Detection of Microscopic Neuroblastoma in Marrow by Histology, Immunocytology, and Reverse Transcription-PCR of Multiple Molecular Markers

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INTRODUCTION

In children with NB, marrow metastasis is a strong prognostic predictor of clinical outcome with serious therapeutic implications. When marrow disease is microscopic, conventional sampling by HIST has limited sensitivity in comparison with IC (1–4). Molecular detection of tumor markers by RT-PCR offers unique sensitivity and specificity. Candidates in the past were often limited to serologically reactive entities, either carried on the surface of or released by tumor cells. Most tumor-associated cytoplasmic or nuclear antigens remain unexplored. Expression cloning strategies using CTLs as probes have identified targets that are potentially useful for immunotherapy (5). Of particular interest are families of genes named MAGE (5, 6), BAGE (7), and GAGE (8). They encode distinct tumor-associated antigens that are recognizable by CTLs. These antigens are expressed in human tumors of diverse histological types but are silent in normal adult tissues, except testis and placenta (9). Several groups have reported expression of MAGE and BAGE genes in NB (10–13). GAGE has broad expression among human NBs, and it is a very specific and sensitive marker for detecting NB in BM and PB (14). Among patients with malignant melanoma, GAGE positivity of BM and/or PB was predictive of survival (15). It may also have utility for measuring microscopic metastasis of breast cancer in lymph nodes (15).

Here, we explored the use of multiple molecular markers for tumor detection, namely GAGE, MAGE-1, MAGE-2, MAGE-3, and MAGE-4, to overcome antigen heterogeneity. We next examined a large cohort of BM from mostly stage 4 NB patients, screening for NB by HIST, IC, and RT-PCR of molecular markers. Because these families of genes are expressed by a wide spectrum of human cancers, including melanoma (5, 8, 16), lung CA (17), breast CA (18), head and neck CA (19), esophageal CA (20), hepatocellular CA (21), urinary bladder CA (22), and gastric CA (23), our findings may have broader clinical implications.

PATIENTS AND METHODS

Patients. Patients with NB evaluated at Memorial Sloan-Kettering Cancer Center were diagnosed and staged in accordance with the International Neuroblastoma Staging System (24). Comprehensive extent-of-disease examinations included computed tomography, 99mTc bone scan, 131I-metaiodobenzylguanidine scan, bilateral BM aspirates (four sites) and biopsies (two...
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The integrity of mRNA was verified by PCR amplification of clonal antibodies as described previously (4). The specificity and sensitivity of IC were previously reported (4).

Tumor samples obtained at surgery were snap frozen for RNA extraction. Total cellular RNA was extracted and cDNA was synthesized as described previously (29).

MONOCLONAL ANTIBODIES

Mononuclear cells were isolated from BMs for these RT-PCR studies. Two hundred fifty-nine BM examinations (aspirate by Wright-Giemsa and biopsy by H&E staining), (b) IC using heparinized BM pooled from four aspiration sites, and (c) molecular detection of tumor markers by RT-PCR.

RESULTS

Expression of GAGE, MAGE, and TH mRNA in Normal BM and PB. By RT-PCR and chemiluminescent detection, BM and PB of normal donors were tested for the presence of these tumor markers. Expression was detected in 0 of 15 BM samples and 0 of 16 PB samples by GAGE mRNA; in 0 of 9 BM samples and 0 of 9 PB samples by MAGE-1, MAGE-2, MAGE-3, and MAGE-4; and in 0 of 17 BM samples and 1 of 17 PB samples by TH. The exquisite sensitivity of molecular detection by GAGE is illustrated in Fig. 1.

