Molecular Detection of Metastatic Melanoma Cells in Cerebrospinal Fluid in Melanoma Patients

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Melanoma frequently metastasizes to the central nervous system (CNS). The diagnosis of CNS metastases typically is made following the onset of clinical symptoms. Thus, more sensitive diagnostic approaches are needed to identify subclinical CNS metastases. Currently, standard cytologic analysis of the cerebrospinal fluid (CSF) is limited by its poor sensitivity. A more sensitive assay was therefore developed using multiple reverse transcriptase-polymerase chain reaction (RT-PCR) markers. CSF was collected and assessed by RT-PCR for three known melanoma-associated markers (MAGE-3, MART-1, and tyrosinase) to detect occult metastatic melanoma cells in the CSF of 37 American Joint Committee on Cancer (AJCC) stage IV melanoma patients. Cytologic analysis of CSF was performed on all patients, and immunohistochemistry (IHC) analysis was performed on 33 CSF samples using anti-S100 and anti-HMB-45 antibodies. Only one patient (3%) had tumor-positive CSF cytology and IHC upon entry into the study, whereas 12 patients (32%) were positive for at least one RT-PCR marker. The correlation between CSF RT-PCR positivity of MART-1 and/or MAGE-3 and the development of CNS metastases at 3 mo was significant (p = 0.04). Fifteen of 37 patients (41%) had either positive MRI and/or positive RT-PCR results. Multimarker RT-PCR is more informative and sensitive than cytology/IHC in assessing the CSF of melanoma patients.

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M melanoma patients with distant metastatic disease often succumb to the development of metastases to the CNS (Wasserstrom et al., 1982; de la Monte et al., 1983; Retas and Gershuny, 1988; Saha et al., 1994; Meyers and Balch, 1998). Cutaneous melanoma has a known propensity to frequently metastasize to the CNS. Brain metastases are identified often (up to 54%) on routine postmortem examination in advanced stage melanoma patients (de la Monte et al., 1983; Saha et al., 1994; Meyers and Balch, 1998). In most cases, the detection of CNS metastases is associated with a very poor prognosis (Meyers and Balch, 1998). Presently, the majority of CNS metastases are identified during routine staging radiographs, or are due to the onset of clinically significant neurologic symptoms. Both clinically symptomatic and radiographically detectable lesions usually require metastatic CNS tumors with a diameter of > 1 cm.

Prolonged survival in patients with limited CNS disease is now possible with the use of new treatment modalities (Legha et al., 1998; Sampson et al., 1998; Sharpless and Das Gupta, 1998; Levine et al., 1999; O’Day et al., 1999; Wen and Loefferl, 1999; Wroński and Arbit, 2000), although the early identification of patients with minimal CNS metastatic disease continues to be problematic.

Currently, the CSF is monitored for occult metastatic tumor cells using standard cytology methods (Bigner, 1992; Bach et al., 1993; Freilich et al., 1995; Galtz et al., 1998; Straathof et al., 1999); however, the efficiency and sensitivity of CSF cytology is very low, and thus is of limited clinical utility (Foulds et al., 1999). Prior studies by our laboratory, and by others, have demonstrated that melanoma cells can be detected in body fluids using a multimarker reverse transcriptase-polymerase chain reaction (RT-PCR) assay, and that this assay can be used to effectively monitor for the development of subclinical metastasis and tumor progression (Hoon et al., 1995, 2000; Kuo et al., 1998; Mellado et al., 1999). The use of a multimarker RT-PCR assay has also been shown to increase the sensitivity of detecting occult disease as compared with a single-marker system (Hoon et al., 1995; Sarantou et al., 1997; Bostick et al., 1999). In this pilot study, we applied a multimarker melanoma RT-PCR assay to detect occult melanoma cells in the CSF of AJCC stage IV patients. The main objective of this study was to determine if RT-PCR analysis of CSF could improve diagnostic sensitivity over conventional cytology/IHC, using mRNA markers for tyrosinase, MART-1, and MAGE-3.

MATERIALS AND METHODS

Patient characteristics A total of 37 patients were studied, including 26 males and 11 females with a median age of 43 y (range: 16–83 y). Five of 37 patients (14%) had metastasis to one organ site, 15 (41%) had metastases to two sites, and 17 patients (46%) had metastases to three or
more sites. Five of 37 patients (14%) had a prior history of treated CNS metastases, including four (11%) with brain metastases and one (3%) with brain and leptomeningeal metastases.

