Rapid Detection of MYCN Gene Amplification and Telomerase Expression in Neuroblastoma

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ABSTRACT

Amplification of the MYCN gene and high telomerase activity predict a poor prognosis for the patients with neuroblastoma. We used PCR techniques for rapid detection of MYCN gene amplification and human telomerase reverse transcriptase (hTERT) expression in neuroblastoma specimens. The detection of MYCN gene amplification is based on differential PCR in which three primer pairs were used to coamplify a 180-bp fragment of target MYCN gene with two reference gene fragments, a 237-bp of p53 exon 7 and a 120-bp of β-globin exon 3, in a single tube of 40 surgically resected tumor samples. MYCN amplification was identified by this differential PCR in all 10 samples carrying more than 10 copies (already known to have MYCN gene amplification by Southern blot analysis). There were no false-negative or false-positive cases, and the relative intensity of MYCN bands in the differential PCR correlated significantly with the copy number determined by Southern blot analysis (γ = 0.99, P < 0.0001). This protocol was also applicable in the biopsy or aspirated samples, as well as the paraffin-embedded tissues, and in detecting intratumoral heterogeneity. Using RT-PCR procedures, hTERT mRNA expression was detectable in all 13 tumors with high telomerase activity. These nonradioisotopic PCR-based protocols for detecting MYCN gene amplification and hTERT mRNA expression are rapid and reliable and are likely to be useful to determine the biological behavior of neuroblastoma.

INTRODUCTION

Neuroblastoma arises from the embryonal neural crest and is the most common solid tumor in children, affecting ~1 in 7000 individuals (1). Clinically, neuroblastoma tumors show remarkable biological heterogeneity, resulting in favorable prognosis in some instances and unfavorable prognosis due to aggressive growth, despite multimodal therapy, in other instances (2, 3). To predict the biological behavior of an individual tumor more precisely, several parameters, such as MYCN amplification (>10 copies), DNA ploidy and deletion of the short arm of chromosome 1, NTRK1 expression, and telomerase activity, have been proposed to predict prognosis of neuroblastoma patients (4–9). Among them, MYCN amplification is frequently used as a clinical indicator of poor prognosis. In Japan, for the advanced cases with the MYCN-amplified tumors (>10 copies), intensive chemotherapeutic regimens are used (10). Recently, we reported that high telomerase activity correlated significantly with a poor prognosis of neuroblastoma patients (11). High telomerase activity is also considered to be a useful prognosis-predicting indicator as well as MYCN gene amplification (12).

Clinical usefulness of molecular analysis as a prognostic factor depends on reliability, rapidity, and simplicity of the procedure. Usually, the detection of gene amplification is carried out by Southern blot or dot blot hybridization procedures, which require microgram quantities of high quality DNA and several days to obtain the results. These procedures are difficult to apply to small tumor samples obtained by biopsy or aspiration and paraffin-embedded archival tissues because of the difficulties to obtain enough quantity/quality of DNA. These techniques are also unsuitable for rapid detection of gene amplification, which are used for determining for the appropriate chemotherapeutic regimen. The rapid detection of MYCN gene amplification, especially in small samples obtained by aspiration or biopsy, has stimulated considerable interest in improved clinical management of neuroblastoma patients.

