ldb1, a LIM domain–binding protein, and a basic helix-loop-helix protein TAL1, which was identified as an oncogene at the translocation breakpoint in T-cell ALL (4–7). From the analogy with the LMO1 or LMO2 transcriptional machinery in T-cell ALL, we searched for the similar complex in the neuronal system by using the different subsets of primary neuroblastoma and the cell lines in comparison with the T-cell ALL cell lines. As a result, the neuronal-specific pattern of expression was observed in *LMO3*, *Ldb2*, *HEN1*, and *HEN2*, among which *LMO3* and *HEN2* were significantly highly expressed in the unfavorable subset of neuroblastomas with *MYCN* amplification compared with the favorable subset. This result strongly suggested that *LMO3* may function in collaboration with *HEN2* in advanced stages of neuroblastoma. Indeed, both genes were coexpressed only in neuroblastoma derived-cell lines, not in other tumor-derived ones, suggesting that their expression is lineage specific. Furthermore, LMO3 and HEN2 physically interacted in mammalian cells, albeit with weak interaction between LMO3 and HEN1 (35). Thus, these results also suggest that LMO3 and HEN2 form a neuronal cassette mimicking the hematopoietic complex composed of LMO2 and TAL1 and regulate the growth of neuroblastoma.

The neuronal-specific basic helix-loop-helix transcription factors, HEN1 and HEN2, were originally identified from the cDNA library of a neuroblastoma cell line based on cross-hybridization with TAL1 (14, 15). Their expression was restricted to the developing nervous system and a neuroblastoma cell line. However, their function has long been unclear. Recently, Bao et al. have reported that HEN1 interacts with LMO proteins by yeast two-hybrid screen and that *Xenopus* HEN1, in concert with XLMO3, is a critical regulator of neurogenesis (35). This prompted us to test our hypothesis both *in vitro* and *in vivo*. As the results, we found that the SH-SY5Y neuroblastoma cells stably overexpressing LMO3, presumably by acting with endogenous HEN2, gained rapid cell growth in the culture medium with 10% or 1% serum, in the soft agar medium, and in nude mice. These suggested that LMO3 is a neuronal-specific oncogene in neuroblastoma, without any rearrangement of the *LMO3* gene (data not shown). However, we failed to establish a stable SH-SY5Y cell line transfected with HEN2. It is presumed that overexpression of HEN2 might have caused cell death or growth arrest in the cells, albeit the reason is elusive.

The double transgenic mice overexpressing LMO2 and TAL1 displayed a more rapid development of leukemia compared with those overexpressing LMO2 alone, suggesting that LMO2 and TAL1 act synergistically through their complex formation in the development of leukemia (13). Of note, Ono et al. reported that LMO2 and TAL1 act as cofactors for GATA3 to induce the expression of the *retinaldehyde dehydrogenase 2* gene in T-cell ALL (39). On the other hand, a stable complex comprising LMO2, TAL1, and GATA1 was required to promote erythroid differentiation (32). Therefore, LMO3 and HEN2 may also form a nuclear complex, including family members of GATA to regulate cell growth and differentiation in neuroblastoma. Our preliminary data have suggested that GATA2, GATA3, GATA4, and GATA6 are highly expressed in neuroblastoma cell lines, among which GATA4 and GATA6 are predominantly coexpressed in neuroblastoma cell lines compared with T-cell ALL lines. Thus, LMO3 and HEN2, in collaboration with GATA and Ldb families, may play a role in determining cell fate in both neural development and neuroblastoma genesis, although this hypothesis needs to be elucidated. Recently, it has been shown that LMO3 enhanced the ability of HEN1 through the physical interaction to transactivate the expression of *Neurogenin-1* as well as *NeuroD* and thereby induced the neuronal differentiation in frog embryos (35). We tested if this is the case in the neuroblastoma cells. However, our preliminary results suggested that the LMO3/HEN2 complex does not transactivate the *Neurogenin-1* as well as *NeuroD* promoter in neuroblastoma cell lines,[5] although it is unclear if the complex could work in normal neuronal development. Thus, like LMO2, alterations in the LMO3-containing transcriptional complex might differentially regulate expression of the downstream target genes closely involved in neuronal differentiation or tumor formation.

[5] Unpublished data.

It is striking that high levels of expression of both *LMO3* and *HEN2* are significantly associated with the poor prognosis in primary neuroblastomas. This clearly reflects how importantly both genes are functioning in the progression of neuroblastoma *in vivo*. Of interest, expression of either gene is well correlated with *MYCN* amplification, raising the possibility that they might be the downstream targets of MYCN. However, we could not confirm it in human neuroblastoma cell line SH-EP in which MYCN was regulated under the control of the rTet-inducible expression system (40). In agreement with this, cDNA microarray-based screening for the genes induced in the *MYCN*-amplified neuroblastoma cells thus far failed to detect either LMO3 or HEN2 (41, 42). The link between LMO family molecules and the other oncogenes or tumor suppressor genes is also important. Despite the lack of prognostic significance, LMO4 overexpressed in breast cancer seems to be indispensable in the mammary carcinogenesis because it interacts with both BRCA1 and CtIP to repress the BRCA1 function (10). This suggests that, similarly to LMO4, LMO3 may also have the interacting partners related to the tumorigenesis. Thus, LMO3 and HEN2 as well as their associated molecules might be good candidates for the future targets of the therapy against aggressive neuroblastomas.

