Quantitation of Marrow Disease in Neuroblastoma by Real-Time Reverse Transcription-PCR[1]

Irene Y. Cheung[2] and Nai-Kong V. Cheung

Department of Pediatrics, Memorial Sloan-Kettering Cancer Center, New York, New York 10021

ABSTRACT

Purpose: **GD2 is abundantly expressed in neuroblastoma (NB). GD2 synthesis is dependent on key enzyme β 1,4-*N*-acetylgalactosaminyltransferase (GD2 synthase). We explore the potential of GD2 synthase mRNA as a molecular marker of minimal residual disease by first comparing it quantitatively with immunocytology and then testing its clinical utility.**

Experimental Design: **A real-time reverse transcription-PCR assay to quantify mRNA of GD2 synthase was developed. Quantitation was normalized to endogenous control glyceraldehyde-3-phosphate dehydrogenase in a multiplex PCR.**

Results: **The upper limit of normal was defined by 31 normal marrow and blood samples, achieving a sensitivity of one NB cell in 10^6 normal mononuclear cells. When 155 bone marrows from 100 NB patients were studied, GD2 synthase mRNA levels correlated well with the number of GD2-positive cells, as measured by immunocytology using anti-GD2 antibodies ($r = 0.96$). This is the first demonstration of the quantitative relationship between a specific mRNA and the actual number of tumor cells. In a pilot study, the level of this transcript in sequential marrow samples of five stage 4 NB patients correlated closely with their clinical status. At 24 months after diagnosis, available remission bone marrows from patients with advanced NB diagnosed at >1 year of age initially treated with protocols N6 and N7 at Memorial Sloan-Kettering Cancer Center ($n = 44$) were analyzed for GD2 synthase mRNA. Positivity was strongly associated with progression-free ($P < 0.005$) and overall survival ($P < 0.001$).**

Conclusions: **Measurement of tumor cells by real-time quantitative reverse transcription-PCR of GD2 synthase has potential clinical utility, especially for the detection of minimal residual disease.**

INTRODUCTION

As the thoroughfare for cellular trafficking, blood and BM[3] harbor tumor micrometastases that seed organs distant from the primary site. The presence of these occult tumor cells has grave prognostic significance in various malignancies, including breast cancer (1), colon cancer (2), stomach cancer (3), lung cancer (4), melanoma (5), and leukemia (6). For the childhood cancer NB, BM disease is associated with an unfavorable outcome (7, 8). Histological examinations by H&E stain of marrow biopsies and Wright-Giemsa stain of marrow aspirates have been the standard techniques to monitor BM disease. However, these techniques can grossly underestimate its prevalence in NB, even if multiple sites are tested (9). Because of its superior sensitivity, immunocytology using monoclonal antibodies can complement and augment morphological methods of tumor detection (9, 10). The use of molecular markers of NB has also been explored to detect BM disease, in particular, the lineage-specific gene transcript tyrosine hydroxylase, which is the first and rate-limiting enzyme in the catecholamine biosynthesis pathway. This transcript is useful in detecting tumor cells in the BM and blood (11). However, its expression may be down-regulated in some cells (12). More recently, cancer-testis antigen *GAGE* has been found to be comparable in sensitivity to immunocytology in detecting NB cells in the BM (13). Molecular detection by RT-PCR has thus far been qualitative. We reason that quantitative PCR measurements of tumor cells over the course of treatment in the marrow and blood can provide substantially more information, allowing comparisons to be made between treatment phases and approaches, as well as among patients.

The emergence of real-time quantitative PCR technology has facilitated the evaluation of microscopic tumors. PCR detection of clonal genomic immunoglobulin H gene rearrangement in B-cell malignancies, including multiple myelomas (14), acute lymphoblastic leukemia (15), and chronic myelogenous leukemia (16), has redefined the meaning of remission and will likely change the way we manage these diseases. In addition, real-time quantitative PCR has been used successfully in measuring EBV viral DNA, a powerful predictor of tumor recurrence in nasopharyngeal carcinoma (17). This technique compares favorably with nested competitive RT-PCR in sensitivity, linearity, and reproducibility (18). In NB, the choice of GD2 synthase for the real-time quantitative RT-PCR assay is particularly attractive because GD2 is expressed homogeneously in NB of all stages. GD2 density in NB cells is high ($5–10 \times 10^6$ molecules/cell; Ref. 19), and this glycosphingolipid antigen is

Received 1/12/01; revised 3/15/01; accepted 3/22/01.

The costs of publication of this article were defrayed in part by the payment of page charges. This article must therefore be hereby marked *advertisement* in accordance with 18 U.S.C. Section 1734 solely to indicate this fact.

[1] Supported in part by grants from the NIH (Grant CA61017), the Robert Steel Foundation, the Justin Zahn Fund, JP's Wish Fund, Katie-Find-a-Cure Fund, and the Pediatric Cancer Foundation.

[2] To whom requests for reprints should be addressed, at Department of Pediatrics, Memorial Sloan-Kettering Cancer Center, 1275 York Avenue, New York, NY 10021. Phone: (212) 639-7747; Fax: (212) 744-2245; E-mail: cheungi@mskcc.org.

[3] The abbreviations used are: BM, bone marrow; NB, neuroblastoma; MRD, minimal residual disease; RT-PCR, reverse transcription-PCR; GAPDH, glyceraldehyde-3-phosphate dehydrogenase; UNG, uracil-*N*-glycosylase; SE, standard error.

rarely lost after anti-GD2 therapy (20). GD2 synthesis is dependent on a key enzyme, β1,4-*N*-acetylgalactosaminyltransferase (GD2/GM2 synthase), that catalyzes the transfer of β1,4-*N*-acetylgalactosamine to precursor gangliosides GD3/GM3, respectively (21). In this report, the enzyme will be termed GD2 synthase for clarity, and not GD2/GM2 synthase. Its gene transcript level was found to correlate with the enzyme activity, as well as GD2 expression in individual cell lines (22). By competitive RT-PCR, GD2 synthase mRNA expression was enhanced in some gastric and colon carcinomas when compared with normal mucosa (23).

