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Original Paper

Detection of Neuroblastoma Cells in CD34$^+$ Selected Peripheral Stem Cells Using a Combination of Tyrosine Hydroxylase Nested RT–PCR and Anti-Ganglioside G_{D2} Immunocytochemistry

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A sensitive assay was developed for the detection of neuroblastoma cell contamination in CD34$^+$ selected and unseparated peripheral blood stem cells (PBSC) used for autologous transplantation in stage 4 neuroblastoma patients. Specifically, we established a non-radioactive nested cDNA–PCR (nPCR) for detection of tyrosine hydroxylase (*TH*) gene expression combined with anti-disialoganglioside G_{D2} immunocytochemistry with the murine monoclonal antibody (MAb) 14G2a. Sensitivities of *TH* nPCR determined with a number of neuroblastoma cell lines and PBSCs correlated to cell line dependent basal *TH* gene expression levels and ranged from 1:10^4 to 1:10^6. The sensitivity obtained by immunocytochemistry was 1:10^5. We observed the highest PBSC contamination rate of 47% (18/38) among 38 PBSC specimens exclusively obtained from stage 4 neuroblastoma patients by using *TH* nPCR and G_{D2} immunocytochemistry in combination. Furthermore, a clinically applied purging method, CD34$^+$ selection by immunoabsorption (CD34$^+$ purity 42.4%), was used on 16 PBSCs. 10/16 (63%) preparations were contaminated prior to CD34$^+$ selection and 56% (9/16) remained contaminated. A significant reduction of neuroblastoma cell contamination by CD34$^+$ selection was not detectable, but the absolute amount of re-infused tumour cells was decreased due to 100-fold smaller cell counts of CD34$^+$ selected grafts used for transplantation. 22 PBSC preparations were used for transplantation. A Kaplan–Meier analysis showed an event-free survival probability of 0.56 ± 0.22 ($n = 9$) in the group with contaminated PBSCs versus 0.88 ± 0.12 ($n = 8$) with no detectable neuroblastoma-cell contamination. Our data suggest that the combined use of *TH* nPCR and G_{D2} immunocytochemistry is optimal to detect contamination and monitor purging strategies. © **1997 Elsevier Science Ltd.**

Key words: neuroblastoma, CD34$^+$ selection, peripheral blood stem cells, autologous transplantation, purging, minimal residual disease, nested PCR, tyrosine hydroxylase, anti-G_{D2} immunocytochemistry
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INTRODUCTION

NEUROBLASTOMA IS the most common solid, extracranial childhood malignancy with a yearly incidence of 7–10 per million. The tumour originates from the sympathetic nervous tissue and is characterised by catecholamine production in 92% of biopsy-proven diagnosis [1]. More than 40% of the patients initially present with disseminated stage 4 disease including bone marrow metastasis with very poor prognosis with an event-free survival probability of 0.27 ± 0.05 after 4 years (German neuroblastoma 90 trial). Current treatment modalities include debulking surgery, first-line chemotherapy

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and consolidation with high-dose chemo(radio)therapy and autologous transplantation which is commonly applied in stage 4 neuroblastoma patients [2–5].

Fifty per cent of bone marrow harvests of stage 4 neuroblastoma patients taken prior to high-dose chemotherapy and autologous transplantation have been found to be contaminated [6]. Therefore, depletion of tumour cells from bone marrow with detectable neuroblastoma cell contamination has been attempted with different panels of murine monoclonal antibodies (MAbs) in combination with goat anti-murine MAb-coated magnetic microspheres [2, 5]. In this regard, autologous transplantation with peripheral blood stem cells (PBSC) has been proposed, since PBSCs are considered to be contaminated less frequently by tumour cells than bone marrow [7]. PBSC collection is usually performed after debulking surgery and first-line polychemotherapy protocols whenever a significant reduction of tumour load is attained. However, some patients still have detectable residual disease according to standard diagnostic tests (e.g. [^{123}I] mIBG or [^{123}I] anti-G_{D2} MAb scintigraphy) at the time of PBSC collection. Ever since detection systems have been improved, neuroblastoma cells have also been found in 14% of PBSC preparations by immunocytological methods [8] and up to 26% by immunocytology and clonogenic analysis used in combination [9]. To overcome the problem of contamination, $CD34^+$ selection has been proposed as a purging method. Log 3 depletion rates have been reported in an experimental system containing 70–90% pure $CD34^+$ cells obtained from bone marrow that was artificially contaminated with 7–10% neuroblastoma cells [10]. However, a purging effect by $CD34^+$ selection of PBSC preparations used for autologous transplantation has yet to be elucidated.

