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Molecular Clonality of In-Transit Melanoma Metastasis

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In-transit melanoma is characterized by an aggressive pattern of recurrence that is associated with a poorer prognosis. Because in-transit melanoma is considered to result from the intralymphatic trapping of melanoma cells between the primary tumor and regional lymph nodes, it provides an excellent model to assess genetic events associated with early metastasis. The hypothesis of this study was to determine whether in-transit metastases are clonal in origin and therefore, may have specific genetic alterations uniquely associated with this disease and the development of early metastasis. This was assessed using loss of heterozygosity (LOH) analysis for specific DNA microsatellite loci. Seventy-nine paraffin-embedded in-transit melanoma lesions from 25 patients (range, 2 to 9 lesions per patient; average, 3.4 lesions per patient) were assessed for LOH using eight microsatellite DNA markers on six chromosomes. In 19 of 25 patients (76%) LOH was demonstrated for at least one marker. The most frequent microsatellite marker demonstrating LOH was D9S157 (56%). Using LOH microsatellite markers to assess intertumor heterogeneity, six of 79 tumors (7.6%) demonstrated different profiles when compared to other lesions from the same patient. In-transit metastases from those patients demonstrating intertumor heterogeneity were further assessed using laser capture microdissection and DNA analysis, and revealed no significant intratumor heterogeneity. In conclusion, LOH was frequently observed in in-transit melanoma metastasis. Based on LOH analysis, in-transit metastases are clonal in origin. The establishment of clinically successful in-transit melanoma metastasis requires specific genetic events that seem to be unique and homogeneous for each patient. ***(Am J Pathol 2001, 158:1371–1378)***

In-transit melanoma is characterized as a distressing pattern of melanoma recurrence and is associated with progressive disease culminating in systemic metastasis and death.[1] Locoregional recurrence is the most common site of metastases after treatment for primary melanoma and 12 to 22% of patients who relapse are at risk for developing this aggressive form of disease.[2–4] Factors predisposing patients to the development of in-transit recurrence are mostly pathological and include the anatomical location of the primary tumor (extremity *versus* trunk) and whether the primary lymph node basin is positive for tumor clinically or histologically.[5] Furthermore, the number of positive lymph nodes and their proximity to the primary tumor are also associated with an increased risk for in-transit recurrence.[6] These observations seem to support the clinical findings that in-transit melanoma is a result of arrested tumor emboli in potentially congested intradermal lymphatic vessels situated between the primary lesion and the first major lymph node basin. At present, no molecular markers exist to identify patients at risk for developing this form of disease. More so, genetic events characterizing this specific disease entity have not been previously described.

Treatment of this disease form is difficult as patients are plagued with locally recurrent, chronic, and medically refractory lesions that may number anywhere from one to >100. In addition, in-transit recurrence is often indicative of occult systemic disease progression and therefore suggests why more aggressive forms of locoregional therapy (ie, limb perfusion, amputation) are for the most part ineffective in prolonging overall patient survival.[7–10] Because of the cutaneous nature of in-transit recurrence these tumors are quite readily accessible even at small sizes (<1 cm). This unique type of metastatic disease recurrence provides an excellent *in vivo* model to study molecular events associated with locoregional tumor metastasis, a phenomenon in melanoma that is often a harbinger of systemic disease progression. Many common genetic aberrations such loss of heterozygosity (LOH) of DNA microsatellites have been reported in melanoma.[11–14] Numerous studies have shown that primary melanomas most frequently demonstrate LOH on chromosome 9 in the region of the *p16INK4* gene, a tumor suppressor gene involved in the development of melanoma.[15,16] Some have noted these findings to occur in primary tumors of all depths of invasion suggesting this

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event as an early occurrence in the tumor progression sequence.[13] In addition, LOH on chromosome 10q in the region of the potential tumor suppressor gene *PTEN/MMC1* was also noted to occur commonly in thin primary melanomas suggesting that this was an early event as well.[12] However, others have demonstrated that LOH in this same region occurs more commonly in advanced stage tumor samples or more aggressive primary tumors, suggesting a later role in tumor progression.[17,18] Another contentious tumor suppressor gene locus has been confined to a narrow region on 6q where significant LOH has been found to occur commonly in metastatic tumors and to a lesser degree in primary lesions >1.5 mm thick suggesting a relationship to melanoma progression.[12,19]