We hypothesize that GD2 synthase mRNA may potentially be a useful molecular marker for the detection of NB in the BM or blood. To date, there has been only one report on the detection of the GD2 synthase transcript by RT-PCR and Southern blot analysis of melanoma cell lines and in the blood of some patients with advanced stages of malignant melanoma (24). The objective of this study is to measure GD2 synthase mRNA by real-time quantitative RT-PCR and to use it as a marker of tumor cells in the BM of NB patients. We want to determine the quantitative relationship between transcription level and the percentage of GD2-positive tumor cells as enumerated by immunocytology. The clinical significance of marrow GD2 synthase will be tested among patients in clinical remission. We will also correlate the serial levels of transcript in individual patients with their clinical status.

PATIENTS AND METHODS

Patients

NB patients evaluated at Memorial Sloan-Kettering Cancer Center were diagnosed and staged in accordance with the International NB Staging System (25). Serial BM samplings were obtained as part of disease evaluation while the patient was being treated, with the approval of the institutional review board of Memorial Hospital. Each marrow examination generally consisted of six samplings [two biopsies (right and left posterior iliac crest) and four aspirates (right and left anterior iliac crest, right and left posterior iliac crest obtained from six different sites of the iliac crests)]. Details of the procedure were described previously (9).

Immunocytology

Freshly collected heparinized BM pooled from four aspiration sites was separated by Ficoll centrifugation. Mononucleated cells were incubated with a panel of anti-GD2 monoclonal antibodies, followed by a reaction with a fluoresceinated anti-mouse IgG+IgM antibody. GD2-positive tumor cells were examined and enumerated by a trained technician using a fluorescence microscope. Quantitation was expressed as the number of GD2-positive cells/total number of mononuclear cells in each counting chamber. Negative detection was defined as $\leq$0.001% (9).

RNA Extraction and cDNA Synthesis

Cryopreserved BM mononuclear cells were used. Total RNAs were extracted, and reverse transcription was performed as described previously (26).

Real-Time Quantitative PCR

Background. Relative quantitation of GD2 synthase mRNA was achieved by means of the ABI Prism 7700 Sequence Detection System (Applied Biosystems, Foster City, CA). In TaqMan real-time quantitation technology (27–29), the 5′ exonuclease activity of the Taq polymerase cleaves and releases the hybridization probe that was labeled with a fluorescent reporter dye. This fluorogenic probe is specific for the target sequence, thereby generating a fluorescence signal that is specific and directly proportional to the amount of PCR product synthesized. PCR reactions are characterized by the time point during cycling when amplification of the PCR product is first detected, rather than by the amount of product accumulated after a fixed number of cycles. Because the amount of product at the exponential phase of the PCR is proportional to the initial copy number of the target, the more abundant the starting quantity of a target, the earlier the PCR amplification will be detected by means of the fluorescence signal. In this technology, the target quantity is measured by identifying the threshold cycle number (C_T), *i.e.*, when the fluorescence signal crosses a preset detection threshold. The laser detector of the Prism 7700 monitors the cycle-to-cycle change in fluorescence signal on-line. The fewer cycles it takes to reach a detectable level of fluorescence, the greater the initial copy number.

Measurement of GD2 synthase transcript was based on two reporter dyes with the largest difference in emission wavelength maxima, namely, 6-FAM for GD2 synthase and VIC for GAPDH, our endogenous reference to control for difference in RNA extraction and cDNA synthesis. After optimization by limiting the primer concentrations of the more abundant target GAPDH, multiplex PCR became the standard assay, resulting in higher throughput and reducing the effect of pipetting errors.

The primers and probe for GD2 synthase were designed using the applications-based primer design software Primer Express (Applied Biosystems, ABI). The probe spanned an intron, thereby avoiding the amplification of contaminating genomic DNA present in the sample. For GD2 synthase, the sense primer was 5′-GACAAGCCAGAGCGCGTTA-3′, and the antisense primer was 5′-TACTTGAGACACGGCCAGGTT-3′. The probe was FAM-5′-AACCAGCCCTTGCCGAAGGGC-3′ (99 bp). For GAPDH, the sense primer was 5′-GAAGGTGAAGGTCGGAGTC-3′, and the antisense primer was 5′-GAAGATGGTGATGGGATTTC-3′. The probe was VIC-5′-CAAGCTTCCCGTTCTCAGCC-3′ (226 bp). GD2 synthase and GAPDH designs were based the sequence from GenBank, accession numbers NM_001478 and J04038, respectively. Primers and probes were synthesized by ABI.

Procedure. In each 25-μl MicroAmp optical tube (ABI), 2 μl of cDNA template were added to a PCR reaction mix. This mixture included the Taqman master mix (ABI) containing 5 mM $MgCl_2$; 200 μM each of dATP, dCTP, dGTP, and dUTP; 0.05 unit/μl AmpliTaq Gold DNA polymerase; and 0.01 unit/μl AmpErase UNG to prevent PCR product carryover, as well as a passive reference dye, ROX. This reference dye provides an internal reference to which the reporter dye signal can be normalized, compensating for the fluorescence between wells and between experiments caused by pipetting errors or instrument variability. Also included in the mixture was 300 nM each of

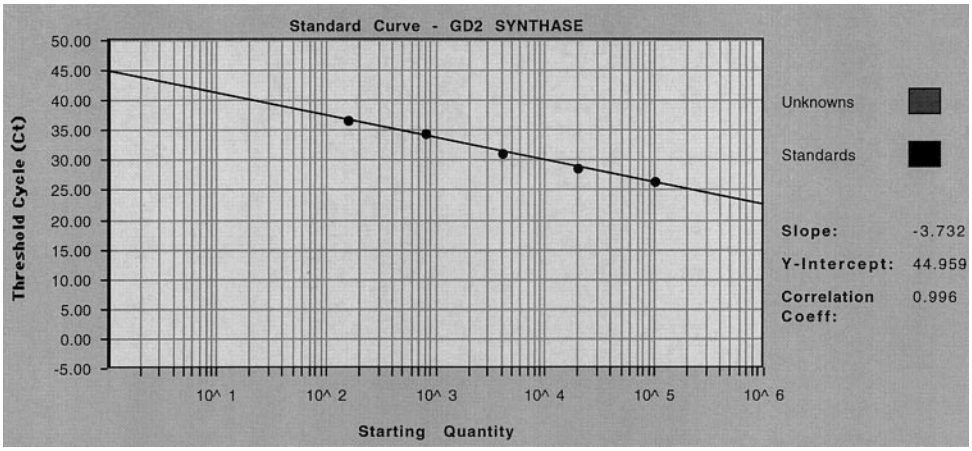

Fig. 1 A representative standard curve of real-time quantitative RT-PCR of GD2 synthase mRNA was derived from serially diluted cDNAs of NB cell line NMB7.

