Chromosomal Gains and Losses in Primary Cutaneous Melanomas Detected by Comparative Genomic Hybridization

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ABSTRACT

The analysis of genetic changes in primary cutaneous melanoma has been limited by the need for fixation for diagnostic purposes. However, comparative genomic hybridization is able to analyze such specimens. We have applied comparative genomic hybridization to 32 primary melanomas to discover and map genomic regions with aberrant DNA copy numbers. The analysis was performed on native, nonamplified DNA extracted from manually dissected tumor sections. Loss of chromosome 9 was detected in 81% of the tumors, most commonly affecting the p arm. Additional common losses occurred on chromosomes 10 (63%), 6q (28%), and 8p (22%). Gains in copy number involved chromosomes 7 (50%), 8 (34%), 6p (28%), 1q (25%), 20 (13%), 17 (13%), and 2 (13%). Amplifications indicating areas harboring potential oncogenes were seen at 4q12, 5p14.3-pter, 7q33-qter, 8q12-13, 11q13.3-14.2, and 17q25. Correlations among the regions with copy number changes indicate that losses of chromosomes 9 and 10 occur early on in melanoma progression, whereas gains of chromosome 7 occur later. This sequence of events was further substantiated by an intratumoral comparison of copy number changes in areas with radial and vertical growth phase patterns. The overall pattern of regions affected by copy number changes is consistent with cytogenetic data from metastatic melanoma; however, the frequencies of involvement differ, providing further insight into the course of genetic events.

INTRODUCTION

The incidence and mortality of melanoma is increasing in the United States, Australia, and Europe (1). Cytogenetic, molecular genetic, and linkage studies have led to the discovery of genetic changes involved in the pathogenesis of melanoma (2, 3). However, as useful as these findings are for understanding the inheritance of melanoma predisposition, our knowledge of genetic events in sporadic melanoma is still scarce. Cytogenetic studies reveal a plethora of chromosomal aberrations in melanoma with some consistent patterns (4, 5). However, most of these data derive from metastatic disease (6–9). Because melanoma is only curable in its early phase, an understanding of the genetic alterations in primary tumors is of great interest, because it may permit an evaluation of the risk of metastasis in a primary melanoma and help to distinguish potential precursors from benign lesions.

In this study, we report CGH3 analysis (10) of chromosomal gains and losses in primary cutaneous melanomas from 32 patients. We show that the predominant pattern of chromosomal imbalances is mostly similar to the findings in metastatic melanoma, but that some additional chromosomal regions are aberrant that might harbor genes relevant for the pathogenesis of melanoma. Comparisons of data from patients with melanomas of varying thickness and from different regions within tumors suggest a sequence of changes in which losses of chromosomes 9 and 10 are the earliest imbalances, whereas gains of chromosome 7 occur later in tumor progression.

MATERIALS AND METHODS

Tumor Material. Primary cutaneous formalin-fixed, paraffin-embedded melanomas from 18 patients and frozen primary melanomas from 14 patients were randomly retrieved from the archives of the Department of Dermatology (University Hospital, Würzburg, Germany) and the Dermatopathology Section, Departments of Pathology and Dermatology (University of California San Francisco, San Francisco, CA). The frozen tissue was from tumors that were macroscopically dissected by a dermatopathologist immediately after excision. The thickest parts of these tumors were fixed in formalin and used for histology, whereas the remainder was embedded in OCT (Tissue-Tek; Miles Inc., Elkhart, IN), snap-frozen in liquid nitrogen, and stored at −80°C. The tumor thickness was determined according to Breslow (11).

DNA Preparation

Paraffin Material. Sections (30 μm) were cut, with a 5-μm section for H&E every five sections. The unstained 30-μm sections were put onto glass slides and microdissected without deparaffinizing. Frozen Tissue. Sections (20 μm) were cut on a cryostat; fixed in 70, 85, and 100% ethanol for 5 min each; and stored at 4°C until microdissection. Every five sections, a 5-μm section was cut and stained with H&E to guide the microdissection.

