

Detection of Low Numbers of Neuroblastoma Cells *In Vitro*

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Abstract

Neuroblastoma is a tumour derived from the sympathoadrenal progenitors of the neural crest. It is one of the most malignant solid tumours in childhood with an annual incidence of 9.4 per 10⁶ children under 15 years of age. Recent studies suggest that immunocytological detection of neuroblastoma cells in bone marrow and circulating neuroblasts during treatment may predict clinical outcome and correlate with tumour relapse. The present methods of diagnosing metastasis in neuroblastoma include histological, biochemical and immunohistological analysis. Morphological distinction between tumour cells and primitive lymphoblasts in bone marrow is often difficult, and these methods may also not always be sensitive enough for early detection of the residual and minimally circulating tumor cells. A sensitive assay for detection of such residual cells using two tissue-specific markers, *NFM* and *SYN*, by reverse transcriptase-polymerase chain reaction (RT-PCR) is reported here. Analysis of the specificity of this assay in three neuroblastoma cell lines, namely *IMR 32*, *SK-N-SH* and *SY5Y* showed positive expression while control peripheral blood mononuclear cells (*HL 60*) were negative. In reconstituted cell spiking tests, this method has the ability to detect 1-10³ neuroblastoma cell in 10⁷ normal peripheral blood mononuclear cells (*PBMC*), as shown by serial dilution and limiting dilution. The *NFM* marker was found to be a more sensitive marker. The specificity and sensitivity of this technique makes it suitable for future application in detection of minimally disseminated tumour cells in neuroblastoma patients.

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Introduction

Neuroblastoma is a tumour derived from the primitive sympathetic neuroblasts and is one of the most common extracranial solid tumours of childhood. The annual worldwide incidence is 9.4 per 10⁶ children under 15 years of age.¹ A wide range of prognosis is possible which closely correlates with the extent of tumour spread at diagnosis and the age of the patient.² Neuroblastoma can be staged into five clinical stages from I to IV-S according to the clinical manifestation³ and TNM (tumour, node and metastases) criteria.⁴ Tumours of localised distribution are grouped as stage I and II, whereas those with remote metastases are grouped as stage III and IV. Although stage IV-S tumours are metastatic, they have a better prognostic outcome than stage IV tumours and can spontaneously regress.⁵ In patients above one year of age, there is almost 100% survival rate in localised tumours as compared to the poor prognosis in non-localised disseminated tumours. In advanced stage tumours, the proto-oncogene, N-myc, has been found to be amplified.^{6,7} The

link between amplification of the N-myc gene in neuroblastoma and poor prognosis has been well documented.^{8,9}

Although prognosis of this malignancy has improved with advances in medical management, the overall 5-year survival is still about 55%.¹ Besides staging and age, prognostic markers used in delineating subgroups of patients with different outcomes include histopathological features, tumour cytogenetic findings, serum ferritin level, tumour DNA content, level of expression and copy number of N-myc, level of catecholamines and neuron-specific enolase.¹⁰⁻¹² Recent studies also suggest that immunocytological detection of residual neuroblastoma cells in bone marrow and the presence of minimally circulating neuroblasts during treatment may predict clinical outcome and correlate with tumour relapse.^{13,14} Hence, such sensitive detection of small number of neuroblast cells in peripheral blood or bone marrow may be important for disease diagnosis, prognosis and monitoring. The present techniques used in diagnosing metastasis in neuroblastoma are not sen-

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sitive enough for early detection of such residual and minimally circulating tumour cells. Thus, a sensitive technique using reverse transcriptase-polymerase chain reaction (RT-PCR) amplification assay could facilitate such detection.¹⁵ Because no tissue-specific abnormality is present in neuroblastoma and the known cytogenetic and molecular abnormalities associated with neuroblastoma such as loss of heterozygosities and partial deletions of chromosome 1p are not suitable as reliable genetic markers for such assay, a tissue-specific gene expression has to be used as marker for neuroblastoma.^{16,17}

In this study, the expressions of neurofilament M, an intermediate filament component of neuron cells¹⁸ and synaptophysin, a phosphoprotein localised to the cytoplasmic membrane of synaptic vesicles found in differentiating neurons,¹⁹ were used as markers for *in vitro* detection of minimally disseminated neuroblastoma cells. This RT-PCR based detection technique using the two markers was tested on neuroblastoma cell lines and normal peripheral blood mononuclear cell (PBMC) line as a preliminary investigation to assess whether this method is suitable for further application in detection of disseminated tumour cells from neuroblastoma patients.