**Specimen collection** This study was approved by the institutional review board at Saint John’s Health Center and the John Wayne Cancer Institute. Specimens of CSF were collected from study patients by standard lumbar puncture (LP). Thirty-seven patients with stage IV malignant melanoma underwent an LP to identify melanoma cells in the CSF using conventional cytologic analysis and RT-PCR. Four of these patients had MRI evidence of metastases upon entry into the study, and one additional patient had previously been treated for CNS metastases with resection followed by gamma knife therapy. Briefly, CSF was obtained, with informed consent, using a standard LP needle inserted at the posterior L4-5 interpace. After establishing opening pressures, CSF was collected for a standard chemistry panel, complete cell count. An additional 2–4 ml of CSF was obtained for standard cytologic examination to rule out micrometastases. The CSF fluid was then cytopun, air dried, and stained with Wright’s stain for cytology analysis by the pathology department. Cell filter and cytopun slides were then subjected to IHC analysis using anti-S100 and anti-HMB 45 antibodies. An additional 5 ml of CSF was set aside for RT-PCR analysis.

**RNA extraction** Total RNA from melanoma cell lines (positive controls) and peripheral blood lymphocytes (PBL) from healthy volunteers (negative controls) were extracted using Tri-Reagent (Molecular Research Center, Cincinnati, OH) as previously described (Bostick et al., 1999). CSF was first concentrated 10-fold using a Centricon-10 concentrator (Amicon, Beverly, MA). The CSF concentrate was then processed using Tri-Reagent LS for liquid samples. Precipitation of CSF RNA was performed using isopropanol supplemented with Pellet Paint Co-Precipitant (Novagen, WI).

**RT-PCR assay** The RT-PCR assay was carried out as previously described (Bostick et al., 1999). One microgram of RNA was used for all patient samples in the study, including the control samples. RNA was incubated at 70°C for 5 min and then put on ice before the addition of RT reaction reagents. The RNA and added RT reagents were then incubated at 37°C for 2 h and then at 95°C for 5 min. All RT reactions were carried out with oligo-dT priming. PCR thermocycling conditions for tyrosinase, MAGE-3, and MART-1 mRNA detection were set up as follows: 1 cycle of denaturing at 95°C for 5 min; followed by 35 cycles of 95°C for 1 min, 55°C for 1 min, and 72°C for 1 min; followed by a final primer sequence extension incubation at 72°C for 10 min. RT-PCR conditions were set up in a thermocycler (Hybaid, Middlesex, U.K.). The RT-PCR cDNA products of tyrosinase, MAGE-3, and MART-1 were detected by electrophoresis, followed by Southern blot analysis using specific individual marker probes (Bostick et al., 1999). Detection of the specific marker cDNA band by Southern blot was considered a positive RT-PCR result. Tumor cell lines and tumor biopsy specimens known to be positive for these markers were included in the assay as positive RT-PCR controls for each experiment. Negative controls consisted of reagents without RNA and normal PBL RNA in each experiment.

**RESULTS**

**Patient characteristics** Patients entered into the study with brain metastases were eligible if they had fewer than five lesions, all of which were < 1 cm in diameter by magnetic resonance imaging (MRI). Patients with larger brain lesions were required to undergo definitive therapy with surgery or stereotactic radiosurgery, followed by a 4-wk period without the development of new CNS lesions. Pre-study evaluation included a complete history, physical examination, and routine laboratory tests. Baseline staging of metastatic disease was assessed by computed tomography (CT) scans of the chest, abdomen, and pelvis, and MRI of the brain. Collectively, the study patients demonstrated widely metastatic disease, with 87% having melanoma metastases disseminated to at least two organ sites. Four patients with a history of treated brain metastases, confirmed radiographically, were entered into this study. Of these four, one patient had previously undergone resection, followed by gamma knife radiosurgery treatment for brain recurrence. No evidence of CNS disease progression was present on the prestudy MRI in this patient, and the patient remains alive and free of evaluable CNS disease at this time.

**Cytology/IHC and RT-PCR analysis** Patients underwent LP upon accrual into the study and the CSF was collected for cytologic and IHC analysis, and for RT-PCR. In four of the 37 patients, sufficient CSF was not obtainable to allow for IHC analysis. Conventional cytologic analysis was performed on all 37 patients. Cytologic analysis identified only one patient of 37 (3%) with melanoma cells in the CSF. This same patient was the only positive patient (3%) among 33 patients tested by IHC staining. This patient was also one of the four patients with MRI evidence of active CNS metastases upon entry into the study, and expressed all three RT-PCR markers in the CSF.

RT-PCR analysis of all 37 CSF specimens was performed for three well-characterized melanoma mRNA markers: tyrosinase, MAGE-3, and MART-1. Of these 37 patients, eight (22%), eight (22%), and six (16%) were positive for tyrosinase, MAGE-3, and MART-1, respectively. Overall, 12 of the 37 (32%) patients were found to express at least one marker. Five (14%) patients were positive for one marker, four (11%) patients were positive for two markers, and three (8%) were positive for three markers. Seven of 37 patients (19%) were RT-PCR positive for two or more markers (Table 1). An additional patient with CSF that was RT-PCR positive as well as cytologically and IHC negative at the onset of this study subsequently became both cytologically and IHC positive 6 mo later.

