Molecular Detection of GAGE Expression in Peripheral Blood and Bone Marrow: Utility as a Tumor Marker for Neuroblastoma

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ABSTRACT

The GAGE family of tumor-associated antigens is present in a wide spectrum of human tumors but is highly restricted among normal tissues except to the testis. By reverse transcription-PCR, GAGE expression was detected in 55 of 67 neuroblastomas (NBs; 8 of 12 stage 1, 13 of 13 stage 2, 9 of 12 stage 3, 7 of 12 stage 4S, and 18 of 18 stage 4), 5 of 5 Ewing’s and peripheral neuroectodermal tumors, and 11 of 11 tumor cell lines (9 NBs, 1 peripheral neuroectodermal tumor, and 1 melanoma). In contrast, 5 of 6 normal tissues (normal testis was positive), 18 of 18 NB-negative bone marrow (BM; 9 normal, 6 non-NB remission, and 3 stage-2 NB), and 9 of 10 NB-negative peripheral blood (PB; 9 normal and 1 stage 2B) were undetectable. In 18 patients with widespread NB under treatment, GAGE expression in paired samples of BM and PB was 89% concordant. Both correlated strongly with disease measured by conventional methods, including marrow histology or immunocytoology, bone scan, meta-iodo-benzylguanidine scan, computed tomography/magnetic resonance imaging, and urine vanillylmandelic acid/homovanillic acid. When serial samples from 14 patients with stage 4 NB were studied, BM from 7 of 7 patients at diagnosis and 14 of 14 patients (25 samples) on treatment were positive for GAGE. Thirteen patients were in continual remission off therapy, and their GAGE expression (12 BM and 9 PB) was undetectable at follow-up. When compared to molecular detection of tyrosine hydroxylase mRNA, GAGE may offer added sensitivity in detecting NB in both BM and PB. The GAGE family of antigens may be potential tumor markers of minimal residual disease.

INTRODUCTION

Metastasis to BM and minimal residual disease are two major hurdles for cancer cure. Conventional histology has not provided the sensitivity and specificity necessary for its detection. Using immunocytoology, marrow metastasis in a variety of cancers can now be routinely measured at a sensitivity of 1 in 1 × 10^5 nucleated cells (1–3). An improvement in quantifying minimal tumor cells may help in the therapeutic assessment of anticancer drugs and is necessary for comparing the increasingly efficient purging techniques (4). However, the sensitivity of immunocytoLOGY is probably pushed to its limit. The recent identification of tumor-specific genes and translocations has unveiled new possibilities. The molecular detection of minimal residual disease has been applied in leukemia (5, 6), Ewing’s sarcoma (7), breast cancer (8), prostate cancer (9), and NB (10, 11). The choice of tumor marker and methodology will undoubtedly influence the success of this approach. Marker heterogeneity within a tumor population is oftentimes not known. For example, TH mRNA is a sensitive and specific marker by RT-PCR in NB (10–12). However, because of the feedback regulation of catecholamine synthesis, TH gene expression is inversely correlated with that of the noradrenaline transporter in human NB (13). MIBG enters tumor cells via the noradrenaline transporter. Because most neuroblastomas have strong MIBG uptake, TH gene expression may be down-regulated in some cells and thus not detectable by RT-PCR. Another consideration in the choice of tumor marker is their tissue distribution. Although most markers explored have restricted expression in normal tissues, any expression in PB or BM can be problematic. For example, PGP 9.5, a neuron-specific neural cell adhesion molecule isoform, is present in normal marrow and, therefore, has limited clinical utility as a marker of minimal residual NB (14).

