Sensitivity of Fluorescence In Situ Hybridization for Melanoma Diagnosis Using RREB1, MYB, Cep6, and 11q13 Probes in Melanoma Subtypes

Pedram Gerami, MD; Mariam Mafee, BS; Teekay Lurtsbarapa, MD; Joan Guitart, MD; Zahra Haghighat, MD; Marissa Newman, MD

Objective: To evaluate the diagnostic sensitivity of fluorescence in situ hybridization (FISH) using probes targeting 6p25, 6q23, 11q13, and Cep6 in melanoma subtypes.

Design: Blinded comparison of chromosomal copy number changes detected using FISH targeting 6p25, 6q23, 11q13, and Cep6 in benign nevi and melanoma subtypes.

Setting: Dermatopathology Laboratory, Department of Dermatology, Northwestern University, Chicago, Illinois.

Participants: One hundred ten individuals with benign nevi and 123 with melanoma (70 superficial spreading, 28 lentigo maligna, 22 nodular, and 3 acral lentiginous melanomas).

Main Outcome Measures: Sensitivity of previously developed criteria using FISH using probes targeting 6p25, 6q23, 11q13, and Cep6 in the melanoma subtypes.

Results: Overall, sensitivity was 83.0% and specificity was 94.0%. The 6p25 gain criterion had the highest sensitivity overall and in each subtype. The assay was most sensitive in the subgroups of nodular and acral melanomas and least sensitive in the superficial spreading subtype. The 11q13 gain was more commonly seen in chronically sun-damaged skin and infrequently in non–chronically sun-damaged skin.

Conclusions: Heterogeneous changes in melanoma occur at the molecular level, and the changes are different among melanoma subtypes. Clonal abnormalities in chromosome 6 with increased copies of the short arm relative to the long arm are common in all melanoma subtypes, suggesting that isochromosome 6 is common in all variants of cutaneous melanoma subtypes. An increase in copy number of 11q13 is most frequent in chronically sun-damaged melanomas.

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of FISH using the previously mentioned probes in the melanoma subtypes from both classification systems. Furthermore, we determined the sensitivity of each particular probe in these subtypes.

**METHODS**

After receiving proper approval from the Northwestern University (NU) institutional review board and the NU cancer center, we studied 123 melanomas and 110 nevi from the NU dermatopathology unit using FISH. Eighty-three melanomas and 86 nevi were cases also used in the previous study by Gerami et al; the remaining 40 melanomas and 24 nevi were new cases added to this study. Histologic sections from all the cases were evaluated by 2 dermatopathologists (P.G. and J.G.). Only cases with strong consensus of diagnosis were included in this study. The method we used for grading dysplasia and for subtyping the melanomas was commensurate with that previously discussed extensively in the literature. The nevi consisted of Spitz nevi; dysplastic nevi with severe, moderate, or mild atypia; and common acquired nevi with no dysplasia. The 123 melanomas consisted of 70 superficial spreading types, 28 lentigo maligna types, 22 nodular types, and 3 acral lentiginous types. Designation of non–chronically sun damaged (NCSD) vs chronically sun damaged (CSD) was accomplished according to site and solar elastosis. Melanomas from a sun-exposed site on the body showing dense bands of solar elastosis on histologic examination were considered CSD. Of the 123 primary melanomas, 72 were from NCSD skin, 48 were from CSD skin, and 3 were acral lentiginous melanomas.

Fluorescence in situ hybridization targeting 4 distinct loci was performed on all the patients. The 4 loci and the specific genes targeted by the probe were 6p25 (RREB1), 6q23 (MYB), 11q13 (cyclin D1), and Cep6 (the centromeric portion of chromosome 6).

Sections 5-µm thick were mounted on positively charged slides (SuperFrost Plus; ThermoShandon, Pittsburgh, Pennsylvania), baked at 56°C overnight, deparaffinized, submerged in 1× salt sodium citrate pH 6.3 at 80°C for 35 minutes, and washed in water for 3 minutes. After protease digestion (pepsin, 4 mg/mL; 0.2N hydrochloride) at 37°C for 15 minutes, sections were rinsed in water for 3 minutes, passed through graded ethanol, and dried. Hybridizations were performed at 37°C for 16 to 18 hours in an automated co-denaturation oven (HYBrite or ThermoBrite Denaturation/Hybridization System; Abbott Molecular Inc, Des Plaines, Illinois) according to the manufacturer’s instructions. Sections were placed in washing buffer (2× salt sodium citrate/0.3% NP-40) (Abbott Molecular Inc) at room temperature for 2 to 10 minutes to remove the coverslips and then were immersed in 73°C washing buffer for 2 minutes, dried, and mounted with DAPI I antifade solution (Abbott Molecular Inc). Table 1. Criteria for FISH