Microdissection was carried out manually under a dissecting microscope. Depending on the size of the tumor, 20–60 unstained sections were used, and regions with a high density of tumor cells were separated from normal cells. The dissected tumor parts were collected in tubes and deparaffinized by washing with xylene and ethanol. DNA extraction and labeling was performed as described by Isola et al. (12). Briefly, tissue was incubated until complete digestion (3–7 days) with proteinase K (Life Technologies, Inc., Gaithersburg, MD) in a buffer containing 50 mM Tris (pH 8.5), 1 mM EDTA, and 0.5% Tween 20. DNA was extracted with phenol-chloroform-isomylalcohol (25: 24:1, v/v), precipitated with 7.5 M ammonium acetate and 100% ethanol, and resuspended in water. The amount of DNA obtained ranged from 3–50 μg.

CGH and Digital Image Analysis. All tumors were measured both with the tumor DNA labeled with fluorescein-12-dUTP (DuPont, Inc., Boston, MA) and reference DNA labeled with Texas red-5-dUTP (standard labeling) and with the labeling reversed. Labeling was performed by nick translation. Nick translation conditions were adjusted so that the maximal probe size after labeling ranged between 800 and 1500 bp. The hybridization mixture consisted of 200–1000 ng of labeled tumor DNA, 200 ng of inversely labeled sex-matched normal human reference DNA from peripheral blood lymphocytes, and 25 μg of human Cot-1 DNA (Life Technologies, Inc.) dissolved in 10 μl of hybridization buffer [50% formamide, 10% dextrose sulfate, and 2× SSC (pH 7.0)]. Hybridization was carried out for 2–3 days at 37°C to normal metaphases (13). All samples were investigated with a single batch of metaphase slides. Slides were washed three times in a washing solution [50% formamide in 2× SSC (pH 7.0)] at 45°C, once in phosphate buffer [0.1 M NaH2PO4, 0.1 M Na2HPO4, and 0.1% NP40 (pH 8.0)], and once in distilled water (both for 10 min at room temperature). Slides were counterstained with 4',6-diamidino-2-phenylindole in an antifade solution. Hybridization quality was evaluated by the signal strength, the smoothness of the signal distribution and 10 min at room temperature. Slides were counterstained with 4',6-diamidino-2-phenylindole in an antifade solution. Hybridization quality was evaluated by the signal strength, the smoothness of the signal distribution and 10 min at room temperature. Slides were counterstained with 4',6-diamidino-2-phenylindole in an antifade solution. Hybridization quality was evaluated by the signal strength, the smoothness of the signal distribution and 10 min at room temperature. Slides were counterstained with 4',6-diamidino-2-phenylindole in an antifade solution. Hybridization quality was evaluated by the signal strength, the smoothness of the signal distribution and 10 min at room temperature. Slides were counterstained with 4',6-diamidino-2-phenylindole in an antifade solution. Hybridization quality was evaluated by the signal strength, the smoothness of the signal distribution and 10 min at room temperature. Slides were counterstained with 4',6-diamidino-2-phenylindole in an antifade solution. Hybridization quality was evaluated by the signal strength, the smoothness of the signal distribution and 10 min at room temperature. Slides were counterstained with 4',6-diamidino-2-phenylindole in an antifade solution. Hybridization quality was evaluated by the signal strength, the smoothness of the signal distribution and 10 min at room temperature. Slides were counterstained with 4',6-diamidino-2-phenylindole in an antifade solution. Hybridization quality was evaluated by the signal strength, the smoothness of the signal distribution and 10 min at room temperature. Slides were counterstained with 4',6-diamidino-2-phenylindole in an antifade solution. Hybridization quality was evaluated by the signal strength, the smoothness of the signal distribution and 10 min at room temperature.
ments that did not fulfill the above-mentioned quality criteria were repeated until good data were obtained. Tumors that did not yield good hybridizations after repeated attempts were not included in the study (four additional melanomas). Digital images were collected from five metaphases with a Photometrics charge-coupled-device camera (Microimager 1400; Xillix Technologies, Vancouver, British Columbia, Canada). The average tumor:reference fluorescence ratios along each chromosome were calculated with custom CGH analysis software. Measurements were made on at least four copies of each autosome.

