Real-Time Analysis of Tyrosine Hydroxylase Gene Expression: A Sensitive and Semiquantitative Marker for Minimal Residual Disease Detection of Neuroblastoma

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ABSTRACT

Purpose: The purpose of this study was to establish a sensitive and semiquantitative method for the detection of minimal residual disease of neuroblastoma, the most common solid tumor in childhood.

Experimental Design: Analysis was performed on a molecular level by reverse transcription-PCR using a new, real-time detection method. We measured two genes simultaneously, tyrosine hydroxylase (TH) as the target gene and glyceraldehyde-3-phosphate dehydrogenase as a reference gene, in blood and bone marrow samples at diagnosis and after follow-up from six patients with neuroblastoma, one patient with ganglioneuroma, and one patient with ganglioneuroblastoma.

Results: The sensitivity of the assay was 1:10⁶ peripheral WBCs. Four patients with stage IV neuroblastoma and one patient with stage III neuroblastoma were scored positive. The other stage III patient and the other two patients with ganglioneuroma and ganglioneuroblastoma followed by acute lymphoblastic leukemia, respectively, were scored negative. Control bone marrow aspirates were also negative. The TH assay is more sensitive than immunohistochemical detection, and the results of the TH assay corresponded with the results of MYCN amplification.

Conclusions: The described TH assay is specific, sensitive, and semiquantitative and can be used for the detection of neuroblastoma cell involvement in bone marrow and blood at diagnosis and during therapy. Furthermore, the TH assay is a possible prognostic marker for neuroblastoma.

INTRODUCTION

Neuroblastoma is the most common solid tumor in children (1). The tumor stage is classified according to Brodeur (2), ranging from localized tumors (stage I) with good outcome to tumors with bone or bone marrow metastases (stage IV) with poor prognosis. Treatment of patients with stage IV neuroblastoma consists of chemotherapy, resection of tumor, and/or local irradiation followed by autologous bone marrow transplantation (3–6).

Detection of neuroblastoma cell involvement in bone marrow is of great importance for treatment and clinical outcome (7). Staining methods are widely used to detect tumor cells, but immunocytochemical and immunohistochemical investigation is often difficult, and with these less sensitive methods, residual and circulating tumor cells can be missed (8, 9). To study minimal residual disease, highly sensitive methods are inevitable. A promising method is RT-PCR (3).

Neuroblastomas are characterized by elevated levels of catecholamine production. The first and rate-limiting step in the synthesis of catecholamines is catalyzed by TH. Therefore, this enzyme is used as a target for the detection of neuroblastoma. An increased expression of the TH gene measured with RT-PCR is indicative of the presence of neuroblastoma cells.

Detection of neuroblastoma on a molecular level has been described by several groups (10–14). Our purpose was to investigate whether introduction of a new method based on the 5’ nuclease assay (15) and the ABI PRISM 7700 sequence detector (16, 17) enables us to perform highly sensitive and semiquantitative detection of neuroblastoma cells in bone marrow and blood at diagnosis and after follow-up. Recent reports about real-time RT-PCR demonstrated the advantages of this method compared with conventional methods (18, 19).

Besides this, the method might have prognostic value. Investigation of biological features of patients with neuroblastoma is commonly performed for prognostic reasons. MYCN amplification, which is a biological factor with prognostic significance, is correlated with a poor prognosis (20, 21). Because the detection of increased expression of TH might have prognostic value (22–25), we looked at the possible role and clinical significance of the relationship between a biological feature...
(MYCN amplification) and the molecular information obtained with the described TH assay.

MATERIALS AND METHODS

Patients. Six patients with neuroblastoma, one patient with ganglioneuroma, and one patient with ganglioneuroblastoma followed by ALL were included in this study. Patient characteristics are given in Table 3. Control samples were BMAs from five children with ALL in complete remission.

