X Inactivation, DNA Deletion, and Microsatellite Instability in Common Acquired Melanocytic Nevi

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ABSTRACT
We have investigated several molecular characteristics of common acquired melanocytic nevi to clarify their relationship to malignant melanoma, which is characterized by clonality and the progressive accumulation of DNA deletions. Twenty-four common acquired nevi were subjected to analysis for loss of heterozygosity at four loci on chromosome 9p and six loci on 10q that are commonly deleted in melanoma, but no deletions were seen. X inactivation analysis was performed in lesions from females, using the methylation-sensitive restriction HpaII site in the CAG microsatellite repeat (HUMARA) in exon 1 of the androgen receptor (AR) gene. In 14 melanomas, 11 (92%) were confirmed to have skewed X inactivation, consistent with monoclonality, as were 16 (80%) of 20 benign nevi. One nevus (5%) and 4 (33%) of 12 melanomas also showed loss of heterozygosity at HUMARA. One nevus showed an additional allele, consistent with low level microsatellite instability, at one of the 11 loci that were examined. Common melanocytic nevi, therefore, arise by apparently clonal proliferation, but they do not share chromosomal deletions that are characteristic of melanoma. However, skewed X inactivation patterns were seen in some samples of adjacent microdissected normal epidermis.

INTRODUCTION
Neoplasms are initially monoclonal with respect to the genotype of the founding cell but become genetically and epigenetically heterogeneous as subsequent mutations occur in descendant subclones (1–3). In contrast, hamartomas and hyperplastic, nonneoplastic proliferations arise from multiple founding cells and will, therefore, reflect any genetic or stable epigenetic heterogeneity in those founders. In normal individuals, chromosomal X inactivation is a marker of epigenetic heterogeneity that has been used to distinguish “monoclonal” (neoplastic) from “polyclonal” (nonneoplastic or hamartomatous) proliferations (4).

Malignant nevus (common moles) are morphologically and biologically benign collections of melanocytes (5, 6). They occur congenitally and, especially in fair-skinned individuals in sunny environments, also arise and regress sporadically throughout life. High mole count is a strong risk factor for melanoma development, and some melanomas arise in preexisting nevi, which suggests that nevi may lie along a pathway of benign-to-malignant progression, driven by somatic mutation (7). Alternatively, acquired melanocytic nevi may consist of genetically normal melanocytes, proliferating because of the lack of growth-restraining proximity to epidermal keratinocytes (8).

Evidence on the clonality of melanocytic nevi is conflicting. A Japanese study (9) found polyclonal (mixed X inactivation status) X inactivation patterns in nevi, whereas melanomas showed the expected monoclonal (skewed X inactivation status) pattern. In contrast, an Australian study (10) found that 79% of (predominantly) junctional nevi tested showed a “clonal” (skewed X inactivation) pattern in a similar assay. An American study also similarly demonstrated a clonal pattern in intradermal nevi (11). We show X inactivation data from a study of intradermal and compound nevi, further supporting previous findings of a skewed X inactivation pattern in common acquired nevi, consistent with clonality.

Malignant melanoma, like many other neoplasms, is characterized by extensive chromosomal deletions. Losses of 9p and 10q are among the most frequent events and occur in a proportion of primary tumors (12–18). The CDKN2A locus appears to be the primary target of 9p deletions, and encodes the tumor suppressor genes p16INK4A and p14ARF (19). We examined 9p and 10q regions for deletions in a cohort of compound and dermal nevi and show that their loss plays little, if any, role in the proliferation of these lesions.

High-level MSI3 is a feature of cancers associated with defects in DNA mismatch repair, such as hereditary non-polyposis colorectal cancer (HNPCC; Ref. 20). So-called low-level MSI appears to be a distinct phenomenon of unknown cause and pathogenic significance and has been reported in a number of cancers, including melanomas and melanocytic nevi (21). We show in this study an instance of low-level MSI at one locus (D10S187) in a common acquired nevus.

Received 4/20/01; revised 9/12/01; accepted 9/13/01.

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1 This research was part of a joint program between Westmead Institute for Cancer Research and the Melanoma and Skin Cancer Research Institute, University of Sydney. It was supported by the New South Wales Cancer Council, the University of Sydney Cancer Research Fund, and the Melanoma Foundation of the University of Sydney.

