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Detection of Tyrosine Hydroxylase mRNA and Minimal Neuroblastoma Cells by the Reverse Transcription–Polymerase Chain Reaction

Hiroyuki Naito, Noboru Kuzumaki, Jun-ichi Uchino, Ryoji Kobayashi, Takaaki Shikano, Yorikazu Ishikawa and Shuzo Matsumoto

To facilitate the diagnosis of bone marrow metastasis in neuroblastoma, we have developed a method of amplifying and detecting the tyrosine hydroxylase (TH) mRNA sequence in bone marrow cells using a combination of reverse transcription and the polymerase chain reaction (RT/PCR). By this method, the sequence of TH was detected clearly in the neuroblastoma tissues of all 6 patients and not detected in the bone marrow cells of any of the 9 negative control children. In a reconstitution experiment, 1 neuroblastoma cell per 100 000 normal bone marrow cells could be detected, thus indicating the great sensitivity of this method. Based on these results, this technique may be of value in the diagnosis and treatment follow-up of bone marrow metastasis of neuroblastoma.
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INTRODUCTION

NEUROBLASTOMA IS one of the most common malignant solid tumours of childhood. It originates in the neural crest, grows rapidly into a huge mass and frequently metastasises to bone marrow. Since disseminated neuroblastoma still carries a poor prognosis, massive therapy followed by allogeneic or autologous bone marrow transplantation has become the current modality of consolidation treatment in such patients. It is important to detect tumour cells in the bone marrow of neuroblastoma patients in order to diagnose the disease, monitor its course during remission or relapse, and to confirm that pretherapy bone marrow stored for autologous transplantation is tumour-free when harvested from a patient. The present means of diagnosing bone marrow metastasis in neuroblastoma include histological, biochemical, and immunohistological analysis. Morphological distinction between tumour cells and primitive lymphoblasts can be difficult, and when only a small proportion of the cells are aberrant, it can be difficult to arrive at a diagnosis by these methods.

Neuroblastoma is characterised by the secretion of catecholamines in approximately 95% of patients [1]. Therefore, it is expected that almost all neuroblastomas produce tyrosine hydroxylase (TH), the first enzyme in the pathway of catecholamine biosynthesis. Expression of the TH gene is regulated in a tissue-specific manner during neonatal development and differentiation [2, 3]. Therefore, if TH mRNA is detected in the bone marrow cells of neuroblastoma patients, we can regard them as having bone marrow metastasis. In this report we exploited the combined method of reverse transcription and polymerase chain reaction (RT/PCR) to detect the mRNA of TH in bone marrow cells and demonstrated that this was a potential tool for detecting minimal bone marrow metastasis in neuroblastoma.

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Table 1. Clinical data of patients

Data	Patients 1	2	3	4*	5	6
Age	8 mo	9 mo	8 mo	22 yr	1 yr	1 yr
Sex	F	M	M	F	M	F
Site	Rt adr	Rt adr	RP	RP	Rt RP	Rt RP
Stage	II	I	II	IV	I	III
Urinary (μg/mg Cr)						
VMA	20–23	35–45	23	8	17–20	11–12
HVA	34	44–60	25	20	21–24	16–28
Tissue	Rosette NB	Com GNB	Round cell NB	PD GNB	Composite GNB	Composite GNB

*Recurrence.
adr = adrenal, RP = retroperitoneal, VMA = vanilmandelic acid, HVA = homovanillic acid, Rosette = rosette forming type, PD = poorly-differentiated type, NB = neuroblastoma, GNB = ganglioneuroblastoma.

MATERIALS AND METHODS

Samples

We used a human neuroblastoma cell line IMR32 and tumour tissues of all 6 neuroblastoma patients operated at Hokkaido University Hospital between June 1989 and January 1990. Details concerning the tumour tissues we used are shown in Table 1. We used the Evans' system for staging of neuroblastoma [4]. The negative controls consisted of bone marrow aspirates from the unilateral anterior iliac spine of 8 paediatric patients with haematological malignancy in remission and one with transient neutropenia. Bone marrow aspirates of 3 patients with neuroblastoma (stage I : 2 patients, stage IV : 1 patient) were analysed, but they showed no histological evidence of neuroblastoma cells in their bone marrow.

Preparation of RNA

Buffy coat cells were prepared from bone marrow. After lysing the remaining erythrocytes in 155 mmol/l NH_4Cl, 10 mmol/l $KHCO_3$, and 0.1 mmol/l EDTA (pH 7.4), the white blood cells (WBC) were washed once in ice-cold phosphate-buffered saline (PBS). Total RNA was isolated from the cell line and clinical samples by acid guanidinium thiocyanate/phenol–chloroform extraction as described by Chomczynski and Sacchi [5].

