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Advances in Brief

Prognostic Significance of Circulating Microsatellite Markers in the Plasma of Melanoma Patients

Bret Taback, Yoshiyuki Fujiwara, He-Jing Wang, Leland J. Foshag, Donald L. Morton, and Dave S. B. Hoon[1]

Department of Molecular Oncology [B. T., Y. F., D. S. B. H.] and Division of Biostatistics [H-J. W.], John Wayne Cancer Institute, Saint John's Health Center, Santa Monica, California 90404 [L. J. F., D. L. M.]

Abstract

Purpose: **Multiple genetic alterations including loss of heterozygosity (LOH) occur commonly in melanoma tumors. We demonstrated previously free-circulating DNA microsatellites with LOH in the blood of melanoma patients. These LOH markers in plasma may be useful as surrogates for subclinical disease progression. The purpose of this study was to determine whether the presence of circulating tumor microsatellite markers in the preoperative blood from patients with melanoma has prognostic utility.**

Experimental Design: **Plasma was analyzed for the presence of LOH at six chromosome regions, which are common for allelic loss in melanoma tumors, in 57 patients undergoing surgical resection of all of the clinically apparent disease.**

Results: **LOH was detected in 32 of 57 patients (56%). Both LOH incidence and frequency correlated with advancing American Joint Committee on Cancer stage. In patients with American Joint Committee on Cancer stage III, the presence of LOH as an independent variable in preoperative plasma was significantly associated ($P = 0.05$) with an increased risk of death. Furthermore, LOH at microsatellite marker D1S228 in the plasma of patients with advanced disease correlated significantly ($P = 0.0009$) with a poorer survival after surgical resection. LOH commonly found in melanoma tumors can be successfully identified in the plasma of a patient, providing a potentially less invasive route for following genetic changes that serve as molecular surrogates for assessing subclinical disease progression.**

Conclusions: **This study provides evidence that blood testing for circulating tumor genetic markers may provide valuable prognostic information and guide future therapy.**

Introduction

Melanoma is often characterized by its unpredictable pattern of recurrence and potential for a highly aggressive disease course. Presently, criteria comprising the T, N, and M staging classification is most commonly used to assess patient risk. However, this staging system in melanoma does not account for the presence of subclinical disease, which may adversely impact survival (1–4). Furthermore, the inherent biological diversity among tumors from same-stage patients may result in different rates of progression and therapeutic responses not accounted for by current staging methods. Genetic-based testing offers the opportunity to perform molecular profiling of a tumor, which may predict its biological behavior, individualize patient care, and, therefore, provide supplemental prognostic value to the current staging system. Moreover, because this information can be obtained from minimal disease states, patient care decisions may eventually be made earlier in the disease course with the potential of having a greater impact on overall survival.

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[1] To whom requests for reprints should be addressed, at Department of Molecular Oncology, John Wayne Cancer Institute, 2200 Santa Monica Boulevard, Santa Monica, CA 90404. Fax: (310) 449-5282; E-mail: hoon@jwci.org.

Certain genetic events have been identified that appear to be consistently related to melanoma progression (5–8). Particularly, LOH[2] of DNA microsatellites has been shown to occur commonly among various alleles at specific chromosome loci, such as at *9p21*, in primary melanomas (6, 8). Studies have demonstrated potential prognostic significance for several of these findings. LOH on chromosome 6q has been reported to occur most commonly with thicker primary melanomas suggesting a relation with a more invasive tumor type (8, 9). Clinical correlations have associated LOH on 10q in primary tumors with a worse prognosis (10). LOH on the short arm of chromosome 1 has been noted to occur later in melanoma progression and, therefore, may play a role as a marker of more advanced disease (11). The findings of additional LOH in more advanced tumors (*i.e.*, highly invasive primary lesions and advanced metastasis) suggests that these additional events, not always present in early stages of primary tumors, may be associated with, or representative of, more aggressive tumors that may be of prognostic value (5, 12–14). However, obtaining tumor tissue for genetic analysis may not always be logistically accessible nor is this a practical approach for serially assessing those ongoing tumor genetic events that occur during disease progression.

We have demonstrated previously the detection of multiple microsatellites containing LOH in the blood of melanoma patients, which reflect those genetic alterations that have occurred in their tumors (primary and metastatic; Refs. 15, 16). The frequency of these circulating LOH markers was found to be quite common and was increased in patients with advanced disease. Other investigators have shown that microsatellites containing LOH can be identified in the blood from patients with breast, head and neck, and lung cancer (17–20). To date, no major study in solid nonviral-related tumors has determined any significant clinical utility or prognostic value of these free-circulating microsatellite markers with LOH.