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3 The abbreviations used are: MSI, microsatellite instability; PBL, peripheral blood lymphocyte; LOH, loss of heterozygosity.
MATERIALS AND METHODS

Sample Tissue and DNA Preparation. Thirty-six formalin-fixed, paraffin-embedded, clinically raised, intradermal and compound, benign melanocytic nevi were retrieved from the archives of the Westmead Hospital Department of Anatomical Pathology and reviewed by one of us (A. R. C.) to confirm the diagnosis. Paired normal (epidermal keratinocyte) and lesion tissue from 12 males and 12 females was microdissected from 5–10-μm sections of specimens (see Fig. 1 for example of microdissection) that had been dewaxed and H&E stained. It is estimated that contamination by stromal cells would not have exceeded 20% of total cells sampled. Samples were digested for 3–5 days at 37°C in Buffer S [10 mM Tris (pH 7.5), 10 mM EDTA (pH 8.0), and 50 mM NaCl] containing 200 μg/ml proteinase K, were phenol extracted, and ethanol precipitated. An abbreviated protocol was used for an additional 12 samples from females, which were assayed only for X inactivation status and in which nevus cells comprised more than 60% of the tissue in the block. Approximately 20 × 10-μm sections were deparaffinized in an Eppendorf tube with octane and methanol (22). Proteinase digestion was carried out in Buffer S at 50°C overnight; the samples were then heated to 95°C for 15 min to inactivate the proteinase K and were microfuged for 5 min to remove debris and supernatant collected to a fresh tube.

DNA was extracted, by standard methods, from fourteen matched PBL and sporadic metastatic melanoma samples from female patients, which were used as positive controls for the X-inactivation assay.

PCR Protocols and Electrophoresis. PCR primers were purchased as lyophilized solution from Sigma Chemical Co., Genosys, Australia. Optimal DNA dilutions for PCR were determined empirically for each sample. Microsatellite markers were in general amplified in two stages, this being necessary partly because of degraded template quality and the fact that the addition of a fluorescent label tends to reduce the ease of amplifying DNA. The PCR protocol was 95°C at 3 min; followed by 5 cycles of 95°C for 1 min, 1–2 min annealing and 1–2 min extension at 72°C. This was followed by 15 cycles of 95°C for 30 sec, 30 s to 1 min annealing and 45 s to 1 min extension at 72°C; followed by a final extension of 72°C for 5 min. Reactions were in 10-μl final volume in PCR buffer [50 mM KCl, 10 mM Tris (pH 8.5), and 1.5 mM MgCl₂] using diluted unlabeled primers (at 2.5 pmol/μl), 200 μM each dNTP, 1.2 μg/μl BSA (23), and 1 unit of Taq DNA polymerase. A 1-μl aliquot was then added to tubes for a similar PCR in 10-μl final volume using 5’-HEX forward-labeled primers. HEX primers require PCR buffer [50 mM KCl, 10 mM Tris (pH range, 8.5–10.0), and 1.5–3.0 mM MgCl₂] and step-length optimization with archival DNA. PCR was performed at 95°C for 3 min, followed by 5 cycles of 95°C for 30 s, annealing for 30 s to 1 min, and extension at 72°C for 20 s to 1 min (shortest possible step lengths are preferable), followed by a final extension of 72°C for 5 min.

The forward primer for the androgen receptor (AR) exon 1 trinucleotide repeat locus HUMARA (4) was 5’-HEX-labeled and shortened from published sequence at the 3’ end to 5’-
GCTGTGAAGGTGCTGT-3'. Reverse primer was as per published sequence: 5'-TCCAGAATCTGTTCCAGGCT- GC-3'. DNA was added to tubes containing 15 μl of [master mix] [50 mM KCl, 10 mM Tris (pH 9.5), and 1.5 mM MgCl2, 1.2 μg/μl BSA, 3 ng/μl each PCR primer, and 200 μM each dNTP]. After 30 min at 95°C, 5 μl of master mix containing 2 units of Taq DNA polymerase was added and PCR carried out as follows: at 95°C for 3 min; 5 cycles of 95°C for 1 min, 52°C for 2 min, and 72°C for 2 min; followed by 35 cycles of 95°C for 30 s, 52°C for 45 s, and 72°C for 45 s; with a final extension of 72°C for 5 min in a Corbett Research FTS-960 thermal sequencer. All of the PCR reactions were repeated for confirmation in duplicate results.