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Clinical significance of serum NM23-H1 protein in neuroblastoma

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

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

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

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

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

Materials and Methods

Patients and controls

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

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

Venous blood samples

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

ELISA for human NM23-H1 protein

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

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

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

Statistical analysis

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

Results

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

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

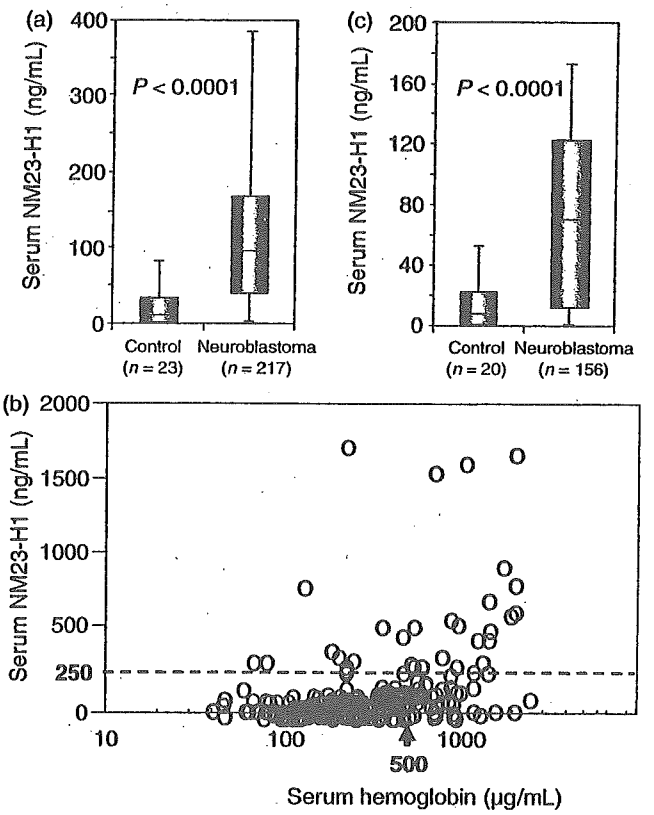

Fig. 1. Serum NM23-H1 levels in patients with neuroblastoma and in control children. (a) Box plots of NM23-H1 serum levels for 217 patients with neuroblastoma and 23 control children with any serum hemoglobin levels. (b) Relationship between the serum levels of NM23-H1 and hemoglobin in all samples examined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

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

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

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

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

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

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

Serum NM23-H1 levels and overall survival

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

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

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

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

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

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

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

Discussion

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

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

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

CI, confidence interval.

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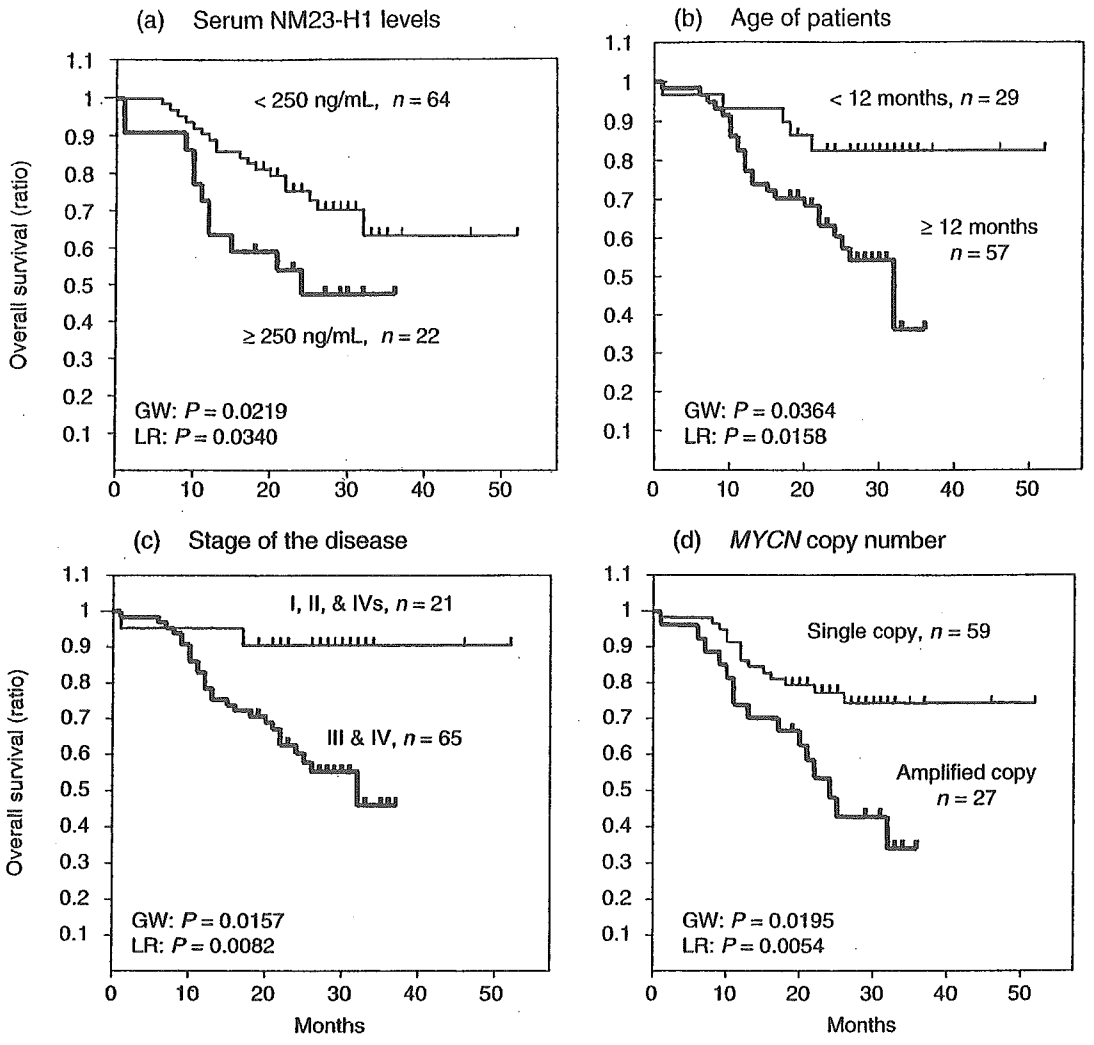

