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# Detection of the PGP9.5 and Tyrosine Hydroxylase mRNAs for Minimal Residual Neuroblastoma Cells in Bone Marrow and Peripheral Blood

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YANAGISAWA YAMBE, T., SASAHARA, Y., FUJIE, H., OHASHI, Y., MINEGISHI, M., ITANO, M., MORITA, S., TSUCHIYA, S., HAYASHI, Y., OHI, R. and KONNO, T. *Detection of the PGP9.5 and Tyrosine Hydroxylase mRNAs for Minimal Residual Neuroblastoma Cells in Bone Marrow and Peripheral Blood.* Tohoku J. Exp. Med., 1998, **184** (3), 229-240 —— The "touchdown" polymerase chain reaction (PCR) technique has been applied to analyze expression of the neuron-specific protein, PGP9.5, and tyrosine hydroxylase (TH) genes for detection of minimal residual neuroblastoma cells in bone marrow and peripheral blood. PGP9.5 and TH gene products were not detected in any normal samples ($n=72$) examined. However, in patients more than 1 year of age with stage III and IV neuroblastoma PGP9.5 mRNA was detected in six of seven bone marrow samples and in four of eight peripheral blood samples, and TH mRNA in four of seven and three of eight, respectively. The detection sensitivity was up to $10^{-6}$ to $10^{-7}$ $\mu$g of total cellular RNA for PGP9.5 and $10^{-4}$ $\mu$g for TH. Among forty bone marrow specimens from nineteen patients with neuroblastoma both PGP9.5 and TH mRNAs were detected in six, and only PGP9.5 mRNA was detected in ten. Since detection of PGP9.5 and TH gene transcripts by the "touchdown" PCR was highly specific and sensitive, it might be most informative at present to carry out both PGP9.5 and TH mRNA assays for minimal residual neuroblastoma cells in blood and bone marrow.
———— PGP9.5; tyrosine hydroxylase; neuroblastoma; minimal residual cells
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Neuroblastoma is one of the most common solid malignancies of children (Crist and Kun 1991). Its prognosis depends on the clinical stage, defined in

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terms of tumor extension and metastasis (Cassady 1990; Brodeur et al. 1993), and bone marrow involvement as another predictor of clinical outcome irrespective of the stage (Moss et al. 1991). Recently, intensive myeloablative treatment followed by autologous bone marrow transplantation or peripheral blood stem cell transplantation has been employed for advanced neuroblastoma, but there is concern with contamination of minimal residual neuroblastoma cells in the harvests and infusates which could bring relapse of the disease after transplantation (Pole et al. 1991; Leibundgut et al. 1993; Matthay et al. 1993). In terms of minimal residual tumor cell detection, improved assays have been applied for clinical use (Moss et al. 1991). Immunocytologic analysis with monoclonal antibodies specific for various tumor cell markers has proved more sensitive to detect metastatic tumor cells in bone marrow than standard microscopic analysis (Moss et al. 1991). Recently, reverse transcriptase (RT) polymerase chain reaction (PCR) analysis amplifying mRNAs of neuron-specific protein gene product, PGP9.5, and tyrosine hydroxylase (TH) has been demonstrated to be useful for detection of minimal residual neuroblastoma cells (Miyajima et al. 1991; Naito et al. 1991; Mattano et al. 1992; Burchill et al. 1994). An assay system for PGP9.5 proved sensitive enough to detect a single neuroblastoma cell in $10^7$ mononuclear blood cells (MNCs) (Mattano et al. 1992), but similar assay methods for TH have been reported to be less sensitive (Miyajima et al. 1991; Naito et al. 1991; Burchill et al. 1994). In this study we carried out PGP9.5 and TH mRNA assays by RT-PCR with the same bone marrow and peripheral blood samples in order to establish the clinical applicability of these tests for detection of minimal residual neuroblastoma cells.