PCR is a powerful procedure to amplify small amounts of DNA or mRNA for molecular analyses. This procedure is very beneficial for several reasons: (a) only small specimen amounts are required; (b) the detection procedure is rapid; and (c) partially degraded DNA may be analyzed. Recently, PCR-based detection of gene amplification has been demonstrated for several oncogenes (13–20). Although it is difficult to evaluate the exact gene copy numbers using PCR, some studies used radioisotopes or laser densitometers to estimate the gene copy number. However, almost all neuroblastomas with amplified MYCN gene have been reported to have more than 10 copies (4–6). Thus, it may be easy to distinguish tumors with the MYCN amplification (>10 copies) from tumors without it by PCR. To detect MYCN gene amplification in neuroblastoma quickly without using radioisotopes for diagnostic and therapeutic usefulness, we established a differential PCR protocol. This procedure
Fig. 1 Detection of MYCN gene amplification in frozen neuroblastoma tissue samples by Southern blot analysis and differential PCR. A. Southern blot analysis of three primary neuroblastomas. As an internal control, the filters were hybridized at the same time with a 32P-labeled β-globin probe. Densitometric analysis revealed that these cases harbored a single, 10, and 40 copies of the MYCN gene, respectively. B and C, ethidium bromide staining of separately amplified three fragments (conventional PCR, B) and coamplification (differential PCR, C) of p53 exon 7, MYCN, and β-globin exon 3 using primary neuroblastoma DNAs isolated from the case with a single copy of the MYCN gene and the case with 40 copies of the MYCN gene. The PCR product bands of single-copy genes were visualized after 21 cycles, whereas the band of tumor DNA with amplified MYCN gene was already visualized after 17 cycles, resulting to a shift of earlier cycles. D, kinetics of conventional or differential PCR of p53 exon 7, MYCN, and β-globin exon 3 using a primary neuroblastoma DNA with a single copy of the MYCN gene and two primary neuroblastoma DNAs known to harbor 10 and 40 copies of the MYCN gene. The intensity of each band with ethidium bromide staining was measured by CCD imaging sensor after 15–33 cycles of amplification. The kinetics of PCR of these fragments showed an exponential amplification phase during 17–27 cycles and a plateau phase after 29 cycles. In the tumor DNAs with the MYCN gene amplification, the linear phase of MYCN fragment shifted to earlier cycles. Except for a slight inhibition of β-globin exon 3 in differential PCR, the intensities of PCR-product bands in differential PCR were similar to those in conventional PCR.
could also be applied for partially degraded DNA isolated from archival paraffin-embedded and formalin-fixed sections. Histological examination of neuroblastoma specimens sometimes reveals hemorrhage, necrosis, and fibrosis, as well as tumor heterogeneity, resulting from maturational and growth cycle differences and from effects of chemotherapeutic agents. The use of this protocol for archival paraffin-embedded sections under histological assessment enables us to evaluate MYCN amplification in the restricted area with viable tumor cells, even in samples containing abundant noncancerous stromal or nonviable tumor cells, as well as to detect the intratumoral heterogeneity of MYCN amplification.

The PCR-based, highly sensitive telomerase assay called telomeric repeat amplification protocol assay (21) is a new tumor marker for cancer diagnosis and biological features (11, 22). More recently, the hTERT/hEST2 gene encoding the catalytic subunit of human telomerase was isolated (23, 24). Unlike other components of telomerase, such as human telomerase RNA (25) or telomerase-associated protein (26), mRNA expression of hTERT reportedly correlates well with telomerase activity.

Thus, existence of both MYCN gene amplification and hTERT expression in neuroblastoma cases may provide synergetic information as indicators of poor prognosis. Here, we present data on hTERT expression in neuroblastoma cases and reliability and usable molecular analyses.

MATERIALS AND METHODS

Tissues. We obtained human neuroblastoma tissues immediately after surgery and stored each specimen at −80°C. Thirty-one surgically resected tumor specimens that carried a single copy of the MYCN gene and 10 specimens that carried multiple (3–200) copies of the MYCN gene (11, 27) were analyzed in this study. In addition, three needle biopsies and seven bone marrow aspiration samples were obtained from patients with disseminated neuroblastomas. These 10 patients underwent needle biopsy or bone marrow aspiration for diagnosis, and after histological and molecular analysis, they received the appropriate chemotherapeutic regimen. The neuroblastoma cell line, TS-N-2, which carries eight copies of the MYCN gene, was analyzed as a positive control. In addition, we analyzed archival formalin-fixed and paraffin-embedded tissues in 6 of 41 cases whose frozen tissues had been examined for Southern blot analysis and in an additional 6 cases whose frozen tissues were unavailable.

