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Technical Advance

Ganglioside GM2/GD2 Synthetase mRNA Is a Marker for Detection of Infrequent Neuroblastoma Cells in Bone Marrow

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GalNAcβ1-4(NeuAcα2-3)Galβ1-4Glcβ1-Cer (GM2)/GalNAcβ1-4(NeuAcα2-8NeuAcα2-3)Galβ1-4Glcβ1-1Cer (GD2) synthetase [β-1,4-*N*-acetyl-galactosaminyl transferase (GalNAc-T)] mRNA, which encodes a key glycosyltransferase for ganglioside GD2 synthesis, was assessed as a molecular marker for detecting metastatic neuroblastoma cells in bone marrow (BM). GalNAc-T mRNA expression by neuroblastoma cell lines ($n = 15$), primary untreated neuroblastoma tumors ($n = 29$), morphologically normal BM ($n = 22$), peripheral blood stem cells ($n = 10$) from patients with cancers other than neuroblastoma, and blood mononuclear cells from normal donors ($n = 17$) was assessed by using reverse transcriptase-polymerase chain reaction (RT-PCR) and electrochemiluminescence detection assay (RT-PCR/ECL). BM harvested from 15 neuroblastoma patients was tested before and after *ex vivo* immunomagnetic bead purging, and results were compared to immunocytological analysis of the same specimens. All neuroblastoma cell lines (mean, 653×10^3 ECL units) and primary tumors (mean, 683×10^3 ECL units) were positive for significant expression of GalNAc-T mRNA compared to normal blood and BM cells. The RT-PCR/ECL assay could detect GalNAc-T mRNA in 100 pg of total RNA, and in a mixture of one neuroblastoma cell among 10^7 normal BM or blood cells. Eight of 15 autologous BM cells harvested from patients with neuroblastoma had tumor cells detectable by immunocytology, and all 15 were positive for GalNAc-T mRNA. After *ex vivo* purging, none of the BM cells was immunocytology-positive, but six remained positive by the RT-PCR/ECL assay. GalNAc-T mRNA provides a specific and sensitive molecular marker for RT-PCR/ECL detection of infrequent neuroblastoma cells in BM. ***(Am J Pathol 2001, 159:493–500)***

Neuroblastoma, the most common extracranial cancer in children, is derived from the neural crest. Approximately 45% of patients have high-risk, metastatic disease (stage 4, International Neuroblastoma Staging System) at diagnosis, and 86% of these have bone marrow (BM) involvement when assessed by immunocytology.[1] High-dose, myeloablative chemo-radiotherapy followed by BM- or blood-derived hematopoietic stem cell rescue (autologous hematopoietic stem cell transplant, AHSCT) is increasingly used to treat these patients and has been shown to improve outcome in a randomized study, especially if followed by 13-*cis*-retinoic acid therapy.[2]

Development of sensitive and specific methods to detect rare tumor cells in BM or peripheral blood is important both for risk assessment at diagnosis and for evaluating response to therapy. In addition, testing of autologous stem cell preparations used for AHSCT is essential, because contaminating tumor cells could cause recurrence of disease after infusion.[3] We routinely perform immunocytology with five anti-neuroblastoma monoclonal antibodies, including one against ganglioside GalNAcβ1-4(NeuAcα2-8NeuAcα2-3)Galβ1-4Glcβ1-1Cer (GD2), that do not react with hematopoietic cells.[1] This method can detect one neuroblastoma cell among 10^5 normal mononuclear cells with very high specificity based on combined morphological and immunostaining

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Table 1. Expression of GalNAc-T mRNA by Neuroblastoma Cell Lines*

Cell line	Tumor status*	Tumor site†	MYCN status‡	Drug sensitivity or resistance§ P, C, M, D, E	GD2, % positive¶	GD2, binding index¶	GalNAc-T mRNA‖ ($\times 10^3$ ECL U)
SMS-KAN	Dx	PRI	A	R, S, S, S, S	98	39	238
SMS-KANR	PD	BM	A	R, S, S, S, S	96	19	879
SMS-KCN	Dx	PRI	A	R, S, S, S, S	98	62	609
SMS-KCNR	PD	BM	A	R, S, S, S, S	99	55	1042
CHLA-15	Dx	PRI	N	R, S, S, S, S	99	66	771
CHLA-20	PD	PRI	N	R, R, S, R, S	98	46	567
SMS-SAN	Dx	BM	A	S, S, S, S, S	96	38	788
SK-N-BE(2)	PD	BM	A	R, S, R, R, R	95	27	615
SMS-LHN	PD	PRI	N	S, S, S, S, S	98	40	496
LA-N-6	PD	BM	N	R, R, R, R, R	2	.08	549
CHLA-8	PDBMT	PRI	A	R, S, S, S, S	98	50	862
CHLA-51	PDBMT	BM	A	S, S, S, S, S	99	39	330
CHAL-79	PDBMT	PRI	A	R, R, R, R, R	96	47	965
CHLA-90	PDBMT	BM	N	R, R, R, R, R	25	3	814
CHLA-134	PDBMT	BM	A	R, R, R, R, R	99	36	446