GD2 synthase forward and reverse primers, 200 nM GD2 synthase (FAM) probe, and 40 nM each GAPDH primer, and 100 nM GAPDH (VIC) probe. Each tube was covered with a MicroAmp optical cap. Every PCR run included a five-point standard to generate standard curves for GD2 synthase and GAPDH, plus a no template control. Samples were often run in duplicate PCR experiments.

Using the ABI Prism 7700 Sequence Detector, the initial PCR began with a 50°C, 2-min step to optimize UNG activity, followed by a 95°C, 10-min step to activate AmpliTaq Gold DNA polymerase and UNG deactivation. Then, 40 cycles at 95°C for 15 s and 60°C for 1 min were performed. The entire PCR took 2 h to complete, with no post-PCR handling.

Calculation

For each unknown test sample, the amount of GD2 synthase and endogenous reference GAPDH was determined from the respective standard curve. Dividing the GD2 synthase level by the GAPDH level resulted in a normalized GD2 synthase value. Quantitation of 31 normal BM and peripheral blood samples established the threshold below which the quantitative value was considered background. The variation in quantitation from experiment to experiment was within 15%.

Statistical Analysis

Prognostic importance of clinical variables was evaluated by Cox regression using univariate and multivariate analyses. Patient survival was estimated by the Kaplan-Meier method, and survival comparisons between groups were made using the log-rank test.

RESULTS

Specificity and Sensitivity of Real-Time Quantitative RT-PCR of the GD2 Synthase Transcript. A multiplex assay was established by generating two reproducible standard curves, one for GD2 synthase mRNA and one for GAPDH. The cDNA standard (100,000 arbitrary units) was derived from NB cell line NMB7 and reverse transcribed in the same manner as the test samples. The standard was serially diluted to obtain a linear dynamic range of >5 logs (Fig. 1). The amplification plots of cDNA standards of GD2 synthase and GAPDH are illustrated in Fig. 2, *a* and *b*. The GD2 synthase transcript level was expressed as a multiple of GAPDH expression. The threshold level, *i.e.*, the upper limit of normal (mean + 2 SE) of GD2 synthase mRNA, was established using a total of 31 normal BM and peripheral blood samples. Mean GD2 synthase/GAPDH was 0.98, and SE was 1.60. Thus, the calculated threshold of the normalized GD2 synthase was 4.18 (rounded to 5.0 for this analysis). Levels below 5.0 were defined as negative. The sensitivity of this assay was established by spiking NMB7 cells at ratios ranging from 1–10,000 tumor cells per million normal marrow mononuclear cells. The quantitative values are tabulated in Table 1. The level of GD2 synthase transcript for a tumor content of 1 cell/10^6 cells was 9.54 ± 1.74.

Quantitation of GD2 Synthase mRNA in 155 BM Samples from 100 NB Patients. The level of GD2 synthase mRNA was correlated with the number of GD2-positive tumor cells as enumerated by immunocytology. In this real-time quantitative RT-PCR, levels greater than 5.0 were defined as positive, whereas measurements of >0.001% GD2-positive cells by immunocytology were deemed positive. Only marrows positive for both GD2 synthase and immunocytology were included in this correlation analysis. As shown in Fig. 3, a linear relationship was found between these two independent measurements ($r = 0.96$).

There was concordance in both positivity and negativity between GD2 synthase mRNA by real-time quantitative RT-PCR and immunocytology. Agreement was 74% (115 of 155 BM samples; Table 2). Thirty-two samples were GD2 synthase positive and immunocytology negative. Eight samples were GD2 synthase negative and immunocytology positive. Interestingly, the BM aspirates examined in seven of the eight samples mentioned above were also negative by histological examination.

Patterns of GD2 Synthase mRNA Measurement of Patients with Serial BM Samplings. In a pilot study, sequential BMs from five stage 4 NB patients were studied throughout the course of their treatment and follow-up (Table 3). The expression levels of GD2 synthase correlated closely with the patient's