Immunohistochemical Analysis. Formalin-fixed, paraffin-embedded, 4-μm-thick tissue sections were deparaffinized, rehydrated, and treated with 3% H2O2 for 30 min, followed by rinsing in PBS. For antigen retrieval, the sections were microwaved in citrate buffer (pH 6) twice for 5 min and placed at room temperature for 20 min, followed by rinsing in PBS. After preincubation with 20% normal goat serum (Vector Laboratories), sections were incubated with biotinylated goat antirabbit serum (1:200; Vector Laboratories) in PBS for 30 min, rinsed in PBS, and incubated for 45 min with horseradish peroxidase-labeled avidin-biotin complex (ABC standard, 1:100; Vector Laboratories). After rinsing with PBS, peroxidase was visualized with 0.05% diaminobenzidine (Sigma, St. Louis, MO) and 0.15% H2O2 in PBS for 5 min. Finally, the sections were rinsed in tap water, intensified with copper (31 mM CuSO4 and 400 mM NaCl), counterstained in Mayer's hematoxylin, rinsed in tap water, dehydrated, and mounted with Pertex (Histolab, Göteborg, Sweden).

Cell Line and Spiking of Cells in Blood. We used the neuroblastoma cell line IMR-32 (American Type Culture Collection) to set up the procedure. The cells were grown in DMEM (Life Technologies, Inc.) plus 10% FCS (Integro) and 2 mM L-glutamine (Life Technologies, Inc.) for 10 min, overnight incubation was performed with the primary antibody rabbit antihuman synaptophysin (DAKO, Glostrup, Denmark) diluted 1:50 in 0.05 M Tris-HCl, 0.1 M NaCl, and 15 mM NaN3 (pH = 7.2). After rinsing in PBS, the slides were incubated with biotinylated goat antirabbit serum (1:200; Vector Laboratories) in PBS for 30 min, rinsed in PBS, and incubated for 45 min with horseradish peroxidase-labeled avidin-biotin complex (ABC standard, 1:100; Vector Laboratories). After rinsing with PBS, peroxidase was visualized with 0.05% diaminobenzidine (Sigma, St. Louis, MO) and 0.15% H2O2 in PBS for 5 min. Finally, the sections were rinsed in tap water, intensiﬁed with copper (31 mM CuSO4 and 400 mM NaCl), counterstained in Mayer’s hematoxylin, rinsed in tap water, dehydrated, and mounted with Pertex (Histolab, Göteborg, Sweden).

Sample Preparation, Isolation, and Reverse Transcription of mRNA. One volume of bone marrow section or (spiked) blood was mixed with 10 volumes of DNA/RNA Stabilization Reagent for Blood/Bone Marrow (Roche Diagnostics).

The conditions for the PCR were 2 min at 50°C; cycling parameters were 15 s at 95°C and 1 min at 60°C (40 cycles), hold at 15°C.

Sequences of the used primers and probes are listed in Table 1. The primers for TH are located in exons not inﬂuenced by alternative splicing. To avoid contamination of genomic DNA, the reverse primers for both TH and GAPDH are located in successive exons.

Plan of Investigation. We wanted to establish a sensitive, specific, and semiquantitative method for the detection of neuroblastoma cell involvement in PB, BMA, and BMB (7) samples on mRNA level. Furthermore, these TH assay results are compared with results of immunohistochemical analysis to...
investigate whether the TH assay can be a useful and better method for the detection of minimal residual disease. Finally, results of MYCN amplification are compared with TH assay results to investigate the possible prognostic value of the TH assay.

RESULTS

Immunohistochemical Analysis

Cristabiopsies for histological examination were performed in six of eight patients. In patients 4 and 8, cristabiopsies were not performed. In patient 5, immunohistochemistry was performed in BMA and in very small and therefore underrepresentative cristabiopsies. The results were negative. In patient 2, the BMB was also relatively small.