Many attempts have been made to identify neuroblastoma cells among normal haematopoietic cells, including cytology (sensitivity $5{:}10^2$), catecholamine fluorescence ($1{:}10^3$), immunoflow cytometry ($1{:}10^4$–$1{:}10^5$) and immunocytochemistry using MAbs with various specificities including anti-95kD glycoprotein (BW 575), anti-ganglioside G_{D2} (126-4) (BW 625), anti-Thy 1 (390), anti-neuron specific enolase, anti-HSAN 1.2 and anti-neural antigen (459). Sensitivities achieved by the use of MAbs panels have varied from $1{:}10^3$ [11], $1{:}10^4$ [12] and $1{:}10^5$ [6, 13, 14]. Recently, molecular biological techniques applying the reverse transcriptase–polymerase chain reaction (RT–PCR) have been introduced. Various neuroblastoma specific gene transcripts have been exploited for the detection of neuroblastoma cells among haematopoietic cells, including neuroendocrine protein PGP 9.5 mRNA [15] and mRNA of tyrosine hydroxylase (TH) mRNA, the first step enzyme of catecholamine synthesis [16–18]. RT–PCR approaches have enhanced the sensitivity of neuroblastoma cell detection by achieving a range of $1{:}10^5$–$1{:}10^6$ with [^{32}P]-labelled oligonucleotides for sensitive PCR product identification after gel electrophoresis. However, no data are available thus far regarding the influence of variations in basal *TH* gene expression levels among neuroblastoma tumour cells. An additional consideration in this regard is that not all neuroblastoma tumours produce catecholamines [19] and express the *TH* gene, which could be detected by other independent markers.

Here we describe a highly sensitive and specific neuroblastoma cell detection system that makes use of *TH* gene product analysis by nested RT–PCR, without the need for radioactive isotopes. Together with the use of G_{D2} immunostaining, this approach markedly increased the sensitivity of neuroblastoma cell detection in PBSC preparations used for autologous transplantation in our centre. An evaluation of the impact of contamination on event-free survival probability post-transplantation indicated the need of a purging strategy for PBSCs used for autologous transplantation of neuroblastoma patients.

MATERIALS AND METHODS

Materials

TAQ-DNA-polymerase and RAV2 reverse transcriptase were purchased from Amersham (Braunschweig, Germany). Restriction enzymes, deoxynucleotides and hexanucleotide random primers were obtained from Boehringer (Mannheim, Germany) and the PCR primers from MWG Biotech (Erlangen, Germany). Monoclonal anti-G_{D2} antibody 14G2a (mouse) was kindly provided by R. Reisfeld, The Scripps Research Institute (La Jolla, California, U.S.A.). The alkaline phosphatase anti-alkaline phosphatase (APAAP) staining kit was purchased from DAKO Corporation (Santa Barbara, California, U.S.A.). All other chemicals and supplies were of the highest grade available and were obtained from commercial sources.

Reconstitution experiments and patients

PBSCs of healthy donors were used for reconstitution experiments. Neuroblastoma cells from SK-N-SH-, IMR 32- and Kelly cell lines were diluted stepwise until $1{:}10^7$, respectively. The SiMa cell line that expresses high levels of the *TH* gene [20] was diluted $1{:}10^8$. Each serial dilution experiment was performed in triplicate. Patient samples were collected from stage 4 neuroblastoma patients who were eligible for high-dose chemotherapy with autologous stem cell rescue. All patients were pre-treated according to the German neuroblastoma protocols. PBSC collection was performed after mobilisation with cyclophosphamide (4 g/m^2) and G-CSF (10 μg/kg) or GM-CSF (250 μg/m^2) alone in the cell separator Fenwall CS 3000, as described previously [21]. Samples of PBSCs from 38 patients were investigated, 19 of which were purged by $CD34^+$ selection with an immunoabsorption method (Cellpro). The $CD34^+$ content was $1.0 \pm 0.9\%$ in the untreated preparation and could be enriched up to $42.4 \pm 19.9\%$. Samples to be analysed were obtained from purged, non-purged and unabsorbed cell fractions. Patients were transplanted with either 1×10^8 PBSCs/kg body weight or 1×10^6 CD34+ selected PBSCs/kg body weight.