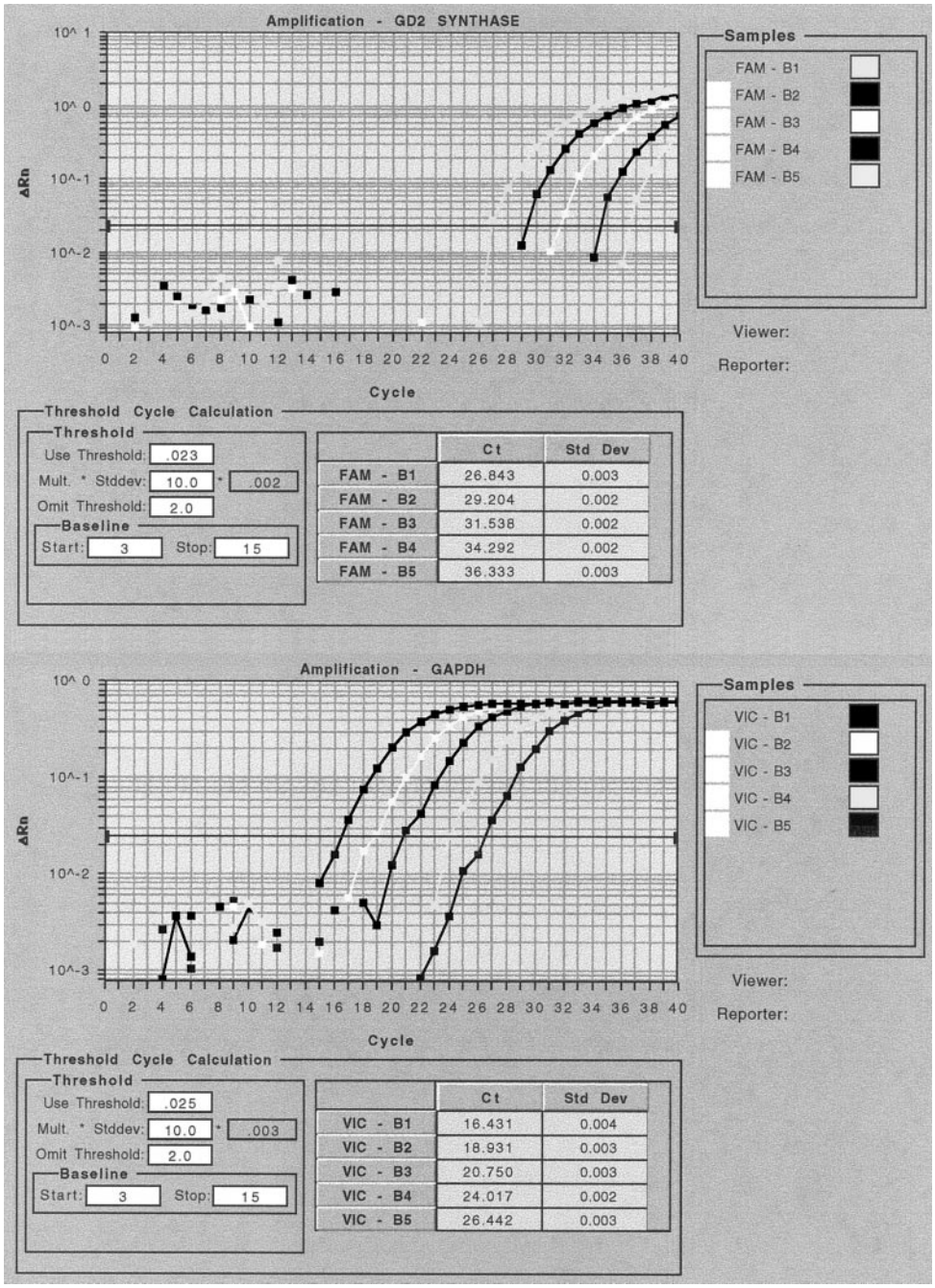

Fig. 2 Amplification plot of GD2 synthase (*a*) and GAPDH (*b*) standard curve.

clinical status. Patients 1–3 are alive and progression free. Patients 4 and 5 have succumbed to NB and died.

Prognostic Significance of GD2 Synthase Transcript on the Survival of Patients with NB. At 24 months after diagnosis, available remission BMs from patients with advanced NB diagnosed at >1 year of age initially treated with protocol N6 ($n = 19$) and protocol N7 ($n = 25$) were analyzed for GD2 synthase mRNA by real-time quantitative RT-PCR. Eight of 19 (42%) N6 patients and 5 of 25 (20%) N7 patients were positive, as defined by the threshold of 5.0. In total, 30% of the samples

Table 1 Sensitivity of real-time quantitative RT-PCR of GD2 synthase mRNA

Ratio of tumor cells to normal marrow cells	GD2 synthase/GAPDH
10^{-2}	581.91 ± 62.41[a]
10^{-3}	93.24 ± 4.75
10^{-4}	24.18 ± 2.06
10^{-5}	12.18 ± 1.51
10^{-6}	9.54 ± 1.74

[a] Mean ± SE of two experiments.

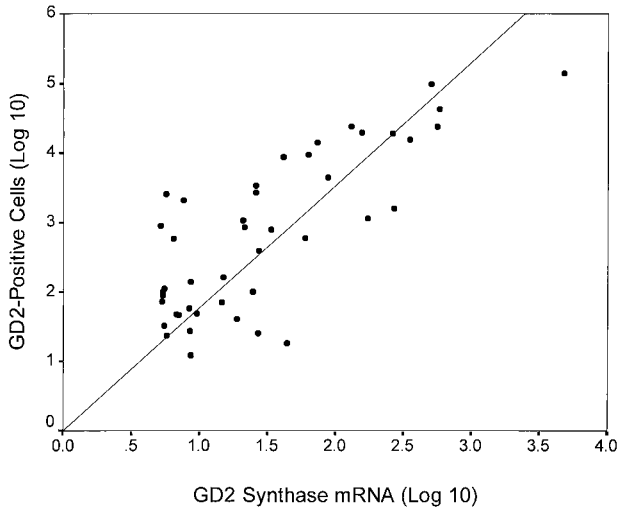

Fig. 3 Correlation between quantitation by immunocytology and GD2 synthase mRNA. Both axes were log(10). Immunocytology was expressed as the number of GD2-positive cells/10^6 marrow mononuclear cells. GD2 synthase mRNA was measured as described in "Patients and Methods." Correlation coefficient = 0.96.

(13 of 44 samples) were positive for GD2 synthase mRNA. Figs. 4 and 5 showed a strong correlation between GD2 synthase positivity at 24 months after diagnosis and adverse clinical outcome for both progression-free survival ($P < 0.005$) and overall survival ($P < 0.001$).

