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

Lambert H. J. Lambooy, Corrie E. M. Gidding, Lambert P. van den Heuvel, Christina A. Hulsbergen-van de Kaa, Marjolijn Ligtenberg, Jos P. M. Bökkerink, and Ronney A. De Abreu

Departments of Pediatrics [L. H. J. L., C. E. M. G., L. P. v. d. H., J. P. M. B., R. A. D. A.], Pathology [C. A. H. d. K.], and Pathology and Human Genetics [M. L.], University Medical Center Nijmegen, 6500 HB Nijmegen, the Netherlands

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 5 min, followed by rinsing in PBS. For antigen retrieval, the sections were microwaved in citrate buffer (pH 6.0) 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) 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 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 (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 Glutamax I (Life Technologies, Inc.).

**Sample Preparation, Isolation, and Reverse Transcription of mRNA.** One volume of bone marrow sample or (spiked) blood was mixed with 10 volumes of DNA/RNA Stabilization Reagent for Blood/Bone Marrow (Roche Diagnostics). The isolation of mRNA was performed with the mRNA Isolation Kit for Blood/Bone Marrow (Roche Diagnostics). Briefly, total nucleic acids are bound to magnetic glass particles, and after washing and eluting, the mRNA fraction is isolated by means of hybridization to biotin-labeled oligo(dT) and captured by streptavidin-coated magnetic particles, followed by magnetic separation. Patient sample handling did not include density gradient centrifugation because this might result in loss of cells (26).

The isolated mRNA was reverse transcribed at 37°C for 1 h. The final concentrations in the reaction (24 μl) were 1X PCR Buffer II (Perkin-Elmer, Foster City, CA), 1 mM deoxyribonucleotide triphosphate (Life Technologies, Inc.), 4 mM MgCl2 (Life Technologies, Inc.), 0.5 μg of Random Hexamer Primer (Promega), 20 units of RNase OUT (Life Technologies, Inc.), and 130 units of Superscript II (RNase H- Reverse Transcriptase; Life Technologies, Inc.).

**Real-time PCR.** The method is based on the 5’ nuclease assay (15) and the ABI PRISM 7700 sequence detector (16, 17). Briefly, the method uses a dual-labeled fluorogenic probe that will specifically hybridize between the primers. The probe contains a reporter (FAM in case of GAPDH or VIC in case of TH) at the 5’ end and a quencher (TAMRA) at the 3’ end. While the probe is intact, the quencher suppresses the emission spectrum of the reporter. During the annealing stage, the probe will hybridize, and the 5’ nuclease activity from the polymerase will degrade the probe during polymerization, resulting in an increase in fluorescent emission. The fluorescence is continuously measured with an ABI PRISM 7700 sequence detector (Perkin-Elmer). Therefore, the reactions are monitored in real time. After a number of PCR cycles, the fluorescence is increased to a point above the threshold (chosen based on the variability of the baseline data). The point at which the amplification plot crosses the threshold is defined as the Ct value (Fig. 1).

This method offers the possibility to perform multiplex PCR, simultaneous detection of the expression of more than one gene in the same tube, using specific sets of primers and specific probes with different fluorogenic labels for each gene to be investigated. We used this multiplex PCR for detection of expression of our target gene TH and the reference gene GAPDH. Measuring the expression of the reference gene provides information about the integrity and amount of mRNA present in the tested samples.

**TH Assay.** PCR samples were prepared as follows. Two μl of cDNA were transferred into MicroAmp Reaction Tubes (Perkin-Elmer) that already contained 48 μl of reaction mixture. The components of the reaction mixture were prepared with the TaqMan PCR Core Reagent Kit (Perkin-Elmer), and the tubes were capped with MicroAmp Caps (Perkin-Elmer). The final concentrations of the reaction components were 0.5 unit of amperase, 200 μM dATP, 200 μM dCTP, 200 μM dGTP, 400 μM dUTP, 1X TaqMan Buffer A, 4 mM MgCl2, 1.25 units of AmpliTaq Gold, 40 nM GAPDH forward primer, 40 nM GAPDH reverse primer, 240 nM GAPDH probe, 800 nM TH forward primer, 800 nM TH reverse primer, and 200 nM TH probe.

Primer concentrations must be adjusted to obtain accurate cycle threshold values for both genes. Care was taken to limit the primer concentrations for the reference gene because amplification of the reference gene must be stopped before limiting the common reactants available for amplification of the target gene.

The conditions for the PCR were 2 min at 50°C and 10 min at 95°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.

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.