Controls and Threshold Definitions. Normal DNA and DNA from tumor cell lines with known aberrations were used as controls. The definition of aberrant regions was performed in two stages: (a) threshold levels were set at tumor:reference fluorescent ratios of 0.80 and 1.2 (14), and any region in which the ratio exceeded these thresholds with either the standard- or reverse-labeled hybridizations was called abnormal; (b) however, because potential copy number changes that occurred only in a subset of cells would be reduced in magnitude, we also applied an additional, narrower threshold interval of 0.85–1.15. These values are based on a series of 15 comparisons of normal DNA samples in which the ratios were never outside the range of 0.9–1.1. Because the narrower thresholds have a greater chance of falsely indicating aberrations, we required ratio profiles for both the standard- and reverse-labeled hybridizations to be outside the 0.85–1.15 interval for a region to be called abnormal.

Using this definition of copy number change on cases that fulfill the criteria of hybridization quality outlined above, the test-retest reproducibility was 100%.

Statistics. Contingency table analysis and ANOVA were used to analyze the relationship between the frequency of copy number changes, tumor thickness, and patient age.

RESULTS

CGH analysis was performed directly on genomic DNA extracted from microdissected parts of primary melanomas. This avoided potential artifactual copy number changes that could be introduced by genomic amplification strategies such as PCR with degenerated primers but necessitated the use of tumors that had areas with a high density of neoplastic cells that could be separated from clusters of normal cells by manual dissection. To be certain that enough DNA (several micrograms) could be conveniently isolated, we had to analyze thicker tumors (mean thickness, 4.5 mm; range, 0.6–13 mm). A total of 30 of 32 (94%) tumors showed losses or gains of DNA sequence copy number, indicating that significant proportions of tumor cells were present in the dissected specimens. Both lesions that did not show changes were rather thin (0.6 and 1.2 mm), broad SSMs with some patches of a lichenoid lymphocytic infiltrate. It is thus possible that those tumors did not contain any copy number changes (potentially because they were thin), or that copy number changes were undetectable due to dilution by normal cells still present in the samples, which is more probable. That it is generally possible to detect copy number changes in thin lesions by our approach is demonstrated by two cases with 1.0- and 1.2-mm thickness as well as by the cases in which the radial growth portions of tumors were studied separately from the vertical growth phase (see below). No significant inflammatory infiltrates were present in these successful analyses.

Overall, losses were slightly more common than gains (99 losses versus 85 gains). On average, there were 5.3 aberrations/tumor (range, 0–14 aberrations/tumor). A summary of the copy number changes by chromosome is depicted in Fig. 1. The most common alteration was a loss of chromosome 9, which occurred in 26 cases (81%). The minimal common lost area was 9p22–pter; however, 24 cases (75%) involved the 9p21 region. Additional chromosomes with frequent losses were chromosomes 10 (63%), 6q (28%), and 8p (22%). Losses of chromosome 10 usually involved the entire chromosome, although

![Fig. 1. Chromosomal localization of DNA-sequence copy number changes in 32 primary melanomas. Lines to the right of the chromosome ideograms represent gains, and lines to the left of the chromosome ideograms represent losses. Bold lines indicate amplifications. The bold lines on the left of chromosomes 9 and 10 represent 10 cases with total loss of chromosome 9 or 10.](cancerres.aacrjournals.org)
six cases showed losses involving only the q arm. Losses of 6q and 8p were often accompanied by gains on the other arm, possibly indicating isochromosome formation. Gains most commonly involved chromosomes 7 (50%), 8 (34%), 6p (28%), 1q (25%), 20 (13%), 17 (13%), and 2 (13%). On chromosome 7, gains were clustered around at least two different regions, one flanking the centromere and the other close to the q telomere. In one case, a high-level gain of 7q33–pter was observed (Fig. 2C). On chromosome 8, gains most commonly involved the q arm, with the distal region being involved more commonly. One case showed a high-level gain of the entire q arm with a peak at 8q12–13 (Fig. 2D). For chromosome 6, the gains mostly involved the entire p arm, although two tumors showed gains of only the telomeric part of the p arm. Gains on chromosomes 17 and 20 mostly involved the q arms; one case showed a high-level gain of the telomeric part of 17q (Fig. 2F). Additional high-level gains were seen at 4q12, 5p14.3–pter, and 11q13.3–14.2 (Fig. 2, A, B, and E).

To detect recurring patterns of concurrent aberrations, we performed pairwise comparisons of all changes that occurred in at least five cases. All eight cases with a 1q gain also showed losses on chromosome 10 (P = 0.011), and five of these eight cases showed a concurrent gain of chromosome 6p.