Materials and Methods

Cell Lines and Culture Of Cells

Three neuroblastoma cell lines, IMR-32 (ATCC No: CCL 127), SK-N-SH (ATCC No: HTB 11) and SY5Y, were cultured in RPMI 1640 medium supplemented with 10% heat-inactivated fetal calf serum and antibiotics in 5% CO₂ at 37°C. IMR-32 has a neuroblastic phenotype while SK-N-SH is composed of two different type of cells, flat epithelium-like cells and neuroblastic cells with short cytoplasmic processes. SY5Y was derived from SK-N-SH and is composed of only neuroblastic cells. A control cell line, HL60, from normal peripheral mononuclear blood was similarly cultured.

RNA Extraction

Total cellular RNA was isolated by the acid guanidinium thiocyanate/phenol-chloroform extraction method as previously described.²⁰ The extracted RNA was dissolved in diethyl pyrocarbonate (DEPC)-treated water and its purity measured by optical densities at 260 nm and 280 nm.

Reverse transcriptase-polymerase chain reaction (RT-PCR)

Detection of neuroblastoma cells by RT-PCR was carried out using RNA extracted from IMR-32, SK-N-SH and SY5Y. Reverse transcriptase enzyme was used to transcribe cDNA from target mRNA using primers covering nucleotides 878 - 898 of human neurofilament M (NFM) gene¹⁸ and nucleotides 113 - 131 of human

synaptophysin (SYN) cDNA.²¹ A control for the RT-PCR reaction was carried out using primers for the β -actin gene.²² The sequences of these primers are listed in Table I. Two μ g of total RNA was incubated with 300 ng of random hexanucleotide primers in a total volume of 8 μ l DEPC-treated water at 65°C for 10 min. The denatured RNA was then added to the RT reaction mixture and incubated at 37°C for 60 min. The RT reaction mixture consisted of 4 μ l of 5' RT buffer (250 mM Tris, pH 8.3), 375 mM KCl, 15 mM Mg Cl₂), 1 μ l 200 mM DTT, 1 μ l 5 mM deoxynucleotide triphosphate mixture (Pharmacia Biotech, Sweden), 1 μ l 200 units MMLV reverse transcriptase (Gibco BRL Life Technologies, Inc., USA). The RT product was then amplified by PCR using 5 μ l 10' PCR buffer (Perkin Elmer, USA), 0.5 μ g each primer and 3 units AmpliTaq (Perkin Elmer Cetus, USA) in a total volume of 50 μ l. PCR amplification conditions were as follows: 35 cycles of denaturation at 94°C for 1 min, 60°C for 1 min and extension at 72°C for 3 min with an initial denaturation at 94°C for 7 min and a final extension at 72°C for 7 min. 10 μ l of the PCR products was electrophoresed on a 2% agarose gel in 1' Tris/borate/EDTA (TBE) buffer and visualised

TABLE I: PRIMER SEQUENCES FOR MARKERS IN THIS STUDY

Marker	Primer	Sequence
NFM	Forward	5'-AAT GCC GCT ACG CCA AGC TCA -3'
	Reverse	5'-CAG TGG CCC AGT GAT GCT TCC T -3'
SYN	Forward	5'-TTG CCA CAT GCG GCA GCT AC -3'
	Reverse	5'-TTG GCC ATA GCC TTG CTG C -3'
β -actin	Forward	5'-ATC TGG CAC CAC ACC TTC TAC AAT GAG CTG CG -3'
	Reverse	5'-CGT CAT ACT CCT GCT TGC TGA TCC ACA TCT GC -3'

on a transilluminator after staining in ethidium bromide (0.5 μ l/ml).

Sensitivity Of the RT-PCR Test

To test the sensitivity of this technique for the detection of neuroblastoma cells in blood, reconstituted cell spiking experiments, were carried out using mixes of tumour cells with a fixed population of normal peripheral blood mononuclear cells (HL60). Serial dilutions of as low as 1 tumour cell in 10⁷ HL 60 cells were carried out. RNA was extracted from these mixes and RT-PCR performed as described above.

