11q23 Allelic Loss Is Associated with Regional Lymph Node Metastasis in Melanoma

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INTRODUCTION

As with other solid tumors, cutaneous melanoma formation and tumor progression appear to result from the accumulation of multiple genetic lesions, and several models for this process have been proposed on the basis of karyotypic analyses (1) and LOH studies (2). However, more recent data show genetically heterogeneous tumor cell populations at early stages of melanoma progression (3), including primary tumors (4).

Loss of genetic material of the long arm of chromosome 11 (11q) has been demonstrated to occur at frequencies ranging from 17% (5) to 32% (6) in primary melanoma and from 29% (2) to 38% (7) and 39% (6) in metastatic melanoma. We recently delineated two distinct putative tumor suppressor loci on chromosome 11q mapping to 11q23.1-q23.2 (in a region of approximately 5 Mb) and 11q23.3 (in a region of approximately 3 Mb) in melanoma (6). The first region overlaps or includes chromosomal regions of common allelic loss that have been defined in breast, bladder, nasopharyngeal, prostate, and lung cancers, whereas the second overlaps or includes regions with frequent allelic loss in lung, breast, ovarian, nasopharyngeal, and prostate cancers. These findings are supported by functional studies in a number of different human cancer systems such as breast and squamous cell skin cancers and melanoma, where suppression of tumorigenicity was demonstrated by microcell-mediated chromosome transfer of all or parts of a normal chromosome 11 into these tumor cell lines (reviewed in Ref. 6).

A recently cloned candidate tumor suppressor gene, PPP2R1B, with somatic alterations in human lung and colon cancers maps to 11q22–24 (8). Furthermore, Murakami et al. (9) localized tumor suppressor activity important in non-small cell lung cancer to 11q23 by YAC complementation.

In contrast to many other tumor systems, the subsequent progression stages of melanoma such as primary tumor, in-transit metastasis, regional lymph node metastasis, and distant organ metastasis are clinically more obvious, and tumor tissue is more easily accessible and can therefore be individually investigated. However, there are sparse reports to date regarding the stage during progression of individual melanomas at which allelic loss in general and 11q loss in particular occur (3, 10). These might help to define the function of individual putative tumor suppressor loci and allow evaluation of their value as prognostic markers. Comparably, karyotypic alterations of chromosome 11 have been reported in advanced-stage melanoma but not in primary tumors (reviewed in Ref. 11) and have been associated with a less favorable clinical prognosis (12).

To establish the point in time of melanoma tumorigenesis at which two putative 11q tumor suppressor loci become relevant, we investigated allelic loss (LOH) in both chromosomal regions in tumors of progressing patients from whom tumors...
samples of multiple progression steps (such as primary tumor, in-transit metastasis, regional lymph node metastasis, and distant metastasis) were available for analysis. We also tested the hypothesis of tumor cell heterogeneity at an early progression stage by microdissecting and analyzing different parts of one individual primary tumor that appeared to consist of histomorphologically distinct areas of tumor cell growth.

MATERIALS AND METHODS

Patients and Tissues. DNA was extracted from microdissected fresh tumor tissue samples, formalin-fixed paraffin-embedded tissue, or peripheral blood lymphocytes obtained from 23 patients selected for availability of tumor tissue of at least two different clinical melanoma progression stages using standard protocols (10). Of the 23 patients, tumor tissue from primary tumor and in-transit metastasis was available from 2 patients; tumor tissue from primary tumor and regional lymph node metastasis was available from 7 patients; tumor tissue from regional lymph node metastasis and distant metastasis was available from 1 patient; tumor tissue from primary tumor, in-transit metastasis, and regional lymph node metastasis was available from 10 patients; tumor tissue from primary tumor, regional lymph node metastasis, and distant metastasis was available from 2 patients; tumor tissue from in-transit metastasis and distant metastasis was available from 1 patient. For several patients, we examined tumor tissue (particularly for in-transit metastases) excised at different time points within that tumor stage separately (i.e., several subsequently occurring in-transit metastases). The primary tumor of one patient (patient 6) was microdissected further from thick tumor slices to obtain DNA from five histomorphologically different areas of tumor growth for separate LOH analysis. LOH results for 6 of 102 tumor samples at markers APOC3 and D11S925 have been included in a previous study (6).