*Time cell line established: DX, at diagnosis, PD, at disease progression during or after chemotherapy, PDBMT, at disease progression after high-dose chemoradiotherapy supported by autologous BM transplantation.

†Site of tumor from which cell line derived: PRI, primary; BM, bone marrow.

‡MYCN gene status, A, amplified; N, nonamplified.

§Sensitivity (S) or resistance (R) to cisplatin (P), carboplatin (C), melphalan (M), doxorubicin (D), and etoposide (E). Sensitive and resistant cell lines had lethal concentration 90 (LC90) values for a given chemotherapeutic agent that was less (S) or greater than (R) the peak plasma level for bolus injection (melphalan, >10,000 ng/ml) or steady-state concentration for continuous infusion (cisplatin, >100; carboplatin, >3000; doxorubicin, >60; etoposide, >1000 ng/ml) in patients.[13]

¶GD2 expression was analyzed by flow cytometry using murine mAb 14G2a and goat anti-mouse (H + L) F(ab′)2 − FITC. GD2-positive cells were defined as those cells with fluorescence greater than background (control IgG2 followed by goat anti-mouse (H + L) F(ab′)2 − FITC). The binding index was calculated from the mean fluorescence and percent positive cells as follows: binding index = (mean fluorescence × % positive cells)/100.

‖GalNAc-T mRNA level is expressed as ECL U. Neuroblastoma cell lines were assessed by RT-PCR/ECL. The positive cut-off point for this study was 42×10^3 ECL U.

criteria. Quantifying tumor cells at diagnosis, at 12 weeks after diagnosis, and at the conclusion of initial chemotherapy, before myeloablative therapy/AHSCT, predicts outcome in patients with stage 4 disease.[4] Reverse transcriptase-polymerase chain reaction (RT-PCR) analysis may further improve detection of rare neuroblastoma cells and thus provide clinically important information. There presently are no known neuroblastoma-specific gene rearrangements that can be used for PCR or RT-PCR analysis, and so detection of neural-associated mRNAs by RT-PCR is being evaluated. RT-PCR assays for neural-associated gene products including protein gene product 9.5 (PGP9.5),[5,6] tyrosine hydroxylase,[6–10] and GAGE[11,12] have been reported for detecting neuroblastoma cells in BM or blood, but their clinical utility has not been fully evaluated. A highly sensitive and specific marker for RT-PCR analysis will be very valuable if the gene is highly expressed and there is limited heterogeneity among tumor cells.

Neural crest-derived tumor cells such as melanoma and neuroblastoma express high levels of gangliosides on their surface.[13–17] GD2 ganglioside found frequently on neuroectodermal-derived tumor cells was originally reported as an oncofetal antigen (OFA-I-2).[17] Nearly all neuroblastomas express a high level of GD2,[14,16] which is minimally or not expressed by normal cells of nonneural origin. β-1,4-*N*-acetyl-galactosaminyl transferase (GalNAc-T; EC2.4.1.92), which also is referred to as GalNAcβ1-4(NeuAcα2-3)Galβ1-4Glcβ1-Cer (GM2)/GD2 synthetase, has a key role in biosynthesis of GM2 and GD2, and its activity is correlated with GM2 and/or GD2 expression.[14,18–20] We hypothesized that GalNAc-T mRNA would be a sensitive and specific molecular marker for detecting small numbers of neural crest-derived tumor cells. This was confirmed for melanoma, where RT-PCR analysis for GalNAc-T mRNA was shown to be highly sensitive in detecting infrequent tumor cells in blood.[20] Here, we show that a combined RT-PCR and electrochemiluminescence detection assay [RT-PCR/electrochemiluminescence (ECL)] for GalNAc-T mRNA detects rare neuroblastoma cells in BM. Comparison of GalNAc-T RT-PCR/ECL and immunocytology assays indicates that GalNAc-T RT-PCR/ECL analysis is more sensitive in detecting neuroblastoma cells than immunocytology.

