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Early Clinical Evaluation of Neuroblastoma Cell Detection by Reverse Transcriptase-Polymerase Chain Reaction (RT-PCR) for Tyrosine Hydroxylase mRNA

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Disseminating disease in neuroblastoma is of considerable clinical importance. Detection of circulating neuroblastoma cells using tyrosine hydroxylase (TH) as a tissue-specific target for reverse transcriptase-polymerase chain reaction has proved to be a sensitive and specific method for the detection of contaminating tumour cells in peripheral blood. The aim of this study was to report the early clinical observations made using this technology in neuroblastoma patient blood samples. A strong association was found between the detection of neuroblastoma cells in circulation with the detection of neuroblastoma in bone marrow. This method may be of use to monitor disease status and identify early signs of relapse in clinically disease-free patients. These results show that RT-PCR detection of *TH* mRNA is a relatively noninvasive, sensitive method for the detection of circulating tumour cells in neuroblastoma patients.

Key words: neuroblastoma, disseminating disease, tyrosine hydroxylase, reverse transcriptase-polymerase chain reaction

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INTRODUCTION

NEUROBLASTOMA shows a wide range of clinical behaviour, from localised tumours with a good prognosis to highly metastatic aggressive tumours with a very unfavourable outcome, despite intensive treatment. Recent reports suggest detection of neuroblastoma cells in bone marrow and circulating blood may predict clinical outcome and correlate with tumour relapse [1–3]. Neuroblastoma disease assessment is normally reliant on extensive staging investigations that include clinical and radiological procedures, with routine bone marrow sampling [4]. Analysis of neuroblastoma cell infiltration in peripheral blood may be a useful noninvasive method of assessing patient disease status, although until recently, methods for the detection of neuroblastoma cells in peripheral blood have not been available. Since catecholamines are secreted by 98% of neuroblastoma tumours, the first enzyme in the catecholamine synthesis pathway, tyrosine hydroxylase (TH), has been used as a target for reverse transcriptase-polymerase chain reaction (RT-PCR) to detect neuroblastoma cells. Using this method, detection of 1–10 neuroblastoma cells in 1×10^7 normal blood cells has been possible in cell spiking experiments [5]. This level of detection is an order of magnitude more sensitive than detection of neuroblastoma cells in peripheral blood using more conventional immunohistochemical techniques [6]. This increase in sensitivity compares well with detection of neuroblastoma cells using RT-PCR for *TH* in bone marrow compared with immunocytological techniques [7]. In the present study, we used RT-PCR for TH to detect neuroblastoma cells in blood samples from neuroblastoma patients.

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MATERIALS AND METHODS

Blood samples

Patients' blood samples were collected from several United Kingdom Children's Cancer Study Group Centres and analysed for circulating neuroblastoma cells using RT-PCR for *TH* mRNA. Blood samples were collected in EDTA, in 2 ml aliquots and frozen at −80°C until required for RNA extraction. Blood samples were taken from patients at diagnosis, during treatment and at the end of treatment. Blood samples were also taken at the time of clinical relapse and from some patients on long-term follow-up. Blood samples from 18 children attending hospital for routine surgical operations were obtained as age matched controls. Institutional ethical approval was obtained and parental consent given for all children from whom blood was taken (controls and neuroblastoma patients). Neuroblastoma staging was made according to the International Neuroblastoma Staging System (INSS) [4].

RNA extraction

Total cellular RNA was extracted from the IMR-32 neuroblastoma cell line, normal whole blood or patient whole blood

using Ultraspec™ RNA (Biogenesis, Bournemouth, U.K.), as previously described [5]. Blood samples were taken from the freezer directly into Ultraspec™ and allowed to thaw in this solution to reduce degradation of RNA by RNAses. The amount of RNA used in each RT-PCR was 5 μg. This RNA was DNAse (Pharmacia Biosystems Ltd, Milton Keynes, U.K.) treated for 15 min at 37°C to remove any contaminating DNA prior to RT-PCR. Recovered RNA and its purity were measured by O.D. at 260 and 280 nm. The quality of isolated RNA was confirmed by RT-PCR analysis for the housekeeping gene, glyceraldehyde-3-phosphate dehydrogenase (*GAPD*).