<table>
<thead>
<tr>
<th>Gene (Ref.)</th>
<th>Primer sequences</th>
<th>Size of PCR product (bp)</th>
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</thead>
<tbody>
<tr>
<td>GAGE (8)</td>
<td>Sense: 5'-AGACGCTAAGCAGCT3'</td>
<td>239</td>
</tr>
<tr>
<td></td>
<td>Antisense: 5'-CTATTGACACATCTTTA-3'</td>
<td>421</td>
</tr>
<tr>
<td>MAGE-1 (29)</td>
<td>Sense: 5'-CAGGAGACCCGCTAGG-3'</td>
<td>230</td>
</tr>
<tr>
<td></td>
<td>Antisense: 5'-AAATGAGCCGCGAGGCT-3'</td>
<td>423</td>
</tr>
<tr>
<td>MAGE-2 (30)</td>
<td>Sense: 5'-GAAGGAGGAACCTCCTTCA-3'</td>
<td>446</td>
</tr>
<tr>
<td></td>
<td>Antisense: 5'-GAAGGAGGAACCTCCTTCA-3'</td>
<td>299</td>
</tr>
<tr>
<td>MAGE-3 (31)</td>
<td>Sense: 5'-GAAGGAGGAACCTCCTTCA-3'</td>
<td>333</td>
</tr>
<tr>
<td></td>
<td>Antisense: 5'-GAAGGAGGAACCTCCTTCA-3'</td>
<td>533</td>
</tr>
<tr>
<td>MAGE-4 (22)</td>
<td>Sense: 5'-GAAGGAGGAACCTCCTTCA-3'</td>
<td>533</td>
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<tr>
<td></td>
<td>Antisense: 5'-GAAGGAGGAACCTCCTTCA-3'</td>
<td>533</td>
</tr>
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Fig. 1  GAGE detection of a 2-fold serial dilution, from 1:2 (Lane 1) to 1:2^19 (Lane 19), of RT-PCR product from 0.01 μg of total RNA extracted from NB cell line LA-N-1-55N. Lane M = biotin-labeled MW marker. A. ethidium bromide staining; B, chemiluminescence of biotin-labeled PCR product. Arrow, GAGE-specific fragment (239 bp).
tests readily detected NB cells in the BM at time of diagnosis ofd, most (39 of 52) examined at the time of remission. All four were positive for NB by more than one of the four detection patients. Among the 259 BM samples from 99 patients, 207 were positive for NB by all four detection markers. Combining $MAGE$ (Table 4). Detection by HIST was comparable to $GAGE$ and TH detection in three BM samples from stage 4 of $GAGE$ antigen GD2-positive tumor cells, we measured gene expression in $MAGE$ and TH, an established NB marker. Fig. 2 illustrates $GAGE$ and TH detection in three BM samples from stage 4 patients. Among the 259 BM samples from 99 patients, 207 were positive for NB by all four methods, most (39 of 52) examined at the time of remission. All four tests readily detected NB cells in the BM at time of diagnosis (Table 4). Detection by HIST was comparable to $GAGE$ and IC at relapse, but was only 30% (19 of 64) positive on treatment and 16% (17 of 106) positive off treatment. Overall TH expression was also low. There was 38% (24 of 64) positivity during treatment, which dropped to 13% at time of clinical remission (i.e., prior to relapse) when minimal residual NB was of most concern. In contrast, 84% (54 of 64) of BMs sampled during chemotherapy were positive by $GAGE$, and 66% (42 of 64) were positive by IC; 34 BM samples were positive by both $GAGE$ and IC. At time of clinical remission, $GAGE$ and IC provided excellent detection, with 65% (69 of 106) and 49% (52 of 106) positivity, respectively. Twenty-seven marrows had detectable NB by both $GAGE$ and IC.

**Agreement among Detection Methods.** We expect that concomitant positivity by multiple techniques could strengthen the credence of a positive finding. Table 5 summarizes marrow positivity by one, two, three, or all four tests, stratified according to sample timing. As expected, the number of concurrently positive samples was highest at the time of gross disease, e.g., at diagnosis, lower when sampled during chemotherapy, and lowest during clinical remission, a time when NB was minimal. In Table 6, we compared positivity by HIST versus positivity by one, two, or all three tests (IC, $GAGE$, and TH). Again, when there was extensive marrow involvement, such as at time of diagnosis and relapse, even by the stringent criteria of positivity of all three tests, there was general agreement with HIST. Because our analysis was restricted to only sample positivity, no correlation with clinical outcome was attempted.