Although a model of melanoma tumorigenesis based on allelic losses has been proposed, clinicopathological correlations are lacking.[12,20] For the most part, many of these studies are small and the tumors evaluated were either primary lesions or advanced metastasis (from random sites) obtained arbitrarily to perform genetic studies.[19,21,22] The extensive diversity of tumor samples assessed among these studies often varied by stage as well as their sites of metastasis limiting any correlation of these molecular events to clinicopathological factors of disease. This may be particularly relevant in malignant cutaneous melanoma as the staging system was recently amended to reflect the impact site of metastasis has on survival.[23,24] Furthermore, because advanced metastasis may contain additional LOH events (ie, distal arm of 1p and 11q23) not identified or infrequent in primary tumors, the role of these findings is unknown.[25,26] Particularly, of interest is at which point in the course of tumor progression do these events occur and are they clinicopathologically relevant. Identification of those genetic events associated with early stages of metastasis offers the advantage of establishing potential tumor markers to assess patient risk and monitor subclinical disease. To date, no study has assessed the genetic changes specifically associated with in-transit. The present study was undertaken to identify incidence and location of allelic loses in in-transit metastasis, a specific clinical entity associated with an early and aggressive form of melanoma progression.

In this report, we analyzed 79 paraffin-embedded in-transit melanoma tumors from 25 patients for LOH using eight microsatellite markers on six different chromosomes. In addition, in 10 patients matched primary tumor paraffin-embedded blocks were available for DNA analysis. Results were assessed for molecular clonality. Patient tumors discordant for LOH genotyping were assessed for intratumor heterogeneity using laser capture microdissection (LCM).

Materials and Methods

Specimens

Seventy-nine in-transit melanoma tumors from 25 patients (range, 2 to 9 lesions per patient; average, 3) diagnosed with AJCC stage III in-transit melanoma were collected from the Department of Pathology at the St. John's Health Center. Anatomical locations of the in-transit tumors in evaluated patients were as follows: two trunk, five head and neck, and 18 extremity. Tissues were selected from patients seen at the John Wayne Cancer Institute from 1991 to 2000 who had paraffin blocks available for at least two or more in-transit melanoma tumors. In addition, 10 patients had matched primary tumor paraffin-embedded blocks available for assessment.

Microdissection and DNA Isolation

Ten-μm sections were cut from paraffin-embedded tumors and floated onto glass slides. Tumor lesions were microdissected from adjacent normal tissue to avoid contamination. Pathological review was performed on all specimens using matching hematoxylin and eosin-stained slides for comparison.

DNA was extracted using QIAamp tissue kit (Qiagen, Valencia, CA). Control DNA was obtained from either peripheral blood lymphocytes (PBLs) using the QIAamp blood kit or from histologically confirmed normal tissue from the same surgical specimen.

Microsatellite-Polymerase Chain Reaction (PCR) LOH Analysis

Eight primer sets on six different chromosomes were selected for PCR amplification. These markers were selected because they showed a high incidence of LOH either in primary melanoma tumors and/or advanced metastasis.[13,14,22] The following FAM-labeled microsatellite markers were used in this study: D1S214 at 1p36.3, D1S228 at 1p36, D3S1293 at 3pter-3p24.2, D6S264 at 6q25.2-q27, D9S157 at 9p23-p22, D9S304 at 9p21, D10S212 at 10q26.12-q26.13, and D11S2000 at 11q22-q23. PCR primer sets for specific allele loci were obtained from Research Genetics, Inc. (Huntsville, AL). Genomic DNA (~50 ng) extracted from paraffin-embedded tumors and matching PBLs was amplified using PCR in a 25-μl reaction volume, containing 10 mmol/L Tris-HCl, pH 8.3, 10 mmol/L KCl, 1.5 mmol/L $MgCl_2$, 200 μmol/L deoxynucleotide-triphosphates, 0.25 μmol/L forward primer, 0.25 μmol/L reverse primer and 0.5 U of AmpliTaq Gold DNA polymerase (Perkin Elmer, Norwalk, CT). PCR cycles consisted of 30 seconds at 94°C, 30 seconds at 50 to 56°C depending on the primer sets, and 90 seconds at 72°C for a total of 40 cycles. This was followed by a 5-minute final extension at 72°C. PCR products were electrophoresed on a 6% denaturing polyacrylamide gel. The gel was scanned by a fluorescent/optical GenomyxSC scanner (Beckman Coulter, Inc., Fullerton, CA). Densitometry was performed on the gel images and analyzed using ClaritySC 3.0 software (Media Cybernetics, Silver Spring, MD). The tumor was scored as exhibiting LOH if there was a 50% or greater reduction in signal intensity of one allele when compared to the respective allele in the corresponding normal DNA.[14]

Table 1. Microsatellite Analysis of In-Transit Melanoma

Microsatellite markers	Chromosome location	LOH frequency*
D1S214	1p36.3	6/19 (32%)
D1S228	1p36	2/19 (11%)
D3S1293	3pter-3p24.2	2/21 (10%)
D6S264	6q25.2-q27	4/18 (22%)
D9S157	9p23-p22	9/16 (56%)
D9S304	9p21	8/17 (47%)
D10S212	10q26.12-13	6/17 (35%)
D11S2000	11q22-q23	9/23 (39%)

*Frequency of LOH in informative cases.