Reverse transcriptase-polymerase chain reaction (RT-PCR)

RT-PCR for *TH* was performed as previously described [5]. For each sample, an RT negative control (RT enzyme absent) was included. RNA from the neuroblastoma cell line IMR-32 was included as a positive control for *TH* RT-PCR in each experiment. The identity of RT-PCR products was confirmed by either Southern blotting or liquid hybridisation with a ^{32}P-end-labelled oligonucleotide probe specific to *TH*. For Southern blotting and liquid hybridisation, RT-PCR products of an unrelated gene were included in the analysis to confirm specificity of hybridisation. RT-PCR products were sequenced as previously described [5].

Bone marrow metastasis and urinary catecholamine levels

Bone marrow status was assessed by bilateral post-iliac crest aspirates and trephines at diagnosis, during treatment and at suspected clinical relapse. Urinary catecholamine levels were measured as previously described [8]. Additional staging investigations were in line with INSS recommendations.

RESULTS

RT-PCR detection of neuroblastoma cells

Following RT-PCR of mRNA from IMR-32 cells for *TH* the expected single band of 180bp was identified (Figure 1a). This was shown to be amplified *TH* by Southern blotting and liquid hybridisation using a ^{32}P end-labelled oligonucleotide probe specific to *TH* (Figure 1b). The amplified 180bp fragment was sequenced and confirmed as being that of the *TH* gene by comparison with the published *TH* sequence (data not shown).

Control blood analysis

In all the control blood samples analysed, no *TH* RT-PCR products were identified under the described conditions by ethidium bromide staining (Figure 2), Southern or liquid hybridisation with the [^{32}P] labelled *TH* oligonucleotide. The integrity of RNA was confirmed by RT-PCR for *GAPD*, which generated a 433bp product (Figure 2).

Patient blood analysis

(*i*) *At diagnosis.* Blood samples were analysed from 23 patients at diagnosis, 13 of these samples were positive for *TH* mRNA (Table 1). 11/13 of these positive samples were from stage 4 patients (Figures 1 and 3), 10 of whom had bone marrow involvement demonstrated histologically. Of the other two *TH*-positive patient blood samples, one was from a patient with stage 4s disease, and the other from a patient initially staged as stage 3, who on review was found to have low level (less than 5%) bone marrow involvement. Therefore, 12 patients had evidence of bone marrow disease; all these patients had elevated urinary catecholamines.

10 patient blood samples were negative for *TH* mRNA at diagnosis and remained negative throughout and at the end of therapy. This group of patients included 8 stage 4 patients, 1 stage 2 and 1 stage 3. Of the stage 4 patients, 5 had no evidence of bone marrow metastasis but had elevated catecholamine levels. Three stage 4 patients had evidence of bone marrow disease demonstrated histologically, one of whom had normal catecholamines. Both the stage 2 and 3 patients had no evidence of bone marrow disease and normal urinary catecholamines.

