A new human neuroblastoma cell line: Biological characteristics, cytogenetics and N-myc oncogene analysis.

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Abstract

A new human neuroblastoma cell line designated NB-39-nu has been established from a human neuroblastoma serially heterotransplanted in athymic nude mice. The cell line was potentially adrenergic, since intraperitoneal inoculation of NB-39-nu into athymic mice produced a neuroblastoma of rosette-fibrillary type with tyrosine hydroxylase-immunoreactivity.

Population doubling time was 41 hrs in the MEM medium and 22 hrs in the RPMI-1640 medium. Modal chromosome number was 83. Karyotypic analysis revealed the structural aberrations of #1 and #2 chromosomes and homogeneously staining regions were inserted within the long arm of chromosome #10. Ultrastructurally the cell line was mainly immature neuroblastic cells but a few of moderately differentiated neuroblastic cells were also included. Dibutyryl cyclic AMP, retinoic acid and cholera toxin but not nerve growth factor induced outgrowth of neurites without any detectable biochemical differentiation. Oncogene analysis indicated amplification of N-myc DNA and over-expression of its mRNA. The level of N-myc mRNA, however, did not change after the treatment of the cell line with dibutyryl cyclic AMP or retinoic acid.

Key words: neuroblastoma cell line, ultrastructure, cytogenetics, HSR, N-myc

INTRODUCTION

A considerable number of cell lines derived from a human neuroblastoma has been established and reported in the literature for these 20 years¹-¹³. And the cell lines have been used for the studies on induction of differentiation¹³-²², chromosomes¹³-²⁵, production of peptides or enzymes²⁴-²⁵ and other biological characteristics²⁶-²⁷. On the other hand, three groups²⁸-⁴⁰ have reported an amplification of N-myc DNA in human neuroblastomas, and moreover, its
amplification is frequently observed in neuroblastoma in the advanced stage i.e., stage III and IV but not in the early stages I to II (1). The recent paper of Tsuda et al. (2) also described that two thirds of stage IV neuroblastomas had N-myc amplification irrespective of cancer chemotherapy or not. Almost all neuroblastomas successfully cultured in vitro or heterotransplanted in athymic nude mice show the N-myc gene amplification, although a few of cell lines do not (3) and a human neuroblastoma which reveals the gene amplification in vivo but not in vitro is also reported (4). On the contrary, most neuroblastomas derived from early disease, stage I and II, do not contain amplified N-myc gene except for approximately 25% of stage II tumors (4, 5). Moreover, such neuroblastomas of early disease hardly grow in vitro or in athymic mice. From these observations, the amplification of N-myc oncogene seems to favor ex vivo proliferation of neuroblastoma cells. The precise correlation between the gene amplification and growth of neuroblastoma, however, has not been clarified.

In this paper we report here a new cell line of human neuroblastoma biologically characterized and its N-myc oncogene analysis.

MATERIALS AND METHODS

Cell culture: Tumor tissue was obtained from serially heterotransplanted human neuroblastoma NB-39 in athymic mice at the 10th passage generation. NB-39 was derived from a surgically removed neuroblastoma, rosette-fibrillary type, stage IV, in the left adrenal gland of a 1-year and 10-month-old male infant. Preoperative chemotherapy or radiation was not done. Levels of urine vanillyl mandelic acid and homovanillic acid of the patient were within normal limits. The tumor tissue was minced in RPMI-1640 (IBL, Fujioka, Japan) or MEM (IBL) medium by scissors into small pieces. Fifteen to 20 pieces were seeded in a 35 mm tissue culture dish (Corning, New York, USA) containing 1.0 ml of RPMI-1640 or MEM supplemented with 15% fetal bovine serum (Filtron, Australia). One week later, tumor cells and fibroblasts were immigrated from the explants and three weeks later the first transfer was possible in both RPMI-1640 and MEN cultures. The transfer was made by cell dispersion with 0.25% trypsin (Sigma, St. Louis, USA) plus 0.02% EDTA (Sigma). Afterwards it could be subcultivated every 5 to 7 days and fibroblasts were eradicated by the 6th passage generation, and was designated NB-39-nu. The concentration of fetal bovine serum was lowered from 15% to 10% thereafter.

Determination of proliferation rate: Proliferation rate of the cultured cells was measured by the replicate culture method of Katsuta et al. (6).

Electron microscopy: For thin section electron microscopy, the cell pellets at the 10th passage generation were fixed in 1.25% glutaraldehyde followed by 1% OsO4, dehydration, and embedded in epoxy resin. Sections were cut on an LKB automicrotome (LKB, West Germany), contrast-stained with uranyl acetate and lead citrate, and examined in a Hitachi H-800 electron microscope (Hitachi, Tokyo).

