ORIGINAL ARTICLE

# Distinct Sets of Genetic Alterations in Melanoma

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## ABSTRACT

### BACKGROUND

Exposure to ultraviolet light is a major causative factor in melanoma, although the relationship between risk and exposure is complex. We hypothesized that the clinical heterogeneity is explained by genetically distinct types of melanoma with different susceptibility to ultraviolet light.

### METHODS

We compared genome-wide alterations in the number of copies of DNA and mutational status of *BRAF* and *N-RAS* in 126 melanomas from four groups in which the degree of exposure to ultraviolet light differs: 30 melanomas from skin with chronic sun-induced damage and 40 melanomas from skin without such damage; 36 melanomas from palms, soles, and subungual (acral) sites; and 20 mucosal melanomas.

### RESULTS

We found significant differences in the frequencies of regional changes in the number of copies of DNA and mutation frequencies in *BRAF* among the four groups of melanomas. Samples could be correctly classified into the four groups with 70 percent accuracy on the basis of the changes in the number of copies of genomic DNA. In two-way comparisons, melanomas arising on skin with signs of chronic sun-induced damage and skin without such signs could be correctly classified with 84 percent accuracy. Acral melanoma could be distinguished from mucosal melanoma with 89 percent accuracy. Eighty-one percent of melanomas on skin without chronic sun-induced damage had mutations in *BRAF* or *N-RAS*; the majority of melanomas in the other groups had mutations in neither gene. Melanomas with wild-type *BRAF* or *N-RAS* frequently had increases in the number of copies of the genes for cyclin-dependent kinase 4 (*CDK4*) and cyclin D1 (*CCND1*), downstream components of the RAS–BRAF pathway.

### CONCLUSIONS

The genetic alterations identified in melanomas at different sites and with different levels of sun exposure indicate that there are distinct genetic pathways in the development of melanoma and implicate *CDK4* and *CCND1* as independent oncogenes in melanomas without mutations in *BRAF* or *N-RAS*.

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THE RISING INCIDENCE OF MELANOMA and lack of effective treatments for advanced disease represent an important public health problem.[1] Exposure to the sun is generally accepted as a major causative factor.[1-3] However, its mechanism is unknown, and the role of exposure to ultraviolet light is complex and has some paradoxical features. For example, in light-skinned people, the group that is predominantly affected by melanoma, tumors are most common on areas that are intermittently exposed to the sun, such as the trunk, arms, and legs, rather than on areas that are chronically exposed to the sun, such as the face. Also, several studies have shown that indoor workers have a higher risk of melanoma than outdoor workers,[4,5] leading some authorities to suggest that chronic exposure to ultraviolet light exerts a protective effect. A small proportion of melanomas arise without obvious exposure to ultraviolet light, because they affect sites that are relatively or absolutely protected, such as the palms and soles (acral melanoma) and mucosal membranes. Finally, genes such as *BRAF* and *N-RAS* that are commonly mutated in melanoma do not show typical ultraviolet "fingerprint" mutations.[6,7]

There has been an ongoing debate about whether this complexity could in part be due to the existence of several distinct types of melanoma. One proposal, based on histologic growth patterns, describes four "histogenetic" types of melanoma: superficial spreading, lentigo maligna, nodular, and acral lentiginous melanoma.[8,9] However, the use of this classification is controversial[10] and has not been broadly adopted in clinical practice, primarily because a substantial number of melanomas do not fit the classic types and the histogenetic type is not an independent prognostic factor.[11,12] A more recent hypothesis suggests that these tumors be classified according to divergent pathways, because patients with melanomas of the head and neck differ from patients with melanomas on the trunk in having higher levels of expression of TP53 protein, a higher frequency of associated nonmelanoma skin cancers, and lower numbers of melanocytic nevi.[13-15]