Materials and Methods

Cell Lines and Tumor Specimens

Fifteen neuroblastoma cell lines that were established from patients at diagnosis or at disease progression were studied (Table 1).[21] They included five cell lines derived from tumors that recurred after AHSCT (CHLA-8, CHLA-51, CHLA-79, CHLA-90, and CHLA-134) and three pairs of cell lines obtained from the same patients at diagnosis and after disease progression (SMS-KAN and SMS-KANR, SMS-KCN and SMS-KCNR, and CHLA-15 and CHLA-20). Most cell lines were maintained in RPMI-1640 medium containing 2 mmol/L L-glutamine (Life Technologies, Grand Island, NY) and 10% heat-inactivated fetal calf serum (Omega Scientific, Tarzana, CA). Cell lines CHLA-15, CHLA-20, CHLA-79, CHLA-90, and CHLA-134 required Iscove's modified Dulbecco's medium (Life

Table 2. Expression of GalNAc-T mRNA by Untreated Primary Neuroblastoma Tumors

Clinical stage	Patient	Age at Dx (mos)	MYCN	Pathology	GalNAc-T mRNA* ($\times 10^3$ ECL U)	Mean ECL U for each clinical stage ($\times 10^3$ ECL U)
1	1	1.0	N	F	676	
	4	99.6	N	F	478	504
	2	0.1	N	F	358	
2	5	8.3	N	F	780	
	8	27.8	N	U	750	
	7	3.4	N	NT†	317	
	6	1.1	N	F	857	626
	9	12.4	N	F	489	
	10	55.0	N	F	564	
3	11	47.0	A	U	644	
	12	53.3	A	U	313	
	15	14.0	N	F	542	
	13	3.2	N	NT†	901	693
	16	99.9	N	N	979	
	14	1.0	N	F	778	
4	18	37.6	A	U	1115	
	19	53.9	A	U	685	
	20	27.7	A	U	589	
	17	5.2	A	U	938	
	23	41.7	N	U	752	
	24	16.0	N	F	962	
	25	162.3	N	U	827	772
	26	76.3	N	U	634	
	21	7.5	N	F	501	
	27	18.3	N	U	741	
	22	6.0	N	F	731	
	28	52.7	N	U	792	
4s	29	0.1	N	U	997	
	30	0.8	N	F	430	

Primary tumors assessed were from patients who had not received previous treatment before surgery. Clinical stage (International Neuroblastoma Staging System), age at diagnosis (months), MYCN gene status (A, amplified; NA, nonamplified), and histopathological classification (F, favorable; U, unfavorable histology) are indicated. In comparing controls to individual stage, there was significant differences, $P \leq 0.005$ (Student's *t*-test, two-tailed).

*Neuroblastoma tumors were assessed by RT-PCR/ECL for GalNAc-T mRNA expression. The positive cut-off point for this study was 42×10^3 ECL U.

†NT indicates "not tested."

Technologies, Grand Island, NY). Cells were cultured in a 5% CO_2, humidified incubator at 37°C.

Twenty-nine primary untreated neuroblastomas were obtained at diagnosis from surgical specimens and were snap-frozen and shipped on dry ice to the Children's Cancer Group Neuroblastoma Biology Resource Laboratory at Children's Hospital Los Angeles where they were maintained at −80°C. These included stage 1, 2, 3, 4, and 4-S tumors (Table 2).[22] The diagnosis of neuroblastoma was verified pathologically, and tumors were classified into histopathological risk groups according to the Shimada classification (Table 2).[23] In addition, *MYCN* gene status (amplified or nonamplified) was determined.[24] Consent was obtained for use of tumor tissue for research.

Control Blood and BM Cells

Blood mononuclear cells from 17 normal adult volunteers were used as controls. Blood (10 ml) was collected in sodium citrate-containing vacutainer tubes as previously described.[20] Mononuclear cells were separated by using a hypotonic density gradient solution.[20] BM ($n = 7$) and peripheral blood stem cells (PBSC) ($n = 10$) were harvested from children with solid tumors other than neuroblastoma, and they did not have evident contamination by tumor cells. Additional BM ($n = 15$) from adult American Joint Committee on Cancer (AJCC) stage I breast cancer patients ($n = 12$) at the John Wayne Cancer Institute, and healthy adult donors ($n = 3$) (BioWittaker, Walkersville, MD) were also assessed. Mononuclear cells derived from small aliquots of these samples were cryopreserved in dimethylsulfoxide and stored in liquid nitrogen before use. Consent was obtained from donors and/or their parents for use of their cells in this research.