Heterotransplantation: One x10^7 cells at the 29th passage generation in the RPMI-1640 medium were heterotransplanted into the subcutis or in the peritoneal cavity of athymic mice, 6-week-old, female (Nihon CLEA, Kawasaki, Japan). Growth rate of the resultant sc tumor was estimated by the method of Ovejera et al. (7). The tumor tissue was also examined histologically and immunohistochemically.

Cytogenetic analysis: Mitotic cells were collected after 4 hours exposure to 0.03 μ g/ml colcemid (Sigma). Chromosome preparations were made with a conventional air-drying method and analysed banding procedure. The best banded metaphases were photographed and karyotyped according to the International System for Human Cytogenetic Nomenclature (ISCN, 1978).

Induction of morphological differentiation: NB-39-nu cells were treated with 0.1-2.0 mM dibutyryl cyclic AMP (dbcAMP) (Sigma), 0.1-10 μ g/ml cholera toxin (Sigma), 10^{-4} -10^{-5} M retinoic acid (Sigma), or 10^{-3} -10^{-4} M 2.5s nerve growth factor (Takara Shuzo, Kyoto). After treatment, the cells were monitored for 7 days in an inverted phase contrast microscopy to show morphologic-
al differentiation or not.

Immunocytochemistry: The cell pellets of NB-39-nu from control and dbcAMP-treated groups at the 24th passage generation and the nude mouse tumor tissues induced by NB-39-nu were fixed in 10% formalin or Bouin's solution, embedded in paraffin wax and sectioned. The sections were immunostained with antibodies against tyrosine hydroxylase (gift from Dr. Kumanishi, Niigata University), chromogranin A (Immunonuclear, Minesota, USA), VIP (gift from Dr. Yanaihara, Shizuoka Pharmaceutical College), neuropeptide Y (gift from Dr. Ito, Niigata University) and serotonin (gift from Dr. Yui, Niigata University) by the method of peroxidase antiperoxidase complex after Sternberger et al. Choline acetyltransferase in the cryostat sections of cell pellet and S-100 immunoreactivity in the paraffin sections were examined with use of the respective monoclonal antibody (Boehringer-Mannheim-Yamanouchi, Tokyo; JIMRO, Takasaki, Japan) by the indirect immunoperoxidase method.

Assay of N-myc DNA and RNA: High molecular weight DNA was prepared from $3 \times 10^8$ cells at the 29th passage generation as described by Hughes et al. Ten micrograms of DNA was digested with EcoR1 restriction endonuclease, and the digests were size-fractionated by agarose gel electrophoresis and transferred to the Biodyne membrane filter (Pall). The N-myc oncogene probe was the 1.0kbp EcoR1-BamH1 fragment of pNb-1. The intensity of amplification was calculated by comparing the serially diluted NB-39-nu DNA with human placental DNA. The 2 kb $\beta$-actin fragment was excised from pAl according to the method of Cleveland et al. Cellular RNA was extracted and processed for the dot blot analysis or Northern transfer hybridization.

RESULTS

NB-39-nu cells were adeherent and polygonal with occasional short cytoplasmic processes. Population doubling time calculated from the growth curves was 41 hrs in RPMI-1640 medium and 22 hrs in MEM medium. Modal chromosome number at the 15th and 19th passage generation was 83 and distribution of chromosome number was shown in Fig. 1. In later passages (80-100th generation), modal chromosome number was almost same as that at the 19th passage. Karyotypic analysis showed that there were numerical variations of each chromosome and many structural rearrangements of chromosomes in this cell line. Particularly characteristic findings were the structural aberrations of #1 and #2 chromosomes and homogeneously staining regions (HSRs) inserted within the long arm of chromosome #10 (Fig. 2, M3), which were seen in almost all cells observed. Double minutes were not present. The designation of all the identified or

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![Fig. 1](image1.png)

**Fig. 1** Distribution pattern of chromosome number. The modal number is 83 at the 19th passage generation.

![Fig. 2](image2.png)

**Fig. 2** Karyotypic analysis reveals frequent marker chromosomes (M1-M21) and homogeneously staining regions in the marker chromosome M3 (arrows).
A NEW HUMAN NEUROBLASTOMA CELL LINE

Table 1 Designations of identified or partially identified structurally abnormal marker chromosomes of NB-39-nu cell line