DNA Analysis. DNA samples were analyzed for LOH in the two target regions defined previously on 11q23 (6) using polymorphic microsatellite repeats at the following six loci: four markers [D11S1786 (11q23.1), D11S1885 (11q23.1), APOC3 (11q23.1–q23.2), and D11S998 (11q23)] are located at chromosome bands 11q23.1–11q23.2 (referred to as region 1), whereas the other two markers, D11S4132 and D11S925, both map to 11q23.3 (referred to as region 2; marker positions from centromere to telomere according to the Genome Database (The Johns Hopkins University, Baltimore, MD); Refs. (9 and 13).

As an internal control, all samples were also analyzed for LOH at other chromosome arms previously reported to be either frequently affected by LOH, such as 9p (D9S954 and D9S958), or infrequently affected by LOH, such as 17p (p53-CA; reviewed in Ref. 1). Conditions for denaturation, annealing, and elongation were as described in the original publications. PCR products were resolved by electrophoresis on 6–8% formamide/polyacrylamide gels and visualized by a nonradioactive silver-staining technique (14). To ensure reproducibility, at least two independent reactions were performed and evaluated per sample.

LOH Analysis. Evaluation of LOH was performed by comparing the intensities of the two alleles in informative cases: only cases where simple visual inspection was sufficient to easily discriminate between LOH or retention of constitutional heterozygosity were included. In some cases where the alleles were clear and well separated, these conclusions were verified by scanning densitometry, and LOH was imputed by a signal loss of one allele of ≥50%.

RESULTS

We detected overall 11q LOH for any marker at any stage in 20 of 23 (87%) patients. Nineteen of 23 (83%) patients had allelic loss at a marker(s) in region 1 (11q23.1–23.2), and 13 of 19 (68%) informative patients had allelic loss at a marker(s) in region 2 (11q23.3); 4 patients were constitutionally homozygous and thus were not informative for both markers tested in the latter region. We detected concomitant LOH in both regions in 12 patients, and we found exclusive loss in region 1 in 3 patients and exclusive loss in region 2 in 1 patient. Three of the 12 patients with concomitant LOH in both regions retained constitutional heterozygosity at loci located in between lost markers. Overall LOH frequencies at the other chromosome arms tested as internal controls were 69% at 9p and 15% at 17p.

In 21 primary tumors, the overall rate of allelic loss was 38% at both regions of 11q combined and 38% and 29% in regions 1 and 2, respectively (Table 1).

At in-transit metastasis, 62% of informative patients showed allelic loss in either region, and 62% and 36% of informative patients showed allelic loss in regions 1 and 2,
LOH at Lymph Node Metastasis in Melanoma

Table 2  11q23.1–q23.2 (regional 1) and 11q23.3 (region 2) allelic loss is associated with regional lymph node metastasis in melanoma

<table>
<thead>
<tr>
<th>Patient no.</th>
<th>Primary tumor (region 1/region 2)</th>
<th>In-transit metastasis (region 1/region 2)</th>
<th>Regional lymph node metastasis (region 1/region 2)</th>
<th>Distant metastasis (region 1/region 2)</th>
</tr>
</thead>
<tbody>
<tr>
<td>15</td>
<td>○/○*</td>
<td>●/□</td>
<td>○/○</td>
<td>N/A</td>
</tr>
<tr>
<td>22</td>
<td>○/NI</td>
<td>●/NI</td>
<td>●/NI</td>
<td>N/A</td>
</tr>
<tr>
<td>12</td>
<td>○/○</td>
<td>●/□</td>
<td>●/□</td>
<td>N/A</td>
</tr>
<tr>
<td>18</td>
<td>N/A</td>
<td>○/○</td>
<td>○/NI</td>
<td>N/A</td>
</tr>
<tr>
<td>3</td>
<td>○/NI</td>
<td>●/□</td>
<td>●/□</td>
<td>N/A</td>
</tr>
<tr>
<td>6</td>
<td>AS/○</td>
<td>○/○</td>
<td>●/□</td>
<td>N/A</td>
</tr>
<tr>
<td>9</td>
<td>○/○</td>
<td>○/○</td>
<td>●/□</td>
<td>N/A</td>
</tr>
<tr>
<td>4</td>
<td>○/○</td>
<td>N/A</td>
<td>○/□</td>
<td>N/A</td>
</tr>
<tr>
<td>19</td>
<td>○/NI</td>
<td>N/A</td>
<td>○/NI</td>
<td>N/A</td>
</tr>
<tr>
<td>23</td>
<td>○/○</td>
<td>N/A</td>
<td>○/□</td>
<td>N/A</td>
</tr>
<tr>
<td>14</td>
<td>N/A</td>
<td>N/A</td>
<td>○/□</td>
<td>N/A</td>
</tr>
</tbody>
</table>