## Materials and Methods

### *Blood, bone marrow and tissues*

Peripheral blood and bone marrow specimens available for the present study were partly obtained from individuals with neuroblastoma or other diseases for diagnostic purposes after receipt of informed consent from the patients themselves and/or their parents. MNCs were prepared from heparinized peripheral and bone marrow blood using Ficoll-Paque (Pharmacia, Uppsala, Sweden) and kept frozen in medium containing 10% dimethyl sulfoxide at −80°C until examined. Neuroblastoma tissues were obtained surgically and similarly stored at −80°C until examined.

### *Cell lines*

The various cell lines used are listed in Table 1 along with their origins. IMR32 was provided by the Japanese Cancer Research Resources Bank (Tokyo) and SKN-SH by Dr. Sugimoto, Kyoto Prefectural University. Other cell lines were from our Institute. The cells were all maintained in RPMI-1640 medium supplemented with 5% heat inactivated fetal calf serum.

TABLE 1. *A list of the cell lines examined*

| Cells | Origin | Cells | Origin |
|---|---|---|---|
| IMR32 | NB | HPB | T-ALL |
| GOTO | NB | HUT-78 | ATL |
| SKN-SH | NB | HT-102 | ATL |
| TN-1 | NB | MT-1 | ATL |
| TN-2 | NB | THP-4 | Pre-BALL |
| TN-3 | NB | THP-7 | Pre-BALL |
| NB-1 | NB | THP-9 | NHL |
| Tunaki | NB | RAJI | BL |
| THP-6 | T-ALL | BJAB | BL |
| HSB | T-ALL | THP-1 | AMoL |
| CEM | T-ALL | K562 | CML-Mbl |
| JURKAT | T-ALL | KG-1 | AML |
| JM | T-ALL | HL60 | APL |
| MOLT | T-ALL | U-937 | NHL-histio |
| P12 | T-ALL | | |

ALL, acute lymphoblastic leukemia; AMoL, acute monoblastic leukemia; APL, acute promyelocytic leukemia; AML, acute myelogenous leukemia; ATL, adult T-cell leukemia; BL, Burkitt lymphoma; CML-Mbl, chronic myelogenous leukemia-myeloblast; NHL, non-Hodgikin lymphoma; NHL-histio, NHL-histiocytic; NB, neuroblastoma; pre-BALL, Precursor-B cell ALL; T-ALL, T-cell ALL.

*RNA extraction*

Total cellular RNA was extracted from cell lines and tumor tissues by the guanidium thiocyanate/cesium chloride method (Sambrook et al. 1989), and from bone marrow or peripheral MNCs by a modification of the Meltzer's method (Meltzer et al. 1990). Extracted RNA was diluted to a concentration of 1 $\mu$g/$\mu$l with dimethyl pyrocarbonate (DEPC) -treated water.

*PGP9.5 RT-PCR*

The RT reaction mixture comprised 2 $\mu$l of 10×PCR buffer (500 mM KCl, 100 mM Tris-HCl, pH 8.8, 15 mM $MgCl_2$, 2.1% Triton X-100), 1 $\mu$l of 20 mM deoxynucleotide triphosphate mixture (Takara, Ohtsu), 0.5 $\mu$l of 40 u/$\mu$l Ribonuclease inhibitor (Toyobo, Tokyo), 10 pmol PGP9.5 RT-specific primer, 5′-TGTTTCACAAGTACT-3′, 10 pmol of $\beta$2-microglobulin(MG) 3′ primer (Gehly et al. 1991), 1 $\mu$g of RNA pretreated at 65°C for 5 minutes and DEPC-treated water to a total volume of 20 $\mu$l. One hundred units of Moloney murine leukemia virus reverse transcriptase (MoMLV-RT; BRL, Bethesda, MD, USA) were added and the reaction was allowed to proceed at 37°C for 60 minutes. $\beta$2-MG cDNA was transcribed as an internal control. The PCR mixture consisted of 8 $\mu$l of 10×