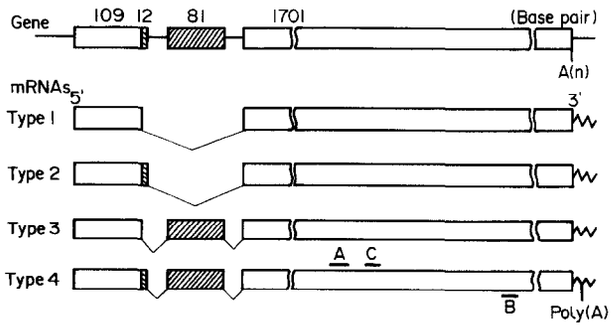

Fig. 1. Structure of four types of human TH mRNA and location of diagnostic oligonucleotides. (A) 5'-TGTCAGAGCTGGACAAGT-3', forward primer identical to a segment of the fifth exon of TH. (B) 3'-CGATGGCCCTTCTGTTATAG-5', reverse primer complementary to a segment of the eighth exon of TH. (C) 5'-GTTCGACCCTGACCTGGACT-3', a sequence between A and B used to probe the region amplified.

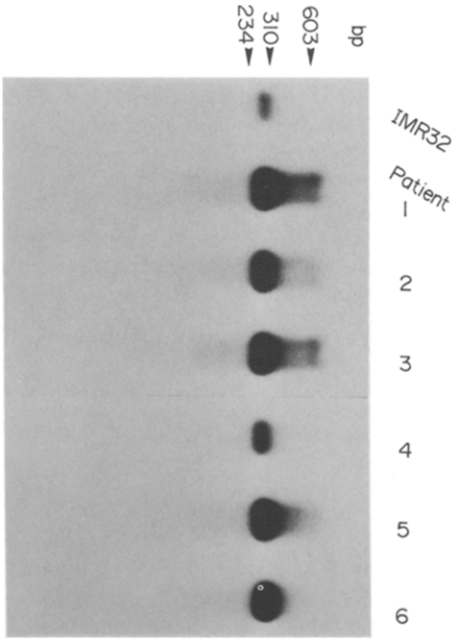

Fig. 2. Detection of TH-specific mRNA amplification products after gel electrophoresis and Southern blotting. Exposure time = 5 hours.

Synthetic oligonucleotides

Figure 1 depicts the mRNA structure and location of oligonucleotides A, B and C, used for TH mRNA detection. Oligonucleotides were synthesised on a Cyclone DNA Synthesizer (Milligen). The sequence information for generating the primers was taken from the literatures [6, 7]. Two 20-base oligonucleotides were used as primers: primer TH(+), 5'-TGTCAGAGCTGGACAAGTGT-3', was derived from the sequence of the fifth exon of the human TH gene and primer TH(−), 3'-CGATGGCCCTTCTGTTATAG-5', was complimentary to the sequence of the eighth exon of the same gene. In the presence of the TH mRNA, 299 base pair (bp) cDNA fragments were expected to be amplified.

Combined RT/PCR method

Samples of total cellular RNA 1 μg were dispensed in 20 μl of reverse transcription buffer containing 10 pmol of primer TH(−), 1 mmol/l of dNTPs (dATP, dCTP, dTTP), 250 μmol/l of dGTP, 750 μmol/l of 7-deaza-2'-deoxyguanosine 5'-triphosphate (dc^7GTP), 20 units of AMV reverse transcriptase (Boehringer Mannheim), and 20 units of RNasin (Wako) and incubated at 43°C for 1 hour. The sample was then suspended in 80 μl of PCR buffer containing 50 pmol of primer TH(+), 40 pmol of primer TH(−) and 2.5 units of Thermus Aquatics DNA polymerase (Boehringer Mannheim). PCR was carried out with a DNA Thermal cycler (Perkin–Elmer Cetus). PCR (denaturation at 94°C for 1 minute, anealing at 55°C for 1½ minutes and extention at 72°C for 2 minutes) was performed for 60 cycles, with a further 2.5 units of the polymerase added after 30 cycles. 5 μl of the reaction product was run on 3% NuSieve/1% agarose minigel in 1 × Tris/borate/EDTA(TBE) buffer. The gel was then denatured, neutralised and transferred to a nylon filter (Biodyne A, Pall). Membrane filters were hybridised with random-primer-labeled TH cDNA probe in