**MRI and RT-PCR analysis** Of the 37 patients assessed, all underwent MRI prior to therapy. Of these, only four patients (11%) were shown to have detectable CNS metastases by MRI at the onset of this study. Of these four patients, two were RT-PCR positive. A third MRI-positive patient, who was RT-PCR negative, had received stereotactic radiosurgery treatment just prior to CSF analysis.

There were four patients who subsequently developed MRI-detectable leptomeningeal metastases during treatment. Two of these four patients were MRI positive for parenchymal CNS metastases at the start of the study. One of these patients was also positive by cytology and IHC and by all three RT-PCR markers. The other MRI-positive patient who later developed leptomeningeal disease was RT-PCR negative upon accrual, but a subsequent CSF specimen was RT-PCR positive 3 mo later. This
The correlations of CSF RT-PCR positivity with occult CNS disease may allow for the application of more specific therapies at a more favorable stage of CNS involvement. This approach would achieve the establishment of radiographically or clinically detectable lesions (Wasserstrom et al, 1982). Computed tomography alone is not sensitive enough to confirm or exclude leptomeningeal metastases, whereas MRI with gadolinium enhancement is somewhat more sensitive (Fouladi et al, 1999; Straathof et al, 1999; Van Oostenbrugge and Twijnstra, 1999). Despite its inefficiency, the use of standard CSF cytology is still considered one of the diagnostic procedures of choice. CSF cytology remains a very insensitive assay despite the multiple CSF samples that are generally required from an individual patient.

MRI and CT are routinely used to evaluate patients for the presence of metastatic melanoma to the brain; however, these studies are limited by their ability to detect lesions < 1 cm. Four of the patients assessed in this study were MRI positive for CNS metastases. MRI effectively identified CNS metastases in 11% of patients, whereas 32% of study patients had positive CSF by multimarker RT-PCR. A total of 15 of 37 patients (41%) had either positive MRI and/or positive RT-PCR results. Based upon the results of this pilot study, the combination of RT-PCR and MRI together may be more informative than CSF cytology/IHC and MRI in these patients.

The assessment by RT-PCR of CSF fluid appeared to be more sensitive for CNS disease involvement than either MRI or cytology. At 4 y following accrual, 23 of 37 patients (62%) have already developed CNS metastases. Nineteen (51%) had metastases to the brain, three (8%) had metastases to the brain and leptomeninges, and one (3%) had leptomeningeal spread. In assessing the patients who eventually developed CNS metastatic disease during follow-up, CSF analysis by RT-PCR could detect approximately 50% of these patients based upon only a single time point analysis. The monitoring efficacy of RT-PCR could potentially be improved with more CSF samplings at different stages of treatment. Limitations inherent in such a serial sampling protocol in patients with subclinical CNS disease include patient compliance issues and the rare complications associated with multiple LP. A larger trial incorporating serial CSF samplings for cytologic and RT-PCR analysis is needed to further validate this study.

As the multidisciplinary treatment of advanced melanoma continues to improve, and in particular for patients with visceral melanoma metastases, more patients are likely to present with CNS metastases. The longer the duration of metastatic disease in the host, the greater the potential for melanoma CNS metastases to occur. The incidence of melanoma CNS metastases is high, and the poor sensitivity of currently used CSF cytologic studies for early CNS metastasis is well known. Therefore, new techniques are needed to improve the sensitivity of detecting subclinical metastasis to the CNS.

DISCUSSION

This study reports the use of RT-PCR analysis of CSF to identify occult metastatic melanoma cells, and to compare RT-PCR to the diagnostic “gold standards” of cytology/IHC and MRI. The use of multimarker RT-PCR, with specific melanoma-associated markers improves sensitivity over single-marker RT-PCR.

In this study, the overall detection rate of melanoma cells in the CSF by RT-PCR was 32% compared with only 3% by cytology/IHC. The accuracy and sensitivity of standard cytologic and IHC studies of CSF is known to be very poor (Jorda et al, 1998; Fouladi et al, 1999; Straathof et al, 1999). The ability to accurately identify occult tumor cells in the CNS by RT-PCR analysis (i.e., prior to the establishment of radiographically or clinically detectable lesions) may allow for the application of more specific therapies at a more favorable stage of CNS involvement. This approach would achieve its greatest value in high risk patients with clinically undetectable CNS disease. A larger study would be necessary to validate correlations of CSF RT-PCR positivity with occult CNS disease of the parenchyma alone versus those patients with leptomeningeal disease.

Four patients in this study had MRI-documented cerebral metastases, and two of these were RT-PCR positive. One of these four patients was positive for cytology, IHC and for all three RT-PCR markers, and was the only patient of 37 with cytologic and RT-PCR markers, and was the only patient of 37 with cytologic and IHC. The accuracy and sensitivity of standard cytologic and IHC analysis (Fig 1), revealed a clear trend towards greater survival among the 25 patients not expressing melanoma RT-PCR markers in their CSF as compared with the 12 patients expressing one or more of these markers, although this trend did not reach statistical significance (p = 0.07).

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