Evaluation of the Clonal Origin of Multiple Primary Melanomas Using Molecular Profiling

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Numerous investigations have been conducted using molecular profiling to evaluate the possible clonal origin of second malignancies in various cancer types. However, to date no study assessing clonality of multiple primaries has been conducted in melanoma. In this investigation using patients treated at a specialist melanoma treatment center, we compared the somatic mutational profiles of pairs of melanomas designated as independent on the basis of thorough assessment of their clinical and pathologic characteristics. We used a set of highly polymorphic genetic markers selected on the basis of their chromosomal positions and the frequencies of reported allelic losses at these genetic loci. Our statistical testing strategy showed no significant evidence of clonal origin of the two primaries in 17 of the 19 patients examined. The results suggest that most second melanomas designated as independent primary tumors on the basis of their clinicopathologic features are indeed independent occurrences of the disease, supporting the validity of the criteria used by experienced pathologists in distinguishing new primaries from metastases.

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INTRODUCTION

In recent years, many investigative studies using new molecular technologies have sought to distinguish independent primary cancers from metastases in a more definitive manner than is possible by routine assessment of clinical and pathologic features. These have been conducted in various organ systems using molecular profiling of cells from pairs of tumors from individual patients, and a large literature of these studies has been developed, most prominently in the area of head and neck cancer (Ha and Califano, 2003) and bladder cancer (Hafner *et al.*, 2002), the two sites in which second malignancies are common. Typically, this has involved the examination of the tumors for somatic mutations in genes that are frequently altered in cancers of the type under investigation, by examining microsatellite instability or loss of heterozygosity (LOH) at mutational hot spots in which LOH occurs frequently. The similarities of the genetic events in both tumors are then examined to determine whether they appear to be closely matched. If so, the tumors are considered to be ''clonal'', that is deriving from a single cell that experienced the pivotal mutations before seeding both tumors.

Molecular studies of clonality have been prominent in understanding the development of smoking-related aerodigestive cancers. The concept of field cancerization postulates distinct tumors developing independently due to a common, regional exposure to the carcinogen (Slaughter *et al.*, 1953). Molecular studies, however, have demonstrated that frequently these subsequent primaries are in fact clonally related (Worsham *et al.*, 1995; Bedi *et al.*, 1996; Scholes *et al.*, 1998). A contrasting picture emerges from studies of contralateral cancer of the breast and lung. Authors of these studies have generally reached the conclusion that the tumors are typically independent for contralateral breast cancers (Kollias *et al.*, 2000; Janschek *et al.*, 2001; Stenmark-Askmalm *et al.*, 2001; Imyanitov *et al.*, 2002; Tse *et al.*, 2003; Chunder *et al.*, 2004; Regitnig *et al.*, 2004; Schlechter *et al.*, 2004), although corresponding studies of new ipsilateral breast cancers indicate that these are predominately of clonal origin (Goldstein *et al.*, 2005a, b). Studies in lung cancer have been conducted using microsatellite markers to distinguish microsatellite instability (Leong *et al.*, 1998; Huang *et al.*, 2001; Shin *et al.*, 2001; Dacic *et al.*, 2005; Geurts *et al.*, 2005), and several have tested mutations in *TP53* and/or K-ras (Sozzi *et al.*, 1995; Lau *et al.*, 1997; Hiroshima *et al.*, 1998; Holst *et al.*, 1998; Matsuzoe *et al.*, 1999; Shimizu *et al.*, 2000; Shin *et al.*, 2001; van Rens *et al.*,

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Abbreviations: LOH, loss of heterozygosity

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2002; Murase *et al.*, 2003). These studies have evaluated clonality in a range of clinical settings, including the comparison of synchronous or metachronous multiple primaries, comparisons of primaries with metastatic tumors, and comparison of head and neck primaries with solitary lung nodules that may or may not be metastases. The results of these studies are mixed, but similarly to breast cancer the evidence appears to suggest that contralateral lung tumors are predominantly of independent origin.

Our study was stimulated by the importance of this issue for interpreting findings from epidemiologic studies of melanoma. Melanoma is a relevant model for the study of clonality because the reported frequency of second primary melanoma is high: melanoma patients experience a rate of occurrence of melanoma about seven to eight times greater than the age-matched general population (Begg, 2001). Furthermore, it is not uncommon for individual patients to develop several primary melanomas. These patients provide a rich potential resource for cancer epidemiologic research (Neugut *et al.*, 1999). Patients with second primaries are increasingly used in epidemiologic case–control studies (see for example, Berwick *et al.*, 2006; Kanetsky *et al.*, 2006; Millikan *et al.*, 2006; Orlow *et al.*, 2007; Concannon *et al.*, 2008). Risk factors occur with greater frequency in these patients than in patients with a single malignancy or in population controls. As a consequence, epidemiologic studies using second primaries can possess greatly enhanced statistical power compared with conventional studies, especially for the study of rare, highly penetrant genetic risk factors (Begg and Berwick, 1997). These types of studies rely on the assumption that individuals recruited on the basis of the diagnosis of a second primary tumor have truly experienced a cancer diagnosis twice (Begg *et al.*, 2006). However, it is plausible that a significant subset of these second- and higher order primaries involve clonal recurrences of the initial primary tumor, misdiagnosed as independent second primaries.

There are several criteria for classifying a new melanoma as an independent primary. The strongest evidence in favor of a primary tumor is the presence of an associated precursor lesion (melanocytic nevus or *in situ* melanoma). Additional criteria to differentiate metastatic and primary lesions include location, grouping, invasion of lymphatic capillaries, and the presence of a brisk inflammatory cell infiltrate, although some of these characteristics may be shared by both primaries and metastatic melanomas (Heenan and Clay, 1991; Bengoechea-Beeby *et al.*, 1993). For pathologists familiar with the spectrum of pathologic features of melanocytic tumors it is usually not difficult to establish a pathologic diagnosis of primary cutaneous melanoma, particularly if the diagnosis is made in the context of an appropriate clinical history. However, it can be difficult or even impossible to determine whether a melanoma is a primary tumor or a metastasis on the basis of histologic characteristics alone (Guerriere-Kovach *et al.*, 2004). This is particularly the case for melanomas involving the dermis devoid of an *in situ* component in the overlying epidermis or other associated precursor lesions. Such tumors may be diagnosed incorrectly as metastatic melanoma on pathologic assessment. Conversely, some metastatic melanomas can show prominent epidermotropism, mimicking a primary tumor (Abernethy *et al.*, 1994; White and Hitchcock, 1998; Swetter *et al.*, 2004). In some instances, the clinical features may be the only clues to the recognition that the tumor is, in fact, a metastasis.