Bone Marrow from Patients with Neuroblastoma

Aliquots of BM harvested from 15 high-risk neuroblastoma patients were evaluated by immunocytology and by GalNAc-T RT-PCR/ECL before and after *ex vivo* purging using magnetic immunobeads.[25] All samples were cryopreserved in liquid nitrogen vapor in Liebovitz's L15 medium (Irvine Scientific, Santa Ana, CA) containing 1.5% Hetastarch, 2.5% human serum albumin, and 10% dimethyl sulfoxide. Informed consent was obtained for use of a small aliquot of these BM cells for research purposes.

RT-PCR and Electrochemiluminescence (ECL) Assay

Total cellular RNA was extracted from tumor cell lines, tumors, blood, and BM mononuclear cells using the TRIzol reagent (Life Technologies) according to the manufacturer's instructions and was treated with RNase-free DNase (Life Technologies). The quality of isolated RNA was confirmed by both the appearance of ribosomal RNA bands and RT-PCR analysis for the housekeeping gene porphobilinogen deaminase.[26] The RT-PCR assay was performed as previously described.[20] Briefly, RT was performed with oligo-dT primers on the amount of total RNA specified for Moloney murine leukemia virus RT (Promega, Madison, WI).[20] RNA was incubated at 70°C for 5 minutes and then put on ice before addition of RT reaction reagents. RT reagents were added, and the mixture was incubated at 37°C for 2 hours and then at 95°C for 5 minutes. The PCR conditions were as follows: 1 cycle of denaturing at 95°C for 5 minutes followed by 35 cycles of 95°C for 1 minute, 65°C for 1 minute, and 72°C for 1 minute before a final primer sequence extension incubation at 72°C for 10 minutes. RT-PCR conditions were set up in a TouchDown thermocycler (Hybaid, Middlesex, UK).

Primer and Probe Synthesis

Primers and probe sequences were designed for detection of specific mRNA by using Oligo Primer Analysis Software, version 5.0 by National Biomedical Systems (Plymouth, MN). To avoid amplification of genomic DNA, primers were designed to target cDNA amplification by selecting gene-specific primer sequences on different exons. Oligonucleotide primers were synthesized and purified at Genosys (Woodland, TX). The GalNAc-T primers used were: sense 5′-CCA ACT CAA CAG GCA ACT AC-3′ and antisense: 5′-GAT CAT AAC GGA GGA AGG TC-3′ resulting in a RT-PCR cDNA product of 230 bp.[20] The sense primer was labeled with biotin for avidin magnetic bead capturing in the ECL assay.[27]

Hybridization probe sequence spanning the exon-exon junction was selected to ensure detection of only RT-PCR cDNA-specific products. Probe was labeled with Tris (2,2-bipyridine) ruthenium (II) and synthesized by The Midland Certified Reagent Co. (Midland, TX). The following antisense probe sequence was synthesized: 5′-ruthenium-GTTGTACTGGGCTCCCTGGGGT-3′.

ECL Analysis of cDNA Products

Amplified PCR products (5 μl) were mixed in a final volume of 50 μl of 1× PCR buffer (Promega, Madison, WI) and 10 pmol of ruthenium-labeled GalNAc-T probe. Products were then denatured and hybridized at 65°C. Twenty-five μl of each resulting hybrid solution was added to 50 μl (0.125 mg/ml) of M280 streptavidin-coated Dynabeads (Dynal, Oslo, Norway) and vortexed for 30 minutes. After the addition of 300 μl of Origen Assay Buffer (Igen International Inc., Gaithersburg, MD), the Origen Analyzer (Igen International Inc.) was set to run the samples and record ECL signals.[27] Results were expressed as ECL units (ECL U), and positive specimens were determined if the level of ECL U was greater than the cut-off point. The positive cut-off point for determining GalNAc-T mRNA-positive samples was three standard deviations more than the mean ECL U of multiple negative control samples assessed in each assay. Three standard deviations more than the mean of negative controls was considered a significant positive result. For each assay, at least two positive controls (melanoma cell lines), at least four negative controls (normal blood mononuclear cells and/or normal BM cells), and reagent controls (reagent alone without RNA or cDNA) for the RT-PCR/ECL assay were included. The assessment of patients' BM, tumor specimens, and tumor cell lines were performed in the same manner as described above. Each assay contained its own set of positive and negative controls. Assays were normalized for data presentation.