* ○, retained constitutional heterozygosity; ●, loss (LOH); N/A, no tumor tissue available or no tumor of that stage occurred; AS, allele shift; NI, not informative.

In-transit metastases with LOH developed after regional lymph node metastases in these two patients.

Table 3  LOH at 11q23 occurs at lymph node metastasis in two patients with progressing melanoma and subsequently developing in-transit metastases do not show LOH

<table>
<thead>
<tr>
<th>Patient/tumor</th>
<th>Type of tumor</th>
<th>Datea (mo/yr)</th>
<th>1786b (11q23.1)</th>
<th>1885b (11q23.1)</th>
<th>APOC3 (11q23.1–23.2)</th>
<th>1998 (11q23)</th>
<th>4132 (11q23.3)</th>
<th>925 (11q23.3)</th>
</tr>
</thead>
<tbody>
<tr>
<td>6AI-IV</td>
<td>ALM, 15 mm, Clark V I–IV epitheloid-solid parts</td>
<td>5/97</td>
<td>○/○</td>
<td>NI</td>
<td>○</td>
<td>NI</td>
<td>NI</td>
<td>○</td>
</tr>
<tr>
<td>6Asp</td>
<td>Invasive spindle-shaped part of primary tumor</td>
<td>5/97</td>
<td>AS</td>
<td>NI</td>
<td>○</td>
<td>NI</td>
<td>NI</td>
<td>○</td>
</tr>
<tr>
<td>6B</td>
<td>Regional lymph node metastasis</td>
<td>7/97</td>
<td>●</td>
<td>NI</td>
<td>●</td>
<td>NI</td>
<td>NI</td>
<td>●</td>
</tr>
<tr>
<td>6C</td>
<td>In-transit metastasis</td>
<td>8/97</td>
<td>○</td>
<td>NI</td>
<td>○</td>
<td>NI</td>
<td>NI</td>
<td>○</td>
</tr>
<tr>
<td>6E</td>
<td>In-transit metastasis</td>
<td>1/98</td>
<td>○</td>
<td>NI</td>
<td>○</td>
<td>NI</td>
<td>NI</td>
<td>○</td>
</tr>
<tr>
<td>9A</td>
<td>SSM, 2, 1 mm, Clark V</td>
<td>1/96</td>
<td>○</td>
<td>○</td>
<td>○</td>
<td>○</td>
<td>○</td>
<td>○</td>
</tr>
<tr>
<td>9B</td>
<td>Regional lymph node metastasis</td>
<td>2/96</td>
<td>●</td>
<td>●</td>
<td>●</td>
<td>●</td>
<td>●</td>
<td>●</td>
</tr>
<tr>
<td>9C</td>
<td>In-transit metastasis</td>
<td>8/96</td>
<td>○</td>
<td>○</td>
<td>○</td>
<td>○</td>
<td>○</td>
<td>○</td>
</tr>
</tbody>
</table>

Type of tumor: ALM, acral lentiginous melanoma; SSM, superficial spreading melanoma; tumor thickness in mm, Clark level.

a Date of excision.
b 1786, markers from centromere to telomere, D11S omitted, chromosomal band location in parentheses.
*c ○, retained constitutional heterozyzogosity; NI, not informative; AS, allele shift; ●, LOH.

respectively. All patients with region 2 LOH in the primary tumor or in-transit metastases also displayed losses in region 1.

