Plasma midkine level is a prognostic factor for human neuroblastoma

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Neuroblastoma is the third-most-common solid tumor of childhood. To date, no reliable blood marker for neuroblastoma has been established. The growth factor midkine is highly expressed in human carcinomas and its knockdown leads to tumor growth suppression in animal models. The present study evaluated the plasma midkine level in human neuroblastoma patients. Plasma samples were obtained from patients found through mass screening, as well as from sporadic neuroblastoma patients. The total number of cases examined was 756. Among them, prognostic information was available for 175 sporadic cases and 287 mass-screening cases. Midkine levels were significantly higher in neuroblastoma patients, including both mass-screening cases and sporadic cases, than in non-tumor controls ($P < 0.0001$). The midkine level was significantly correlated with the statuses of *MYCN* amplification, *TRKA* expression, ploidy, stage and age ($P < 0.0001$, < 0.0001, $= 0.004$, < 0.0001 and < 0.0001, respectively), which are known prognostic factors for neuroblastoma. There was a striking correlation between high plasma midkine level and poor prognosis ($P < 0.0001$). Within sporadic cases, the midkine level was also strikingly higher than in non-tumor controls ($P < 0.0001$), and correlated with the statuses of *MYCN* amplification and stage ($P = 0.0005$ and $= 0.003$, respectively). There was a significant correlation between high plasma midkine level and poor prognosis ($P = 0.04$). Taken together, the present data indicate that plasma midkine level is a prognostic factor for human neuroblastoma. (*Cancer Sci* 2008; 99: 2070–2074)

Neuroblastoma (NBL) is the third-most-common malignant tumor of childhood, accounting for 15% of cancer-related death.[1] In spite of an enormous amount of research devoted to curing this disease, its prognosis remains poor. NBL has several established prognostic factors, i.e. *MYCN* amplification, *TRKA* expression level, ploidy, stage and age.[1,2] Cases with tumors with an amplified *MYCN* gene, low *TRKA* expression or diploidy show poor prognosis. Cases at stage 3 or 4, or at ages older than 18 months also show poor prognosis. Since molecular fingerprints within tumor tissues, such as *MYCN* amplification, *TRKA* expression level and ploidy, require a tumor biopsy or its removal, a blood marker for NBL has long been awaited.[1,2] A blood marker would not only be useful for the initial diagnosis but would also be beneficial for the sequential monitoring of the tumor status.

The growth factor midkine (MK) was originally found in embryonal carcinoma cells, and has been implicated in cancer development.[3–5] MK is highly and frequently expressed in human carcinomas, including Wilms' tumor, tumors of the digestive tract, brain tumors, urinary bladder tumors and breast tumors, whereas its expression is scarcely detected in normal adult tissues.[6–10] Strong MK expression is also detected in pre-cancerous stages of human colorectal cancer and human prostate cancer.[11,12] Knockdown of MK expression leads to suppression of xenografted tumors of mouse colorectal cancer cells and human prostate cancer cells.[13,14]

We previously reported that the plasma MK level was correlated with the values of established prognostic factors through a study of 220 cases, including 82 non-mass-screening (sporadic) cases and 122 mass-screening cases.[15] However, in that study, information on the prognosis of patients was too limited to determine whether the plasma MK level could be a prognostic factor. In the present study, we measured plasma MK levels of 756 NBL cases, which consisted of 286 sporadic cases, 387 mass-screening cases and 83 unknown cases. Among them, prognostic information was available for 175 sporadic cases and 287 mass-screening cases. This enabled us to evaluate the plasma MK level as a prognostic factor.

Mass screening for NBL started in 1985 in Japan, but was discontinued in 2004 because of the lack of apparent beneficial effects on the cure rate of NBL. Mass-screening cases are grouped into the favorable prognosis group, and most of the mass-screening cases are thought to have spontaneously regressed. Therefore, nowadays, sporadic NBL patients are the major subject of therapy. However, information obtained from mass-screening cases has been useful, especially to understand tumor phenotype with favorable prognosis. This is the reason why we enrolled 387 mass-screening patients in this study. Accordingly, we evaluated plasma MK levels in two categories: first, the entire set of NBL cases including mass-screening cases and sporadic cases; and second, the set of sporadic cases.