PCR buffer, in addition to total volume of RT-PCR product, 50 pmol of forward (5′-AGATCAACCCCGAGATGCTGAACAAAGTG CTG-3′) and reverse (3′-ATTAGGCTGCCTTGCAGAGAGCCACGGCAGAGAA-5′) primers, respectively, in a total volume of 100 $\mu$l. Two units of Vent DNA polymerase (New England Biolabs, Beverly, MA, USA) were added, and the PCR was performed under the conditions described by Don et al. (1991); denaturing at 94°C for 40 seconds, annealing at 65°C for 30 seconds, and extension at 72°C for 60 seconds at the start, and then the annealing temperature was decreased 0.5°C every cycle from 65°C to a "touchdown" temperature at 55°C, at which 20 cycles were then carried out. At the end of each round of 40 cycles, further extension at 72°C for 10 minutes was included. PCR was carried out with a DNA thermal cycler, Cetus 9,600 (Perkin Elmer Cetus, Vaterstatten, Germany). For each sample, 10 $\mu$l of the PCR product was electrophoresed on a 2% agarose gel (Funakoshi, Tokyo) and stained with ethidium bromide.

*TH RT-PCR*

The RT reaction was carried out according to the method described by Naito et al. (1991) with 10 pmol of TH(−) primer (3′-CGATGGCCCTTCTGTTATAG-5′) but 100 units of MoMLV-RT for AMV-RT and addition of Ribonuclease Inhibitor, and incubation at 37°C for 60 minutes. The PCR mixture consisted of 10 $\mu$l of 10×PCR buffer (100 mM KCl, 100 mM $(NH_4)_2$ $SO_4$, 200 mM Tris-HCl, pH 8.8, 20 mM $MgSO_4$, 0.1% Triton X-100), 50 pmol TH(+) primer (5′-TGTCAGAGCTGGACAAGTGT-3′) and 40 pmol TH(−) primer and 20 $\mu$l of RT product in a total volume of 100 $\mu$l. Two units of Vent DNA polymerase were added and the reaction was allowed to proceed under the conditions of "touch-down" PCR as described above, but at the "touchdown" temperature of 55°C, 40 cycles were carried out. 10 $\mu$l of each PCR product was electrophoresed on a 2% agarose gel and stained with ethidium bromide for visualization.

*Southern blot analysis*

Agarose gels were transferred overnight onto nylon membranes (Boehringer Mannheim, Mannheim, Germany) with the use of transfer buffer (2 M NaCl, 1 M Tris HCl) by an established procedure (Rolfs 1992). Oligonucleotide probes for PGP9.5 (5′-CACGCAGTGGCCA ATAATCAAGACAAACTGGGA-3′) (Mattano et al. 1992) and TH (5′-GTTCGACCCTGACCTGGACT-3′) (Naito et al. 1991) were labelled with digoxigenin (DIG) and hybridized by using a DIG DNA Labelling and Detection Kit (Boehringer Mannheim) according to the manufacturer's instructions. The membranes were washed and the hybrids were visualized on Kodak XAR-5 film (Eastman Kodak, Rochestor, NY, USA).

## RESULTS

### *Expression of PGP9.5 and TH mRNAs*

RT-PCR for PGP9.5 and TH mRNAs in total cellular RNAs from neuroblastoma cell lines showed amplification of a 650 nucleotide (nt) fragment (the expected product length for PGP9.5, 653 nt) and a 300 nt fragment (the expected product for TH, 299 nt) (Figs. 1a, 1b). Southern blot analysis with specific DIG labelled oligonucleotide probes confirmed the identity of the fragments as PGP9.5 (Fig. 2) and TH (data not shown). Assay results with various specimens are shown in Table 2. All the neuroblastoma cell lines ($n=8$) and tissues ($n=21$) examined were positive for both PGP9.5 and TH mRNAs. RT-PCR of cellular RNA from metastatic bone marrow MNCs containing microscopically detectable neuroblastoma cells ($n=4$) showed positive bands corresponding to the expected products for PGP9.5 and TH. In contrast, leukemia ($n=17$) and lymphoma ($n=4$) cell lines were all negative for PGP9.5 and TH. Peripheral ($n=42$) and bone marrow ($n=30$) MNCs from patients with leukemia, lymphoma or non-malignant hematologic diseases were shown to express neither PGP9.5 nor TH genes at a detectable level.