Table 1 DNA sequences and locations of the primers and probes used in the PCR

<table>
<thead>
<tr>
<th>Gene</th>
<th>Primer/probe</th>
<th>Location</th>
</tr>
</thead>
<tbody>
<tr>
<td><strong>GAPDH</strong></td>
<td>Forward primer: 5'-CAACAGCCTCAAGATCATCAGC-3' &lt;br&gt; Reverse primer: 5'-TGGCATGACTGTGGTGTGAG-3' &lt;br&gt; Probe: 5'-(FAM)CCTGGCCAAGGTCATCCATGACAAC (TAMRA)-3'</td>
<td>Exon6/exon7/exon8</td>
</tr>
<tr>
<td><strong>TH</strong></td>
<td>Forward primer: 5'-TCATCACCTGTACCAAGTT-3' &lt;br&gt; Reverse primer: 5'-GGTGCCGTGCTGTACT-3' &lt;br&gt; Probe: 5'-VICAGCGAGAAGCTGATTGCTGAGA(TAMRA)-3'</td>
<td>Exon5/exon6</td>
</tr>
</tbody>
</table>

*Primers and probes were designed according to the sequence reported in PubMed [GenBank accession no. J04038 (GAPDH gene) and accession no. NM_000360 (TH gene)]. Reverse primers are located in different exons to exclude the amplification of genomic DNA.

Fig. 1 Sensitivity of the real-time RT-PCR method: dilutions of IMR-32 cells in PB. The normalized fluorescence for detection of TH (A) and GAPDH (B) is shown as a function of cycle number.
with a sensitivity of 1:10^6 peripheral WBCs. The presence of results as demonstrated by their CT values (Table 3).

Integrity of the mRNA from all patient samples was shown). Integrity of the mRNA from all patient samples was confirmed by PCR for GAPDH. All samples showed similar shown). Integrity of the mRNA from all patient samples was confirmed by PCR for GAPDH. All samples showed similar shown). Integrity of the mRNA from all patient samples was confirmed by PCR for GAPDH. All samples showed similar

\[ \Delta C_T = C_T (TH) - C_T (GAPDH) \]

In the unspiked sample, no expression of TH was detected, so no \( \Delta C_T \) could be calculated.

In the same tubes, the integrity and relative amount of mRNA were tested by the expression of GAPDH. All samples showed almost the same results, as illustrated in Fig. 1B and demonstrated by their individual \( C_T \) values (Table 2). There is an inverse correlation between \( C_T \) and amount of target: low amount of target corresponds with a higher \( C_T \) value; and samples with higher amounts of target will have lower \( C_T \) values.

Calculation of the \( \Delta C_T \) values [\( \Delta C_T = C_T (TH) - C_T (GAPDH) \)] demonstrated that a \( C_T \) value of 18 corresponded with a sensitivity of 1:10^6 peripheral WBCs. The presence of increased amounts of neuroblastoma cells in samples, with ratios of 1:10^5 and 1:10^6 peripheral WBCs, corresponded with \( \Delta C_T \) values of 16 and 12, respectively.

**TH Assay Results of Our Patient Group**

The established TH assay was used to investigate different samples (PB, BMA, and BMB) of our patient group, together with BMA samples from control patients, i.e., patients with ALL (n = 5). An example of the detection of TH gene expression from patient samples is demonstrated in Fig. 2.

In all experiments, a positive control was included (IMR-32 cell line) that showed consistent \( C_T \) values (data not shown). Integrity of the mRNA from all patient samples was confirmed by PCR for GAPDH. All samples showed similar results as demonstrated by their \( C_T \) values (Table 3).

**Detection of Minimal Residual Disease: Immunohistochemistry versus TH Assay**

Immunohistochemical investigation of BMB samples from patients with stage III neuroblastoma (patients 1 and 2) demonstrated no neuroblastoma cell involvement, whereas a positive TH assay result was obtained in the BMB and PB samples from patient 2 (Table 3). This patient died of complications of treatment.

Investigation of samples from patients with stage IV neuroblastoma demonstrated that both methods were positive for patient 3. This patient died of disease. In patient 5, the initial BMB sample showed massive tumor infiltration in the H&E staining, but after five courses of chemotherapy, immunohistochemistry failed to show residual tumor cells, whereas the TH assay was still positive. This patient also died of disease.

Both methods detected neuroblastoma cell involvement in samples from patient 6 after follow-up. Immunohistochemically investigated samples were negative after 8 courses of chemotherapy, whereas the TH assay became negative after 11 courses of chemotherapy. Immunohistochemistry after 11 courses showed one solitary positive cell, which could not be retrieved in the H&E staining and therefore could not be ascertained to be a tumor cell. This patient has been in remission for 2 years.