DISCUSSION

As the focus of curative strategies shifts to MRD, objective quantitation of occult tumor cells becomes critically important. For NB, the efficacy of various therapeutic approaches is often based on BM response. In particular, autologous marrow/stem cell transplantation is predicated on the marrow being free of tumor cells (30). As in other metastatic cancers, BM is often the first site of relapse and is not uncommonly the only site of metastasis for NB. Large retrospective studies have clearly demonstrated the importance of marrow remission in ensuring long-term survival (31). As therapy becomes more effective, being able to quantify tumor cells in sequential follow-up BM samples will help define the quality of remission by serving as a surrogate marker for tumor response. Patients in solid remission may be spared further chemotherapy and its leukemia risk. Sensitive methods to detect NB cells can further provide better timing for marrow collection before autologous stem cell transplant. At time of MRD, the absence of detectable molecular marker may serve as a surrogate end point for the adjuvant treatment strategy. Moreover, quantitative information on BM disease will help detect relapse earlier and improve the patient's chance of survival.

Immunocytology using specific antibodies against GD2 has been successful in detecting and quantifying tumor cells in the BM (9, 10). However, only freshly collected samples can be used. This technique is also labor intensive because it requires counting cells under the microscope. In contrast, molecular monitoring of residual tumor cells by RT-PCR uses cryopreserved mononuclear cells, and experiments can be repeated multiple times and for multiple markers as they are being developed. Sensitivity of tumor cell detection by immunocytology is 1 in 10^5 cells, whereas in RT-PCR, it is 1 in 10^6 cells. However, molecular-based MRD assays can be hampered by false positive results due to the fact that tumor-specific markers that detect rare tumor cells can also be present in nontumor cells. In addition, there is the process of illegitimate transcription, *i.e.*, the transcription of any gene in any cell type. Tissue-specific markers can also lead to false positive PCR results if normal cells are introduced in the circulation after invasive procedures.

Table 2 Concordance in molecular *versus* immunological detection of GD2 in the BM samples

	Immunocytology		
Real-time RT-PCR	Positive	Negative	Total
Positive	45	32	77
Negative	8	70	78
Total	53	102	155

In our laboratory, cancer-testis antigens *MAGE* (13), *GAGE* (26), *SSX* (32), *BAGE*, and *NYESO* (data not shown) have been examined to evaluate their potentials as markers for MRD. Among these antigens, *GAGE* was found to be a superior molecular marker because its presence in the blood and BM had prognostic importance in disease progression and survival for patients with advanced NB (33) and melanoma (34). *GAGE* detection was based on RT-PCR and chemiluminescence, where positivity was identified by a PCR product of the appropriate size, and confirmed by Southern blotting.

Development of a real-time quantitative RT-PCR assay broadens the potential for monitoring of MRD in NB. The advantages of real-time quantitation are numerous. With a wide linear dynamic range and superior sensitivity and accuracy, it allows good intra-assay and interassay reproducibility. Additional attractions include high throughput capacity, speed, and elimination of lengthy post-PCR handling steps, preventing potential carryover contamination. In this study, a new molecular marker, GD2 synthase, was explored to measure NB cells in the BM by real-time quantitative RT-PCR. Over the last 15 years, our laboratory has routinely used anti-GD2 immunocytology to enumerate NB cells in the BM of patients. Whereas anti-GD2 monoclonal antibodies are specific for the oligosaccharide moiety of the antigen GD2, real-time quantitation of GD2 synthase mRNA provides us with information on the expression of the enzyme. The excellent quantitative correlation between these two measurements suggests a close relationship between enzyme GD2 synthase and antigen GD2 in individual tumor cells and among patients. Thus, comparison over time in the same patient can be made, as well as a comparison through different phases of treatment and among patients. This report is the first demonstration of a quantitative relationship between a specific mRNA and the actual number of tumor cells. Because of the inherent sensitivity of RT-PCR, we were not surprised to find more samples that were GD2 synthase positive and immunocytology negative. These were unlikely to be false positive

Table 3 Relative quantitation of GD2 synthase mRNA of sequential samples in the BMs of stage 4 NB patients during the course of treatment and follow-up[a]

	Patient				
Timeline	1	2	3	4	5
At diagnosis	24.7 (0 m)[b]	21.0 (0 m)	107.1 (0 m)	4098.3 (0 m)	350.3 (0 m)
During treatment	12.0 (6 m)	0 (6 m)	9.9 (6 m)	37.4 (6 m)	15.0 (1 m)
Clinical remission	1.8 (12 m)	0 (12 m)	0 (12 m)	0 (13 m)	2.2 (10 m)
Follow-up	4.2 (17 m)	0 (18 m)	0 (18 m)	50.8 (17 m)	8.5 (13 m)
Follow-up	1.1 (24 m)	0 (31 m)	0 (24 m)	16.2 (24 m)	
Relapse				25.8 (30 m)	546.3 (18 m)
Relapse				26.1 (41 m)	
Status	PF[c] (73 m)	PF (83 m)	PF (100 m)	Dead (50 m)	Dead (22 m)

[a] Relative quantitation was expressed as GD2 synthase/GAPDH with a detection threshold of 5.0, as described in "Patients and Methods."
[b] Months (m) after diagnosis.
[c] PF, progression free.

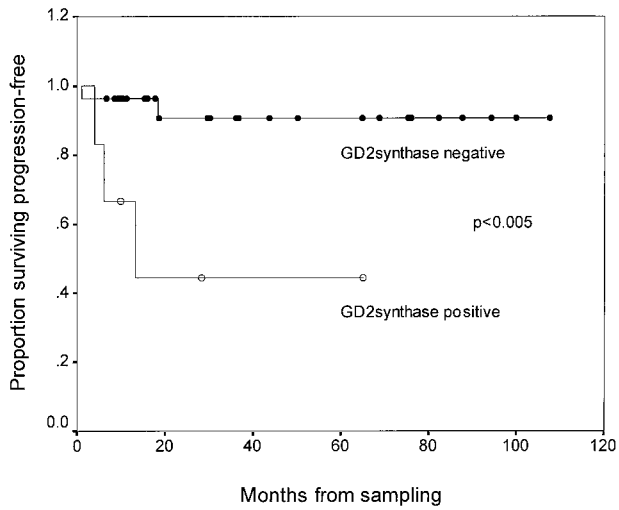

Fig. 4 Relationship between GD2 synthase mRNA and Kaplan-Meier analysis of progression-free survival from remission marrows sampled at 24 months after diagnosis.