Genetic studies have provided support for this dual-pathway hypothesis concerning melanomas on skin exposed to the sun. *BRAF* mutations are common only in melanomas arising in areas intermittently exposed to the sun and are rare in melanomas on skin that is chronically exposed to the sun or on acral skin and mucosal membranes that are seldom or never exposed to the sun.[16] In addition, several studies have shown that melanomas of the palms and soles and mucosal membranes have distinctive patterns of chromosomal aberrations as compared with those at other sites.[17-19] Understanding whether the heterogeneity of melanoma with respect to the site, degree of exposure to the sun, and histologic characteristics is caused by biologically distinct types of melanoma is of great clinical importance, because it is likely to result in separate targeted therapeutic approaches and prevention strategies. To shed light on this area, we analyzed 126 primary melanomas classified into four groups on the basis of their location and degree of exposure to the sun. Our analysis included a genome-wide assessment of the differences in the number of copies of DNA that used array-based comparative genomic hybridization[20,21] and a focused analysis of signaling pathways that are markedly altered in melanoma (Fig. 1).

## METHODS

### TUMOR SPECIMENS

We collected archival, paraffin-embedded primary melanomas that had an invasive component in which tumor cells predominated over stromal cells from seven centers: the Dermatopathology Section of the Department of Pathology and Dermatology, University of California, San Francisco; the Department of Dermatology, Kumamoto University School of Medicine, Kumamoto, Japan; the Department of Pathology, Memorial Sloan-Kettering Cancer Center, New York; DermPath, Friedrichshafen, Germany; the Department of Dermatology, Seoul National University College of Medicine, Seoul, South Korea; the Department of Dermatology, Tohoku University Graduate School of Medicine, Sendai, Japan; and the Department of Dermatology, University of Würzburg, Würzburg, Germany. The study was approved by the institutional review board of the University of California, San Francisco. We obtained roughly similar numbers of four types of tumors: 36 specimens of acral melanoma, defined as melanoma occurring on the non–hair-bearing skin of the palms or soles or under the nails; 20 specimens of mucosal melanoma, defined as tumors arising on mucosal membranes; 30 specimens of melanoma arising from skin with chronic sun-induced damage; and 40 specimens of melanoma arising from skin without chronic sun-induced damage. The distinction between the last

