(Prognostic risk grouping)

米国 Children's Cancer Group (CCG) study で取り扱われた手術後5年経過し，その間の予後が判明している腫瘍 (CCG-3881, low and intermediate risk group および CCG-3891, high risk group)500 例について，INPC による組織分類，発生年齢と disease free survival (DFS) の解析によって年齢を加味した組織型と予後との関連が以下のように決定された．

（1）神経芽腫について

i) high MKI を示す腫瘍は，腫瘍細胞の分化度，年令に拘わらず常に予後不良グループである．

ii) Undifferentiated NB は年齢に関係なく予後不良グループである．

iii) 1.5 歳以上の NB，poorly differentiated は予後不良グループである．

iv) 1.5 歳以上で発生した中等度 MKI を示す NB は予後不良グループである．

v) 上記以外の腫瘍は予後良好群である．

（2）GNB・nodular について

発生年齢と結節を形成する神経芽腫成分の分化度および MKI の程度によって予後が決定される．すなわち神経芽腫成分が上記（i）から（iv）までに相当する際は予後不良であるが，それ以外は予後良好群である．

（3）GNB・intermixed type, GN は年齢にかかわらず予後良好群である．

2. INPC と従来の分類との関連について

日本病理学会小児腫瘍組織分類委員会[6]の神経芽腫分類との比較は表3に，さらに同分類と Shimada 分類[7]との対応は表4に示した

おわりに

INPC の概要について概説した．本分類は，従来の分類とほぼ対応しており，理解されやすい．先に記したようにすでに多くの国々でこの組織分類に基づいて神経芽腫が分類されており，標準的な分類法として確立されつつある．わが国でもこのほど改訂された日本病理学会小児腫瘍分類委員会による小児腫瘍組織分類アトラス「神経芽腫群腫瘍」(2004 年 3 月金原出版刊行)[5]でも INPC の分類を全面的に取り入れて詳細に解説した．従ってわが国においても本腫瘍の標準的な病理診断は今後 INPC に準じて行われることになる．なお，INPC の詳細はこのアトラスを参照して頂きたい．本分類が病理医をはじめわが国の小児腫瘍を携わる方々全てに広く理解され，活用されることを強く望むものである．

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Unusual Chromaffin Cell Differentiation of a Neuroblastoma After Chemotherapy and Radiotherapy: Report of an Autopsy Case With Immunohistochemical Evaluations

[Case Report]

Miyauchi, Jun MD*; Kiyotani, Chikako MD†; Shioda, Yoko MD†; Kumagai, Masaaki MD†; Honna, Toshiro MD‡; Matsuoka, Kentaro MD*; Masaki, Hidekazu MD §; Aiba, Motohiko MD[IPN]; Hata, Jun MD¶; Tsunematsu, Yukiko MD†

From the Departments of *Clinical Laboratory, †Hematology/Oncology, ‡Surgery, and § Radiology, National Children's Hospital, Tokyo, Japan; ¶National Research Institute for Child Health and Development, Tokyo, Japan; and [IPN]Department of Pathology, Tokyo Women's Medical University Daini Hospital, Tokyo, Japan.

Reprints (present address): Jun Miyauchi, MD, Department of Clinical Laboratory, National Center for Child Health and Development, 2-10-1 Okura, Setagaya-ku, Tokyo 157-8535, Japan (e-mail: miyauchi_j@nechd.go.jp).

Abstract:

Neuroblastomas are derived from neural crest cells that are capable of multilineage differentiation. Ganglionic neuronal differentiation of childhood neuroblastoma is seen with increasing age, leading to more differentiated tumors called ganglioneuroblastomas or ganglioneuromas. Despite the fact that neuroblastomas most often arise from the adrenal medulla, chromaffin-cell differentiation in neuroblastomas is not widely recognized. Tumor cells with a chromaffin-cell nature have only been detected using histochemical techniques in neuroblastoma cell lines or focal areas of certain in vivo tumors. We describe a neuroblastoma that exhibited an unusual differentiation toward chromaffin cells in a patient that had been treated with surgery, intensive chemotherapy, and radiotherapy. Although a biopsy specimen of the retroperitoneal primary tumor was extensively necrotic, possibly because of a previous chemotherapy regimen, surgically resected metastatic tumors of bilateral ovaries were viable and diagnosed as poorly differentiated neuroblastomas according to the International Neuroblastoma Pathology Classification system. However, metastatic tumors of bilateral lungs

examined at the time of autopsy exhibited histologic features similar to those of a pheochromocytoma/paraganglioma, and immunohistochemical examinations demonstrated that these tumors were composed of extra-adrenal chromaffin cells. This case confirms that neuroblastomas in childhood can transform into pheochromocytoma/paraganglioma-like tumors under special conditions.

Neuroblastoma is the most common extracranial solid cancer that occurs during infancy and childhood.[4] Neuroblastomas are derived from embryonic neural crest cells [20] and can differentiate along the sympathetic neuronal cell pathway with increasing age. Depending on the degree of differentiation and the amount of Schwannian stromal components, neuroblastic tumors are classified into three major categories: neuroblastoma, ganglioneuroblastoma, and ganglioneuroma.[22,23] In contrast to these well-known neuronal differentiation patterns, chromaffin-cell differentiation in neuroblastomas is not widely recognized, although some investigators have histochemically demonstrated a chromaffin nature in neuroblastoma cell lines [7] as well as in focal areas of extra-adrenal tumors.[9,12,13] Despite these observations, the differentiation of neuroblastomas exclusively toward chromaffin cells is extremely rare, and only one such tumor has been previously described.[15] Here, we report a case of childhood neuroblastoma originating from the retroperitoneum with bilateral ovary metastases that were histologically diagnosed as ordinary neuroblastoma. An autopsy, performed after intensive chemotherapy and radiotherapy, revealed metastatic tumors of the lung consisting of differentiated chromaffin cells.

CASE REPORT

The patient was a 4-year-old girl who was admitted to a hospital in China complaining of abdominal distension. Histologic examinations of needle biopsy specimens from the abdominal tumor, serum analysis data (including an elevation in vanillylmandelic acid), and radiographic evaluations suggested the presence of a stage 3 neuroblastoma (International Neuroblastoma Staging System [5]). A urinary test performed in the neuroblastoma mass screening program in Japan when the patient was 6 months old had been negative. After receiving one course of chemotherapy (cyclophosphamide, 0.53 g/m^2; vincristine, 0.66 mg/m^2 ×2; THP-adriamycin, 40 mg/m^2), she was transferred to our hospital about 1 month after the onset.

A meta-iodobenzyl-guanidine scintigram revealed accumulations of radioactivity in the left renal hilus, pelvic cavity (later determined to be the left ovary), paraaortic lymph nodes, and spinal bone marrow. A computed tomographic (CT) scan revealed a retroperitoneal primary tumor and a large mass lesion in the pelvic cavity. The tumor markers were elevated as follows: neuron specific enolase = 12.3 ng/mL (normal range, <=6.0 ng/mL), vanillylmandelic acid = 255.6 μg/mg Cr (normal range, 3.5–15 μg/mg Cr), and homovanillic acid = 121.9 μg/mg Cr (normal range, 4.5–20 μg/mg Cr). Her left ovary was surgically resected because of massive enlargement, suggesting tumor metastasis, and a biopsy specimen was taken from the retroperitoneal primary tumor. The N-*myc* gene was not amplified in the resected tumor sample. After surgery, four courses of chemotherapy according to the Regimen 98A$_3$ protocol (cyclophosphamide, 1.2g/m^2 ×2; vincristine, 1.5 mg/m^2 ×1; THP-adriamycin, 40 mg/m^2 ×1; cisplatin, 25 mg/m^2 ×5 *c.i.*) of the Study Group of Japan [21] were performed, but the meta-iodobenzyl-guanidine scintigram remained positive. Following the fifth course of chemotherapy, the retroperitoneal primary tumor, the metastatic tumor in the right ovary, and the lymph nodes were surgically removed 6 months after the initial surgery. Although the values of all the

tumor markers decreased to within a normal range thereafter, the meta-iodobenzyl-guanidine scintigram still revealed an uptake of radioactivity, so an additional four courses of chemotherapy and radiation therapy were performed. The patient was scheduled to receive a bone marrow transplantation, but a chest x-ray and CT scan revealed multiple, fine, nodular lesions in bilateral lungs, and values of her tumor markers began to increase once again. After treatment with total body irradiation (12 Gy), the patient received a stem cell transplantation using umbilical cord blood, but she died of complications from the transplantation, including graft-versus-host disease and a brain hemorrhage, at the age of 5 years.