Here we report that the plasma MK level is a prognostic factor for NBL.

Materials and Methods

Plasma samples. Clinical data of 756 neuroblastoma patients are summarized in Table 1. The same archive samples were used as those without malignant tumors ($n = 17$; eleven were <1-year old and six were >1-year old).[15]

Enzyme-linked immunoassay for human MK. An enzyme-linked immunoassay for human MK was performed as described previously.[16] Briefly, human MK was produced using *Pichia pastoris* GS115 by transfection with a human MK expression vector, which was constructed into pHIL-D4 (Invitrogen, Carlsbad, CA, USA). This yeast-produced human MK was used to immunize rabbits and chickens to raise antibodies. The rabbit antihuman MK antibody (50 μL of 5.5 μg/mL in 50 mM Tris HCl (pH 8.2), 0.15 M NaCl, 0.1% NaN_3) was coated onto the wells of microtiter plates (Polysorp plates, Nunc, Rochester, New York, USA) for 20 h at room temperature. After washing with 0.05% Tween-20 in phosphate-buffered saline (PBS), the wells were

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Table 1. Plasma samples

	n
No malignant tumors	17
<1 year	11
>1 year	6
Neuroblastomas	756
Stage 1,2,4S	372
Stage 3,4	330
Unknown	54
MYCN amplification –	643
MYCN amplification +	97
Unknown	16
High *TrkA* expression	425
Low *TrkA* expression	159
Unknown	172
Mass screening	387
Sporadic	286
Stage 1,2,4S	62
Stage 3,4	209
Unknown	15
MYCN amplification –	207
MYCN amplification +	73
Unknown	6
High *TrkA* expression	109
Low *TrkA* expression	113
Unknown	64
Hyperdiploidy/pentaploidy	96
Diploidy/tetraploidy	136
Unknown	54
<18 months	101
>18 months	183
Unknown	2
Unknown	83
Hyperdiploidy/pentaploidy	379
Diploidy/tetraploidy	263
Unknown	114
<18 months	506
>18 months	242
Unknown	8

blocked with 300 μL of 0.1% casein, 0.01% Microcide I (aMReSCO) in PBS for 20 h at 37 C. Plasma samples (10 μL each) were mixed with 100 μL of 50 mM Tris HCL (pH 8.4), 0.5 M KCl, 0.1% casein, 0.5% bovine serum albumin (BSA), 0.01% Microcide I and 0.1 μg/mL peroxidase-labeled chicken antihuman MK antibody. Aliquots of 50 μL of this mixture were added to wells prepared as described above, and further subjected to chromogenic detection at optical density at 450 nm (OD_{450}) using tetramethylbenzidine as the substrate. This assay system shows linearity from 0 to 5 ng/mL of MK, and there is no cross-reaction with pleiotrophin, a close homolog of MK.[5]

Statistical analysis. The Kruskal–Wallis test was used to evaluate the statistical differences between stages. The Mann–Whitney *U*-test was used to further evaluate the difference between the two groups. The Mann–Whitney *U*-test was used for analysis of the other prognostic factors. Survival time was measured from the date of initial diagnosis to the date of death or last contact. The Kaplan–Meier method was used to compare survival between the groups defined by plasma MK levels, and survival differences were analyzed using the log-rank test. All analyses were carried out using StatView for Windows (ver. 5.0; SAS Institute, Cary, NC, USA). $P < 0.05$ was considered statistically significant.