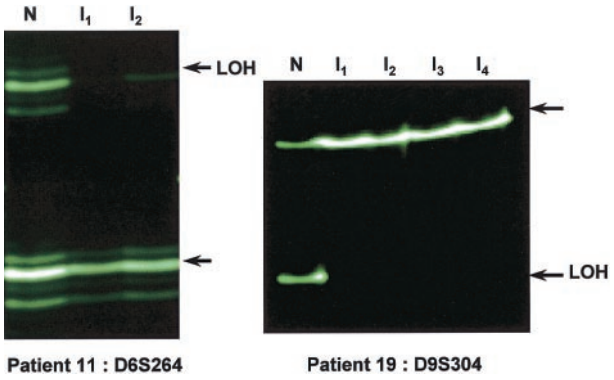

Figure 1. Representative analysis of LOH in in-transit melanoma metastasis (patients 11 and 19) for microsatellite markers (D6S264 and D9S304). Analysis was performed on DNA obtained from paired paraffin-embedded in-transit tumors (I_1, I_2, and so forth) and normal tissue from the same histopathological section or PBLs (N). **Arrows** denote respective alleles; LOH marked.

LCM and DNA Extraction

Patients demonstrating discordant LOH profiles were assessed for intratumor heterogeneity using LCM. Three random and discrete regions from each tumor were selected for LCM and DNA extraction. Amplitude, pulse duration, and number of hits were adjusted to capture $\sim 4 \times 10^6$ μm^3 of tissue. DNA was isolated with 100 μl of Proteinase K (0.04% of Proteinase K, 10 mmol/L of Tris-HCL, pH 8.0, 1 mmol/L of 1% Tween) at 42°C overnight, followed by heat-denaturing of Proteinase K at 95°C for 10 minutes. PCR and LOH analysis was performed as previously described.

Results

LOH Analysis of In-Transit Melanoma Lesions

Microsatellite analysis of DNA isolated from 79 in-transit melanoma lesions obtained from 25 patients was performed using eight microsatellite markers on six chromosomes. In 19 of 25 patients (76%) LOH was demonstrated for at least one microsatellite marker. In the informative cases, D9S157 showed the highest LOH frequency (56%) followed by D9S304 (47%), D11S2000 (39%), D10S212 (35%), and D1S214 (32%) (Table 1). Representative LOH in in-transit melanoma lesions as compared with respective control lymphocyte DNA is shown in Figure 1.

On average each in-transit lesion demonstrated LOH for two chromosome loci. An analysis of the incidence of LOH in in-transit metastasis was as follows: 17 lesions (22%) demonstrated no LOH for any of the markers assessed, 13 lesions (17%) demonstrated LOH for one marker, 24 lesions (30%) for two markers, 20 lesions (25%) for three markers, one lesion (1%) for four markers, and four lesions (5%) for five markers (Table 2).

Intertumor Heterogeneity Analysis

Only six of the 79 lesions (7.6%) analyzed demonstrated different LOH profiles as compared to other lesions from the same patient. Results were repeated to confirm the findings. This lack of heterogeneity among in-transit lesions occurred in six patients. There was no specific microsatellite marker associated with this intertumor heterogeneity. Four of six patients demonstrated a differential loss or retention of one microsatellite marker per lesion in comparison to their other lesions. In two patients, two additional LOH events occurred: one patient demonstrated allelic loss at loci D1S214 and D1S228, most likely indicating a large deletion on the short arm of chromosome 1 and the other patient demonstrated allelic loss at two different chromosomal markers (D6S264 and D11S2000) (Table 3). Interestingly, in both patients, the additional LOH occurred in the same in-transit lesion. To determine whether these in-transit tumors were composed of cells with LOH genotypes matching either of the differing in-transit tumors, intratumor heterogeneity analysis was performed using LCM.