In general, patients with metastatic melanoma have shown inconsistent responses to various therapeutic interventions, which may be attributed to inaccurate assessment of tumor burden and/or heterogeneity among different patient tumor genotypes. At present, surgery remains the mainstay for treating primary tumors and has shown limited success in treating patients with isolated distant metastasis (21–23). However, identification of those select same-stage patients who derive significant benefit from additional interventions is required to improve therapeutic appropriateness. The purpose of this pilot study was to assess the clinical utility of LOH detected in the blood as prognostic markers in melanoma patients undergoing surgical resection for all of the clinically detectable disease.

Materials and Methods

Plasma Sample Collection and DNA Isolation. Five ml of blood were collected in sodium citrate tubes (Becton Dickinson, Franklin Lakes, NJ) from

[2] The abbreviations used are: AJCC, American Joint Commission on Cancer; LOH, loss of heterozygosity.

57 patients of various AJCC stages (7 stage I, 12 stage II, 27 stage III, and 11 stage IV patients) undergoing surgical resection of their melanoma at the John Wayne Cancer Clinic, Saint John's Health Center. All of the patients signed an informed consent form approved by the Institutional Review Board. All of the patients had limited disease based on physical exam and preoperative imaging (which may have included computed tomography, magnetic resonance imaging, and/or positron emission tomography scan) and underwent complete resection of all of the clinically apparent disease. Patient staging was based on postoperative analysis. Blood was collected within 2 weeks before surgery. Blood was drawn, and plasma was immediately separated from cells by centrifugation (1000 × *g*; 15 min), filtered through a 13-mm serum filter (Fisher Scientific, Pittsburgh, PA) to remove any potential contaminating cells. Plasma was aliquoted and cryopreserved at −30°C. Whole blood was spotted on FTA blood stain cards (Fitzco, Minneapolis, MN) for normal genomic DNA controls isolation at a later date. DNA was extracted from plasma using QIAamp extraction kit (Qiagen, CA) as described previously (15, 16). DNA was extracted from 1 ml of plasma from each patient for the study.

Microsatellite Markers, PCR, and LOH Analysis. LOH analysis on plasma was performed without knowledge of clinical criteria or outcome of patients. Eight microsatellite markers on six different chromosomes were selected based on our previous studies on melanoma tumors demonstrating LOH: D1S228 at *1p36*; D1S214 at *1p36.3*; D3S1293 at *3p-3p24.2*; D6S264 at *6q25.2–6q27*; D9S157 at *9p23-p22*; D9S161 at *9p21*; D10S216 at *10q24-q26*; and D11S925 at *11q23.3–11q24*. All of the primer sets were obtained from Research Genetics (Huntsville, AL), and sense primers were labeled with a fluorescent dye: 5-(and-6)-carboxyfluorescein FAM. Genomic DNA (~50 ng) was amplified by PCR in 25-μl reactions containing 1 × PCR buffer (Perkin-Elmer, Foster City, CA), 6 pmol of each primer, 1 unit of Taq DNA polymerase, 2.5 μm deoxynucleotide triphosphates, and 1.5 mM of $MgCl_2$. Forty PCR cycles were performed with each cycle consisting of 30 s at 94°C, 30 s at 50–56°C, and 90 s at 72°C, followed by a final extension step of 72°C for 5 min as described previously (15).

PCR products were electrophoresed on 6% denaturing polyacrylamide gel containing 7.7 M urea at 1600 V for 2 h. Genomyx SC scanner (Beckman Coulter, Fullerton, CA) was used to image the fluorescence-labeled PCR products, and densitometric analysis was performed with ClaritySC software (Media Cybernetics, Silver Spring, MD). Intensity calculations and comparisons of the specific alleles in normal control and respective plasma DNA of the patients were performed to evaluate for LOH. The LOH was defined if a >50% reduction of intensity was noted in one allele when compared with the respective allele in the matched-paired lymphocytes (15, 24).

Statistics. Spearman correlation was estimated to correlate the association of number of markers and known melanoma prognostic factors and AJCC stage. Multivariate analysis using the Cox proportional hazard regression model was performed to examine the association of clinicopathological risk factors and LOH markers with survival. Wald test was used for determining the statistical significance, and the risk ratio was determined at the 95% CI in this model. AJCC stage, Breslow thickness, gender, age, and LOH were assessed unless otherwise noted using the Cox proportional hazard regression model. Survival curves were constructed using the Kaplan-Meier method, and log-rank test was used to test the equality of the curves. A 0.05 two-sided significance level was used for determination of statistical significance (25).

