Molecular Diagnostics of Melanoma Fine-Needle Aspirates

A Cytology-Histology Correlation Study

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Abstract

Patients with advanced-stage melanoma harboring a BRAF mutation are candidates for BRAF inhibition as a therapeutic strategy. The use of fine-needle aspiration (FNA) to diagnose metastatic melanoma is increasing. Studies examining the predictive value of BRAF mutation analysis on melanoma FNAs via correlation with follow-up excision findings are lacking. We examined 37 consecutive FNA cases of metastatic melanoma in which the aspirated lesion was subsequently excised. DNA was purified from Diff-Quik–stained FNA smears and tissue blocks from corresponding excisions in parallel. BRAF mutation status was successfully obtained from both specimen types in 34 (92%) of 37 cases. BRAF mutations were detected in 12 (35%) of 34 cases—11 V600E and 1 V600K. Results of BRAF mutational analysis were concordant in all 34 FNA smear/tissue excision pairs.

Thus, melanoma FNA for molecular diagnostics represents a rapid, minimally invasive, and effective management strategy in this era of precision medicine.

Despite decreased incidence rates for the most common malignancies such as lung, colorectal, and prostate cancer, the incidence of melanoma in the United States continues to increase.1 According to the 2011 Surveillance Epidemiology and End Results (SEER) data, approximately 70,230 individuals were diagnosed with melanoma.2 In 2012, an estimated 76,250 individuals will be diagnosed with melanoma and 9,180 will die of the disease.3 Despite representing fewer than 5% of skin cancer diagnoses, melanoma is the cause of more than 75% of deaths attributed to skin cancer annually.4 Surgery is often effective in the treatment of patients with locoregional disease; however, at least 12% of patients have distant metastases.2

Before 2011, no systemic therapy was found to affect the overall survival rate for patients with metastatic melanoma, which was reflected by a median survival of approximately 8 months.5,6 In 2011, 2 new therapies were approved by the United States Food and Drug Administration for metastatic melanoma: ipilimumab (CTLA-4 inhibitor) and vemurafenib (BRAF inhibitor).7 Recent insights into the molecular pathogenesis of melanoma have highlighted the role of activating BRAF mutations, which are present in approximately 29% to 66% of cases.8-13 The 2 most common mutations involve codon 600, leading to either a V600E or V600K amino acid substitution, the former being more prevalent than the latter.13,14 Vemurafenib is a potent inhibitor of BRAF V600E-mutated melanoma and a vital new therapeutic option for patients harboring BRAF mutations. An interim analysis was conducted of the phase III trial (BRIM-3) on 675 patients with melanomas that were positive for BRAF V600E mutations.
The patients were randomly selected to receive vemurafenib or dacarbazine, and a statistically significant advantage was seen in overall survival, with a relative risk reduction of 63% in the risk of death compared with dacarbazine \( P < .001 \).\(^{15}\)

Fine-needle aspiration (FNA) represents a rapid, relatively inexpensive, minimally invasive means to sample and diagnose metastases in patients with melanoma and potentially prevent more invasive procedures such as surgical excisions.\(^{16-19}\) In advanced-stage melanoma patients with deep-seated lesions that are less accessible for surgical sampling or removal, FNA samples may represent the only opportunity to obtain tissue for pathologic diagnosis and molecular diagnostic analysis for \( \textit{BRAF} \) mutations. We and others have previously demonstrated that FNA material represents an effective platform for \( \textit{BRAF} \) mutation testing.\(^{20,21}\)

