Loss of Expression of the Metastasis Suppressor Gene KiSS1 during Melanoma Progression and Its Association with LOH of Chromosome 6q16.3-q23

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Abstract

KiSS1 is a putative melanoma metastasis suppressor gene, the expression of which may be regulated by another gene(s) mapping to chromosome 6q16.3-q23. To additionally elucidate the role of KiSS1 in the progression of human melanoma in vivo, we examined KiSS1 mRNA expression in 51 melanocytic tumors with various stages of progression by in situ hybridization. We also examined a correlation between loss of KiSS1 mRNA expression and loss of heterozygosity (LOH) of 6q16.3-q23 in 27 melanoma metastases. All of the four nevocellular nevi and eight primary melanomas <4 mm in thickness showed KiSS1 mRNA expression, whereas only 50% (6 of 12) of primary melanomas >4 mm in thickness expressed KiSS1. Loss of KiSS1 mRNA was equally frequent in metastases; 44% (12 of 27) of tumors lost KiSS1 expression. LOH of 6q16.3-q23 was observed in 52% (14 of 27) of metastases. There was a strong association between LOH and loss of KiSS1 expression (P = 0.03); nine metastases with LOH of 6q16.3-q23 lost KiSS1 expression, whereas 10 tumors with no LOH showed positive KiSS1 mRNA expression. The findings in this study show, for the first time, KiSS1 downregulation during the progression of melanoma in vivo and strongly suggest that inactivation of a tumor suppressor gene(s) mapping to 6q16.3-q23 by deletion or mutation coupled with LOH may lead to the downregulation of KiSS1.

Introduction

Cytogenetic analysis and LOH1 studies in melanoma metastases have demonstrated that chromosomes 1, 6, and 10 are the most frequent sites of chromosome alterations in advanced stage disease (1, 2). In particular, the long arm of chromosome 6 is preferentially lost during the progression of the majority of melanomas, suggesting the existence of a metastasis suppressor gene(s) important for melanoma metastasis on 6q (3–6). Direct experimental evidence for a metastasis suppressor gene(s) on 6q has been provided by showing suppression of metastasis after introduction of a normal copy of chromosome 6 into highly metastatic human melanoma cell lines (7, 8). By the use of differential display and subtractive hybridization, a novel cDNA designated as KiSS1 was isolated from melanoma cells that had been suppressed for metastatic potential by the introduction of human chromosome 6 (9). Transfection and constitutive expression of the full-length KiSS1 cDNA suppressed metastasis of human melanoma and breast carcinoma cells, strongly suggesting that KiSS1 is a novel metastasis suppressor (9–11). Surprisingly, however, KiSS1 mapped to 1q32-41 by fluorescence in situ hybridization, the region not implicated in melanoma progression (12). Evidence from subsequent experiments suggests that the expression of KiSS1 is very likely to be regulated by a gene(s) localized in the 40-cM region between 6q16.3-q23 (13).

Although the function of the protein encoded by KiSS1 is unknown, the KiSS1 gene product has recently been shown to repress 92-kDa type IV collagenase (MMP-9) expression by effecting reduced NF-κB binding to the promoter (14). Furthermore, very recent investigations have shown that KiSS1 encodes a COOH-terminally amidated peptide with 54 amino acid residues, which is a ligand of a novel human G-protein-coupled receptor (named AXOR12 and hOT7T175, respectively; Refs. 15 and 16). The peptide ligand named as “metastin” by one of the investigators inhibits chemotaxis and invasion of hOT7T175-transfected Chinese hamster ovary cells in vitro and attenuates pulmonary metastasis of hOT7T175-transfected B16-BL6 melanomas in vivo (16). These findings show functional mechanisms by which KiSS1 may act as a metastasis suppressor gene.