RNA isolation

Total RNA was isolated from cells lysed in guanidinium isothiocyanate followed by caesium chloride centrifugation [22]. RNA concentration and purity were determined spectrophotometrically.

cDNA synthesis and polymerase chain reaction (PCR)

RNA (1 μg) was converted to cDNA in a final volume of 20 μl as described previously [23]. cDNA equivalent (400 ng) was used for *TH*-cDNA amplification and 200 ng was taken for amplification of the internal standard glyceraldehyde-3-phosphate dehydrogenase (*GAPDH*). PCR was performed in a final volume of 50 μl containing 10 mM Tris pH 8.0, 50 mM KCl, 1.5 mM $MgCl_2$, 0.1 mg/ml gelatin, 200 mM of dGTP, dATP, dTTP, dCTP each, 2 μM of the amplimers

and 2.5 U TAQ-DNA-polymerase (Amersham Buchler, Braunschweig, Germany). To exclude contamination with PCR products, samples to be used for cDNA synthesis or PCR, respectively, were prepared by using separate solutions, pipettes and centrifuges. As a negative control, water instead of RNA or the reverse transcriptase, respectively, were examined at fixed time intervals. Reaction conditions for PCR were 96°C for 15 s, 55°C for 30 s and 72°C for 90 s (Thermocycler 60, Bio-Med, Theres, Germany). After PCR, 10 µl were applied for polyacrylamide gel electrophoresis (8% acrylamide, 0.25% bis(acrylamide)). Subsequently, gels were stained with ethidium bromide and signal intensities were digitalised by the 'CS-1 Videoimager' (Cybertech, Berlin, Germany) and analysed densitometrically with the 'WIN-CAM' software (Cybertech, Berlin, Germany). The identities of PCR products were established by estimating the molecular weights of the amplified material before and after digestion with restriction enzymes. PCR was performed in the exponential range of the amplification kinetics of *GAPDH* and *TH* primer pair as described previously [20]. Genomic DNA and mRNA sequence information of human tyrosine hydroxylase was used for primer generation to establish tyrosine hydroxylase nested reverse transcriptase PCR (*TH* nPCR) [24, 25]. A previously described, primer pair A,B (Figure 1) located in the constant *TH* mRNA region was used for external amplification [16] (*TH* type I, position: 509–807, 299 bp, 30 cycles). A second amplification was induced with 3 µl product and internal primers C,D (*TH* type I, position: 546–640, 95 bp, 30 cycles).

Immunostaining with anti-G_{D2} antibody

Alkaline phosphatase anti-alkaline phosphatase (APAAP) staining was performed by using a DAKO APAAP kit system (DAKO Corporation, Santa Barbara, California, U.S.A.). Briefly, adhesion areas of microslides were coated with 4–7×10^4 cells by cytocentrifugation at 400*g* for 5 min. Non-specific binding was blocked by incubation with serum for 30 min. Cells were incubated with mouse anti-G_{D2} MAb 14G2a for 60 min. The bridging rabbit anti-mouse MAb and murine APAAP immunocomplex were incubated for 30 min. Substrate was added for 20 min to induce red dye formation. Antibody, immunocomplex and substrate incubations were at room temperature. G_{D2}-positive neuroblastoma cells (positive control) stained red, whereas PBSCs (negative control) remained unstained. Microscopic examination of a number of fields corresponding to 10^7 cells was performed. G_{D2}-positive (red) cells were expressed as a percentage of the total cell number investigated.