Assay Sensitivity of in Vitro *Tumor Cell Dilutions*

Sensitivity of the RT-PCR assay was assessed by seeding SK-N-BE(2) neuroblastoma cells (stage 4, *MYCN* amplified) into PBSCs or BM cells at concentrations of one tumor cell in 10^4 to 10^6 nucleated normal cells (see Figure 2). Dilutions were prepared as follows: 1000, 100, and 10 tumor cells were seeded in 10 million normal cells to get 10^{-4}, 10^{-5}, and 10^{-6} dilution, respectively. For 10^{-7} dilution 10 tumor cells were seeded in 100 million normal cells. The cells were pelleted, total RNA was prepared, and then the GalNAc-T RT-PCR/ECL assay was performed.

Results

GalNAc-T mRNA Expression by Neuroblastoma Cell Lines and Tumors

Optimal and stringent conditions were established for the primers and RT-PCR conditions for GalNAc-T mRNA amplification. PCR conditions were standardized and uniform throughout the studies, and equal amounts of cDNA were used in each experiment. Under these conditions, all 15 neuroblastoma cell lines were positive for GalNAc-T mRNA at levels ranging from 238 to 1042 $\times 10^3$ ECL U (mean of negative controls $22 \times 10^3 \pm$ SEM 2.5×10^3 ECL U) (Table 1). Expression was not related to the tumor site from which the cell line was derived, *MYCN* gene status, or drug sensitivity. Interestingly, LA-N-6 and CHLA-90 cell lines that had little or no cell surface GD2 detected by flow cytometry had substantial levels of GalNAc-T mRNA (Table 1). All 29 primary neuroblastoma tumors were positive for GalNAc-T mRNA at levels ranging from 313 to 997 $\times 10^3$ ECL U (mean of negative control $22 \times 10^3 \pm$ SEM 3×10^3 ECL U) (Table 2). There was no clear relation of GalNAc-T mRNA expression to stage or age at diagnosis, to *MYCN* gene status, or to histopathology (Table 2). The mean ECL U of mononuclear cell samples from 17 normal donors was 22×10^3

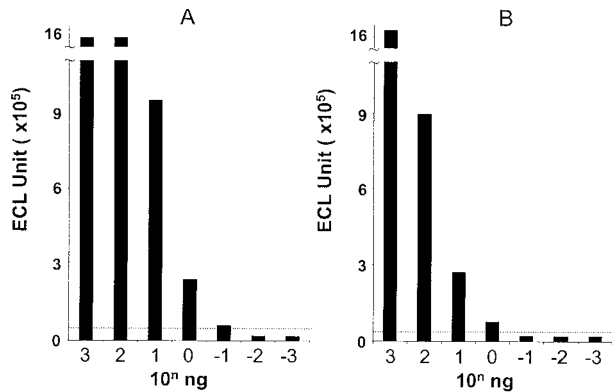

Figure 1. Representative studies of neuroblastoma cell RNA-dilution analysis. Total RNA from neuroblastoma cell lines CHLA-51 (stage 4, *MYCN* nonamplified) (**A**) and CHLA-8 (stage 4, *MYCN* amplified) (**B**) was serially diluted 10-fold from 10^3 ng to 10^{-3} ng, and assessed for GalNAc-T mRNA by the RT-PCR/ECL assay. The positive cut-off point (**dotted line**) for this experiment was 42×10^3 ECL U. The negative controls for the mRNA dilution assay included four normal donor PBL with mean ECL U of $27 \times 10^3 \pm$ SEM 2.5×10^3 ECL U.