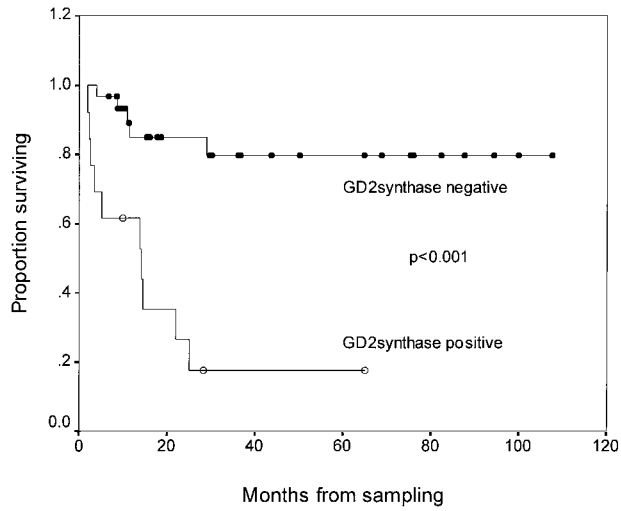

Fig. 5 Relationship between GD2 synthase mRNA and Kaplan-Meier analysis of survival from remission marrows sampled at 24 months after diagnosis.

samples because these patients had other evidence of disease, although some of their marrows were histologically negative.

Clinical utility of this method was demonstrated by first examining sequential BM samples of individual NB patients. The level of transcript agreed with their clinical disease status, and it correlated closely with the tumor burden in the marrow, as measured by immunocytology (data not shown). Secondly, we tested the importance of remission marrow GD2 synthase mRNA among patients 2 years after diagnosis. This cohort had previously been monitored for *GAGE* expression by RT-PCR and chemiluminescence. *GAGE* positivity of their marrows at 24 months after diagnosis was strongly correlated with disease progression and death (33). GD2 synthase expression was also significantly associated with patient outcome. Interestingly, there was a statistically lower percentage of GD2 synthase-positive marrows among patients entered in protocol N7 compared with those entered in protocol N6. Because these two protocols were essentially identical, except for the inclusion of ^{131}I-labeled monoclonal antibody 3F8 in N7, this difference may be a reflection of the treatment efficacy of the two protocols.

This newly developed quantitative RT-PCR assay has many potential clinical utilities. By quantifying tumor cells in the marrow during the course of treatment and follow-up, substantially more information on disease status will be gained. Because of the intensified use of topoisomerase II inhibitors and alkylators, there is an increase in the incidence of secondary leukemia among NB patients (35). Patients early on in solid remission may not need further chemotherapy. Adjuvant therapies, such as monoclonal antibody, oral VP16, or *cis*-retinoic acid, often make gradual and not quantum changes in MRD, which by definition is beyond the sensitivity of conventional histological or radiographic techniques. The efficacy and duration of adjuvant treatment, which can only be assessed retrospectively after an extended period of clinical follow-up, may now be determined by using the level of GD2 synthase transcript as the surrogate end point. Patients who may be at risk for relapse, albeit systemic or in central nervous system, may benefit from earlier intervention/prophylaxis if an elevation in the molecular marker in BM, blood, or cerebrospinal fluid is noted.

Moreover, better timing for marrow or stem cell collection may be possible. Because GD2 is present in other malignancies, including osteosarcoma (36), soft tissue sarcoma (37), medulloblastoma and high-grade astrocytoma (38, 39), retinoblastoma (40), melanoma (41, 42), and small cell lung cancer (43), this quantitative assay may have broader clinical applications.

REFERENCES

1. Diel, I. J., Kaufmann, M., Costa, S. D., Holle, R., von Minckwitz, G., Solomayer, E. F., Kaul, S., and Bastert, G. Micrometastatic breast cancer cells in bone marrow at primary surgery: prognostic value in comparison with nodal status. J. Natl. Cancer Inst. (Bethesda), *88:* 1652–1658, 1996.

2. Lindemann, F., Schlimok, G., Dirschedl, P., Witte, J., and Riethmueller, G. Prognostic significance of micrometastatic tumour cells in bone marrow of colorectal cancer patients. Lancet, *340:* 685–689, 1992.

3. Jauch, K. W., Heiss, M. M., Gruetzner, U., Funke, I., Pantel, K., Babic, R., Eissner, H. J., Giethmueller, G., and Schildberg, F. W. Prognostic significance of bone marrow micrometastases in patients with gastric cancer. J. Clin. Oncol., *14:* 1810–1817, 1996.

4. Pantel, K., Izbicki, J., Passlick, B., Angstwurm, M., Haussinger, K., Thetter, O., and Riethmueller, G. Frequency and prognostic significance of isolated tumour cells in bone marrow of patients with non-small cell lung cancer without overt metastases. Lancet, *347:* 649–653, 1996.

5. Ghossein, R., Coit, D., Brennan, M., Zhang, Z. F., Wang, Y., Bhattacharya, S., Houghton, A., and Rosai, J. Prognostic significance of peripheral blood and bone marrow tyrosinase messenger RNA in malignant melanoma. Clin. Cancer Res., *4:* 419–428, 1998.

6. Gruhn, B., Hongeng, S., Yi, H., Hancock, M. L., Rubnitz, J. E., Neale, G. A., and Kitchingman, G. R. Minimal residual disease after intensive induction therapy in childhood acute lymphoblastic leukemia predicts outcome. Leukemia (Baltimore), *12:* 675–681, 1998.

7. Hartman, O., Valteau-Couanet, D., Vassal, G., Lapierre, V., Brugieres, L., Delgado, R., Couanet, D., Lumbroso, J., and Benhamou, E. Prognostic factors in metastatic neuroblastoma in patients over 1 year of age treated with high-dose chemotherapy and stem cell transplantation: a multivariate analysis in 218 patients treated in a single institution. Bone Marrow Transplant., *23:* 789–795, 1999.