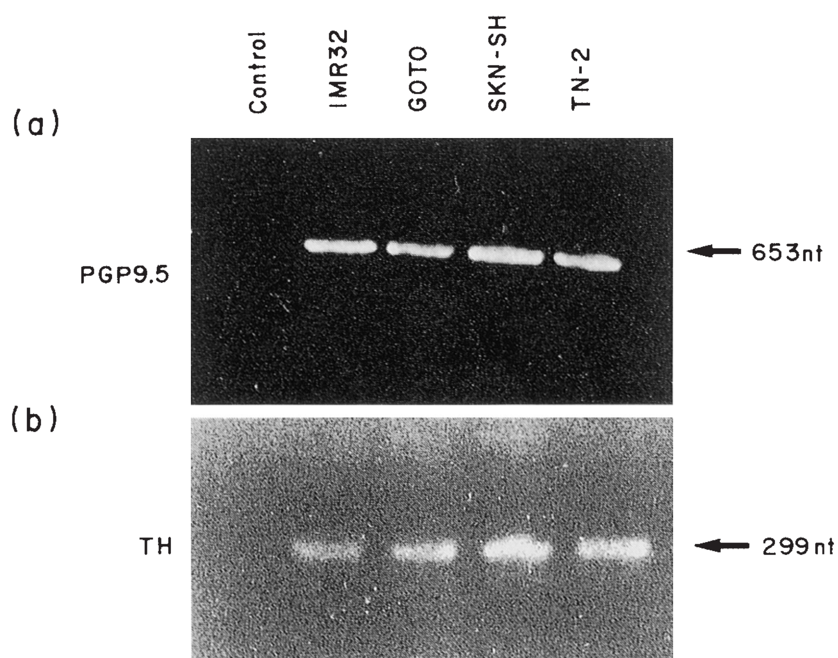


Fig. 1. RT-PCR analysis of PGP9.5 (a) and tyrosine hydroxylase (TH) (b) expression in neuroblastoma cell lines. IMR32, GOTO, SKN-SH and TN-2 were used as representative neuroblastoma cell lines. Control is a monocytic leukemia cell line, THP-1. RT-PCR for PGP9.5 produces cDNA of a 653 nucleotide (nt) fragment (a) and for TH a 299 nucleotide fragment (b) as indicated by the arrows.

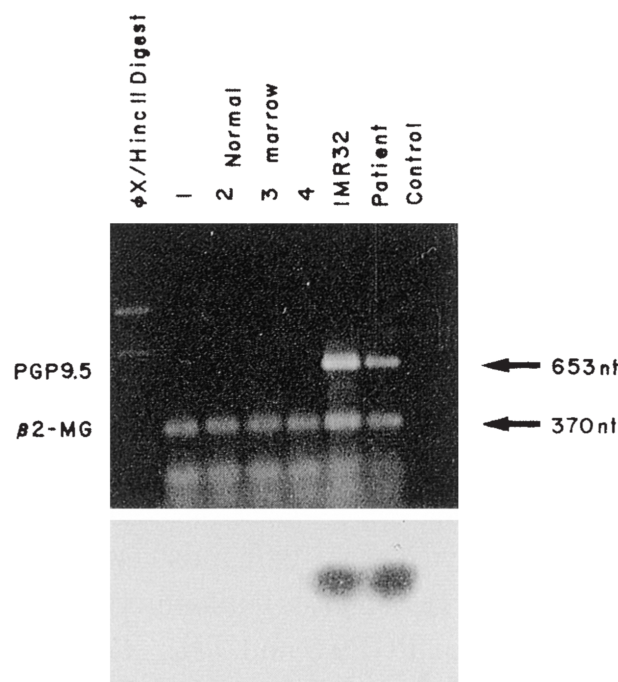


Fig. 2. Southern blot analysis of the RT-PCR product for PGP9.5. Normal bone marrow samples (lanes 2 to 5), IMR32 neuroblastoma cell line (lane 6), a bone marrow sample from a patient with bone marrow metastasis (lane 7) and a monocytic leukemia cell line, THP-1 (lane 7) were subjected to RT-PCR analysis (upper figure) and then to Southern blot analysis (lower figure) with an oligonucleotide probe for PGP9.5. $\phi$X/Hinc II Digest is a molecular weight marker. The 653 nucleotide fragment is a cDNA of PGP9.5 and the 370 nucleotide fragment is that of $\beta$2-microglobulin ($\beta$2-MG) to confirm the integrity of all cDNA samples.