Intratumor Heterogeneity Analysis

Intratumor heterogeneity was assessed among the 26 lesions in the six patients who demonstrated intertumor heterogeneity. Tumor cells were captured from three different regions for each tumor, DNA was extracted, and PCR was performed to assess for LOH at the discordant microsatellite markers (Figure 2). These findings were then compared to the original LOH status of the tumor obtained from whole tumor DNA analysis. Six of 26 (23%) in-transit lesions demonstrated intratumor heterogeneity for at least one microsatellite marker (Table 4). All patients, except one, in which intertumor heterogeneity was identified maintained one in-transit metastasis that when assessed, demonstrated intratumor heterogeneity for the

Table 2. Frequency of Microsatellite Markers for LOH

Number of markers	LOH frequency* (n = 79)
0	17 (22%)
1	13 (17%)
2	24 (30%)
3	20 (25%)
4	1 (1%)
5	4 (5%)

*Frequency of LOH in informative cases.

Table 3. Intertumor Heterogeneity Among In-Transit Metastases from Melanoma Patients

Patient	Microsatellite marker	LOH profiles of in-transit melanoma metastasis								
		I_1	I_2	I_3	I_4	I_5	I_6	I_7	I_8	I_9
7	D9S304	R	L	L	L					
10	D1S214	L	R	R	R					
18	D10S212	R	L							
19	D1S214	R	R	R	L					
	D1S228	R	R	R	L					
20	D6S264	R	L	R						
	D11S2000	R	L	R						
23	D11S2000	L	L	L	L	L	R	L	L	L

$I_{(n)}$, in-transit metastasis; R, retained; L, LOH.

allele in question. In the two patients demonstrating intertumor heterogeneity for two LOH markers, intratumor heterogeneity was identified in the same in-transit metastasis for each marker. In general, minimal intratumor heterogeneity was noted for those in-transit metastases demonstrating intertumor heterogeneity.

LOH Analysis in Matched Primary and In-Transit Lesion Pairs

In 10 patients corresponding primary tumor blocks were available for LOH assessment. Five patients (50%) demonstrated the acquisition of an additional LOH in an in-transit metastasis not noted in the primary tumor (Table 5). In four of these patients, in-transit tumors demonstrated LOH at one additional marker, and one patient's tumor for two additional markers. No intratumor heterogeneity was noted in any of the three primary tumors (patients 7, 18, and 20) where the corresponding metastasis demonstrated intertumor heterogeneity.

Among the 79 in-transit melanoma metastases assessed using eight microsatellite markers, six demonstrated different LOH profiles (four at one marker and two at two markers). In only eight instances was the LOH status different as compared to 624 occurrences in which the LOH findings were similar. Therefore, the probability that an in-transit metastasis would vary in its LOH status for any of the molecular markers that were assessed with respect to its corresponding in-transit tumor is 1.3% (8 of 632). These findings provide genetic evidence for clonality among metastasis associated with this exclusive clinicopathological disease.