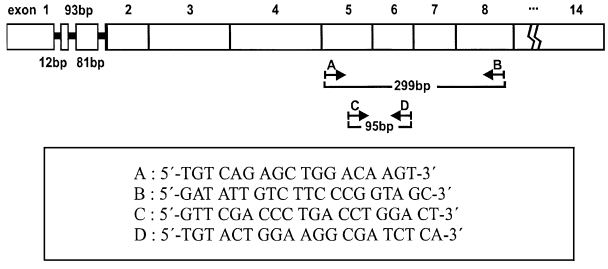

Figure 1. Regions selected for tyrosine hydroxylase reverse transcriptase–nested polymerase chain reaction (*TH* nPCR). *TH* mRNA (top) is characterised by a constant (exons 3–14) and variable (exons 1–2) region. The combination of two possible inserts (12 bp, 81 bp) implicates four possible splicing products [24, 25]. Primers are located in the constant region, leading to a 299 bp fragment (1. PCR) and a 95 bp fragment (nested PCR). Both external (A,B) and internal (C,D) primers were generated from sequences of different exons to avoid interference with possible genomic DNA contamination.

Statistics

The chi-square test was used to analyse possible correlations of anti-G_{D2} immunostaining and *TH* nPCR. The event-free survival probability of transplanted patients was calculated by the Kaplan–Meier analysis. Two-sided Student's *t*-test (95% confidence interval) was applied to test the statistical significance of differences in event-free survival probabilities. A P value of < 0.05 was considered to be significant.

RESULTS

Prior to neuroblastoma cell reconstitution experiments, the analysis of basal *TH* gene expression levels of different neuroblastoma cell lines estimated by semiquantitative PCR indicated increasing signal intensities of *TH* relative to *GAPDH*: SK-N-SH (32 ± 6%) < IMR 32 (88 ± 10%) < Kelly (103 ± 16%) < SiMa (110 ± 14%). The *TH* gene expression signal was not detectable in SK-N-LO and LS cells. However, *TH* gene expression signals of this single PCR approach required a drastic enhancement of intensity for detection of minimal neuroblastoma cell contamination. Therefore, *TH* nPCR was established, which resulted in uniform *TH* signals of high intensities (420 ± 30%) in SK-N- SH, IMR 32, Kelly, SiMa and also SK-N-LO cell lines. Only LS cells lacked the *TH* signal. The 4 cell lines with varying basal *TH* gene expression levels were used to evaluate the sensitivity of *TH* nPCR.

In dilution experiments with SK-N-SH cells, concentrations higher than $1{:}10^3$ were detectable (data not shown). Increasing sensitivities were found for the IMR 32, Kelly and SiMa cell lines (Figure 2). A characteristic of the reconstitution experiments by 10-fold serial dilution was a complete loss of signals at a distinct dilution step. With Kelly cells, the cut-off cell concentration was between $1{:}10^5$ and $1{:}10^6$. Stepwise dilution from $1{:}10^5$ to $1{:}10^6$ resulted in a distinct decrease in *TH* signal intensity at $4{:}10^6$ (Figure 2). In comparison, the sensitivity of anti-G_{D2} immunocytochemistry was found in the range of $1{:}10^5$ when determined in dilution experiments with Kelly cells that express relatively large amounts of G_{D2}. In order to demonstrate the specificity of this method, 20 bone marrow samples and 18 peripheral mononuclear cell (PMNC) preparations from healthy donors were investigated by *TH* nPCR as negative control. *TH* gene expression signals were not detectable in any of these samples (data not shown). However, each of 7 diagnostic bone marrow samples of stage 4 neuroblastoma patients with microscopic bone marrow involvement used as positive control showed a specific *TH* nPCR signal (data not shown).

PBSC samples obtained from 38 neuroblastoma stage 4 patients were investigated (Figure 3, Table 1). Signals of samples from 2 patients with purged PBSC preparations are shown in Figure 3. Patient 1 showed no signal in any preparation, whereas a *TH* nPCR signal was found after analysis of an unseparated PBSC preparation from patient 2, which was no longer detectable after $CD34^+$ selection. The unabsorbed cell fraction was also *TH* nPCR positive.