Results

Specificity Of Markers

Following RT-PCR amplification of RNA extracted from the neuroblastoma cell lines, a 392 bp product was obtained using the primers for NFM (Fig. 1) while SYN primers yielded a 785 bp product (Fig. 2). It was ob-

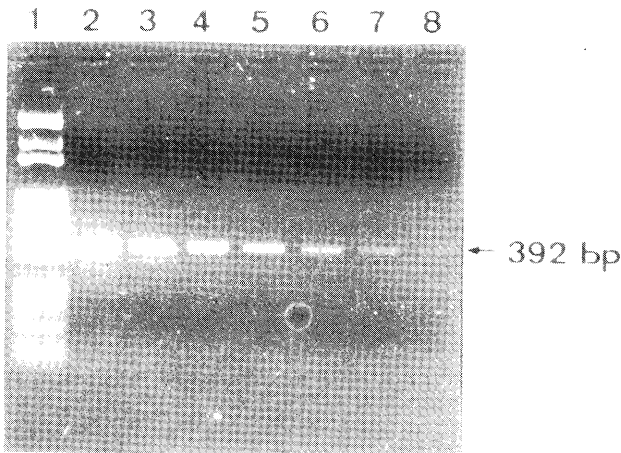


Fig. 1. RT-PCR assay using NFM marker. Lane 1: molecular weight marker, pGEM (Promega, USA); lanes 2-8: dilutions of IMR-32 cells shown in Table II. There was a decrease in intensity of the 392bp band with increasing dilutions of tumour cells.

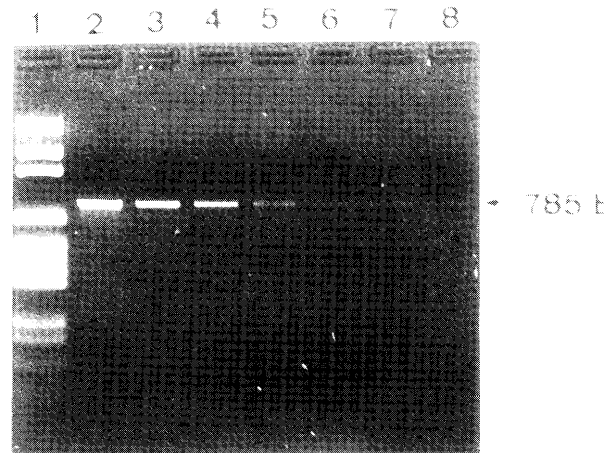


Fig. 2. RT-PCR assay using SYN marker. Lane 1: molecular weight marker, pGEM (Promega, USA); lanes 2-8: dilutions of IMR-32 cells as shown in Table III. There was a decrease in intensity of the 785bp band with increasing dilutions of tumour cells.

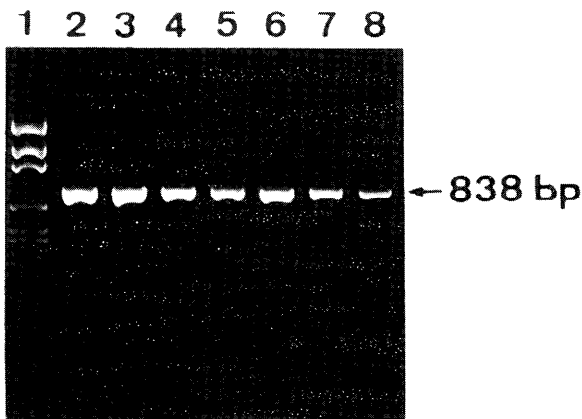


Fig. 3. Control RT-PCR assay using β -actin marker. Lane 1: molecular weight marker, pGEM (Promega, USA); lanes 2-8: dilutions of HL60 cells from 10^6 to 10^1 cells. A 838bp amplified product was observed corresponding to the expression of β -actin in HL60 cell line.

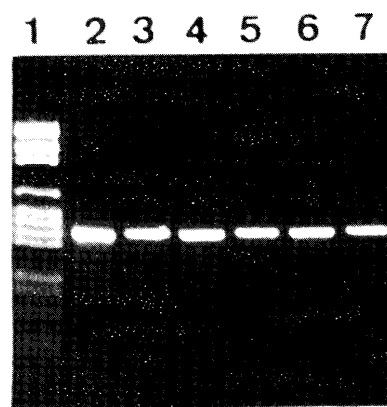


Fig. 4. Sensitivity of NFM marker in reconstituted cell spiking experiment. Serial dilutions of IMR-32 cell lines in fixed concentration of 10^7 HL60 cells were prepared as in Table IV. Lane 1: molecular weight marker, pGEM (Promega, USA); lanes 2-7: serial dilutions of IMR-32 cells from 10^5 cells to 1 cell as in Table IV.