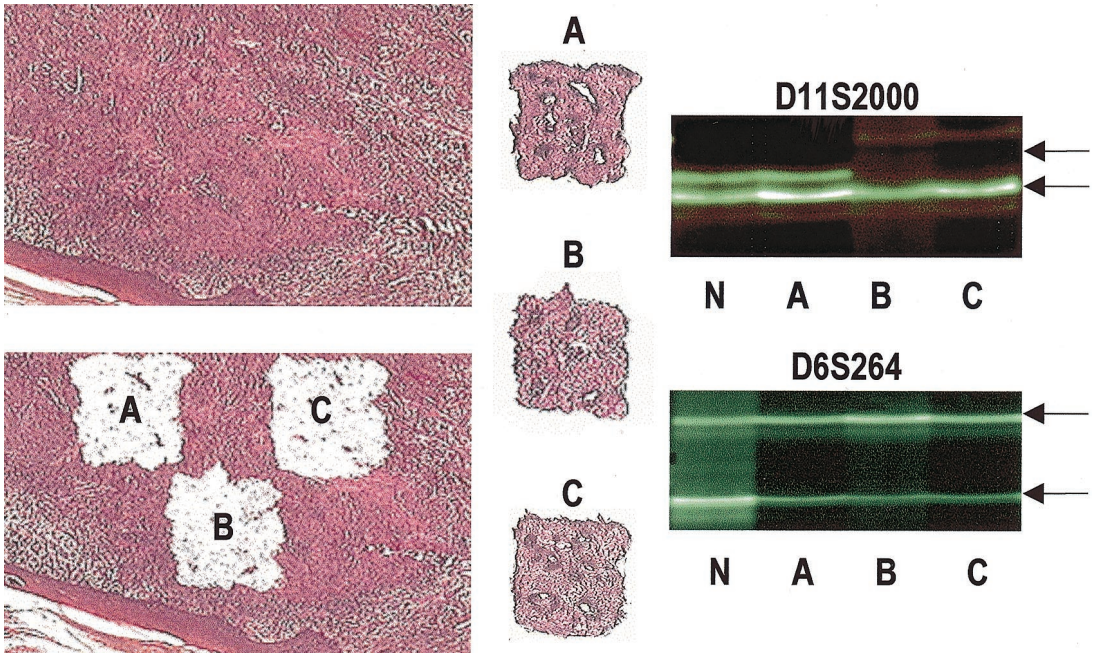

Figure 2. In-transit melanoma metastasis (H&E stain; original magnification, ×10) demonstrating intratumor heterogeneity (**top right**) or homogeneity (**bottom right**), characterized as the retention or loss of alleles at two microsatellite loci (D11S2000 and D6S264). Representative tumor tissue specimens (**middle: A**, **B**, and **C**) were obtained from three regions of an in-transit tumor (**left**) using LCM. N, normal tissue or PBLs. **Arrows** denote respective alleles; LOH for D11S2000 was noted in **B** and **C** (**top bands**), and for D6S264 in **A**, **B**, and **C** (**bottom bands**).

Table 4. Intratumor Heterogeneity within In-Transit Metastases in Patients with Intertumor Heterogeneity

Patient	Microsatellite marker	LOH profiles of in-transit melanoma metastasis $\left(\frac{\text{Total tumor}}{\text{Microdissected regions}}\right)$								
		I_1	I_2	I_3	I_4	I_5	I_6	I_7	I_8	I_9
7	D9S304	R RRR	L LRL	L LLL	L LLL					
10	D1S214	L LLL	R LRR	R RRR	R RRR					
18	D10S212	R RRR	L LRL							
19	D1S214	R RRR	R RRR	R RLR	L LLL					
	D1S228	R RRR	R RRR	R RRL	L LLL					
20	D6S264	R RRR	L LLL	R RRR						
	D11S2000	R RRR	L RLL	R RRR						
23	D11S2000	L LLL	L LLL	L LLL	L LLL	L LLL	R LRR	L LLL	L LLL	L LLL

$I_{(n)}$, in-transit metastasis; R, retained; L, LOH.

Discussion

In-transit melanoma is a specific pathological event associated with a unique clinical presentation. Although it occurs infrequently, its presence is associated with a worse prognosis. This may be because this clinical entity represents the successful establishment of tumor metastasis. Because these lesions are an early development in the progression of melanoma metastasis, their cutaneous presentation makes them an ideal and easily accessible source to evaluate those genetic changes that occur during disease dissemination. Furthermore identifying those genetic events that permit the development of stable metastasis may provide molecular markers associated with specific disease patterns that can be used early in the disease course to assess patients' risk for recurrence. Frequent allelic losses have been demonstrated in primary melanomas as well as in metastatic lesions.[13,22] However, little is known of the genotypic aberrations associated with early stages of melanoma metastasis. Our hypothesis was that if a primary tumor's genotype is homogenous for cells with a metastatic phenotype then in-transit metastasis would not demonstrate significant intertumor heterogeneity. Conversely if those metastatic cell types comprising a primary tumor are genetically heterogeneous, or the acquisition of additional genetic changes occurs early after the metastatic event then the corresponding clinical metastasis should manifest relative significant heterogeneity with each other. If the former is the case then molecular diagnostic testing should more appropriately focus on genotyping the early metastases as opposed to the primary tumor. This approach may provide more accurate prognostic information and offers a potential to more selectively target systemic treatments.

Currently most studies assessing tumor genetics often infer findings of chromosomal alterations identified from primary tumors or more advanced metastatic lesions with a tumor's metastatic potential. At present however, correlation with clinical disease progression and stage are lacking in melanoma. Significant heterogeneity may exist among primary tumors whereby, only a minority of cells possesses the metastatic phenotype. Furthermore, studies often evaluated a small number of tumors, which were arbitrarily obtained at different stages of disease, and/or from different organ sites of metastasis. There are limited studies assessing specific pathological stages of melanomas with regards to genotype and their progression.

In this study, LOH analysis was performed to identify those genetic events associated with in-transit metastasis, a clinicopathological marker of early-stage disease progression. Because in-transit tumors are locally recurrent lesions that clinically seem to have a similar biological behavior to treatment and/or progression, LOH assessment offers the opportunity to identify those genotypic events associated with this disease phenomenon and determine whether molecular clonality exists for this metastatic phenotype. This modality allows for molecular assessment of these lesions through an additional dimension by determining whether there is significant intratumor heterogeneity present among lesions that appear clinicopathologically identical.