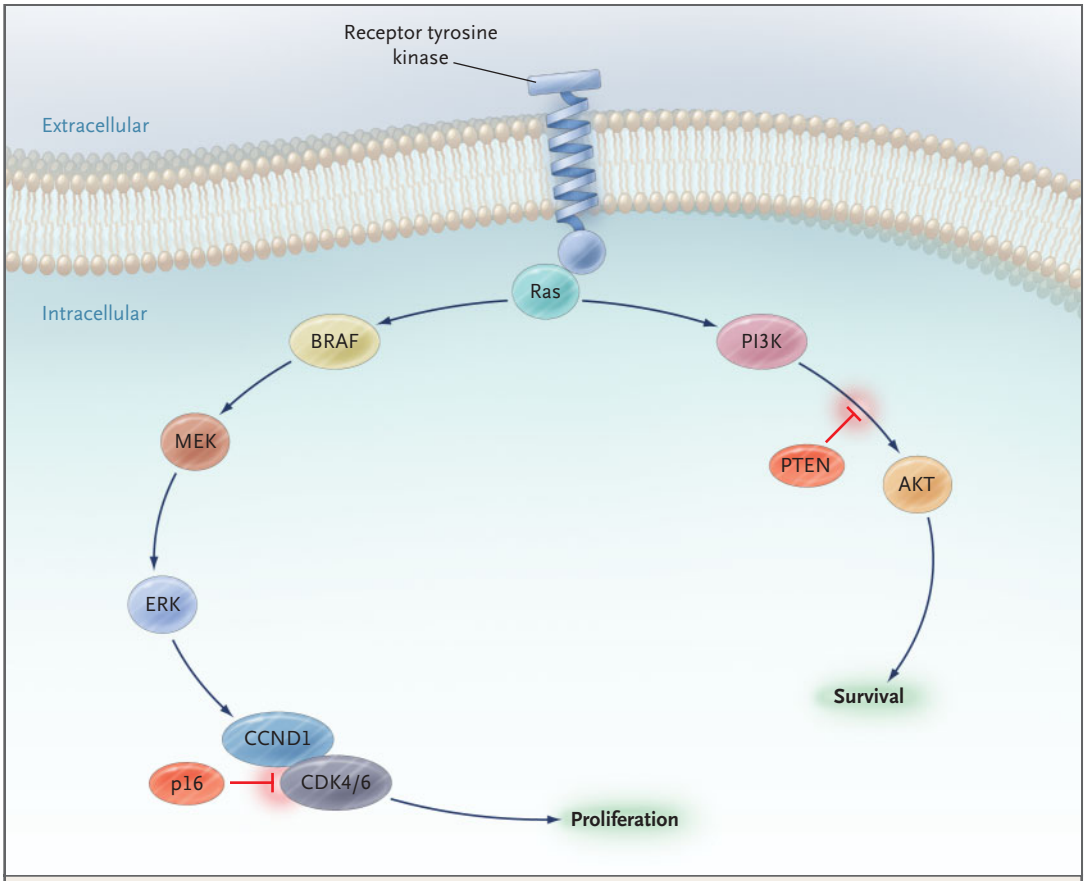



**Figure 1. The Mitogen-Activated Protein (MAP) Kinase and Phosphatidylinositol 3' Kinase (PI3K) Pathways.**
Signals from receptor tyrosine kinases can promote proliferation through the MAP kinase pathway (left branch) and survival through the PI3 kinase pathway (right branch).

two groups was based solely on the presence or absence on microscopy of marked solar elastosis of the dermis surrounding the melanomas. In all but a few cases, melanomas associated with chronic sun-induced damage occurred on the face and melanomas that were not associated with chronic sun-induced damage occurred on the trunk, arms, and legs (Table 1; further details are provided in the Supplementary Appendix, available with the full text of this article at www. nejm.org).

### EXPERIMENTAL METHODS

DNA for comparative genomic hybridization was extracted from tumor-bearing tissue as described previously.[22] Array-based comparative genomic hybridization was carried out on 600 to 2000 ng of genomic DNA, labeled by random priming, as previously described.[23] Data points of low quality, as assessed by a large standard deviation between replicate spots on each array, were rejected. Clones that had missing data in more than 25 percent of the samples in the group as a whole or in 50 percent in any individual group were excluded from further analysis (the Supplementary Appendix provides details of primary data processing). The data set used for comparative genomic hybridization has been deposited in the Gene Expression Omnibus (accession number, GSE2631; available at www.ncbi.nlm.nih.gov/geo/).

Immunohistochemical analysis was performed on tissue microarrays as described previously with the use of standard protocols and the use of 3-amino-9-ethylcarbazole as a chromagen according to the manufacturer's specifications.[24] The following antibodies were used: monoclonal antibody AM29 against cyclin D1 (CCND1; catalog number, 18-0220; Zymed) in a 1:200 dilution, as described previously[25]; monoclonal antibody Ab-4 against cyclin-