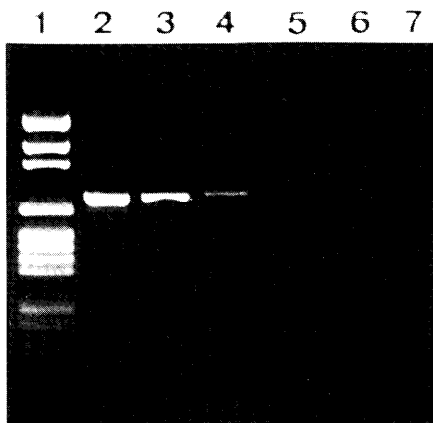


Fig. 5. Sensitivity of SYN marker in reconstituted cell spiking experiment. Serial dilutions of IMR-32 cell lines in fixed concentration of 10^7 HL60 cells were prepared as in Table IV. Lane 1: molecular weight marker, pGEM (Promega, USA); lanes 2-7: serial dilutions of IMR-32 cells from 10^5 cells to 1 cell as in Table IV.

served that there was a decrease in intensity of the amplification products on the ethidium stained gels with decreasing concentrations of neuroblastoma cells.

As shown in Figures 1 and 2, amplification products from both NFM and SYN markers were obtained even at dilutions of as low as 1 to 10 cells from the IMR-32 cell line. Similar results were obtained from the other two cell lines, SY5Y and SK-N-SH as shown in Tables II and III. The tissue-specific expression of these two markers was confirmed by the observation that no similar amplification could be obtained from the normal peripheral blood mononuclear cell line, HL60 (Tables II and III). To confirm that this was not due to actual failure of the RT-PCR process, a control pair of primers for the amplification of β -actin gene was used and a 838 bp amplification product was observed from HL60 cells (Fig. 3). The β -actin gene is ubiquitously expressed in all cell types. Similar results were obtained from the other two cell lines, SY5Y and SK-N-SH (Fig. 4). In addition, the specificity of the RT-PCR products from the NFM and SYN markers were confirmed from dot blot hybridisation with the respective NFM and SYN cDNA probes (results not shown).

TABLE II: SPECIFICITY OF EXPRESSION OF NFM MARKER

No. of cells	10 ⁶	10 ⁵	10 ⁴	10 ³	10 ²	10 ¹	1*
IMR-32	+	+	+	+	+	+	±
SY5Y	+	+	+	+	+	+	●
SK-N-SH	+	+	+	+	+	+	±
HL60							

* Faint amplification products observed in some cases. This dilution was repeated to obtain 10 sample mixes each. According to Poisson limiting dilution statistics, 70% of such mixes would contain a single tumour cell while 30% would not.

TABLE III: SPECIFICITY OF EXPRESSION OF SYN MARKER

No. of cells	10 ⁶	10 ⁵	10 ⁴	10 ³	10 ²	10 ¹	1*
IMR-32	+	+	+	+	+	+	±
SY5Y	+	+	+	+	+	+	±
SK-N-SH	+	+	+	+	+	+	±
HL60							

* Faint amplification products observed in some cases. This dilution was repeated to obtain 10 sample mixes each. According to Poisson limiting dilution statistics, 70% of such mixes would contain a single tumour cell while 30% would not.

TABLEIV: SENSITIVITY OF NFM AND SYN MARKERS IN IMR-32 CELL LINES*

IMR-32	10 ⁵	10 ⁴	10 ³	10 ²	10 ¹	1
HL60	10 ⁷	10 ⁷	10 ⁷	10 ⁷	10 ⁷	10 ⁷
NFM	+	+	+	+	+	+
SYN	+	+	+			

* Serial dilutions of IMR-32 cells were prepared in a fixed population of 10⁷ normal peripheral mononuclear blood cells from control cell line, HL60. RT-PCR was performed from extracted RNA from these mixes and results of amplification products were analysed by agarose gel electrophoresis.

Sensitivity of RT-PCR Assay

In reconstituted cell spiking experiments, it was possible to detect the expression of the NFM and SYN markers from mixtures of decreasing neuroblastoma cells in fixed number of 10⁷ normal peripheral blood mononuclear cells, HL60 (Fig. 5). In all three neuroblastoma cell lines, the sensitivity of detection of expression of the NFM marker was as high as 1 cell per 10⁷ normal HL60 cells but the SYN marker was found to be less sensitive being detected at 10 in 10⁷ cells from SK-N-SH cell line and at 10³ in 10⁷ from both IMR-32 and SY5Y cell lines (Tables IV, V and VI). To verify the detection sensitivity at the level of 1 tumour cell in 10⁷ HL60 cells for the expression of NFM marker, ten sample mixes at that dilution were prepared and RT-PCR carried out using the extracted RNA. These results were reproducible in six of the samples and are consistent with Poisson limiting dilution statistics which predict that 70% of such mixes would contain a single tumour cell, whereas 30% would not.