<table>
<thead>
<tr>
<th>Criteriona</th>
<th>Criterion Is Positive if</th>
</tr>
</thead>
<tbody>
<tr>
<td>RREB gain</td>
<td>&gt;29% of cells have &gt;2 copies of RREB</td>
</tr>
<tr>
<td>RREB/Cep6 ratio</td>
<td>&gt;55% of cells have more copies of RREB than of Cep6, indicating gains in the short arm of chromosome 6 relative to the centromeric portion of chromosome 6</td>
</tr>
<tr>
<td>MYB/Cep6 ratio</td>
<td>&gt;40% of cells have fewer copies of MYB than of Cep6, indicating loss in the long arm of chromosome 6 relative to the centromeric portion of chromosome 6</td>
</tr>
<tr>
<td>11q13 gain</td>
<td>&gt;39% of cells have &gt;2 copies of 11q13</td>
</tr>
</tbody>
</table>

Abbreviation: FISH, fluorescence in situ hybridization.

a If any of these criteria are met, the case is considered positive using FISH.

Table 2. Characteristic Features of Melanoma Subtypes

<table>
<thead>
<tr>
<th>Characteristic</th>
<th>All (N=123)</th>
<th>Superficial Spreading (n=70)</th>
<th>Lentigo Maligna (n=28)</th>
<th>Nodular (n=22)</th>
<th>Acral Lentiginous (n=3)</th>
<th>CSD (n=48)</th>
<th>NCSD (n=72)</th>
</tr>
</thead>
<tbody>
<tr>
<td>Age, mean, y</td>
<td>58.8</td>
<td>53.6</td>
<td>12.0</td>
<td>7.0</td>
<td>2.0</td>
<td>20.0</td>
<td>35.0</td>
</tr>
<tr>
<td>Location, %</td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Extremities</td>
<td>35.9</td>
<td>18.6</td>
<td>3.6</td>
<td>50.0</td>
<td>0</td>
<td>6.2</td>
<td>56.9</td>
</tr>
<tr>
<td>Head and neck</td>
<td>35.0</td>
<td>45.7</td>
<td>82.1</td>
<td>31.8</td>
<td>0</td>
<td>66.7</td>
<td>15.3</td>
</tr>
</tbody>
</table>

Abbreviations: CSD, chronically sun damaged; NCSD, non–chronically sun damaged.

Table 3. Probe Sensitivities in Melanoma Subtypes

<table>
<thead>
<tr>
<th>Variable</th>
<th>All (N=123)</th>
<th>Superficial Spreading (n=70)</th>
<th>Lentigo Maligna (n=28)</th>
<th>Nodular (n=22)</th>
<th>Acral Lentiginous (n=3)</th>
<th>CSD (n=48)</th>
<th>NCSD (n=72)</th>
</tr>
</thead>
<tbody>
<tr>
<td>Overall sensitivity, %</td>
<td>83.0</td>
<td>81.4</td>
<td>82.1</td>
<td>90.9</td>
<td>90.0</td>
<td>83.3</td>
<td>81.9</td>
</tr>
<tr>
<td>6p25&gt;Cep6 sensitivity, %</td>
<td>48.8</td>
<td>50.0</td>
<td>42.9</td>
<td>45.5</td>
<td>100.0</td>
<td>45.8</td>
<td>48.6</td>
</tr>
<tr>
<td>6q25 gain sensitivity, %</td>
<td>74.8</td>
<td>72.9</td>
<td>67.9</td>
<td>81.8</td>
<td>100.0</td>
<td>70.8</td>
<td>75.0</td>
</tr>
<tr>
<td>6q23&gt;Cep6 sensitivity, %</td>
<td>24.4</td>
<td>20.0</td>
<td>21.4</td>
<td>31.8</td>
<td>100.0</td>
<td>18.8</td>
<td>25.0</td>
</tr>
<tr>
<td>11q13 gain sensitivity, %</td>
<td>30.1</td>
<td>20.0</td>
<td>35.7</td>
<td>54.5</td>
<td>33.3</td>
<td>41.7</td>
<td>22.2</td>
</tr>
</tbody>
</table>