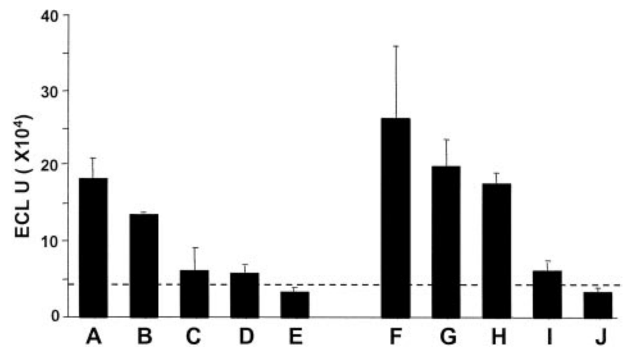

Figure 2. Detection of neuroblastoma cells diluted into PBSC or BM cells. SK-N-BE(2) neuroblastoma cells were seeded into normal (tumor-free) PBSC or cells from normal (tumor-free) BM at one tumor cell diluted in 10^4 to 10^7 PBSC or BM cells. Representative studies are shown; the mean data from two separate RT-PCR studies are shown with SEM. The positive cut-off point 3 SD more than the mean of normal controls (**dotted line**) for this study was 42×10^3 ECL U. **A–D:** One SK-N-BE(2) cell in 10^4, 10^5, 10^6, and 10^7 normal BM cells, respectively. **F–I:** One SK-N-BE(2) cells in 10^4, 10^5, 10^6, and 10^7 normal PBSC, respectively. **E** and **J:** Normal BM (10^7) cells and normal PBSC alone, respectively. SK-N-BE(2) cells alone (positive control) was $>75 \times 10^4$ ECL units.

± SEM 1.6×10^3 ECL U. In morphologically normal BM cells and PBSCs from children with solid tumors other than neuroblastoma ($n = 17$), the mean ECL U was $18 \times 10^3 \pm$ SEM 0.7×10^3 ECL U. For BM from breast cancer AJCC stage I patients ($n = 12$), the mean ECL U was $12 \times 10^3 \pm$ SEM 1.8×10^3 ECL U.

GalNAc-T mRNA Detection Sensitivity

To assess the sensitivity of GalNAc-T mRNA detection, RNA from two representative neuroblastoma cells lines (CHLA-51, stage 4, *MYCN* nonamplified; CHLA-8, stage 4, *MYCN* amplified) was serially diluted in molecular grade water and analyzed (Figure 1). GalNAc-T mRNA could be detected consistently in as low as 100 pg of total RNA (Figure 1).

We also assessed the sensitivity of the assay by assessing *in vitro* dilutions of tumor cells in normal PBSCs and BM cells followed by RNA extraction and RT-PCR. The amount of GalNAc-T mRNA detected increased linearly with the number of tumor cells seeded but did not correlate absolutely with the number of tumor cells present (Figure 2). The assay could detect as low as one neuroblastoma cell in 10^7 normal cells as demonstrated by higher ECL U compared to PBSCs and BM cells without added SK-N-BE(2) neuroblastoma cells. The tumor-free controls had ECL U similar to the negative controls (mean of $22 \times 10^3 \pm$ SEM 2.5×10^3 ECL U) and below the assay positive cut-off point of 42×10^3 ECL U. (Figure 2).

Assessment of Bone Marrow before and after ex Vivo *Immunomagnetic Bead Purging*

The specificity and sensitivity of the GalNAc-T RT-PCR/ECL assay was further evaluated by analyzing BMs harvested from 15 children with neuroblastoma for AHSCT (Table 3). These BM cells were purged *ex vivo* with immunomagnetic beads using a monoclonal antibody mixture that included one directed against GD2. Aliquots of BM from before and after purging were analyzed by immunocytology and RT-PCR/ECL. Samples were blinded so that immunocytology and purging information was not known by individuals performing the RT-PCR/ECL assay. Before purging, 8 of the 15 BMs had detectable neuroblastoma cells by immunocytology at a frequency of 1 to 70 per 10^5 nucleated BM cells; whereas, after purging, none were positive. By RT-PCR/ECL, all samples were positive before purging with GalNAc-T mRNA ranging from 162 to 411×10^3 ECL U. After purging, GalNAc-T mRNA detection ranged from 16 to 275×10^3 ECL U, with 6 of 15 BM cell remaining positive. The analyses were run three times, and results were consistent with minimal deviation. Within the limits of this small study, there was no significant correlation between the number of tumor cells detected by immunocytology and the level of GalNAc-T mRNA in BM before purging. This study indicates that the GalNAc-T RT-PCR/ECL assay is more sensitive than immunocytology in identifying neuroblastoma cells in BM. In all cases, BM after purging showed significant reduction of RT-PCR/ECL values, furthermore, in nine cases the RT-PCR/ECL values were reduced to below background level for the BM after purging. The clinical follow-up of these AJCC stage IV patients showed that all six of the patients with GalNAc-T mRNA-positive BM after purging had developed disease progression within 3 years. Three patients whose BM were GalNAc-T-negative after purging did not have disease progression.