<table>
<thead>
<tr>
<th>Marker number</th>
<th>Structural designation</th>
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<tbody>
<tr>
<td>M1</td>
<td>t(1;17)(1q21;1q26)</td>
</tr>
<tr>
<td>M2</td>
<td>t(2;11)(2p15;11q23)</td>
</tr>
<tr>
<td>M3</td>
<td>11p33 deleted</td>
</tr>
<tr>
<td>M4</td>
<td>t(1;17)(1q21;1q26)</td>
</tr>
<tr>
<td>M5</td>
<td>11p15.5 deleted</td>
</tr>
<tr>
<td>M6</td>
<td>t(1;17)(1q21;1q26)</td>
</tr>
<tr>
<td>M7</td>
<td>t(1;17)(1q21;1q26)</td>
</tr>
<tr>
<td>M8</td>
<td>t(1;17)(1q21;1q26)</td>
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<tr>
<td>M9</td>
<td>t(1;17)(1q21;1q26)</td>
</tr>
<tr>
<td>M10</td>
<td>t(9;22)(q34;q11)</td>
</tr>
<tr>
<td>M11</td>
<td>del(11)(11q13)</td>
</tr>
<tr>
<td>M12</td>
<td>del(20)(20q13)</td>
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<tr>
<td>M13</td>
<td>del(21)(21q22)</td>
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Fig. 3 Transmission electron micrograph of a NB-39-nu cell. A large, indented nucleus and well developed cell organelles are noticed.

Fig. 4 Electronmicrographs showing budding of c-type virus particle (a), a single cilium (b), a secretory granule (c), and numerous secretory granules in a club-like cytoplasmic process of the ip tumor of NB-39-nu (d) (a, x 50,000; b and c, x 30,000; d, x 10,000). bar in a = 0.1 μm; bars in b to d = 0.2 μm.

- 9 -

The tumor cells were taken in every eight nude

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<td>M10</td>
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M12 - M19 unidentified marker chromosomes
mice and the tumors appeared one month later. Doubling time of the tumor weight was about 5 days. The histology of both tumors grown in nude mice after sc and ip transplantation of NB-39-nu cells was neuroblastoma of rosette-fibrillary type (Fig. 5, histology of the ip tumor not shown) which was compatible with the primary tumor of the patient.

NB-39-nu cells showed morphological change, that is elongation of neurite-like processes under treatment with dbcAMP, cholera toxin and retinoic acid. Among them, dbcAMP was most potent and about 40% of cells differentiated even 24 hours after treatment (Fig. 6a). Retinoic acid induced morphological differentiation 3 days after treatment in the range of concentrations used (Fig. 6b), but toxic for the cells after 7-day-treatment. Cholera toxin exhibited the same tendency as the retinoic acid (Fig. 6c) being toxic for the cells on the 7th day of treatment. On the other hand, nerve growth factor even at the concentration of $10^{-4}$ M could induce only slight morphological changes mentioned above (Fig. 6d). In the morphologically differentiated cells, neurotubules were observed in neuritic processes but very few of neurosecretory granules were encountered (Fig. 4c). Immunocytochemistry of control and differentiated NB-39-nu cells after treatment with dbcAMP or retinoic acid also failed to show positively immunostained cells with every antibodies used. The ip tumor, however, contained

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**Fig. 5** Histology of the tumor induced in an athymic nude mouse by sc heterotransplantation of NB-39-nu. Occasional rosette formation can be seen (a) (hematoxylin and eosine, original magnification, x 100). bar = 50 μm. Immunoreactive cells with antityrosine hydrxylase (b) and antichromogranin A antibody (c) are detected only in the ip tumor of NB-39-nu (b, c:x 200). bar = 25 μm.

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**Fig. 6** Morphological changes induced by dbcAMP (a, 1mM, 48 h), retinoic acid (b, $10^{-4}$ M, 3 d) and cholera toxin (c, 1 μg/ml, 5 d). dbcAMP seems to be most potent in induction of morphological differentiation. NGF has no effect on the cells (d, $10^{-4}$ M, 5 d). (a:d:original magnification, x 100). bars in a−d = 50 μm.
A NEW HUMAN NEUROBLASTOMA CELL LINE

about 3.2% of argyrophilic cells with Grimelius staining and numerous neurosecretory granules especially in the cytoplasmic process at the ultrastructural level (Fig. 4d). Immunohistochemistry of the tumor also revealed about 14.5% of tyrosine hydroxylase immunoreactive cells and about 4.8% of chromogranin A immunoreactive cells, respectively (Figs. 5b and c), while the sc tumor harbored only 0.3% of argyrophilic cells and no immunoreactive cells with the antibodies used.

About 200 fold amplification of N-myc DNA was noted and elevated level of N-myc mRNA was confirmed (Fig. 7). Thiele, C.J. et al.\textsuperscript{15} presented the data that expression of N-myc mRNA decreased after treatment of the neuroblastoma cell line with retinoic acid. When NB-39-nu was differentiated with dbcAMP or retinoic acid, in contrary to their results, the level of N-myc mRNA remained constant (Fig. 8).