METHODS

Immunohistochemistry

An indirect immunohistochemical analysis was performed using formalin-fixed, paraffin-embedded tissue sections and the standardized streptavidin-biotin peroxidase complex method (DAKO-LSAB; Dako Japan, Kyoto, Japan) with 3,3'-diaminobenzidine as a chromogen. When required, antigen retrieval was performed according to the manufacturer' s instructions. The sources and clones of the primary antibodies that were used are listed in Table 1.

Table 1. Panel of Primary Antibodies Used in Immunohistochemistry

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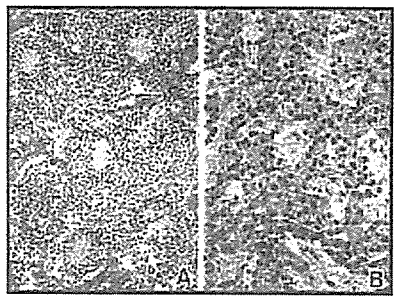
RESULTS

Pathologic Findings of the Surgical Materials

The left ovarian tumor, surgically resected when the patient was 4 years old, measured 12.5 × 11.5 × 8.0 cm in diameter and weighed 610 g. The cut surface of the tumor was solid, grayish brown in color, with scattered foci of necrosis. The tumor consisted of small neuroblasts with round nuclei, forming neuropils and rosettes (Fig. 1A), corresponding to a poorly differentiated neuroblastoma according to the International Neuroblastoma Pathology Classification system,[22,23] although this evaluation was made after the patient had received chemotherapy. The tumor contained thin fibrovascular stroma that partially formed a lobular architecture. The biopsy material from the retroperitoneal primary tumor, obtained simultaneously at the time of surgery, consisted of necrotic tissue with no viable tumor cells, possibly due to the chemotherapy performed in China.

FIGURE 1. Histology of the tumors. **A:** The metastatic left ovarian tumor consists of small round cells forming neuropils and rosettes, a typical histologic appearance of neuroblastoma (original magnification, ×36). **B:** The metastatic lung tumor observed at autopsy consists of compact sheets of cells with a



deeply basophilic cytoplasm and fibrovascular stroma, a histologic appearance similar to that of pheochromocytoma/paraganglioma (original magnification, ×60).

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The right ovarian tumor, resected 6 months later, measured 4.0 × 2.7 × 2.5 cm in diameter and weighed 18 g, with its cut surface being solid and grayish brown in color. The tumor consisted of compact nests of small round neuroblasts forming neuropils, with few rosettes, and was histologically similar to the left ovarian tumor. The retroperitoneal primary tumor, resected together with the right ovary, measured 4 × 3 cm in diameter on the cut surface, exhibited extensive necrosis with calcification and hemosiderosis, and contained only tiny nests of viable neuroblastoma cells at the periphery. The left adrenal gland adjacent to the primary tumor was intact with no tumor involvement, indicating that the tumor had arisen from the retroperitoneum.

Autopsy Findings

An autopsy was performed about 8 hours after the death of the patient. Extensive dissemination of the tumor was present in bilateral lungs. The metastatic lung tumors consisted of small nodules, measuring up to 5 mm in diameter, located mainly on the pleura and in the interlobular connective tissue. The tumors were composed of solid, compact sheets of epithelial-like cells with a deeply basophilic cytoplasm and single, round to oval nuclei (Fig. 1B). The tumor stroma consisted of fine vascular channels surrounded by a small amount of fibrous tissue. These characteristics are similar to those for pheochromocytoma and paraganglioma. Neuroblasts, ganglion cells, and Schwann cells were absent. Microscopic tumor metastases were also found in the left kidney, pancreatic head, and paraaortic lymph nodes. The histologies of these metastases were basically similar to those in the lung, although degenerative changes and/or necrosis made a definite histologic diagnosis difficult.

Immunohistochemistry

Immunohistochemistry showed that the left ovarian metastatic tumor, resected during the initial surgery, was positive for Bcl-2. The tumor was also positive for chromogranin A (CGA) (Fig. 2A), synaptophysin (Syn), and CD57 (HNK-1), but only in the neuropils at the center of the rosettes. Dopamine [beta]-hydroxylase (D[beta]H), tyrosine hydroxylase (TH), and insulin-like growth factor II (IGF-II) stained weakly and/or focally positive, while phenylethanolamine N-methyltransferase (PNMT) stained negative (Table 2). These results are consistent with the characteristics of a neuroblastoma. On the other hand, the metastatic lung tumors exhibited strong and diffuse positive staining for CGA (Fig. 2B), Syn, and TH, weak to moderate positive staining for D[beta]H and IGF-II, a very weak staining reaction for CD57, and negative staining

for Bcl-2 and PNMT (Table 2). These immunohistochemical staining patterns indicated that the metastatic lung tumors were composed of chromaffin cells with an extra-adrenal phenotype. The right ovarian tumor, resected during the second surgery, exhibited positive staining for both ganglion cell and extra-adrenal chromaffin cell markers (Table 2), although the histology of the tumor resembled that of the left ovarian neuroblastoma.

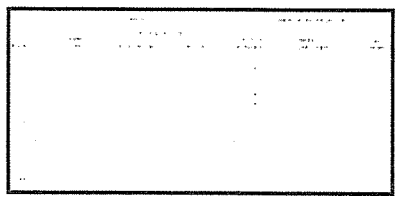

Table 2. Results of Immunohistochemistry*

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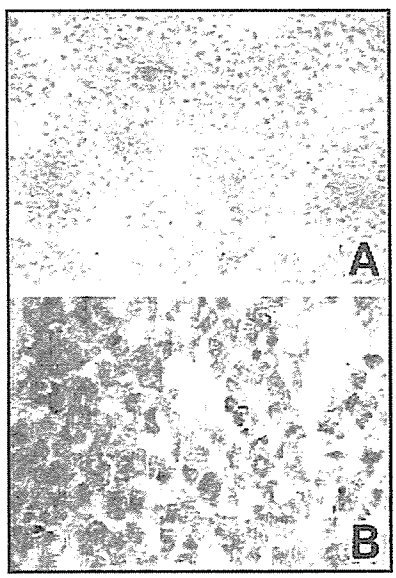

FIGURE 2. Immunohistochemical staining of chromogranin A (CGA). **A:** The metastatic left ovarian tumor is positive for CGA only in the neuropils at the center of the rosettes (original magnification, ×50). **B:** The metastatic lung tumor exhibits strong, diffuse cytoplasmic staining for CGA (original magnification, ×50).