Discussion

This study indicates that detecting infrequent neuroblastoma cells in BM or blood by their expression of GalNAc-T mRNA, a neural tissue-associated enzyme that is essential for ganglioside GD2 synthesis, may have considerable clinical application. High expression of Gal-

Table 3. GalNAc-T mRNA Expression in BM before and after *ex Vivo* Immunomagnetic Bead Purging

Patient	Before/after purging	Immunocytology (No. of tumor cells)	GalNAc-T mRNA* ($\times 10^3$ ECL U)	
1	Before	0	266	+
	After	0	27	−
2	Before	38	268	+
	After	0	16	−
3	Before	0	405	+
	After	0	112	+
4	Before	0	162	+
	After	0	35	−
5	Before	0	228	+
	After	0	17	−
6	Before	0	297	+
	After	0	164	+
7	Before	1	411	+
	After	0	275	+
8	Before	0	222	+
	After	0	86	+
9	Before	1	332	+
	After	0	16	−
10	Before	7	248	+
	After	0	18	−
11	Before	70	228	+
	After	0	46	+
12	Before	1	198	+
	After	0	19	−
13	Before	9	237	+
	After	0	16	−
14	Before	0	285	+
	After	0	18	−
15	Before	6	295	+
	After	0	106	+

*BM was assessed by RT-PCR/ECL (1 μg RNA/reaction) for GalNAc-T mRNA expression. The positive cut-off point for this study was 42×10^3 ECL U.

A "+" indicates above the cut-off point, and "−" indicates below.

NAc-T mRNA was detected by a RT-PCR/ECL assay in all 15 neuroblastoma cell lines and all 29 primary untreated tumor specimens. Neuroblastoma cell lines are derived from the most aggressive neuroblastomas,[28] and the panel of 15 tested includes a spectrum with respect to primary and BM metastasis derivation, therapy before derivation, *MYCN* gene status, drug sensitivity, and cell surface GD2 expression. These cell lines represent the type of neuroblastomas for which detection of rare tumor cells in BM or blood is important. The 29 primary untreated neuroblastomas tested are representative of the disease with respect to stage and age at diagnosis, *MYCN* gene status, and histopathology. Of importance, high-risk, metastatic neuroblastomas (stage 4, *MYCN* amplified and nonamplified) expressed GalNAc-T mRNA at levels equal to regional (stage 3) and localized tumors (stages 1 and 2).

The sensitivity and specificity of detecting neuroblastoma cells with the GalNAc-T RT-PCR/ECL assay are excellent. In the *in vitro* model experiments, the assay can detect GalNAc-T mRNA in as low as 100 pg of total RNA, and can detect one tumor cell per 10^7 normal nucleated cells. All eight BMs from patients that contained neuroblastoma cells detectable by immunocytology (1 to 70 tumor cells per 10^5 nucleated cells) also had significant levels of GalNAc-T mRNA. In addition, a GalNAc-T mRNA signal was detected in seven BMs that did not have detectable tumor cells by immunocytology. *Ex vivo* immunomagnetic bead purging of these 15 BMs, which is directed at removing neuroblastoma cells while preserving essential BM stem cells, decreased GalNAc-T mRNA from easily detectable to control levels in 9 of 15 BMs, providing further evidence of specificity. Thus, the data indicate that RT-PCR assessment of GalNAc-T mRNA is a sensitive and specific semiquantitative method for detecting small numbers of neuroblastoma cells in BM or blood.

Expression of other neural tissue-associated genes has been evaluated for detecting neuroblastoma cells in marrow or blood using RT-PCR by other groups. These include PGP9.5,[5,6] tyrosine hydroxylase,[6–9] MAGE,[11] and GAGE[11,12] genes. We have evaluated expression of these genes in neuroblastoma cell lines and primary tumors with quantitative and qualitative RT-PCR and with Northern analysis and found that PGP9.5 and tyrosine hydroxylase are expressed by virtually all neuroblastomas, whereas MAGE and GAGE genes are less frequently expressed.[29,30] Expression of PGP9.5 is consistent and at a higher level than tyrosine hydroxylase.[29,30]

Detection of rare neuroblastoma cells in BM or blood is likely to be important for assessing risk at diagnosis and for evaluating response to therapy. The latter, if highly correlated with outcome, could provide surrogate information for therapeutic decisions. Immunocytological evaluation of BM from patients with stage 3 and 4 disease provides prognostic information at diagnosis as well as during therapy in that patients with the highest number of tumor cells have the worst outcome.[1,4] Although immunocytology can identify one tumor cell among 10^5 normal mononuclear cells, many patients with stage 4 disease who do not have tumor cells detectable by this test during therapy still have a poor outcome. RT-PCR detection of GalNAc-T mRNA is more sensitive than standard immunocytology, but the clinical value of detecting neuroblastoma cells at a level of a few cells in BM or blood remains to be determined. Thus, performance of both RT-PCR/ECL and immunocytology analyses will determine whether some of those patients whose BM are negative by immunocytology are positive by RT-PCR. Clinical follow-up will then determine whether outcome is different for patients with RT-PCR detectable *versus* nondetectable tumor cells.

