筑波大学

博士（医学）学位論文
Chromosomal abnormalities associated with prognosis and clinical feature of neuroblastoma

（神経芽腫の予後および臨床像に関連した染色体上のDNAコピー数異常）

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筑波大学大学院博士課程医学研究科

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INTRODUCTION

Neuroblastoma is one of the most common malignant tumors of childhood and characterized by variable clinical behaviors\(^1\)\(^2\). Progression to the fatal disease is most common in children over 1 year of age with bone metastasis. These cases show the 5-year survival rate below 30-40%\(^1\)\(^3\). Spontaneous regression usually occurs in infants (under 1 year of age), and the metastases confined to the liver, bone marrow, and/or skin\(^1\)\(^2\)\(^4\) is most common in infant cases. Hereditary factors might play more important role in oncogenesis of malignant tumors in children like retinoblastoma and neuroblastoma than that in adults. Elucidation of molecular mechanism in oncogenesis of neuroblastoma has been tried, however, the molecular mechanisms of regression and progression of the disease are still unclear.

In Japan, for early diagnosis and proper treatment against progressive disease, in 1985, mass screening (MS) in infants aged 6 months by detecting urinary vanillylmandelic acid was introduced. After that, the incidence of the neuroblastoma aged less than 1 year has increased\(^5\). Nevertheless, the survival rate in patients with bone metastasis has not been improved\(^5\). It is known that many neuroblastomas detected by MS show good prognosis and some, not all, of patients found by MS do not require any treatment\(^5\)\(^6\). The regressive neuroblastoma is considered not to be same as progressive tumor in biological characteristics in spite of showing malignant pathological features of neuroblastoma. Thus, neuroblastoma should have several oncogenic pathways.

Prognostic factors such as MYCN amplification\(^7\)\(^8\), 1p loss of
heterozygosity (LOH)\textsuperscript{9,10} and alterations in DNA ploidy\textsuperscript{11} are not enough to predict the prognosis of the advanced-stage neuroblastoma showing resistance to aggressive chemotherapy because of certain population of fatal patients without these poor prognostic factors was found. And differences in clinical features of neuroblastomas can not be explained only by these genetic changes.

Clarification of molecular and biological differences between progressive and regressive diseases and identification of a novel genetic alteration strictly associated with fatal disease would be useful for appropriate treatment and for an understanding of oncogenic pathways of neuroblastomas.

While Southern blotting, microsatellite analysis and polymerase chain reaction-single strand conformation polymorphism (PCR-SSCP) are used in analysis of the known genes and/or a part of chromosomal regions, these methods have a limitation in genome-wide analysis of unknown region in a greater part of human genome. Furthermore, sufficient size of primary tumor and normal specimens and high quality of DNA obtained by open biopsy is required. Comparative genomic hybridization (CGH) analysis allowed us genome-wide assessment of abnormalities in the relative copy number of DNA sequences in solid tumors which showed a low mitotic index\textsuperscript{12,13}. This technique enables us to survey the whole genome in a single hybridization using one microgram DNA from tumor, and to map the possible amplified and deleted genes simultaneously onto the chromosomal region in solid tumors. Chromosomal copy number abnormalities in neuroblastoma by CGH have been described in several reports\textsuperscript{14–17}. These studies have
shown gain at 17q, a novel DNA copy number abnormality related to the advanced-stage neuroblastoma, however they did not mentioned the association with fatal disease resistant to chemotherapy. No data including DNA copy number abnormalities related to regression and progression of the disease has been prevented in previous reports due to a lack of MS case.

In this study, to clarify the differences in genetic background between regressive and progressive neuroblastomas, regressive neuroblastomas including MS cases and fatal and progressive neuroblastomas were analyzed by CGH. In Chapter I, a novel chromosomal gain strongly associated with fatal neuroblastoma resistant to chemotherapy revealed by analysis of poor prognostic neuroblastomas with bone metastasis is described (1). In Chapter II, two types of chromosomal imbalance pattern associated with regressive and progressive diseases revealed by CGH profile are described (2).

Footnotes
(1) Hirai, M., et al., Genes Chromosomes and Cancer, revised.
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Chapter I

1q23 gain is associated with progressive neuroblastoma resistant to aggressive treatment
ABSTRACT

Neuroblastoma is one of the most common malignant tumors of childhood and is characterized by regressive and progressive disease. Genetic factors that define progression of neuroblastomas are still unknown. We performed comparative genomic hybridization (CGH) on 27 neuroblastomas and dual-color fluorescence in situ hybridization (FISH) to identify genetic aberrations associated with progressive neuroblastoma showing resistance to aggressive treatment. 17q21-q25 gains and MYCN amplification were associated with stage 4 neuroblastomas; however, these genetic aberrations had no significant relation to the progression of stage 4 neuroblastomas. A novel chromosomal gain at 1q21-q25 was found in 8 of 16 cases (50%) of stage 4 neuroblastoma. Gain of 1q21-q25 was observed in all of the progressive cases (8/8), which showed resistance to chemotherapy, including 5 fatal neuroblastomas in stage 4, whereas 1q21-q25 gain was not found in any of the 8 remission cases in stage 4. Survival analysis also showed that 1q21-q25 gain was associated with a poor outcome. High xenotransplantability in nude mice was observed for the tumors with 1q21-q25 gain (4/5; 80%). These data reveal that 1q21-q25 gain is strongly associated with progression of stage 4 neuroblastoma. Furthermore, by dual-color FISH analysis using cosmid clones, the 1q21-q25 gain was narrowed to increase in DNA copy number on 1q23 in the fatal type of stage 4 neuroblastoma showing this gain. These results suggest that DNA amplification at 1q23 may play a role in the development of progressive neuroblastoma in an advanced stage.
INTRODUCTION

Neuroblastoma is a malignant tumor of childhood originating from embryonal neural-crest tissue. Neuroblastoma has a variable clinical course, including progression to fatal disease, maturation to ganglioneuroblastoma or ganglioneuroma, and spontaneous regression (de Lorimier et al., 1990; Pritchard et al., 1994). It is classified by the stage of the disease and the age of the patient at diagnosis, because its prognosis depends on the region of metastasis and the age of onset. Staging based on the International Neuroblastoma Staging System (INSS) (Brodeur et al., 1993) is employed to provide prognostic information and to determine criteria for treatment. Spontaneous regression is more frequent in stages 1, 2, and 4S than in stage 4 and usually occurs in infants (de Lorimier et al., 1990; Brodeur et al., 1993; Pritchard et al., 1994). The 5-year survival rate for infant cases in stages 1-3 and 4S is 80-95%, and some of these patients require no treatment (de Lorimier et al., 1990; Evans et al., 1996). On the other hand, the 5-year survival rate for stage 4 cases is below 30-40%, and the progressive disease in stage 4 is resistant to aggressive treatments such as chemotherapy and irradiation (de Lorimier et al., 1990; Iwafuchi et al., 1996).