Fig. 2. Overall survival curves for 86 patients with neuroblastoma who were found clinically. (a) Overall survival curves for 22 patients with a serum NM23-H1 level ≥ 250 ng/mL, and for 64 patients with a level < 250 ng/mL. (b) Overall survival curves for 57 patients 12 months of age or older, and for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

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

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

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

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

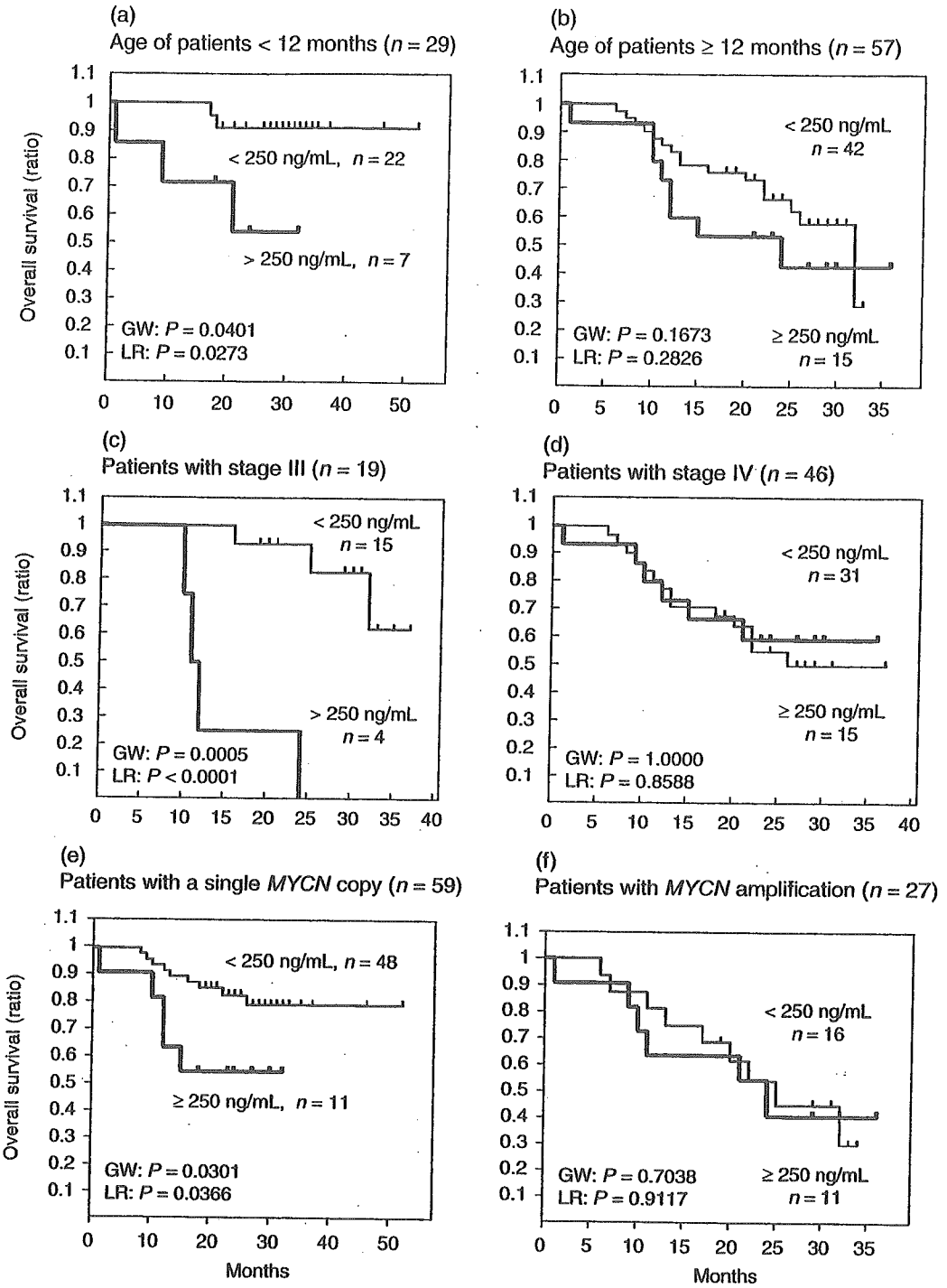

Fig. 3. Clinical significance of the serum NM23-H1 levels in the groups classified according to the age of the patients, or stage of the disease, or copy number of *MYCN*. (a) Survival curves for seven patients with a serum NM23-H1 level of ≥ 250 ng/mL, and for 22 patients with a level < 250 ng/mL. Both groups of patients were younger than 12 months of age. (b) Survival curves for 15 patients with a serum NM23-H1 level of ≥ 250 ng/mL, and for 42 patients with a level < 250 ng/mL. Both groups of patients were 12 months old or older. (c) Survival curves for four patients with a serum NM23-H1 level of ≥ 250 ng/mL, and for 15 patients with a level < 250 ng/mL. Both groups of patients were at stage 3 of the disease. (d) Survival curves for 15 patients with the serum NM23-H1 level ≥ 250 ng/mL, and for 31 patients with the level < 250 ng/mL. Both groups of patients were at stage 4 of the disease. (e) Survival curves for 11 patients with a serum NM23-H1 level of ≥ 250 ng/mL, and for 48 patients with a level < 250 ng/mL. Both groups of patients had a single copy of *MYCN*. (f) Survival curves for 11 patients with a serum NM23-H1 level of ≥ 250 ng/mL, and for 16 patients with a level < 250 ng/mL. Both groups of patients had *MYCN* amplification in the tumor. GW, generalized Wilcoxon's test; LR, log-rank test.

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and serum NSE is a useful marker for patients with advanced neuroblastoma in whom the elevated levels are associated with a poor outcome.[27] The disialoganglioside GD2 is found on the surface of most neuroblastoma cells, and elevated plasma levels have been found in patients.[28] Nevertheless, none of these markers is used at present to predict clinical outcomes or to choose treatment protocols. Therefore, serum NM23-H1 levels might be useful for clinical purposes.