**Table 1. Characteristics of the Four Types of Melanoma.***

| Group | No. of Samples | Tumor Thickness | Sex† | | Age | Mutant *BRAF*‡ | Mutant *RAS*‡ | Wild-Type *BRAF* and *RAS*‡ | Common Chromosomal Aberrations§ |
|---|---|---|---|---|---|---|---|---|---|
| | | | Male | Female | | | | | |
| | | *mm* | *no. of patients* | | *yr* | | *no. (%)* | | |
| **Melanomas on skin without chronic sun-induced damage** | | | | | | | | | |
| Trunk | 20 | | 12 | 8 | | 14 (78) | 3 (17) | 1 (6) | Increased copy no. (6p, 7, 8q, 17q, 20q) |
| Median | | 3.6 | | | 65 | | | | Reduced copy no. (9p, **10,** 21q) |
| Range | | 1.2–7.5 | | | 28–82 | | | | |
| Leg | 12 | | 4 | 7 | | 4 (36) | 3 (27) | 4 (36) | |
| Median | | 2.8 | | | 69 | | | | |
| Range | | 1.2–5.0 | | | 21–84 | | | | |
| Arm | 6 | | 4 | 2 | | 2 (33) | 2 (33) | 2 (33) | |
| Median | | 4.0 | | | 70 | | | | |
| Range | | 1.8–11.7 | | | 11–85 | | | | |
| Head | 1 | | 0 | 1 | | 1 (100) | 0 | 0 | |
| Median | | 2.7 | | | 38 | | | | |
| Range | | | | | | | | | |
| Data missing | 1 | | 0 | 1 | | 1 (100) | 0 | 0 | |
| Median | | 3.7 | | | 75 | | | | |
| Range | | | | | | | | | |
| Total | 40 | | 20 | 19 | | 22 (59) | 8 (22) | 7 (19) | |
| Median | | 3.1 | | | 65 | | | | |
| Range | | 1.2–11.7 | | | 11–85 | | | | |
| **Melanomas on skin with chronic sun-induced damage** | | | | | | | | | |
| Head | 21 | | 9 | 10 | | 2 (10) | 4 (20) | 14 (70) | Increased copy no. (6p, 11q13, 17q, 20q) |
| Median | | 3.0 | | | 76 | | | | Reduced copy no. (6q, 8p, 9p, 13, 21q) |
| Range | | 1.0–7.5 | | | 52–94 | | | | |
| Trunk | 3 | | 3 | 0 | | 0 | 0 | 1 (100) | |
| Median | | 1.7 | | | 78 | | | | |
| Range | | 1.4–6.2 | | | 76–84 | | | | |
| Arm | 3 | | 2 | 1 | | 0 | 0 | 3 (100) | |
| Median | | 3.8 | | | 73 | | | | |
| Range | | 3.3–6.0 | | | 67–83 | | | | |

| | | | | | | | | |
|---|---|---|---|---|---|---|---|---|
| Data missing | 3 | | 1 | 1 | | 1 (33) | 0 | 2 (67) | |
| Median | | 3.0 | | | 78 | | | | |
| Range | | 2.0–3.8 | | | 73–82 | | | | |
| Total | 30 | | 15 | 12 | | | | | |
| Median | | 3.0 | | | 76 | 3 (11) | 4 (15) | 20 (74) | |
| Range | | 1.0–7.5 | | | 52–94 | | | | |
| **Mucosal melanoma** | | | | | | | | | |
| Total | 20 | | 7 | 13 | | 2 (11) | 1 (5) | 16 (84) | Increased copy no. (**1q, 6p,** 7, 8q, **11q13, 17q,** 20q) |
| Median | | 5.0 | | | 68 | | | | Amplification (1q31, 4q12, **12q14**) |
| Range | | 3.8–45.0 | | | 38–83 | | | | Reduced copy no. (**3q,** 4q, 6q, **8p,** 9p, **10, 11p,** 11q, 21q) |
| **Acral melanoma** | | | | | | | | | |
| Sole | 27 | | 14 | 11 | | 3 (14) | 3 (14) | 16 (73) | Increased copy no. (**6p,** 7, 8q, 17q, 20q) |
| Median | | 2.9 | | | 76 | | | | Amplification (5p15, 5p13, **11q13,** 12q14) |
| Range | | 1.2–8.0 | | | 34–90 | | | | Reduced copy no. (6q, 9p, **10,** 11q, 21q) |
| Subungual | 7 | | 5 | 2 | | 3 (43) | 0 | 4 (57) | |
| Median | | 4.6 | | | 66 | | | | |
| Range | | 2.0–10.0 | | | 42–77 | | | | |
| Palm | 1 | | 1 | 0 | | 1 (100) | 0 | 0 | |
| Median | | 1.0 | | | 62 | | | | |
| Range | | | | | | | | | |
| Data missing | 1 | | NA | NA | NA | NA | NA | NA | |
| Median | | 1.9 | | | | | | | |
| Range | | | | | | | | | |
| Total | 36 | | 20 | 13 | | 7 (23) | 3 (10) | 20 (67) | |
| Median | | 3.0 | | | 73 | | | | |
| Range | | 1.0–10.0 | | | 34–90 | | | | |

* NA denotes not available.
† Data about sex were missing for some patients.
‡ Percentages indicate the proportions of samples sequenced successfully. Because of rounding not all percentages total 100.
§ Common chromosomal aberrations are those that occur in at least 20 percent (reduced copy no. or increased copy no. or at least 10 percent amplification) of samples within individual groups. Boldfaced regions occur at significantly different frequencies between groups ($P<0.05$).