Cell blocks prepared from FNA needle rinses are commonly used for ancillary molecular and immunocytochemical studies. Nonetheless, it is not uncommon for cell blocks to exhibit insufficient cellularity for these purposes. As an alternative to FNA cell blocks, we previously reported that both freshly prepared and archived, decoverslipped, Diff-Quik–stained cytologic direct smears represent robust sources of cellular material for \( \textit{EGFR} \) and \( \textit{KRAS} \) mutational analysis in non–small cell lung carcinoma FNAs.\(^{22}\) We then used this method to assess for the presence or absence of \( \textit{BRAF} \) mutations in 15 FNA cases of metastatic melanoma.\(^{20}\) DNA isolated from archived, decoverslipped, Diff-Quik–stained direct smears was suitable for \( \textit{BRAF} \) mutation analysis, and in one instance, a false-negative \( \textit{BRAF} \) mutation assay result was observed in 1 cell block preparation compared with the corresponding direct smear. These studies established the feasibility of molecular testing on FNA samples; however, a question remains: “Are \( \textit{BRAF} \) mutation results obtained from melanoma FNA samples representative of the larger metastatic melanoma excision?”\(^{23}\)

The aims of the current study were 2-fold. First, we sought to expand on our previous study by analyzing Diff-Quik–stained direct smears prepared from additional FNA cases of metastatic melanoma for \( \textit{BRAF} \) mutations. Our second objective was to investigate the predictive value of \( \textit{BRAF} \) mutation status in FNA samples of metastatic melanoma by performing parallel mutational analysis on surgical excisions of the previously aspirated lesions. We found perfect correlation in \( \textit{BRAF} \) mutation status between paired FNA smears and the surgical resection specimens, thereby highlighting the usefulness of FNA-based molecular testing for treating patients with metastatic melanoma.

Materials and Methods

The study was approved by the institutional review board at the University of Michigan, Ann Arbor. The electronic pathology database was searched to identify FNA samples that were diagnosed as positive for melanoma. For each case, the database was then searched to identify follow-up excision specimens. The FNA cytology reports along with the surgical pathology reports of the corresponding excision specimens were examined to correlate the anatomic sites from which the FNA samples were obtained with subsequent surgical excision sites. For the purpose of this study, 37 consecutive cases of metastatic melanoma FNA in which the aspirated lesion was subsequently excised were examined. In all cases, the FNA procedures were performed by cytopathologists in the palpable FNA clinic. Slides for both the FNAs and subsequent excisions were retrieved.

Diff-Quik–stained cytologic direct smears, 1 smear per FNA case, were decoverslipped in xylene as previously reported.\(^{20,22}\) Decoverslipped smears were reviewed (K.D.B. and M.H.R.) and the area containing the highest proportion of tumor cells was marked on the underside of each slide using a marking pen. The size of the marked area and the percentage of tumor cellularity in each area were recorded. Genomic DNA was extracted from cells manually microdissected from the marked areas, ranging from 24 to 100 mm\(^2\).

H&E slides for each follow-up surgical resection specimen were reviewed (M.H.R.). A representative formalin-fixed paraffin-embedded (FFPE) tissue block that exhibited the highest percentage of tumor cellularity was selected for \( \textit{BRAF} \) mutational analysis. Genomic DNA extraction from the FFPE tissue blocks was performed on the BioRobot EZ1 instrument (Qiagen, Valencia, CA) using the paraffin section protocol. One to 2 sections of 10-μm thickness were used for each case. DNA was eluted in a final volume of 25 μL of TE buffer (10 mmol/L tris [hydroxymethyl] ammonium-chloride and 0.5 mmol/L EDTA; pH 9.0).

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\( \textit{BRAF} \) V600E and V600K mutations were assessed using a multiplexed allele-specific polymerase chain reaction (PCR) method. The assay uses individual allele-specific primers for the V600E and V600K mutations along with a control primer set in a single-tube PCR. The control primers amplify a portion of \( \textit{BRAF} \) exon 15 to generate a 155-base pair (bp) product that runs at 151 bp on capillary electrophoresis (CE). This reaction ensures the integrity of the specimen DNA. Also included are 2 allele-specific forward primers: one that is specific for the \( \textit{BRAF} \) c.1799T>A (V600E) mutation and one for the c.1798_1799GT>AA (V600K) mutation. The primers were demonstrated to express high specificity such that the allele-specific amplification products were generated only from specimens that harbored a
V600E or V600K mutation. When either mutation is present, the associated primer pairs with the same reverse primer used in the control PCR reaction to generate a 126-bp (120 bp on CE) product in the case of a V600E mutation, or a 129-bp (123 bp on CE) amplicon in the case of a V600K mutation. The reverse primer used for the production of both the control and allele-specific PCR products is labeled with the FAM (fluorescein) dye. After PCR amplification, the PCR products were resolved using CE on an Applied Biosystems 3130xl genetic analyzer (Applied Biosystems, Foster City, CA). Only specimens exhibiting adequate amplification product in the control reaction were considered interpretable for the presence or absence of the V600E and V600K amplification products. Analytic sensitivity for the detection of V600E and V600K mutations was determined to be between 2.5% and 5% mutant allele using this method.