**DISCUSSION**

Tumor heterogeneity is a critical variable in cancer screening and the design of therapeutic strategies. When it occurs within individuals (e.g., primary versus metastatic) or among...
patients, it severely limits the clinical utility of single antigen/method in tumor detection. The advantage of multiple markers in monitoring circulating tumor cells has been shown in malignant melanoma (27). In NB, we attempt to use multiple molecular markers to overcome tumor heterogeneity plus independent tests to improve sensitivity. When we examined primary tumors of different stages, marker positivity improved from 82% by GAGE alone to 88% by one of the four MAGE genes to 95% when five markers were tested. Thirty-nine of 67 tumors expressed three to four MAGE genes, and only 17 of 67 expressed all four MAGE genes and GAGE. In contrast to previous reports, we found a much higher frequency of MAGE expression in NB (12, 13) and did not confirm the correlation between clinical stage and MAGE (13). MAGE expression in these previous studies was generally low (13% for MAGE-1 and 39% for MAGE-3) when compared to our findings (51% for MAGE-1 and 76% for MAGE-3). This was due to the difference in the sensitivity of detecting PCR products (ethidium bromide staining versus chemiluminescence).

In the clinical management of NB, neuroblasts in the BM signal advanced stage, and their absence is a requisite for successful application of autologous marrow or stem cell transplantation. It is useful to have a series of sensitive screening tests available. However, there was no evidence-based rationale for an efficient algorithm of tests to ascertain the presence of NB in the BM. In this report, by examining 259 BMs using four detection techniques, we concluded that GAGE followed by IC provided an efficient strategy to detect NBs. Critical to the sensitivity of the RT-PCR assay is the adoption of a chemiluminescent method to detect biotin-labeled PCR products, avoiding radiation hazard or carcinogens commonly used in comparable assays (see Fig. 1). However, false positivity of normal BM and PB samples can plague ultrasensitive methods. In this study, blood from one normal volunteer had detectable GAGE. By increasing the sample size of normal subjects, we should be able to ascertain if false positivity is a potential concern, or whether this particular donor was not a true "normal."

Sensitivity of TH RT-PCR in this report was similar to that found by Miyajima et al. (28) at the time of diagnosis. However, although 16 of 28 marrow samples obtained during remission were positive in their study, we found only 14 positive samples in a much larger remission sample size of 145 (Table 4). One possible explanation was the quality of remission between these two study populations. Despite this difference, 69 of 145 were positive by GAGE and 52 of 145 were positive by IC, suggesting that GAGE and IC have superior sensitivity over TH when samples were obtained from patients in remission off chemotherapy. Similarly, sensitivity of HIST was inferior to GAGE or IC. These findings were not unexpected because GAGE and G362 expression were found in most, if not all, stage 4 NBs. In addition, G362 expression was homogeneous among NB cells and between tumor sites, a safeguard against false negativity because of tumor heterogeneity.

In addition to overcoming antigen heterogeneity by testing multiple markers (e.g., GAGE, TH by RT-PCR, and G362 by IC) and increasing sensitivity by multiple independent methods (IC, HIST, and RT-PCR), specificity can be optimized if more stringent criteria are used for tumor detection, such as requiring at least two tests to be simultaneously positive. By this criterion, 48 of 64 (75%) marrow samples in this study were positive for NB patients on chemotherapy, and 40 of 106 (38%) were positive for patients off chemotherapy (Table 5). In contrast, positivity by HIST alone was 19 of 64 (30%) for patients on treatment and 17 of 106 (16%) for patients off treatment (Table 6). It appears that HIST consistently underdetects NB in marrow in >50% of cases. Therefore, it is not surprising that, despite achieving complete clinical remission, more than half of these patients can succumb ultimately to recurrence of marrow disease. With the availability of more sensitive and specific measurements of NB in the marrow, we can now determine with greater precision the relative cleanliness of autologous stem cell harvest, the appropriate timing of stem cell cryopreservation, and the efficacy of purging techniques, as well as myeloablative or other adjuvant therapies.

**ACKNOWLEDGMENTS**

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**REFERENCES**


Detection of microscopic neuroblastoma in marrow by histology, immunocytology, and reverse transcription-PCR of multiple molecular markers.

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