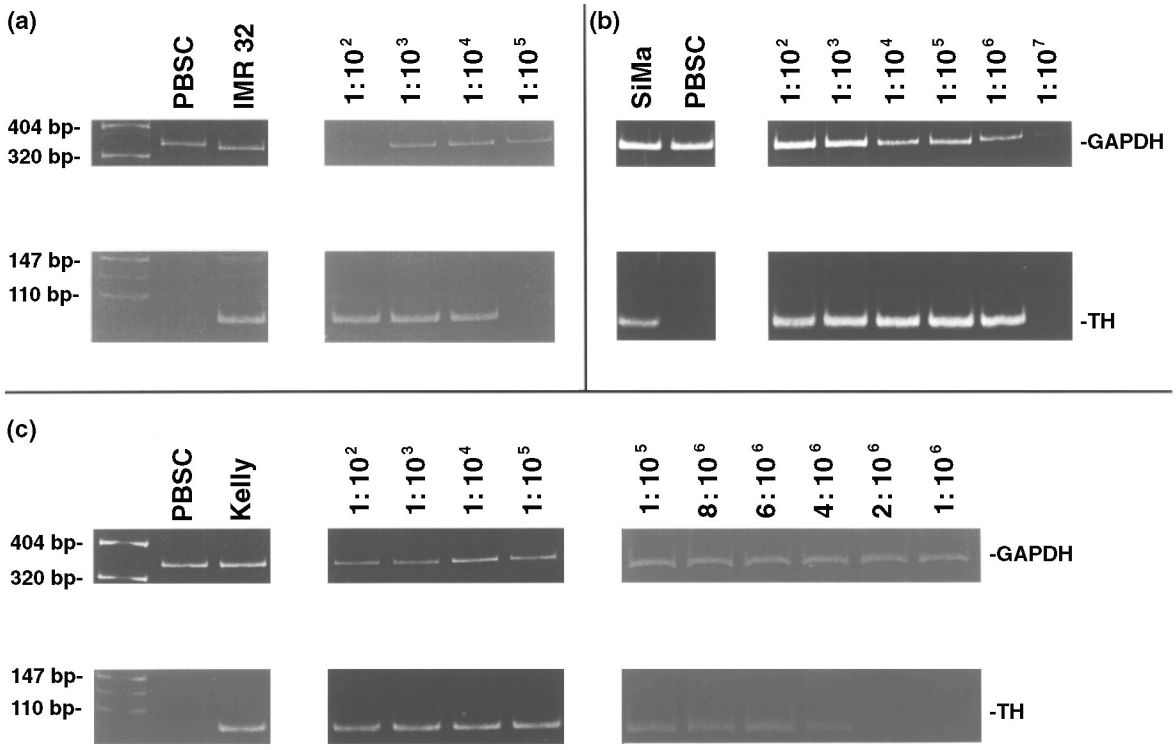

Figure 2. Reconstitution experiments of neuroblastoma cells in PBSCs of healthy donors by *TH* RT–nPCR. The sensitivity using neuroblastoma cell lines with distinct *TH* gene expression (IMR 32, SiMa, Kelly) was determined. With factor 10 dilution, signals of uniform intensities were lost below a distinct cut-off concentration (a, b). Reduction of Kelly cell concentration induced a decreased signal intensity at 4:10⁶ (c). Left lane = molecular weight marker.

Probes generated specific *TH* nPCR signals or showed red-stained G_{D2}–positive cells (Tables 1 and 2) in 18/38 unseparated PBSCs corresponding to a total contamination rate of 47%. 14/36 (39%) samples exclusively examined by *TH* nPCR, generated *TH* gene expression signals (Table 2). 11/31 (35%) probes analysed by G_{D2} immunocytochemistry were found to be contaminated with red-stained cells. Twenty-nine samples of unseparated PBSCs were investigated simultaneously (*values, Table 2) by both test systems. Among these, 12/29 (41%) had *TH* signals and 10/29 (34%) exhibited red-stained cells with anti-G_{D2} MAb. These results (Table 2) indicate a positive correlation (*TH* nPCR versus G_{D2} staining chi-square = 5.15, $n = 29$, $P = 0.02$).