Characterization of genetic changes in neuroblastoma, such as MYCN amplification, alterations in DNA ploidy, and deletion of 1p in tumors, has shown an association with the stage or clinical course of neuroblastoma (Brodeur et al., 1984; Seeger et al., 1985; Christiansen and Lampert, 1988; Look et al., 1990; Brodeur et al., 1992; Caron et al., 1996). Cytogenetic approaches
have been used to elucidate chromosomal abnormalities correlated with the clinical course (Hayashi et al., 1989). Involvement of trisomies for chromosome arms 1q and 17q in tumor progression of neuroblastoma has been reported (Gilbert et al., 1984). Comparative genomic hybridization (CGH) allowed a genome-wide assessment of abnormalities in the relative copy number of DNA sequences in solid tumors (Kallioniemi et al., 1992, 1994). Recent analysis of chromosomal copy number abnormalities by CGH have shown frequent 17q gain in neuroblastomas (Altura et al., 1997; Brinkschmidt et al., 1997; Lastowska et al., 1997a, 1997c; Plantaz et al., 1997;) and association of 17q21-q25 gain with advanced-stage neuroblastoma (Brinkschmidt et al., 1997). Association of 17q gain with unfavorable neuroblastoma has been shown by survival analysis (Lastowska et al., 1997c).

In spite of numerous genetic abnormalities with prognostic relevance found in neuroblastomas, the prognosis of advanced-stage neuroblastoma is difficult to predict, and the mechanisms of disease progression and of the acquisition of resistance against aggressive treatment are not understood. Identification of genetic alterations that are strictly associated with fatal disease in stage 4 neuroblastoma would be helpful for an understanding of the molecular mechanisms of development of the progressive disease. Such findings may lead to the establishment of appropriate therapies and means for predicting the prognosis of advanced neuroblastomas based on genetic information. Therefore, we analyzed neuroblastomas by CGH and fluorescence in situ hybridization (FISH) and identified genetic abnormalities in order to elucidate the gene strongly associated with progressive neuroblastoma.
MATERIALS AND METHODS

Tumor Specimens

Twenty-seven tumor specimens of neuroblastoma, including 25 primary tumors, 1 metastatic tumor of bone, and one tumor transplanted in nude mice were analyzed. All tumors were consecutive samples obtained by open biopsy before chemotherapy and radiotherapy. The stage of each patient was categorized according to the INSS (Brodeur et al., 1993). Sixteen cases were categorized in stage 4, and 11 cases were in stages 1-3 or 4S. All patients in stage 1-3 and 4S were infants who are alive with no evidence of disease. Eight cases in stage 1-3 were found by mass screening. DNA was extracted from frozen tissue according to standard procedures (Sambrook et al., 1989).

Comparative Genomic Hybridization

CGH was performed by indirect methods according to previously described protocols, with slight modifications (Kallioniemi et al., 1992, 1994). Tumor DNA was labeled with biotin 16-dUTP (Gibco BRL, Gaithersburg, MD) and reference DNA, which had been isolated from healthy human male lymphocytes, was labeled with digoxigenin 11-dUTP (Boehringer Mannheim, Mannheim, Germany) by nick translation. The reaction conditions were optimized for each sample so that labeled fragments (after denaturation at 95°C) ranging between 300 and 1000 bp in size were obtained. The mixture which contained 600 nanograms of each labeled DNA from tumor and normal lymphocytes and 15 μg of human Cot1 DNA (Gibco BRL) in 15 μl of hybridization buffer (50% formamide, 10% dextran sulfate, and 2×SSC) was hybridized to normal metaphase
cells (Vysis, Downers Grove, IL; FML Lab., Tokyo, Japan) (at 37°C for 3 days). After hybridization, the metaphase slides were incubated with fluorescein (FITC)-conjugated avidin (Dupont NEN) and anti-digoxigenin antibody conjugated with tetramethylrhodamine (TRITC) (Dupont NEN). Chromosomes were counterstained with 4',6-diamidino-2-phenylindole dihydrochloride (DAPI) (Molecular Probes, Eugene, OR). For each experiment, we included CGH using normal DNA as a reference probe and normal DNA as a test probe to check the quality of the CGH experiment. For experimental quality controls, we performed CGH on cell lines in which 9p21 was deleted, and on primary tumors of neuroblastoma in which MYCN amplification (2p24) and 1p LOH (1p35-ter) have been found (data not shown).

**Digital Image Analysis**

We used a digital image analysis system based on a fluorescence microscope with a double bandpass filter (Leica, Wetzlar, Germany) and a charge-coupled-device camera (Applied Imaging, Santa Clara, California) to acquire images of fluorescence on the metaphase cells. Three-color images representing the tumor DNA (green), reference DNA (red) and DAPI counterstaining from 10 metaphase cells per hybridization were collected. Green-to-red fluorescence ratios along individual chromosomes were assessed with Cytovision software (Applied Imaging). To increase the accuracy of the average of fluorescence ratio values, 95% confidence intervals were calculated. Chromosomal imbalances were detected on the basis of deviation of the ratio profile from the balanced value (green/red = 1.00). The values 1.25 and 0.75 were used as diagnostic cut-off levels for gain and loss, respectively (du
Manoir et al., 1995).

**Dual-Color FISH Analysis**

Dual-color FISH analysis of interphase nuclei prepared from paraffin-embedded primary tumors and fresh tumors, which had been inoculated in nude mice, was performed according to standard procedures (Nederlof et al., 1990; Hyytinen et al., 1994). FITC-labeled cosmid DNA and a digoxigenin-labeled pericentromeric chromosome 1-specific probe, D1Z5 (ONCOR, Gaithersburg, MD), were used to check DNA copy number. Cosmid clones mapped on 1q were provided by the National Institute of Infectious Disease (Tokyo, Japan). After hybridization, nuclei were incubated with a TRITC-labeled anti-digoxigenin antibody (Dupont NEN) to visualize the centromeric probe. For the accurate FISH analysis, especially, for reduction the influence of signals in background, we adjust the consistency of nuclei to be one nuclear with in the field at 600 magnifications of a microscope, and we counted the FITC signals on the G2 phase nuclei which had more than two D1Z5 signals. Therefore, the number of nuclei having countable signals were 20 in each experiment.

**Tumor Xenografts into Nude Mice, Loss of Heterozygosity (LOH), and Southern Blot Analyses**

The amount of 5 cubic millimeters of minced primary tumor was planted onto subcutaneous tissue and fascia of the back of 5-week old nude mouse (BALB/c-nu/nu) (Nippon Kurea, tokyo, Japan). Xenotransplantability was judged by tumor growth within 3 weeks in the first and second generation mice. On failure cases, the transplanted tumor was disappeared within a month in the first generation. Analysis of 1p LOH (Hayashi et al., 1989;
Maris et al., 1995; Caron et al., 1996) and Southern blotting of MYCN (Seeger et al., 1985; Brodeur et al., 1992) were performed as described. To confirm DNA amplification on 1q23, Southern blotting of genome DNA from fatal neuroblastoma was performed using cosmid DNA (CYS1-40 at 1q23) as a probe.

**Statistical Analysis**

Overall survival was calculated by use of Kaplan-Meier estimation (Kaplan and Meier, 1958). We used the log-rank test to evaluate the relationship between 1q21-q25 status and clinical features.