### Results

Thirty-seven consecutive palpable FNA samples of metastatic melanoma from 37 patients, in which the aspirated lesion was subsequently excised, were examined in this study. Twenty-nine of the lesions were lymph nodes and 8 were subcutaneous soft tissue lesions. The anatomic site of each lesion is listed in Table 1. The archived, coverslipped Diff-Quik–stained cytologic direct smears were stored for 14 to 53 months in the pathology slide archives. After decoverslipping the smears in xylene, manually microdissected areas on the smears ranged from 24 to 100 mm². The percentage of tumor cellularity in these areas was greater than 90% tumor cells in 31 cases, 80% to 90% in 3 cases, and 60% to 70% in 1 case. The age of the archived FFPE blocks for the excised lesions ranged from 13 to 52 months. Based on review of H&E-stained sections from these blocks, the percentage of tumor cellularity in these areas was greater than 90% tumor cells in 31 cases, 80% to 90% in 3 cases, and 60% to 70% in 1 case. The age of the archived FFPE blocks for the excised lesions ranged from 13 to 52 months. Based on review of H&E-stained sections from these blocks, the percentage of tumor cellularity in these areas was greater than 90% tumor cells in 31 cases, 80% to 90% in 3 cases, and 60% to 70% in 1 case.
cellularity across the entire section was estimated to be more than 90%, between 50% and 90%, and between 10% and 50% tumor cellularity in 23, 10, and 4 cases, respectively (Table 1). PCR amplification using genomic DNA isolated from Diff-Quik–stained FNA smears and FFPE excision tissue blocks was successful in 36 (97%) and 35 (95%) of 37 cases, respectively. In 34 (92%) of 37 cases, the BRAF mutation status was successfully obtained from both the FNA and the corresponding excision specimen. BRAF mutations were detected in 12 (35%) of these 34 cases. The V600E mutation was most commonly observed and was present in 11 cases of these pairs that were evaluable (Table 1). One case harbored a V600K mutation. A wild-type result was obtained in the remaining 22 cases. In total, BRAF mutation testing results were concordant for all 34 FNA direct smear/FFPE excision block pairs that were evaluable (Table 1).

BRAF mutation testing results were indeterminate in only 3 cases because of PCR amplification failures. One failure occurred in a direct smear (case 10; Table 1); BRAF mutational analysis on the corresponding FFPE block from the surgically excised lesion revealed the absence of a mutation in this case. The other 2 PCR amplification failures were observed in FFPE tissue blocks (cases 19 and 31; Table 1). In these instances, a wild-type result was observed in the BRAF mutational analyses performed using the corresponding direct smears. Each of the 3 failed samples was associated with more than 90% tumor cellularity (Table 1), and was 21, 34, and 37 months in age, respectively.

Discussion

With the increasing importance of molecular diagnostics in prognostication and guiding the treatment of patients with malignant neoplasms, this testing becomes most relevant in high-stage, surgically unresectable disease. In this setting, minimally invasive, small biopsy procedures can be desirable, and in some instances represent the only means to obtain tissue specimens for diagnosis and molecular testing. In this regard, FNA represents a highly effective method because on-site assessments for cellular adequacy allow for not only the verification of tumor cell acquisition but also for real-time, judicious triaging of cellular material for diagnostically, prognostically, and therapeutically relevant ancillary studies.