Clinical staging at the time of PBSC collection was available in 33/38 cases (Table 1). Twelve patients were in complete remission (CR) and 7/12 (58%) showed *TH* nPCR signals or red-stained G_{D2}-positive cells in the unseparated PBSC preparation. Twenty-one had clinically detectable residual disease (PR) according to standard diagnostic tests, including [^{123}I]mIBG or [^{123}I] anti-G_{D2} MAb scintigram (Table 1). Only 8/21 (38%) specimens of original PBSC material showed either *TH* nPCR or G_{D2} signals.

Sixteen PBSC preparations were subsequently purged by CD34$^+$-selection and re-analysed by both methods (Table 1). 10/16 (63%) samples were initially either G_{D2} or *TH* nPCR positive. After CD34$^+$ selection (average CD34$^+$ purity: 42.4%), 9/16 (56%) samples remained contaminated. However, the absolute tumour cell number re-infused into the patient was significantly lower when using CD34+ selected PBSCs are used due to the 100-fold smaller total cell counts of the graft used for transplantation. Although CD34$^+$ selected PBSCs of patient 12 showed a *TH* nPCR signal, it was absent in the unseparated PBSC preparation probably due to RNA degradation indicated by a weak *GAPDH* signal, even though 2–3-fold more cDNA was used for amplification. *TH* nPCR signals of probes from patients 4, 6, 7 and 8 disappeared after the purging procedure.

23/38 PBSCs preparations were given following high-dose chemotherapy (Table 1). One patient (No. 26) died 65 days post-transplant due to fulminant cytomegaly virus infection and was therefore excluded from further analysis. The distribution of events including probabilities of event-free survival (*P*EFS) are shown in Table 3 and Figure 4. Kaplan–Meier analysis showed an overall *P*EFS of 0.62 ± 0.12 ($n = 22$).

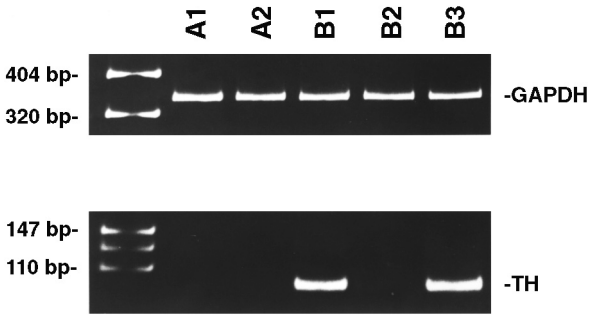

Figure 3. *TH* RT–nPCR with PBSC preparations from two patients (a, b). (1) Unseparated PBSCs; (2) CD34$^+$ selected PBSCs; (3) unabsorbed cell fraction (only performed to confirm the *TH* signal in sample 1). Left lane = molecular weight marker.

Table 1. *Investigation of unseparated and CD34$^+$ selected PBSCs using* TH *nPCR and G_{D2} immunocytochemistry*

Patients			PBSCs, unseparated		PBSCs, CD34$^+$ selected		Remission at the time of collection
			TH nPCR	G_{D2} staining	*TH* nPCR	G_{D2} staining	
1	T		+	–	–	–	PR
2	T		+	–	+	–	CR
3			+	–	+	–	n.a.
4			+	–	–	–	PR
5			+	+	+	+	PR
6			+	+	–	+	n.a.
7			+	+	–	+	PR
8	T		+	+	–	+	PR
9	T	E	–	+	–	+	CR
10			–	+	–	+	PR
11	T		–	+	–	n.d.	CR
12			–	–	+	–	n.a.
13	T		–	–	–	–	PR
14	T	E	–	–*	–	–	PR
15			–	–	–	–	PR
16			–	–	–	–	CR
17			–	–	–	–	PR
18			–	–	n.d.	–	n.a.
19	T		n.d.	–	n.d.	–	CR
20	T	E	+	n.d.			n.a.
21	T		+	n.d.			CR
22	T	E	+	–			CR
23			+	+			PR
24			+	+			PR
25	T		+	+			CR
26	T	E#	n.d.	+			CR
27	T		–	–*			CR
28	T		+	–			PR
29	T		–	–			PR
30	T		–	–			PR
31	T		–	–			CR
32	T		–	–			CR
33			–	–			PR
34	T	E	–	n.d.			PR
35	T		–	n.d.			PR
36	T	E	–	n.d.			PR
37			–	n.d.			PR
38	T	E	–	n.d.			PR

T = transplanted patients; E = event; * = high background signal; n.d. = not done; n.a.-not available; CR = complete remission; PR = partial remission (including VGPR = very good PR); # = death from cytomegaly virus infection.