The elevated serum level of NM23-H1 was correlated with a poor prognostic feature, namely, *MYCN* amplification (Table 1). Godfrid *et al.* identified genes that are part of the *MYCN* downstream pathway using SAGE libraries of *MYCN* transfected and control neuroblastoma cell lines.[13] The chromosome 17q genes *NM23-H1* and *NM23-H2* were strongly induced in *MYCN*-expressing cells. A striking correlation between *MYCN* amplification and mRNA or protein expression of both *NM23* genes was found in the cell lines. The present multivariate analysis showed no influence of serum NM23-H1 level on overall survival, and this finding might be caused by the overlap of patients with *MYCN* amplification with those with a high serum level of NM23-H1. However, within the group of patients with a single copy of *MYCN*, patients with a higher level of NM23-H1 had a worse outcome (Fig. 3e). The findings suggest that *NYCN* amplification may influence serum NM23-H1 levels as well as clinical outcome, and that neuroblastomas with a single copy of *MYCN* and a higher serum NM23-H1 level may have had a mutation or an increased copy number of the *NM23-H1* gene.[6,24,29] *MYCN* overexpression in some neuroblastomas with a single copy of *MYCN* may have resulted in higher serum NM23-H1 levels and a poor outcome; however, a recent study showed that *MYCN* overexpression did not affect the prognosis of advanced-stage neuroblastomas with a single *MYCN* copy.[30]

In patients with NHL and AML, it is thought that serum NM23-H1 protein is produced directly by the tumor cells, and its serum level depends on the total mass of malignant cells overexpressing *NM23-H1*.[14] High concentrations of NM23 protein were found in the serum and body fluid of patients with lung cancer overexpressing the *NM23* genes.[31] Tumor cells may secrete this protein through some unknown mechanism, because there is no signal peptide sequence for secretion in the NM23 molecule. Serum NM23-H1 in patients with neuroblastoma might be derived from tumor cells and might be induced by *MYCN* amplification/overexpression or by *NM23-H1* overexpression independent of *MYCN*.

The serum level of NM23-H1 protein is clinically useful as an important prognostic factor in NHL or AML, and the present study showed that the protein could be a factor predicting an outcome of patients with neuroblastoma. It would be interesting to examine whether the serum NM23-H1 level generally predicts a poor outcome for patients with other tumors. The mechanisms by which the NM23-H1 protein is secreted into the serum and how it affects patient outcome are unclear. We are now studying the possibility that a high concentration of serum NM23-H1 may positively affect tumor cell growth or negatively affect normal cells.

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ORIGINAL PAPER

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Granulocytic sarcoma of the spine in a child without bone marrow involvement: a case report and literature review

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Abstract We report a 2-year-old Japanese boy without bone marrow involvement who developed a primary granulocytic sarcoma in his spinal canal. Tumour cells were positive for myeloperoxidase, MIC2, CD56 and, CD68 on formalin-fixed, paraffin-embedded tissue sections and CD13, CD33, CD45, and CD64 on acetone-fixed fresh frozen sections. Nine months after the initiation of treatment, the tumour had significantly regressed and the patient was able to walk with help. *Conclusion*: Our patient is the youngest case of granulocytic sarcoma of the spine without bone marrow involvement. Immunohistochemical methods are very helpful in establishing a diagnosis of granulocytic sarcoma.

Keywords Granulocytic sarcoma · Myeloid sarcoma · Spinal cord compression · Without bone marrow involvement

Abbreviations AML: acute myeloid leukaemia · GS: granulocytic sarcoma

Introduction

Granulocytic sarcoma (GS), also termed extramedullary myeloid tumour, myeloid sarcoma or chloroma, is a malignant, solid tumour consisting of myeloblasts or immature myeloid cells occurring in extramedullary sites [1]. GS has often been described in association with acute myeloid leukaemia (AML), chronic myeloid leukaemia, or myeloproliferative disorders [1]; however, GS rarely presents in the absence of other haematological disease. Many of these cases are misdiagnosed as small round cell tumours such as malignant lymphoma, rhabdmyosarcoma or Ewing sarcoma [1]. GS may occur in almost any part of the body, but is most commonly seen in the skin, lymph nodes, and bone [1, 21,26]. Spinal cord compression is an uncommon symptom.

Here we report the youngest case of a primary GS occurring in the spinal canal and causing severe spinal compression in a child without bone marrow involvement. The importance of immunohistochemical studies in the diagnosis of GS without bone marrow involvement is discussed.

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Case report

A 2-year-old Japanese boy was taken to a local hospital complaining of external genital and lower extremity pain for a month; the patient had difficulty standing or walking. As MRI showed an epidural mass filling the spinal canal below the L3 level and extending into the left abdominal cavity (Fig. 1a, b), he was referred to our hospital for further evaluation and treatment. Although an open biopsy was planned to enable a diagnosis, his mother refused the procedure and the patient was discharged because of her decision. Three months later, however, the patient's lower extremities became paralysed and he developed bladder and bowel problems; he was thus readmitted to our hospital. On physical examination at the time of the second admission, he could not move his legs by himself and his lower extremities were more atrophic than at the previous examination. The deep tendon reflexes in his lower extremities were disturbed. A sensory assessment revealed hyperaesthesia below the level of L3. A complete blood count at the time of the second admission