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DISCUSSION

The adrenal medulla is derived from neural crest cells with a multilineage differentiation potential and is composed of heterogeneous cell types, including mostly chromaffin cells and a minor population of ganglion cells and Schwann-like supporting cells.8,20 Reflecting this complex cellular composition, pheochromocytoma, an adrenal medullary tumor of chromaffin cells usually seen in adults, occasionally exhibits a mixed phenotype, such as pheochromocytoma admixed with ganglioneuroma, ganglioneuroblastoma, neuroblastoma, Schwannoma, or melanocytic tumors.1-3,6,10,14,16,18,19,24 These tumors are called "composite" or "compound" tumors. On the other hand, childhood neuroblastomas are known

to differentiate along a sympathetic neuronal cell pathway with increasing age. However, the potential of neuroblastomas to differentiate along two lineages, not only neuronal cells but also chromaffin cells, has been demonstrated in neuroblastoma cell lines 7 and certain in vivo neuroblastomas 9,12,13: tumor cells with a chromaffin cell nature, detected as CGA- or IGF-II-positive cells, have been focally found in extra-adrenal neuroblastomas with a lobular architecture.12 The metastatic lung tumors in our patient arose from the extra-adrenal retroperitoneum and exhibited a lobular architecture and chromaffin cell differentiation, consistent with the above observation. Nevertheless, the conversion of a neuroblastoma to a pheochromocytoma/paraganglioma-like tumor, as seen in our case, is extremely unusual; to the best of our knowledge, a similar transformation has been described in only 1 other patient.9

Three cell types in the sympathetic neuroendocrine system, namely, adrenal and extra-adrenal chromaffin cells and sympathetic ganglion cells, can be differentiated using histochemical markers (Table 2). IGF-II is expressed in chromaffin cells but not in sympathetic neuronal cells.12,13 CGA and Syn are also useful markers of chromaffin cells, since they are strongly and diffusely expressed in chromaffin cells but only focally and weakly expressed in neuroblasts and ganglion cells.24 Bcl-2 is a marker of sympathetic neurons and is expressed in all neuroblastomas, whereas tumor cells undergoing neuroendocrine differentiation lose this antigen.11 CD57 (HNK-1) is a marker of fetal adrenal medullary ganglion cells, and its expression in neuroblastomas is uniformly associated with ganglionic differentiation but is lost with differentiation along chromaffin cell lineage.8,9,13 TH is present in all catecholamine-producing cells, while D[beta]H is expressed only in norepinephrine-producing cells and PNMT is only expressed in human adrenal medulla cells that convert norepinephrine to epinephrine.20,24 The immunohistochemical results in the present case showed that the metastatic lung tumors were of an extra-adrenal chromaffin cell lineage, although IGF-II was only weakly labeled. On the other hand, the left ovarian tumor, resected during the initial surgery, expressed antigens that were associated with ganglionic differentiation, consistent with a diagnosis of neuroblastoma. Interestingly, the histology of the right ovarian tumor, resected 6 months after the first surgery, was that of an ordinary neuroblastoma, while an immunohistochemical characterization revealed the features of both ganglionic and extra-adrenal chromaffin cells. Thus, this tumor exhibited intermediate characteristics of neuroblasts and chromaffin cells, indicating that the functional maturation of the chromaffin cells preceded any morphologic transformation.

The occurrence of an extra-adrenal pheochromocytoma as a secondary malignancy in an adolescent 15 years after the patient had been treated for a neuroblastoma has been documented.17 The pheochromocytomatous tumors in our patient, however, occurred as disseminated multiple lesions in bilateral lungs during the course of therapy, indicating that these tumors were metastatic but not a secondary malignancy. The reason why this neuroblastoma patient exhibited such an unusual differentiation pattern in her metastatic lesions, leading to a histology similar to that of a pheochromocytoma/paraganglioma, is not clear. Since the patient received intensive chemotherapy and radiotherapy, these therapies may have caused the unusual differentiation pattern by selecting special tumor clones with the capability of differentiating toward chromaffin cells. Similar phenotypic conversion has been observed in another patient 15: among the tumors that were treated with a monoclonal antibody against ganglioside G_{D2}, which is abundantly expressed on neuroblastomas, the only tumor that lost G_{D2} expression underwent pheochromocytomatous conversion, indicating a

possible association between tumor differentiation and the therapeutic treatment. The neuroblastoma in the present case was also unusual in that it metastasized to bilateral ovaries. A special genetic background may have been involved in the unique aspects of this case.

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Key Words: neuroblastoma; pheochromocytoma; paraganglioma; differentiation; chromaffin cell

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特　集

小児固形腫瘍の治療戦略

3. 神経芽腫の手術—これまでとこれから

筑波大学臨床医学系小児外科

金子　道夫

キーワード　神経芽腫，機能温存，臨床試験，グループスタディ

I. 内容要旨

筑波大学では1985年以降，乳児神経芽腫にも進行神経芽腫にも軽減された手術を一貫して施行してきた．85年以降のマススクリーニング発見乳児例40例では，非手術の1例を除き原発巣切除＋リンパ節サンプリングを施行し，全例無病生存，手術による合併症は全く認められない．一方，進行神経芽腫には厚生省班研究プロトコールで治療を行い，初期治療終了後，次に引き続く化学療法に影響を与えないよう，原発巣切除と2cm以上のリンパ節切除・サンプリングを行い，ほぼ全例に術中照射を併用した．これまで20例にこのような手術を施行し，局所再発は4例で，stage 4の5年生存率は70%，10年生存率は62%と良好であった．局所再発が死因となった症例はなかった．現時点では系統的リンパ節郭清を伴う徹底した切除が手術の主流であるが，2005年より局所治療を超大量化学療法終了後，すなわち治療の最後に行う治療方式の臨床試験が行われる．手術も軽減化コンセプトで施行することとなった．化学療法と局所治療を分けて施行し，お互いの干渉を避けるこのプロトコールコンセプトにより，局所治療の進行神経芽腫全体の治療に占める意義が明らかにされる可能性がある．

II. はじめに

神経芽腫の治療は限局性腫瘍には手術が，転移性腫瘍には化学療法で原発巣・転移巣，特に後者をコントロール後に腫瘍全摘とリンパ節郭清が行われ，施設によりその後に放射線治療や幹細胞移植を伴った抗癌剤大量療法が行われている．我が国では限局性腫瘍であっても遺残腫瘍がある場合には化学療法が行われてきたが，最近の欧米での限局性腫瘍に対する治療方針は多少の遺残腫瘍があっても，手術のみで90%以上の長期生存が得られており，手術のみで十分であると報告されている．1985年に全国的に施行開始された神経芽腫マススクリーニングは2004年に中止となったが，その理由として進行症例の減少，少なくとも神経芽腫の死亡例の大幅な減少が得られなかったこととに加え，発見された神経芽腫患者に行われた治療が結果的に過大となった可能性が指摘された．そこで，現時点での神経芽腫治療の問題点特に外科治療の問題点とこれからの解決法について述べる．

III. 神経芽腫の手術の現状と問題点

1　限局性腫瘍と乳児神経芽腫での問題点

乳児神経芽腫では進展症例は少なく，手術可能であることが多い．しかし，椎間孔から脊柱管内に広範囲に進展したり，stage 3では腹部の主要血管を取り巻いて摘出困難な場合も少なからずある．しかし，治療成績はstage 1，2のみでなく，stage 3もMYCN増幅がない限りほぼ100%近い．乳児例ではマススクリーニング症例を中心にグループスタディが行われ，まずstage 1，2で治療の軽減がはかられた．さらに最近はstage 3でも化学療法の軽減化スタディが進行中である．乳児期の治療成績が良いのは腫瘍自体がもつ特性によるところが大きく，治療の要素は相対的に少ない．これらの患者群に全摘を目指した過大な手術をする必要性は乏しい．できるだけ小さな侵襲で原発腫瘍を切除する，