In the light of these issues it is surprising that the clonal relationship between first and second primary melanomas has not been investigated earlier using molecular techniques. Clonality has been examined for ''in-transit'' melanoma metastases by investigating LOH at eight candidate loci in the primary tumors and the lymphatic metastases, demonstrating a close concordance of the genetic fingerprints of lesions derived from the same patient (Nakayama *et al.*, 2001). A more recent study compared X-chromosome inactivation and LOH in five loci between primary melanomas and their corresponding metastases, and the results revealed that the majority of melanoma metastases share a common clonal origin with the matched primary tumor (Katona *et al.*, 2007). Furthermore, a group of investigators led by Bastian *et al.* (1998, 1999, 2000, 2003) has conducted a series of studies examining copy number changes in melanomas and benign nevi using array comparative genomic hybridization techniques (Curtin *et al.*, 2005). They showed that the benign nevi exhibited very few copy number abnormalities relative to the malignant tumors, confirming the potential value of molecular profiling as a diagnostic tool in differentiating benign from malignant melanocytic tumors. However, to our knowledge, no studies have been conducted that seek to challenge the validity of the diagnosis of new primary melanomas as independent occurrences of cancer.

Determining whether a melanoma is a primary or a metastasis is of critical clinical importance. In contrast to a new primary, metastatic disease is rarely curable. Furthermore, primary melanomas and melanoma metastases are managed clinically in quite different ways. Also, as noted above, the distinction is important for the validity of epidemiologic studies of multiple primary cancers.

RESULTS

We compared the mutational profiles of pairs of presumptively independent primary melanomas for each of a series of 19 patients who had been treated at the Sydney Melanoma Unit, Royal Prince Alfred Hospital in Sydney, Australia. These comparisons were on the basis of 26 highly polymorphic markers (Table 1). LOH is represented in the table by black triangular symbols, with the direction of the symbol distinguishing losses on the short versus long allele. Thus, concordant black triangles indicate losses of the same allele at the same locus and represent potentially clonal mutations, though clearly such concordances could occur independently on the two tumors by chance. Likewise, independent mutations could occur in either tumor even if the tumors shared a clonal origin. To assess the evidence favoring clonality, we used a statistical test that determines whether

the number of concordant mutations exceeds the number expected on the basis of chance.

For most of the cases, the patterns of LOH appear to be random. The results of the statistical tests displayed at the bottom of the table indicate that only 2 of the 19 cases have statistically significant evidence of clonal relatedness, $P=0.01$ for case no. 34 and $P=0.04$ for case no. 30. As we used a statistical test with a significance level of 5% we expect one ''significant'' finding when we perform about 20 independent tests. Case no. 34 has relatively few mutations, three on the first tumor (T1) and two in the second tumor (T2), with the two common mutations occurring on the same allele. Case no. 30 showed genetic alterations in both tumors for seven of the markers, with six of these seven occurring on the same allele. Interestingly, case no. 34 involved two synchronously occurring melanomas, both on the trunk, and both superficial spreading melanomas (clinical details of all cases are provided in Table 3). In contrast, case no. 30 involved tumors that occurred 2.4 years apart in distinct anatomic locations and with different cell types. These data suggest that most of these tumor pairs are independent, confirming the pathologic diagnoses, though we cannot rule out the possibility that one or two are clonal.

To verify that our testing procedure has the potential to detect tumor pairs whose origin is clonal we also examined 13 metastatic tumors from five patients (one patient had four metastases and another had three), obtained from archival material from Memorial Sloan-Kettering Cancer Center in New York. As shown in Table 2, 10 of the 12 comparisons of these definitively clonal pairings demonstrated statistically significant evidence of clonal relatedness (sensitivity = 83%), with eight of the pairings producing strongly significant ($P<0.01$) findings.

DISCUSSION

Although a number of studies of the possible clonal origin of double malignancies have been conducted to date, none to our knowledge have involved double primary melanomas. This absence may be due to the fact that most dermatopathologists do not perceive the misdiagnosis of a metastasis as a second primary as a likely occurrence or as a diagnostic problem. However, the high incidence of reported multiple primaries in this disease could be due in part to the misdiagnosis of metastases as independent primaries. Our study was constructed to provide preliminary evidence on this issue. The results would appear to support the conclusion that most second primary melanomas diagnosed on the basis of their clinical and pathologic characteristics are indeed independent occurrences of the disease.

It is of interest to examine more closely the two cases that showed patterns suggestive of clonal relatedness. Case no. 30

Table 1. Mutational patterns observed in patients with multiple primary melanomas