Both methods were negative for samples from patient 7 (ganglioneuroma).

No comparison could be made for patients 4 (stage IV) and 8 (ganglioneuroblastoma followed by ALL) because no immunohistochemical examination was performed.

**Potential Prognostic Value: Comparison of the TH Assay with MYCN Amplification**

Both patients with stage III neuroblastoma (patients 1 and 2) had an amplified MYCN gene status, but only patient 2 was positive with the TH assay (Table 3). Patient 2 died of complications of treatment.

Three of four patients with stage IV neuroblastoma (patients 3–5) were positive with the TH assay and had an amplified MYCN gene status. All three patients had a bad outcome.
Real-Time Analysis of TH Gene Expression

MYCN amplification was not detected in patient 6; however, as mentioned, the TH assay was initially positive and became negative after 11 courses of chemotherapy. This patient has been in remission for 2 years.

No MYCN amplification and no bone marrow involvement by neuroblastoma cells was detected in samples from patient 7 (ganglioneuroma) and patient 8 (ganglioneuroblastoma followed by ALL).

DISCUSSION

Detection of neuroblastoma cells by RT-PCR for TH has been described by several groups (10–14). These methods contain post-PCR manipulations, which are time-consuming and laborious, and they introduce the risk of carry-over contamination. Recently, a method was developed (16, 17) to overcome these limitations of post-PCR sample handling. We used this method for the detection of expression of TH and GAPDH in BMA, BMB, and PB from patients with neuroblastoma. Unfortunately, for some patients, no samples were available at various time points because these patients had already been treated at the start of the study.

Sensitivity. The sensitivity of this method was determined by spiking with IMR-32 cells (a neuroblastoma cell line) and turned out to be 1:10⁶ peripheral WBCs. This illustrates a better result than other groups have found: Moss et al. (27, 28) reported a detection of 1 tumor cell in 10⁵ mononuclear cells with immunocytochemistry; and Faulkner (29) found a similar sensitivity. A possible reason for this lower detection limit may be the difficulty in distinguishing neuroblastoma cells from other cells in the matrix (30).

Catecholamine Concentration and TH Assay. Normal catecholamine levels were found in patients negative for the TH assay. Only the results from patient 6 deviated: although no increased catecholamine concentrations were found after follow-up, expression of TH was detected until completion of 11 courses of chemotherapy. These results indicate a higher sensitivity; however, to understand these findings, more comprehensive studies are needed.

Observation of an elevated catecholamine production was accompanied in all but one case by a positive TH assay. The exception was patient 7, who had a ganglioneuroma. These tumors, however, can be admixed with pheochromocytoma and may cause catecholamine production without an increased TH expression (31). TH assay results are therefore more specific for the detection of neuroblastoma cell involvement than the measurement of catecholamine production.

Minimal Residual Disease. Immunohistochemical investigation is not sensitive enough to detect residual and circulating tumor cells (9). To demonstrate that the TH assay is more sensitive for the detection of minimal residual disease, we compared the results of immunohistochemical analysis with the TH assay results. Four patients demonstrated the same result for both methods: patients 1 and 7 were negative; and patients 3 and 6 were positive. Immunohistochemical investigation did not demonstrate neuroblastoma cell involvement in patient 2 at diagnosis and in patient 5 after follow-up. A positive TH assay result for these patients in combination with the clinical outcome for these patients clearly demonstrated the increased sensitivity of the TH assay. The results from patient 6 were less unequivocal but also seemed indicative of an increased sensitivity of the TH assay; however, taking into consideration the fact that this patient is in remission, the clinical significance of this result is unclear. Therefore, larger patient groups have to be studied to elucidate the clinical significance of results such as the ones from patient 6. Other investigators confirm the potential clinical utility of real-time PCR as a detection method for tumor cell involvement (19). The TH assay is therefore a highly effective tool for the molecular monitoring of minimal residual disease, although accurate definitions of molecular remission and molecular relapse have to be established to understand the biological and clinical significance of minimal residual disease.