An accurate cytologic diagnosis of metastatic melanoma can allow for timely, appropriate staging of patients with this disease and guide subsequent steps in patient treatment. Significant strides have recently been made in our understanding of this disease; specifically, up to 66% of melanomas harbor an activating BRAF mutation that represents a viable target for BRAF-targeted therapy with vemurafenib. FNA samples of metastatic melanoma are often cellular, thereby enabling cytopathologists to reliably diagnose metastatic melanoma. Currently, in this era of precision medicine, cytopathologists are often charged with the task of obtaining sufficient FNA material not only for cytidagnosis but also for relevant ancillary molecular studies.

In this study, we expanded on our previous findings and the findings of others by demonstrating that FNA material from metastatic melanoma lesions is amenable to BRAF mutation analysis. We detected activating BRAF mutations in 12 (32.4%) of 37 overall patients with metastatic melanoma, consistent with prior studies that reported the presence of BRAF mutations in 29% to 66% of melanomas. The majority of BRAF mutations were V600E substitutions; 1 V600K mutation was also detected. This is consistent with our prior observation and the observations of others that the V600E mutation is detected more commonly than V600K in melanoma. Most importantly, we observed a 100% concordance between the mutational status of FNA samples and that of subsequent surgical excisions of these previously aspirated metastases. Specifically, paired BRAF mutation assay results were available in 34 of 37 patients in our study; in all 34 instances, we found no discordance between the BRAF mutation status in the FNA sample and that in the excised, previously aspirated lesion. Of note, paired mutational assay results were not available in 3 of the 37 patients because of PCR amplification failures. An indeterminate BRAF mutation result was observed in 1 (3%) and 2 (5%) of 37 FNA and excision samples, respectively. The reasons for these failures are unclear and in our study did not seem to correlate with the age of the archived material or tumor cellularity. PCR amplification failures are occasionally observed in both cytologic and surgical specimens but at a similarly low rate to what we observed. Billah and colleagues reported their experience in molecular analysis of lung carcinoma FNAs for EGFR and KRAS mutations; smears and cell blocks represented sources of cellular material at their institution, and they observed PCR failures in 22 (11%) of 195 cases. Overall, our results lend further support to the notion that the information obtained from FNA samples is highly representative of the biological properties of sampled melanoma lesions. We extend this to now demonstrate equivalency in BRAF mutation testing results between FNAs and surgical excisions. Because FNA can be performed rapidly in an outpatient setting without anesthesia and is associated with minimal complications, successful FNA sampling is safe and efficient and can prevent unnecessary surgical procedures.

Cell blocks have represented the traditional platform by which cellular material from FNA needle rinses are prepared for ancillary studies. Nonetheless, the occasional instances
Image 1 V600E mutation detected in a fine-needle aspiration (FNA) and subsequent excision specimen (case 36; Table 1). A, Representative photomicrograph of a decoverslipped Diff-Quik–stained smear (×400) analyzed for BRAF mutational analysis. B, Capillary electrophoresis reveals the presence of a V600E mutation in the FNA sample. C, Photomicrograph of an H&E-stained section (×400) of the subsequently excised lesion. D, Capillary electrophoresis reveals the presence of the V600E mutation in the excision specimen. The left gray bands in the capillary electropherograms indicate the fragment locations of the V600E and V600K mutation-specific polymerase chain reaction (PCR) amplicons. The right gray band indicates the fragment location of the control PCR product.