Case#	12		13		14		15		16		19		20		22		23		25	
Marker	T1	T2	T1	T2	T1	T2	T1	T2	T1	T2	T1	T2	T1	T2	T1	T2	T1	T2	T1	T2
D1S214	–	–	▲	▲	–	–	–	–	–	–	–	–	I	I	I	▲	I	▲	▼	I
D1S2766			I	I	–	–	–	–	I	▼	I	I	▲	I	▲	▲	I	I	▲	I
D1S2882	–	–	–	–	I	▲	I	I	–	–	–	–	▼	I	–	–	–	–	▼	▼
D2S131																				
D2S139					I	▼	–	–	I	▲	I	I	–	–	–	–	I	I	I	▼
D2S2182	I	I	▼	I	–	–	–	–			I	▼	▼	I	–	–	I	▲	▲	▲
D2S2291																				
D2S206	–	–	–	–	I	▼	▼	▲	▼	▼	–	–	I	▲	▼	I	–	–	–	–
D3S1293	I	I	▲	▲	▼	I	▼	▲	I	▼	I	▲	▲	I	▼	I	▲	▲	I	I
D4S1543					▲	▲	▲	I	–	–	▲	I	▲	I	–	–	▲	▲	I	▲
D6S1043			▼	▲	I	▲					–	–	I	I	–	–			I	I
D6S275					–	–					▲	▲								
D6S457					I	I	▲	I			I	I								
D7S1824	I	▼	▲	▼	I	▲	▼	▼			–	–	▲	I	▲	▲	▼	▼	▲	▲
D8S1104	I	I	▲	I	▼	I	I	I	I	I	–	–	–	–	I	I	–	–	▲	▼
D9S157	I	▲	▲	▲	–	–	▲	I			I	I	–	–	▲	▲	▼	▲	▲	▲
D9S304	I	▲	▼	▲	▼	I	I	▼			–	–	I	I	▼	I	▼	▲	▼	▲
D10S185	–	–	–	–			–	–			–	–	I	I	–	–	I	▲		
D10S212	I	I			–	–	–	–	I	▲	I	I	▼	I			–	–	▲	▲
D10S676	▲	▼	–	–	▲	▲	▲	▼			▼	I	▲	▲			I	▲	▼	▼
D11S1998	–	–	▼	▼	–	–	–	–	I	▲	I	I	–	–	–	–	▲	▲	▼	I
D11S2000									–	–	–	–					–	–		
D13S153																				
D17S786	I	▲	▼	▼	▲	▲	▲	▼	–	–	I	I	▲	I	▼	▼	I	▼	▲	I
TP53	–	–	▲	▲	–	–	▲	▲	–	–	▼	▲	▲	I	I	▼	–	–	–	–
D17S1322	I	▼	▼	▲	▼	▲	▲	▼	I	▲	–	–	▲	I	▼	I	I	▼	–	–
P-values	1.00		0.28		0.46		0.89		0.44		0.51		0.57		0.12		0.21		0.11	

Table 1. Continued

Case#	26		27		28		29		30		31		32		33		34	
Marker	T1	T2	T1	T2	T1	T2	T1	T2	T1	T2	T1	T2	T1	T2	T1	T2	T1	T2
D1S214	▼	I	▲	▲	▲	▲	–	–	▼	▼	I	I	–	–	I	▼	▲	▲
D1S2766	▲	▲	▲	I	I	I	I	▼	▲	▲	I	▲	▲	I	I	I	I	I
D1S2882	▼	▼	–	–	▲	▲	▲	I	–	–	I	▲	▲	I	I	▲	▲	▲
D2S131																		
D2S139	▲	▲	I	I	▼	▲	▼	I	–	–	▼	▲	▼	I	I	▼	I	I
D2S2182	I	▲	–	–	▲	I	▲	I	▲	I	▼	▲	▼	I	▲	▲	▼	I
D2S2291																		
D2S206	▲	▲	–	–	–	–	I	I	I	▼	▲	▲	I	I	I	I	I	I
D3S1293	I	▲	▼	I	I	I	I	▼	I	I	I	I	I	I	I	I	I	I
D4S1543	▲	▲	▲	▲	I	I	▼	I	▲	I	–	–	▲	I	I	I	–	–
D6S1043	–	–	–	–	–	–	–	–	▲	▲	▼	▼	I	▲	–	–	–	–
D6S275	–	–	–	–	▼	I									I	I	I	I
D6S457							▼	▲					I	I	I	I	I	I
D7S1824	▼	▼	–	–	▲	I	▲	▲	–	–	▼	I	▼	▲	I	I	–	–
D8S1104	▲	I	▼	I	–	–	I	I	–	–	–	–	▲	I	I	I	I	I
D9S157	▼	▲	I	I	▲	▲	▼	I	▲	I	I	I	I	▲	I	▼	I	I
D9S304	▼	I	I	I	▲	▲	–	–	▲	▲	I	I	▲	I	I	▼	–	–
D10S185	–	–	▲	▲					▼	I	▼	▲	–	–			–	–
D10S212	–	–	–	–	▼	I	▼	I	–	–	▲	▲	▼	I	–	–	I	I
D10S676	▲	▼	▼	I	I	▼	▼	▲	I	I	▲	▲	▼	I	–	–	I	I
D11S1998	–	–	–	–	▼	I	▲	▼	▼	▲	–	–	I	I	I	▲	–	–
D11S2000	▲	I			I	I					–	–			I	▲	–	–
D13S153																		
D17S786	I	▼	I	▼	I	▲	▲	I	▲	▲	▼	I	I	▲	I	I	I	I
TP53	I	▲	▼	▲	▼	▼	▼	▲	▼	▼	–	–	–	–	I	I	I	I
D17S1322	–	–	–	–	–	–	▼	I	–	–	▼	I	–	–	▼	I	–	–
P-values	0.26		0.20		0.06		0.97		0.04		0.29		1.00		0.38		0.01	

LOH, loss of heterozygosity.
▲ LOH (short allele); ▼ LOH (long allele); I informative/no change; – non-informative.
We used the term LOH for simplicity, but gain of the contralateral allele has been observed for some markers by us and others (Bastian *et al.*, 1998; White and Hitchcock, 1998; Curtin *et al.*, 2005).

exhibited concordant LOH at six separate genetic loci, yet the tumors have different cell types, occurred 2.4 years apart, and were located in distant anatomic sites. A re-inspection of the pathological characteristics indicated that both tumors had significant epidermal components extending beyond bulky dermal components. Case no. 34 had only two concordant mutations, but the overall patterns were very similar, that is most of the loci exhibited no mutations on either tumor. The two tumors were synchronous, with the same cell type in the same general anatomic site, the chest, although the tumors were in the left and right portions of the chest, and well apart. Re-examination of the pathology in recut sections showed that the two tumors were mostly epidermal, and thus appeared pathologically to be independent primaries. As we expect one false-positive finding for every 20 statistical tests performed at the 5% significance level, the observation of only two significant results in this set of 19 is broadly consistent with the conclusion that few, if any, of these melanoma pairs, and very few in general, are of clonal origin.