dependent kinase N2A (CDKN2A; catalog number, MS-887-P1; Laboratory Vision) in a 1:25 dilution; a polyclonal antibody against phosphorylated Akt (Ser473; catalog number, 9277S; Cell Signaling Technology) in a 1:80 dilution; and E10 monoclonal antibody against phosphorylated extracellular-signal–regulated kinase (ERK, Thr202/Tyr204; catalog number, 9106S; Cell Signaling Technology) in a 1:80 dilution. The intensity of staining was scored on a scale on which a score of 0 indicated the lowest intensity and a score of 4 the highest intensity. Scores of 2 or greater were considered positive.

We used a polymerase-chain-reaction (PCR) assay to amplify DNA that included *BRAF* codon 600 (113 specimens) and codons 12, 13, and 61 of *N-RAS* (113 specimens) and *H-RAS* and *K-RAS* (95 specimens each). PCR products were purified with the use of ExoSAP-IT (USB), sequenced directly with the use of specific primers, and analyzed with the use of an ABI Prism 3700 DNA Analyzer (Applied Biosystems).

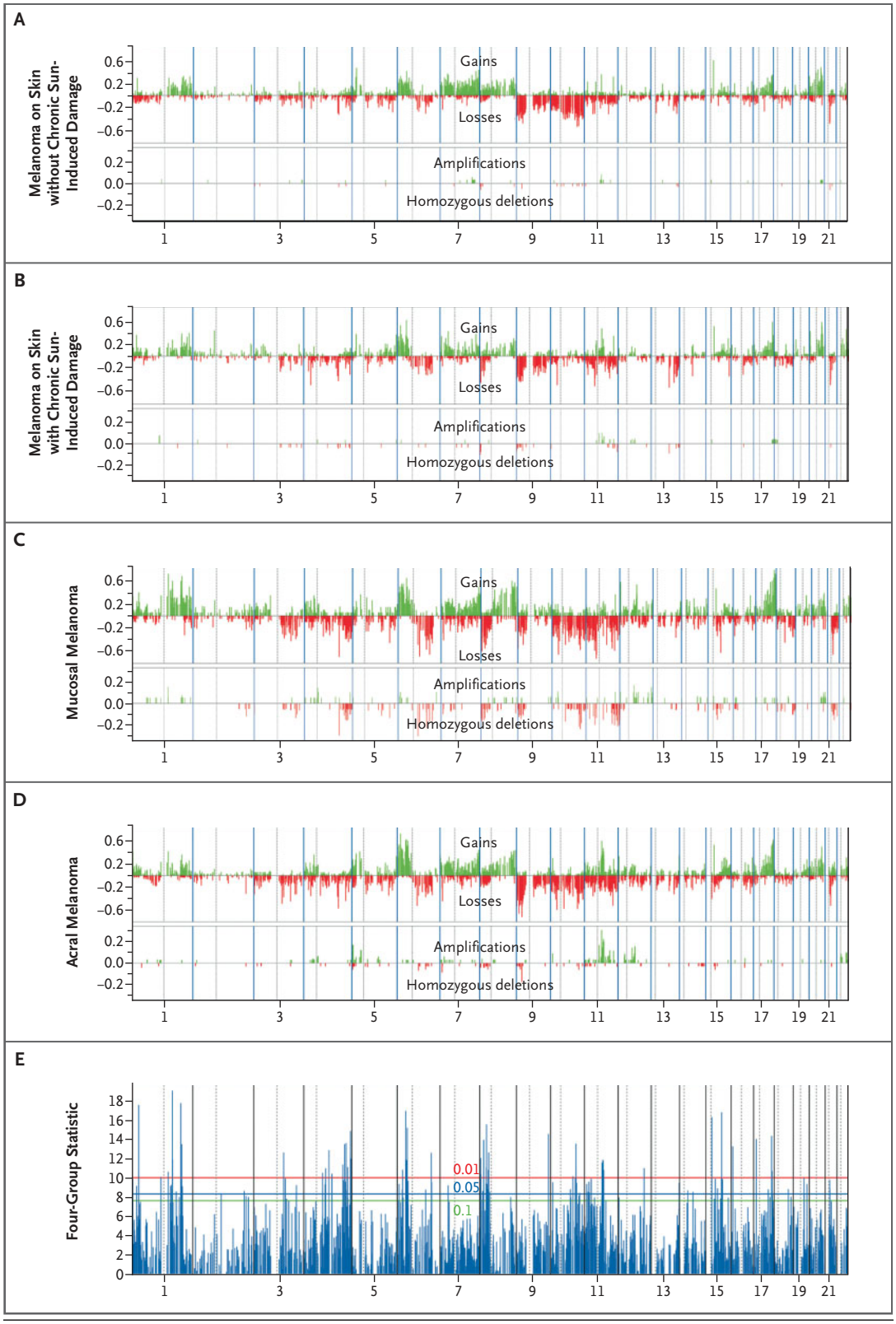
**Figure 2 (facing page). Changes in the Number of Copies of DNA in Subgroups of Melanoma.**