Abbreviations: CSD, chronically sun damaged; NCSD, non–chronically sun damaged.
Slides were evaluated using an epifluorescence microscope equipped with DAPI, aqua, gold, greenV2, and red single band-pass filter sets (Abbott Molecular Inc). Each slide was reviewed by 1 of 3 reviewers (P.G., T.L., and M.N.). All reviewers received similar training and used the same standard method as described herein. Three total reviewers, including 1 attending dermatopathologist and 2 research fellows, were involved in the enumeration process. The reviewers used a similar protocol and were blinded to the histologic diagnosis (Table 1). The slides were quickly scanned under each of the 4 filters to detect any areas with gains or losses in any of the DNA loci being evaluated. Areas with the most significant copy number changes were selected for enumeration. If an area was selected for enumeration, a minimum of 10 adjacent cells from that area were selected for evaluation. If the area was rich in aberrations, then 30 adjacent cells were evaluated from the same site. Otherwise, a maximum of 3 separate sites in the sections were evaluated to obtain 30 enumerated cells. The areas were analyzed using high-power magnification (×64 or ×100 objective). Cells with highly overlapping borders were not counted owing to concern for falsely elevated signal counts as a result of overlapping cells. Nuclei that showed no signals for more than 1 probe were not analyzed. Samples in which 30 nuclei could not be evaluated were excluded from the study.

Criteria for the melanoma were discussed and determined previously and include greater than 55% of cells with more copies of 6p25 than of Cep6 (6p25/Cep6), greater than 29% of cells with more than 2 copies of 6p25, greater than 40% of cells with fewer copies of 6q23 than of Cep6 (6q23/Cep6), and greater than 38% of cells with more than 2 copies of 11q13 (Table 1). The sensitivity of the assay was determined using the histopathologic findings as a gold standard. The sensitivity in this case equals the total number of melanoma cases that tested positively by the assay divided by the total number of melanoma cases tested in each specified histologic subtype.

<table>
<thead>
<tr>
<th>Chromosome 6, abnormal %</th>
<th>All</th>
<th>Superficial Spreading</th>
<th>Lentigo Maligna</th>
<th>Nodular</th>
<th>Acral Lentiginous</th>
<th>CSD</th>
<th>NCSD</th>
</tr>
</thead>
<tbody>
<tr>
<td>79.7</td>
<td>75.7</td>
<td>78.6</td>
<td>90.9</td>
<td>100.0</td>
<td>81.3</td>
<td>81.9</td>
<td></td>
</tr>
<tr>
<td>30.1</td>
<td>20.0</td>
<td>35.7</td>
<td>54.5</td>
<td>33.0</td>
<td>41.7</td>
<td>22.2</td>
<td></td>
</tr>
</tbody>
</table>

Abbreviations: CSD, chronically sun damaged; NCSD, non-chronically sun damaged.

RESULTS

A total of 123 melanomas consisting of 70 superficial spreading, 28 lentigo maligna, 22 nodular, and 3 acral lentiginous melanomas were evaluated in addition to 110 benign nevi consisting of 29 common nevi; 19 mildly dysplastic; 23 moderately dysplastic, and 16 severely dysplastic nevi; 10 Spitz nevi; 10 epithelioid blue nevi; 1 clonal nevus; 1 recurrent nevus; and 1 spindle cell nevus of Reed (Table 2). Overall, sensitivity was 83% and specificity was 94%. Among the 123 melanomas, the 6p25 gain criterion showed the highest sensitivity of 74.8%. The 6p25>Cep6 criterion was positive in 48.8% of all cases, the 11q13 gain criterion in 30.1%, and 6q23>Cep6 in 24.4% (Table 3 and Table 4). Fifty-seven percent of the 123 cases were positive for 2 or more criteria.