Our study has technical, epidemiological, and statistical limitations. We obtained specimens from both primaries for 19 cases, but these cases were selected based on the availability of sufficient tissue samples. This opportunistic selection of cases, and the small sample size, limits our ability to estimate accurately the proportion of cases that may be misdiagnosed. Furthermore, the cases were obtained from a specialized melanoma treatment center where the pathologic reviews were accomplished by dermatopathologists specializing in melanoma, and where full clinical histories were also available. All such information is rarely available to either clinicians or pathologists at the time of initial diagnosis in routine clinical practice and hence misdiagnosis of a metastasis as a primary may be somewhat more common in everyday clinical practice, particularly outside of specialist centers. Nonetheless, most patients with a second skin melanoma designated as a second primary have clinical courses consistent with a new primary and more favorable than would be expected for stage IV melanomas. For a subset of markers we encountered amplification failures. This could be the result of primers being unable to anneal to their specific sequences due to homozygous deletions or duplications, but more likely the high rate of failures encountered for D2S131, D2S2291, D6S275, D6S457, D10S185, and D13S153 was due to the suboptimal quality of the DNA.

Table 2. Mutational patterns observed in controls with metastases

Control#	1			2				3		4		6	
Marker	T1	T2	T3	T1	T2	T3	T4	T1	T2	T1	T2	T1	T2
D1S214	I	I	I	–	–	–	–	I	I	–	–	–	–
D1S2766	–	–	–	I	I	I	I	–	–	I	I	I	I
D1S2882	▲	▲	▲	I	▲	I	I	I	I	–	–		
D2S131				I	I	I	I	–	–	I	▲		
D2S139	▲	▲	I	I	I	▼	I	I	I	I	I	▼	▲
D2S2182	▲	I	▲	I	I	I	I	I	I	I	▼	▼	▼
D2S2291	–	–	–	–	–	–	–	I	I	I	I	–	–
D2S206	I	I	I	I	I	I	I	I	I	I	I	▲	▲
D3S1293	I	I	I	I	I	I	I	I	I	▲	I	▲	▲
D4S1543	I	I	I	–	–	–	–	I	I	–	–		
D6S1043	I	I	I	–	–	–	–	–	–	I	I	▼	▼
D6S275	▼	I	I	▼	▼	▼	▼	I	I	I	I		
D6S457	I	I	▼	▼	▼	▼	▼	I	I	–	–		
D7S1824	I	▼	▲	I	I	I	I	I	▼	–	–	I	I
D8S1104	I	I	▼	–	–	–	–	–	–	–	–	–	–
D9S157	▲	▲	▲	▼	I	I	▼	I	I	▲	I	I	▲
D9S304	–	–	–	–	–	–	–	I	I	I	I	▲	▲
D10S185	–	–	–	–	–	–	–	–	–	▼	I		
D10S212	I	▼	I	▼	▼	▼	▼	I	I	▼	▼	–	–
D10S676	▲	▲	I	–	–	–	–	I	I	▼	▼	I	▼
D11S1998	–	–	–	I	I	I	I	I	I	–	–	▲	▲
D11S2000	▼	I	▼	I	▼	▼	▼	I	I	▼	▼	▼	▼
D13S153				I	I	I	I	▼	I	▼	I		
D17S786	I	I	▲	I	I	I	I	–	–	–	–	▲	▲
TP53	▲	I	▲	–	–	–	–	–	–	I	I	–	–
D17S1322	I	I	▼	–	–	–	–	–	–	–	–	I	I
P-values	See note (1)			See note (2)				1.0		0.05		<0.01	

(1) P=0.01 (T1 vs T2); P=0.02 (T1 vs T3); P=0.50 (T2 vs T3).
(2) $P<0.01$ (T1 vs T2); $P<0.01$ (T1 vs T3); $P<0.01$ (T1 vs T4); $P<0.01$ (T2 vs T3); $P<0.01$ (T2 vs T4); $P<0.01$ (T3 vs T4).

Melanoma is a disease that is frequently characterized by small tumors. We had hoped as part of this study to conduct array comparative genomic hybridization on all pairs of samples as an alternative genomic approach to profiling the tumors. However, sufficient DNA of high molecular weight suitable for array comparative genomic hybridization for both tumors in the pair was available only for four cases (data not shown). In general, for a technology of this nature to be applicable in a clinical diagnostic setting, we would need a minimum of 0.6 μg of high-molecular-weight DNA from each tumor and counterpart normal sample if extracted from fresh frozen tissue, or 1.5 μg of DNA extracted from formalin-fixed, paraffin-embedded tissue. Such quantities will typically not be available from both tumors. With a PCR-based method such as the one presented here, one would require no more than 0.5 μg of DNA if testing a relatively high number of microsatellite repeats, and approximately 0.2 μg when working with fresh-frozen tissue.

We employed a statistical test that was designed specifically for the purpose of detecting clonal relatedness (Begg *et al.*, 2007). This test is based on the simplifying assumptions that the mutations at different loci are independent, that the probabilities of mutations are similar for each locus, and that each allele is equally likely to experience a mutation. Each of these assumptions is clearly approximate. Validation studies show that the test is robust to modest departures from the latter two assumptions (Begg *et al.*, 2007). In fact, in our presumptively independent cases, 66% of the losses that occurred in both members of the tumor pair occurred on the same allele. This modest preponderance of concordances could be the result of clonality in some of the pairs (such as case nos. 30 and 34), but it may also be explained by the possibility that allelic changes do not occur with equal probability for the alleles at specific genetic loci, especially if located within or nearby a gene involved in the development of the tumor. If this is true, then we expect to see a modest correlation in mutational profiles even for independent tumors. The statistical power of the test is, of course, dependent on the number of independent genetic markers evaluated. In practice, one could increase power by examining more loci for allelic gains and losses and by testing for the presence of common point mutations such as the V600E variant on the *BRAF* gene.

In summary, our study provides evidence that most melanomas that are classified as independent second primaries on the basis of comprehensive clinicopathologic analysis in a specialist melanoma treatment center are indeed independent occurrences of melanoma. In clinical use, this technology could, on present evidence, be a supplement to but not a replacement for detailed clinical and pathologic evaluation of the lesions.

MATERIALS AND METHODS

Case selection

Archival specimens of sufficient quality for analysis were obtained from two independent primary melanomas for each of a series of 19 patients who had been treated at the Sydney Melanoma Unit, Royal Prince Alfred Hospital in Sydney, Australia. These cases were selected on the basis of the availability of specimens from both tumors with dimensions (based on diameter and thickness) that were likely to provide sufficient DNA for analysis. Clinical and pathologic details are reported in Table 3. In 13 patients, the tumor pairs occurred in the same general anatomic region and in 16 pairs the tumors were of the same histologic type. In 10 of these patients, the lesions mapped both to the same anatomic region and had the same histologic subtype. For comparison, we also utilized 13 ''known'' metastatic lesions in five patients with melanoma available as archived material at the Memorial Sloan-Kettering Cancer Center in New York. One patient had four synchronous tumors to the leg (control no. 2, Table 2), whereas another patient had three related tumors (control no. 1). The study was approved by the Institutional Review Boards at the Royal Prince Alfred Hospital and Memorial Sloan-Kettering Cancer Center. The study was conducted according to the Declaration of Helsinki Principles.

