Significance of Survivin mRNA Expression in Prognosis of Neuroblastoma

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Neuroblastoma (NB) is the most common malignant solid tumor in childhood, and among all childhood malignancies is second in prevalence only to leukemia. In NB we need to both make an accurate diagnosis and rapidly analyze the expression of genetic prognostic factors such as MYCN, H-ras, and trkA. Moreover, it has recently become important to analyze the expression of survivin mRNA, a member of the inhibitor of apoptosis protein family. Expression of the survivin gene is related to tumorigenesis and inhibition of apoptosis in some malignant tumors. We investigated its expression by reverse transcription-polymerase chain reaction (RT-PCR) in NB cell lines (SK-N-SH, NB-39, and IMR-32), two normal blood cell samples, and 13 clinical NB tumor samples. All three NB cell lines had high levels of mRNA expression for this gene, but normal blood cells had no expression. We detected expression of survivin mRNA in 7 of the 13 NB tumor samples (54%). Two NB patients were in stage I disease, 6 in stage II, and 5 in stage IV. Quantitative analysis by RT-PCR revealed that the ratio between survivin mRNA and human glyceraldehyde-3-phosphate dehydrogenase (h-GAPDH) mRNA was very low in stages I and II (0—0.017). In contrast, in advanced NBs (stage IV) the ratio was much higher (0—0.050). The prognoses of the three patients in the advanced stage who had high ratios of expression were poor. A high level of expression of survivin mRNA indicates a high grade of malignancy, high likelihood of recurrence, and poor prognosis.

Key words survivin; reverse transcription-polymerase chain reaction; prognostic factor; neuroblastoma

Neuroblastoma (NB) is a very common malignant solid tumor in childhood. Prognosis in NB patients tends to vary greatly, and many studies have demonstrated that both clinical and molecular biological factors are correlated with outcome.1) For example, patients under the age of 1 year at diagnosis usually have good prognoses, but those diagnosed over the age of 1 year have poor prognoses.2) Increased expression of the molecular biological factors MYCN, H-ras and trkA is well known in NB.3—11)

Recently, there has been great interest in apoptosis, or programmed cell death, the mechanism by which cells essentially suicide.12) Many inhibitors of apoptosis are known to contribute to tumorigenicity and increased spread of tumor cells.13) Survivin is a recently described member of the inhibitor of apoptosis protein (IAP) family.14) This gene exists on chromosome 17q and inhibits apoptosis by blocking the effects of caspase-9, which is activated in extrinsic and intrinsic pathways.14—17) Survivin is expressed in many malignant tumors, including breast, lung, stomach, colon and pancreatic cancers, bladder tumors, malignant lymphoma, and NB.18) It is not usually present in normal tissues and is rarely found in mature tissues.17) Thus, survivin expression is likely to be an important prognostic factor in tumor malignancy, and we considered that survivin mRNA expression would be useful in determining tumor malignancy and prognosis in NB.

We therefore used reverse transcription-polymerase chain reaction (RT-PCR) to investigate the expression of survivin mRNA in NB cell lines, normal blood cell samples, and clinical NB tumor samples.

Here, we describe how the degree of expression of survivin mRNA is a very useful prognostic indicator.

MATERIALS AND METHODS

Cell Lines, Clinical NB Tumor Samples, and Normal Blood Cell Samples Three NB cell lines (IMR-32,19,20) SK-N-SH,20,21) and NB-3920) were examined. They were cultured in RPMI 1640 medium supplemented with 10% fetal bovine serum, 100 U/ml penicillin, 0.1 mg/ml streptomycin, and 2 g/l sodium bicarbonate under 5% CO2 at 37 °C. Two normal adult blood cell samples and 13 clinical NB tumor samples were also examined. Two of the tumor samples were from recurrent tumors. These tumor tissues had been stored at −80 °C since collection. The clinical diagnoses for these patients had been made by histopathology. Informed consent was obtained from all patients before the study began.