In the past, tumor markers have primarily been identified by serological methods. Expression cloning strategies using CTLs as probes have only been exploited recently (15). In melanoma patients, CTLs have recognized distinct families of antigens encoded by genes MAGE-1 (15), MAGE-3 (16), BAGE (17), and GAGE (18). These antigens are expressed in human tumors of various histological types but are silent in normal adult tissues except testis. GAGE gene expression (GAGE-1 and GAGE-2) has been detected in about 12–25% of various adult cancers including melanoma, non-small lung cancer, sarcoma, bladder cancers, and head and neck tumors (18). In contrast to

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3 The abbreviations used are: BM, bone marrow; NB, neuroblastoma; TH, tyrosine hydroxylase; RT-PCR, reverse transcription-PCR; MIBG, [131I]meta-iodo-benzylguanidine; PB, peripheral blood; MSKCC, Memorial Sloan-Kettering Cancer Center; VMA, vanillylmandelic acid; HVA, homovanillic acid; PNET, peripheral neuroectodermal tumor; CTL, cytolytic T lymphocytes.
MAGE and BAGE genes (19–21), the expression of GAGE has not been described in NB. In this report, we used RT-PCR and a sensitive chemiluminescent detection technique to determine its expression among human NB tissues of all clinical stages. Because of its restricted tissue distribution, we also test its utility as a molecular marker for minimal disease in PB and BM.

PATIENTS AND METHODS

Patients

Patients with NB evaluated at MSKCC were diagnosed and staged in accordance with the international neuroblastoma staging system (22). Comprehensive extent-of-disease evaluations included computed tomography, 99mTc bone scan, MiBG scan, bilateral BM aspirates (four sites) and biopsies (two sites), and measurement of urinary and serum tumor markers. Urine VMA and HVA were determined by high-pressure liquid chromatography. Mononuclear cells were purified from PB and BM samples for immunocytology and RT-PCR. Immunocytochemistry was carried out using a panel of anti-G_{122} monoclonal antibodies (23). Patients with stage 4 NB were treated with intensive chemotherapy as described previously (24, 25).

Cell Lines

Tumor cell lines SK-Mel-1 (melanoma), SK-N-MC (PNET), and IMR32 (NB) were obtained from the American Type Culture Collection (Rockville, MD). LAN-1 was from Dr. Robert Seeger (Children's Hospital of Los Angeles, Los Angeles, CA), and 55N and 5S were from Dr. June Biedler (MSKCC); NMB7 and IMR6 were from Dr. S. K. Liao of McMaster University (Hamilton, Ontario, Canada). The NB cell lines SK-N-WD, SK-N-JD, and SK-N-MM were established at MSKCC.

Preparation of Total RNA

Tumor tissue cellular RNA was isolated by a guanidine thiocyanate-phenol-chloroform extraction method using Tri Reagent (Molecular Research Center, Cincinnati, OH). The concentration of total RNA was determined by spectrophotometry, and 2 μg of RNA were used RT-PCR. Mononuclear cells from BM and PB were isolated by gradient centrifugation through Ficoll-Paque (Pharmacia Biotech, Piscataway, NJ) and resuspended in RPMI 1640 (JRH Biosciences, Lenexa, KS) containing 10% bovine serum (JRH). Upon centrifugation at 4°C for 5 min at 1500 rpm, the pelleted cells were washed with cold PBS treated with diethylpyrocarbonate from Sigma Chemical Co. (St. Louis, MO), and total cellular RNA was extracted with Tri Reagent.

RT-PCR

RT. In a final volume of 20 μl, total RNA was reverse transcribed in the presence of 200 units of Moloney murine leukemia virus reverse transcriptase (Life Technologies, Inc., Gaithersburg, MD) and oligo(dT)$_{15}$ primer (at 2 μM final concentration; Promega Corp., Madison, WI), 1 μM each deoxynucleotide triphosphate (Pharmacia Biotech, Inc.), 20 units RNase inhibitor (Promega), and 1X RT buffer [50 mM Tris-HCl (pH 8.3), 75 mM KCl, and 3 mM MgCl$_2$], as well as 10 mM DTT (Life Technologies, Inc.). The reaction mixture was incubated at 37°C for 60 min and terminated by heating at 90°C for 5 min. For negative controls, identical experiments were carried out without reverse transcriptase and oligo(dT)$_{15}$ primer.