TABLEV: SENSITIVITY OF NFM AND SYN MARKERS IN SY5Y CELL LINES*

SY5Y	10 ⁵	10 ⁴	10 ³	10 ²	10 ¹	1
HL60	10 ⁷	10 ⁷	10 ⁷	10 ⁷	10 ⁷	10 ⁷
NFM	+	+	+	+	+	+
SYN	+	+	+			

* Serial dilutions of SY5Y cells were prepared in a fixed population of 10⁷ normal peripheral mononuclear blood cells from control cell line, HL60. RT-PCR was performed from extracted RNA from these mixes and results of amplification products were analysed by agarose gel electrophoresis.

TABLE VI: SENSITIVITY OF NFM AND SYN MARKERS IN SK-N-SH CELL LINES*

SK-N-SH	10 ⁵	10 ⁴	10 ³	10 ²	10 ¹	1
HL60	10 ⁷	10 ⁷	10 ⁷	10 ⁷	10 ⁷	10 ⁷
NFM	+	+	+	+	+	+
SYN	+	+	+	+	+	

* Serial dilutions of SK-N-SH cells were prepared in a fixed population of 10⁷ normal peripheral mononuclear blood cells from control cell line, HL60. RT-PCR was performed from extracted RNA from these mixes and results of amplification products were analysed by agarose gel electrophoresis.

Discussion

Detection of neuroblastoma cells in peripheral blood and bone marrow of patients at diagnosis and relapse have conventionally been detected by histological, biochemical and immunohistological analysis.¹⁰⁻¹² Recently, the use of monoclonal antibodies against tumour surface antigen on neuroblastoma cells has improved the detection sensitivity to approximately 1 tumour cell per 10⁵ mononuclear cells.²³⁻²⁵ However, some degree of false positivity have been observed with all the monoclonal antibodies currently in use.²⁶ N-myc amplification has also been used as a marker for disease staging by some research groups but it is not useful for the detection of minimally disseminating neuroblastoma. This is because the expression of N-myc without amplification is also found in certain undifferentiated cells²⁷⁻²⁹ since N-myc is involved in cell development and is not specific for neural cells alone. In addition, in terms of specificity, N-myc amplification is found in only approximately 30% of all neuroblastoma patients.³⁰ The use of RT-PCR using specific markers for detection of small numbers of circulating neuroblastoma has also been previously attempted. One marker recently described is PGP9.5, a neurone-specific enolase-related protein.³¹ It was observed to have a sensitivity of 1 cell in 10⁷ blood mononuclear cells. However, this marker was reported not to be specific for neuroblastoma cells as it is also expressed in other neurally derived tissues. Tyroxine hydroxylase, the first enzyme in the catecholamine pathway, has also been proposed for use in neuroblastoma monitoring.³² As catecholamines are secreted by 98% of all neuroblastomas, the use of RT-

PCR to assay tyroxine hydroxylase mRNA was thought to be a useful marker. Although this technique was also found to be highly sensitive in detecting as low as 1-10 neuroblastoma cells per 10^7 blood cells, the heterogeneous expression of tyrosine hydroxylase by neuroblastoma cells does not permit quantitative cell number comparisons between patients by measurements of RT-PCR products of transcription of this marker.

In this present study, the feasibility of using NFM and SYN as possible markers to detect neuroblastoma cells was assessed. The specificity of expression of both markers in neuroblastoma cell lines was established by the positive detection of amplification products in all three tumour cell lines but not in the control peripheral blood mononuclear cell line, HL60. Using the RT-PCR assay, the level of sensitivity was 1 in 10^7 cells using the NFM marker and between 10 and 10^3 in 10^7 cells using the SYN marker. Such titration of concentration of cells while not permitting absolute quantitative comparisons of marker expression due to the nature of the RT-PCR technology, has nonetheless been used by other researchers as an indication of the sensitivity of the technique.^{31,33} Thus, the results indicate that the NFM marker was more sensitive than the SYN marker in detecting lower concentrations of circulating neuroblastoma cells. In addition, this RT-PCR technique described for use is a non-invasive, non-radioactive, sensitive and simple method that can be applied for use in detection of small numbers of neuroblastoma cells. Although the expressions of NFM and SYN markers were tested *in vitro* only on cultured neuroblastoma cell lines, the successful and sensitive detection of NFM marker from these cell lines using the RT-PCR technique indicates that this is a promising technique to test on naturally disseminating cells. Further investigation is thus required to confirm these preliminary observations on tumour cells from clinical specimens such as blood and bone marrow biopsy samples taken from patients at diagnosis, during treatment, or at relapse. This would also enable correlation of the sensitivity of such neuroblastoma markers with clinical data and staging, follow-up and relapse prediction, the results of which would help in evaluating the clinical significance of residual circulating tumour cells with respect to prognosis, early detection of relapse and in the screening of peripheral stem cell harvests prior to autologous infusion.

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