RNA Extraction Total RNA from the three cell lines and 13 NB tumor samples was extracted with TRIzol reagent (Gibco BAL) by the acid-guanidium-phenol chloroform extraction method.22) Total RNA from the two normal blood cell samples was extracted with TRIzol LS Reagent (Gibco BAL) by acid-guanidium-phenol chloroform extraction method.16)

Reverse Transcription-Polymerase Chain Reaction For determination of survivin mRNA expression, total RNA (1 μg) was reverse-transcribed in a 10 μl reaction mixture with a first strand cDNA synthesis kit (Rever Tra-α-Ⅲ(TM), Toyobo). RT was performed with Oligo-dT. The mixture was incubated at 48 °C for 30 min, followed by annealing at 95 °C.

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for 10 min and then holding at 4°C. For MYCN mRNA expression, the mixture was incubated at 42°C for 50 min, followed by annealing at 72°C for 15 min and then holding at 4°C. For trkA, the mixture was incubated at 42°C for 20 min, followed by annealing at 99°C for 5 min and then holding at 4°C. PCR amplification was carried out in 10× reaction mixture containing 1.2 pmol of the respective primer. We used a KOD-Plus PCR kit (Toyobo). The PCR cycling conditions were as follows: for survivin, initial denaturation at 48°C for 30 min, 10 min at 95°C, followed by 25 cycles at 95°C for 15 s and 60°C for 1 min; for MYCN, initial denaturation at 95°C for 10 min, followed by 25 cycles at 94°C for 15 s, 57°C for 5 s, and 72°C for 10 s; for trkA, initial denaturation at 94°C for 2 min, followed by 25 cycles of denaturing at 94°C for 15 s, annealing at 60°C for 90 s, extension at 68°C for 20 s, and then holding at 4°C.

We used human glyceraldehyde-3-phosphate dehydrogenase (h-GAPDH) as an internal marker and NB cell lines (IMR-32, NB-39, and SK-N-SH) as positive controls. The primer sequences are listed in Table 1.

Analysis and Quantities of PCR Products PCR products were electrophoresed through 2.0% agarose gel, stained with ethidium bromide (Wako), and visualized under a UV lamp. We used a bioanalyzer (Agilent Technologies) to accurately determine band sizes (Fig. 1). For each sample we determined the ratios of survivin/h-GAPDH mRNA, MYCN/h-GAPDH mRNA, and trkA/h-GAPDH mRNA.

RESULTS AND DISCUSSION

We used RT-PCR to analyze survivin mRNA expression in three cell lines. Fig. 1 shows the result of bioanalyzer. Electrophoresis was used to approximate the bands and the bioanalyzer was used to determine accurate band sizes. The band size for survivin mRNA was 261 bp, and that of h-GAPDH as an internal marker was 209 bp. Survivin mRNA was expressed in all three NB cell lines (IMR-32, NB-39, and SK-N-SH). Expression of MYCN and trkA mRNA in the cell lines, as determined by bioanalyzer, is also shown in Fig. 1. Fig. 2 shows the expression of mRNA of survivin, MYCN and trkA relative to that of h-GAPDH mRNA in each cell line. Expression of MYCN mRNA was recognized in NB-39 and IMR-32, and that of trkA mRNA in NB-39 and IMR-32, but in SK-N-SH there was no expression of MYCN or trkA mRNA. The two normal adult blood cell samples did not express survivin mRNA (Fig. 3). Therefore, expression of survivin mRNA was apparent only in the NB cell lines. Survivin mRNA was detected in 7 of the 13 tumor samples (54%)