DNA Isolation. From 0.1–0.5 g of frozen tissue specimens and 10⁶ of human adult peripheral mononuclear cells, genomic DNA was extracted using proteinase K, followed by phenol/chloroform extraction as described previously (28). For needle biopsy samples (0.01 g) and bone marrow aspiration samples (10⁶ cells), DNA was extracted from the remaining part of the samples after pathological examination. Concentration of the extracted DNA was measured by spectrophotometry or electrophoresis. For archival formalin-fixed, paraffin-embedded tissues, DNA was extracted after a xylene extraction step. These tissues were fixed in 10% buffered formalin for 24 h at room temperature, paraffin-embedded, and then processed according to routine pathological procedures. Several 5–10-μm sections from these tissues were cut using a microtome. A 1-ml aliquot of xylene was added into a 1.5-ml tube containing these tumor sections, rocked for 5 min, and spun for 5 min. The pellet was washed three times with 1 ml of ethanol and spun for 5 min. The supernatant was then discarded, and the pellet was dried in a desiccator for at least 30 min and dissolved in a 5–20-μl aliquot of water. In two tumors, which showed histopathological heterogeneity of tumor cells, we microdissected a 6-μm section into two or more tubes, following the tumor cell distribution.

Southern Blot Analysis. When enough DNA was obtained, 2 μg of genomic DNA derived from frozen tissues were digested to completion with 10 units of EcoRI, electrophoresed on a 1.0% agarose gel, and then blotted onto a nitrocellulose filter. The filter was hybridized to an MYCN probe (PN-MYC1; Oncor, Inc., Gaithersburg, MD) and a β-globin probe and then autoradiographed as described previously (6, 27).

PCR Primers. The MYCN primers were: MYCN-3, 5'-GGT AGT ATT CGT CCC ATT GGC A-3', and MYCN-4, 5'-GTG AAT CAG GTT GAG TTC ATT G-3'. These primers amplify a 178-bp segment of 3’ lesion downstream of the MYCN exon 3 (29). The p53 gene and the β-globin gene were used as the single-copy reference genes. The p53 exon 7 primers were described by Hsu et al. (30), amplifying a 237-bp fragment. The β-globin exon 3 primers were: β-1, 5'-GTG TGC TGG CCC ATC ACT TT-3'; and β-19, 5’-CAA GAA AGC GAG CTT AGT GA-3', amplifying a 120-bp segment of the β-globin exon 3 (31).

PCR Condition. PCR was carried out in a final volume of 50 μl using Thermal Sequence Cycler (Iwaki, Tokyo, Japan). The PCR mixture contains genomic DNA sample (10–50 ng), 50 mM KCl, 10 mM Tris-HCl (pH 8.3), 1.5 mM MgCl₂, 0.25 mM deoxynucleotide (Takara, Kyoto, Japan), 50 mM Tris-hydrochloride (pH 8.3), 75 mM KCl, 3 mM MgCl₂, 10 mM DTT, 20 units of RNase inhibitor (RNasin; Promega, Madison, WI), and 200 units of Moloney murine leukemia virus RNaseH-reverse transcriptase (Superscript; Life Technologies, Inc., Gaithersburg, MD). The reaction mixture was incubated at 37°C for 60 min. One μl of solution was amplified by PCR using primers specific to hTERT and GADPH, as reported previously (23). The PCR conditions and sequences were as follows:

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3 The abbreviations used are: hTERT, human telomerase reverse transcriptase; RT-PCR, reverse transcription-PCR; GADPH, glyceraldehyde-3-phosphate dehydrogenase.
mixture contains 50 mM KCl, 10 mM Tris-HCl (pH 8.3), 1.5 mM MgCl₂, 0.25 mM deoxynucleotide triphosphate, 0.5 μM of each primer, and 2 units of Taq polymerase (Amplitaq Gold; Perkin-Elmer, Foster City, CA). PCR was carried out in a final volume of 50 μl using a Program Temp Control System PC-800 (Astec, Fukuoka, Japan). After 12 min of incubation at 95°C, hTERT mRNA was amplified for 35 cycles (95°C for 60 s, 60°C for 60 s, and 72°C for 70 s), and GADPH was amplified for 20 cycles (95°C for 60 s, 60°C for 60 s, and 72°C for 70 s), followed by extension of 7 min at 72°C.

**Estimation of PCR and RT-PCR products.** After PCR or RT-PCR, 10 μl of each sample were electrophoresed on a 12% polyacrylamide gel or 2% agarose gel and stained with 0.2% ethidium bromide. The intensity of each band was visually evaluated as well as by CCD image sensor (Densitograph AE-6900-F; Atto, Japan) under UV illumination.