Two histograms are shown for each group of melanoma (Panels A, B, C, and D) and for the four groups combined (Panel E). In each panel, the upper plots show low-level gains (green) and losses (red), and the lower plots show amplifications (green) and homozygous deletions (red) for each type. Vertical dashed lines represent the location of the centromere. The x axis represents genomic position, with the bacterial artificial chromosomes ordered according to position in the genome beginning at 1p and ending at 22. The y axis represents the fraction of the samples with a given clone altered. Panel E shows the statistical differences between the changes in the number of copies in the four groups. The magnitude of the F statistic is shown as the height of the vertical bars, and their global significance is indicated with horizontal dashed lines that show the maxT-adjusted P-value cutoffs (red, P=0.01; blue, P=0.05; and green, P=0.1).

### STATISTICAL ANALYSIS

The experimental variability of each sample included in the array for comparative genomic hybridization was assessed as described previously.[26] For each hybridization, the median absolute deviation, a robust estimate of the standard deviation, was calculated for each portion of the genome found to have a constant number of copies, and the median of those values was used to estimate the noise level of the measurement. Changes in the numbers of copies of DNA within chromosomes and of entire chromosomes were identified by applying the unsupervised hidden-Markov-model procedure as previously described[26,27] and used to assess overall genomic instability.[23] Microarray elements with absolute base-2 logarithm ($\log_2$) ratios greater than 2.5 times the median absolute deviation were classified as aberrant (having gains or losses in numbers of copies). The proportion of the genome altered in each sample was computed as the proportion of aberrant clones. Genomic regions represented by microarray elements were declared to be homozygously lost if their $\log_2$ ratio was less than −0.9. The height of the peak representing the increase in the number of copies and the narrowness of the affected region relative to its flanking segments were used to define high-level amplification (details are provided in the Supplementary Appendix). Amplicons were defined as contiguous regions of amplification. Missing values were imputed with the use of the lowess approach, which is a robust, locally linear regression method.[28] The F statistic, corrected for multiple testing with the use of maxT-adjusted permutation-based P values (i.e., adjusted for multiple testing in a way that strongly controls the probability of declaring false positives), was used to assess the significance of differences between the ratios of individual clones among the four groups of melanomas.[29] Adjusted P values of less than 0.05 were considered to indicate statistical significance, ensuring the probability of at least one false discovery of no more than 0.05 and thus providing strong control of the family-wise error rate.[29]

We compared individual clones containing genes of interest between two or more groups using the Kruskal–Wallis test. We used Fisher's exact test to evaluate the two-by-two tables comparing mutational status or cluster assignments among the four groups. The rate of misclassification of the classifiers was assessed with the use of the leave-one-out cross-validation method by repeatedly applying a diagonal linear discriminant analysis classifier while varying the number of features used by the predictor.[30] Features were reselected at each cross-validation step with the use of F-statistic ranking. Hence, only samples in the most current training set were used, thus eliminating potential downward bias of the resulting error rate.[30] The reported leave-one-out error rates correspond to the predictor with the number of features resulting in the best performance of the classifier. All calculations were done in R statistical language.[31]