Table 1. PCR Primers

<table>
<thead>
<tr>
<th>Gene</th>
<th>Sequence</th>
<th>Detected size (bp)</th>
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</thead>
<tbody>
<tr>
<td>survivin</td>
<td>5’-AAG AAC TGG CCC TTC TTG GA-3’</td>
<td>261</td>
</tr>
<tr>
<td>survivin</td>
<td>5’-GGC TCT TTC TCT GTG CAG T’-3’</td>
<td></td>
</tr>
<tr>
<td>MYCN</td>
<td>5’-GAC CAC AAG GCC CTC AGT AC-3’</td>
<td>240</td>
</tr>
<tr>
<td>MYCN</td>
<td>5’-GTC GAT GGG AAG GCA TCG TT-3’</td>
<td></td>
</tr>
<tr>
<td>trkA</td>
<td>5’-TGG AGA AGA AGG AGG AAA CA-3’</td>
<td>412</td>
</tr>
<tr>
<td>trkA</td>
<td>5’-GCC TTG ACA GCC ACC ACC AF-3’</td>
<td></td>
</tr>
<tr>
<td>h-GAPDH</td>
<td>5’-TCC TCT GAC TTC AAC AGC GAC ACC-3’</td>
<td>209</td>
</tr>
<tr>
<td>h-GAPDH</td>
<td>5’-TCT TTC TTC TTC TTG TGC TCT TG-3’</td>
<td></td>
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</table>

Fig. 1. Expression of Survivin, MYCN and trkA mRNAs in NB Cell Lines by RT-PCR
Bands detected by bioanalyzer. Lane 1: marker; lane 2: SK-N-SH; lane 3: 1 NB-39, lane 4: IMR-32. The sizes of the PCR products were 261 bp (survivin), 241 bp (MYCN), 412 bp (trkA), and 209 bp (h-GAPDH).

Fig. 2. Expression of Survivin, MYCN and trkA mRNAs in NB Cell Lines (SK-N-SH, NB-39, and IMR-32)
The relative expression of each gene is given as the ratio of its mRNA expression to that of h-GAPDH.
are shown in Fig. 4. Three of the 5 stage IVA (advanced tumor samples and the expression levels plotted. The results mRNA was analyzed quantitatively by RT-PCR in the 13 (Table 2). The ratio between survivin mRNA and h-GAPDH. 13

![Fig. 3. Expression of Survivin mRNA in Normal Blood Cell Samples by RT-PCR](image)

Bands detected by bioanalyzer. Lane 1: marker; lane 2: male, 24 years; lane 3: female, 23 years. The sizes of RT-PCR products were 261 bp (survivin) and 209 bp (h-GAPDH).

### Table 2. Relationship between Expression of Survivin, MYCN, and trkA and Clinical Prognosis in NB Patients

<table>
<thead>
<tr>
<th>Tissue</th>
<th>Stage</th>
<th>Age</th>
<th>Survivin</th>
<th>MYCN</th>
<th>trkA</th>
<th>Recurrence and death</th>
</tr>
</thead>
<tbody>
<tr>
<td>T1</td>
<td>I</td>
<td>8 m</td>
<td>+</td>
<td>-</td>
<td>+</td>
<td>-</td>
</tr>
<tr>
<td>T2</td>
<td>I</td>
<td>9 m</td>
<td>-</td>
<td>-</td>
<td>-</td>
<td>+</td>
</tr>
<tr>
<td>T3</td>
<td>II</td>
<td>2 m</td>
<td>-</td>
<td>-</td>
<td>-</td>
<td>+</td>
</tr>
<tr>
<td>T4</td>
<td>II</td>
<td>8 m</td>
<td>+</td>
<td>+</td>
<td>-</td>
<td>-</td>
</tr>
<tr>
<td>T5</td>
<td>II</td>
<td>7 m</td>
<td>+</td>
<td>+</td>
<td>-</td>
<td>-</td>
</tr>
<tr>
<td>T6</td>
<td>II</td>
<td>4 m</td>
<td>-</td>
<td>-</td>
<td>-</td>
<td>-</td>
</tr>
<tr>
<td>T7</td>
<td>II</td>
<td>8 m</td>
<td>-</td>
<td>-</td>
<td>-</td>
<td>-</td>
</tr>
<tr>
<td>T8</td>
<td>II</td>
<td>7 m</td>
<td>-</td>
<td>-</td>
<td>-</td>
<td>-</td>
</tr>
<tr>
<td>T9</td>
<td>IVa</td>
<td>11 y</td>
<td>+</td>
<td>+</td>
<td>+</td>
<td>+</td>
</tr>
<tr>
<td>T10</td>
<td>IVa</td>
<td>6 y</td>
<td>-</td>
<td>-</td>
<td>-</td>
<td>-</td>
</tr>
<tr>
<td>T11</td>
<td>IVa</td>
<td>2 y</td>
<td>-</td>
<td>-</td>
<td>-</td>
<td>-</td>
</tr>
<tr>
<td>T12</td>
<td>IVa</td>
<td>5 y</td>
<td>+</td>
<td>+</td>
<td>-</td>
<td>-</td>
</tr>
<tr>
<td>T13</td>
<td>IVa</td>
<td>5 y</td>
<td>+</td>
<td>-</td>
<td>-</td>
<td>-</td>
</tr>
</tbody>
</table>