### Table 1 MYCN gene amplification and telomerase expression in surgically resected neuroblastoma tissue samples

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* Stage classification according to Evans (36).

b The copy number of the MYCN gene had been determined by Southern blot analysis and reported previously except for five cases, shown in column one by * (10, 26).

c The result of our differential PCR protocol estimated visually. +, the intensity of the MYCN band was distinctly stronger than those of the reference genes; −, the intensity of the MYCN band was similar to those of the reference genes.

d Relative intensity of the MYCN band in differential PCR was calculated by the equation: 2 × (MYCN height)/(β-globin height + p53 height) using a CCD image sensor.

e Telomerase activity classified by serial dilution of the extract as reported previously (11). High, detectable using the extract containing 0.06 μg of protein; Low, detectable using the extract containing 6 μg of protein but undetectable using 0.06 μg of protein; Nil, undetectable using the extract containing 6 μg of protein. TRAP, telomeric repeat amplification protocol.

f +, visually detectable after 35 cycles of amplification.
MYCN Amplification Estimated by Differential PCR

The Kinetics of Conventional and Differential PCR. In preliminary experiments, we separately amplified the three fragments of p53 exon 7, MYCN, and β-globin exon 3 from human adult peripheral mononuclear cell DNA, a primary neuroblastoma DNA known to harbor a single copy of the MYCN gene, and two primary neuroblastoma DNAs known to harbor 10 and 40 copies of the MYCN gene (Fig. 1A). The intensity of each PCR product band was quantified using a CCD image sensor. The estimation of PCR products after 15, 17, 19, 21, 23, 25, 27, 29, 31, and 33 cycles revealed a linear exponential phase and plateau phase for these three genes. The PCR product bands derived from a single copy of these genes were visualized after 21 cycles, whereas the bands derived from tumor DNA with amplified MYCN gene were already visualized after only 17 cycles, also resulting in a shift of the linear phase (Fig. 1B). We next coamplified these three segments in a single reaction tube (so-called differential PCR) using the same DNA samples (Fig. 1C). The PCR product bands derived from a single copy of these genes were also visualized after 21 cycles in the differential PCR. In the tumor sample with amplified MYCN gene, the linear phase also shifted to earlier cycles of the exponential phase. A slight inhibition occurred on the amplification of β-globin exon 3 in differential PCR. The difference of the intensity between the bands of the MYCN gene and the single-copy reference genes was obvious after 23 cycles. After 29 cycles, the PCR products reached the plateau phase (Fig. 1D). Because the amounts of the PCR products derived from the MYCN and the single-copy reference genes reflected template DNA concentration until 27 cycles of amplification using 50 ng of genomic DNA, our standard PCR protocol used 25 cycles of amplification using ~25 ng of genomic DNA in the present study.

For 40 cases with frozen tissue samples, the copy number of the MYCN gene had been determined by Southern blot analysis (Table 1) as reported previously (11, 27), except for five cases. This analysis using β-globin probe as an internal control revealed that 10 tumors had the amplified MYCN gene (3–200 copies), and 30 had a single-copy MYCN gene. In these 30 tumor samples without MYCN gene amplification, the amounts of the PCR products of the MYCN gene in the differential PCR protocol were similar to those of single-copy reference genes. In 10 tumor samples with amplified MYCN gene (≥3 copies), the intensity of the MYCN band was distinctly stronger than those of the single-copy reference genes. The results of visual evaluation and densitometry calculations are shown in Table 1. Both the sensitivity and specificity of detecting MYCN gene amplification visually by differential PCR were 100%. To determine the minimal copy number of detectable MYCN gene amplification in the present differential PCR protocol, we prepared DNA samples with two, three, four, and five copies of the MYCN gene by mixing the DNA samples with and without MYCN gene amplification. In DNA samples with three, four, five, and eight copies of the MYCN gene, the visual intensities of the MYCN bands were distinctly and progressively stronger than those of single-copy reference genes, and these were confirmed by using densitometry (data not shown).