**Influence of Tumor Thickness on Copy Number Changes.**
Overall, there was an increase in the number of aberrations in thicker tumors; however, its statistical significance was low (P = 0.34). When tumors with a thickness of <3.0 mm (n = 9) was compared with tumors with a thickness of ≥3.0 mm (n = 23), losses of chromosomes 9 and 10 occurred with comparable frequency in both groups, whereas a gain of chromosome 7 was found significantly more frequently in thicker lesions (P = 0.05). Gains of 1q and 17 as well as losses of 6q were also more frequent in the thicker tumors, but the difference was not significant at the 0.05 level.

This suggests that losses of chromosomes 9 and 10 occur earlier in tumor progression than the other changes. The fact that the loss of 9p was the only aberration detectable in two lesions and that the loss of 9p or 9q together with a total loss of chromosome 10 was the only aberration in two additional tumors also supports that interpretation. In contrast, in all 16 cases in which a gain of chromosome 7 occurred, accompanying losses of 9p or of the entire chromosome 9 were present, indicating that gains of chromosome 7 occur after the losses of chromosome 9.

**Age Dependency of Copy Number Changes.** A strong correlation between patient age and the number of aberrations was found (P = 0.007). Interestingly, the additional aberrations found in older subjects were not distributed entirely at random but seemed to form clusters. Gains of chromosomes 5 and 17 and losses of chromosome 18 occurred exclusively in patients older than 70 years of age (Table 2).

**Intratumoral Heterogeneity of Copy Number Changes.** In seven of the tumors, up to four morphologically distinct regions were dissected and analyzed separately (Table 1). In cases 3, 20, 29, 30, and 33, tumor parts in the radial growth phase were compared with parts in the vertical growth phase (nodular parts). In general, the nodular parts tended to show more copy number changes than the radial parts. However, in four cases, the radial part showed no detectable aberrations, which could be explained by contaminating lymphocytes or keratinocytes, so that aberrations in the radial growth phase may be underestimated. As shown in case 23, in which two separate blocks of the same melanoma were studied, a high proportion of inflammatory cells in one tumor part can obscure changes that are clearly detectable in other areas of the same tumor with a more pure population of tumor cells.

Case 9 was a SSM which had two separate exophytic nodules situated within a common zone of radial growth phase melanoma. As indicated, both nodules showed an identical complex pattern of copy number changes. Case 33 was an SSM with a broad radial part and two separate nodules, so that a total of four different areas were studied (Fig. 3). Three of these areas, both nodules and the right radial part, showed copy number changes. In the right radial part and right nodular parts, the sole abnormality was loss of chromosome 9p. The left nodule showed loss of chromosome 10 in addition to 9p (Table 1).

In a total of three cases, copy number (cases 20, 30, and 33) changes were detected in the radial growth phase, all showing a loss of 9p or of the entire chromosome 9. The corresponding nodular parts showed additional changes, i.e., loss of chromosome 13 or 10 or gain of chromosomes 7 and 8.

**Copy Number Changes and Histological Tumor Type.** Because tumors were not selected for equal representations of histogenetic types, our power for the comparison of aberrations among melanoma subtypes is limited. However, some aberrations showed a strikingly unequal distribution among tumor types. Of the three LMMs, two showed gains of chromosomes 17 compared to only 1 of 21 SSMs (P = 0.035). As LMM occurs in older people, this association might be confounded by the described age effect for chromosome 17. That there might, however, be a true association of particular changes with...
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Table 1 Comparison of DNA sequence copy number changes within different parts of seven melanomas