A total of 81.4% of the 70 cases of superficial spreading melanoma were positive by at least 1 of the 4 FISH criteria. The 6p25 gain criterion showed the highest sensitivity in this subset, with 72.9% of the melanoma cases testing positively (Figure 1). The 6p25>Cep6 criterion was positive in 50.0%, whereas the 11q13 and

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**Table 4. Abnormalities in Chromosomes 6 and 11 in Melanoma Subtypes**

<table>
<thead>
<tr>
<th>Chromosome</th>
<th>All</th>
<th>Superficial Spreading</th>
<th>Lentigo Maligna</th>
<th>Nodular</th>
<th>Acral Lentiginous</th>
<th>CSD</th>
<th>NCSD</th>
</tr>
</thead>
<tbody>
<tr>
<td>6p25</td>
<td>79.7</td>
<td>75.7</td>
<td>78.6</td>
<td>90.9</td>
<td>100.0</td>
<td>81.3</td>
<td>81.9</td>
</tr>
<tr>
<td>6q23</td>
<td>30.1</td>
<td>20.0</td>
<td>35.7</td>
<td>54.5</td>
<td>33.0</td>
<td>41.7</td>
<td>22.2</td>
</tr>
</tbody>
</table>

**Figure 1.** Superficial spreading melanoma with prominent gains in 6p25. A, Hematoxylin-eosin–stained sections of the melanoma (original magnification ×400). B, The respective fluorescence in situ hybridization study under the red channel (RREB1/red signal); arrowheads indicate the increasing copies of 6p25 (original magnification ×600).
6q23< Cep6 criteria each showed sensitivity of 20.0% in the superficial spreading melanomas.

An overall sensitivity of 82.1% was seen in the 28 lentigo maligna melanoma cases. The 6p25 gain criterion was the most commonly met criterion in this group as well, with a positive result in 67.9% of cases. The 6p25>Cep6 criterion had the second highest sensitivity at 42.9%. The 11q13 gain had a sensitivity of 35.7% and the 6q23<Cep6 criterion of 21.4%.

The probe set performed with a sensitivity of 90.9% in the 22 nodular melanomas tested (Figure 2). A positive FISH result by the 6p25 gain criterion was seen in 81.8% of the cases, the 6p25>Cep6 criterion was seen in 45.5%, and the 11q13 criterion was seen in 54.5%. The 6q23<Cep6 criterion had the lowest sensitivity in this group, being positive in 31.8% of the 22 cases.

The criteria also showed variable sensitivity depending on the degree of solar damage. Of the 123 total melanomas, 72 were considered melanomas of NCSD skin, 48 were melanomas of CSD skin, and 3 were acral lentiginous melanomas. Overall sensitivity for the NCSD cases was 81.9%, with the 6p25 gain criterion being the most sensitive at 75.0%, followed by 6p25>Cep6 at 48.6%, 6q23>Cep6 at 25.0%, and 11q13 at 22.2%. In the CSD melanomas, overall sensitivity was 83.3%, with the 6p25 gain criterion having the highest sensitivity of 70.8%, followed by 6p25>Cep6 at 45.8%, 11q13 at 41.7%, and 6q23>Cep6 at 18.8% (Figure 3). Of the 3 acral lentiginous melanomas, 1 was positive for all 4 criteria and the other 2 were positive for all the criteria except for 11q13 (Figure 4).

**COMMENT**

Cytogenetic analysis of melanocytic neoplasms using FISH has emerged as an important diagnostic tool. Previously, we showed that FISH analysis of formalin-fixed, paraffin-embedded tissue using probes targeting 6p25, 6q23, 11q13, and Cep6 can distinguish between melanomas and benign nevi with high sensitivity and specificity. Because melanocytic neoplasms are heterogeneous in the molecular pathways leading to melanoma, different histologic subtypes have a stronger propensity for specific chromosomal aberrations. In this study, we found that the sensitivity of the probe set was 81.4% among the superficial spreading melanomas. This was the lowest sensitivity among the 4 melanoma subtypes recognized by the World Health Organization. Among the various probes, 6p25 was the most sensitive, with 6p25 gain being positive in 74.8% of the cases. The 6p25 gain criterion was also the most sensitive in each of the different subtypes. Overall, 72.9% of all melanomas tested met the criterion for gain in 6p25, 6p25>Cep6, or loss in 6q23<Cep6, highlighting the high frequency of a chromosome 6 imbalance in all the melanoma subtypes tested. This imbalance is likely related to an isochromosome, which contains 2 copies of the same chromosomal arm. The sensitivity could likely be improved by adding FISH probes targeting other loci of importance in melanoma, such as 9p21, 7q34, and 10q23.