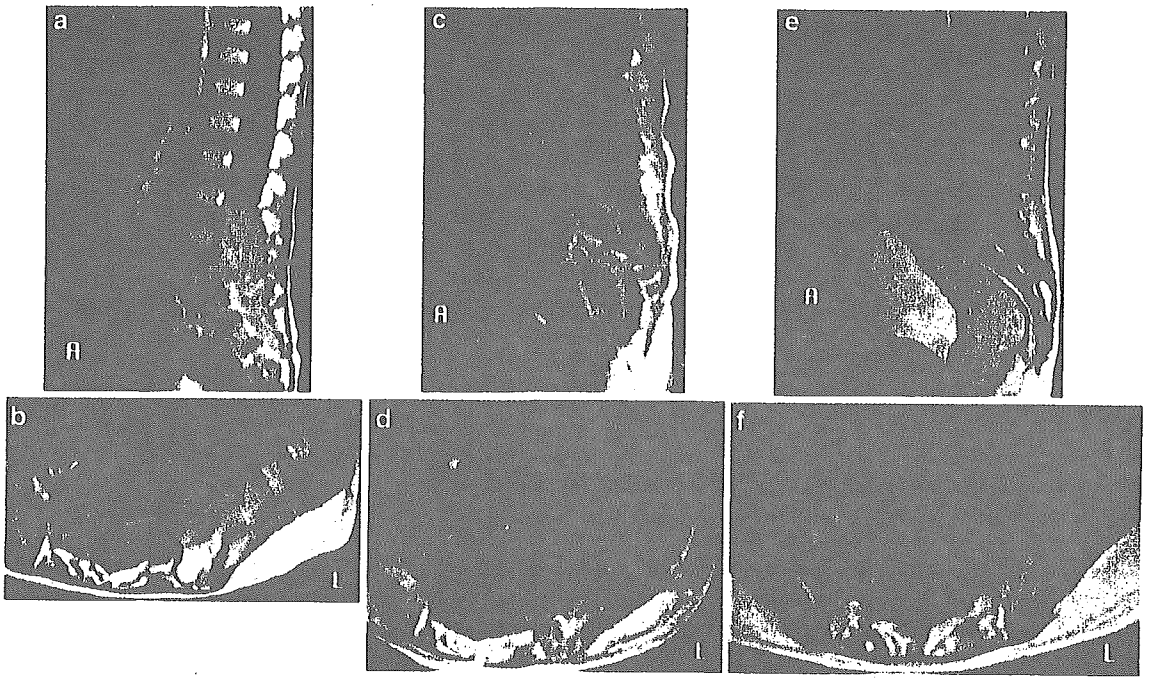

Fig. 1 a,b At the time of the initial admission, sagittal and coronal T1-weighted MRI scans showed an epidural mass filling the spinal canal below L3 and extending into the left abdominal cavity. **c,d** At the time of the second admission, the tumour had increased in size, compared to the images obtained 3 months earlier. **e,f** The size of the tumour shrunk remarkably after six courses of chemotherapy

showed a white blood cell count of 10,500 cells/μl with a normal differential, an haemoglobin level of 12.4 g/dl, and a platelet count of 289,000 cells/μl. Bone marrow aspiration showed normocellular bone marrow with no evidence of leukaemia. An MRI examination showed that the tumour had increased in size (Fig. 1c, d). An open biopsy was performed and a small round-cell-like tumour was observed in the biopsy sample. To establish a definitive diagnosis, immunohistochemical studies were performed. At this time, the tumour cells tested positive for myeloperoxidase, MIC2 (CD99), CD56, and CD68 and negative for c-Kit (CD117) and CD43 using immunohistochemical techniques on formalin-fixed, paraffin-embedded tissue sections (Fig. 2). Additional immunohistochemical studies using acetone-fixed, fresh-frozen sections revealed that the tumour cells were also positive for CD13, CD33, and CD64, all of which are markers for myeloid-lineage cells, and CD45 but negative for CD3 and CD79a (Fig. 3). Therefore, a histopathologicla diagnosis of GS was made.

First of all, we performed three courses of AML chemotherapy using etoposide, cytarabine, mitoxantron, and idarubicin (1st course: etoposide 150 mg/m^2, days 1–5, cytrabine 200 mg/m^2, days 6–12, mitoxantron 5 mg/m^2, days 6–10; 2nd course: cytarabine 3 g/m^2×2/day, days 1–3, etoposide 100 mg/m^2, days 1–5, idarubicin 10 mg/m^2, day 1; 3rd course: etoposide 150 mg/m^2, days 1–3, cytarabine 200 mg/m^2, days 4–8, mitxantron 5 mg/m^2, days 4–6). Despite this regimen, an apparent regression of the tumour was not observed. We then changed the chemotherapy to four additional courses of ifomide, pirarubicin, etoposide, and carboplatin (4th-7th course: ifomide 3 g/m^2, days 1, 2, pirarubicin 30 mg/m^2, days 4, 5, etoposide 400 mg/m^2, day 3, carboplatin 100 mg/m^2, days 1–5). In total, seven courses of chemotherapy were performed 9 months after the initiation of treatment. A subsequent MRI examination showed that the tumour had significantly regressed (Fig. 1e, f), and the patient was able to walk again with assistance.

Discussion

GS without bone marrow involvement is rare and only a few cases have presented with spinal involvement. Yamauchi et al. [26] summarised 74 GS patients without bone marrow involvement, two of their own and 72 previously reported cases; 13 out of 102 tumours (13%) in these patients had head or spinal cord involvement. Tsimberidou et al. [21] summarised GS patients without bone marrow involvement treated at the MD Anderson Cancer Center between 1990 and 2002 and reported that 4 out of 21 patients (19%) with GS without bone marrow involvement had CNS involvement. Our literature survey found only 25 GS cases without bone marrow involvement causing spinal cord compression since 1950, including the above reports (Table 1) [2, 3, 5, 7, 8, 9, 10, 11, 12, 13, 15, 16, 17, 18, 19, 20, 22, 23, 24, 25,28]. The age distribution of these patients ranged from 12 to 73 years (mean 32.2 years). Since our patient was 2 years old, he is the youngest GS patient without bone marrow involvement of the spine to be reported. 1

In our literature review including our case, the symptoms of GS causing spinal cord compression were variable depending on the patient and tumour location. Local pains were present in 88% of the patients, with