To additionally elucidate the role of KiSS1 in the progression of human melanocytic tumors in vivo, we examined KiSS1 mRNA expression in various stages of progression by in situ hybridization. Furthermore, to test the hypothesis that expression of KiSS1 is regulated by a gene(s) mapping to 6q16.3-q23, we also examined a correlation between loss of KiSS1 mRNA expression and LOH of 6q16.3-q23 in melanoma metastases.

Materials and Methods

Tissue Samples. Twenty primary tumors and 27 metastases were obtained from 41 surgical patients with melanoma (25 males and 16 females with ages ranging from 31 to 84 years). In 5 patients, both primary and metastatic tumors were available. The types of melanoma included 24 acral lentiginous melanomas, nine nodular melanomas, four superficial spreading melanomas, two lentigo maligna melanomas, one mucosal melanoma, and one melanoma of unknown subtype. Eight of the 20 primary tumors were <4 mm in thickness (1.3–3 mm; median, 1.8 mm), and the remaining 12 primary tumors were >4 mm in thickness (4.2–23 mm; median, 5.5 mm). Metastatic tumors consisted of 10 lymph node metastases, 12 in-transit metastases, and five distant skin metastases. In 1 patient, both lymph node and skin metastases were examined. Four lesions of nevocellular nevi were also included.

Partial tissue samples were fixed in 10% neutral buffered formaldehyde and processed for routine pathology. The remaining tissue was embedded in Tissue-Tek OCT compound (Miles, IN), snap frozen in liquid nitrogen, and stored at −70°C until use. Ten serial 6-μm sections were cut from each frozen tissue embedded in OCT compound. The sections were placed on silan-coated glass slides (Dako Japan, Kyoto, Japan). One section was stained with H&E, and the remaining sections were subjected to in situ hybridization and DNA extraction for PCR-microsatellite LOH analysis.

Probes Preparation. Probes of cDNA for the human KiSS1 were synthesized by PCR based on GenBank nucleotide sequence (accession no. U43527). Amplified cDNA of 282 bp (nucleotide number 124–405) was cloned into pBluescript vector (Stratagene, La Jolla, CA) and linearized with XbaI for the sense probe and HindIII for the antisense probe. We confirmed that the sequence for this clone was identical to the human KiSS1 sequence. DIG-labeled cRNA probes were made using the DIG RNA Labeling Kit (Boehringer Mannheim, Mannheim, Germany) following the protocol provided by the manufacturer. The labeling efficiency was tested by dot blots of the probes at increasing dilutions on nitrocellulose membranes and detection by anti-DIG antibody and substrates, as described below.
In Situ Hybridization. Cryostat sections (6 µm) were washed with diethyl pyrocarbonate-treated PBS and fixed in freshly prepared 4% paraformaldehyde (Wako, Tokyo, Japan) in 0.1 M PBS for 5 min. After washing in three changes of PBS, the slides were treated with 0.1 M triethanolamine + 0.25% acetic anhydride (volume for volume; both from Sigma Chemical Co., Poole, United Kingdom) for 10 min. The slides were prehybridized for 1 h at 42°C with 60 µl of hybridization buffer. The hybridization buffer comprised 50% formamide, 4 SSC, 1 Denhardt’s solution, 125 µg/ml RNA, and heat-denatured 100 µg/ml salmon sperm DNA (Sigma Chemical Co.). The mixture was drained from the slide and 30 µl of the hybridization buffer containing 1:100 diluted DIG-labeled cRNA probe for 20 h at 42°C. The sections were washed once with 2 × SSC at 65°C, once with 2 × SSC at 50°C, twice with 0.1 × SSC at 50°C, and treated with 100 ng/ml RNase A for 30 min at 37°C. Then, immunological detection was performed using the DIG Nucleic Acid Detection Kit (Boehringer Mannheim) following the manufacturer’s instructions. Briefly, the sections were washed for 5 min in buffer 1 [0.1 M Tris-HCl and 0.15 M NaCl (pH 7.5)] followed by 60 min in buffer 1 containing 1% blocking agent. Anti-DIG antibody diluted to 1:500 in buffer 1 was added to the sections and incubated for 30 min at room temperature. Chromogenic reactions were carried out in the presence of nitroblue tetrazolium chloride and 5-bromo-4-chloro-3-indolyl-phosphate, and the slides were counterstained with methyl-green. The stained slides were evaluated by two independent observers and scored as diffusely positive (++) when the percentage of stained melanoma cells in the entire lesion was >75%, heterogeneously positive (+) when the percentage was between 25 and 75%, and negative (−) when the percentage was <25%. The sections stained with the sense probe were used as controls for nonspecific hybridization.