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Michio Kaneko
Department of Pediatric Surgery, University of Tsukuba, Tsukuba, Japan

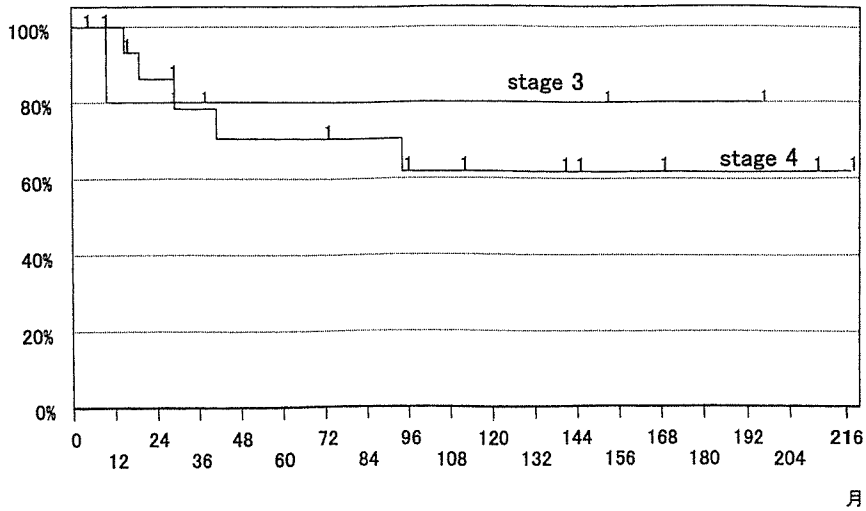

図　病期3，4　手術例の生存曲線

それ以上の手術が必要かどうかは疑わしい．stage 1，2に関しては全摘(通常容易)，stage 3でも簡単な切除でよいと考えられるが，前向き臨床試験が必要である．筑波大学では1985年以降40例のマススクリーニング症例に対し，当初から原発腫瘍切除＋リンパ節サンプリングという手術方針で，化学療法は乳児グループスタディに従って行った[1]．その結果，腹腔動脈を取り囲むstage 3で化学療法を先行して腫瘍がほぼ消失した非手術例1例を除く39例に手術が行われた．腎はすべて温存され，再発・再手術を認めず，全例後障害なく無病生存している．

非マススクリーニング例では頸部縦隔の2例が気道圧迫で手術を行い，うち1例が急速な再増殖による気道圧迫で窒息し，7年後に肺炎で死亡した．3カ月以下の急速進展例では手術を含め治療に難渋する症例がある．しかし，乳児期の神経芽腫の手術を含めた治療の基本は，治療し過ぎないことであろう．

2　進行症例に対する手術　—筑波大学の経験を中心に—

腹部原発の進行神経芽腫は原発巣とリンパ節が一塊となり大動脈とその主要分岐を取り巻くように進展し，全摘はきわめて困難である．いわゆる癌の根治手術は不可能である．化学療法により腫瘍が縮小すると易出血性であった腫瘍は硬化して出血は少なくなるが非常に硬くなり，脈管からの剝離は難しく，血管の外膜層で剝離するのがもっとも容易で，腫瘍の「全摘」に近い手術が可能である．現在，我が国でも欧米でもこのような手術が行われている．しかし，それでも全摘は困難で手術による障害も多くなる．

筑波大学では85年から厚生労働省班研究プロトコールに従って化学療法を行い，少なくとも3クール以降，多くは6クールを終了してから手術を行っている．術中照射を併用し，手術法は原発巣全摘，リンパ節は主要な血管を損傷しないようにしてなるべく切除，90年頃からは大きいものをサンプリングするにとどめた．手術した22例での結果は，大きなリンパ節が摘出困難な2例で，骨転移などが遠隔再発したときに残存リンパ節の増大がみられた．また，照射野外からの再発が手術野まで進展した例など，結局4例で「局所再発」が認められた．術後2年以上の観察で腫瘍死は6例，stage 4の5年生存率70%，10年生存率が61%であった(図)．「局所再発が」死亡の原因となった症例はなかった．腫瘍の腎進展で無機能となった1例を除き，全例で両側腎が温存され，2例の腸閉塞を除き合併症，後障害を認めていない．また，術中照射による椎体，脊髄の障害もない．

IV. 進行神経芽腫に対する適切な手術をめざして

欧米でも進行例に系統的リンパ節郭清を行う意義を疑問視する外科医は少なからず存在する．これまで，適切な局所治療の臨床試験は困難とされていたが，2005年から開始される進行神経芽腫に対する臨床試験では局所治療を治療の最後におき，手術の方式も統一した．これにより，局所治療の意義を明らかにし，さらに，治療の最後に摘出した腫瘍の病理組織と再発との関連

を見ることができるプロトコールとした．以下にそのプロトコールの外科治療の部分を掲載する．

1　外科治療

神経芽腫の診断・治療において外科治療の果たす役割は生検による組織採取にとどまらず，化学療法や放射線療法とならび根治的治療に関わる積極的な役割を担っていることは本腫瘍を扱う治療医の多くが認めるところである．しかし，進行神経芽腫に対する外科治療の実際は個々の症例やそれぞれの外科医によりばらつきがあり，わが国では進行神経芽腫自体の症例数が多くないこともあり，エビデンスに基づいた同腫瘍の外科治療指針を示すことができない．従って，そのエビデンスを作ることもこのプロトコールの目的とするところである．

神経芽腫では発生部位やリスク分類に応じた綿密な治療戦略を立てることが重要である．従って，治療前の腫瘍生検は従来から必須とされてきた．一方，high-risk 群に分類される 1 歳以上の病期 3，4 の神経芽腫は，化学療法で縮小が得られても完全摘除が非常に難しく，厳密には完全摘除は不可能といっても誤りではない．しかし，多くの施設でより高い根治性を目指して可能な限り切除を行うことに努力してきた[2)~5)]．神経芽腫の進展様式からいって，完全切除を目指す手術は血管およびその周囲の神経組織，リンパ管の損傷を伴い，術後腹部主要臓器の血行障害，腸管の運動障害，術後の大量リンパ瘻，呼吸不全の問題を常に伴っていた[6)7)]．特に，腎の血行障害は化学療法剤の変更，減量，さらに引き続いて施行される大量化学療法のリスクを大きく左右する．手術のこれらの問題は術後化学療法の開始時期の遅れ，投与抗癌剤の減量，腎障害など，術後治療のばらつきの大きな要因になり，適切な治療法を明らかにする臨床試験の遂行を妨げる大きな要因となった．

いくつかの施設では術中照射法や術後照射と組み合わせることにより，より侵襲の少ない手術とする治療方針をとり，それにより局所再発率を高めることはないとの報告が見られる[8)~10)]．局所療法を可能な限り同一化して，プロトコール全体での治療の揺らぎを小さくした．

2　外科治療に関する一般的事項

1）生検

治療に先立って組織学的診断と同時に腫瘍の生物学的特性の評価や遺伝子検索のため，凍結保存などが行えるよう可能な限り十分量の組織の採取を行う．すなわち少なくとも 1cm 角相当の腫瘍を採取することが望ましく，針生検による腫瘍採取は本ガイドラインでは推奨しない．以下省略．

2）原発巣摘除と機能温存

進行神経芽腫は治療成績がまだ不良とはいえ，5 年生存率は 40% に達する．従って，局所再発の頻度を上げることなく機能温存を考慮することは重要である．また，治療中の合併症を少なくし，治療を予定通り完遂するためにも短期合併症を防止することは治療成績向上に資すると考えられる．以下に進行神経芽腫の外科治療ガイドラインを掲げる．