In this study, 62 in-transit lesions in 19 patients demonstrated LOH for at least one microsatellite marker. Furthermore, only six tumors (one tumor from each of six patients) demonstrated additional LOH as compared to other lesions from the same patients. This study demonstrates the relatively conserved LOH pattern among the multiple sets of in-transit melanoma lesions analyzed, confirming a molecular clonality to an individual patient's tumor metastasis.

To assess intratumor heterogeneity among the six patients who had at least one lesion with a discordant LOH genotype all 26 in-transit lesions were microdissected by LCM for DNA isolation from three different regions within each tumor. LOH from these areas was compared to that obtained from the total tumor, in each instance, the LOH

Table 5. LOH Profile in Paired Primary and In-Transit Tumors

Patient	Tumor	Microsatellite marker							
		D1S214	D1S228	D3S1293	D6S264	D9S157	D10S212	D11S200	D9S304
7	P	R	R	H	H	R	R	L	R
	I_1	R	R	H	H	R	R	L	R
	I_2	R	R	H	H	R	R	L	L
	I_3	R	R	H	H	R	R	L	L
	I_4	R	R	H	H	R	R	L	L
8	P	R	R	R	R	R	R	R	H
	I_1	R	R	R	R	R	R	R	H
	I_2	R	R	R	R	R	R	R	H
11	P	H	H	R	R	L	R	R	R
	I_1	H	H	R	R	L	R	R	R
	I_2	H	H	R	R	L	R	R	R
13	P	H	R	R	R	L	H	H	H
	I_1	H	R	R	R	L	H	H	H
	I_2	H	R	R	R	L	H	H	H
14	P	H	R	R	R	H	H	R	R
	I_1	H	R	R	R	H	H	R	R
	I_2	H	R	R	R	H	H	R	R
	I_3	H	R	R	R	H	H	R	R
15	P	L	L	R	H	H	R	R	H
	I_1	L	L	R	H	H	L	R	H
	I_2	L	L	R	H	H	L	R	H
	I_3	L	L	R	H	H	L	R	H
	I_4	L	L	R	H	H	L	R	H
16	P	R	R	R	R	H	R	R	R
	I_1	R	R	R	R	H	L	R	R
	I_2	R	R	R	R	H	L	R	R
	I_3	R	R	R	R	H	L	R	R
	I_4	R	R	R	R	H	L	R	R
	I_5	R	R	R	R	H	L	R	R
18	P	R	R	H	R	H	R	R	H
	I_1	R	R	H	R	H	R	R	H
	I_2	R	R	H	R	H	L	R	H
20	P	R	R	R	R	H	H	R	R
	I_1	R	R	R	R	H	H	R	R
	I_2	R	R	R	L	H	H	L	R
	I_3	R	R	R	R	H	H	R	R
22	P	R	R	H	R	R	L	R	R
	I_1	R	R	H	R	R	L	R	R
	I_2	R	R	H	R	R	L	R	R

P, primary; $I_{(n)}$, in-transit; R, retained; L, LOH.

status of the total tumor (retained or lost) was reflected by the majority of the microdissected region. These findings demonstrate that the LOH profile of a primary tumor reflects that of the majority of cells composing an individual tumor and that no allele status (retained or loss) was dominant. Our assessment of in-transit metastasis demonstrates minimal intratumor heterogeneity.

The most frequently occurring and well-studied microsatellite loci in primary melanoma is 9p23.[27,28] This was also the most common allelic loss noted in in-transit tumors. This event seems to occur early in the development of melanoma and its presence is conserved in in-transit disease as well. The next most frequent site of LOH was D9S304, also in the area of 9p23. This supports the finding that primary tumors with larger deletions in the 9p23 region have a higher risk of metastasis when compared to those with fewer losses.[21,29] Our finding of substantial LOH in the 11q21–23 region for in-transit lesions is in agreement with others suggesting this loss is a later event in tumor progression and may more likely be involved in tumor metastasis rather than cancer initiation.[26,30] LOH on chromosome 1p in early stage tumors is a rare event.[25] However, we found significant LOH in this region in in-transit metastasis. In our previous study we found an increased incidence of LOH in this region in metastatic lesions.[14] Furthermore, we detected a trend between plasma DNA microsatellites with LOH in this region and clinical progression of disease. Our finding of a relatively lower incidence of LOH on chromosome 3 and 6 indicates that this event occurs much later during melanoma tumor progression or lesions with deletions in these regions metastasize preferentially through a hematogenous route and/or do not form stable cutaneous metastasis. Particularly, LOH at markers D1S228, D3S1293, and D6S264 occurred with a lower frequency (11%, 10%, and 22%, respectively) in in-transit tumors as compared to our findings from 24 tumors obtained from patients with stage IV disease (46%, 46%, and 50%, respectively)

(unpublished data). Interestingly, only one of the 10 primary tumors in this study contained LOH for any of these three markers. This raises the question of whether the acquisition of LOH at any of these loci occurs much later in tumor progression.