Microdissection and DNA Extraction. Microdissection and DNA extraction were carried out using the Pinpoint Slide DNA Isolation System (Zymo Research, Orange, CA). The cryostat sections mounted on glass slides were placed on an inverted microscope. Adhesive solution was then applied to a tumor area with the aid of matching He&E-stained section. The tumor tissue embedded within the dried solution was then lifted with a scalpel blade, transferred into a microfuge tube, and digested for 4 h at 55°C in buffer containing proteinase K. The lysate was heated at 95°C for 10 min to inactivate proteinase K. Aliquots of 1 µl of this lysate were used directly for PCR. Control DNA was obtained from either peripheral blood lymphocytes using the DNA Extraction Kit (Stratagene) or normal tissue DNA (Sigma Chemical Co.). The mixture was drained from the slide and 30 µl of the hybridization buffer containing 1:100 diluted DIG-labeled cRNA probe for 20 h at 42°C. The sections were washed once with 2 × SSC at 65°C, once with 2 × SSC at 50°C, twice with 0.1 × SSC at 50°C, and treated with 100 ng/ml RNase A for 30 min at 37°C. Then, immunological detection was performed using the DIG Nucleic Acid Detection Kit (Boehringer Mannheim) following the manufacturer’s instructions. Briefly, the sections were washed for 5 min in buffer 1 [0.1 M Tris-HCl and 0.15 M NaCl (pH 7.5)] followed by 60 min in buffer 1 containing 1% blocking agent. Anti-DIG antibody diluted to 1:500 in buffer 1 was added to the sections and incubated for 30 min at room temperature. Chromogenic reactions were carried out in the presence of nitroblue tetrazolium chloride and 5-bromo-4-chloro-3-indolyl-phosphate, and the slides were counterstained with methyl-green. The stained slides were evaluated by two independent observers and scored as diffusely positive (++) when the percentage of stained melanoma cells in the entire lesion was >75%, heterogeneously positive (+) when the percentage was between 25 and 75%, and negative (−) when the percentage was <25%. The sections stained with the sense probe were used as controls for nonspecific hybridization.

Microsatellite-PCR LOH Analysis. A total of 13 microsatellite polymorphisms that are mapped to chromosome 6q16.3 were used for analysis. All of the primers for PCR amplification were obtained from Research Genetics (Huntsville, AL). One of each primer pair was end-labeled with 32P-ATP. PCR amplification was performed in reaction buffer containing 0.1 mM each oligonucleotide primer, 0.2 mM each deoxynucleotide-triphosphate, 1.5 mM MgCl2, and 0.5 unit of Taq DNA polymerase (Promega, Madison, WI) in a final volume of 10 µl. PCR cycles consisted of 1 cycle at 94°C for 3 min followed by 29 cycles of 1 min at 94°C, 1 min at 55°C, and 1 min at 72°C. PCR products were separated through 6% denaturing polyacrylamide gels. All samples showing two distinct allelic bands in normal DNA were considered to be informative. The signal intensities for all informative samples were examined visually by two independent observers. LOH was scored as positive when a clear reduction (>75%) in signal intensity was detected in one of the alleles in the tumor DNA compared with the same allele in the paired normal DNA.

Statistics. Two-by-two tables were compared using the χ2 test.