Table 3. Clinical and histologic characteristics of multiple melanomas

	First primary			Second primary			
Case ID	Thickness	Site	Histology	Thickness	Site	Histology	Interval[1]
12	2.1 mm	H/N	SSM	1.3 mm	H/N	SSM	9.5 years
13	4.6 mm	Trunk	SSM	0.5 mm	Trunk	SSM	Synch
14	0.9 mm	Arm	SSM	1.7 mm	H/N	SSM	1.2 years
15	1.2 mm	Trunk	SSM	2.7 mm	Trunk	NM	1.3 years
16	1.2 mm	Arm	SSM	0.8 mm	Trunk	SSM	7.1 years
19	1.7 mm	H/N	LMM	2.5 mm	H/N	NM	6.2 years
20	1.2 mm	Trunk	SSM	1.8 mm	H/N	SSM	0.8 years
22	1.7 mm	Trunk	SSM	0.9 mm	Trunk	SSM	0.3 years
23	1.3 mm	Leg	SSM	1.9 mm	Leg	SSM	6.4 years
25	0.6 mm	Arm	SSM	0.9 mm	Arm	SSM	0.6 years
26	2.2 mm	Trunk	SSM	0.4 mm	Trunk	SSM	Synch
27	0.5 mm	Trunk	SSM	1.0 mm	Trunk	NM	4.1 years
28	0.6 mm	Trunk	SSM	1.8 mm	Trunk	SSM	4.2 years
29	0.6 mm	Trunk	SSM	1.1 mm	Trunk	SSM	Synch
30	5.7 mm	Trunk	NM	8.0 mm	Leg	LMM	2.4 years
31	0.7 mm	Leg	SSM	1.0 mm	Arm	SSM	Synch
32	1.2 mm	Trunk	SSM	1.7 mm	Trunk	SSM	1.0 years
33	0.5 mm	Trunk	SSM	0.5 mm	Leg	SSM	Synch
34	0.7 mm	Trunk	SSM	0.5 mm	Trunk	SSM	Synch

H/N, head and neck; LMM, lentigo malignant melanoma; NM, nodular melanoma; SSM, superficial spreading melanoma.
[1]Time (years) between diagnoses; Synch, synchronous dates of diagnosis.

Marker selection

We chose 26 highly polymorphic genetic markers; these were selected on the basis of their chromosomal positions and their reported or expected allelic loss (Table 4) (Thompson *et al.*, 1995; Nakayama *et al.*, 2001; Shirasaki *et al.*, 2001; Massi *et al.*, 2002; Pollock *et al.*, 2003; Uribe *et al.*, 2005). Eleven of these markers map to eight different chromosomes and have earlier shown a high incidence of LOH or microsatellite instability (>30%) either in primary or metastatic melanomas (35,46–48): D1S214 (1p36.3), D2S2182 (2p16), D2S2291 (2p16), D6S275 (6q15–q16), D6S457 (6q21–q23.2), D9S304 (9p21), D9S157 (9p23–p22), D10S212 (10q26.12–13), D11S2000 (11q22–q23), D13S153 (13q14), D17S786 (17p13), and D17S1322 (17q21). The heterozygosity of these markers ranged from 20 to 62% in published studies (Bengoechea-Beeby *et al.*, 1993; Thompson *et al.*, 1995; Shirasaki *et al.*, 2001; Pollock *et al.*, 2003) and from 61 to 92% according to the Centre d'Etude du Polymorphisme Humain database (version v2.1 last accessed on 8 April 2008). The following six markers with heterozygosities between 69 and 87% (Centre d'Etude du Polymorphisme Humain) have not been tested earlier in melanomas but were selected because they map to chromosomal arms found by Curtin *et al* (2005) to be altered: D6S1043 (6q16), D7S1824 (7q34), D8S1104 (8p11), D10S676 (10q22), D11S1998 (11q23), and a pentanucleotide repeat within the *TP53* gene (17p13). An additional set of five markers on three different chromosomes showed 19–23% LOH in melanoma cases as reported by Uribe *et al.* (2005): D10S185 (10q23.3), D2S139 (2p12), D2S131 (2p22–25), and D2S206 (2q33–37). Finally, four more markers were selected that were at known or suspected oncogene or tumor suppressor gene sites. These markers are D4S1543 on 4q13 (*c-Kit* maps to 4q11–12), D1S2882 and D1S2766, which map to the smallest overlapping deletion (SRO1) suspected to harbor a new melanoma tumor suppressor gene (Walker *et al.*, 2004), and D3S1293 on 3p22, which maps near *TGFBR2* (Nakayama *et al.*, 2001), with heterozygosities between 67 and 74% (Centre d'Etude du Polymorphisme Humain).

Sample preparation and DNA extraction

DNA was extracted from the tumor area contained in 20–30 × 5-μm-thick formalin-fixed paraffin-embedded tissue sections placed on uncharged glass slides. A hematoxylin–eosin-stained slide was used to confirm the presence of tumor and to differentiate between tumor and normal adjacent cells. These areas were then isolated and scraped into separate Eppendorf tubes with sterile scalpels. Tissues were deparaffinized with xylene and DNA extracted with the QIAamp Micro Kit (Qiagen Inc., Valencia, CA) following the manufacturer's recommendations. The DNA quantity and quality were determined by measuring the A_{260}, A_{280}, and A_{230} with a NanoDrop ND1000 spectrophotometer (Nanodrop, Wilmington, DE).