In five of 10 patients with primary tumors available for analysis additional LOH events were noted to occur in the corresponding in-transit metastasis. Although the sample size is limited, this acquired LOH between the primary tumor and its in-transit supports the view that additional LOH may be required for a tumor to establish a successful metastasis. The low incidence of intertumor heterogeneity identified among in-transit lesions suggests that multiple viable metastasis to at least one organ site (in this instance skin) are clonal. This is the first study to demonstrate the molecular clonality of metastatic melanoma tumors in a clinically defined patient population (early stage III in-transit disease). This may account for the clinicopathological finding that many of these in-transit lesions behave similarly. Therefore, following occult metastasis progression using recently described methods to detect circulating serum/plasma microsatellites may be possible once the genetic profile of the metastatic clone is characterized.[14]

LOH at D10S212 occurred commonly in in-transit metastasis (35%) and was the most frequently lost allele in comparison to paired primary tumor samples. Only one primary tumor demonstrated LOH at this locus. This uncommon occurrence in our study may be because of the limited number of matched-pair primary tumors available for analysis. Similarly, others have reported an increased incidence of LOH on 10q in distant metastasis. Because no single molecular marker consistently demonstrated LOH for each patient's in-transit tumor, combinations of markers may prove more useful in tumor profiling.

An additional issue we are currently investigating is whether site of metastasis selects for a particular tumor genotype. In no instance was LOH noted in the primary lesion but not present in the corresponding in-transit metastasis. This finding demonstrates the utility of LOH analysis as molecular markers for following tumor progression throughout a patient's disease course.

In conclusion this study demonstrates the frequent occurrence of LOH in in-transit melanoma metastasis. Although primary tumors may be heterogeneous in nature, those cells that are successful in establishing clinically significant metastasis seem to have a homogenous genotype in in-transit melanoma. For each patient, identification of the genotypic profile of early stage metastasis can potentially be interpreted to more accurately stage tumor progression. Because melanoma metastasis can be dynamic and evolving the clinical significance of these genetic alterations requires prospective studies that are currently in progress.