With the exception of patient 5, we found the same TH assay result in BMB and BMA sample combinations (n = 4). This strongly indicates that BMA samples alone could be sufficient for screening and follow-up for neuroblastoma cell involvement; however, this has to be studied more extensively. An additional advantage of examination of BMA samples is the fact that they are less invasive for the patient than BMB samples.

Prognostic Value. Results of MYCN amplification, a prognostic factor for neuroblastoma, were compared with the results of the TH assay. Although MYCN amplification was only performed in the primary tumor at diagnosis, whereas detection
Table 3  Patient characteristics and results from all patient samples

<table>
<thead>
<tr>
<th>Patient no.</th>
<th>Age at diagnosis (mo)</th>
<th>Diagnosis (tumor stage)</th>
<th>Treatment at sampling time</th>
<th>Catecholamine concentration (in urine)</th>
<th>Material</th>
<th>CT</th>
<th>ΔC&lt;sub&gt;T&lt;/sub&gt;</th>
<th>TH</th>
<th>TH score&lt;sup&gt;b&lt;/sup&gt;</th>
<th>Immunohistochemical detection of neuroblastoma cells&lt;sup&gt;c&lt;/sup&gt;</th>
<th>MYCN amplification&lt;sup&gt;d&lt;/sup&gt;</th>
<th>Clinical outcome</th>
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<tbody>
<tr>
<td>1</td>
<td>76</td>
<td>Neuroblastoma (III)</td>
<td>Untreated</td>
<td>BMB Normal</td>
<td>BMA</td>
<td>15</td>
<td>37</td>
<td>22</td>
<td>-</td>
<td>-</td>
<td>+</td>
<td>CR</td>
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<tr>
<td>2</td>
<td>19</td>
<td>Neuroblastoma (III)</td>
<td>Untreated</td>
<td>BMB Elevated</td>
<td>BMA</td>
<td>15</td>
<td>34</td>
<td>19</td>
<td>+</td>
<td>-</td>
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<td>DC</td>
</tr>
<tr>
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<td>BMB Elevated</td>
<td>BMA</td>
<td>16</td>
<td>29</td>
<td>13</td>
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<td>NT</td>
<td>+</td>
<td>DOD</td>
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<tr>
<td>4</td>
<td>36</td>
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<td>Untreated</td>
<td>BMB Elevated</td>
<td>BMA</td>
<td>17</td>
<td>18</td>
<td>1</td>
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<td></td>
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<td></td>
<td>After 7 courses with VECI</td>
<td>BMB Elevated</td>
<td>BMA</td>
<td>16</td>
<td>28</td>
<td>12</td>
<td>+</td>
<td>NT</td>
<td>NT</td>
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<td></td>
<td></td>
<td></td>
<td></td>
<td>BMA Elevated</td>
<td>BMA</td>
<td>16</td>
<td>39</td>
<td>23</td>
<td>-</td>
<td>NT</td>
<td>+</td>
<td>DOD</td>
</tr>
<tr>
<td>5</td>
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<td>Neuroblastoma (IV)</td>
<td>After 5 courses with VECI</td>
<td>BMB Elevated</td>
<td>BMA</td>
<td>20</td>
<td>36</td>
<td>16</td>
<td>+</td>
<td>NT</td>
<td>-</td>
<td>DOD</td>
</tr>
<tr>
<td>6</td>
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<td>After 5 courses with VECI</td>
<td>BMB Elevated</td>
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<td>17</td>
<td>29</td>
<td>12</td>
<td>+</td>
<td>NT</td>
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<td></td>
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<td>After 7 courses VECI</td>
<td>BMB Normal</td>
<td>BMA</td>
<td>16</td>
<td>34</td>
<td>18</td>
<td>+</td>
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<td></td>
<td>After 8 courses with VECI</td>
<td>BMB Normal</td>
<td>BMA</td>
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<td>32</td>
<td>12</td>
<td>+</td>
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<td>After 10 courses with VECI</td>
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<td>32</td>
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<td>+</td>
<td>NT</td>
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<td></td>
<td>After 11 courses with VECI</td>
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<td>BMA</td>
<td>15</td>
<td>37</td>
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<td>38</td>
<td>21</td>
<td>-</td>
<td>NT</td>
<td>-</td>
<td>CR</td>
</tr>
<tr>
<td>8</td>
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<td>Untreated</td>
<td>BMB Normal</td>
<td>BMA</td>
<td>20</td>
<td>39</td>
<td>23</td>
<td>-</td>
<td>NT</td>
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<td>CR</td>
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<td>BMA Elevated</td>
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<td>BMA Elevated</td>
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<td>BMA Elevated</td>
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</table>