**RESULTS**

**Chromosomal Aberrations Associated with Stage 4 Neuroblastoma**

Neuroblastomas in stage 4 showed more chromosome aberrations than did earlier-stage neuroblastomas. Table 1 shows the relationship of chromosomal copy number abnormalities to the stage of neuroblastoma. Frequent chromosomal loss occurred at 1p34-pter (6/27; 22%, 6/16; 38% in stage 4) and 11q21-q25 (6/27; 22%, 5/16; 31% in stage 4), but no chromosomal loss was found in stages 1-3 and 4S except for 1 case with 11q21-q25 loss (data not shown). In stage 4 neuroblastosmas, we observed frequent chromosomal gains at 17q21-q25 (16/16;100%), 1q32-q43 (10/16; 63%), 2p23-p24 (9/16; 56%), and 1q21-q25 (8/16; 50%). Gains of chromosome 17 material were the most common in neuroblastomas (25 of 27 cases; 92%), however, this highly frequent 17q gain included the entire region of chromosome 17 or arm of 17q (7/27; 26%) showing straight profile in stage 1-3 and 4S. Gain of 17q21-q25 with local deviation limited to this region was found in 18 cases (18/27; 67%, 16/16; 100% in stage 4) (Table 1 and
The frequency of 17q21–q25 gain in stage 4 was significantly higher than in stages 1–3 and 4S (Fisher's exact test; \( P=0.000012 \)) (Table 1). We found two distinct regions on 1q showing increased DNA copy numbers at 1q32–q43 and 1q21–q25. Figure 1A and B show increased and decreased DNA copy numbers and representative profiles of CGH on chromosome 1 in dead cases in stage 4 (cases 1 and 18). Frequencies of 1q32–q43 and 1q21–q25 gains in neuroblastomas were 52% (14/27) and 33% (9/27), respectively. Six cases (1 case in stage 2B and 5 cases in stage 4) showed both of these gains, 8 cases (1 case in stage 1, 2 cases in stage 2 and 5 cases in stage 4) showed 1q32–q43 gain without 1q21–q25 gain, and 3 cases in stage 4 showed 1q21–q25 gain without 1q32–q43 gain (Table 1 and Fig. 1). Gain of 1q21–q25 was found in 8 of 16 (50%) cases in stage 4, whereas none of the stage 4S cases and only one of the stage 1–3 cases showed this gain. The frequency of 1q21–q25 gain in stage 4 was significantly higher than in stages 1–3 and 4S (\( P=0.033 \)). Gain of 1q32–q43 was observed preferentially in stage 4, but no statistical difference in frequency among clinical stages was observed (\( P>0.05 \)). Gain of 2p23–p24 and MYCN amplification were observed only in stage 4 cases (9/16; 56%), and associated with the advanced stage of neuroblastoma (\( P=0.024 \)) (Table 1). These cases had both 2p23–p24 gain and MYCN amplification, and the cases without 2p23–p24 gain did not have MYCN amplification.

**Association of 1q21–q25 Gain with Progression of Neuroblastoma**

Table 2 shows clinical aspects of stage 4 cases, 1q21–q25 gain, 1q32–q43 gain, MYCN amplification, and 1p LOH in the present study. Six patients, who are living over 1 year without
evidence of disease after treatment for 2 years, were categorized as being in complete remission (CR). Five patients died of tumor showing progression against the aggressive chemotherapy for advanced neuroblastoma. Five patients are still under treatment, and 3 of them have been showing progression or relapse of disease against the chemotherapy. These 3 patients under treatment and 5 dead patients were categorized as being in progressive disease (PD). Two out of 5 patients under treatment have showed a good response to the chemotherapy, and were categorized as being in under treatment with evidence of remission (UT) in this paper. As shown in Table 2, all of the PD cases (8/8; 100%) but none of the UT/CR cases (0/8; 0%) in stage 4 showed 1q21-q25 gain. Transplantation of tumors into nude mice was performed in 8 cases in stage 4 and 9 cases in stages 1-3 and 4S. Four tumors from PD cases with 1q21-q25 gain in stage 4 had xenotransplantability, whereas none of the neuroblastomas without 1q21-q25 gain had xenotransplantability (Table 2). Gain of 1q32-q43 were observed in 5 of 8 PD cases and 5 of 8 UT/CR cases. Gain of 2p23-p24 and MYCN amplification were observed in 6 of 8 PD cases and 3 of 8 UT/CR cases. Loss of 1p35-p36 were observed in 4 of 8 PD cases and 2 of 8 UT/CR cases. LOH of 1p was detected in 3 of 8 cases examined in stage 4 (cases 18, 21, and 23). These 3 cases showed 1p35-p36 loss by CGH. Table 3 summarizes the association of clinical status with chromosomal abnormalities. Gain of 1q21-q25 was strongly associated with PD in the stage 4 neuroblastomas (P=0.000078). On the other hand, no significant differences in the frequencies of 17q21-q25 gain, 1q32-q43 gain and 2p23-p24 gain including MYCN amplification
among clinical subtypes were observed (P>0.05).

Figure 2A and B showed the overall survivals of two groups of patients, with 1q21-q25 gain and without 1q21-q25 gain. Significant differences in survival between cases with 1q21-q25 gain and those without 1q21-q25 gain were observed in all patients (the log-rank test; P=0.03) (Fig. 2A), and also in stage 4 patients (P=0.02) (Fig. 2B). No significant difference in survival between cases with 1q32-q43 gain and those without 1q32-q43 gain were observed in all patients (P>0.05) (Fig. 2C).

**Increase in DNA Copy Number on Chromosome 1q23 in Progressive Neuroblastoma**

To determine the region responsible for the 1q21-q25 gain, 22 cosmid clones mapped on 1q were employed in dual-color FISH analysis. Figure 3 shows the chromosomal location of cosmids and representative results of FISH analysis in dead case (case 7). Fourteen clones located at 1q21-q22, 3 clones located at 1q23, and 5 clones located at 1q24-q25 were used. These chromosomal locations have been confirmed by FISH (Kugoh et al., 1995). Interphase nuclei of tumor xenografts in nude mice at the first generation (cases 1, 7, and 5) and primary tumor specimens (case 1 and 9) were analyzed. These tumors showed 1q21-q25 gain on CGH analysis. Signals of CYS1-40 were 2.3-fold in cases 1, 7 and 9 and 2.2-fold in case 5 to D125 signals per cell. Signals of CYS1-68 were 3.1-fold in case 1, 3.0-fold in case 7 and 2.0-fold in case 9 to D125 signals per cell. In all cases analyzed by FISH, cosmid clones other than CYS1-40 and CYS1-68 did not show increases in signal to D125 (Table 4 and Fig. 4). In Figure 4, the results of FISH analysis are summarized. Two cosmid clones,
CYS1-40 and CYS1-68, showed increased signals on interphase nuclei from tumors, and the other 20 cosmid DNAs showed no signal increase in FISH analysis. We confirmed the chromosomal location of CYS1-40 and CYS1-68 at 1q23 by FISH using a normal-lymphocyte metaphase cells (data not shown). We confirmed DNA amplification at 1q23 by Southern blotting of genome DNA from tumor of dead case (case 1) using fragment of cosmid DNA (CYS1-40) as a probe. Intensity of band hybridized with cosmid DNA in tumor was increased 5-fold than that in normal human placenta (Fig. 3D).

**DISCUSSION**

We present here 1q21-q25 gain in the progressive type of stage 4 neuroblastoma, which is characterized by rapid tumor growth and resistance to aggressive treatment. One case with 1q21-q25 gain in stage 2B required surgical treatment and additional courses of chemotherapy in spite of being an infant case. Survival analysis by Kaplan-Meier estimation indicates that 1q21-q25 gain was significantly associated with a poor outcome.