References

1. Wong J, Cagle L, Kopald K, Swisher S, Morton D: Natural history and selective management of in transit melanoma. J Surg Oncol 1990, 44:146–150
2. Fusi S, Ariyan S, Sternlicht A: Data on first recurrence after treatment for malignant melanoma in a large patient population. Plast Reconstr Surg 1993, 91:94–98
3. Milton GW, Shaw HM, Farago GA, McCarthy WH: Tumour thickness and the site and time of first recurrence in cutaneous malignant melanoma (stage I). Br J Surg 1980, 67:543–546
4. McCarthy WH, Shaw HM, Thompson JF, Milton GW: Time and frequency of recurrence of cutaneous stage I malignant melanoma with guidelines for follow-up study. Surg Gynecol Obstet 1988, 166:497–502
5. Cascinelli N, Bufalino R, Marolda R, Belli F, Nava M, Galluzzo D, Santinami M, Levene A: Regional non-nodal metastases of cutaneous melanoma. Eur J Surg Oncol 1986, 12:175–180
6. Calabro A, Singletary SE, Carrasco CH, Legha SS: Intraarterial infusion chemotherapy in regionally advanced malignant melanoma. J Surg Oncol 1990, 43:239–244
7. Jaques DP, Coit DG, Brennan MF: Major amputation for advanced malignant melanoma. Surg Gynecol Obstet 1989, 169:1–6
8. Ebskov LB: Major amputation for malignant melanoma: an epidemiological study. J Surg Oncol 1993, 52:89–91
9. Cumberlin R, De Moss E, Lassus M, Friedman M: Isolation perfusion for malignant melanoma of the extremity: a review. J Clin Oncol 1985, 3:1022–1031
10. Coit DG: Hyperthermic isolation limb perfusion for malignant melanoma: a review. Cancer Invest 1992, 10:277–284
11. Healy E, Rehman I, Angus B, Rees L: Loss of heterozygosity in sporadic primary cutaneous melanoma. Genes Cancer 1995, 12: 152–156
12. Healy E, Belgaid C, Takata M, Harrison D, Zhu NW, Burd DAR, Rigby HS, Matthews JNS, Rees JL: Prognostic significance of allelic losses in primary melanoma. Oncogene 1998, 16:2213–2218
13. Healy E, Belgaid C, Takata M, Vahlquist A, Rehman I, Rigby H, Rees J: Allelotypes of primary cutaneous melanoma and benign melanocytic nevi. Cancer Res 1996, 56:589–593
14. Fujiwara Y, Chi DDJ, Wang H, Keleman P, Morton DL, Turner R, Hoon DSB: Plasma DNA microsatellites as tumor-specific markers and indicators of tumor progression in melanoma patients. Cancer Res 1999, 59:1567–1571
15. Haluska F, Hodi F: Molecular genetics of familial cutaneous melanoma. J Clin Oncol 1998, 16:670–682
16. Hussussian C, Struewing J, Goldstein A, Higgins P, Ally D, Sheahan M, Clark W, Tucker M, Dracopoli N: Germline p16 mutations in familial melanoma. Nat Genet 1994, 8:15–21
17. Herbst RA, Weiss J, Ehnis A, Cavenee WK, Arden KC: Loss of heterozygosity for 10q22-10qter in malignant melanoma progression. Cancer Res 1994, 54:3111–3114
18. Petris K, Keller G, Chimenti S, Amantea A, Kerl H, Hofler H: Microsatellite instability and loss of heterozygosity in melanoma. J Invest Dermatol 1995, 105:625–628
19. Millikin D, Meese E, Vogelstein B, Witkowski C, Trent J: Loss of heterozygosity for loci on the long arm of chromosome 6 in human malignant melanoma. Cancer Res 1991, 51:5449–5453
20. Walker GJ, Palmer JM, Walters MK, Hayward NK: A genetic model of melanoma tumorigenesis based on allelic losses. Genes Chromosom Cancer 1995, 2:134–141
21. Holland EA, Beaton SC, Edwards BG, Kefford RF, Mann GJ: Loss of heterozygosity and homozygosity deletions on 9p21–p22 in melanoma. Oncogene 1994, 9:1361–1365
22. Morita R, Fujimoto A, Hatta N, Takehara K, Takata M: Comparison of genetic profiles between primary melanomas and their metastases reveals genetic alterations and clonal evolution during progression. J Invest Dermatol 1998, 111:919–924
23. Balch C, Buzaid A, Atkins M, Cascinelli N, Coit D, Fleming I, Houghton Jr A, Kirkwood J, Mihm M, Morton D, Reintgen D, Ross MI, Sober A, Soong S-J, Thompson JA, Thompson JF, Gershenwald JE, McMasters KE: A new American Joint Committee on Cancer staging system for cutaneous melanoma. Cancer 2000, 88:1484–1491
24. Barth A, Wanek L, Morton D: Prognostic factors in 1,521 melanoma patients with distant metastases. J Am Coll Surg 1995, 181: 193–201
25. Dracopoli N, Harnett P, Bale S, Stanger B, Tucker M, Housman D, Kefford R: Loss of alleles from the distal short arm of chromosome 1

occurs late in melanoma tumor progression. Proc Natl Acad Sci USA 1989, 86:4614–4618
26. Robertson G, Goldberg E, Lugo T, Fountain J: Functional localization of a melanoma tumor suppressor gene to a small (< or = 2 Mb) region on 11q23. Oncogene 1999, 18:3173–3180
27. Fountain J, Bale S, Housman D, Dracopoli N: Genetics of melanoma. Cancer Surv 1990, 9:645–671
28. Fountain JW, Karayiorgou M, Ernstoff MS, Kirkwood JM, Vlock DR, Titus-Ernstoff L, Bouchard B, Vijayasaradhi S, Houghton AN, Lahti J, Kidd VJ, Housman DE, Dracopouli NC: Homozygous deletions within human chromosome band 9p21 in melanoma. Proc Natl Acad Sci USA 1992, 89:10557–10561
29. Puig S, Ruiz A, Lazaro C, Castel T, Lynch M, Palou J, Vilalta A, Weissenbach J, Mascaro J-M, Estivill X: Chromosome 9p deletions in cutaneous malignant melanoma tumors: the minimal deleted region involves markers outside the p16 (CDKN2) gene. Am J Hum Genet 1995, 57:395–402
30. Herbst R, Larson A, Weiss J, Cavenee W, Hampton G, Arden K: A defined region of loss of heterozygosity at 11q23 in cutaneous malignant melanoma. Cancer Res 1995, 55:2494–2496