PCR. The oligonucleotides used as PCR primers for detecting the entire GAGE family were published previously by Van den Eynde et al. (18): sense primer, 5'-AGACGCTAGCTAGGCTC-3'; and antisense primer, 5'-CCATCAGGACACATCTTCA-3'. Both primers were 5'-end labeled with biotin (Integrated DNA Technologies, Coralville, IA). PCR with these GAGE-specific primers produced an amplified DNA fragment of 239 bp. Two oligonucleotides used as PCR primers for detecting TH gene expression were reported previously by Naito et al. (26): sense primer, 5'-TGTCAGAAGCTTACACGAAGTTGC-3'; and antisense primer, 5'-GATTATGCTCTCCGGAAGTGAC-3'. Both primers were 5'-end labeled with biotin (Oligos Etc., Wilsonville, OR). PCR with these TH-specific primers produced an amplified DNA fragment of 299 bp. The integrity of mRNA isolated from these samples was confirmed by PCR using primers specific for human β$_2$-microglobulin with an amplified DNA fragment of 333 bp: sense primer, 5'-CTCGCCGCTACTCTTCTTCTTG-3'; and antisense primer, 5'-GCTCATGTGTCATGTCCTGACCCCACCTTA-3'.

PCR mixture contained 5 μl of 10X PCR buffer [100 mM Tris-HCl (pH 8.3), 500 mM KCl, and 15 mM MgCl$_2$] from Perkin-Elmer (Foster City, CA), 0.4 μl of 25 mM deoxynucleotide triphosphate, 0.3 μl of each oligonucleotide primer (20 μM), 0.2 μl of AmpliTaq DNA polymerase (5 units/μl; Perkin-Elmer), and one-twentieth of the cDNA synthesis solution, as well as nanopure water to achieve a volume of 50 μl.

GAGE-PCR. PCR was performed in an Omnigene Thermal Cycler (Hybaid, Teddington Middlesex, United Kingdom). After the first denaturation step at 94°C for 3 min, 29 cycles of amplification were carried out with denaturation at 94°C for 1 min, annealing at 55°C for 2 min, and extension at 72°C for 3 min. A final extension at 72°C for 8 min completed the PCR reaction.

TH-PCR. Thirty cycles of amplification were carried out with denaturation at 92°C for 1 min, annealing at 59°C for 1 min, and extension at 72°C for 2 min.

Chemiluminescent Detection of PCR Products

PCR products were electrophoresed on 1.5–2% high-melting agarose gel (Fisher Scientific, Pittsburgh, PA) with BioMarker Low-Biotin DNA molecular weight standards (BioVentures, Murfreesboro, TN) and transferred to Zeta-Probe nylon blotting membrane (Bio-Rad, Hercules, CA) by means of a semidry Southern-light protocol (Bedford, MA). When compared to ethidium bromide staining, chemiluminescent detection was more than 500-fold more sensitive. Using primers without biotin end-label, some PCR products were purified and sequenced using the Automated Nucleotide Sequencing System Model 373 (Applied Biosystems, Foster City, CA). The sequences agreed with the GAGE cDNA sequences available from GenBank.
Table 1  GAGE expression in cell lines, tumors, and normal tissues

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Statistical Analysis
Correlations of disease detection by GAGE and other modalities and the concordance between PB-GAGE and BM-GAGE were tested by Fisher’s Exact test.

RESULTS

GAGE Expression in Cell Lines, Tumors, and Normal Tissues. GAGE expression was strong by RT-PCR and chemiluminescence in 11 of 11 tumor cell lines (Table 1). These included one melanoma (SK-Mel-1), one PNET (SK-M-NC), and nine NBs (LAN-i, 55N, 55, IMR6, 1MR32, NMB7, SK-N-WD, SK-N-JD, and SK-N-MM). Among frozen NB tumors from 67 patients, 55 were positive for GAGE expression: 8 of 12 stage 1, 13 of 13 stage 2, 9 of 12 stage 3, 18 of 18 stage 4, and only 7 of 12 stage 4S. Of these 67 tumors, 5 were obtained at second-look surgery after induction chemotherapy, and they were all positive. Although there was no correlation of stage with expression, negative localized tumors (four stage 1, and three stage 3) tended to have mixed ganglioneuroma-NB components. Fig. 1 illustrates GAGE expression in a representative panel of tumors from all clinical stages of neuroblastoma, cell lines, and a normal BM. Among normal tissues examined (tonsil, liver, cerebellum, thyroid, lung, and testis), only testis showed expression, as was reported previously (Ref. 18; Table 1).