## RESULTS

### DISTINCT SETS OF GENOMIC ABERRATIONS AMONG MELANOMA SUBGROUPS

Array-based comparative genomic hybridization showed that several genomic regions had changes in the number of copies of DNA in all four groups of melanoma (Fig. 2). In addition, there were marked differences in aberrant genomic regions among the groups (Fig. 2 and Table 1). These differences were most pronounced between melanomas on skin that was relatively or absolutely protected from the sun (acral and mucosal melanomas) and melanomas on skin with various degrees of exposure to the sun. Specifically, acral or mucosal melanomas had a significantly higher degree of chromosomal aberrations, as assessed by the overall proportion of the genome affected by gains or losses of DNA (P=0.004 by the Kruskal–Wallis test), changes in the total number of copy-number transitions within chromosomes (P<0.001 by the Kruskal–Wallis test), and changes in the number of amplicons (P<0.001 by the Kruskal–Wallis test) (Fig. 3). Amplifications were found in 89 percent of acral melanomas and 85 percent of mucosal melanomas, but they involved different genomic regions in the two groups (Fig. 2 and Table 1). Amplifications were infrequent in the group of melanomas on skin with chronic sun-induced damage and the group on skin without chronic sun-induced damage. In addition to these differences in the degree of genomic instability, there were differences in aberration patterns among the four groups (Fig. 2).

Table 1 shows the common genomic aberrations and the regions that were affected with different frequencies in each group after adjustment for multiple testing. On the basis of the changes in the number of copies of DNA alone, samples could be correctly classified into the four groups with an overall accuracy of 70 percent. Two-way classification of acral and mucosal melanomas correctly classified 50 of 56 samples (89 percent). Most intriguingly, two-way classification of the group of melanomas on skin with chronic sun-induced damage and the group on skin without chronic sun-induced damage led to the correct classification of 59 of 70 samples (84 percent). Unsupervised agglomerative hierarchical clustering separated the group of melanomas that were on skin with chronic sun-induced damage and the group on skin without chronic sun-induced damage that had been exposed to sun into two major clusters (P<0.001 by Fisher's exact test) (Fig. 3C). Frequent focused gains involving the *CCND1* locus (P=0.001), losses involving chromosome 4q (P=0.004), and gains involving regions of chromosome 22 (P=0.004) were significantly more common in the group with chronic sun-induced damage than in the group without such damage; the latter group had more frequent losses involving chromosome 10q (P=0.002). All P values were calculated with the use of a maxT-adjusted permutation-based t-test as described above and are reported for the most significant changes of the relevant chromosomal regions.

### ALTERATION OF THE MITOGEN-ACTIVATED PROTEIN KINASE PATHWAY

Mutations in *BRAF* were significantly more common in the group of melanomas that were on skin without chronic sun-induced damage than in the other three groups (P<0.001 by Fisher's exact test) (Fig. 4F), confirming our previous report.[16] All mutations in *RAS* genes were found in *N-RAS* and occurred only in samples without *BRAF* mutations. We did not observe a significant association between *N-RAS* mutations and melanoma subtypes (P=0.4 by Fisher's exact test) (Fig. 4F).

Immunohistochemical analysis showed that samples without mutations in *BRAF* or *N-RAS* less frequently expressed phosphorylated ERK than did samples with such mutations, suggesting that in samples with wild-type *BRAF* and *N-RAS*, the pathway was not activated upstream of phosphorylated ERK (see the Supplementary Appendix). One downstream gene, *CCND1*, resided in the most common genomic region affected by focused amplifications (Fig. 4A). The increases in the number of copies of *CCND1* were inversely correlated with mutations in *BRAF* (P=0.008 by the Kruskal–Wallis test), independently of the type of melanoma (Fig. 4D). Specimens that had increased levels of expression of *CCND1* on immunohistochemical analysis had without exception either mutations in *BRAF* or *N-RAS* or an increased number of copies of *CCND1* (see the Supplementary Appendix). This observation and the strong inverse correlation between *BRAF* mutations and the increase in the number of copies of *CCND1* suggest that the elevation in the levels of CCND1 protein as a result of either mutations in upstream genes or increases in the gene dosage represents a crucial event driving progression in melanoma.