Image 2 V600K mutation detected in a fine-needle aspiration (FNA) and subsequent excision specimen (case 2; Table 1). A, Representative photomicrograph of a decoverslipped Diff-Quik–stained smear (×400) analyzed for BRAF mutational analysis. B, Capillary electrophoresis reveals the presence of a V600K mutation in the FNA sample. C, Photomicrograph of an H&E-stained section (×400) of the subsequently excised lesion. D, Capillary electrophoresis reveals the presence of the V600K mutation in the excision specimen. The left gray bands in the capillary electropherograms indicate the fragment locations of the V600E and V600K mutation-specific polymerase chain reaction (PCR) amplicons. The right gray band indicates the fragment location of the control PCR product.
in which cell blocks are acellular or exhibit sparse cellularity represent a significant impediment to the performance of molecular diagnostic studies. In this study, similar to our previous study, we chose to use Diff-Quik–stained direct smears, instead of cell blocks, prepared from melanoma FNA samples for BRAF mutation analysis. The advantages of this approach center around 2 aspects. First, the use of stained smears allows for a more direct approach of selecting tissue for molecular analysis. Specifically, the pathologist can select the areas of the slides that are enriched in tumor cells, manually microdissect the cells from these areas, and use the material to isolate tumor-enriched DNA. Second, the use of cell blocks is fraught with additional limitations besides the previously mentioned limitation of unpredictable cellularity. For instance, cell blocks prepared from needle rinses represent a pooled specimen derived from multiple needle passes. Sufficient dilution of tumor cells by background benign elements, such as lymphocytes, can thereby potentially lead to false-negative results on molecular assays. Furthermore, cell block cellularity is often assessed in an extrapolative fashion by examining an H&E-stained section prepared from the cell block. The percentage of tumor cells in deeper sections of the cell block used for molecular testing is inferred but not actually known.

In our study, archived decoverslipped smears represented the source of FNA cellular material for BRAF mutation analysis. Similar to our previous observations and those of Killian and colleagues, the act of decoverslipping smears does not appear to have a significant deleterious effect on the performance of DNA-based molecular tests. Nonetheless, the decoverslapping process can be time-consuming. Thus, to ensure that BRAF mutation status for an FNA sample can be obtained expeditiously, it would be more prudent for cytopathologists to anticipate that additional material will be needed for molecular analysis by preparing at least 1 extra Diff-Quik–stained smear from a given needle pass. This can be accomplished simply by distributing the contents of an FNA pass over multiple smears. An extra, uncoverslipped Diff-Quik–stained smear can then be immediately submitted to the molecular diagnostics laboratory for cellular microdissection, DNA isolation, and mutation testing. The advantage of this approach is that it is more time efficient to use cytologic smears for this purpose than FFPE cell blocks and tissue excision specimens. Specifically, preparation of FFPE blocks first requires processing in a tissue processor. Next, paraffin scrolls or unstained tissue sections must be prepared. The formalin-fixed nature of these tissues, along with the required deparaffinization steps, results in a
significant increase in time required for DNA extraction. In contrast, the protocol of isolating DNA from cytologic smears is more direct, requires fewer steps, and can be accomplished within several hours. This difference has significant implications for turnaround time for obtaining molecular diagnostic results. Furthermore, the crosslinking of biomolecules such as nucleic acids by formalin can complicate the isolation of DNA and introduce polymerase “blocks” during PCR.23 Diff-Quik–stained smears are not vulnerable to these complicating factors because air-dried direct smears are not exposed to formalin during the staining process.

It should be noted that in our study we used an allele-specific PCR amplification-based fragment analysis assay. The Cobas 4800 BRAF V600 Mutation Test (Roche Diagnostics, Indianapolis, IN) was not applied to our specimens; nonetheless, the 2 assays are similar in that they both involve PCR amplification using genomic DNA. Furthermore, the analytic sensitivity of the Cobas 4800 assay was reported to be 5% mutant allele;26, the analytic sensitivity of our assay is similar (2.5%-5% mutant allele). The differences between the 2 assays is that our test is designed to detect and distinguish the V600E and V600K mutations whereas the Cobas 4800 test can detect but does not differentiate between these mutations. Overall, we hypothesize that the Cobas 4800 test could be effectively applied to DNA obtained from FNA smears, and that the mutation concordance rates between FNA and excised tissue material would be similar. Finally, our study involved a retrospective analysis of archived patient material; the patients in our cohort were either clinically observed or received other modes of treatment. None of the patients were treated with vemurafenib. Thus, vemurafenib treatment response data were not available for this cohort of patients.

In summary, use of FNA material, especially cytologic direct smears, for molecular analysis can further expedite the effective treatment of patients with metastatic melanoma.

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References