For survivin: +, low mRNA expression; ++, high expression; −, no expression. For MYCN and trkA: +, mRNA expression; −, no mRNA expression. For recurrence and death: +, occurred; −, did not occur.

![Fig. 4. Expression of Survivin mRNA in NB Tumor Samples](image)

Survivin mRNA abundance is expressed as a ratio to that of h-GAPDH mRNA. □: diagnosis at <1 year; alive; △: diagnosis at ≥1 year; alive; ■: diagnosis at ≥1 year; tumor recurrence, died.

(Table 2). The ratio between survivin mRNA and h-GAPDH mRNA was analyzed quantitatively by RT-PCR in the 13 tumor samples and the expression levels plotted. The results are shown in Fig. 4. Three of the 5 stage IVa (advanced stage) patients had higher relative expression of survivin mRNA (0.029—0.050) than did patients in the earlier clinical stages (I, II). These three patients in stage IVa all had recurrences and died. In contrast, in the other 10 patients the relative expression was low (0—0.017), and all 10 were alive without recurrence. Therefore, a ratio of about 0.02 is the cut-off point between good and poor prognosis (Fig. 4).

Moreover, we also determined the levels of expression of MYCN and trkA mRNA in the 13 patients (Table 2). Abnormal amplification and expression of MYCN and no, or low levels of, expression of trkA mRNA are well known to occur in advanced NB patients with poor prognoses.\(^3\)\(^{-5}\),\(^7\)\(^{-10}\) For T1—T8 patients (equivalent to early stage I and II, under the age of 1 year at diagnosis, and disease found by mass screening), 7 of the 8 patients except T6 had no MYCN mRNA expression and were positive for trkA mRNA expression. T1, T4, T5, and T6 patients expressed survivin mRNA, but the relative level of expression was low. All 8 patients were alive without recurrence. In contrast, the advanced NB patients (stage IVa: T9, T12, and T13) had relatively high levels of survivin mRNA expression; they developed recurrences and died, even though the T12 and T13 patients expressed trkA mRNA. This means that a high level of expression of survivin mRNA indicates a high grade of tumor malignancy, high likelihood of recurrence, and poor prognosis.

Survivin is a novel member of the IAP family and is expressed not only in several apoptosis-regulated fetal tissues, but also in a few adult differentiated tissues.\(^14\)\(^{-17}\) Furthermore, survivin is overexpressed in most common human cancers.\(^14\)\(^{-17}\) Survivin mRNA was expressed in all NB cell lines and was also expressed in 7 of 13 clinical NB tumor samples. It was not expressed in normal blood cell samples. Three of the 13 NB patients had unfavorable outcomes and had high levels of expression of survivin mRNA. Although we still need to analyze a greater number of advanced NB tumor samples, relative expression of survivin mRNA appears promising as a prognostic indicator in NB patients.

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