*MYCN* amplification and 1p LOH are accepted as major poor prognostic factors (Seeger et al., 1985; Brodeur et al., 1992). In this study, *MYCN* amplification and 1p LOH were strongly associated with stage 4 disease, whereas had no significant association with PD cases in stage 4. The present study suggests that 1q21-q25 gain has a stronger association with progressive disease in advanced neuroblastoma than do other poor prognostic factors (Tables 2 and 3). It is suggested that 1q21-q25 gain might include an candidate poor prognostic factor with relationship
to resistance to chemotherapy.

High xenotransplantability observed in tumors showing 1q21-q25 gain suggests an association of the malignant potential of these tumors with this chromosomal aberration. It is well known that oncogene activation by DNA amplification and subsequent enhanced expression contribute to cancer development by positive modulation of growth and of malignancy phenotypes.

We narrowed 1q21-q25 gain in the fatal type of neuroblastoma to increase in DNA copy number on 1q23. Previous cytogenetic studies have shown a correlation of chromosome 1q trisomy with progressive neuroblastoma (Gilbert et al., 1984). FISH data using a chromosome 1-specific probe and cosmid DNA probes on 1q in the present study did not suggest trisomy of chromosome 1 in progressive stage 4 neuroblastoma.

The frequency (92%) of gains of chromosome 17 material in neuroblastoma in the present study is similar to that in previous reports (Brinkschmidt et al., 1997; Lastowska et al., 1997a; Plantaz et al., 1997). Recent CGH studies have also shown association of 17q gain with high-grade neuroblastomas and with a poor prognosis (Meddeb et al., 1996; Brinkschmidt et al., 1997; Lastowska et al., 1997c; Plantaz et al., 1997;). In the present study, the frequency of 17q21-q25 gain was significantly higher in stage 4 than in stages 1-3 and 4S, however, we found that all stage 4 neuroblastomas, including PD, UT, and CR cases, showed 17q21-q25 gain. These results suggest that 17q21-q25 gain is related to the stage of neuroblastoma rather than the progressive phenotype in stage 4 neuroblastoma characterized by resistance to aggressive treatment. Gain of 17q might be an
earlier event than 1q21-q25 gain in the development of the advanced neuroblastoma. Chromosome 17 rearrangements and unbalanced 1p;17q translocation in neuroblastomas result in gain of 17q and loss of 1p (Gilbert et al., 1984; Van Roy et al., 1994; Lastowska et al., 1997b, 1997c). In addition to MYCN amplification and 1p LOH, 17q gain may be a prognostic marker associated with clinical staging.

Gain of 1q32-q43 was frequently found next to 17q21-q25 gain in neuroblastomas. However, this gain had no significant association with advanced stage disease and with PD cases. We suggest that 1q32-q43 gain might be an early event in the development of neuroblastoma.

These two distinct regions of gain at 1q have not been reported in previous CGH studies of neuroblastoma (Meddeb et al., 1996; Brinkschmidt et al., 1997; Lastowska et al., 1997a; Plantaz et al., 1997;). Some of these investigators have made the threshold of gain and loss less severe, therefore two peaks of gain on 1q might be missed and confused with entire 1q gain in previous studies. We evaluated the gains on 1q carefully by repeated experiments using both direct and indirect labeling methods. Early stage neuroblastomas found by mass screening in infancy showing hyperdiploid karyotypes and spontaneous differentiation or regression showed straight and entire chromosomal abnormalities (submitting). We have an idea that gains and losses with regional peaks on chromosomes should been discriminated from whole chromosomal abnormalities on CGH profiles.

In the present study, a novel gain of 1q21-q25 indicated a strong association with progressive and fatal neuroblastoma in
stage 4 that shows resistance to aggressive treatment. *MYCN* amplification (Brodeur et al., 1984; Seeger et al., 1985), 1p LOH (Christiansen and Lampert, 1988; Hayashi et al., 1989), and TRK-A expression (Nakagawara et al., 1993) are well-known prognostic factors of neuroblastoma. Chromosomal gain at 1q21-q25 was limited in PD cases; therefore, if confirmed in a large-scale study, 1q21-q25 gain may be a possible prognostic marker for fatal disease which needs more aggressive treatment at the outset. Molecular cloning and characterization of the responsible genes on 1q23 would clarify pathways of progression of neuroblastoma and shed light on effective therapy against fatal neuroblastoma.

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Figure Legends

Figure 1. Genetic imbalances on chromosome 1, 11 and 17 in 27 neuroblastomas. The line on the left and the line on the right of each chromosome ideogram represent loss and gain of genetic material, respectively. The black lines indicate stage 4 cases and the gray lines indicate stages 1-3 and 4S cases. Shaded box shows heterochromatic region, which was not interpretable because of high suppression of hybridization by Cot1 DNA (Kallioniemi et al., 1994; Lastowska et al., 1997). On CGH profiles, the green-to-red ratios in fluorescence are plotted along the chromosome ideograms with 95% confidence intervals. The intensity ratio of a balanced copy number, shown by a vertical bar, was calculated from the mode of the intensity ratio. For each case, the thresholds for over- and under-representation are represented by the fourth line (value of 1.25) and by the second line (value of 0.75), respectively. A) Regions of chromosomal gain and loss on chromosome 1. B) Two distinct regions of 1q gain on CGH profiles. Case 18: 1q21-q31 gain, 1p34.2-pter loss. Case 1: 1q21-q25 and 1q32-q43 gain. C) Regions of chromosomal gain and loss on chromosome 11, with representative CGH profile of 11q21-q25 loss (case 18). D) Regions of chromosomal gain on chromosome 17, with representative CGH profile of 17q21-q25 loss (case 18).

Figure 2. A) Overall survival of two groups of patients, with 1q21-q25 gain and without 1q21-q25 gain. Survival curves for 9 cases with 1q21-q25 gain compared with 18 cases without 1q21-q25 gain in all stages of neuroblastoma. B) Survival curves for 8
cases with 1q21-q25 gain compared with 8 cases without 1q21-q25 gain in stage 4 neuroblastomas. C) Overall survival of two groups of patients with 1q32-q43 gain and without 1q32-q43 gain. Survival curves for 14 cases with 1q32-q43 gain compared with 13 cases without 1q32-q43 gain in all stages of neuroblastoma.

Figure 3. Increase in DNA copy number at 1q23 in fatal stage 4 neuroblastoma. A) Location of 22 cosmid clones mapped on 1q used in FISH analysis. B), C) Detection of DNA copy number increase in stage 4 neuroblastoma (case 1). The chromosome 1 pericentromeric probe (D125) was labeled with TRITC (red), and the probes for the 1q23 region were 1q23 cosmids, CYS1-40 (B) and CYS1-68 (C), were labeled with FITC (green). In B, 4 green and 2 red signals are shown. In C, 6 green and 2 red signals are shown. D) DNA amplification at 1q23 in fatal neuroblastoma detected by Southern blotting. Genomic DNA from neuroblastoma of dead patient (case 1) and normal human placenta DNA were diluted keeping the total DNA to 2 micrograms, and separated on agarose gel. Tumor, genomic DNA from tumor of case 1; Normal, genomic DNA from normal human placenta. DNA fragment of cosmid CYS1-40, digested by BamHI indicating 3 kb (arrow), was detected.