3）摘出術ガイドライン

1）原発巣に関して

原発部位に関わらず，原則として周囲臓器をできるだけ温存して原発巣を全摘出する．原発巣と一塊になったリンパ節は原発巣とともに切除を目指す．

1-1　副腎，後腹膜原発

肝・腎に関しては，手術時に viable と見られる浸潤がある場合は，部分合併切除を行う．

1-1-2　機能のある腎は温存する．腎血管を巻き込んでいて剝離が困難な場合，腫瘍被膜内切除にて腎血管を温存し，腎合併切除を極力避ける．腎動脈の攣縮にはキシロカインを浸したガーゼで包み，攣縮を軽減しつつ手術を続行し，腎温存に努める．

1-1-3　広範な腎実質浸潤がある場合には，腎合併切除をする．腎合併切除を行っても，腫瘍全摘出困難な場合は，腎を温存して，できるだけ腫瘍切除を行う．

1-1-4　腹腔動脈や上腸間膜動脈などの腹部大動脈からの主要な血管を巻き込んでいて剝離が困難な場合は，腫瘍被膜内切除にて血管を温存してできるだけ腫瘍を切除するものとする．

1-1-5　脾臓への直接浸潤，あるいは，脾動静脈を巻き込んでいる場合，5 歳以上の症例では，脾合併切除による腫瘍摘出を行ってもよいが，5 歳未満の症例では，脾温存によるできるかぎりの腫瘍切除とする．

縦隔・頸部・仙骨前については省略．

2）リンパ節に関して

2-1-1　原則として系統的リンパ節郭清は行わないものとする．

2-1-2　転移リンパ節と思われる 2.0cm 以上のリンパ節は切除する．それ以下の大きさであっても肉眼・触診上で viable と見られる腫瘍があると考えられるリンパ節は切除する．

2-1-3　2.0cm 以上のリンパ節の腫大したリンパ節が

手術時にない場合，治療前に転移の見られた部位のリンパ節サンプリングを行う．

3) 外科登録と central review

本プロトコールに従い治療を行い生検以上の手術を行った場合，担当の外科医は手術記録と外科登録用紙(原発部位，病期，初回手術の内容などを記載)を手術後10日以内に事務局あてに送付する．

V. おわりに

神経芽腫治療における手術や放射線治療の意義付けは非常に困難であった．そこでこれら局所治療を最後に行う厳密な臨床試験により，局所治療の意義と局所残存腫瘍の再発に対する意義を明らかにすることができれば画期的なことと考えられる．その後に，局所治療の時期・方法を randomized clinical trial ができれば，進行神経芽腫治療における局所治療の意義はより一層明らかになる．

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PAST AND FUTURE ROLE OF SURGERY IN NEUROBLASTOMA

Michio Kaneko

Department of Pediatric Surgery, University of Tsukuba, Tsukuba, Japan

The significance of surgery in the treatment of neuroblastoma remains unresolved. Moderate surgical resection has been employed in both infantile and advanced neuroblastoma since 1985 at the University of Tsukuba Hospital. Resection of the original tumor accompanied by lymph node sampling was performed in 39 cases of infantile neuroblastoma detected by mass screening. The patients are alive without tumor and with no treatment sequelae. In 20 patients with advanced neuroblastoma aged 1 year or older, the same surgery was performed with intraoperative radiation after more than 5 cycles of induction chemotherapy. Four patients experienced local recurrence with recurrence of remote metastases which were the cause of patient death. The overall survival rate of patients with stage 4 neuroblastoma was 70% at 5 years and 62% at 10 years. A clinical trial in which local therapy will be performed after myeloablative chemotherapy will be carried out from 2005 to evaluate separately the efficacy of chemotherapy and local therapy avoiding their direct interference. This will elucidate the value of local therapy and also moderate surgical resection in the treatment of advanced neuroblastoma.

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ORIGINAL REPORT

Prediction of *MYCN* Amplification in Neuroblastoma Using Serum DNA and Real-Time Quantitative Polymerase Chain Reaction

Takahiro Gotoh, Hajime Hosoi, Tomoko Iehara, Yasumichi Kuwahara, Shinya Osone, Kunihiko Tsuchiya, Miki Ohira, Akira Nakagawara, Hiroshi Kuroda, and Tohru Sugimoto

From the Department of Pediatrics, Kyoto Prefectural University of Medicine, Graduate School of Medical Science, Kyoto; and Division of Biochemistry, Chiba Cancer Center Research Institute, Chiba, Japan.

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Address reprint requests to Takahiro Gotoh, MD, PhD, Department of Pediatrics, Kyoto Prefectural University of Medicine, Graduate School of Medical Science, Kamigyo-ku, Kyoto 602-8566, Japan; e-mail: takahiro_email:@yahoo.co.jp.

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ABSTRACT

Purpose

MYCN amplification (MNA) indicates a poor prognosis in neuroblastoma (NB) and is routinely assayed for therapy stratification. We aimed to develop a diagnostic tool to predict *MYCN* status using serum DNA, which, in cancer patients, predominantly originates from tumor-released DNA.

Patients and Methods

Using DNA-based real-time quantitative polymerase chain reaction, we simultaneously quantified *MYCN* (2p24) and a reference gene, *NAGK* (2p12), and evaluated *MYCN* copy number as an *MYCN/NAGK* (*M/N*) ratio in 87 NB patients whose *MYCN* status had been determined by Southern blotting. Of these patients, 17 had *MYCN*-amplified NB, and 70 had nonamplified NB.

Results

The serum *M/N* ratio in the MNA group (median, 199.32; range, 17.1 to 901.6; 99% CI, 107.0 to 528.7) was significantly ($P < .001$) higher than the ratio in the non-MNA group (median, 0.87; range, 0.25 to 4.6; 99% CI, 0.82 to 1.26; Mann-Whitney *U* test). The sensitivity and specificity of the serum *M/N* ratio as a diagnostic test were both 100% when the serum *M/N* ratio cutoff was set at 10.0. Among six MNA patients whose clinical courses were followed, the serum ratios decreased to the normal range in the patients in remission (n = 3), whereas the ratios increased to high levels in the patients who relapsed (n = 2) or failed to achieve remission (n = 1).

Conclusion

Measurement of the serum *M/N* ratio seems to be a promising method for accurately assessing *MYCN* status in NB, although a larger set of patients needs to be examined to confirm this result.

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INTRODUCTION

Neuroblastoma (NB) is the most common extracranial solid tumor in children and is characterized by a wide range of clinical behaviors, from spontaneous regression to rapid progression with a fatal outcome. The clinical heterogeneity has been reported to be associated with a variety of biologic features of NB. One such aberration, *MYCN* amplification (MNA; ie, creation of multiple copies of the *MYCN* gene in the nuclei of tumor cells), is strongly associated with rapid tumor progression and a poor outcome. MNA is detected in 4% of patients in the early stages of NB, 8% of patients in stage 4S, and approximately 30% of patients in advanced stages. Currently, assessment of *MYCN* status is essential for determining therapy stratification in NB.[1-6] Having rapid access to selected biologic data for each tumor has become increasingly important in

routing patients to appropriate therapies. Several years ago, fluorescence in situ hybridization (FISH) replaced Southern blotting as the most accurate and timely way of evaluating tumors for MNA. Using FISH, the turnaround time for results was shortened from weeks to days, making its use in clinical trials realistic.