Results

LOH was detected in 32 of 57 (56%) preoperative plasma samples assessed from informative patients. In the majority of those patients, 14 of 32 (44%), LOH was present for only one marker. Eleven patients demonstrated LOH for two microsatellite markers (34%), 5 patients for three markers (16%), and 2 patients for four markers (6%). In 25 patients (44%), LOH was not identified at any of the markers tested. LOH was most commonly identified at microsatellite marker D11S925 occurring in 11 of 35 informative patients (31%). The incidence of LOH at D9S157 and D9S161 were both 24%, occurring in 10 of 41 informative patients and 9 of 37 informative patients, respectively. LOH in informative patients occurred with decreasing frequency at D10S216 (23%), D6S264 (21%), D1S214 (20%), D1S228 (18%), and D3S1293 (9%), respectively. No LOH was detected in any of the plasma samples collected from the blood of 40 normal donors.

Microsatellite Marker Correlation with AJCC Stage. Microsatellite markers for LOH were assessed for correlation to the following clinicopathological prognostic parameters: age, gender, anatomical site of the primary tumor, Clark level, Breslow thickness, and AJCC stage. Only stage correlated significantly with the overall incidence and frequency of LOH in plasma, as well as the specific marker demonstrating LOH. An increasing incidence of LOH correlated significantly with advanced AJCC stage ($P = 0.004$). Five of 19 (26%) patients with stage I or II disease demonstrated LOH for at least one marker whereas 17 of 27 (63%) stage III patients and 10 of 11 (91%) patients with stage IV disease had positive plasma samples for LOH. As expected, the frequency of microsatellite markers containing LOH significantly increased from stage I to IV ($P = 0.0001$). Most noticeable, was the increase in frequency of LOH for specific microsatellite markers D9S157 and D1S228. D9S157 was detected in 0 of 14 stage I and II patients, whereas the plasma of 4 of 19 (21%) stage III and 6 of 8 (75%) stage IV patients contained LOH for this marker. Similarly, D1S228 was present in the plasma of only 1 of 14 (7%) stage I and II patients, whereas 2 of 19 (11%) stage III and 4 of 6 (67%) stage IV patients demonstrated allelic loss at this loci.

Microsatellite Marker Correlation with Survival. To determine whether microsatellite markers for LOH demonstrated any prognostic utility, marker expression was correlated to patient survival after surgery. Median follow-up was 21 months. Relapse in stage I and II patients was uncommon during our follow-up period. No stage I and only one stage II patient developed a recurrence. No LOH was detected in the preoperative plasma from the patient who developed the early recurrence. Longer follow-up is necessary to determine whether these plasma DNA markers demonstrate any prognostic utility in these early-stage patients. In contrast, patients with advanced disease demonstrated a poorer prognosis with 7 of 27 (26%) stage III and 5 of 11 (45%) stage IV patients expiring postoperatively during the follow-up period. There was a significant correlation between LOH presence, survival, and AJCC stage (Fig. 1).

The detection of LOH for any of the microsatellite markers assessed was associated with a progressively worse prognosis in patients with advanced disease. Particularly, AJCC stage III patients expressing LOH in their plasma had a significantly increased risk of death as compared with same-stage patients without LOH (Wald test; $P = 0.05$; risk ratio, 3.14; CI, 1.0 and 10.1). Specific microsatellite markers were evaluated for correlation with overall patient survival to

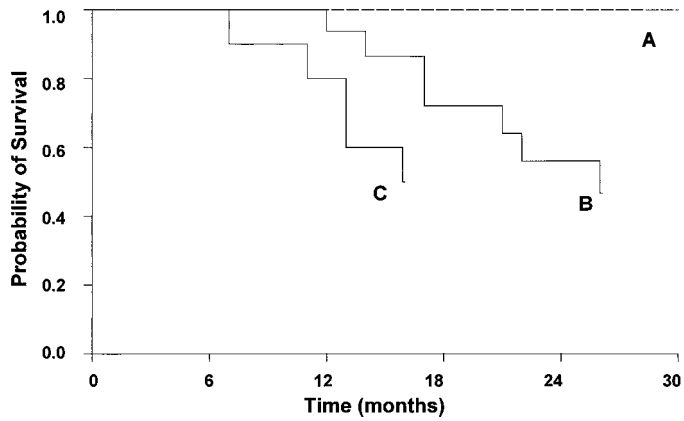

Fig. 1. Correlation of ≥1 microsatellite marker with LOH in plasma and overall survival. *Group A*, AJCC stage III and IV LOH (−). *Group B*, AJCC stage III patients that were ≥1 marker with LOH (+). *Group C*, AJCC stage IV patients that were ≥1 marker with LOH (+). Kaplan-Meier curves demonstrate that all three categories were significantly different (Log rank test; $P = 0.0023$).