Table 4. Microsatellite markers for the study of melanoma clonality

Chromosome	Location	Marker	Genes nearby	Het.	LOH (%)[1]	Het.[2]	Primer forward (5′3′)[3]	Primer reverse (5′3′)[3]
1	p36.3	D1S214	*BW2, BMND3, SCCD*	19/79 (24%)[(b)]	6/19 (32%)	74%	CCGAATGACAAGGTGAGACT	AATGTTGTTTCCAAAGTGGC
	p22	D1S2766	*CYR61*	Not clear[(a)]	Not clear	74%	CTCAGCCTAGTGCAGCC	GCTTAAACCCATGATTGGTAT
	p31	D1S2882	*BCL10*	Not clear[(a)]	Not clear	74%	AATGAAAATTGTAGTA CCTGTTTCG	CTTGCTAAGGATGATAGCCTC
2	p22–p25	D2S131	*ITGB1BP1*	21/29 (72%)[(c)]	4/21 (19%)	86%	TTTACTGCTGAGACAACCCA	GTATAGGAGCCACACCCCTG
	p16	D2S2182	*MSH2*	Not clear[(d)]	60% MSI	75%	GCTCGAAAAATGATTTGATCC	GGCTAAGCCTAGATGCTTGA
	p16	D2S2291	*MSH2*	Not clear[(d)]	60% MSI	75%	TGTCAACAGTGGCTAATCATC	TTAGAAATATGGCTGCCAGG
	p12	D2S139	*DFNA43*	21/29 (72%)[(c)]	4/21 (19%)	83%	AGCTCAAAGCAAATGCATGC	AAATTGCGAAACTGTGGCTT
	q33–q37	D2S206	*PARK11*	9/29 (31%)[(c)]	2/9 (22%)	86%	TTAAAAATTAAGTAGGC TTTTGGTT	GTCCTCATGTGTTTATGCTGT
3	p22	D3S1293	*TGFBR2*	21/25 (84%)[(b)]	2/21 (10%)	74%	ACTCACAGAGCCTTCACA	CATGGAAATAGAACAGGGT
4	q13	D4S1543	*KIT, SULT1E1*			67%	TTCCAGCAATAGGGATGGAGTC	CGAAAGTAGTTAATATGG CTTCCGA
6	q15–q16	D6S275	KiSS1 regulators	Not clear[(e)]	(52%)	86%	TAATTTCACATACAGGCCCT	AATGAACACGCTCTAAGGAT
	q21–q23.2	D6S457	*TCF21*	Not clear[(e)]	(52%)	82%	ATTGGCAATAGTTACGAATTA	GGCATTTGTGGAGTGG
	q16	D6S1043	*RRAGD, CASP8AP2*			83%	CAAGGATGGGTGGATCAATA	TTGTATGAGCCACTTCCCAT
7	q34	D7S1824	*ADCK2, BRAF*			87%	GTTTGATTCAGTCAGTGG	TGGGATAGAACAGAATAG
8	p11	D8S1104	*PLAT, ADRB3*			78%	TCAGCTATGAGAAAAGTTGAATG	GACCCTTGTTTGTGTACGGT
9	p21	D9S304	*CDKN2A*	17/79 (22%)[(b)]	8/17 (47%)	86%	GTGCACCTCTACACCCAGAC	TGTGCCCACACACATCTATC
	p22–p23	D9S157	*PTCH, SH3GL2*	16/79 (20%)[(b)]	9/16 (56%)	87%	AGCAAGGCAAGCCACATTTC	TGGGGATGCCCAGATAA CTATATC
10	q23.3	D10S185	*PTEN*	13/29 (45%)[(c)]	3/13 (23%)	82%	TCCTATGCTTTCATTTGCCA	CAAGACACACGATGTGCCAG
	q26.12–13	D10S212	*MKI67*	17/79 (22%)[(b)]	6/17 (35%)	66%	GAAGTAAAGCAAGTTCT ATCCACG	TCTGTGTACGTTGAAAATCCC
	q22	D10S676	*LRRC20*			90%	GAGAACAGACCCCCAAATCT	ATTTCAGTTTTACTATGTGCATGC
11	q22–q23	D11S2000	*ATM*	23/79 (29%)[(b)]	9/23 (39%)	92%	AGTAGAGAACAAAACAC TGTGGC	TTTGAAGATCTGTGAAATGTGC
	q23	D11S1998	*SCN2B*	23/27 (85%)[(f)]	20/23 (87%)	69%	AGCCATCAACTAGCTTTCCC	GGGAGGCACCAACAGATG
13	q14	RBi2 / D13S153	*RB1*	15/29 (52%)[(c)]	6/15 (40%)	64%	AGCATTGTTTCATGTTGGTG	CAGCAGTGAAGGTCTAAGCC
17	p13	D17S786	*TP53*	18/29 (62%)[(c)]	5/18 (28%)	82%	TACAGGGATAGGTAGCCGAG	GGATTTGGGCTCTTTTGTAA
	q21	D17S1322	*BRCA1*	12/29 (41%)[(c)]	4/12 (33%)	67%	CTAGCCTGGGCAACAAACGA	GCAGGAAGCAGGAATGGAAC
	p13	*TP53*	*TP53*			72%	GAATCCGGGAGGAGGTTG	AACAGCTCCTTTAATGGCAG

Het., Heterozygosity; LOH, loss of heterozygosity as reported by others: (a) Walker *et al.* (2004); (b) Nakayama *et al.* (2001); (c) Uribe *et al.* (2005); (d) Massi *et al.* (2002); (e) Shirasaki *et al.* (2001); (f) Herbst *et al.* (2000).
[1]LOH/informative cases (%).
[2]Heterozygosity as reported by the Centre d'Etude du Polymorphisme Humain (CEPH), except for marker D17S1322, reported by the GDB.
[3]Primer sequences obtained from NCBI-UniSTS (STS, sequence-tagged site), Primer3 online tool (http://frodo.wi.mit.edu/cgi-bin/primer3/primer3_www.cgi), and Cawkwell *et al.* (1994).