At regional lymph node metastasis, we found LOH of either region in 71% of patients, with 67% of patients having loss in region 1 and 53% of patients having loss in region 2. At this stage, we identified one patient that had loss exclusively in region 2, all other patients with region 2 LOH also had loss in region 1.

At distant organ metastasis, all four patients investigated showed LOH at region 1, and all three of these four patients that were informative at a marker(s) in region 2 displayed allelic loss there as well.

LOH results of all patients for whom separately analyzed tumor tissues excised at different time points within the same clinical stage (i.e., several subsequently developing in-transit metastases) were identical at the respective stage are only counted once in Tables 1 and 2.

In 3 of 23 patients, we found no 11q LOH at any stage or marker tested. LOH was consistently detected through all stages tested and included the primary tumor in 8 of 23 patients. In the remaining 12 patients, the first clinical progression step at which LOH was detected was as follows (Table 2): (a) in-transit metastasis (2 patients); (b) regional lymph node metastasis (8 patients); and (c) distant metastasis (2 patients). Two of the eight patients with first 11q LOH at regional lymph node metastasis showed LOH in in-transit metastases, but these developed 4 and 9 months after resection of the lymph node metastases (patients 16 and 3). Most notably, two other patients in this group of eight patients retained constitutional heterozygosity in in-transit metastases that occurred 2 and 7 months after regional lymph node metastases that had already displayed loss (detailed results for both patients 6 and 9 are shown in Table 3, and exemplary LOH analyses of patient 9 are depicted in Fig. 1). Taken together, in 8 of 12 (67%) patients with 11q allelic loss during tumor progression, regional lymph node metastasis was the first clinical progression stage at which this loss was detected.

Finally, we microdissected and individually analyzed several areas of a primary tumor of a patient (patient 6) in whom we
had detected LOH in a regional lymph node metastasis but not in the primary tumor or in two different in-transit metastases that appeared 2 and 7 months after the regional lymph node metastasis was resected (Table 3). Here we did not find any definite LOH within four different but histomorphologically similar tumor areas characterized by solid epithelioid tumor cell growth (6AI in Fig. 2a, c, and d). However, we detected an allele shift presenting as a newly appearing allele (Fig. 2d, allele 3) in a histomorphologically distinct area of infiltrating spindle-shaped tumor cell growth (6Asp in Fig. 2a, b, and d) at marker APOC3 (11q23.1–q23.2). The same parental chromosome was affected by LOH in a subsequently occurring lymph node metastasis (6B in Fig. 2d).

DISCUSSION

In this study, we aimed to establish the point in time during clinical melanoma tumorigenesis and tumor progression at which two putative 11q tumor suppressor loci become relevant. Therefore, we investigated allelic loss in both chromosomal regions in tumors of progressing patients from whom tumor samples of multiple progression steps were available for analysis. Due to this selection bias, the overall frequency of LOH (87%) is substantially higher than that seen in previous studies by us [35% (6, 11)] and others [17%, 29%, and 38% (2, 5, 7)] that investigated unselected random patient populations. LOH rates were as expected in the other chromosomal areas tested as internal controls; a low frequency of 15% at marker p53-CA located within the p53 gene on 17p13 is consistent with data published previously (15). In contrast, we found 69% LOH at markers D9S171 and D9S958 on 9p21, a chromosomal region known to be frequently affected by allelic loss in melanoma (1).

At first glance, the constant rise of the frequency of LOH with clinical progress of the patient could be ascribed simply to growing genetic instability. For clarification of this issue, the results of the three patients with no 11q LOH at all and eight patients with LOH throughout all stages (including the primary tumor for all of these patients) are less helpful than the results of the 12 patients with LOH occurring at some stage during clinical progression (Table 2). In this latter group, we found an association between 11q LOH and regional lymph node metastasis because this was the first clinical progression stage at which allelic loss was detected in 67% of these patients. This is supported by the finding that, in contrast to 11q LOH occurring first at regional lymph node metastasis, 9p21 LOH investigated as an internal control had already occurred in the primary tumor in all but one case and was then detected consistently through all clinical stages in all patients affected. This is in agreement with the hypothesis that disturbances of putative tumor suppressor(s) on the short arm of chromosome 9 are early events during melanoma tumorigenesis (1).