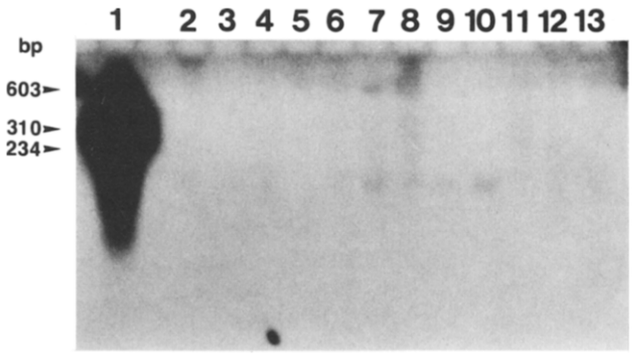

Fig. 3. Specificity of RT/PCR assay. Lane 1, IMR32 cells; lanes 2–10, bone marrow samples from 9 control patients; lanes 11–13, bone marrow samples from 3 neuroblastoma patients. Exposure time = 5 days.

hybridisation buffer (0.9 mol/l NaCl/50 mmol/l NaH_2PO_4/5 mmol/l EDTA/50% deionised formamide/5x Denhardt's solution/0.3% sodium dodecyl sulphate (SDS)/250 μg/ml sonicated salmon sperm DNA) overnight at 42°C. The cDNA probe was generated by the RT/PCR method from one of the neuroblastoma tissues and the origin of the amplified product was confirmed by hybridisation with an oligonucleotide probe derived from the internal part of the amplified fragment (Fig. 1 C). After washing in 2–0.1 × SSC/0.2% SDS at 50°C, the filters were exposed to X-ray film (Fuji) at −70°C.

RESULTS

We first studied the feasibility and specificity of the RT/PCR assay to detect TH mRNAs in neuroblastoma. The specificity of the amplified products was established by the size of the fragments deduced from their electrophoretic mobility (299 bp) and by cDNA probe hybridisation under stringent conditions. By means of the RT/PCR technique, we were able to amplify 299 bp cDNA fragments in IMR32 cells and neuroblastoma tissues from all 6 patients (Fig. 2). Grid quantification analysis showed that the volume of PCR products from each neuroblastoma tissue was equivalent to or more than that of IMR32 cells (1–3.75 times). In contrast, no TH mRNA transcripts were detected in bone marrow samples from any of the 9 control patients nor 3 neuroblastoma patients (Fig. 3).

To determine the sensitivity of the RT/PCR assay, we performed a dilution experiment (Fig. 4). Decreasing numbers of IMR32 cells were mixed with 10^7 normal bone marrow cells, and total RNA was prepared from the cell mixture. After 60 cycles of amplification, we were able to detect one IMR32 cell per 100 000 normal bone marrow cells.

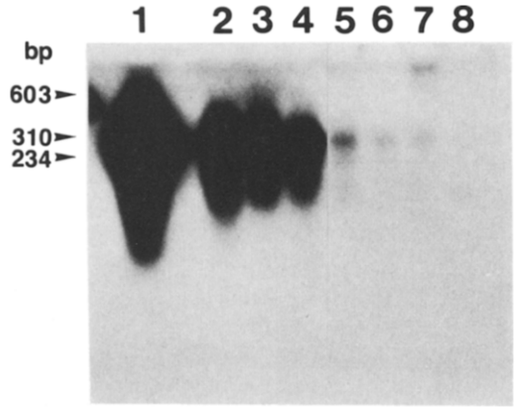

Fig. 4. Sensitivity of RT/PCR assay. Lane 1, IMR32 cells alone; lanes 2–7, dilutions of IMR32 cells in ratios of $1/10^2$, $1/3 \times 10^2$, $1/10^3$, $1/10^4$, $1/3 \times 10^4$ and $1/10^5$ control bone marrow cells; lane 8, control bone marrow cells. Exposure time = 5 days.

DISCUSSION

The standard method used to determine the presence of metastatic tumour cells in bone marrow is the examination of bone marrow aspirates and biopsy specimens by light microscopy. This method is not always accurate. Recently, the use of monoclonal antibodies that react selectively with cells of neuroectodermal origin has further improved diagnostic possibilities. However, the monoclonal antibodies currently in use all have some degree of false positivity [8, 9], so the clinical importance of detecting antibody positive cells in the absence of morphological evidence of infiltration is still debated [10]. The catecholamine fluorescence and tissue culture methods used to identify neurite processes were found to be less sensitive [11]. The clonogenic efficiency of malignant cells in solid tumours is very low, and culture assays are not practicable for detecting residual disease [12]. Therefore, many centres rely on standard cytological examination of marrow smears and histological examination of marrow biopsies to detect bone marrow involvement by tumour cells, though new assays to improve accuracy of detection of neuroblastoma metastasis are needed.