<table>
<thead>
<tr>
<th>Case</th>
<th>Area</th>
<th>Aberrations</th>
</tr>
</thead>
<tbody>
<tr>
<td>3</td>
<td>Radial part</td>
<td>No aberrations</td>
</tr>
<tr>
<td>3</td>
<td>Nodular part</td>
<td>dim (1p, 2, 3, 6q, 9, 10), enh (1q, 6p, 7, 20)</td>
</tr>
<tr>
<td>9</td>
<td>Right nodule</td>
<td>dim (3q, 9p21.2-qter, 10q24.2-qter, 12q13.2-21.3, 11q14.2-qter, 14q), enh (1q, 6p, 7p10-13, 7q10-22)</td>
</tr>
<tr>
<td>9</td>
<td>Left nodule</td>
<td>Identical changes</td>
</tr>
<tr>
<td>20</td>
<td>Radial part</td>
<td>dim (1p21-22, 4p, 4q13-qter, 6q, 8p, 9p, 10, 16q), enh (2p11.2, 8q, 11p14-qter, 11q10-22, 12q23-qter, 15q23-qter), amp (4q12-13.1, 8q12-13, 11q13.3-14.2)</td>
</tr>
<tr>
<td>20</td>
<td>Nodular part</td>
<td>dim (4p, 4q13-qter, 6q, 8p, 9p, 10, 13, 16q), enh (2p11.2, 8q, 11p14-qter, 11q10-22, 12q qter, 15q23-qter), amp (4q12-13.1, 8q12-13, 11q13.3-14.2)</td>
</tr>
<tr>
<td>23</td>
<td>Block 1, central part of nodule</td>
<td>No aberrations</td>
</tr>
<tr>
<td>23</td>
<td>Block 2, entire nodule</td>
<td>dim (9p21-qter, 9p21-qter), enh (8q, 9q)</td>
</tr>
<tr>
<td>29</td>
<td>Radial part</td>
<td>No aberrations</td>
</tr>
<tr>
<td>29</td>
<td>Central nodule</td>
<td>dim (9p, 10, 20p, x), enh (1q, 7, 8)</td>
</tr>
<tr>
<td>29</td>
<td>Peripheral nodule</td>
<td>dim (9p, 10, 20p, x), enh (1q, 7, 8)</td>
</tr>
<tr>
<td>30</td>
<td>Radial part</td>
<td>dim (6q, 9, 10), enh (6p, 16p)</td>
</tr>
<tr>
<td>30</td>
<td>Nodular part</td>
<td>dim (6q, 9, 10), enh (6p, 7, 8, 16p)</td>
</tr>
<tr>
<td>33</td>
<td>Left radial part (1)</td>
<td>No aberrations</td>
</tr>
<tr>
<td>33</td>
<td>Right radial part (2)</td>
<td>dim (9p)</td>
</tr>
<tr>
<td>33</td>
<td>Left nodular part (3)</td>
<td>dim (9p, 10)</td>
</tr>
<tr>
<td>33</td>
<td>Right nodular part (4)</td>
<td>dim (9p)</td>
</tr>
</tbody>
</table>

* dim; losses; enh; gains; amp, amplifications (see Ref. 38). Aberrations that differ in different regions of the same tumor are shown in bold.

* Only one area with dense sheets of tumor cells was sampled.

* This part of the tumor was ulcerated and had areas with dense inflammatory infiltrates.

* The numbers in parentheses indicate the dissected parts depicted in Fig. 3.

Melanoma subtypes are also suggested by the single ALM of the series (Table 1, case 20) that showed a unique combination of changes including several amplifications not seen in the other tumors (Fig. 2, A, D, and E).

**DISCUSSION**

Most of our knowledge of cytogenetic changes in melanoma stems from the analysis of metastases (4, 15). Only limited information on primary tumors is available, which is subject to the inherent methodological difficulties of classical cytogenetics. CGH has proven to be a powerful tool for assessing genomic imbalances in cancer and has the important advantage of being applicable to archival material (16). This technique permitted us to analyze regional variations of DNA copy number in primary cutaneous melanomas. Our data show that CGH of primary melanomas can be performed with native DNA if microdissected parts of relatively pure populations of tumor cells are used as the source of DNA. The hazard of a distortion of the copy number changes potentially inherent in amplification techniques can thereby be avoided. We believe that the two tumors that showed no aberrations in our study probably contained too great a proportion of normal reactive cells for aberrations to be detected by CGH. Therefore, it seems safe to conclude that the vast majority of primary melanomas with a thickness above 1 mm show aberrations detectable with CGH. This might enable CGH to be developed as an adjunctive diagnostic procedure in selected melanocytic tumors.

It is noticeable that the chromosomes most commonly involved in copy number changes in our study (chromosomes 1, 6, 7, 9, and 10) are in agreement with the findings of multiple cytogenetic studies of metastatic melanoma that have reported these chromosomes to be involved in both structural and numerical aberrations (4, 15, 17). Remarkably, our data suggest that losses of chromosomes 9 and 10 occur even more frequently (81 and 63%, respectively) than already suggested by cytogenetic studies and allelotyping approaches (18, 19).