Results

KiSS1 mRNA Expression in Normal Skin, Nevocellular Nevus, Primary Melanoma, and Metastasis (Table 1). Analysis of frozen tissue sections by in situ hybridization showed KiSS1 mRNA expression in the cytoplasm of cells of nevocellular nevus and melanoma. The detection of KiSS1 mRNA was carried out using the DIG-labeled antisense RNA probe. The corresponding sense RNA probe was used as a control of specificity, and all of the control hybridization was negative. Because of the clear hybridization signals, the scoring of sections by two independent observers was identical. In normal skin, hybridization signals for KiSS1 mRNA were detected in pilosebaceous units and eccrine sweat glands. Keratinocytes in normal skin were not labeled with KiSS1. However, in the hyperplastic epidermis overlying nevocellular nevus and primary melanoma, KiSS1 mRNA was frequently detected. Although KiSS1 expression in melanoma was reported in cultured melanocytes using reverse transcription-PCR (9), KiSS1 transcripts were not detected in normal melanocytes by in situ hybridization.

KiSS1 mRNA expression was detected in all four lesions of the nevocellular nevus (Fig. 1A). The intensity of labeling was strong in the cytoplasm of nevus cells located in the reticular dermis. In contrast, nevus cells within the epidermis showed negative or weak hybridization signals. In primary melanomas with variable thickness, 70% (14 of 20) of tumors showed KiSS1 mRNA expression. Although the expression was heterogeneous with only 25–75% melanoma cells positive in 2 tumors, the remaining 12 tumors showed diffuse KiSS1 mRNA expression with >75% of tumor cells positive. All eight primary melanomas <4 mm in thickness (ranged from 1.3 to 3 mm; median, 1.8 mm) showed KiSS1 mRNA expression. In these tumors, nests of proliferating atypical melanocytes within the epidermises were intensely labeled (Fig. 1B). In contrast, loss of KiSS1 mRNA expression was frequently observed in thicker lesions (Fig. 1C). Only 50% (6 of 12) of primary melanomas with a tumor thickness >4 mm (thickness ranged from 4.2 to 23 mm; median, 5.5 mm) showed KiSS1 mRNA expression. Loss of KiSS1 mRNA expression was equally frequent in metastases; 56% (15 of 27) of the lesions showed a diffuse expression with >75% of labeled cells, whereas the expression was lost in the remaining 44% (12 of 27) of metastases.

LOH of 6q16.3–q23 in Melanoma Metastases (Table 2). Previous analyses in cell lines as well as in vivo tumors have provided compelling evidence that at least one metastasis suppressor gene important for melanoma progression resides on chromosome 6 (7, 8, 17). Recently, this putative melanoma metastasis suppressor locus has been mapped to ~40-cM region between D6S488 and D6S314 in 6q16.3-q23 by microcell-mediated chromosomal transfer experiments of a deletion variant of human chromosome 6 to highly metastatic human melanoma cell line C8161 (13). To examine whether the melanoma metastases in vivo actually show LOH of this particular chromosome region, and to additionally refine this metastasis suppressor locus, we carried out PCR-based LOH analysis in 27 metastases by using seven polymorphic loci (D6S268, D6S266, D6S267, D6S287, D6S262, D6S457, and D6S292) mapped within this region, as well as an additional six flanking loci (D6S275, D6S300, D6S468, D6S314, D6S311, and D6S473; Table 2). Overall, 52% (14 of 27) of metastases showed LOH of the 40-cM region and the telomeric, as well as centromeric, flanking regions in 6q16.3-q23 (Fig. 1D), confirming that this region is the frequent site of somatic allelic loss in melanoma metastases. Additional refinement of this metastasis suppressor locus was not possible, however, because all of the 14 metastases except 1 tumor (case 24) showed LOH spanning the entire 40-cM region between D6S468 and D6S314. A skin metastasis from case 24 showed retention of heterozygosity at D6S268 and D6S262 flanked by LOH at the adjacent loci. However, this tumor retained KiSS1 expression.