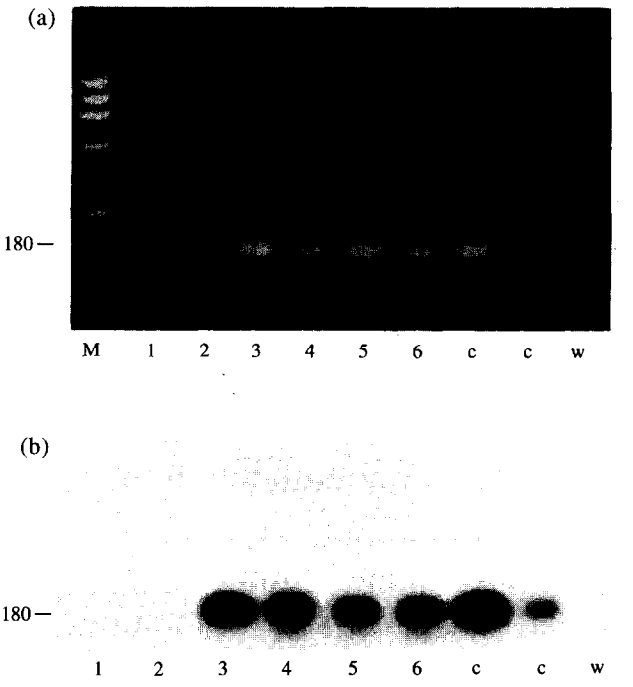

Figure 1. (a) Products of RT-PCR for *TH* mRNA separated by agarose gel electrophoresis and stained with ethidium bromide in 6 patient blood samples (1–6) taken at diagnosis. Samples 3–6 were from stage 4 patients, sample 1 from a stage 3 patient and sample 2 from a stage 2 patient. c = positive control, RT-PCR for *TH* mRNA on 100 pg and 100 ng of IMR-32 cells. (b) Southern blot of gel shown in (a) after hybridisation with ^{32}P-end labelled oligonucleotide probe specific for *TH*. Samples 3–6 were confirmed positive by RT-PCR for *TH* mRNA. M = molecular weight markers, w = water control.

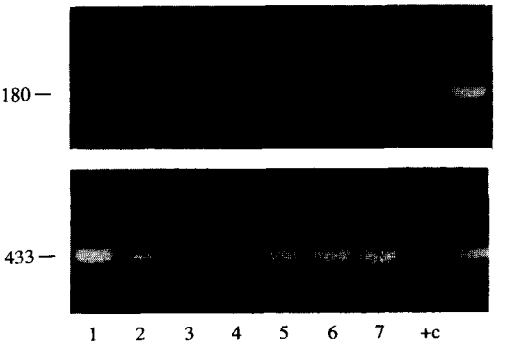

Figure 2. Products of RT-PCR for *TH* mRNA and *GAPD* separated by agarose gel electrophoresis and stained with ethidium bromide in 7 control bloods from children (1–7). RT-PCR for *TH* mRNA generated a 180bp product in the positive control (+c) sample of IMR-32 RNA, but no band was identified in the 7 control samples. RT-PCR for *GAPD* generated a 433bp product in all RNA samples. +c = positive control, RT-PCR for *TH* and *GAPD* mRNA on RNA extracted from IMR-32 cells.

Table 1. Summary of RT-PCR for TH mRNA results in patient blood samples at diagnosis and relapse

	Bone marrow +ve	Bone marrow −ve
At diagnosis		
TH mRNA + (13)	12	1
TH mRNA − (10)	3	7
At relapse		
TH mRNA + (8)	7	1
TH mRNA − (2)	0	2

Results of RT-PCR for *TH* mRNA are scored as "+" if found and "−" if absent. For each blood sample, where possible, three independent RNA extractions were performed and RT-PCR analysis carried out twice on each extraction. At diagnosis, 23 patient samples were analysed, and at relapse 10. Bone marrow metastasis was assessed histologically and scored "+ve" if contaminating neuroblastoma cells were identified and "−ve" if bone marrow was clear.

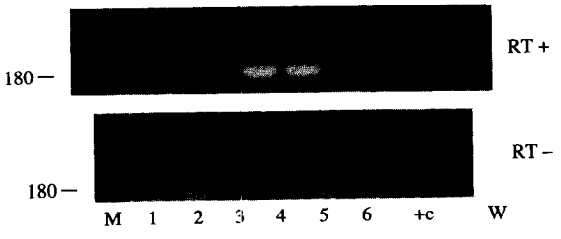

Figure 3. Products of RT-PCR for *TH* mRNA separated by agarose gel electrophoresis and stained with ethidium bromide in 6 patient blood samples taken at diagnosis (1–6, RT+). All the samples shown in this figure were from stage 4 patients. Corresponding negative controls, in which reverse transcriptase enzyme was not included, are also shown (1–6, RT−). +c = positive control, 100 pg, 10 ng mRNA from IMR-32 cells. Samples 4 and 5 were positive by RT-PCR for *TH* mRNA. M = molecular weight markers, W = water control.