This discrepancy could be explained by the difficulties in the detection of numerical aberrations in some complex karyotypes and by problematic assessment of allelic losses in the presence of homozygous deletions or significant contamination of normal cells.

The loss of chromosome 9 in 81% of tumors, including eight of nine cases in which three and fewer aberrations were found and one case in which it was the only aberration, indicates its importance in the development of melanoma. Losses of chromosome 9 seem to occur early on in tumorigenesis as suggested by: (a) their appearance at comparable frequencies in thinner and thicker lesions; (b) their presence in the radial growth phase portion of some tumors where additional changes were present in nodular areas (Table 2); and (c) the findings that some aberrations such as gains of chromosome 7 only occurred in the presence of chromosome 9 losses. An early involvement of chromosome 9 in melanoma tumorigenesis would be in agreement with previous studies, which found losses of chromosome 9 in lesions classified as dysplastic nevi (6). The common deleted area of chromosome 9 was the distal part of the p arm, encompassing 9p21 in most of the cases. Two cases apparently showed losses immediately adjacent to 9p21. However, considering the relatively low resolution of CGH, no definite statement can be made as to whether those losses still contain the p16 gene region or are indicative of an additional
suppressor gene(s) distal from 9p21. This question is being addressed by fluorescence in situ hybridization.

Losses of chromosome 10 were an additional frequent finding in this study. Arguments similar to those for chromosome 9 can be made suggesting that losses of chromosome 10 occur early in tumorigenesis. The most frequently lost area was the q arm, and two cases showed regional losses at 10q21-22 and 10q24-qter, respectively. The latter region has previously been found to be lost or involved in translocations (20), and loss of heterozygosity has been found on chromosome 10q (21, 22).

Copy number changes on chromosome 6 showed gains of the p arm and losses of the q arm. This is in accordance with other studies (4, 17). The cases showing aberrations of chromosome 6 in our study mostly had multiple changes (mean, 5.5 for the p arm and 7.6 for the q arm), indicating that they might occur later in tumor progression. The q arm of chromosome 6 has been shown to harbor a suppressor gene relevant for melanoma pathogenesis (23, 24). In addition, the frequent gain of the p arm suggests that an oncogene involved in melanoma progression resides on the distal part of the p arm of chromosome 6.

Chromosome 7 showed gains in half of the cases. The complex pattern of gains suggests that several regions may be affected. One region involves the distal q arm highlighted by an amplification in one case and a regional gain in another. An additional region of gains focuses around the centromere. The epidermal growth factor receptor gene resides at 7p12-13, and an association between the overexpression of the epidermal growth factor receptor and an increased copy number of chromosome 7 in melanoma has been reported (25). The fact that gains of chromosome 7 occurred mostly in thicker tumors in our series and always presented with other aberrations indicates that it is acquired later in tumor progression, as has been postulated by previous investigators (15, 26).

The p arm of chromosome 8 is frequently lost in a variety of cancers (4). The data of our series suggest that a suppressor gene relevant in melanoma resides in the 8p21–pter region. Gains on chromosome 8 most frequently involved the q arm, with the common gained region being the distal q arm, where the myc oncogene is located. Immunohistochemical studies have suggested an increase of c-myc expression during melanoma progression (27). The amplification of a region at 8q12–13 highlights a locus that might harbor a novel recurrent loci (Table 2). (We note that age-related chromosomal heterogeneity within melanoma would not be detectable by CGH.)

Melanomas in older people usually have a protracted clinical course and thus a longer tumor progression than that in younger patients. This could indicate that there are two separate pathways to clinically manifest tumors: (a) one requiring many minor events and thus taking a long time; and (b) another, more rapid way requiring only a few significant steps.

In conclusion, the recurrent copy number changes identified by CGH in primary melanomas correspond in part to previous cytogenetic and allelic loss data. In addition, our results highlight several novel regions that might harbor other genes involved in melanoma pathogenesis. Our findings suggest that losses of 9p are more prevalent in primary melanomas than previously estimated and are likely to represent an early event in the evolution of sporadic melanoma.

### REFERENCES


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