8. Cotterill, S. J., Pearson, A. D. J., Pritchard, J., Foot, A. B. M., Roald, B., Kohler, J. A., and Imeson, J. Clinical prognostic factors in 1277 patients with neuroblastoma: results of the European Neuroblastoma Study Group "Survey" 1982–1992. Eur. J. Cancer, *36:* 901–908, 2000.

9. Cheung, N. K., Heller, G., Kushner, B. H., Liu, C., and Cheung, I. Y. Detection of metastatic neuroblastoma in bone marrow: when is routine marrow histology insensitive? J. Clin. Oncol., *15:* 2807–2817, 1997.

10. Faulkner, L. B., Tintori, V., Tamburini, A., Paoli, A., Garaventa, A., Viscardi, E., Tucci, F., Lippi, A. A., De Bernardi, B., and Bernini, G. High-sensitivity immunocytologic analysis of neuroblastoma cells in paired blood and marrow samples. J. Hematotherapy, *7:* 361–366, 1998.

11. Miyajima, Y., Kato, K., Numata, S. I., Kudo, K., and Horibe, K. Detection of neuroblastoma cells in bone marrow and peripheral blood at diagnosis by the reverse transcriptase-polymerase chain reaction for tyrosine hydroxylase mRNA. Cancer (Phila.), *75:* 2757–2761, 1995.

12. Lode, H. N., Bruchelt, G., Seitz, G., Gebhardt, S., Gekeler, V., Niethammer, D., and Beck, J. Reverse transcriptase-polymerase chain reaction analysis of monoamine transporters in neuroblastoma cell lines: correlations to meta-iodobenzylguanidine uptake and tyrosine hydroxylase gene expression. Eur. J. Cancer, *31A:* 586–590, 1995.

13. Cheung, I. Y., Barber, D., and Cheung, N. K. V. Detection of microscopic neuroblastoma in marrow by histology, immunocytology, and RT-PCR of multiple molecular markers. Clin. Cancer Res., *4:* 2801–2805, 1998.

14. Ladetto, M., Donovan, J. W., Harig, S., Trojan, A., Poor, C., Schlossnan, R., Anderson, K. C., and Gribben, J. G. Real-time polymerase chain reaction of immunoglobulin rearrangements for quantitative evaluation of minimal residual disease in multiple myeloma. Biol. Blood Marrow Transplant., *6:* 241–253, 2000.

15. Verhagen, O. J., Willemse, M. J., Breunis, W. B., Wijkhuijs, A. J., Jacobs, D. C., Joosten, S. A., van Wering, E. R., van Dongen, J. J., and van der Schoot, C. E. Application of germline IGH probes in real-time quantitative PCR for the detection of minimal residual disease in acute lymphoblastic leukemia. Leukemia (Baltimore), *14:* 1426–1435, 2000.

16. Emig, M., Saussele, S., Wittor, H., Weisser, A., Reiter, A., Willer, A., Berger, U., Hehlmann, R., Cross, N. C., and Hochhaus, A. Accurate and rapid analysis of residual disease in patients with CML using specific fluorescent hybridization probes for real time quantitative RT-PCR. Leukemia (Baltimore), *13:* 1825–1832, 1999.

17. Lo, Y. M. D., Chan, L. Y. S., Chan, A. T. C., Leung, S. F., Lo, K. W., Zhang, J., Lee, J. C. K., Hjelm, M., Johnson, P. J., and Huang, D. P. Quantitative and temporal correlation between circulating cell-free Epstein-Barr virus DNA and tumor recurrence in nasopharyngeal carcinoma. Cancer Res., *59:* 5452–5455, 1999.

18. Wattjes, M. P., Krauter, J., Nagel, S., Heidenreich, O., Ganser, A., and Heil, G. Comparison of nested competitive RT-PCR and real-time RT-PCR for the detection and qualification of AML1/MTG8 fusion transcripts in t(8;21) positive acute myelogenous leukemia. Leukemia (Baltimore), *14:* 329–335, 2000.

19. Wu, Z., Schwartz, E., Seeger, R. C., and Ladisch, S. Expression of GD2 ganglioside by untreated primary human neuroblastomas. Cancer Res., *46:* 440–443, 1986.

20. Kramer, K., Gerald, W., Kushner, B. H., Larson, S. M., Hameed, M., and Cheung, N.K.V. Disialoganglioside GD2 loss following monoclonal antibody therapy is rare in neuroblastoma. Clin. Cancer Res., *4:* 2135–2139, 1998.

21. Furukawa, K., Soejima, H., Niikawa, N., Shiku, H., and Furukawa, K. Genomic organization and chromosomal assignment of the human β1,4-*N*-acetylgalactosaminyltransferase gene. J. Biol. Chem., *271:* 20836–20844, 1996.

22. Yamashiro, S., Ruan, S., Furukawa, K., Tai, T., Lloyd, K. O., Shiku, H., and Furukawa, K. Genetic and enzymatic basis for differential expression of GM2 and GD2 gangliosides in human cancer cell lines. Cancer Res., *53:* 5395–5400, 1993.

23. Yuyama, Y., Dohi, T., Morita, H., Furukawa, K., and Oshima, M. Enhanced expression of GM2/GD2 synthase mRNA in human gastrointestinal cancer. Cancer (Phila.), *75:* 1273–1280, 1995.

24. Kuo, C. T., Bostick, P. J., Irie, R. F., Morton, D. L., Conrad, A. J., and Hoon, D. S. B. Assessment of messenger RNA of β1,4-*N*-acetylgalactosaminyltransferase as a molecular marker for metastatic melanoma. Clin. Cancer Res., *4:* 411–418, 1998.

25. Brodeur, G., Pritchard, J., Berthold, F., Carlen, N. L. T., Castel, V., Castleberry, R. P., DeBernardi, B., Evans, A. E., Favrot, M., Hedborg, F., Kaneko, M., Kemshead, J., Lampert, F., Lee, R. E. J., Look, T., Pearson, A. D. J., Philip, T., Roald, B., Sawada, T., Seeger, R. C., Tsuchida, Y., and Voute, P. A. Revisions of the international criteria for neuroblastoma diagnosis, staging and response to treatment. J. Clin. Oncol., *11:* 1466–1477, 1993.