TABLE 2. *Detection of PGP9.5 and TH mRNAs by RT-PCR in neuroblastoma tissues, cell lines, peripheral blood and bone marrow*

| Specimens | Number examined | Number positive for | |
|---|---|---|---|
| | | PGP9.5 mRNA | TH[c] mRNA |
| Neuroblastoma | | | |
| Cell lines | 8 | 8 | 8 |
| Tumor tissue | 21 | 21 | 21 |
| Bone marrow[a] | 4 | 4 | 4 |
| Leukemia cell lines | 17 | 0 | 0 |
| NHL cell lines | 4 | 0 | 0 |
| Controls[b] | | | |
| Peripheral blood | 42 | 0 | 0 |
| Bone marrow | 30 | 0 | 0 |

[a]Microscopically positive for neuroblastoma cells.

[b]Obtained from patients with leukemia in remission, lymphoma, or other hematological diseases such as aplastic anemia and idiopathic thrombocytopenia.

[c]tyrosine hydroxylase.

*Comparison of sensitivity*

To compare the assay sensitivities for detection of PGP9.5 and TH mRNAs in neuroblastoma cells, total cellular RNAs were extracted from two cell lines, IMR32 and TN-2, and two tumor specimens from different patients. The RNA extracts were serially diluted with cellular RNA from normal bone marrow MNCs. Each dilution was subjected to analysis for PGP9.5 and TH mRNA expression. As shown in Figs. 3a, 3b and Table 3, the RT-PCR used in the present study

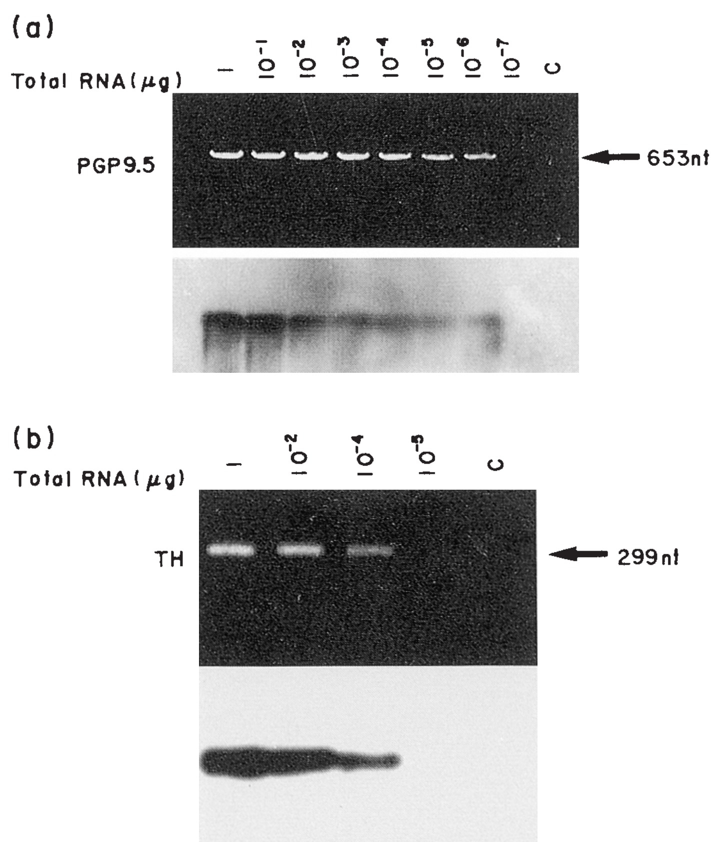


Fig. 3. Sensitivity testing for RT-PCR of PGP9.5 (a) and tyrosine hydroxylase (TH) (b) mRNAs. Total cellular RNA extracted from the IMR32 neuroblastoma cell line was serially diluted with cellular RNA from normal bone marrow MNCs, and each dilution was subjected to RT-PCR and Southern blot analysis as in Fig. 2.