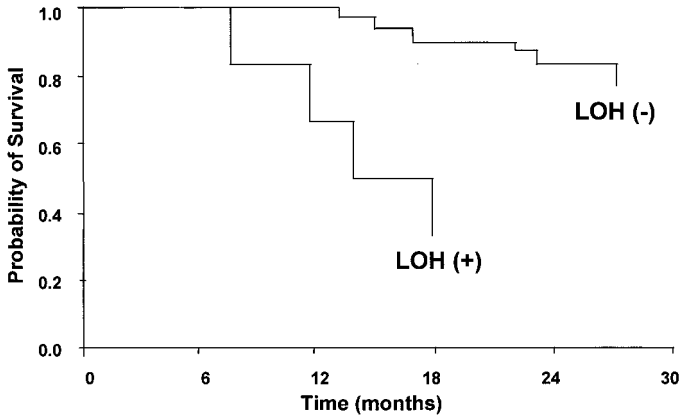

Fig. 2. Correlation of LOH at D1S228 with survival in AJCC stage III/IV patients. Kaplan-Meier curves demonstrate patients with D1S228 LOH are at increased risk (Log rank test; $P = 0.0009$) compared with no LOH at D1S228.

determine prognostic significance. When controlling for stage, LOH on D1S228 was associated with a shorter postoperative survival in advanced-stage patients after surgical resection of all of the clinically apparent disease (Wald test; $P = 0.0009$; risk ratio, 6.2; CI, 1.57 and 24.4; Fig. 2). LOH on D9S157 was associated with a poorer prognosis but did not reach statistical significance in multivariate analysis. However, the combination of LOH at D9S157 and D1S228 was associated with a poorer prognosis when controlling for stage (III/IV) risk (Wald test; $P = 0.044$; risk ratio, 3.92; CI, 1.03 and 14.88; Fig. 3). In those cases where plasma samples did not contain LOH for either of these two microsatellite markers, only 4 of 21 (19%) stage III and none of the two stage IV patients expired, whereas 3 of 6 (50%) stage III and 5 of 9 (56%) stage IV patients expired whose plasma samples were positive for LOH at both of these markers.

Discussion

Once melanoma has metastasized, overall prognosis is generally poor. Methods to more accurately assess this risk include histopathological evaluation of the primary tumor and draining lymph nodes along with serial radiographic imaging studies to survey for distant metastasis. Although these techniques have permitted a more accurate appraisal of tumor biology by precisely staging patients, it is not uncommon for the same-stage patients to have differing disease courses. Improved methods are needed to identify those same stage patients who may benefit from a more aggressive treatment approach. Assessment of tumor DNA may provide more accurate insight into tumor progression and, thus, allow for the identification of molecular surrogates that precede and predict clinical events (26).

We and others have shown that as melanomas progress they acquire additional LOH events, which may be associated with a metastatic or more aggressive phenotype (5, 6, 10, 13, 14, 24). Therefore, identifying those ongoing tumor genetic events early during disease progression may permit a more accurate method of predicting tumor behavior and provide genetic markers with clinical prognostic utility. Studies have attempted to correlate LOH in melanoma tumors with clinical outcome (10, 15). However, this requires the availability of primary tumor tissue that may not always be available for analysis and may not reflect those ongoing genetic events that occur during later stages of disease progression. Evaluating metastasis may be more relevant but currently requires the presence of a significant tumor volume for detection (either clinically or radiographically) and an invasive biopsy procedure, which may be inaccessible and/or associated with the potential for complication. Furthermore, this technique is not practical for continuously evaluating genetic changes during disease progression. Evaluation of circulating DNA microsatellites in the blood of cancer patients offers a convenient method to assess for the presence of subclinical tumor burden and monitor those genetic changes that may provide early insight into the disease status of a patient. However, to date no clinical utility or prognostic value has been determined by these free-circulating DNA markers. Recently, studies have shown other forms of circulating DNA in plasma such as viral DNA of nasopharyngeal carcinoma patients (27) and K-ras mutated DNA in colorectal carcinoma patients (28) have clinical utility outcomes. Recently, we have demonstrated the feasibility of detecting tumor-related LOH in the plasma from patients with melanoma and showed a correlation with disease progression and tumor (15). A 91% correlation (21 of 23 patients) between plasma LOH marker detected and the LOH in the corresponding tumor specimen was observed.