Results

Plasma MK levels of NBL patients and the relationship of plasma MK to established prognostic factors for NBL. The entire set of 756 NBL cases consisted of 387 cases found through mass screening, 286 sporadic NBL cases and 83 unknown cases (Table 1). Plasma MK level of the NBL cases was 23–1 062 520 pg/mL, whereas that of non-tumor controls was 146–517 pg/mL (Fig. 1a). The values of NBL cases were significantly higher than those of controls ($P < 0.0001$). We set the cut-off value average ± 4SD of non-tumor controls at 900 pg/mL (Fig. 1a). The group of cases with levels higher than 900 pg/mL was designated high MK, whereas cases with lower than 900 pg/mL were grouped into low MK.

MYCN amplification, *TRKA* expression level, ploidy, stage and age are well-known prognostic factors for NBL.[1] The values of each factor were determined for all 756 NBL cases. As shown in Figure 1(b–f), MK levels were significantly correlated with all the prognostic factors. Thus, MK levels were significantly higher in *MYCN*-amplified cases ($P < 0.0001$, *versus MYCN*-nonamplified), in cases with low *TRKA* expression ($P < 0.0001$, *versus* high *TRKA* expression), in diploidy cases ($P = 0.004$), in cases at stage 3 and 4 ($P < 0.0001$, *versus* stage 1, 2, and 4S) and in cases older than 18 months ($P < 0.0001$, *versus* younger than 18 months). These groups in which MK levels were high, i.e. *MYCN*-amplified, low *TRKA* expression, diploidy, stage 3 and 4 and older than 18 months, are known to have a poor prognosis. The data indicate close correlations between MK levels and known prognosis factors and are consistent with our previous report.[15]

Figure 2(a) shows Kaplan–Meier survival curves based on plasma MK levels for all NBL cases. A high MK level was closely associated with poor prognosis of NBL patients ($P < 0.0001$), indicating that the MK level alone can be a prognostic factor for NBL patients. It was interesting that a high MK level was associated with poor prognosis within the unfavorable NBL group based on ploidy, i.e. diploidy ($P = 0.02$) (Fig. 2b). This was also the case within favorable NBL groups, that is, groups with *MYCN* non-amplification, age <18 months or high *TRKA* expression ($P = 0.02$, 0.001 or 0.02, respectively), although the survival differences between high MK and low MK were very small (data not shown).

Analysis for sporadic NBL cases. We examined 286 sporadic NBL cases, among which prognostic information was available for only 175. Plasma MK level was significantly higher in sporadic NBL cases than in non-tumor controls ($P < 0.0001$) (Fig. 3a). It was closely related to the values of two prognostic factors, i.e. *MYCN* amplification and stage ($P = 0.0005$ and 0.003, respectively) (Fig. 3b,c), but not to those of age, *TRKA* expression level and ploidy (data not shown).

Kaplan–Meier analysis revealed that a high MK level was correlated with poor prognosis in the sporadic NBL patients ($P = 0.04$) (Fig. 4a). The Kaplan–Meier data on MK was further compared with those on known prognostic factors. Survival based on ploidy exhibited a significant difference ($P = 0.025$) (Fig. 4b). *MYCN* amplification, *TRKA* expression level and stage also showed significant differences ($P = 0.003$, 0.01 and 0.008, respectively), whereas age could not be a prognostic factor for the sporadic NBL cases examined (data not shown). The Cox hazard ratio was 1.71 for MK level, 2.27 for ploidy, 2.70 for *MYCN* amplification, 2.38 for *TRKA* expression and 1.84 for stage.