PCR and fragment-size analysis

Analyses of microsatellites were performed by PCR using primers flanking the repetitive sequence, coupled with fragment-size analysis using a fluorescent label. During assay design, all primer pairs were checked with the Basic Local Alignment Search Tool (Blast, NCBI) to ensure specificity. Specific fragments were amplified in a reaction mix containing 10–15 ng of DNA, 0.5 μM each of the specific forward and reverse primers, 300 μM of dNTP, 0.05 U μl^{-1} of DNA polymerase, and AmpliTaq Buffer II containing 1.5 mM of $MgCl_2$ (Applied Biosystems, Foster City, CA). Specific primer sequences amplified products of 103–247 bp and are listed, together with the cycling conditions, in Table 5. After amplification, the

Table 5. Experimental conditions used for the detection of allelic losses and gains

Marker	Forward primer (5′3′)[1,2]	Reverse primer (5′3′)[1]	Fragment-size range (bp)	Additives[3]	DNA polymerase[4]	No. of cycles	PCR T_a[4] (°C)
D1S214	N*-CCGAATGACAAGGTGAGACT	AATGTTGTTTCCAAAGTGGC	120–142	1 M betaine	HotStart	40	50
D1S2766	H*-CTCAGCCTAGTGCAGCC	GCTTAAACCCATGATTGGTAT	163–195	1 M betaine	AmpliTaq	40	50
D2S131	N*-TTTACTGCTGAGACAACCCA	GTATAGGAGCCACACCCCTG	229–247	1 M betaine	AmpliTaq	40	50
D2S2182	6F*-GCTCGAAAAATGATTTGATCC	GGCTAAGCCTAGATGCTTGA	228–242	1 M betaine	AmpliTaq	40	50
D2S2291	N*-TGTCAACAGTGGCTAATCATC	TTAGAAATATGGCTGCCAGG	233–245	1 M betaine	AmpliTaq	40	50
D2S139	H*-AGCTCAAAGCAAATGCATGC	AAATTGCGAAACTGTGGCTT	175–197	1 M betaine	HotStart	50	50
D2S206	H*-TTAAAAATTAAGTAGG CTTTTGGTT	GTCCTCATGTGTTTATGCTGT	123–151	NA	AmpliTaq	40	55
D4S1543	H*-TTCCAGCAATAGGGA TGGAGTC	CGAAAGTAGTTAATATGG CTTCCGA	144–170	NA	AmpliTaq	40	55
D6S275	N*-TAATTTCACATACAGGCCCT	AATGAACACGCTCTAAGGAT	207–219	1 M betaine	AmpliTaq	50	50
D6S457	N*-ATTGGCAATAGTTACGAATTA	GGCATTTGTGGAGTGG	197–207	2% glycerol	AmpliTaq	10, 10, 25	55, 53, 51
D6S1043	H*-CAAGGATGGGTGGATCAATA	TTGTATGAGCCACTTCCCAT	103–143	1 M betaine	HotStart	40	50
D7S1824	H*-GTTTGATTCAGTCAGTGG	TGGGATAGAACAGAATAG	163–199	1 M betaine	HotStart	40	50
D8S1104	6F*-TCAGCTATGAGAAAAGT TGAATG	GACCCTTGTTTGTGTACGGT	129–141	1 M betaine	HotStart	40	50
D9S304	6F*-GTGCACCTCTACACCCAGAC	TGTGCCCACACACATCTATC	135–175	1 M betaine	AmpliTaq	40	50
D9S157	H*-AGCAAGGCAAGCCACATTTC	TGGGGATGCCCAGATAACTATATC	133–149	2% glycerol	AmpliTaq	10, 10, 25	55, 53, 51
D10S185	H*-TCCTATGCTTTCATTTGCCA	CAAGACACACGATGTGCCAG	143–159	1 M betaine	AmpliTaq	15, 25	55, 53
D10S212	H*-GAAGTAAAGCAAGTTCT ATCCACG	TCTGTGTACGTTGAAAATCCC	189–201	1 M betaine	AmpliTaq	40	50
D10S676	6F*-GAGAACAGACCCCCAAATCT	ATTTCAGTTTTACTATGTGCATGC	175–199	1 M betaine	HotStart	40	50
D11S2000	6F*-AGTAGAGAACAAAACAC TGTGGC	TTTGAAGATCTGTGAAATGTGC	199–235	1 M betaine	HotStart	50	50
D11S1998	N*-AGCCATCAACTAGCTTTCCC	GGGAGGCACCAACAGATG	129–165	1 M betaine	HotStart	40	50
RBi2 / D13S153	6F*-AGCATTGTTTCATGTTGGTG	CAGCAGTGAAGGTCTAAGCC	212–236	1 M betaine	AmpliTaq	15, 25	55, 53
D17S786	H*-TACAGGGATAGGTAGCCGAG	GGATTTGGGCTCTTTTGTAA	135–157	1 M betaine	AmpliTaq	40	50
D17S1322	6F*-CTAGCCTGGGCAACAAACGA	GCAGGAAGCAGGAATGGAAC	~144	1 M betaine	HotStart	50	50
TP53	6F*-GAATCCGGGAGGAGGTTG	AACAGCTCCTTTAATGGCAG	140–175	1 M betaine	HotStart	40	50
D3S1293	6F*-ACTCACAGAGCCTTCACA	CATGGAAATAGAACAGGGT	116–144	1 M betaine	AB AmpliTaq	5, 20, 20	56, 55, 53
D1S2882	6F*-AATGAAAATTGTAGTAC CTGTTTCG	CTTGCTAAGGATGATAGCCTC	224–237	NA	AB AmpliTaq	40	55

NA, not applicable; T_a, annealing temperature.
[1]Primer sequences obtained from UniSTS.
[2]Forward primer modified with a 5′-fluorescent primer: N*, NED; H*, HEX; 6F*, 6FAM.
[3]Amount of additives shown corresponds to the final concentration in the reaction.
[4]AB, AmpliTaq (Applied Biosystems); Hot Start , Qiagen Hot Start Taq polymerase (Qiagen).