Specificity and Sensitivity of GAGE Detection in BM and PB. GAGE was not found by RT-PCR in 18 of 18 NB-negative marrows: 9 of 9 normal volunteers, 6 of 6 non-NB remission patients, and 3 of 3 stage 2 NB patients, as were 9 of 10 NB-negative (8 of 9 normal PB and 1 of 1 stage 2 NB) PB samples. The absence of GAGE expression in blood cells suggests that GAGE might be a potential molecular marker for studying minimal residual disease. To demonstrate its sensitivity, RNAs were extracted from preparations of normal marrow containing NB cells (NMB7) at ratios ranging from 1 NB cell/million BM cells to 10⁵ NB cells/million BM cells. RT-PCR was then performed to detect GAGE expression. The limit of detection was at least 1 neuroblastoma tumor cell in 10⁶ normal marrow cells (Fig. 2).

Detection of GAGE Expression in BM and PB of Neuroblastoma Patients. Simultaneously drawn BM and PB samples from 18 patients with widespread neuroblastoma (1 stage 4S and 17 stage 4) were studied for GAGE and TH gene expression. They were compared to the results of conventional marrow histology, marrow immunocytoLOGY, bone scan, MIBG scan, computed tomography/magnetic resonance imaging, and urine VMA/HVA (Table 2). There was close agreement of GAGE detection between BM and PB samples (P = 0.002) collected from the same patient at the same time. Both BM and PB GAGE expression correlated strongly with other evidence of disease (P = 0.07 and 0.02, respectively). In BM samples, 5 of...
11 TH-negative patients had detectable \textit{GAGE}. All 18 PB samples were TH negative, whereas 9 of them had detectable \textit{GAGE} expression. Among 10 patients with no objective evidence of active disease by clinical criteria, one patient (no. 6) had detectable TH, and 4 patients (nos. 7, 8, 9, and 10) were \textit{GAGE} positive and TH negative in their BM. These four patients had high-risk NB; all were stage 4 diagnosed after 2 years of age with marrow metastases: 3 with distant skeletal metastases, 2 with >40 copies of \textit{N-myc} amplification, 2 with lp deletion, 3 with elevated ferritin, 3 with serum lactic acid dehydrogenase >1500 at diagnosis, all four with unfavorable histology, and one had prior distant bony relapse. Two of these 4 patients were receiving induction chemotherapy; one patient just completed anti-\textit{Gd2} antibody immunotherapy, and one was off all treatment when their BM and PB \textit{GAGE} were studied. The sole patient (no. 11) with recurrent disease in the primary site and negative \textit{GAGE} and TH continued to show no marrow involvement by immunocytochemical or conventional histological examination.

Serial BM and PB samples (total \textit{n} = 53) from 14 patients diagnosed with stage 4 NB and treated with dose-intensive therapy (chemotherapy protocols N5 and N6; Refs. 24 and 25) were studied. \textit{GAGE} expression was detectable in 7 of 7 BM samples obtained at diagnosis and 14 of 14 patients (25 BM samples) while on treatment. Of these 14 patients, one patient relapsed. \textit{GAGE} expression in her BM was transiently negative while the patient was still on therapy. After the marrow \textit{GAGE} became positive, four of five subsequent PB samples over a period of 4 months were also \textit{GAGE} positive before the patient presented with an isolated radiographic relapse in the distal femur on MIBG scan, when conventional marrow histology, immunocytochemistry, bone scan, X-rays, or VMA/HVA measurements were still normal. The other 13 patients have remained in continuous clinical remission, median follow-up of 24 months (range, 5–43 months) from the time of \textit{GAGE} testing and 55 months (range, 20–77 months) from diagnosis. Their \textit{GAGE} expression was undetectable in all 21 samples (12 BM and 9 PB).