Discussion

In the present study, we first evaluated the plasma MK level using the entire set of NBL cases including both the mass screening and sporadic cases. As predicted from our previous data,[15] we found that MK level is correlated with established prognostic factors (*MYCN*, *TRKA*, ploidy, stage and age). Since

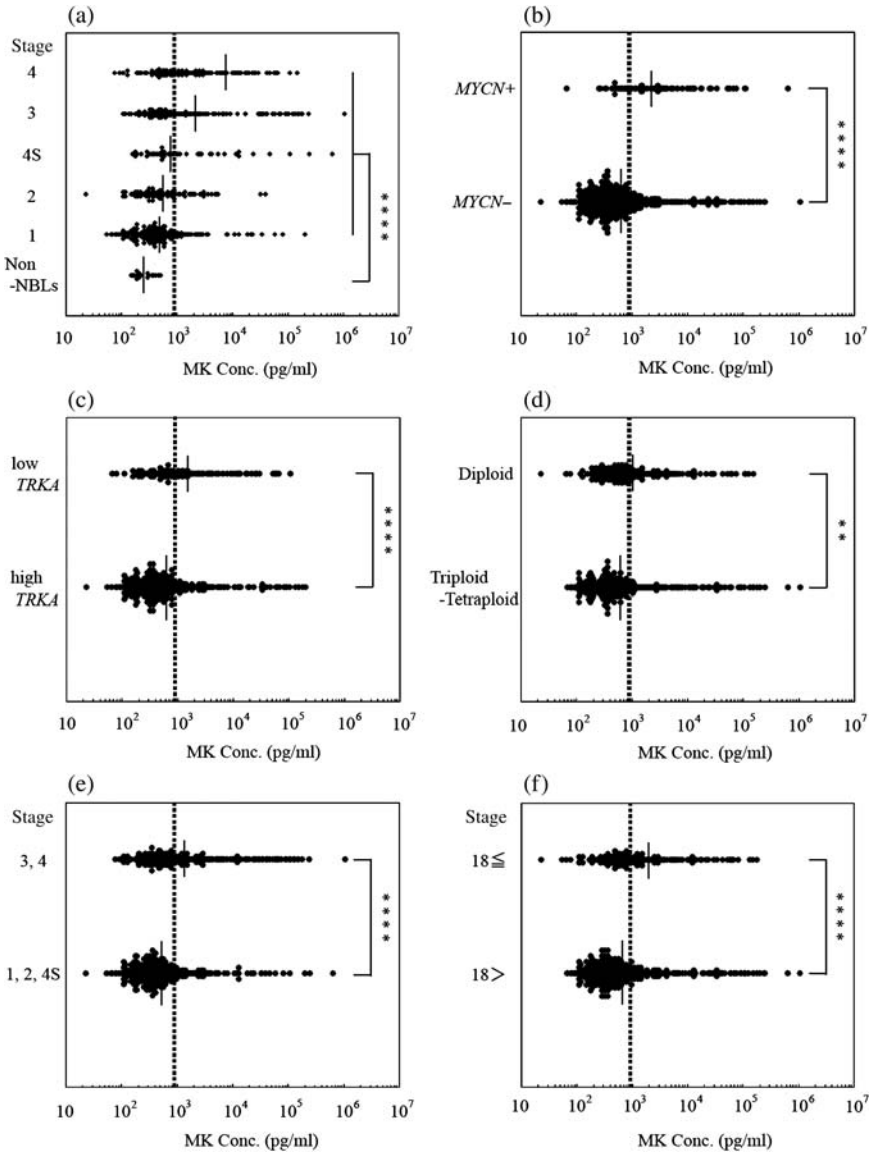

Fig. 1. Plasma midkine (MK) levels of the entire set of neuroblastoma (NBL) cases and the relationship of MK level to established prognostic factors for NBL. Blood MK levels are presented with dots. Each dot represents a NBL patient or a non-NBL control as indicated. (a) MK level distribution of the NBL patients through stages. Non-NBL, non-NBL controls. ****$P < 0.0001$. (b) NBL cases divided into *MYCN* amplification (MYCN+) and nonamplification (MYCN–). ****$P < 0.0001$. (c) NBL cases divided into low *TRKA* expression (low *TRKA*) and high *TRKA* expression (high *TRKA*). ****$P < 0.0001$. (d) NBL cases divided into diploid and triploid/pentaploid. **$P = 0.004$. (e) NBL cases divided into stage 3 or 4 (Stage 3, 4) and stage 1, 2 or 4S (Stage 1, 2, 4S). ****$P < 0.0001$. (f) NBL cases divided into age >18 months and <18 months. ****$P < 0.0001$.