products were loaded onto 2.5% agarose gels stained with ethidium bromide and examined after electrophoresis. The quantity of PCR product obtained was assessed by comparing the band intensities to a mass marker (Invitrogen, Carlsbad, CA). PCR products were diluted to 1–3 ng μl^{-1} and then analyzed by capillary electrophoresis on the ABI 3730xl DNA sequencer (Applied Biosystems) in the presence of a GS500LIZ size standard (Applied Biosystems) and by use of the GeneScan ver3.0 software (Applied Biosystems) to determine product length. The electropherograms were analyzed with Peak Scanner v1.0 software (Applied Biosystems) and the ABI PRISM GeneMapper TM Software version 3.0. Samples were considered informative when two clear allelic peaks were present in the electropherograms of the normal DNA (heterozygous sample), and not informative when only one peak was present (homozygous sample). For the informative sets, the ratios of allele 1 and allele 2 signals were compared in normal (N) and tumor tissue (T) [($N_{allele1}$/$N_{allele2}$): ($T_{allele1}$/$T_{allele2}$)]. This ratio should be close to 1 when no allelic loss has occurred. We note that as the PCR-based microsatellite analysis consists of examination of the relative allelic peak heights, in several instances we cannot distinguish between

loss of an allele and the gain of the contralateral allele. However, most of the markers used map to chromosomal arms deleted in melanoma (Thompson *et al.*, 1995; Bastian *et al.*, 1998) and therefore all allelic changes were designated as LOH. The cutoff to establish whether LOH had occurred was chosen based upon microscopic evaluation of the H&E-stained tissues and considered on a case-to-case basis. As an example, if the tumor sample contained ~20% normal cells, LOH was defined as a 40% reduction or more in the intensity of one of the two alleles in the tumor sample (Figure 1) (Orlow *et al.*, 1994).

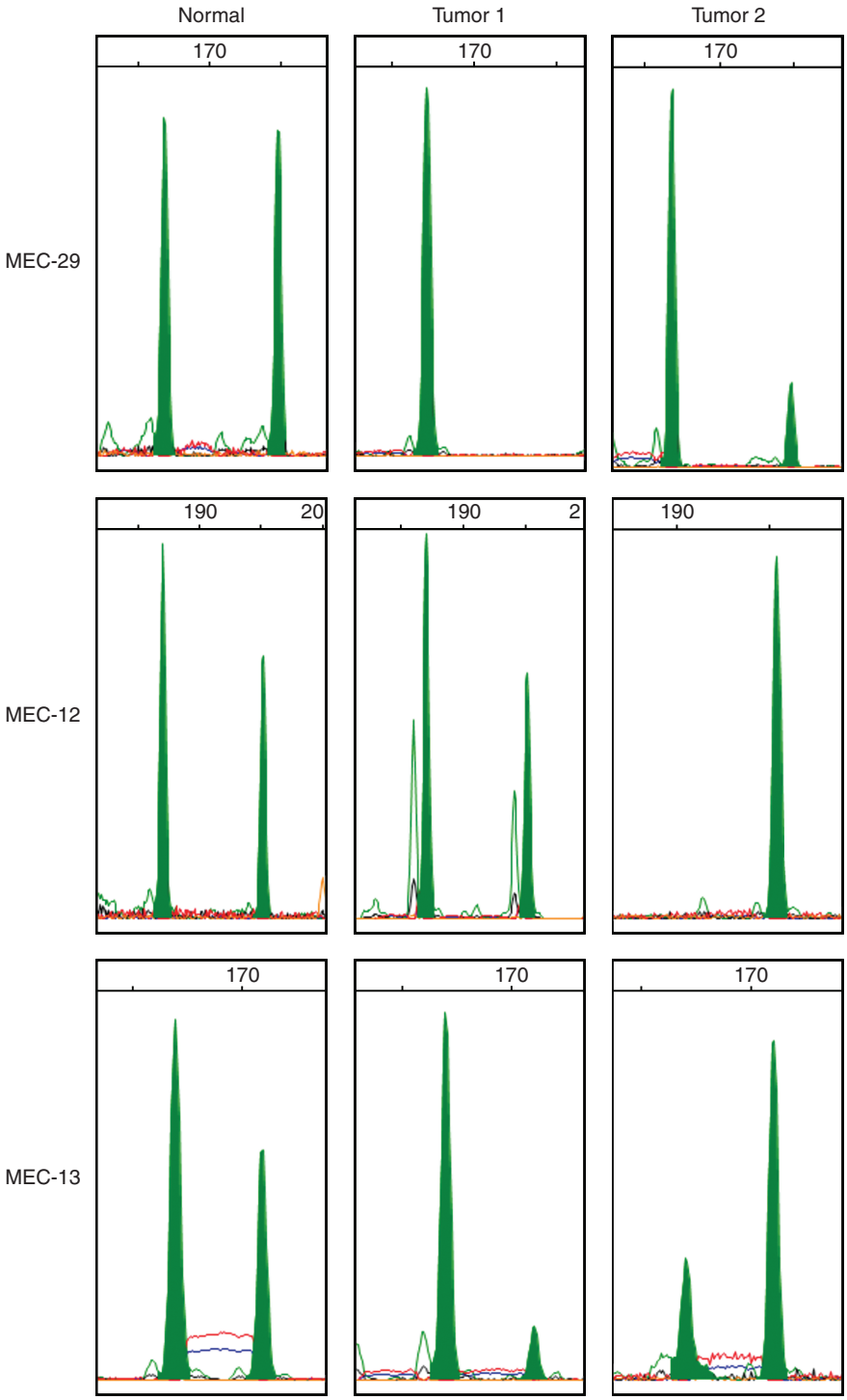

Figure 1. Analysis of allelic gains and losses using fragment-size analysis (FSA). The depicted results correspond to sets of electropherograms obtained for the tetranucleotide-repeat marker D7S1824 for patients in whom the tumor pairs showed concordant allelic loss (case no. 29, loss of the long allele); loss in one of the tumors only (case no. 12); and discordant losses (case no. 13, loss of the long allele in tumor 1 versus loss of the long allele in tumor 2).

Quality control

Careful labeling of study samples and 96-well plates was monitored throughout all procedures. To avoid contamination, DNA extraction and pre-PCR procedures including scraping of cells from the paraffin-embedded tissue were conducted in areas free of PCR products and with dedicated instrumentation, including aerosol-resistant pipette tips and disposable plastic ware. Pipettes were wiped with ethanol and exposed together with plastic ware to UV for 15 minutes before each use. Samples that failed to amplify were repeated at least twice. All results were interpreted at least twice by two laboratory members (D.V.T. and I.O.)

Statistical analysis

The patterns of mutational events in the two tumors were compared using a statistical test designed for this specific purpose (Begg *et al.*, 2007). The test involves counting the total number of concordant mutations that occur on the same parental allele and benchmarking this total against a reference distribution that is based on the assumption that mutations on the two tumors occurred randomly.

CONFLICT OF INTEREST

The authors state no conflict of interest.

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