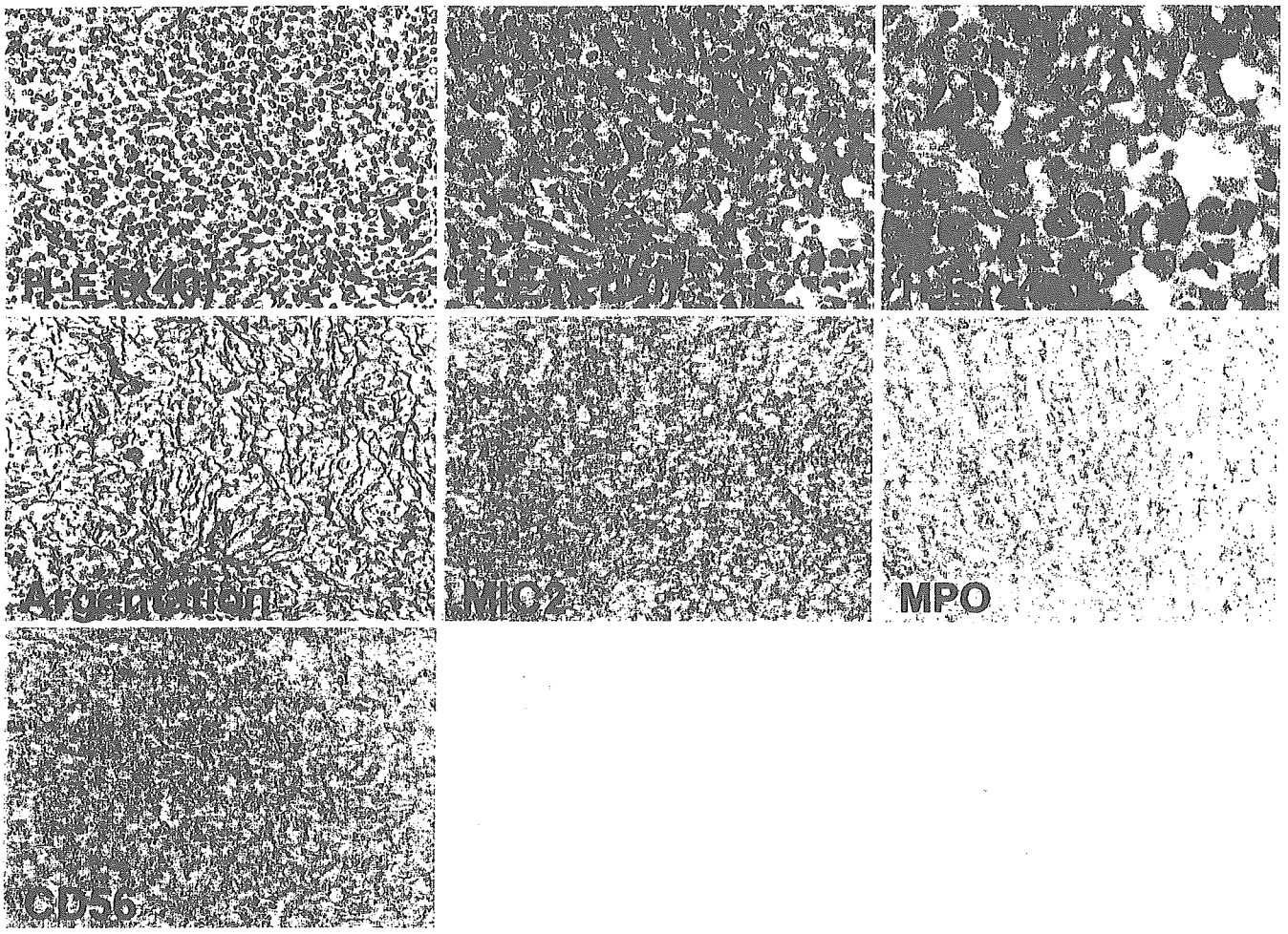

Fig. 2 Haemotoxylin and eosin and immunohistochemical staining of paraffin sections. On the stained sections, myeloid cells with round or oval nuclei, and scanty cytoplasm were generally observed. The nuclei were homogeneous, and exhibited atypia and karyomitosis. The nucleoli were obscure. On argentation, argentaffine fibres were observed twisted around the cells. On immunohistochemical-stained sections, MIC2, myeloperoxidase, CD56, and CD68 tested positive

Fig. 3 Immunohistochemical staining of acetone-fixed, fresh-frozen sections; CD13, CD33, CD45, and CD64 tested positive