<sup>a</sup> ΔC<sub>T</sub> = C<sub>T</sub>(TH) − C<sub>T</sub>(GAPDH).
<sup>b</sup> Samples with ΔC<sub>T</sub> values ≥ 21 were scored negative.
<sup>c</sup> Examination only in BMB.
<sup>d</sup> Examination in primary tumor.
<sup>e</sup> NT, not tested; ND, not detectable; VECI, combination of vincristine, teniposide, carbopolatin, and ifosfamide; CR, complete remission; DOD, died of disease; DC, died of complications of treatment.
<sup>f</sup> Right side.
<sup>g</sup> Left side.
<sup>h</sup> Solitary positive cell, not retrieved in H&E staining.
of circulating tumor cells with the TH assay was performed at diagnosis and after follow-up, striking similarities were found comparing both results.

A negative result for both methods corresponded with a good clinical outcome, and patients who were positive for both methods had a bad outcome. However, patient 1, who had stage III neuroblastoma, demonstrated clinically interesting results: in contradiction to a prognostically unfavorable MYCN gene status, the TH assay was negative. Because the clinical outcome for this patient was good, it is obvious that in this case the TH assay results could be a worthwhile additional predictive factor. Also interesting were the results from patient 6: the TH assay results became negative during follow-up. Furthermore, absence of MYCN amplification and a good clinical outcome were observed. Taking into consideration that our patient group is relatively small, some reservations have to be made regarding the conclusions based on the TH assay results. Despite this, the TH assay results seemed suggestive for a prediction of clinical outcome. More comprehensive studies are required to investigate the real prognostic value of the TH assay.

Recent reports concerning the use of molecular detection for the prediction of disease outcome demonstrated that minimal residual disease detection in bone marrow may predict poor prognosis (24, 32). Other recent reports confirm the use of detection of minimal residual disease as a prognostic marker (23) or demonstrate an association of molecular detection of TH and disease outcome (25). Based on our own results, we believe that it is worthwhile to use the described TH assay as a prognostic marker for neuroblastoma. However, more studies with larger patient groups are required to establish the relationship between and the clinical significance of biological information obtained with MYCN amplification and molecular information obtained with the TH assay. There are indications that persistence of TH gene expression has prognostic value, although the independent prognostic value compared with conventional markers is still unclear (8, 22, 23).

In summary, detection on a molecular level by real-time PCR is highly specific and efficient, and more than one gene can be studied simultaneously. The described TH assay turned out to be a quick and sensitive way to study neuroblastoma cell involvement in bone marrow and/or blood at diagnosis and during follow-up. The sensitivity of the TH assay is higher than that seen with immunohistochemical analysis and thus more suitable for studying minimal residual disease. Applications of the TH assay could be monitoring of the efficiency of tumor cell purging after CD34 selection in autologous hematopoietic stem cell harvests (12, 33). Finally, the TH assay is of possible prognostic value.

REFERENCES

10. Miyajima, Y., Horibe, K., Fukuda, M., Matsumoto, K., Numata, S., Mori, H., and Kato, K. Sequential detection of tumor cells in the peripheral blood and bone marrow of patients with stage IV neuroblastoma by the reverse transcription polymerase chain reaction for tyrosine hydroxylase mRNA. Cancer (Philad.), 77: 1214–1219, 1996.
21. Cohn, S. L., London, W. B., Huang, D., Katzenstein, H. M., Salwen, H. R., Reinhart, T., Madafiglio, J., Marshall, G. M., Norris, M. D., and Haber, M. MYCN expression is not prognostic of adverse...


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