In the three patients in whom we did not detect any 11q LOH at all and the two patients in whom 11q LOH first occurred at distant metastasis, the deletions may be smaller than detectable by the method used. Alternatively, there may be other tumor suppressors in different chromosomal regions involved in tumor progression of these cases.
The detection of retained markers between the two regions lost in 3 of 12 patients with concomitant LOH of both regions and the finding that 3 patients showed loss exclusively in region 1 and 1 patient showed loss exclusively in region 2 confirm our previous delineation of two distinct deletion targets (6). This is also supported by the differences in LOH frequencies between the two regions. It is unlikely that both targets represent the same gene because it would have to cover more than 10 Mb. Moreover, because it was not the aim of the study to further narrow down the previously delineated two deletion targets (6). This is also supported by the differences in LOH frequencies between the two regions. It is unlikely that both targets represent the same gene because it would have to cover more than 10 Mb. Moreover, because it was not the aim of the study to further narrow down the previously delineated two deletion targets and because we investigated four markers at region 1 and only two markers at region 2, the lower frequency of markers examined and the resulting lower overall informativeness in region 2 may also have influenced the lower LOH rates there. However, on the basis of the number of cases and progression stages investigated here, we are unable to draw any further conclusions with respect to differences in timing or function between these two distinct deletion targets.

Morita et al. (3) recently investigated LOH at several chromosome regions in 14 pairs of melanoma primary tumors and metastases and found that LOH at chromosome arms 6q, 8p, 9p, 9q, and 18q were mostly shared between primary tumors and their metastases. However, they detected chromosome 11 LOH in a lymph node metastasis of a patient with a primary melanoma without LOH and speculated that loss of this chromosome may play an important role in the acquisition of metastatic potential in sporadic melanoma. Karyotypic analyses of Morse et al. (16) showed chromosome 11q and/or 17q aberrations exclusively in brain metastases in six of eight patients investigated.

While we were preparing this article, Robertson et al. (17) narrowed the location of a putative melanoma suppressor by a functional assay using microcell hybrids to a 2-Mb candidate region between markers D11S1786 and D11S2077. This region coincides with our region 1. Recently, Murakami et al. (9) localized tumor suppressor activity important in non-small cell lung cancer to 11q23 by YAC complementation. Marker D11S1885 of region 1 is included in the central 700-kb region on 11q23 where these authors suspect all or parts of a putative tumor suppressor gene to reside. Therefore, the tumor suppressor activity shown by both these authors may be the deletion target in region 1. Another recently cloned candidate tumor suppressor gene, PPP2R1B, which has somatic alterations in 15% of primary human lung and colon cancers tested, maps to 11q22–24 (8) and appears to be located centromeric of region 1. This chromosomal area was not included in the study presented here because we did not detect substantial rates of allelic loss at five markers spanning the region of that gene (D11S2000, 11q23 LOH at Lymph Node Metastasis in Melanoma).
Our finding of an allele shift restricted to a histomorphologically distinct area of a primary tumor supports the hypothesis of heterogeneous tumor cell populations at an early stage of melanoma (3) including the primary tumor (4). The fact that two in-transit metastases without this allele shift or LOH developed in this patient after the regional lymph node metastasis with 11q23 LOH had been excised further supports this hypothesis.

In conclusion, our findings indicate that allelic loss of 11q during melanoma progression is associated with regional lymph node metastasis and that this is not the effect of random genetic instability. Inactivation of putative tumor suppressor gene(s) located in these regions may therefore be responsible for progression of the disease to the regional lymph nodes. This marks an important step in clinical melanoma tumor progression for the patients because it substantially reduces the chance of long-term survival. Finally, because most studies to date on 11q LOH in other tumor systems have concentrated on primary tumors, it may be worthwhile to investigate later stages of disease in these malignancies as well to clarify whether this chromosome arm harbors tumor suppressor genes whose inactivation leads to lymph node metastasis as a general pathway.

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