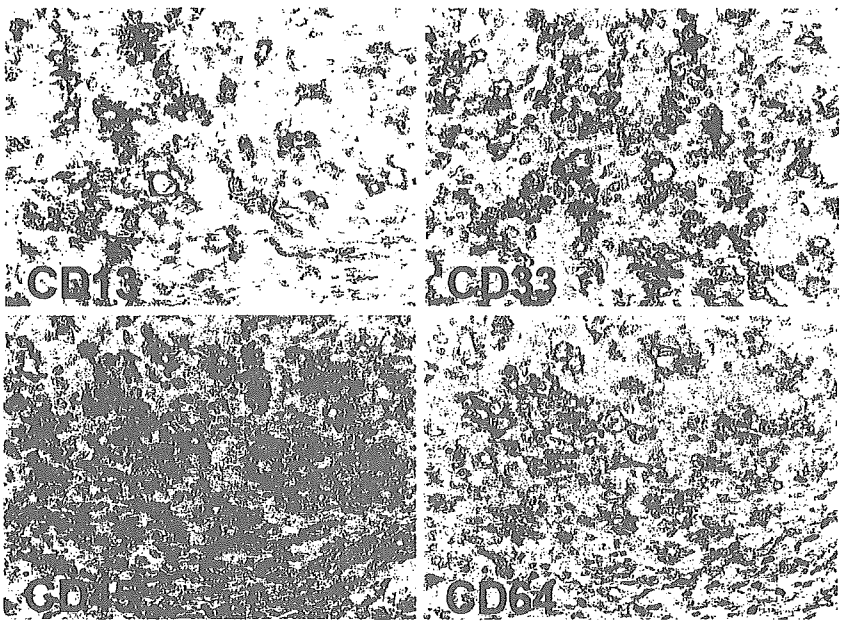

Table 1 Reported cases of GS of the spine without bone marrow involvement

No	Reference	Gender	Age (years)	Site of lesions	Treatment	Progression to leukaemia	Outcome
1	[17]	M	22	T8	Surgical decompression and radiotherapy	Negative	Death at 13 months
2	[24]	F	12	T3–T11	Surgical decompression and radiotherapy	Positive (6 months)	Death at 8 months
3	[12]	M	20	T8	Surgical decompression, radiotherapy, and chemotherapy	Positive (26 months)	Death at 43 months
4	[10]	M	33	T9–T12	Radiotherapy and chemotherapy	Negative	Survival at 14 months
5	[16]	M	21	T4–T8	Surgical decompression	Positive (29 months)	death at 29 months
6	[3]	M	13	T12–L1	Surgical decompression and radiotherapy	Negative	Survival at 72 months
7	[13]	F	29	T9–T12	Surgical decompression, radiotherapy, and chemotherapy and marrow transplantation	Positive (7 months)	Death at 1 year
8	[28]	M	31	T12–L4	Surgical decompression, radiotherapy, chemotherapy, and autologous bone marrow transplantation	Positive (32 days)	Survival at 18 months
9	[18]	M	58	T5–T8	Radiotherapy and chemotherapy	Negative	Death at 3 months
10	[8]	M	70	L2–L3	Surgical decompression, radiotherapy and chemotherapy	Negative	Survival at 3 months
11	[25]	M	49	L3–L5	Chemotherapy	Negative	Not described
12		M	36	S1	Not described	Not described	Not described
13	[23]	M	20	Less than L5	Surgical decompression, and chemotherapy	Positive (5 months)	Survival at 4 months
14	[9]	M	22	T4	Surgical decompression, radiotherapy, chemotherapy, and autologous and HLA-identical bone marrow transplantation	Positive (11 months)	Death at 29 months
15	[19]	M	22	L4–S1	Not described	Positive (4 weeks)	Not described
16	[5]	M	47	C6–C7	Surgical decompression, radiotherapy, and chemotherapy	Negative	Death at 12 months
17		M	49	L3–L4	Surgical decompression and chemotherapy	Negative	Survival at 3 months
18		M	15	L2	Surgical decompression and chemotherapy	Positive (10 months)	Death at 12 months
19	[20]	M	22	L3–S1	Radiotherapy and chemotherapy	Positive (6 weeks)	Death at 4 months
20	[15]	M	29	T2–T4	Surgical decompression, radiotherapy, and chemotherapy	Negative	Survival at 9.5 years
21	[11]	M	73	T4–T6	Surgical decompression, radiotherapy, and chemotherapy	Negative	Death at 4 months
22	[22]	M	13	T11–L1	Surgical decompression	Negative	Death at 12 months
23	[2]	F	35	C4–C5	Surgical decompression, radiotherapy, and chemotherapy	Negative	Survival at 3 months
24	[7]	M	40	S1	Surgical decompression, radiotherapy, and chemotherapy	Negative	Survival at 2 years
25		F	17	T6–T10	Chemotherapy	Negative	Survival at 2 years
26	Present case	M	2	Less than L3	Chemotherapy	Negative	Survival at 1 year

50% of these reporting back pains. Motor deficits ranging from extremity weakness to loss of bowel/bladder function (31%) or paraplegia (27%) were detected in 73% of the patients. Numbness and loss of sensation were recorded in 19% and 35% of the patients, respectively [2, 3, 5, 7, 8, 9, 10, 11, 12, 13, 15, 16, 17, 18, 19, 20, 22, 23, 24, 25,28]. If appropriate early diagnosis and early treatment are done, those symptoms can be reversible. Therefore, GS should be included in the differential diagnosis of extradural spinal cord tumours, regardless of the evidence of leukaemia.

GS is difficult to diagnose in patients without bone marrow involvement because of its rarity. Yamauchi et al. [26], Chen et al. [4], Neiman et al. [16], Eshghabadi et al. [6], and Meis et al. [13] reported that 47%, 48%, 56%, 59%, and 75% of GS patients without bone marrow involvement were initially misdiagnosed, respectively. These cases were most often misdiagnosed as lymphoproliferative disorders. In addition, small round-cell tumours, particularly in children (neuroblastoma, rhabdmyosarcoma, Ewing sarcoma/peripheral neuroectodermal tumour and medulloblastoma) must be included in the differential diagnosis [1]. Immunohistochemical methods have been reported to be helpful in establishing a diagnosis of GS [4,22]. The myelo- and/or monoblasts in GS lesions have antigenic profiles that are similar to the blasts in AML/acute monoblastic leukaemia and express myeloid- and monocytoid-associated antigens, like CD13, CD14, CD33, CD64, CD68, and c-Kit (CD117), as well as lysozyme [1]. In addition,

blasts in the GS also express leukocyte common antigen (CD45), CD43 and MIC2 (CD99). CD45 is useful for distinguishing GS from non-haematopoietic tumours but is not helpful for distinguishing GS from lymphoproliferative disorders because it is expressed in most haematopoietic tumours. CD43 is also expressed in T-cell lymphoma [14]. While MIC2 (CD99) expression has long been used as diagnostic marker for Ewing sarcoma or primitive neuroectodermal tumours; MIC2 (CD99) has also been shown to be expressed by immature myeloid cells and lymphoid cells [14,27]. C-Kit (CD117) is also highly sensitive in GS [4]. In our patient, myeloperoxidase, MIC2 (CD99), CD13, CD33, CD45, CD56, CD64, and CD68 were positive and c-Kit (CD117), CD3, CD43, and CD79a were negative. We emphasise that immunohistochemical methods are very important for the diagnosis of GS, especially the positive reaction of myeloid lineage-specific markers, such as myeloperoxidase, CD33, and CD64. Since some of these markers cannot be examined using formalin-fixed, paraffin-embedded sections, parallel examinations using acetone-fixed, fresh-frozen sections are recommended.