Semiquantitative Evaluation by Using Differential PCR. To evaluate the quantitative accuracy of this protocol, the relative intensity of the MYCN band calculated by the equation of $2 \times (\text{MYCN height})/(\beta$-globin height + p53 height) (16) using a CCD image sensor was compared with the copy number of MYCN gene determined by Southern blot analysis. There was a significant correlation between these methods [MYCN copy number = 1.76 × (Relative intensity of the MYCN gene) − 1.54, γ = 0.99, P < 0.0001]. The copy number of the MYCN gene was estimated by the relative intensity of the MYCN gene using the following equation (MYCN copy number = 1.76 × (Relative intensity of the MYCN band) − 1.54).

Application of the Differential PCR for Small Tissues. In three needle biopsies and seven bone marrow aspiration samples obtained from advanced neuroblastoma patients, we could detect MYCN amplification in two cases [a needle-biopsy sample of a stage III tumor (case 95) and a bone marrow metastatic sample (case 197)] using the differential PCR (Fig. 2A). These results were obtained within 1 day, and on the next day, we started the chemotherapeutic regimen for MYCN-amplified tumors in these two patients. After the attainment of the primary tumor volume reduction or the diminishment of bone marrow metastasis due to chemotherapy, the primary tumors were resected at surgery and revealed to have 100 and 80 copies of the MYCN gene, respectively, by Southern blot analysis (data not shown). The remaining eight cases were treated with chemotherapeutic regimen for advanced tumors without MYCN amplification.
Application for Archival, Formalin-fixed, Paraffin-embedded Tissues. In five formalin-fixed and paraffin-embedded tissue samples whose MYCN copy number was already determined by Southern blot analysis using frozen tissues (Fig. 2B), the intensities of the MYCN bands were distinctly stronger in the samples with MYCN gene amplification after 25 cycles of PCR reaction. Next, we applied this method for five archival paraffin-embedded tissue samples in which the copy number of the MYCN gene was unknown. Among these five samples, two samples (cases U4 and U5) had MYCN gene amplification (Fig. 3).

Fig. 3 Histological analysis of neuroblastoma specimens with heterogeneity of MYCN gene amplification. A, histological examination of two neuroblastoma specimens with intratumoral heterogeneity. The upper section (case 173), which was resected after chemotherapy, contained small islands of residual viable tumor cells (a) with massive fibrotic and necrotic tissues (b). The lower section (case 185) showed intratumoral heterogeneity with so-called nodular lesion (c). B, results of differential PCR using DNAs isolated from paraffin-embedded sections. In case 173, MYCN amplification was undetectable in DNA isolated from the whole paraffin-embedded section (b) but could be detected using DNA isolated from the microdissected small islands of viable tumor cells remained (a). In case 185, MYCN amplification was detected in DNA isolated from the nodular lesion (c) but undetectable in other lesions (d).
cases 2, 5, 36, 77, and 90) had increased hTERT mRNA expression that was detectable in cases 55, 31, 4, 74, and 56. Among these five tissues, cases 55, 31, and 4 had the amplified MYCN gene, and cases 55, 31, 4, and 74 had high telomerase activity (detectable by telomeric repeat amplification protocol assay using the extract containing 0.06 µg of protein). However, none of five cases (cases 2, 5, 36, 77, and 90) had either amplified MYCN gene or high telomerase activity.

2B). Both patients had advanced stages of disease resulting in death, whereas the remaining three patients without detectable MYCN amplification continued to be disease free.

Intratumoral Heterogeneity. Although MYCN amplification could not be detected using DNA isolated from the frozen tissue or the whole paraffin-embedded section in case 173, a surgically resected tumor after chemotherapy, it could be detected using DNA isolated from the microdissected small island of viable tumor cells remaining after our differential PCR method (Fig. 3). In case 185, MYCN gene amplification could not be detected in DNA isolated from frozen tissue. Histopathological examination revealed intratumoral heterogeneity, which was classified as a “nodular type.” Using DNA isolated from this nodular lesion in the paraffin-embedded section, differential PCR showed MYCN gene amplification (Fig. 3). Both of these patients received the intensive chemotherapeutic regimen for the cases with MYCN amplification. Patient 173 has not had recurrence of neuroblastoma, but patient 185 died of recurrent tumor.