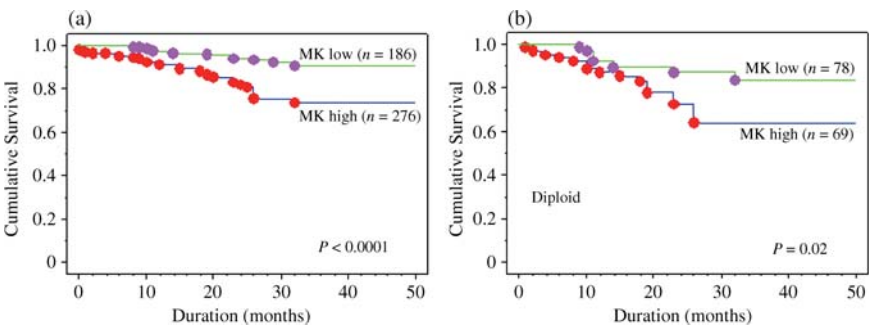

Fig. 2. Kaplan–Meier curves for neuroblastoma (NBL) cases. 'MK low' was defined as a blood midkine (MK) level less than 900 pg/mL, whereas 'MK high' was more than 900 pg/mL. Cumulative survival rates of MK low and high groups were estimated for the entire set of NBL cases (a) and cases with diploidy (b).

mass screening has been discontinued, sporadic NBL are the major subject of therapy. We therefore further evaluated the MK level of only the sporadic cases. Our study revealed that, within sporadic cases, blood MK level alone could be a predictor of prognosis. MK level was also significantly correlated with *MYCN* amplification and stages.

However, blood MK level could not predict prognosis of patients in the intermediate risk group (*MYCN* non-amplification and stage 3 or 4) (data not shown). It could not predict the prognosis of patients within the high-risk group or low-risk group either (data not shown). This indicates that a single molecule may not be satisfactory for predicting the prognosis or judging the precise status of NBL for the decision of therapy, since, like other carcinomas, a complex of molecules is thought to contribute to carcinogenesis and development of NBL.[17,18] There are several blood markers predicting clinical outcome of neuroblastoma patients; i.e. serum lactate dehydrogenase, ferritin, neuron-specific enolase, disialoganglioside GD2 and NM23H1.[19–23] Therefore,

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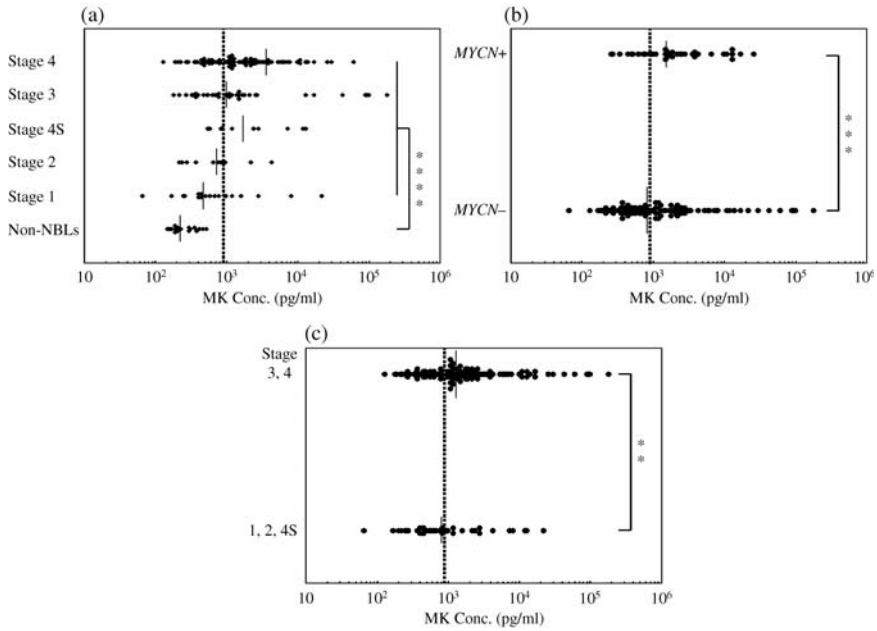