Figure 4. Identification of the region of chromosomal amplification at 1q23 in progressive disease in stage 4, showing 1q21-q25 gain by dual color FISH. Twenty-two cosmids were mapped at 1q21-q22 (14 cosmids), 1q23 (3 cosmids), and 1q24-q25 (5 cosmids). Samples are A) primary tumor from case 1, B) xenograft tumor from case 1, C) xenograft tumor from case 7, D) primary tumor
from case 9, and E) xenograft tumor from case 5. All of these specimens were obtained by open biopsy before treatment and transplanted in nude mice. Black boxes: DNA amplification indicated by increased signals of cosmid. White boxes: absence of amplification. Slashed boxes: not determined.
Fig. 1

C

D

Case 18

Case 18

Case 1

Case 1

A

B
Fig. 2

A

Survival (%)

Time in months

without 1q21-q25 gain
(n=18)

with 1q21-q25 gain
(p=0.03)
(n=9)

B

Survival (%)

Time in months

without 1q21-q25 gain
(n=8)

with 1q21-q25 gain (n=8)
(p=0.02)

C

Survival (%)

Time in months

with 1q32-q43 gain (n=14)

without 1q32-q43 gain (n=13)
A

1q21-q22: 14 cosmids
1q23: 3 cosmids
1q24-q25: 5 cosmids

B CYS1-40

C CYS1-68

D

Tumor
\( \times 1 \) \( \times 5 \) \( \times 25 \)

Normal
\( \times 1 \) \( \times 5 \) \( \times 25 \)

3 kb

Fig. 3
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<th>Location</th>
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<th>B</th>
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</table>

Fig. 4
stages I-3’, 4S (p=0.000012).

Frequency of 1q21-q25 gain in stage 4 is significantly higher than in stages 1-3’. 4S (p<0.024).

Frequency of 2p22-p24 gain in stage 4 is significantly higher than in stages 1-3’. 4S (p<0.033).

Number of cases with chromosomal abnormalities/number of cases examined.

<table>
<thead>
<tr>
<th>Stage</th>
<th>18/27 (67)</th>
<th>16/27 (60)</th>
<th>16/16 (100)</th>
<th>11/11 (100)</th>
<th>Total</th>
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<tr>
<td>gain</td>
<td>6/27 (33)</td>
<td>6/27 (33)</td>
<td>8/16 (50)</td>
<td>8/16 (50)</td>
<td>4</td>
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<tr>
<td>1-3’ `</td>
<td>3/27 (11)</td>
<td>0/27 (0)</td>
<td>2/16 (13)</td>
<td>2/16 (13)</td>
<td>4</td>
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</table>

TABLE 1. Association of Chromosomal Abnormalities with Stage of Neuroblastoma.

<table>
<thead>
<tr>
<th>Stage</th>
<th>1q21-q25 (g)</th>
<th>2p22-p24 (g)</th>
<th>1q21-q25 (g)</th>
<th>1q21-q25 (g)</th>
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<tr>
<td>1-3’ `</td>
<td>17q21-q25</td>
<td>18q21-q25</td>
<td>17q21-q25</td>
<td>18q21-q25</td>
</tr>
<tr>
<td>Follow-up terms of the patient from onset, ( \mu ) and ( \mu ) in this case and the cases with or without ( \mu ) or without ( \mu ) of ( \mu ) ( \mu ) in this case and the cases with or without ( \mu ) or without ( \mu ) of ( \mu ).</td>
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</tbody>
</table>

**Table 2.** Clinical and histologic characters and \( \mu \) findings in Stage 4 Retinoblastomas.
than in CR or NP (p = 0.00078).

progressive disease. Prevalence of 1q21-9q25 gain in PD is statistically higher.

complete remission. "PD" under treatment with evidence of remission. "CR", number of cases with chromosomal abnormality/number of cases examined. CR, table 3. Association of 1q21-9q25 gain with clinical status of stage 4 neuroblastomas.

<table>
<thead>
<tr>
<th>Gain Status</th>
<th>Gain of 1q21-9q25 (%)</th>
<th>Gain of 2p23-p22.3 (%)</th>
<th>Gain of 1q32-9q33 (%)</th>
<th>Gain of 1q21-9q25 (%)</th>
<th>Total</th>
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<td>2 / 2</td>
<td>0 / 2</td>
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<td>16</td>
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<tr>
<td>CR</td>
<td>2 / 2</td>
<td>6 / 6</td>
<td>2 / 6</td>
<td>8 / 8 (100)</td>
<td>16</td>
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<tr>
<td>Control</td>
<td>18 / 16 (90)</td>
<td>6 / 16 (50)</td>
<td>10 / 16 (63)</td>
<td>6 / 16 (50)</td>
<td>50</td>
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</table>

TABLE 3. Association of 1q21-9q25 gain with clinical status of stage 4 neuroblastomas.
<table>
<thead>
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<th>Case</th>
<th>DIZ5-CYTI-40</th>
<th>Mean (S.D.)</th>
<th>DIZ5-CYTI-68</th>
<th>Mean (S.D.)</th>
<th>DIZ5-CYTI-40</th>
<th>Mean (S.D.)</th>
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<td>1.0 T0ld</td>
<td>2.0 (0.040)</td>
<td>6.0 (1.05)</td>
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<td>2.5 (0.65)</td>
<td>nd</td>
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<td>1.0 T0ld</td>
<td>2.0 (0.33)</td>
<td>2.0 (0.44)</td>
<td>nd</td>
<td>2.5 (0.47)</td>
<td>nd</td>
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<tr>
<td>1.0 T0ld</td>
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<td>2.5 (0.80)</td>
<td>nd</td>
<td>2.5 (0.80)</td>
<td>nd</td>
<td>2.3 (0.80)</td>
</tr>
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</table>

TABLE 4: Sign & Number counted on FISH analysis in PRy lyophilized.
Chapter II

Chromosomal imbalance patterns in regressive and progressive neuroblastomas
ABSTRACT

To clarify the differences in genetic backgrounds between regressive and progressive diseases in neuroblastomas, we analyzed 45 cases by comparative genomic hybridization (CGH), including sixteen stage 4 cases with bone metastasis and 29 infant cases (22 mass screening and seven stage 4S cases). We found two types of chromosomal imbalance pattern in these neuroblastomas. All 16 advanced cases showed multiple and regional gains and losses on CGH profile which have been observed in many other cancers in adult, whereas all of spontaneous regressive cases, 6 out of 7 (86%) stage 4S cases, showed whole chromosomal gains and losses without any peak on each chromosome. Interestingly, in 22 cases found by mass screening, 18 (82%) cases showed whole chromosomal abnormalities. All patients with whole chromosomal abnormalities on CGH profile did not have any poor prognostic factor. These patients are alive without evidence of disease. Four mass screening and one stage 4S cases with multiple and regional gains and losses similar to stage 4 cases showed poor prognosis. All of 5 (100%) infant cases with regional gains and losses showed 17q21–q25 gain. Four of these 5 (80%) cases had 11q21–q25 loss. And this loss was the most frequent and remarkable loss in regional chromosomal imbalances. These results indicate the difference in genetic backgrounds related to regressive and progressive disease in neuroblastoma. Classification of neuroblastomas using the chromosomal imbalance patterns in CGH would be useful for management of infant cases and for predictive assessment of prognosis.
INTRODUCTION

Neuroblastoma is one of the most common malignant tumors of childhood and shows variable clinical behaviors: progression to the fatal disease, differentiation to ganglioneuroblastoma or ganglioneuroma, and spontaneous regression\textsuperscript{1,2}. Prognosis of neuroblastoma depends on the lesion of metastasis and the age of onset\textsuperscript{1-3}. Progression to the fatal disease is most common in stage 4 disease (the International Neuroblastoma Staging System (INSS)\textsuperscript{3}) with bone metastasis in children over 1 year of age. Spontaneous regression and differentiation usually occur in infants (under 1 year of age), and it is most common in stage 4S tumors with the metastases confined to the liver, bone marrow, and/or skin.