**DISCUSSION**

Using a sensitive chemiluminescent detection method, \textit{GAGE} expression was found in all stage 4 neuroblastomas. It was present in the bone marrow in all patients with distant disease at diagnosis and were negative during follow-up in patients in continual remission. \textit{GAGE} expression was highly concordant between bone marrow and peripheral blood. Both correlated strongly with evidence of disease measured by other modalities. Given its restriction in both adult and fetal tissues, as well as the wide distribution among human cancers, the \textit{GAGE} family of antigens may potentially provide a useful tumor marker for monitoring marrow and blood metastases at the time of minimal residual disease.

Similar to \textit{MAGE} and \textit{BAGE}, \textit{GAGE} was discovered as melanoma targets for CTLs derived from melanoma patients (18). Because of their specific expression in tumors, these antigens constitute useful targets for specific cancer immunotherapy. Previous attempts to identify tumor markers have used serological methods. Antigen cloning strategies using CTL as "probes" may provide novel repertoires of tumor markers for...
molecular diagnosis. In NB, \textit{MAGE} expression has been detected previously in 30% of cell lines (19, 20). Up to 73% of NB tumors expressed at least one \textit{MAGE} or \textit{BAGE} allele (21). In the initial report of \textit{GAGE}, 12–25% of human tumors (NB not studied) was positive (18). Exploiting the higher sensitivity of \textit{GAGE}, the initial report of tumors expressed at least one \textit{GAGE} molecule was not shown, whereas normal tissues (except testis) remained negative. \textit{GAGE} expression was detected in all NB cell lines as well as all stage 4 tumors. There was no correlation of \textit{GAGE} expression with patient’s age, tumor \textit{N}-myc, CD44, or Trk-A expression (data not shown). The absence of \textit{GAGE} was only found in localized/4S tumors. The cellular function of \textit{GAGE} is presently unknown. Similar to \textit{MAGE} and \textit{BAGE}, its exquisite tissue restriction to the testis and repression in other adult and fetal tissues older than 20 weeks has raised the possibility that these gene products may play a role during early stages of embryonic development (18). Although antigenic peptide YRPRPRRY encoded by \textit{GAGE}-1 is an efficient target for CTL, its MHC restriction makes it unlikely to be of general clinical utility for immunotherapy of neuroblastoma because the receptor HLA-Cw6 molecule is not a common HLA type. In addition, most neuroblastomas have low to no expression of HLA-class I antigens (27). This can render strategies using CTL \textit{in vivo} a daunting task for NB.

On the other hand, \textit{GAGE} gene may be a potentially important marker for minimal residual disease. Close correlation of \textit{GAGE} detection and clinical evidence of neuroblastoma suggests that at the time of minimal residual disease, a significant proportion of circulating tumor cells expresses the \textit{GAGE} gene. Our findings showed concordance of \textit{GAGE} expression in BM and PB, whereas TH expression had greater disparity. None of the 18 PB samples showed TH expression, whereas 50% of them had detectable \textit{GAGE}. Among BM samples, 46% of the TH-negative ones were positive for \textit{GAGE}. Because \textit{GAGE} expression correlated strongly with the presence of disease, it was unlikely that this superior sensitivity over TH was due to false positivity. Other studies have reported higher percentages of PB showing TH expression, although patients in complete remission have a much lower positive rate (14%) in PB compared to BM (57%; Ref. 10). In our study, \textit{GAGE} and TH were compared in 18 patients who were undergoing either intensive chemotherapy or were off therapy, most with minimal disease. Under these limiting conditions, \textit{GAGE} appeared to have better sensitivity than TH. One may speculate that \textit{GAGE} expression is less dependent on whether the cells are stationary metastases (e.g., in BM) or in transit in the PB, whereas other markers (e.g., TH) may be down-regulated when the tumor cells circulate. If TH is expressed in a subset of NB cells separate from those that express \textit{GAGE}, the two probes may complement each other in tumor detection. If \textit{GAGE} is indeed a marker for circulating NB stem lines, its measurement would be particularly useful for timing PB stem cell harvest and evaluating purging efficacies. Given the wide distribution of \textit{GAGE} among human malignancies, it is possible that \textit{GAGE} may be useful as a marker for tumors other than NB. These preliminary results suggest that \textit{GAGE} deserves further testing in prospective clinical trials with a larger number of patients to define its clinical utility.

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