An equal volume of denaturing sample dye [formamide containing 10 mM EDTA (pH 8.0) and bromphenol blue] was added, samples were heat denatured for 3 min at 95°C and snap-chilled, and 2 μl of sample were loaded onto a 6% 29:1 polyacrylamide gel containing 8 M urea and 0.6× Tris-borate EDTA. This was run at 900 V and 40°C in 0.6× Tris-borate EDTA in Corbett Research Gel Scan-2000 DNA analyzer. LOH and clonality were calculated from area-under-curve determination (24). When a microsatellite marker is amplified by PCR, a heterozygous product will contain relatively more of the smaller allele, characteristic of both the microsatellite marker under study and the size separation of the alleles. The relative allelic shift is given by dividing PCR product yields so that the ratios of allele 1 and allele 2 are compared in normal (N) and lesion (L) tissue (Nallele 1 / Nallele 2 + Lallele 1 / Lallele 2). If no loss has occurred, this ratio will be close to 1. A change in allelic ratios of >30% in duplicate samples is conventionally scored as LOH.

X-Inactivation Analysis. For X-inactivation analysis, the test DNA was split into an untreated aliquot and another aliquot subjected to digestion with the methylation-sensitive restriction enzyme HpaII (Promega Corporation, Sydney, Australia) at 37°C overnight and then was heat inactivated at 95°C for 10 min prior to PCR or the HUMARA locus. Relative allelic signal ratio before and after digestion in both normal and lesion tissue was compared. The X-inactivation assay algorithm is analogous to that used for LOH analysis, and a >30% change in allelic ratio in at least duplicate samples for tissue (undigested) and tissue (HpaII-digested) was preselected as a positive result for skewed X inactivation. In practice, all of the samples with skewed X inactivation showed at least a 50% change in this ratio. LOH status of normal and lesion DNA was also assessed, because the X-inactivation status can be determined only with knowledge of the presence or absence of lesion alleles.

RESULTS

LOH in Benign Nevi. As shown in Table 1, no LOH was observed at any of the four 9p and six 10q loci tested. In particular, none of the 23 informative samples was deleted at the D9S162 marker, which is internal to the CDKN2A/p14ARF locus. One of the 11 nevi from females that were informative for LOH showed deletion of the chromosomal-X androgen receptor (HUMARA) locus. In addition, one sample showed a new allele at 1 of the 10 loci examined (D10S187), consistent with low-level MSI (Fig. 2).

| Table 1 Deletion analysis of 24 nevi at 11 microsatellite loci on 9p, 10q, and Xq |
|-----------------|--------|---|---|
| Chromosome | Marker | n | LOH |
| 9p | D9S162 | 24 | 0/20 |
| 9p | IFNA | 24 | 0/17 |
| 9p | D9S942 | 24 | 0/23 |
| 9p | D9S171 | 21 | 0/19 |
| 10q | D10S579 | 23 | 0/13 |
| 10q | D10S597 | 24 | 0/15 |
| 10q | D10S468 | 24 | 0/17 |
| 10q | D10S187 | 23 | 0/18 |
| 10q | D10S214 | 24 | 0/18 |
| 10q | D10S212 | 22 | 0/10 |
| Xq | HUMARA | 12 | 1/11 |

X-Inactivation Analysis of Benign Nevi. Twenty-four nevi from females were assayed for X inactivation. In 12 of these cases, paired normal and lesion DNA samples were obtained (see Fig. 1), although 12 were assayed only as lesion DNA. Paired normal and tumor samples from 14 female patients with metastatic melanoma were assayed as positive controls (Table 1).

In all, 20 of the 24 nevi were informative at the HUMARA locus and 16 (80%) showed reduction of one allele after HpaII predigestion of the template, which indicated nonrandom X inactivation and which was consistent with monoclonality (Fig. 2). The degree of allele reduction ranged from 57 to 82%, with the residuum most likely attributable to nonclonal populations of stromal cells, although minor subclones of nevus cells with different patterns of X inactivation cannot be ruled out. One nevus sample showed LOH at the HUMARA locus, and its template could not be amplified after HpaII digestion.