Only 1 event occurred in a group of 8 patients with G_{D2} and *TH* nPCR negative results. Compared to patients transplanted with contaminated PBSCs (G_{D2} or *TH* nPCR positive, *values, Table 3), the difference was non-significant ($P = 0.08$) (Figure 4).

DISCUSSION

In this study we have monitored tumour cell contamination of PBSCs used for autologous transplantation following high-dose chemotherapy of stage 4 neuroblastoma patients. The contamination rate detected by using *TH* nPCR combined with anti-G_{D2} immunocytochemistry was almost twice that published previously [9]. However, the level of *TH* mRNA expression was found to vary among different neuroblastoma cell lines and can change during neuroblastoma cell differentiation *in vitro* induced by differentiation agents such as retinoic acid [20]. There is clinical evidence that variable *TH* gene expression also occurs *in vivo* among patients with fluctuating catecholamine metabolite excretion detected by urinary vanillylmandelic acid (VMA) and homovanillic acid (HVA) concentrations [26]; this occurred even in 7.5% of neuroblastoma patients where VMA/HVA was not detectable [19]. The variability of basal *TH* mRNA production has a strong impact on the sensitivity of the *TH* nPCR system (Figure 2). Therefore, it is necessary to use other unrelated neuroblastoma markers. The same applies to G_{D2} which is

Table 2. *Results of* TH *nPCR and G_{D2} immunocytochemistry of unseparated PBSCs*

	TH nPCR			
	Positive	Negative	n.d.	Total
G_{D2}				
positive	7*	3*	1	11
negative	5*	14*	1	20
n.d.	2	5		7
Total	14	22	2	38

n.d. = not done, * chi-square = 5.15, $P = 0.02$.

Table 3. Results of TH *nPCR and* G_{D2} *immunocytochemistry of PBSCs versus events and event-free survival (EFS) of transplanted patients*

	TH nPCR						
	Positive		Negative		n.d.	Total	
	n	EFS	*n*	EFS	*n*	*n*	EFS
G_{D2}							
Positive	2		2(1)		0	4(1)	[0.67 ± 0.27] *n* = 4
Negative	3(1)		8(1)	[0.88 ± 0.12]	1	12(2)	[0.61 ± 0.14] *n* = 12
n.d.	2(1)		4(3)			6(4)	
Total	7(2)	[0.63 ± 0.22] *n* = 7(2)	14(5)	[0.61 ± 0.14] *n* = 14	1	22(7)	[0.62 ± 0.12] *n* = 22

n.d. = not done; number of patients who relapsed (events) are indicated in parentheses.

a highly accepted surface marker expressed by most neuroblastoma tumours [1]. Variable G_{D2} expression was also found *in vitro* [27] and *in vivo* [28]. The variation in expression of both markers most likely accounts for differing results with both test systems (Table 1). Therefore, it is better to use these markers in combination for very sensitive detection of minimal residual disease.

Several lines of evidence indicate that reinfusion of tumour cells affected the probability of event-free survival (*P*EFS) in our small number of transplanted patients: First, there was a better although non-significant *P*EFS of patients transplanted with PBSCs without detectable neuroblastoma cell contamination (Figure 4). Second, the better outcome of patients transplanted with uncontaminated PBSCs did not correlate with clinical staging at the time of PBSC collection (Table 1). This finding is further supported by observations that circulating neuroblastoma cells which contaminate PBSCs have clonogeneic properties *in vitro* [9] and thus could well be a major cause of relapse after autologous reinfusion demonstrated by gene marking experiments in AML (acute myeloid leukaemia) and neuroblastoma patients [29, 30]. Therefore, future efforts should focus on developing advanced purging methods for PBSCs to reduce the amount of reinfused tumour cells.