Detecting hTERT Expression by RT-PCR

In the 40 surgically resected cases investigated for MYCN gene amplification, hTERT mRNA expression was also evaluated by RT-PCR. Among them, telomerase activity had already been examined in 35 cases as reported previously (11); 13 showed high telomerase activity (positive using the extract containing 0.06 µg of protein), 20 showed low activity (positive using the extract containing 6 µg of protein but undetectable using the extract containing 0.06 µg of protein), and 2 did not show telomerase activity. Although GAPDH mRNA was similarly amplified after 20 cycles of amplification in all 40 samples, hTERT mRNA was detected in only 21 samples, including all 10 MYCN-amplified tumor samples after 35 cycles of amplification (Table 1; Fig. 4). The hTERT mRNA expression was detected in all 13 tumors with high telomerase activity and in 5 of 23 tumors with low or undetectable activity. Detection of hTERT mRNA expression by RT-PCR correlated significantly with high telomerase activity (P < 0.001). Among the remaining five tumors whose telomerase activity was not examined, three tumors with hTERT expression were advanced stages of disease.

DISCUSSION

Previously, quantitative PCR analyses have been studied for some oncogenes such as neu, epidermal growth factor receptor, and MYCC amplification as well as MYCN gene (13–20). Although PCR techniques are not completely quantitative, this rapid and easy technique is increasingly being used in both research and clinical settings. To detect MYCN gene amplification by semiquantitative PCR without using radioisotopes, we designed primers to amplify a 178-bp segment 3’ downstream of the MYCN gene exon 3. Because deletion of the p53 gene is rare in neuroblastoma (33, 34) and the β-globin gene has been commonly used as an internal control in Southern blot analysis of the MYCN gene (6, 27), we used a 237-bp segment of the p53 gene exon 7 and a 120-bp segment of β-globin gene exon 3 as reference genes. The sizes of these control fragments were designed to sandwich the MYCN fragment in electrophoresis so that we could avoid the over- or underestimation of MYCN gene amplification due to the degradation of template DNA. Consequently, our differential PCR protocol could detect MYCN gene amplification with 100% sensitivity and 100% specificity, both in frozen and paraffin-embedded neuroblastoma samples without using radioisotopes. This approach was considered to be useful for rapidity and reliable analysis of clinical specimens.

The prognosis of patients with neuroblastoma mostly depends on the biological characteristics of tumor cells as well as the stage of tumor. In almost all neuroblastomas with MYCN amplification, the copy numbers ranged between 10 and 300 (4–6, 27). Neuroblastomas with more than 10 copies of the MYCN gene have been reported to correlate with aggressiveness of tumor growth and a poor outcome for patients (4, 5). In Japan, we have tried new chemotherapeutic regimens against advanced neuroblastomas with MYCN gene amplification (≥10 copies), which is more intensive than usual regimens. To start chemotherapy as early as possible, we established the present protocol of differential PCR. This protocol could detect MYCN amplification (≥10 copies) retrospectively in archived and even partially degraded DNA isolated from paraffin-embedded tissues, in a few residual tumor cells after aggressive chemotherapy, and in a small lesion with intratumoral heterogeneity. In the reported cases with intratumoral heterogeneity, case 173 received the intensive chemotherapeutic regimen for the cases with MYCN amplification and remains alive without recurrence. Thus, accurate evaluation of intratumoral heterogeneity using our differential PCR protocol may be useful for the treatment of neuroblastoma patients.

We have reported that high telomerase activity correlated significantly with a poor prognosis for the patients (11, 12). In addition to all MYCN-amplified tumor showing high telomerase activity, the cases with high telomerase activity without MYCN amplification also showed a poor prognosis (12). Thus, high telomerase activity is also a useful prognosis-predicting indicator independent of MYCN amplification. Although telomerase activity in human development was reported recently to be controlled by the two mechanisms, transcriptional regulation of hTERT and alternative splicing...
of hTERT transcripts (35), hTERT mRNA expression detected by RT-PCR correlated significantly with high telomerase activity in neuroblastoma. Because this protocol can be performed within 1 day, detection of hTERT mRNA by RT-PCR may also have clinical usefulness to determine the biological behavior of neuroblastoma.

In conclusion, in the present study, we described a rapid and easy PCR-based protocol for detection of MYCN gene amplification and hTERT mRNA expression in neuroblastoma specimens with good specificity and sensitivity. These protocols may be clinically useful to determine the therapeutic regimen as well as scientifically useful to consider the biological characteristics of neuroblastoma.

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