X Inactivation in Adjacent Epidermis. Finally, X inactivation was also assessed in the adjacent microdissected epidermal keratinocytes of the 10 informative individuals from the nevus cohort. Significant allelic reductions were observed after HpaII digestion in five of the samples of normal skin. Two epidermal keratinocyte samples showed nonrandom X inactivation with reduction of the same allele as the nevus, and two showed reduction of the opposite allele, which indicated independent X-inactivation status. In one case, nonrandom X inactivation was found in the normal epidermal sample, but not in the nevus.

X-Inactivation Analysis and LOH at HUMARA in Malignant Melanoma. Twelve of 14 metastatic melanomas tested were informative at the HUMARA locus (Table 2), and of these, 11 showed an allelic reduction after digestion with HpaII, consistent with monoclonality (Fig. 2), as has previously been reported (9). Interestingly, as has been noted previously (25), most of the blood-derived DNAs from these individuals also showed skewed X inactivation. Such samples are, therefore, not necessarily appropriate “controls” for X inactivation analysis of tumor specimens. In contrast to the nevi, 4 (33%) of 12 informative metastatic melanoma tumors showed LOH at the HUMARA locus. Furthermore, of these 4 samples showing LOH, in each case in which one allele was reduced by LOH, the other was always reduced after HpaII digestion in the clonality assay, leaving only a residual signal from stromal DNA (Fig. 2). In all
of these cases, the residual signal showed an allelic profile similar to that of the undigested control, which indicated random X inactivation. LOH, therefore, targeted only the inactive chromosome X, and this observation is being pursued in additional studies.

DISCUSSION

We found that in 80% of informative benign melanocytic nevi and 92% of metastatic melanomas, the PCR product yield at the HUMARA locus of one allele was significantly reduced if the template were predigested with the methylation-sensitive restriction endonuclease HpaII. This indicates that a majority of cells contributing to the template shared a common pattern of X inactivation that is consistent with the monoclonality of these lesions. These results, obtained with compound and dermal nevi, mirror the findings of another Australian study (10) in which 79% of predominantly junctional nevi were shown to have skewed X inactivation and were considered monoclonal in this way, as was the case in a smaller American sample (11).

The absence of skewed X inactivation in 10–20% of nevi and melanoma samples requires some explanation. The simplest reasons would be restriction-digest failure because of template impurities or excessive stromal contamination that obscured the signal from nevus or melanoma cells in some cases. Variable sensitivity of the PCR assay attributable to uneven methylation patterns in some individuals is also likely to contribute to false negative results, as demonstrated in a recent study (26). It is also still possible, as discussed below, that more than one pattern of X inactivation is represented in the ancestral cells of these nevi.

Despite this uncertainty, several studies, taken together, now show that common acquired nevi, of junctional, dermal, and compound types, show skewed X inactivation and appear monoclonal by this assay, as do melanomas. In contrast, one Japanese study (9) has concluded that all nevi, whether congenital or acquired, were polyclonal (i.e., showed random X inactivation). It may be that the biology of common acquired nevi is somewhat different in Europeans and non-Europeans because of variations in skin pigmentation and the effects of sun exposure.

In this study, we systematically determined the extent of skewed X inactivation in four tissue types: microdissected nevi, epidermal keratinocytes, PBLs, and metastatic melanoma. We observed frequent skewing in all of the groups of samples. Skewed X inactivation of PBLs is common in women and increases in degree and frequency with age (25, 27). We found X-inactivation skewing ratios >3:1 in PBLs of 5 of 9 female metastatic melanoma patients who were of middle age to elderly, consistent with previously published reports. Several studies have noted skewed X inactivation of other normal tissues: endometrium (28), lobules and larger ducts in the breast (29), and gastric mucosa (30). However, in previous studies of nevi, either adjacent normal tissues were not studied (9) or allelic ratios were not reported, and it is, therefore, difficult to interpret their data, especially those from “controls” (10).