In 1985, to find neuroblastomas before dissemination, mass screening (MS) by measuring of urinary catecholamine metabolites in infants aged 6 months was introduced in Japan. After that, incidence of the neuroblastoma aged less than 1 year has increased\textsuperscript{4-6}, and the patients found by MS have been treated. Nevertheless, the survival rate in patients with bone metastasis has not been improved\textsuperscript{4-6}. It is known that many neuroblastomas detected by MS show good prognosis and some, not all, of patients found by MS do not require any treatment\textsuperscript{4-6}. Frequency of neuroblastoma found in adrenal gland of the aborted fetuses has been reported to be 40 times as much as that in the after-birth patients\textsuperscript{7}. Accumulated data suggest that neuroblastomas may have several pathways in oncogenesis and that regressive neuroblastoma would be discriminated from progressive neuroblastoma in biological characteristics.
It should be noticed that several MS cases were dead in infancy due to treatment, whereas, reduced treatment for infant cases results in relapse of tumors. Clarification of genetic differences between progressive and regressive diseases would be useful to identify the regressive cases, which dose not need any treatment, among infant cases. Proper treatment against infant cases could be determined by genetic indicators with clinical markers for prognosis. MYCN amplification and lp loss of heterozygosity (LOH) are potent markers for prognosis in neuroblastoma. However, these markers are not enough to identify the progressive cases among early stage neuroblastomas like MS cases. The mechanisms of tumor regression or progression have not been understood in neuroblastoma. For the analysis of unknown region in a greater part of human genome, comparative genomic hybridization (CGH) is one of most useful technique for genome-wide assessment in solid tumors. CGH analysis enables us to survey the whole genome in a single hybridization using a microgram DNA from tumor, and to map abnormalities in the relative copy number of DNA sequences simultaneously onto the chromosomal region. Recently, CGH analysis in neuroblastoma have been reported14)-19). However, these studies have not described differences among regressive and progressive neuroblastomas because of lacking MS cases.

In this study, we show differences in genetic background between regressive and progressive diseases by analysis of neuroblastomas consisting of poor prognostic cases in stage 4, regressive cases in stage 4S and MS cases.

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MATERIALS AND METHODS

Tumor Specimens

Forty-five tumor specimens of neuroblastoma including 44 primary tumors and 1 metastatic tumor of bone were collected. All tumors were consecutive samples obtained by open biopsy before chemotherapy and radiotherapy. The stage of each patient was categorized according to the INSS\textsuperscript{3}. Twenty-two cases were found by MS; 8 cases in stage 1, 8 cases in stage 2, 4 cases in stage 3, and 2 cases in stage 4S. Seven cases diagnosed before screening age were categorized in stage 4S. Sixteen cases categorized in stage IV with bone metastasis were not found by MS. DNAs were extracted from the frozen tumor tissues according to standard procedures\textsuperscript{21}.

Comparative Genomic Hybridization

CGH was performed by indirect labeling methods according to previously described protocols with slight modifications\textsuperscript{12,13}. Tumor DNA was labeled with biotin 16-dUTP (Gibco BRL, Gaithersburg, MD) and reference DNA, which had been isolated from healthy human placenta, was labeled with digoxigenin 11-dUTP (Boehringer Mannheim, Indianapolis, IN) by nick translation. The reaction conditions were optimized for each sample so that labeled fragments ranging between 300 and 1000 bp in size after denaturation at 95°C were obtained. Six hundred nanograms of each labeled DNA from tumor and placenta were mixed with 15 μg of human Cot1 DNA (Gibco BRL) in 15 μl of hybridization buffer (50% formamide, 10% dextran sulfate, and 2×SSC). The mixture was denatured at 73°C for 5 min, and hybridized to a metaphase cells from peripheral mononuclear cells of healthy donors (Vysis, Downers Grove, IL;
FML labo., Tokyo, Japan). Prior to hybridization, the slides of metaphase cells were denatured at 73°C for 5 min in 70% formamide solution and then dehydrated in ethanol. After hybridization at 37°C for 3 days, the slides were washed in washing buffer (50% formamide, 2×SSC) and incubated with fluorescein (FITC) conjugated avidin (Dupont NEN, Boston, MASS) and anti-digoxigenin antibody conjugated with tetramethylrhodamine (TRITC) (Dupont NEN). Chromosomes were counterstained with 4′,6-diamidino-2-phenylindole dihydrochloride (DAPI) (Molecular Probes Inc., Eugene, OR). For each experiment, we included CGH using normal DNAs as a reference probe and normal DNAs as a test probe to check the quality of CGH experiment. For experimental quality controls, we performed CGH on cell lines in which 9p21 deletion was detected, and on primary tumors of neuroblastoma in which MYCN (on 2p24) amplification and 1p LOH (at 1p35-pter) have been found. The experiment was repeated at least twice for every case and reverse CGH was performed in representative cases to check the quality of the CGH data.

**Digital Image Analysis**

We used a digital image analysis system based on a fluorescence microscope with a double bandpass filter (Leica, Wetzlar, Germany) and a charge-coupled-device camera (Applied Imaging, Santa Clara, California) to acquire images of fluorescence on the metaphase cells. Three-color images representing the tumor DNA (green), reference DNA (red), and DAPI counterstaining from 8 to 10 metaphase cells per hybridization were collected. Green-to-red fluorescence ratio along individual chromosome was assessed with Cytovision software (Applied Imaging). The ratio values were
averaged, and the resulting profile was plotted along each individual chromosome. To increase the accuracy of the average of fluorescence ratio values, 95% confidence intervals were calculated. Chromosomal imbalances were detected on the basis of deviation of the ratio profile from the balanced value (green/red = 1.00). The values 1.25 and 0.75 were used as diagnostic cut-off levels for gain and loss, respectively

**Loss of Heterozygosity (LOH), Southern Blot Analyses, and Analysis of DNA ploidy**

Analysis of 1p LOH was performed in 17 cases as described. Southern blotting of MYCN was performed in all cases as described. Detection of DNA ploidy by fluorescence activated cell sorter (FACS) was performed in 18 cases as described.

**Statistical Analysis**

Overall survival was calculated by use of Kaplan-Meier estimation. We used the log-rank test to evaluate the relationship between chromosomal imbalance patterns and clinical features.