In this study, we describe a real-time polymerase chain reaction (PCR) method for evaluating *MYCN* status that shortens the turnaround for results to just a few hours. Furthermore, to facilitate the evaluation of *MYCN* status of tumors, we used serum DNA for the PCR template, which, in cancer patients, predominantly consists of tumor-released DNA.[7] Quantification of serum DNA has also been proposed as a screening tool for early detection of lung cancer.[8] Several groups were able to detect tumor-related aberrations, such as loss of heterozygosity and mutations in the *p53* gene, using the serum DNA of patients with a malignant tumor.[9-11]

Recently, Combaret et al[12] reported that high levels of *MYCN* DNA were present in the peripheral blood of patients with *MYCN*-amplified NB. However, they evaluated serum *MYCN* (2p24) dosage based on PCR without a reference gene, so their assay could be influenced by the quality of the template DNA or a numerical change of chromosome 2. To avoid these problems, we used DNA-based real-time quantitative PCR and a single copy reference gene, the *N-acetylglucosamine kinase* gene (*NAGK*; 2p12), so that *MYCN* copy number per chromosome 2 could be evaluated as the *MYCN/NAGK* (*M/N*) ratio. *NAGK* was chosen because it is on the same chromosome as *MYCN* but sufficiently distant from the region spanned by the *MYCN* amplicon (2p12 *v* 2p24)[13] that a numerical change in chromosome 2 would not affect the *M/N* ratio. The diagnostic performance of the test was evaluated in patients with an NB whose *MYCN* status had been determined by Southern blotting.

PATIENTS AND METHODS

Subjects

Eighty-seven patients diagnosed with NB at the Hospital of the Kyoto Prefectural University of Medicine and Chiba Cancer Center Research Institute were enrolled onto this study with the informed consent of their parents. The studies were conducted under research protocols approved by each institutional review board. At the time of diagnosis, 44 patients were younger than 1 year, and 43 were between 1 and 13 years of age. Seventeen of the patients had MNA, and 70 patients did not have MNA, as determined by Southern blotting. According to the International Neuroblastoma Staging System,[4] the 17 children with MNA included one patient each in stage 1 and 2B, two in stage 3, and 13 in stage 4, whereas the 70 children without MNA included 22 in stage 1, 18 in stage 2A and 2B, five in stage 4S, seven in stage 3, and 18 in stage 4.

Twelve of the 17 patients with MNA and 33 of the 70 nonamplified patients were also analyzed by dual-color FISH technique, as previously described,[14] using an *MYCN* probe (pNb101) and a chromosome 2 centromere probe (D2Z). FISH results of these patients were consistent with the Southern blotting results, although three of the patients who were diagnosed as non-MNA by Southern blotting were found to have one to four extra copies of the *MYCN* gene relative to the chromosome 2 centromere number by FISH. This low level of amplification has been defined as *MYCN* gain, which is an intermediate stage between MNA and non-MNA.[15] Because the prognostic significance of *MYCN* gain is still unclear, these patients were classified as non-MNA according to the Southern blotting results.

Sample Preparation

Tumor specimens were surgically resected and immediately stored at −80°C. Peripheral blood was obtained from each patient before any therapy and surgery. To avoid contamination of serum DNA by the DNA from WBCs, we prepared serum exclusively from the liquid fraction of clotted blood after centrifugation at 1,000 × *g* for 10 minutes and stored it at −20°C until DNA extraction.

DNA Isolation

DNA was extracted from tissues and serum samples by using the QIAmp tissue and blood kits (Qiagen, GmbH, Hilden, Germany), respectively, according to the manufacturer's protocols. For each patient, 200 μL of the stored serum was used for extraction of free DNA.

Real-Time Quantitative PCR

TaqMan PCR was performed using the ABI Prism 5700 Sequence Detection System (Applied Biosystems, Foster City, CA). The PCR mixture contained TaqMan universal PCR master mix (Applied Biosystems), 200 nmol/L of each primer, and 100 nmol/L of fluorogenic probe. The principle of the TaqMan analysis has been described previously in detail.[16-18] In addition to the *MYCN* sequence, *NAGK* (GenBank accession No. NM 017567) located at 2p12 was simultaneously measured as a single-copy reference gene. The sequence of primers and the TaqMan probe used for *MYCN* and *NAGK* are as follows: *MYCN* forward, 5′-GTGCTCTCCAATTCTCGCCT-3′; *MYCN* reverse, 5′-GATGGCCTAGAGGAGGGCT-3′; *MYCN* probe, 5′-FAM-CACTAAAGTTCCTTCCACCCTCTCCT-TAMRA-3′; *NAGK* forward, 5′-TGGGCAGACACATCGTAGCA-3′; *NAGK* reverse, 5′-CACCTTCACTCCCACCTCAAC-3′; and *NAGK* probe, 5′-VIC-TGTTGCCCGAGATTGACCCGGT-TAMRA-3′. All PCR reactions were performed with one cycle of 95°C for 5 minutes, followed by PCR amplification with 50 cycles of 95°C for 15 seconds and 60°C for 1 minute. Standard curves were constructed in each PCR run with four-fold serial dilutions containing 20, 5, 1.25, 0.3125, and 0.078125 ng/μL of a healthy donor's DNA in addition to 20 ng/μL of salmon sperm DNA, and the dosages of the target genes in each sample were interpolated using these standard curves. The *MYCN* copy number of a sample of DNA was determined by the ratio of the *MYCN* dosage to the *NAGK* dosage (*M/N* ratio). Copy numbers were expressed as the average of two measurements.

Effect of WBC Contamination

To assess the effect of WBC contamination in serum samples on the serum *M/N* ratio, we measured the serum *M/N* ratio using DNA extracted from a series of WBC-contaminated serum samples. The samples were prepared by adding 0, 1 × 10, 1 × 10^2, 1 × 10^3, 1 × 10^4, and 1 × 10^5 of WBCs from a healthy donor to 200 μL of serum from a *MYCN*-amplified patient.

Statistical Methods

The difference in the serum *M/N* ratio between the MNA and non-MNA groups was assessed using the Mann-Whitney *U* test. $P < .05$ was judged as significant.

RESULTS

Serum M/N Ratio As a Predictor of MYCN Status of Tumor

Serum *M/N* ratios could be determined in approximately 4 hours by real-time quantitative PCR. Figure 1 shows the distribution of the serum *M/N* ratio in the MNA and non-MNA groups at the time of diagnosis. The serum *M/N* ratio in the MNA group (n = 17; median, 199.32; range, 17.1 to 901.6; 99% CI, 107.0 to 528.7) was significantly ($P < .001$) higher than the ratio in the non-MNA group (n = 70; median, 0.87; range, 0.25 to 4.6; 99% CI, 0.82 to 1.26). In fact, there was no overlap between the two groups in the limited number of patients examined in this study. As a cutoff for the serum *M/N* ratio to distinguish between MNA and non-MNA patients, we empirically chose a value of 10, which was in the middle of the two ranges. With this value, the sensitivity and specificity of the serum *M/N* ratio as a diagnostic test to distinguish patients with MNA from those without MNA were both 100% for our limited number of patients. That is, the serum *M/N* ratio was in complete agreement with the Southern blotting results. The positive and negative predictive values were 100%. The serum *M/N* ratios were also consistent with results obtained by FISH for 45 of the patients (FISH analyses were performed in 12 of the 17 MNA patients and in 33 of the 70 nonamplified patients). Three of the patients who had one to four extra copies of the *MYCN* gene relative to chromosome 2 centromere number, as determined by FISH, also had slightly elevated serum *M/N* ratios (2.5, 3.3, and 4.6).