Using the RT/PCR assay we were able to detect TH mRNA in all of the neuroblastoma tissues tested. Moreover, the density of the specific bands of all of these samples was higher than that of IMR32. In contrast, no TH mRNA was detected in any of the control bone marrow samples tested by this method. Nor could we detect TH mRNA in the bone marrow of 3 patients with neuroblastoma. We think this is because these patients have no metastasis or too minimal metastasis to be detected by this method. The sensitivity of this PCR method was high enough to detect one neuroblastoma cell mixed in with 100 000 normal bone marrow cells. Because the G+C content of human TH mRNA is high, we were unable to attain this high level of sensitivity until we used the nucleotide analogue dc^7GTP in addition to dGTP as a precursor for DNA synthesis [13]. In our study, proper exposure time to detect the presence or absence of neuroblastoma cells was 5 days and it took 10 days totally to complete this assay. However the RT/PCR technique is economical and relatively simple for a laboratory already using molecular biology techniques. Moreover this high sensitivity and specificity suggest that RT/PCR will be of clinical value in detecting minimal bone marrow metastasis and in assessing the effectiveness of bone marrow purging in the process of autologous bone marrow transplantation in neuroblastoma. Further studies, however, are necessary to determine whether this method can detect minimal bone marrow metastasis in cases in which conventional methods have failed to yield a clear diagnosis. Neuroblastoma can be difficult to differentiate histologically from other small round cell tumours of childhood. These tumours include lymphoma, leukaemia, rhabdomyosarcoma and Ewing's sarcoma. This RT/PCR method may be also useful in differentiating neuroblastoma from other small round cell tumours of childhood that involve the bone marrow.

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Flavone Acetic Acid Potentiates the Induction of Endothelial Procoagulant Activity by Tumour Necrosis Factor

J. Clifford Murray, K. Anne Smith and David M. Stern

Treatment of human umbilical vein endothelial cells with flavone acetic acid (FAA) at 800 μg/ml for 4 h resulted in a 3–11-fold increase in procoagulant activity. This increase was due to enhanced tissue factor expression on the endothelial cell surface, as evidenced by the blocking of the enhanced clotting with antibody to tissue factor, by substitution of normal with factor VII deficient plasma, or by simultaneous treatment of the endothelial cells with cycloheximide or actinomycin D. FAA was not toxic to endothelial cell at concentrations up to 1.6 mg/ml over 4 h. Combined treatment with FAA and tumour necrosis factor α (TNF-α) (100 pg/ml) produced a 675-fold (range 160–1980) increase in tissue factor activity, compared to 5-fold and 50-fold increases for the individual agents respectively. Northern blotting of total RNA from cells treated with the combination of agents or either agent alone, followed by probing with a cDNA to human tissue factor demonstrated a synergistic increase in tissue factor mRNA after combination treatment. *In vivo*, the combination of FAA and TNF-α could be shown to induce greater growth delay in two murine tumours than would be predicted on the basis of the activity of either agent alone.
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INTRODUCTION

TUMOUR NECROSIS factor alpha (TNF-α) causes rapid regression in a number of murine tumour models [1]. Although the mechanism of action of TNF-α is poorly understood, nutrient deprivation due to occlusion of the tumour vasculature is thought to be an important component of its activity [2, 3]. *In vivo*, TNF-α treatment of tumour-bearing mice causes the deposition of insoluble fibrin within the vasculature of the tumour with the formation of occlusive thrombi [2], while *in vitro* treatment of human umbilical vein endothelial cells (HUVEC) or bovine aortic endothelial cells with TNF-α enhances tissue factor expression and suppresses the protein C anticoagulant pathway, thus promoting clot formation [4]. Recent studies have indicated that the tumour vasculature may also represent a target for the novel anticancer agent flavone acetic acid (FAA): not only is there a rapid loss of blood flow in the tumour after FAA treatment [5–7] but this is associated with a consumption coagulopathy, which is absent in non-tumour bearing animals [8]. These data suggest that there may be shared components in the mechanisms of action of TNF-α and FAA,

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