We identified 2 distinct patterns with 6p25 gain. In most patients, 6p25 gains were characteristic of most of the cells in the melanoma, whereas in other cases, despite...
uniform cytologic atypia on histologic sections, only focal islands or nests of cells with 6p25 gain were identified. Therefore, thorough scanning for gains before selecting areas for FISH enumeration is critical. In addition, the presence of this second pattern also may explain the higher percentage of melanoma cases identified with gains in 6p25 using FISH compared with using comparative genomic hybridization.14

The sensitivity of the probe assay was particularly excellent in nodular melanomas at 90.9% and in acral lentiginous melanomas at 100%. This is particularly important because many mimickers of melanoma, such as Spitz tumors, may have a nodular growth pattern; therefore, the high sensitivity in this subgroup is particularly significant. In the nodular group, we found that the 6p25>Cep6 criterion had a sensitivity of 45.5% and that 11q13 had a sensitivity of 54.5%. The acral lentiginous category of melanoma was most likely to meet multiple FISH criteria for melanoma. All 3 acral lentiginous melanoma cases met multiple FISH criteria for melanoma. This is also highly consistent with previous studies15 characterizing acral lentiginous melanomas as having multiple and frequent chromosomal aberrations.

We found that cases with CSD skin have a similar overall sensitivity of 83.8% with this probe set compared with a sensitivity of 81.9% for melanomas on NCSD skin. Gains

Figure 4. Acral lentiginous melanoma with multiple chromosomal aberrations. A, A low-power (original magnification ×40) photomicrograph of an invasive acral lentiginous melanoma. B, A higher-power (original magnification ×400) view of the same melanoma showing nests of atypical cells with prominent pagetoid spread. C, The fluorescence in situ hybridization view showing gains in 11q13 visualized by the green channel (cyclin D1/green signal) (original magnification ×600). D, The fluorescence in situ hybridization view showing gains in 6p25 visualized by the red channel (RREB1/red signal). Arrowheads indicate cells with chromosomal copy number aberrations.
in 6p25 were seen with nearly equal frequency in CSD and NCSD lesions. However, not unexpectedly, the sensitivity for 11q13 was significantly higher in CSD skin as opposed to NCSD skin. In the present series, we defined CSD melanomas of skin similarly to that of Curtin et al. as cases occurring in a chronically sun-exposed distribution and with extensive solar elastosis on light microscopy. Although we had a slightly higher incidence of melanomas of CSD skin outside the head and neck area, such as on the back of the hands and arms, we found a similar frequency of 11q13 gains in this subtype of melanoma as did Curtin et al. The present study confirms the heterogeneous molecular changes in melanoma. We also confirm the findings of a previous study showing high performance of this probe set in distinguishing melanoma from nevi. These data further demonstrate which probes and accompanying criteria are most helpful in the specific subtypes of cases with unequivocal melanomas. We believe that this information can be critically important to physicians performing FISH on melanocytic neoplasms to anticipate and comprehend their results and assist in their analysis of equivocal lesions. However, note that this study exclusively included unequivocal cases. One further limitation of this study is the need for interobserver evaluation in future studies to evaluate the interobserver reliability of the assay. Also, scanning and selection of areas to count can influence these data because gains are easier to identify than are losses. It is possible in cases with 6p25 gain or 11q13 gain that areas with 6q23 loss were overlooked to focus FISH enumeration on areas with gains. Hence, the percentage of cases with 6q23 loss may be underestimated as a natural consequence of the FISH enumeration procedure, although this should not affect overall sensitivity.

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Author Contributions: Dr Gerami had full access to all the data in the study and takes responsibility for the integrity of the data and the accuracy of the data analysis.

Study concept and design: Gerami. Acquisition of data: Gerami, Mafee, Lurtsbarapa, Guitart, Haghighat, and Newman. Analysis and interpretation of data: Gerami. Drafting of the manuscript: Gerami and Mafee. Critical revision of the manuscript for important intellectual content: Gerami, Lurtsbarapa, Guitart, Haghighat, and Newman. Statistical analysis: Gerami. Obtained funding: Gerami. Administrative, technical, and material support: Gerami, Mafee, Lurtsbarapa, Guitart, Haghighat, and Newman.

Study supervision: Gerami.

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REFERENCES