RT-PCR analysis using GalNAc-T mRNA should further improve assessment of autologous stem cells used for hematopoietic reconstitution after myeloablative therapy. The clinical significance of one neuroblastoma cell among 10^5 to 10^7 BMs or PBSCs used for AHSCT is not yet known; however, one per 10^5 could result in infusing 2 to 4×10^4 tumor cells into a 10-kg or 20-kg patient. Although the clonogenicity of neuroblastoma cells in BM or blood is likely to be different for each tumor, the risk of relapse from infused malignant cells could be significant. Neuroblastomas that recurred after autologous BM transplantation with nonpurged BM cells that had been genetically marked with the neo gene demonstrated that infused neuroblastoma cells can establish tumors *in vivo*.[3] Thus, removal of detectable neuroblastoma cells by *ex*

vivo purging may be a critical factor for achieving relapse-free survival after AHSCT. In the current study, all BM cells before purging were GalNAc-T mRNA-positive, including those that were immunocytology-positive. After purging, 9 of the 15 GalNAc-T mRNA-positive BM cells were negative for the signal. A persistent signal after purging could be due to incomplete purging, because the signal for nine samples was decreased to control levels after purging, including five of eight with immunocytologically detected tumor cells in the before purging BM cells. In addition, the level of GalNAc-T mRNA remaining in the six BM cells after purging was decreased by 33 to 80% compared to before purging, suggesting a decrease in tumor cells. Follow up on these and additional patients will determine the prognostic significance of detecting GalNAc-T mRNA in stem cell preparations used for hematopoietic reconstitution.

Anti-GD2 monoclonal antibodies alone or with cytokines have been used to treat neuroblastoma and melanoma patients.[31–33] Recent phase I and II studies of neuroblastoma have suggested an anti-tumor effect, and a phase III randomized study testing the mouse/human chimeric anti-GD2 antibody ch14.18 with GM-CSF and interleukin-2 with or without 13-*cis*-retinoic acid after myeloablative therapy and AHSCT will be performed by the Children's Oncology Group. In addition, a phase I study of a humanized anti-GD2 antibody/interleukin-2 fusion protein is underway. Identification of infrequent neuroblastoma cells in BM or blood using the GalNAc-T RT-PCR assay in these studies could contribute to evaluation of these GD2-directed treatments.

The study demonstrates that a neural tumor-associated enzyme necessary for ganglioside biosynthesis can be used as a diagnostic and follow-up RT-PCR marker. We and others have demonstrated that biochemical detection of gangliosides does not always correlate with monoclonal antibody detection level, and that the monoclonal antibody binding to cell surface gangliosides may underestimate the individual ganglioside expression.[34–36] The sensitivity of antibody detection of cell surface antigen depends highly on epitope exposure and accessibility to antibody. In the current study, we demonstrate that neuroblastoma cell lines (LA-N-6, CHLA-90) with no or little detectable cell surface ganglioside GD2 by flow cytometry, do express GalNAc-T mRNA. Thus, GalNAc-T mRNA provides a sensitive means of identifying neuroblastoma cells independently from expression of cell surface GD2 as detected by monoclonal antibody.

In summary, GalNAc-T mRNA meets criteria for being a useful marker for detecting rare neuroblastoma cells. It is uniformly and highly expressed by primary and metastatic tumor cells, by tumor cells with or without *MYCN* amplification, and by tumor cells resistant or sensitive to chemotherapeutic agents. By contrast, it is expressed at 10- to 100-fold lower levels by normal blood, including hematopoietic stem cells, and BM cells. This marker, in combination with others such as PGP9.5 and tyrosine hydroxylase, should provide a useful panel for evaluating blood and BM cells for the presence of infrequent neuroblastoma cells in patients with high-risk disease. The clinical significance of detecting such cells will need to be assessed in uniformly treated patients.

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