TABLE 3. *Sensitivity of detection of PGP9.5 and TH mRNAs in neuroblastoma cell lines and tumor tissues by RT-PCR*

| | Minimum amount (μg) of RNA for detection of | |
|---|---|---|
| Cells | PGP9.5 mRNA | TH mRNA |
| IMR 32 | $10^{-6}$ | $10^{-4}$ |
| TN-2 | $10^{-6}$ | $10^{-4}$ |
| Tumor tissue 1 | $10^{-7}$ | $10^{-4}$ |
| Tumor tissue 2 | $10^{-6}$ | $10^{-4}$ |

demonstrated PGP9.5 mRNA at dilutions up to $10^{-6}$ to $10^{-7}$ $\mu g$ and TH mRNA up to $10^{-4}$ $\mu g$ of total cellular RNAs from neuroblastoma cell lines or tissues.

*Detection of PGP9.5 and TH mRNAs in peripheral and bone marrow MNCs from patients with neuroblastoma*

Peripheral and bone marrow MNC-specimens from 19 neuroblastoma patients at onset or relapse of the disease were examined for the expression of PGP9.5 and TH mRNAs. As shown in Table 4, only patients with advanced disease at the age of one year or more were positive for PGP9.5 and TH mRNAs. In bone marrow MNCs from 5 patients with stage IV neuroblastoma PGP9.5 mRNA was detected in all at diagnosis or relapse and TH mRNA in only 3 of them. A patient with stage III neuroblastoma was positive for PGP9.5 and TH mRNAs in her bone marrow without any microscopic evidence of metastasis. Peripheral blood MNCs

TABLE 4. *Detection of PGP9.5 and TH mRNAs by RT-PCR in peripheral blood and bone marrow MNCs from patients with neuroblastoma at different stages*

| Stage | Number of patient examined | Number of mRNA positive patients in | | | |
|---|---|---|---|---|---|
| | | Peripheral blood | | Bone marrow | |
| | | PGP 9.5 | TH | PGP 9.5 | TH |
| Patients at age 1 year or more | | | | | |
| II | 1 | 0 | 0 | 0 | 0 |
| III | 2 | 0 | 0 | 1 | 1 |
| IV | 6[a] | 4 | 3 | 5(2)[b] | 3(2)[b] |
| Patients at age less than 1 year | | | | | |
| II | 7 | 0 | 0 | 0 | 0 |
| III | 2 | 0 | 0 | 0 | 0 |
| IV | 1 | 0 | 0 | 0 | 0 |

[a]Bone marrow samples were obtained from only 5 patients.
[b]The number in parenthesis indicates bone marrow specimens containing microscopically detectable neuroblastoma cells.

TABLE 5. *Detection of PGP 9.5 and TH mRNAs by RT-PCR from bone marrow and peripheral blood MNCs of patients with neuroblastoma*

| | |
|---|---|
| Number of MNCs positive for | |
| both PGP 9.5 and TH mRNAs | 6 |
| only PGP 9.5 mRNA | 10 |
| only TH mRNA | 0 |
| Number of MNCs negative for | |
| both PGP 9.5 and TH mRNAs | 24 |
| Total | 40 |

from these patients including one whose bone marrow sample was unavailable were positive for PGP9.5 in 4 and for TH in 3 cases. Patients less than one year of age at diagnosis were all negative for PGP9.5 and TH mRNAs at any disease stages. The majority of the patients in infancy were found by mass screening for neuroblastoma. Among 40 bone marrow and peripheral blood specimens from 19 patients with neuroblastoma at various clinical settings both PGP9.5 and TH mRNAs were detected in 6, and in 10 only PGP9.5 was detected (Table 5).