Fig. 3. Analysis for sporadic neuroblastoma (NBL) cases. Blood midkine (MK) levels of sporadic NBL cases are shown. (a) MK level distribution of the sporadic NBL patients through stages. Non-NBLs, non-NBL controls. ****$P < 0.0001$. (b) Sporadic NBL cases divided into *MYCN* amplification (MYCN+) and non-amplification (MYCN–). ***$P = 0.0005$. (c) Sporadic NBL cases divided into stage 3 or 4 (Stage 3, 4) and stage 1, 2 or 4S (Stage 1, 2, 4S). **$P = 0.003$.

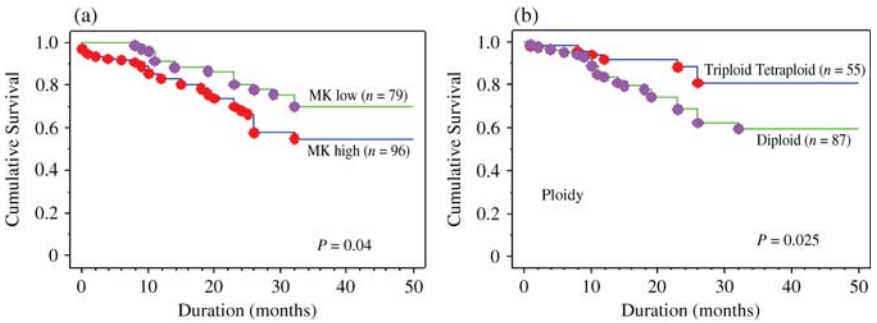

Fig. 4. Kaplan–Meier curves for sporadic neuroblastoma (NBL) cases. Cumulative survival rates of sporadic NBL cases were compared based on the following criteria. (a) Midkine (MK) low or high. (b) Diploid or triploid/pentaploid.

it is reasonable to expect that a combination of the plasma levels of MK and other blood biomarkers will facilitate accurate prognosis and accurate evaluation of tumor status. In addition, many efforts are being made to identify molecular changes associated with NBL with unfavorable prognosis.[17,18] Such studies will provide other biomarkers for NBL.

It is interesting that MK levels of stage 4s were lower in the present study than those in the previous study. Twelve cases were only available for stage 4s in the previous study. In the present study, 39 cases of stage 4s were available for the analysis of the entire set of NBL (Fig. 1a) and 15 cases for the sporadic NBL (Fig. 3a). Therefore, it is conceivable that midkine level deduced in the present study is more reliable because of the increased number of cases analyzed.

This is the first report indicating the plasma MK level as a prognosis factor for a human carcinoma. MK is frequently and highly expressed in malignant tumors regardless of the tissue type,[5] similar to mutations in the p53 gene. An elevated serum MK level is also detected in more than 80% of human adult carcinomas.[16] Although the MK level has not been evaluated as a prognosis factor for human carcinomas except for NBL, further assessment of the MK level will be useful in potentially establishing it as a new biomarker for other carcinomas.

Tumor growth is suppressed by the knockdown of MK expression.[13,14] MK is barely detectable in normal adult tissues. Furthermore, the present study has established that high blood MK level is closely related to poor prognosis, at least in NBL. Therefore, our data also support the idea that MK is a candidate molecular target for cancer therapy. Indeed, MK-deficient mice carrying a *MYCN* transgene show delayed development of NBL as compared with wild-type mice (Kishida and Kadomatsu, unpublished data). A therapy targeting MK for NBL is currently being studied in our laboratory.

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