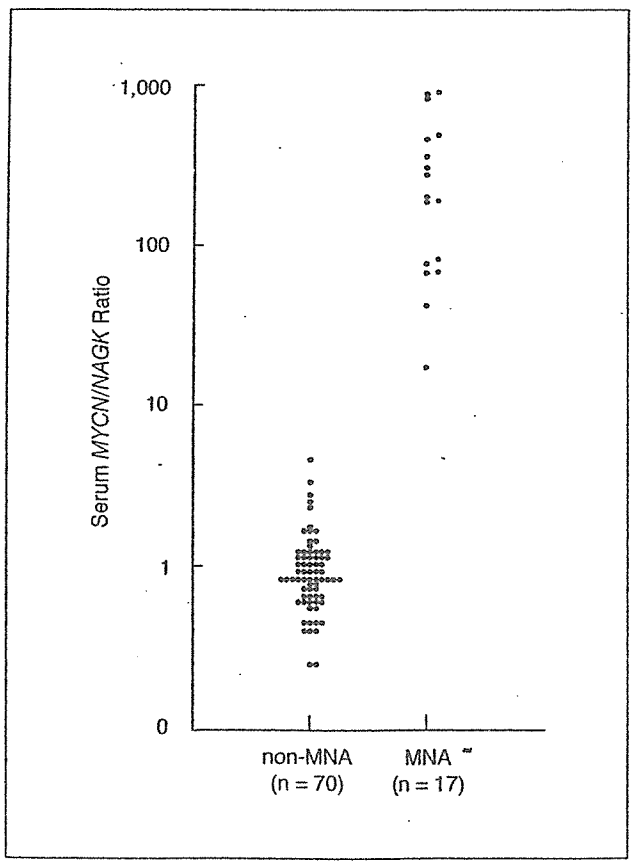

Fig 1. A scatter plot of serum *MYCN/NAGK* ratio in patients with *MYCN*-amplified (MNA) and nonamplified (non-MNA) neuroblastoma. The serum *MYCN/NAGK* ratio was significantly ($P < .001$) higher in the MNA group (median, 199.32; range, 17.1 to 901.6; 99% CI, 107.0 to 528.7) than in the non-MNA group (median, 0.87; range, 0.25 to 4.6; 99% CI, 0.82 to 1.26; Mann-Whitney *U* test).

Change in Serum M/N Ratio Levels During Follow-Up

To evaluate whether an increase in the serum *M/N* ratio can be used as an indicator of relapse, we measured serum *M/N* ratios at several points in the clinical courses of six patients with MNA (Fig 2). In three patients who were in complete remission (patients 1, 2, and 3), the serum *M/N* ratios decreased to the normal range and were consistently low. In contrast, in one patient who failed to achieve remission (patient 4), the serum *M/N* ratio did not decrease to the normal range and remained at a high level until his death. In the other patients who experienced recurrence after remission (patients 5 and 6), the serum *M/N* ratio first decreased to the normal range and then increased beyond the cutoff value by the time of diagnosis.

Effect of WBC Contamination on Serum M/N Ratio

We found that a high serum *M/N* ratio could be masked by the presence of WBC. The *M/N* ratio of serum from an *MYCN*-amplified patient decreased with increasing WBC contamination (Fig 3). When 200 μL of serum was contaminated with 1×10^5 of WBC, corresponding to approximately one fortieth of the WBC concentration in normal whole blood, the serum *M/N* ratio decreased below the cutoff level.

DISCUSSION

Serum markers, such as ferritin,[19] lactic dehydrogenase,[20] and neuron-specific enolase,[21] have been proposed as prognostic markers of NB, although they have shown little prognostic value. Recently, elevated levels of plasma midkine have been reported to correlate with a poor prognosis. However, the significance of this finding is controversial because plasma midkine levels are highest in stage 4S patients.[22] Therefore, a noninvasive assay of tumor-related

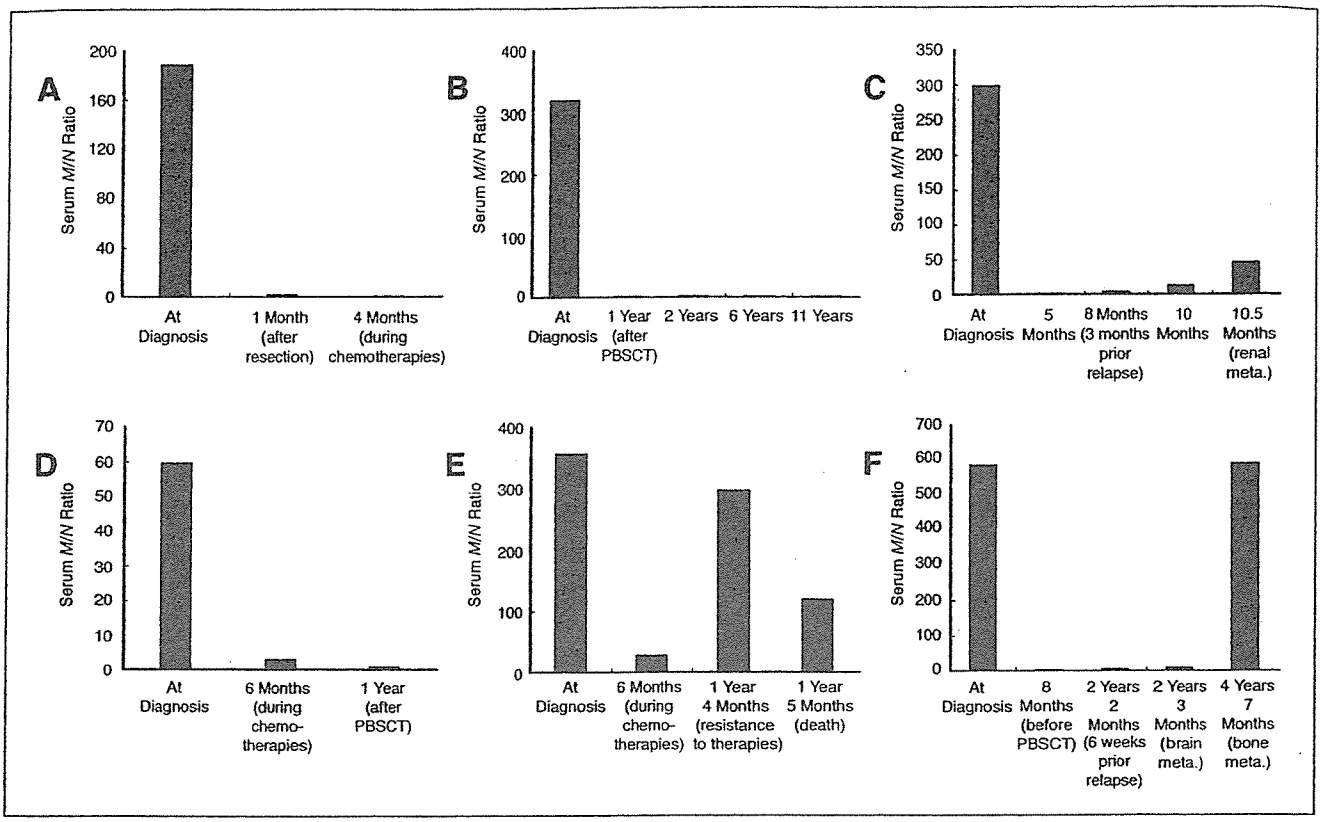

Fig 2. Changes in serum *MYCN/NAGK* (*M/N*) ratio levels of six patients with *MYCN* amplification during follow-up. PBSCT, peripheral-blood stem-cell transfusion; meta., metastasis. (A) Patient 1; (B) patient 3; (C) patient 5; (D) patient 2; (E) patient 4; (F) patient 6.

genetic aberrations using serum DNA is desirable for the assessment of prognosis and therapy stratification at the time of diagnosis. Among the tumor-related genetic aberrations detected in NB, MNA was of greatest interest to us because of its significant prognostic value.