Although no clear relationship between specific treatment modalities and survival was found in a review of the literature [27], most GS patients without bone marrow involvement progress to AML if left untreated [5, 6,26]. In contrast, a reduced risk of developing AML was reported in GS patients without bone marrow involvement receiving chemotherapy for AML. Since early diagnosis followed by appropriate therapy may prevent leukaemic transformation in these cases, GS should be included in the differential diagnosis of extradural spinal cord tumours, regardless of the evidence of leukaemia and immunohistochemical methods are mandatory for a correct diagnosis.

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Case Report

Recurrent yolk sac tumor following resection of a neonatal immature gastric teratoma

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Abstract Gastric teratomas are very rare and usually benign. Only a few cases of gastric teratomas with malignant components have been reported. This report describes recurrence of a yolk sac tumor following resection of a neonatal immature gastric teratoma. Gastric teratoma recurring as a malignant lesion has not been previously reported. Recurrence of immature gastric teratomas should be considered, and a periodic follow-up check with alpha-fetoprotein level should be mandatory.

Keywords Gastric teratoma - Immature teratoma - Yolk sac tumor

Introduction

Gastric teratomas are very rare, with a reported incidence of less than 1% of all pediatric teratomas [*1*]. As of 2002, only 107 cases had been reported in the English literature [*2–7*] and 54 cases in the Japanese literature [*8*, *9*]. Gastric teratoma patients exhibit characteristic differences in gender (90% are male), age (less than 1 year of age), and malignant behavior compared with patients with teratomas originating in other organs. Malignant gastric teratomas are especially rare, with only a few cases reported [*10–12*]. We describe a case of immature gastric teratoma that recurred as yolk sac tumor (YST) 2 years after the resection. To our knowledge, this report is the first of its kind in the literature.

Case report

A 4-day-old male infant was admitted with vomiting and fever. Abdominal examination revealed a firm, elastic, mobile mass with an irregular contour in the left upper quadrant. Upper gastrointestinal tract contrast radiography revealed a gastric tumor with irregular contour (Fig. 1). Computed tomography (CT) showed a calcified, low-density tumor involving the stomach wall and extending toward the retroperitoneum. The serum alpha-fetoprotein (AFP) level was appropriate for age at 80,050 ng/ml, beta human chorionic gonadotropin concentration was <0.1 ng/ml (standard <0.1 ng/ml), and a neuron-specific enolase level was 13.2 ng/ml (standard <10.0 ng/ml) (Table 1).

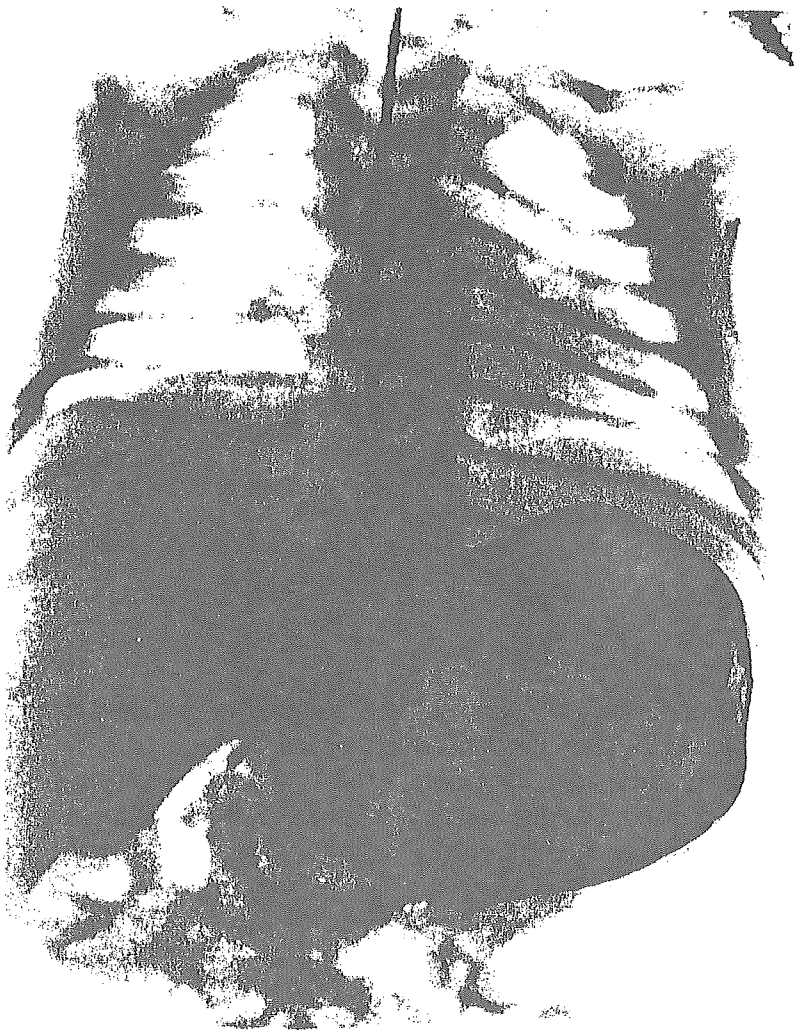

Fig. 1 Upper gastrointestinal contrast study shows a large space-occupying mass with irregular contour in the stomach

Table 1 Serum tumor marker levels on admission (*AFP* alpha-fetoprotein, *HCG* human chorionic gonadotropin, *Beta-HCG* beta human chorionic gonadotropin, *NSE* neuron-specific enolase, *VMA* vanillylmandelic acid, *Cr* creatinine, *HVA* homovanillic acid, *CEA* carcinoembryonic antigen)

	This case	Standard
AFP(day 5)	80,050.0 ng/ml	
HCG	<0.4 mIU/ml	<0.7 mIU/ml