*Illustrative neuroblastoma cases implying clinical significance of simultaneous detection of PGP9.5 and TH mRNAs*

The first patient was a 4-year-old boy with metastatic neuroblastoma at stage IV (Fig. 4). His bone marrow showed microscopic massive infiltration of neuroblastoma cells which showed 7-fold amplification of N-myc gene. At diagnosis not only bone marrow but also peripheral MNCs were found to be positive for PGP9.5 and TH mRNA expression by RT-PCR. After the first course of multidrug chemotherapy, PGP9.5 and TH were still detectable in MNCs from his bone marrow in which neuroblastoma cells were not apparent microscopically. Although TH was no longer detected after the second course of the chemotherapy, PGP9.5 remained positive until just before bone marrow collection for autologous transplantation using CD34 enriched cells. Six months later BM became positive again for PGP9.5 mRNA followed by reappearance of a tumor at the primary site. He died from disseminated neuroblastoma.

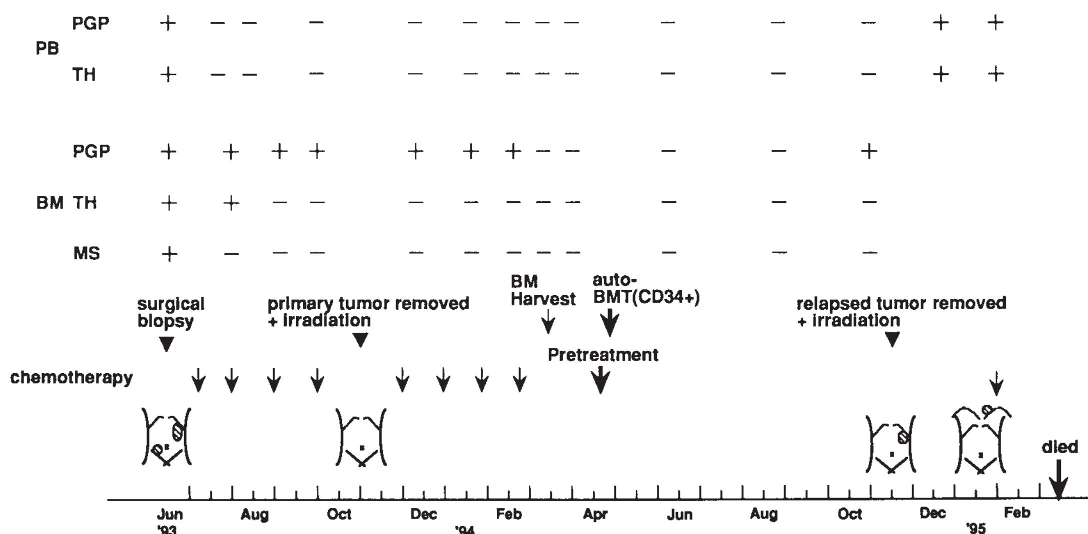


Fig. 4. Clinical course of a patient with stage IV neuroblastoma and detection of PGP9.5 and tyrosine hydroxylase mRNAs. PB, peripheral blood samples; BM, bone marrow samples; PGP, PGP9.5; TH, tyrosine hydroxylase; MS, microscopic metastasis to bone marrow; autoBMT (CD34+), autologous bone marrow transplantation after enrichment of CD34 positive cells.