By using DNA-based real-time quantitative PCR with a single-copy reference gene, we have demonstrated that the *M/N* ratio in serum DNA is a valuable diagnostic tool to discriminate MNA patients from non-MNA patients. The serum *M/N* ratio in the MNA group was significantly higher than the ratio in the non-MNA group, without an overlap. The highest sensitivity (100%), highest specificity (100%), highest positive predictive value (100%), and highest negative predictive value (100%) were obtained with a serum *M/N* ratio cutoff value of 10.0. Furthermore, we found an elevated level of the serum *M/N* ratio in a stage 1 patient and a stage 2B patient with MNA (188.7 and 901.6, respectively), even though the tumor was localized in these patients. This suggests that tumors could release a significant amount of genomic DNA into the systemic circulation even at an early stage. Furthermore, Sozzi et al[23] reported that the concentration of plasma DNA in 84 lung cancer patients was higher than the concentration in 43 controls, regardless of the tumor stage, and suggested that circulating DNA in peripheral blood was an early event in lung carcinogenesis.

Another clinical benefit of the serum *M/N* assay is that it could be used as a marker to monitor therapeutic efficacy and recurrence after therapies. The serum *M/N* ratio decreased to the normal range in the patients in remission but remained at a high level in the patient who failed to achieve remission. Furthermore, in two patients with recurrence after remission, the serum *M/N* ratio initially decreased to the normal range but then increased beyond the cutoff value by the time of diagnosis. The serum *M/N* ratio did not increase to the initial level as long as the metastasis was localized in the brain, but it did increase to the initial level when the patient later developed a bone metastasis (patient 6). This is noteworthy because it suggests that a brain metastasis releases genomic DNA into the systemic circulation less easily than extracranial tumors. If this is confirmed by examination of additional patients, then it is possible that tumors localized in brain could be overlooked with diagnostic assays based on serum DNA.

A possible pitfall of our serum *M/N* assay is that a high serum *M/N* ratio could be reduced by WBC contamination (Fig 3). This could be a result of dilution of tumor DNA with the WBC DNA, which would be expected to have an *M/N* ratio of 1. Therefore, the importance of removing WBCs from serum should be addressed in diagnostic assays

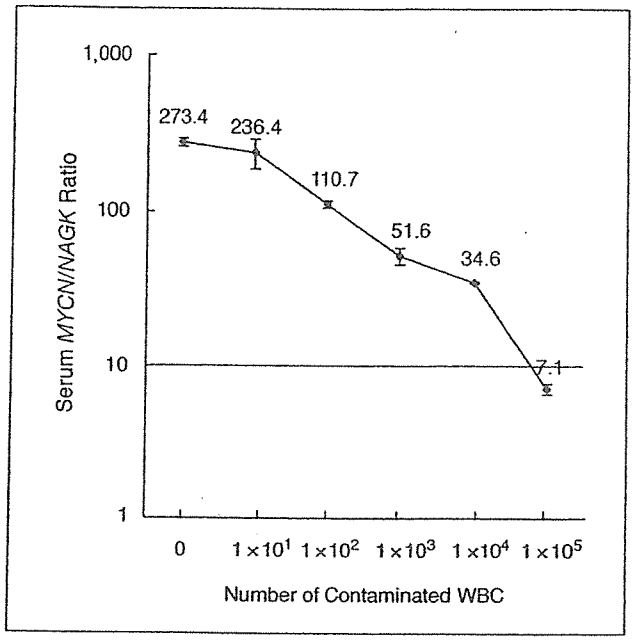

Fig 3. Influence of WBC contamination in serum samples on the serum *MYCN/NAGK* ratio. Data are presented as the mean ± standard deviation of duplicate measurements. The transverse line represents a *MYCN/NAGK* ratio cutoff value of 10.0.

that use serum DNA. For the same reason, a predominance of any nontumor DNA in serum may lower an elevated *M/N* ratio of an *MYCN*-amplified patient. However, this assay can be accurate on the premise that, in cancer patients, serum DNA predominantly consists of tumor-released DNA.[7] In addition, the use of serum DNA as a diagnostic tool in lung cancer patients has resulted in a diversity of findings, suggesting that these differences likely reflect variations in the manner in which the blood specimens were collected and handled and variations in the methods by which the assay were conducted.[24] Therefore, it is necessary to standardize the serum collection procedure to ensure that different laboratories obtain the same result with a given blood sample. An additional high-speed centrifugation step (16,000 × *g* for 5 minutes) was found to eliminate cellular contamination even after thawing of stored samples.[25] By using the appropriate centrifugation methods, we believe that WBC-free serum can be reliably achieved.

Although a large set of patients needs to be studied to verify the accuracy of this assay and to set an appropriate cutoff, our results are promising and need to be further tested. The advantages of this method are that it takes only 4 hours and much less effort than FISH and Southern blotting, which should make this assay an alternative to these other methods for determining *MYCN* status. A third advantage is that the serum *M/N* ratio seems to be a promising indicator of therapeutic efficacy and relapse in the follow-up of patients with MNA, although more patients need to be examined to confirm its reliability.

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Authors' Disclosures of Potential Conflicts of Interest

The authors indicated no potential conflicts of interest.

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Mini-review

Diversity in neuroblastomas and discrimination of the risk to progress

Takeo Tanaka[a,*], Tomoko Iehara[b], Tohru Sugimoto[b], Minoru Hamasaki[c], Satoshi Teramukai[d], Yoshiaki Tsuchida[e], Michio Kaneko[e], Tadashi Sawada[b,d]

[a]*Department of Pediatrics and Division of Clinical Research, National Hospital Organization, Kure Medical Center, Hiroshima, Japan*
[b]*Department of Pediatrics, Kyoto Prefectural University of Medicine, Kyoto, Japan*
[c]*Department of Pathology, Shizuoka Children's Hospital, Shizuoka, Japan*
[d]*The Japanese Infantile Neuroblastoma Cooperative Study*
[e]*The Japanese Advanced Neuroblastoma Cooperative Study*

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Abstract

The clinical diversity of Neuroblastomas (NBs) was discriminated into three groups with high sensitivity and specificity to patient's outcome. The '*high risk*' NB is defined with any of following conditions, MYCN amplification or unfavorable histology of International Neuroblastoma Pathological Classification (INPC) or low Ha-ras/trk A expression. The '*low risk*' NB is defined with all following conditions, single copy of MYCN and INPC favorable histology and high Ha-ras/trk A expression and localized tumor. The remaining NBs were classified into '*intermediate risk*' ones. According to these criteria, the diversity of the 248 mass-screening NBs was shown with variety progressive risk; 40% were classified in *low risk group*, 25% were in *high risk group* and 35% were in *intermediate risk group*.

Keywords: Neuroblastoma; Risk discrimination; *MYCN*; INPC; Ha-ras; Trk A; Mass-screening.

1. Introduction

Neuroblastoma (NB) is the most common extracranial solid malignancy in childhood. This malignancy shows diversity in their clinical behavior. Recent advances in molecular and genetic approach promote to understand their biology and provide predictors associated with clinical behavior of NBs [1]. MYCN amplification is a powerful predictor with high specificity to aggressiveness of NBs [2], however, the sensitivity is only a half of the patients with poor clinical outcome [3]. Brodeur [4] proposed three types of NBs classified according to several biological markers. The clinical evaluation whether we can get predictive specificity and sensitivity enough to use for the patients has not been done yet.

* Corresponding author. Tel.: +81 823 22 3111; fax: +81 823 22 0478.
E-mail address: ttanaka@kure-nh.go.jp (T. Tanaka).

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