Relationship between the Loss of KiSS1 Expression and LOH of 6q16.3–q23 in Melanoma Metastases (Table 3). In 27 melanoma metastases, 9 tumors that showed LOH of 6q16.3–q23 lost KiSS1 expression, whereas 10 tumors with no LOH showed positive KiSS1 mRNA expression. Despite the retention of 6q16.3–q23, 3 tumors were negative for KiSS1. Expression of KiSS1 was detected in 5

<table>
<thead>
<tr>
<th>Tumor</th>
<th>No. of cases with KiSS1 expression</th>
<th>No. of cases examined</th>
</tr>
</thead>
<tbody>
<tr>
<td>Nevocellular nevus</td>
<td>4 of 4 (100%)</td>
<td>4</td>
</tr>
<tr>
<td>Primary melanomas &lt;4 mm in thickness</td>
<td>8 of 8 (100%)</td>
<td>8</td>
</tr>
<tr>
<td>Primary melanomas &gt;4 mm in thickness</td>
<td>6 of 12 (50%)</td>
<td>12</td>
</tr>
<tr>
<td>Metastasis</td>
<td>15 of 27 (56%)</td>
<td>27</td>
</tr>
</tbody>
</table>

Table 1 Expression of KiSS1 mRNA in melanocytic lesions of different progression stages
tumors that showed LOH of 6q16.3-q23. The association between LOH and loss of KiSS1 expression was statistically significant ($P = 0.03$).

**Discussion**

Since the cloning of KiSS1, a number of experiments using cell lines have provided functional evidence showing KiSS1 as a metastasis suppressor of melanoma and breast carcinoma (9–11, 16). However, the actual expression or loss of KiSS1 in vivo tumors was unknown. In the study described here, we show for the first time that the KiSS1 expression is lost during the progression of melanocytic tumors in vivo; i.e., all of the lesions of benign nevocellular nevus and early stage primary melanoma express KiSS1 mRNA, but the expression is frequently lost in deeply invasive primary melanomas and metastases. Marked decrease of KiSS1 expression in primary melanomas >4 mm in thickness is striking and may in part account for the dramatic fall of survival in patients presented with such fairly thick primary tumors (although these patients are rather rare and constitute <10% of all melanoma patients; Ref. 18). By contrast, the frequency of KiSS1 loss between deeply invasive primary melanomas and metastases is nearly the same (~50%). This is keeping with the notion that deeply invasive vertical growth phase melanoma cells already harbor numerous cytogenetic abnormalities and are metastasis competent (19) and that no major additional genetic changes may be required for additional progression to metastatic dissemination (20).

There is compelling evidence that the long arm of chromosome 6 contains at least four tumor suppressor genes important for melanoma, including SOD2 and AIM1 (reviewed by Welch and Goldberg, Ref. 21). Recently, a novel candidate gene was mapped to a 40-cM region between 6q16.3-q23 by microcell-mediated chromosome transfer of a deletion variant of neomycin-tagged human chromosome 6 into metastatic C8161.9 melanoma subline (13). Because this subline did not express KiSS1 (while it was expressed in the same cell transferred with an intact chromosome 6), it is suggested that a tumor suppressor gene mapping to this region may be a critical regulator of KiSS1 (13). The finding in the present study showing significant associations between loss of KiSS1 mRNA expression and LOH of 6q16.3-q23 in melanoma metastases supports this hypothesis and strongly suggests that inactivation of the tumor suppressor gene mapping to 6q16.3-q23 by deletion or mutation coupled with LOH may lead to the downregulation of KiSS1. A tumor suppressor gene may also be inactivated by homozygous deletion, promoter methylation, or protein inactiva-

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**Table 2** LOH of 6q16.3–23.3 in melanoma metastases