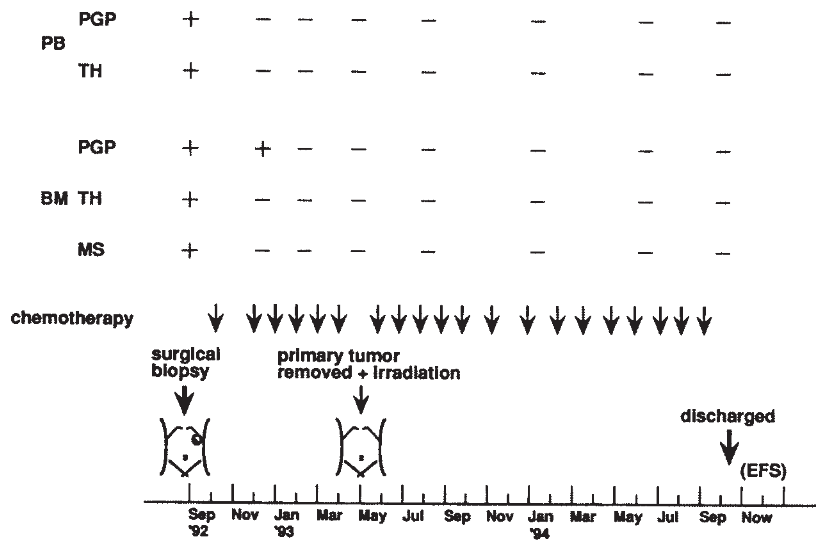


Fig. 5. Clinical course of a patient with stage III neuroblastoma and detection of PGP9.5 and tyrosine hydroxylase mRNAs. See abbreviations in Fig. 4.

The second patient was a 3-year-old girl with a stage IV neuroblastoma (Fig. 5). N-myc gene in her tumor cells was not amplified. Examination of bone marrow smears at diagnosis revealed the presence of neuroblastoma cells with pseudo-rosette formation. At that time peripheral as well as bone marrow MNCs were positive for both PGP9.5 and TH mRNAs. After the second course of a multidrug chemotherapy PGP9.5 mRNA was detectable in MNCs from the bone marrow in which neuroblastoma cells were not microscopically found. Further examination, however, never revealed PGP9.5 and TH mRNAs in either peripheral blood or bone marrow MNCs. After discontinuation of the chemotherapy she is surviving without any evidence of disease recurrence.

## Discussion

In view of the finding that the presence of neuroblastoma cells in bone marrow is closely correlated to the prognosis of the patients irrespective of the stage (Moss et al. 1991), the present study was performed to demonstrate PGP9.5 and TH mRNAs as tumor markers to detect minimal residual neuroblastoma cells in bone marrow and peripheral MNCs. Both gene products have been shown to be expressed in neuroblastoma cells and be useful for detection of metastatic or circulating tumor cells (Miyajima et al. 1991; Naito et al. 1991; Mattano et al. 1992; Burchill et al. 1994). TH RT-PCR was reported to be able to detect a single neuroblastoma cell in $10^5$ normal bone marrow MNCs (Naito et al. 1991), while PGP9.5 RT-PCR was sufficiently sensitive for one tumor cell to be found in $10^7$ control peripheral MNCs (Mattano et al. 1992). Analyses were carried out simultaneously with the same samples to detect PGP9.5 and TH mRNAs in bone

marrow and peripheral MNCs, resulting in confirmation of the sensitivity differences of the previous reports. The sensitivity of PGP9.5 RT-PCR was approximately 100-fold higher than that of TH RT-PCR, when serial dilutions of cellular RNA of neuroblastoma cells and cell lines were subjected to the tests. Low expression of TH mRNA in tumor cells and the high GC content of tumor TH mRNA may be responsible for the lower sensitivity of detection by RT-PCR (Naito et al. 1991).

Normal peripheral and bone marrow MNCs, and leukemia and lymphoma cell lines analyzed by RT-PCR for the expression of PGP9.5 and TH genes were all negative in the present study. Gilbert et al. experienced false positive results with eight of the ten normal blood samples and seven of twelve normal bone marrow samples using highly sensitive nested PCR analysis for PGP9.5 mRNA and suggested low specificity of the assays for detection of minimal residual neuroblastoma cells (Gilbert et al. 1997). However, the same author described that false positive results for PGP9.5 mRNA was not observed after a single round PCR (Gilbert et al. 1997). To improve specificity of PCR assays the "touch-down" PCR method was used in this study (Don et al. 1991). The "touchdown" PCR was a single round PCR method and could detect PGP9.5 mRNA as sensitive as nested PCR (Table 3, Fig. 3). In addition, Vent DNA polymerase which is more thermostable than Taq polymerase (Kong et al. 1993) was used to achieve one step PCR to minimize contamination (DeFilippes 1991). On the other hand detection of TH gene expression was not so sensitive as PGP9.5, but was considered to be very specific to neuroblastoma cells (Gilbert et al. 1997). In conclusion detection of both PGP9.5 and TH mRNAs appears to be the best approach to evaluate minimal residual neuroblastoma cells at the present time.

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