(*ii*) *Sequential from diagnosis.* 12 patients were followed from diagnosis with sequential samples, 6 of whom were positive for *TH* mRNA. Four of these patients had no detectable *TH* mRNA 6–8 weeks into therapy. At the end of therapy, these samples remained negative. One stage 4 patient was positive at diagnosis and remained positive during and at the end of therapy. This patient had bone marrow disease throughout his treatment and has subsequently died. The stage 4s patient, positive for *TH* mRNA at diagnosis, remained positive 1 month after first diagnosis, at this time catecholamine levels remained elevated. This patient received no therapy and 1 year later is well with normal urinary catecholamine levels.

(*iii*) *At relapse.* 8 of 10 patient blood samples taken at the time of clinical relapse were positive for *TH* mRNA (Table 1). 7 of these patients had histological evidence of disease in the bone marrow and elevated urinary catecholamines at the time of relapse. The two *TH*-negative blood samples were from patients with localised relapse, one adrenal and one central nervous system. Both these patients had *TH*-positive tumours, and the patient with an adrenal tumour had elevated catecholamine levels but the patient with a central nervous system relapse had normal levels. Neither had evidence of metastatic disease in the bone marrow. Of the relapsed patients, 9 of the 10 were stage 4 patients and one a stage 3 patient. All relapsed patients have subsequently died.

(*iv*) *At remission.* Blood samples were taken from 27 apparently clinically disease-free patients (post therapy and/or follow-up). Of these, 7 previously diagnosed stage 4 patients were found to be positive for *TH* mRNA, and 5 have subsequently relapsed and died. In one remission patient, sequential samples demonstrated an increased level of *TH* mRNA in blood samples at clinical relapse compared to preclinical relapse (Figure 4). *TH* mRNA has been found in blood samples up to 12 months prior to clinical relapse. At relapse, all these patients had elevated catecholamine levels and evidence of bone marrow infiltration. 2 patients positive for *TH* mRNA in this group have been in clinical remission for up to 2 years.

Of the 20 patient samples negative for *TH* mRNA post treatment or during long-term follow-up, 2 patients have relapsed. One of these patient samples, stage 4 at diagnosis, was positive for *TH* at diagnosis, but at relapse had localised abdominal disease. The other patient had stage 3 disease at diagnosis and remained negative for *TH* mRNA. Both patients had elevated catecholamine levels at the time of clinical relapse.

DISCUSSION

The use of RT-PCR for the detection of small numbers of circulating cells has been previously described [7, 9, 10]. Using *TH* as a target, we have found RT-PCR detection of neuroblastoma cells in peripheral blood to be specific and sensitive [5]. From the clinical data presented in this study, we have found a strong correlation between detection of *TH* mRNA by RT-PCR in peripheral blood and disease status demonstrated by bone marrow infiltration; 92% (12/13) of patients positive for *TH* mRNA at diagnosis and 88% (7/8) of patients positive for *TH* mRNA at relapse had evidence of bone marrow disease. Only 3 patients with bone marrow infiltration had undetectable *TH* mRNA in peripheral blood and only 1 patient positive for *TH* mRNA had no detectable bone marrow disease. Of the 3 patients with bone marrow disease but no detectable *TH* mRNA in peripheral blood, one tumour was a non-catecholamine-secreting tumour, therefore this methodology would not detect these tumour cells. Since the remaining 2 patients had catecholamine-secreting tumours and histological evidence of bone marrow contamination, failure to detect *TH* mRNA in peripheral blood