To evaluate the prognostic significance of circulating DNA microsatellites demonstrating LOH, preoperative blood was assessed from patients with melanoma undergoing surgical resection of all of the clinically evident disease. The presence of LOH in the blood of melanoma patients is a common finding occurring in 56% of patients assessed. The increased incidence of LOH correlated significantly with advanced AJCC stage of disease. Twenty-six percent of early-stage (I and II) patients demonstrated LOH in preoperative blood samples in contrast with 71% of patients with advanced disease (stage III and IV). These findings likely reflect the increased tumor burden associated with more advanced-stage disease. Furthermore, the patient frequency of LOH was significantly greater in those patients with more advanced-stage disease. An average of 0.37 LOH/stage I and II patients was detected, whereas 1.0 LOH/stage III patient and 2.2 LOH/stage IV patient was present. These findings ascribe to similar observations that demonstrate an increased frequency of LOH in metastatic tumors as compared with their respective primary tumors.

After a median follow-up period of 21 months, no stage I and only one stage II patient recurred. No LOH was detected in the preoperative plasma from this patient with early relapse. However, for the majority of these patients the overall outlook is good, and failure after surgical excision of the primary tumor is not common. The assessment of serial bleeds in a larger cohort of patients is currently under investigation to determine the prognostic value of circulating genetic markers in early-stage melanoma.

LOH at microsatellite markers D1S228 and/or D9S157 was associated with a worse prognosis in patients with advanced disease after surgical resection. In addition, LOH at D1S228 maintained predictive

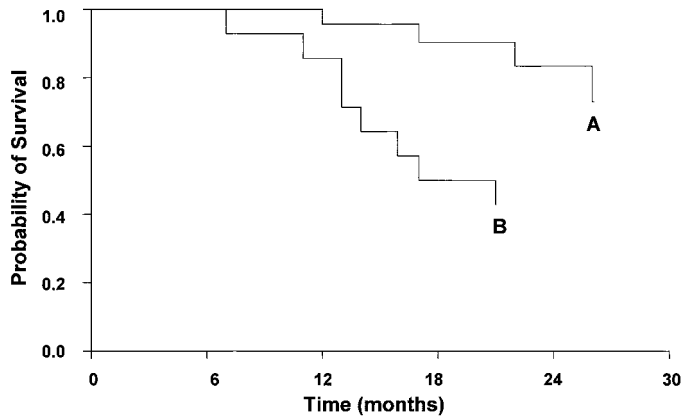

Fig. 3. AJCC stage III/IV patient survival correlation for D1S228 and D9S157 LOH detection in plasma. *Group A,* AJCC stage III/IV LOH (−) for both markers. *Group B,* AJCC stage III/IV LOH (+) for both markers. Kaplan-Meier curves demonstrate patients with LOH in both markers are at significant increased risk (Log rank test; $P = 0.017$) compared with no LOH at either of these markers.

value in multivariate analysis when controlling for stage. These clinical findings appear to be supported by molecular mapping studies demonstrating the presence of potential tumor suppressor genes in these chromosomal regions, and that may influence tumor progression.

D9S157 is isolated to chromosome *9p21–22*, which is one of the most commonly studied deletion regions in melanoma (6–8, 29, 30). It has been shown to occur in up to 60% of cases, and loss of this region has been linked to the tumor suppressor gene CDKN2A that is involved in cell cycle arrest (31) and other potential unidentified tumor suppressor gene(s) (Ref. 32). In one study, evaluating LOH in primary tumors for clinical correlation did not identify a prognostic value for this particular marker. This may be attributable to its frequent expression in lesions of all thickness (10). The LOH markers at 9p may not be useful as prognostic marker(s) alone because of their high frequency, but in combination with other LOH markers they may have more clinical utility (15).

Allelic losses at chromosome 1p have been shown to occur in certain types of tumors such as neuroblastoma, breast cancer, and gastrointestinal tumors and have been associated with a poorer patient prognosis (33–36). In melanoma, LOH on chromosome 1p has been shown to occur later in tumor progression, but the clinical implications have yet to be evaluated. In this study we demonstrate that as a LOH marker in plasma it is significantly associated with a worse outcome in patients with more advanced disease and in combination with other melanoma-specific genetic markers may identify potential aggressive disease.

Presently, methods to stage advanced disease rely on radiographic imaging, and this study provides evidence to suggest that the detection of circulating tumor-associated DNA may aid current staging techniques. Additionally, this will allow for the assessment of a "serial genetic profile" of tumor progression that may prove highly predictive. Genetic markers identified in plasma and associated with tumor progression may provide a rapid method of serially monitoring patients for those genetic events that precede clinical evidence of advancing disease.

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