**RESULTS**

**Two Types of Chromosomal Imbalance Pattern in Neuroblastomas**

We found two types of chromosomal imbalance pattern in neuroblastomas. All 16 cases in stage 4 showed multiple and regional gains and losses on CGH profile. The representative CGH profiles of a dead case in stage 4 were shown in Fig 1A. This regional chromosomal imbalance pattern is generally found in several cancers in adult. Whereas, 8 out of 9 (89%) cases in stage 4S showed whole chromosomal gains and losses with straight lines on CGH profiles without any regional DNA copy number
changes. The representative CGH profiles of a regressive case in stage 4S were shown in Fig 1B. Table 1 shows the clinical and biological characters of 29 infant cases including 22 MS cases and 7 cases in stage 4S. These 29 tumors are expected to show good outcome including spontaneous regression and/or differentiation. Twenty-four out of 29 (83%) cases showed whole chromosomal abnormalities on CGH profile as same as that of regressive stage 4S cases. Eighteen out of 24 (75%) cases showing whole pattern were MS cases. Five cases (cases 4, 43, 14, 269 and 69) showed regional gains and losses in CGH similar to that in stage 4 cases.

**Relevance of Chromosomal Imbalance Pattern to Clinical Characteristics of Neuroblastoma**

Table 2 and 3 show the relevance of CGH profile patterns to the stage and clinical status of neuroblastomas. All of 24 cases showing whole chromosomal abnormalities were categorized in stages 1-3 and 4S. Any case showing whole pattern was not found in stage 4 neuroblastomas. Without any aggressive chemotherapy, these patients with whole pattern in CGH are alive without evidence of disease and categorized as being in complete remission (CR) (Table 3). Eight out of 24 (33%) whole pattern-neuroblastomas were stage 4S disease and showed spontaneous regression of the tumors.

Sixteen out of 21 (76%) cases showing regional gains and losses were categorized in stage 4. Five out of these 21 (24%) cases were infant; one case in stage 1, 3 cases in stage 2 and one case in stage 4S. Eight out of 21 (38%) patients showed progression and/or relapse of tumors, although they had been
treated with an aggressive chemotherapy. These 8 patients were categorized in progressive disease (PD) (Table 3). Five out of these 8 (63%) PD cases were dead within 7-28 months after diagnosis. Four out of 21 (19%) cases showing regional pattern were under treatment with evidence of disease (one case in stage 2B, one case in stage 4S and 2 cases in stage 4). Nine out of 21 (43%) patients were categorized as being in CR after the aggressive chemotherapy for 2 years. No case of 21 regional pattern neuroblastomas showed spontaneous regression and differentiation of the tumor.

Figure 2 shows the overall survival of two groups of patients with whole or regional profile patterns on CGH. Significant difference in survival rate between these two groups was observed (P=0.04). Regional gains and losses in CGH was associated with poor prognostic neuroblastoma. These data indicate that whole chromosomal abnormalities in CGH is associated with good prognostic neuroblastoma.

Relevance of Chromosomal Imbalance Pattern to Prognostic Factors in Neuroblastoma

Table 4 shows the prognostic factors and chromosomal imbalance patterns found in this study. Ten out of 21 (48%) case showing regional gains and losses had MYCN amplification. Loss of 1p35-pter were detected by CGH in 8 out of 21 (38%) cases, and 5 cases with 1p LOH were included in these 8 cases. Seven out of 10 (70%) cases showing regional pattern indicated diploid.

Interestingly, all cases showing whole pattern did not have neither MYCN amplification nor 1p LOH. Seven out of 8 (88%) cases showing whole pattern indicated aneuploidy which had
correlated with good prognosis\textsuperscript{26}. A diploid case showed whole pattern with no DNA copy number change similar to a profile in normal control experiment.

11q21–q25 Loss and 17q21–q25 Gain Detected in Infant Cases Showing Regional Gains and Losses in CGH

Figure 3 summarizes frequent DNA copy number abnormalities on 11q and 17q in neuroblastomas. All 5 (100\%) infant cases (cases 4, 43, 14, 269 and 69; Table 1) with regional abnormalities had 17q21–q25 gain, and 4 out of 5 (80\%) cases had 11q21–q25 loss (cases 4, 43, 14 and 269). 11q21–q25 loss also was found in 5 out of 16 (31\%) cases in stage 4. 11q21–q25 loss was the most frequent loss detected by CGH in neuroblastomas. 17q21–q25 gain also found in all 16 (100\%) cases in stage 4, was the most frequent gain detected by CGH in neuroblastomas as reported previously.

DISCUSSION

We present here two types of chromosomal imbalance pattern on CGH related to prognosis and clinical feature of neuroblastoma. Profile pattern with regional gains and losses was associated with advanced stage and poor prognosis of neuroblastoma. Regional gains and losses on CGH, generally found in many cancers, might be due to DNA amplifications and deletions of genes involved in oncogenesis and/or tumor progression. Whole chromosomal abnormalities found in MS and stage 4S cases showing spontaneous regression have never been reported in other cancers in adult. Whole chromosomal abnormalities might be due to abnormal chromosome number and ploidy changes indicating the different pathway in
tumorigenesis from neuroblastoma showing regional pattern on CGH.

The previous CGH studies in neuroblastomas have not clearly shown differences of chromosomal imbalance patterns relevant to prognosis or clinical feature\textsuperscript{14)-19}. In our study, by analysis for 29 infant cases including 22 MS cases, we clearly showed the differences of chromosomal imbalance patterns in regressive and progressive neuroblastoma.

Recently, some of clinical investigators have tried no treatment for MS cases in stage 1, because MS cases in early stages are considered to be regressive disease\textsuperscript{4)-6}. A few cases in infancy with no treatment of chemotherapy result in a relapse of tumors. Biologically useful marker to find the case which requires aggressive treatment is crucial in effective therapy. Chromosomal imbalance pattern detected in this study might be a potential indicators in decision of proper treatment for infant cases and at early stage. Five infant cases showing regional gains and losses in this study should require careful follow-up; 2 out of 5 cases (cases 269 and 69) have been treated with aggressive chemotherapy, while 3 cases (cases 4, 43 and 14) had been finished the treatment with a few course of the reduced chemotherapy for infant after tumor resection. These cases may have different clinical feature from good prognostic disease in infant.

Gain of 17q21-q25 and loss of 11q21-25 were the most frequent changes in neuroblastomas and might be remarkable markers for disease. The high frequency of 17q gain in neuroblastoma in this study coincides with previous reports.\textsuperscript{14)-17} In our study, 17q21-q25
gain was significantly associated with the stage 4 disease, however, interestingly, all of the neuroblastomas with regional chromosomal imbalance pattern including 5 infant cases in stages 1, 2B and 4S had 17q21-q25 gain. These results suggest that potential oncogenesis on 17q may be involved in initial step of oncogenesis of neuroblastoma. The candidate oncogenes might include nm23 (17q21.3), NGFR (17q21.22) and survivin (17q25)27.

Loss of 11q21-q25 was detected in 9 of 45 (20%) examined cases, in 4 of 29 (14%) infant cases in stages 1-3 and 4S, and in 5 of 16 (31%) cases in stage 4. Nine of 21 (43%) cases showing regional gains and losses and 4 of 5 (80%) infant cases in stages 1, 2 and 4S with regional gains and losses showed 11q21-q25 loss. Loss of 1p35-pter corresponding to 1p LOH was detected in 8 of 45 cases (18%), in 2 of 29 (7%) infant cases in stages 1-3 and 4S and in 6 cases of 16 (38%) in stage 4. Eight cases out of 21 (38%) showing regional gains and losses and 2 of 5 (40%) infant cases in stages 1, 2 and 4S showing regional gains and losses showed 1p35-pter loss. Frequency (80%) of 11q21-q25 loss in infant cases with regional pattern was higher than that of 1p loss (40%). These results suggest that 11q21-q25 loss is an crucial changes in addition to 1p loss and may be one of the most important factors in the initial step of oncogenesis of neuroblastomas.