DISCUSSION

The cell line NB-39-nu reported here is a human neuroblastoma cell line judging from the morphological and karyotypic analysis. It might be non-peptidergic, since no immunoreactivity with antibodies against peptides which are frequently found in human neuroblastomas was detected, and also non-cholinergic because of absence of choline acetyltransferase-immunoreactivity. This cell line, however, is potentially adrenergic, since tyrosine hydroxylase immunoreactivity was demonstrated only in the ip tumor induced in the nude mouse but not in the cells in vitro or in the sc NB-39-nu tumor. The ip environment might be more suitable for NB-39-nu to obtain functio-

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Fig. 7  (A) Amplification of N-myc gene in NB-39-nu. High molecular weight DNA was prepared from NB-39-nu maintained in nude mice (lane 1, 4, and 7), cultured in MEN (lane 2, 5, and 8) or in RPMI-1640 (lane 3, 6 and 9). Placental DNA was used as standard (lane 10). Southern blot analysis was carried out as described in materials and methods. The amount of DNA used was 10 μg (lane 1, 2, 3 and 10), 2 μg (lane 4, 5 and 6) or 0.4 μg (lane 7, 8 and 9).

(B) Overexpression of N-myc mRNA in NB-39-nu. Three μg of polyA RNA was glyoxylated and analyzed as described previously\textsuperscript{40}. The same filter was rehybridized with the β-actin probe. Lane 1: NB-39-nu cultured in MEN medium, lane 2:NB-39-nu cultured in RPMI-1640, lane 3: neuroblastoma cell line NB-1, lane 4:vulval carcinoma cell line A431.

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Fig. 8  Unchanged expression of N-myc mRNA after differentiation. Total cellular RNA was prepared from NB-39-nu cells after treatment with dbcAMP (lane 2:30 min, lane 3:24 h) or retinoic acid treatment (lane 4: 3 h, lane 5:24 h, lane 6:72 h). Lane 1: non-treated cells. Two μg of each total RNA was slot blotted and hybridized with either the N-myc or the β-actin probe.
nal activity than the sc environment, possibly because of higher blood and nutritional supply or some serum factors influencing cellular functions.

This cell line was responsive to dbcAMP, cholera toxin and retinoic acid showing morphological differentiation. Biochemical differentiation, however, was not induced. Recently Tsokos et al.\textsuperscript{11}) have reported that some neuroblastoma cell lines differentiate into not only neuronal but also schwannian and melanocytic cells after treatment with either dbcAMP or retinoic acid. NB-39-nu, however, did not show such differentiation other than neuronal by the dbcAMP or retinoic acid, since S-100 immunoreactivity and ultrastructures indicative of a schwann cell or a melanocyte could not be detected. A single cilium observed in NB-39-nu seems to have not described in the cell lines derived from the ordinary childhood neuroblastomas\textsuperscript{4,11,14}) but a cell line derived from adult neuroblastoma originated from the C8 nerve root has been reported to have a single cilium on the tumor cell.\textsuperscript{15}) This finding implies a possible neuroepithelial nature of NB-39-nu, in spite of its usual neuroblastic morphology in vitro and in nude mice.

Chromosomal analysis indicated hypotetraploidy with modal chromosome number of 83, which was almost stable in later passages. A notable finding on the structural chromosome aberration was the partial deletion in the short arm of chromosome #1. This was first described in human neuroblastoma by Brodeur et al.\textsuperscript{15}) and was observed in about 70% of human neuroblastomas and cell lines, though the tumorigenic significance of chromosome 1p aberration is not yet certain. On the other hand, HSR marker chromosomes as observed in this cell line have been reported to be so associated with the amplification of the oncogene N-myc\textsuperscript{18}). In fact NB-39-nu had amplified N-myc oncogene and the overexpression of N-myc mRNA, which has been noticed in advanced stages\textsuperscript{41,42}). The N-myc oncogene was mapped to chromosome #2 (2p23-24) and the structural chromosome aberration of #2 including this region was observed in this cell line. This finding may support the speculati-
on that the chromosomal rearrangement of #2 might have been the first event initiating a multistep processes leading to amplification of N-myc within HSR\textsuperscript{43}).

In the morphologically differentiated NB-39nu cells or prior to differentiation under treatment of dbcAMP or retinoic acid, over-expression of N-myc mRNA remained constant when surveyed the cells in mass. This result is different from the previous report in which retinoic acid induced differentiation of a neuroblastoma cell line has been accompanied with decrease in N-myc overexpression just prior to occurrence of cellular differentiation\textsuperscript{15}). This discrepancy is hard to explain. The relative amount of N-myc mRNA, however, might remain almost equal to that of the control group, since the frequency of differentiated cells treated with dbcAMP was about 40% and others did not show morphological changes. Clonal selection of dbcAMP sensitive or not-sensitive NB-39-nu might resolve this problem.

In summary, NB-39-nu might be a useful cell line in the research field of the relationship between growth and morphological differentiation, and between chromosomal and N-myc oncogene abnormalities.

Acknowledgement

This work was supported in part by a Grant-in-Aid for Cancer Research from the Ministry of Health and Welfare.

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