26. Cheung, I. Y., and Cheung, N. K. V. Molecular detection of GAGE expression in peripheral blood and bone marrow: utility as a tumor marker for neuroblastoma. Clin. Cancer Res., *3:* 821–826, 1997.

27. Heid, C. A., Stevens, J., Livak, K. J., and Williams, P. M. Real time quantitative PCR. Genome Res., *6:* 986–994, 1996.

28. Gibson, U. E., Heid, C. A., and Williams, P. M. A novel method for real time quantitative RT-PCR. Genome Res., *6:* 995–1001, 1996.

29. Holland, P. M., Abramson, R. D., Watson, R., and Gelfand, D. H. Detection of specific polymerase chain reaction product by utilizing the 5′-3′ exonuclease activity of *Thermus aquaticus* DNA polymerase. Proc. Natl. Acad. Sci. USA, *88:* 7276–7280, 1991.

30. Faulkner, L. B., Garaventa, A., Paoli, A., Tintori, V., Tamburini, A., Lacitignola, L., Veltroni, M., Lo Piccolo, M. S., Viscardi, E., Milanaccio, C., Tondo, A., Spinelli, S., Bernini, G., and De Bernardi, B. *In vivo* cytoreduction studies and cell sorting-enhanced tumor-cell detection in high-risk neuroblastoma patients: implications for leukapheresis strategies. J. Clin. Oncol., *18:* 3829–3836, 2000.

31. Ladenstein, R., Philip, T., Lasset, C., Hartmann, O., Garaventa, A., Pinkerton, R., Michon, J., Pritchard, J., Klingebiel, T., Kremens, B.,

Pearson, A., Coze, C., Paolucci, P., Frappaz, D., Gadner, H., and Chauvin, F. Multivariate analysis of risk factors in stage 4 neuroblastoma patients over the age of one year treated with megatherapy and stem-cell transplantation: a report from the European Bone Marrow Transplantation Solid Tumor Registry. J. Clin. Oncol., *16:* 953–965, 1998.

32. Chi, S. N., Cheung, I. Y., and Cheung, N. K. V. SSX-2 and SSX-4: cancer-testis antigens as tumor markers of neuroblastoma. J. Pediatr. Hematol. Oncol., *22:* 380, 2000.

33. Cheung, I. Y., Chi, S. N., and Cheung, N. K. V. Prognostic significance of GAGE detection in bone marrows on survival of patients with metastatic neuroblastoma. Med. Pediatr. Oncol., *35:* 632–634, 2000.

34. Cheung, I. Y., Cheung, N. K. V., Ghossein, R. A., Satagopan, J. M., Bhattacharya, S., and Coit, D. G. Association between molecular detection of GAGE and survival in patients with malignant melanoma: a retrospective cohort study. Clin. Cancer Res., *5:* 2042–2047, 1999.

35. Kushner, B. H., Cheung, N. K. V., Kramer, K., Heller, G., and Jhanwar, S. C. Neuroblastoma and treatment-related myelodysplasia/leukemia: the Memorial Sloan-Kettering experience and a literature review. J. Clin. Oncol., *16:* 3880–3889, 1998.

36. Heiner, J., Miraldi, F. D., Kallick, S., Makley, J., Smith-Mensah, W. H., Neely, J., and Cheung, N. K. V. *In vivo* targeting of GD2-specific monoclonal antibody in human osteogenic sarcoma xenografts. Cancer Res., *47:* 5377–5381, 1987.

37. Chang, H. R., Cordon-Cardo, C., Houghton, A. N., Cheung, N. K. V., Casper, E. S., and Brennan, M. F. Expression of disialogangliosides GD2 and GD3 by human soft tissue sarcomas. Cancer (Phila.), *70:* 633–638, 1992.

38. Shinoura, N., Dohi, T., Kondo, T., Yoshioka, M., Takakura, K., and Oshima, M. Ganglioside composition and its relation to clinical data in brain tumors. Neurosurgery, *31:* 541–549, 1992.

39. Longee, D. C., Wikstrand, C. J., Mansson, J. E., He, X., Fuller, G. N., Bigner, S. H., Fredman, P., Svennerholm, L., and Bigner, D. D. Disialoganglioside GD2 in human neuroectodermal tumor cell lines and gliomas. Acta Neuropathol., *82:* 45–54, 1991.

40. Saarinen, U. M., Sariola, H., and Hovi, L. Recurrent disseminated retinoblastoma treated by high-dose chemotherapy, total body irradiation, and autologous bone marrow rescue. Am. J. Pediatr. Hematol. Oncol., *13:* 315–319, 1991.

41. Cheresh, D. A., Honsik, C. J., Staffileno, L. K., Jung, G., and Reisfeld, R. A. Disialoganglioside GD3 on human melanoma serves as a relevant target antigen for monoclonal antibody-mediated tumor cytolysis. Proc. Natl. Acad. Sci. USA, *82:* 5155–5159, 1985.

42. Thurin, J., Thurin, M., Herlyn, M., Elder, D. E., Steplewski, Z., Clark, W. H., and Koprowski, H. GD2 ganglioside biosynthesis is a distinct biochemical event in human melanoma tumor progression. FEB Lett., *208:* 17–22, 1986.

43. Grant, S. C., Kostacoglu, L., Kris, M. G., Yeh, S., Larson, S. M., Finn, R. D., Oettgen, H. F., and Cheung, N. K. V. Radioimmunodetection of small-cell lung cancer using the anti-GD2 ganglioside monoclonal antibody 3F8: a pilot trial. Eur. J. Nucl. Med., *23:* 145–149, 1996.

Clinical Cancer Research

Quantitation of Marrow Disease in Neuroblastoma by Real-Time Reverse Transcription-PCR

Irene Y. Cheung and Nai-Kong V. Cheung

Clin Cancer Res 2001;7:1698-1705.