Present study suggests the classification of neuroblastomas using the chromosomal imbalance pattern would be useful to diagnose poor prognostic disease in infant and early stage cases. Further molecular analysis of these chromosomal imbalances would shed light on the study in elucidation of the mechanisms.
in tumorigenesis of neuroblastoma.

REFERENCES


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activity analysis identify two biologically different groups of 4S neuroblastomas. Br J Cancer 181:394-400.


Figure Legends

Figure 1. The CGH profiles of two types of chromosomal imbalance pattern in neuroblastoma. The green-to-red ratios in fluorescence are plotted along the chromosome ideograms with 95% confidence intervals. The intensity ratio of a balanced copy number, shown by a center vertical bar, was calculated from the mode of the intensity ratio. The thresholds for over- and under-representation are shown by the fourth line (value of 1.25) and by the second line (value of 0.75), respectively. Shaded box shows heterochromatic region, which was not interpretable because of high suppression of hybridization by Cot1 DNA*. A) The representative CGH profiles showing regional chromosomal gains and losses. Dead case in stage 4 with 1p35-pter loss, 1p21 loss, 1q21-q31 gain, 2p22-pter gain, 3q24-q36 gain, 6q15-qter loss, 7p15-p22 gain, 11q21-qter loss, 13q21-q31 gain and 17q21-qter gain is shown. B) The representative CGH profiles showing whole chromosomal abnormalities in stage 4S case showing spontaneous regression (case 150). Loss of chromosome 3 and gain of chromosomes 6, 7 and 17 are detected.

Figure 2. The overall survival (Kaplan-Meier estimation) of two groups of patients with whole chromosomal abnormalities and with regional gains and losses on CGH analysis. The log-rank test evaluated the relationship between patients with regional gains and losses on CGH and poor prognosis (P=0.04).
Figure 3. Genetic imbalances on chromosome 11 and 17 in 45 neuroblastomas including MS cases. The lines on the left and the lines on the right of chromosome ideograms represent loss and gain of genetic material at the corresponding regions on each chromosome, respectively. The black line indicates infant cases in stages 1-3 and 4S, and the gray lines indicates stage 4 cases. The lines corresponding to entire regions of whole chromosomes, arms of p or q show whole chromosomal abnormalities. All 5 infant cases in stages 1-3 and 4S with regional chromosomal gains and losses had 17q21-q25 gain which was found in all stage 4 cases. Four out of these 5 cases had 11q21-q25 loss which was found in 5 out of 16 stage 4 cases and was the most frequent loss in neuroblastomas.
Fig. 2

Time in months

(\(p=0.04\))

Regional Pattern

(n=21)

Whole Pattern

(n=24)

Survival (%)
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<th>Sample</th>
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<th>Stage</th>
<th>Ploidy</th>
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<th>1p LOH</th>
<th>CGH</th>
<th>17q21-25</th>
<th>11q21-25</th>
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<td>W</td>
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1MS, mass screening; +, MS case; -, not MS case. 2According to the INSS. 3Determined by FACS. 4MYCN amplification; +, presence and -, absence of amplification. 5CGH profile pattern. 6aneu, aneuploid. 7nd, not determined. 8W, whole chromosomal abnormalities. 9di, diploid. 10R, regional chromosomal abnormalities.
Table 2. Relevance of CGH profile patterns to clinical stages of neuroblastoma

<table>
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<th>stage$^1$</th>
<th>Number of cases</th>
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<td>regional$^2$ (%)</td>
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<td>2</td>
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<td>3</td>
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<tr>
<td>total</td>
<td>21 (76)</td>
<td>24 (0)</td>
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</table>

$^1$According to the INSS; stage 2 includes 2A and 2B.  
$^2$Regional chromosomal abnormalities.  
$^3$Whole chromosomal abnormalities.
Table 3. Relevance of CGH profile patterns to clinical status of neuroblastomas

<table>
<thead>
<tr>
<th>clinical status</th>
<th>regional$^1$(%)</th>
<th>whole$^2$(%)</th>
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<tbody>
<tr>
<td>CR$^3$</td>
<td>9 / 21 (43)</td>
<td>24 / 24 (100)</td>
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<td>PD$^4$</td>
<td>8 / 21 (38)</td>
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<tr>
<td>UT$^5$</td>
<td>4 / 21 (19)</td>
<td>0 / 24 (0)</td>
</tr>
</tbody>
</table>

$^1$Regional chromosomal abnormalities. $^2$Whole chromosomal abnormalities. $^3$CR, complete remission. $^4$PD, progressive disease, including 4 dead cases. $^5$UT, under treatment.
<table>
<thead>
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</thead>
<tbody>
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<td></td>
<td>regional (%)</td>
<td>whole (%)</td>
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<td><strong>MYCN</strong> ³</td>
<td>10 / 21 (48)</td>
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<tr>
<td>1p LOH⁴</td>
<td>5 / 10 (50)</td>
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<tr>
<td>1p loss⁵</td>
<td>8 / 21 (38)</td>
<td>0 / 24 (0)</td>
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<tr>
<td>Ploidy: diploid</td>
<td>7 / 10 (70)</td>
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<tr>
<td>aneuploid</td>
<td>3 / 10 (30)</td>
<td>7 / 8 (88)</td>
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</tbody>
</table>

¹regional chromosomal abnormalities. ²whole chromosomal abnormalities. ³MYCN amplification. ⁴loss of heterozygocity. ⁵1p35-pter loss detected by CGH.
CONCLUSIONS

In this study, DNA copy number abnormalities associated with progressive and regressive diseases were examined in a variety of primary tumors of neuroblastoma.

(i) A novel gain at 1q21-q25 on CGH indicated a strong association with progressive and fatal neuroblastomas in stage 4 which showed resistance to aggressive chemotherapy.

(ii) Gain at 1q21-q25 was narrowed to DNA amplification at 1q23 by FISH and Southern blotting.

(iii) Two types of chromosomal imbalance pattern related to prognosis and clinical feature of neuroblastoma were found. Regional gains and losses was related to the advanced stage and poor prognosis of neuroblastoma. Whole chromosomal abnormalities was predominantly found in infant cases detected by MS and stage 4S cases showing spontaneous regression.

(iv) Gain at 17q21-q25 was detected in all of the neuroblastomas with regional chromosomal imbalance pattern. This alteration may include a potential oncogene possibly activated during initiation of oncogenesis of neuroblastoma.

(v) Loss at 11q21-q25 was the most frequent loss in neuroblastomas with regional chromosomal imbalance pattern and seems to be an important factor in an initial step of oncogenesis of neuroblastoma.

Present findings may have an impact on elucidation of mechanisms in tumorigenesis of regressive and progressive neuroblastomas, if confirmed by prospective study using a large number of neuroblastomas, and would be useful for establishment of proper and effective treatment on neuroblastoma at the outset in infant.
ACKNOWLEDGMENT

I express my heartfelt thanks to Drs. Masanao Miwa and Kazuhiko Uchida for their continuous and kind-hearted encouragement in this work, and for helpful suggestions and critical reading of the manuscript.

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