**Table 2** Analysis of X-inactivation status and LOH at HUMARA

<table>
<thead>
<tr>
<th>Samples</th>
<th>Informative&lt;sup&gt;a&lt;/sup&gt; %</th>
<th>Nonrandom X inactivation&lt;sup&gt;b&lt;/sup&gt; %</th>
<th>LOH</th>
</tr>
</thead>
<tbody>
<tr>
<td>Compound and dermal nevi</td>
<td>24</td>
<td>21</td>
<td>17 (81%)</td>
</tr>
<tr>
<td>Metastatic melanomas</td>
<td>14</td>
<td>12</td>
<td>11 (92%)</td>
</tr>
</tbody>
</table>

<sup>a</sup> Heterozygous at HUMARA locus.

<sup>b</sup> Greater than 30% reduction in relative strength of one allele in lesion as per “Materials and Methods.”
We found one-half (5 of 10) of informative samples showed skewed X-inactivation status in epidermal keratinocytes and that the direction of X-inactivation skewing was independent of nevus cells. Lines of Blaschko, are known to mark domains of homogeneous X inactivation, as revealed by females with chromosome X-linked dermatoses affecting keratinocytes (31, 32). Small tissue samples are likely to be contained within such regions of shared X-inactivation status and to appear clonal by this assay. We presume that the epidermal specimens that did not show skewed X inactivation lay at a boundary between regions of different keratinocyte X inactivation status, but this hypothesis should be tested by serial sampling of large skin specimens. In all cases in which X-inactivation analysis was carried out on melanoma metastases with LOH at HUMARA, it was found that a small residual stromal signal remained that showed a normal allelic ratio. We interpret this as indicating random X inactivation in the admixed stromal tissue.

These complexities highlight a common fallacy in the interpretation of clonality data obtained from the analysis of X inactivation, and indeed a difficulty with usage of the term clonal. Cells that arise from a common ancestor after the latter has completed X inactivation (Lyonization) will normally share a random, mixed pattern of X inactivation. If they do not, then they probably do not share such a common ancestor and are, by that criterion, polyclonal. However, the converse is not true. The presence of such a shared X-inactivation pattern does not prove the monoclonality of a primary tumor (or nevus), because a group of genetically or epigenetically diverse cells could share a pattern of X inactivation long before the onset of tumorigenesis and yet, in theory, contribute subpopulations to it. Although polyclonality can be proven, monoclonality must always be qualified by the method by which it was observed and cannot be used to deduce how early the putative clone arose.

Only one instance of LOH, at the chromosome X HUMARA locus, was found in a total of 245 paired genotypes examined in the nevus cohort (Table 1). An isolated finding of this kind is consistent with occasional reports of infrequent deletions in nevi in previous studies (14, 21).

The deletion results for D9S942, and by implication, nearby CDKN2A, in common acquired nevi, are concordant with results from a recent study (33), in which sporadic primary melanomas were examined for deletion of D9S942. No thin primary melanomas (<0.75 mm thickness), but 35% of thick lesions (>3.0-mm thickness) showed deletion at the D9S942 locus. This suggests that such loss is a relatively late event in melanocyte tumorigenesis. The results here are consistent with the long-proposed model in which nevi represent an early event in a multistep pathway of melanoma tumorigenesis, but of course do not provide positive evidence to support it (6). We found no deletions of 9p or 10q in benign melanocytic nevi, despite their frequent deletion in metastatic melanoma. Sufficient lesions have now been examined in various studies to conclude that these genetic events are unlikely to be of importance in the initiation of neoplasia in common acquired nevi.

Interestingly, 33% of informative paired melanoma samples studied showed LOH at the single chromosomal X locus that was examined (HUMARA). Whereas this rate of loss was consistent with summarized data for deletion of this chromosome (34), it is important to put this apparently modest rate in perspective. Chromosome X is either actually or functionally hemizygous in both males and females; therefore, this rate corresponds to an autosomal deletion frequency of 66%. Furthermore, in 4 of 4 of samples with LOH at HUMARA, we showed that the inactive X chromosome was the target of LOH. This observation has a precedent in ovarian cancer (35) and is being followed up in a larger series, to determine whether significant melanoma tumor suppressor genes, or oncogenes, may reside in this region.

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*Clin Cancer Res* 2001;7:4054-4059.

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