<table>
<thead>
<tr>
<th>Locus</th>
<th>Cytogenetic localization</th>
<th>Mapping distance (cM)</th>
<th>Case no.</th>
</tr>
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<tbody>
<tr>
<td>D6S275</td>
<td>6q16.3–21</td>
<td>102.81</td>
<td>10</td>
</tr>
<tr>
<td>D6S300</td>
<td>6q16.3–21</td>
<td>103.45</td>
<td>12</td>
</tr>
<tr>
<td>D6S468</td>
<td>6q16.3–21</td>
<td>107.88</td>
<td>14</td>
</tr>
<tr>
<td>D6S268</td>
<td>6q16.3–21</td>
<td>114.93</td>
<td>21</td>
</tr>
<tr>
<td>D6S266</td>
<td>6q21–23</td>
<td>120.31</td>
<td>23</td>
</tr>
<tr>
<td>D6S267</td>
<td>6q21–23</td>
<td>121.97</td>
<td>24</td>
</tr>
<tr>
<td>D6S267</td>
<td>6q21–23</td>
<td>121.97</td>
<td>26</td>
</tr>
<tr>
<td>D6S262</td>
<td>6q22.3–23.1</td>
<td>121.97</td>
<td>28</td>
</tr>
<tr>
<td>D6S457</td>
<td>6q21–23.2</td>
<td>130.00</td>
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</tr>
<tr>
<td>D6S292</td>
<td>6q16.3–23.2</td>
<td>136.97</td>
<td>32</td>
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<tr>
<td>D6S314</td>
<td>6q16.3–27</td>
<td>143.40</td>
<td>33</td>
</tr>
<tr>
<td>D6S311</td>
<td>6q21–23.3</td>
<td>147.13</td>
<td>34</td>
</tr>
<tr>
<td>D6S473</td>
<td>6q21–23.3</td>
<td>155.17</td>
<td>38</td>
</tr>
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</table>

* ●, LOH; ○, retention of heterozygosity; *, uninformative; blank, not examined.

* Microsatellite markers deleted in a deletion variant of neomycin-tagged human chromosome 6 (neo6qdel; Ref. 13) are indicated in bold letters.

* Cytogenetic localization and mapping distance of individual markers is according to information obtained at www.marshfieldclinic.org/research/genetics/.
tion that would not involve LOH (22). This may explain why three metastases that did not show LOH of 6q16.3-q23 lost KiSS1 expression. An alternative possibility explaining loss of KiSS1 expression in these tumors might be direct chromosomal alterations involving chromosome 1q32-41 where KiSS1 resides.

The findings in this study confirm the importance of KiSS1 down-regulation in the progression of melanoma in vivo. Recent investigation shows that the KiSS1 gene product is subjected to proteolytic cleavage followed by amide transfer generating a 54 amino acid peptide “metastin,” which is a ligand of an orphan G-protein coupled receptor hOT7T157 (16). Although metastin could be a potential new therapeutic agent for advanced stage of melanoma (16), perhaps we need more information; e.g., whether KiSS1 gene product is properly processed in melanoma cells and whether the receptor hOT7T157 is expressed in melanomas in vivo. The investigation is now underway in our laboratory. Another important issue is the identification of a putative KiSS1 regulator gene mapping to 6q16.3-q23. Our attempt to additionally refine the region by constructing deletion maps of metastic tumor samples failed because of the preferential large deletions in this chromosomal area (23). More sophisticated approaches, such as comparison of mRNA expression profiling between a nonmetastatic neo6 cell line and a metastatic neo6qdel cell line (13) by using DNA microarray, might be necessary.

Acknowledgments

We thank Yuko Yamada and Masako Matsubara for technical assistance.

References


Table 3 Relationship between KiSS1 mRNA expression and LOH of 6q16.3–q23 in melanoma metastases*  

<table>
<thead>
<tr>
<th>KiSS1 mRNA expression</th>
<th>LOH of 6q16.3–q23</th>
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</thead>
<tbody>
<tr>
<td>Positive</td>
<td>Negative</td>
</tr>
<tr>
<td>5</td>
<td>10</td>
</tr>
<tr>
<td>9</td>
<td>3</td>
</tr>
</tbody>
</table>

* P = 0.03.
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