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

<table>
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<tr>
<th>Gene</th>
<th>Primer/probe</th>
<th>Location</th>
</tr>
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<tbody>
<tr>
<td>GAPDH</td>
<td>Forward primer: 5'-CAACAGCCTCAAGATCATCAGC-3'</td>
<td>Exon 6</td>
</tr>
<tr>
<td></td>
<td>Reverse primer: 5'-TGGCATGAGACTTGGGTGATGAG-3'</td>
<td>Exon 7/exon 8</td>
</tr>
<tr>
<td></td>
<td>Probe: 5'-(FAM)CCTGGCCAAGTCCATGGAAGGAC (TAMRA)-3'</td>
<td>Exon 7</td>
</tr>
<tr>
<td>TH</td>
<td>Forward primer: 5'-TCATCACTGGTCACCAAGTT-3'</td>
<td>Exon 5</td>
</tr>
<tr>
<td></td>
<td>Reverse primer: 5'-GGTCGCGGTGTCCGCCTGAC-3'</td>
<td>Exon 6/exon 7</td>
</tr>
<tr>
<td></td>
<td>Probe: 5'-(VIC)AGCGCAGGAGTCTGAGC(A TAMRA)-3'</td>
<td>Exon 6</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.

TH Assay Characteristics

The sensitivity of the TH assay was determined by spiking IMR-32 cells into whole blood. Gene expression was detected as an increase of fluorescence during amplification. The normalized fluorescence was used to demonstrate TH gene expression (Fig. 1A). As little as 10 IMR-32 cells per 10^7 peripheral WBCs were detectable, resulting in a sensitivity of the TH assay of
with a sensitivity of 1:10^6 peripheral WBCs. The presence of 
identified mRNA from all patient samples was 
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 ΔC_T values [ΔC_T = C_T (TH) − C_T 
(GAPDH)] demonstrated that a ΔC_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 
ΔC_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 expres-
sion 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).

#### Determination of the Threshold Detection Limit

Results of our control patients (Table 3; patients 9–13) demonstrated ΔC_T values of 22 and higher. The BMA sample from the 
patient with ganglioneuroma (patient 7) had a ΔC_T value of 21, 
whereas no TH expression could be demonstrated in samples 
from the patient with ganglioneuroblastoma followed by ALL 
(patient 8).

Based on these results, the detection threshold limit was set 
on 21. Patients with ΔC_T values of ≥21 are considered negative.

### Stage III Neuroblastoma Patients

The TH assay of the BMA sample from patient 1 at diagnosis was negative. No elevated catecholamine levels in the urine were detected (Table 3). This patient is in remission.

Patient 2 had elevated catecholamine levels at diagnosis, and the TH assay was positive for the samples of this patient. The patient died of complications of treatment.

### Stage IV Neuroblastoma Patients

Patient 3 had elevated catecholamine levels in the urine at diagnosis. The TH assay was positive for the BMA sample, whereas the PB sample had a negative TH assay result. This patient had a bad outcome. Samples from patient 4 were taken at diagnosis and after follow-up. Catecholamine levels remained elevated after follow-up, and the TH assay was positive for BMA and PB samples. This patient also had a bad outcome. Patient 5 was only investigated after follow-up. At that time, the catecholamine levels were still elevated, and the TH assay was positive for the BMB sample and the BMA sample taken at the right side, whereas the BMA sample taken at the left side and the PB sample were negative. Treatment was not successful, and the patient died of disease. The remaining patient (patient 6) was also only investigated after follow-up. Catecholamine levels at all sampling times were normal, but initially the TH assay was positive in all samples. Samples taken after 11 courses of chemotherapy had a negative TH assay result. Patient 6 has been in complete remission for 2 years.

### 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.

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**Table 2** Spiking PB with IMR-32 cells

<table>
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<tr>
<th>Sample</th>
<th>GAPDH</th>
<th>TH</th>
<th>ΔC_T&lt;sup&gt;a&lt;/sup&gt;</th>
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<tr>
<td>1:10&lt;sup&gt;6&lt;/sup&gt;</td>
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<tr>
<td>1:10&lt;sup&gt;4&lt;/sup&gt;</td>
<td>19</td>
<td>31</td>
<td>12</td>
</tr>
</tbody>
</table>

<sup>a</sup> ΔC_T = C_T (TH) − C_T (GAPDH). In the unspiked sample, no expression of TH was detected, so no ΔC_T could be calculated.

<sup>b</sup> ND, not detectable.
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 neuroblastoma cells was detected in samples from patient 7). These results indicate a higher sensitivity. The results from patient 6 were less 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 equivocal 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 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>
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<th>Patient no.</th>
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<th>Diagnosis (tumor stage)</th>
<th>Treatment at sampling time</th>
<th>Treatment at sampling time</th>
<th>Catecholamine concentration (in urine)</th>
<th>Material</th>
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<th>TH</th>
<th>(\Delta C_T^{a})</th>
<th>(TH) score(^b)</th>
<th>Immunohistochemical detection of neuroblastoma cells(^c)</th>
<th>MYCN amplification(^d)</th>
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\(^a\) \(\Delta C_T = C_T(TH) - C_T(GAPDH)\).

\(^b\) Samples with \(\Delta C_T\) values ≥ 21 were scored negative.

\(^c\) Examination only in BMB.

\(^d\) Examination in primary tumor.

\(^e\) NT, not tested; ND, not detectable; VECI, combination of vincristine, teniposide, carboplatin, and ifosfamide; CR, complete remission; DOD, died of disease; DC, died of complications of treatment.

\(^f\) Right side.

\(^g\) Left side.

\(^h\) 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.

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