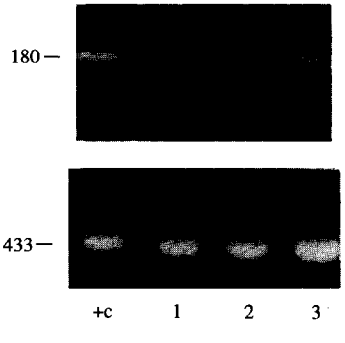

Figure 4. RT-PCR products for *TH* mRNA in serial blood samples taken from a stage 4 patient. 1, clinically disease-free (follow-up sample); 2, 4 months after initial follow-up blood sample; 3, at time of clinical relapse (9 months after initial sample); +c, positive control 100 pg mRNA from IMR-32 cells. Corresponding RT-PCR results for *GAPD* are also shown.

may reflect variations in peripheral blood sampling. It is likely the detection of *TH* mRNA in peripheral blood from a patient with no evidence of bone marrow infiltration histologically also reflects sampling variations, which will become more significant with decreasing numbers of metastatic cells. During subsequent therapy, circulating *TH* mRNA-positive cells were not detectable 6–8 weeks into chemotherapy, which compares with the apparent clearance of tumour cells from peripheral blood detected using immunocytology [2].

Analysis of blood samples from clinically disease-free patients identified circulating tumour cells in 26% (7/27) of patients. On follow-up, 71% (5/7) of these patients subsequently relapsed and died. This is compared to the 10% (2/20) of patients with blood samples negative for *TH* mRNA who relapsed. Detection of neuroblastoma cells in the peripheral blood was made up to 12 months before urinary catecholamine levels began to increase and clinical relapse occurred. This suggests RT-PCR for *TH* mRNA may have a role in early detection of disseminating disease and monitoring of disease status. The method would be of most value in patients where high intensity or alternative therapies could offer an improved patient survival. As yet, in neuroblastoma, early identification of relapse will not ultimately affect patient outcome as improved therapies for stage 4 neuroblastoma are required. Comparative analysis of blood samples before clinical relapse and at the time of relapse demonstrated an increase in circulating *TH* mRNA at relapse, indicating an increase in the numbers of circulating tumour cells. Although comparative data within individuals may indicate changes in circulating tumour cell number, the absolute number of circulating tumour cells cannot be quantified since the transcription level for *TH* mRNA will differ between tumours. Equally, although all the neuroblastoma tumours we have examined express *TH* mRNA [5], circulating tumour cells that do not express *TH* mRNA will not be detected by this methodology. The strong correlation between detection of neuroblastoma cells in peripheral blood and patient outcome suggests that detection of circulating tumour cells in peripheral blood may be a useful tool to evaluate new treatments and treatment regimes.

Urinary catecholamine levels have been used extensively as a diagnostic indicator for neuroblastoma disease [8]. In newly diagnosed patients with elevated urinary catecholamine levels, urinary catecholamines remained elevated until after surgery. Post surgery, levels returned to within the normal range following removal of the tumour mass. Therefore, whilst urinary catecholamines are useful as a diagnostic indicator of neuroblastoma, they are of limited value for the assessment of current disease status, unlike infiltration of bone marrow or peripheral blood with neuroblastoma cells. One of the most significant problems with analysis of blood or bone marrow samples for contaminating tumour cells is sampling error, as the number of target circulating cells decreases this problem increases in significance. The statistical significance of this phenomenon requires comparative analysis of a large number of samples.

In summary, these results demonstrate RT-PCR for *TH* mRNA-expressing neuroblastoma cells in peripheral blood is a useful and relatively noninvasive method for identification of circulating tumours cells. This method can be used to detect small numbers of neuroblastoma cells in bone marrow, and may be of value for the detection of contaminating tumour cells in peripheral stem cell harvests prior to autologous infusion. The precise risk associated with tumour cell contamination of bone marrow and peripheral stem cell harvests is not clear, although contamination can be substantial and vary depending on the time of harvest [2]. Further evaluation of this method and its long-term clinical significance is currently being explored through the United Kingdom Children's Cancer Study Group.

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