In vitro experiments with highly contaminated bone marrow indicated that an effective neuroblastoma cell depletion could be obtained by $CD34^+$ selection [10], a common marker of human haematopoietic precursor cells not expressed in human neuroblastoma cells [31]. Therefore, $CD34^+$ selection was suggested as a purging strategy for clinical PBSC autologous grafts, e.g. by immunoabsorption methods [32]. Such a procedure was used to select $CD34^+$ cells from PBSCs. However, we clearly demonstrated that the combination of *TH* nPCR with anti-G_{D2} immunocytochemistry revealed neuroblastoma cell contamination even in $CD34^+$ selected PBSCs. The $CD34^+$ selection method probably failed to reduce significantly the relative tumour cell concentration because of only a moderate 42.4% purity obtained. However, due to $CD34^+$ enrichment, 1×10^6 cells/kg body weight used for transplantation achieved efficient and successful engraftment. On the basis of the relative neuroblastoma cell contamination rate and the total PBSC counts prior and after $CD34^+$ selection, we calculated a mean log tumour cell depletion of 1.41 ± 0.45 (R. Handgretinger, Children's Hospital, University of Tuebingen). This indicates that a significantly reduced number of tumour cells was re-infused to the patient.

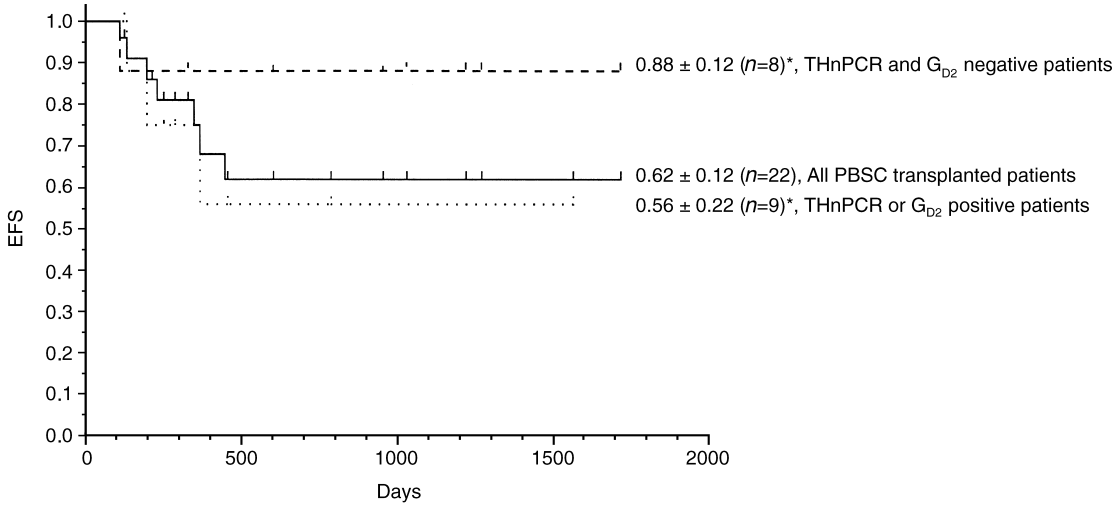

Figure 4. Kaplan–Meier analysis of stage 4 neuroblastoma patients after autologous PBSC transplantation. ***P*****EFS ± standard error are indicated. ****P* **= 0.08. Mean observation time: 640 ± 510 days (minimum 115 days, maximum 1719 days).**

In order to further increase $CD34^+$ purity in PBSCs and optimise purging efficiency, two-step methods combining immunabsorption and magnetic activated cell sorting (MACS) appear promising. Thus, preliminary data ($n = 2$ patients) suggest enrichments > 99.0% with absence of *TH* nPCR and G_{D2} signals and such patients engrafted successfully. Furthermore, direct PBSC purging strategies might be developed, e.g. neuroblastoma cell depletion by anti-G_{D2} immunobeads as performed with autologous bone marrow transplantation [2, 5].

In conclusion, the combined application of *TH* nPCR and anti-G_{D2} immunocytochemistry proved to be a most sensitive tool to allow monitoring of clinical purging strategies. Since PBSC preparations collected from stage 4 neuroblastoma patients were found to be contaminated to a very high extent with tumour cells, purging of such cell preparations is advisable. Consequently, we suggest purging autologous grafts to the point where both *TH* nPCR and G_{D2} immunocytochemistry yield negative results.

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