Interestingly, *CDK4*, whose protein is one of the binding partners of CCND1 and which is located

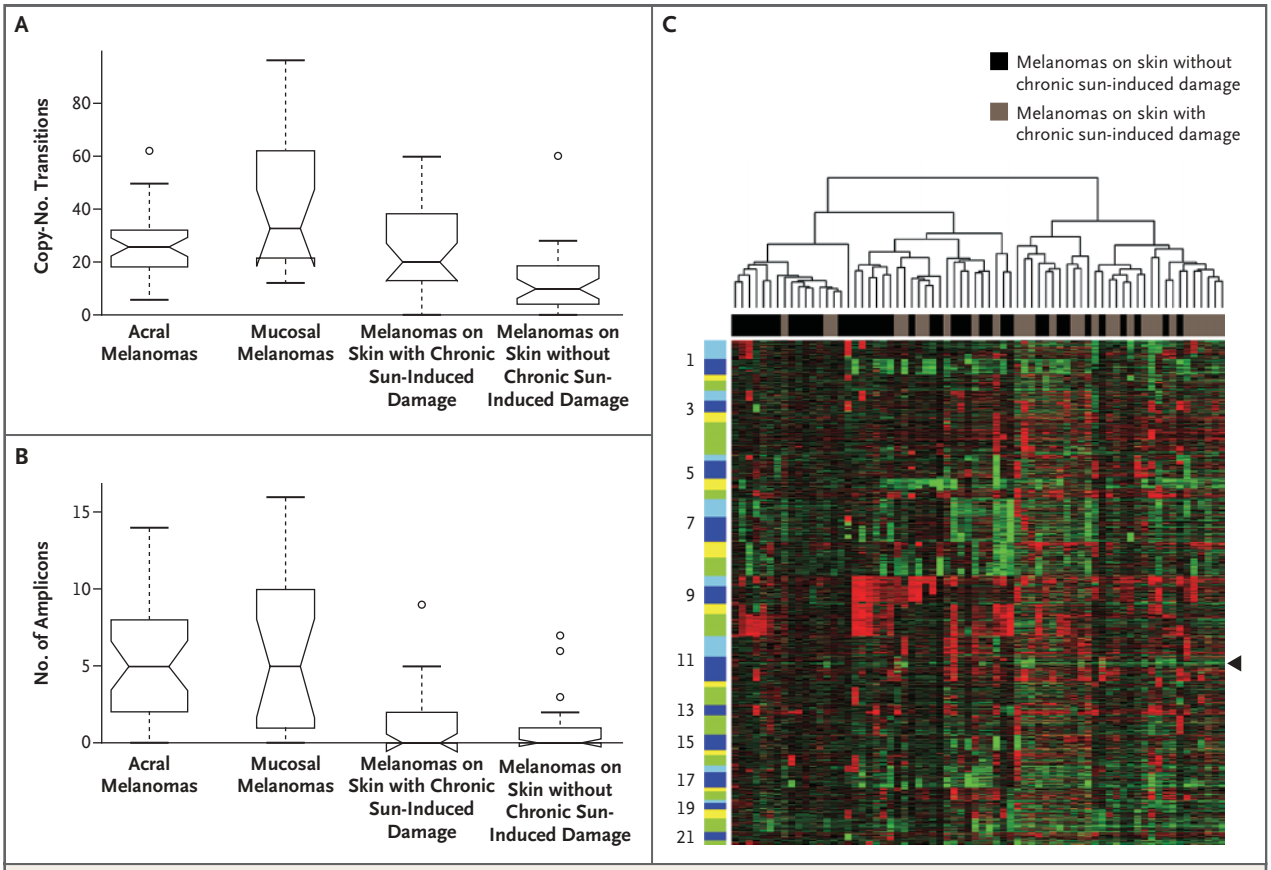



**Figure 3. Qualitative and Quantitative Differences in Chromosomal Aberrations among the Four Groups of Melanoma.**

The box plots indicate significant differences in the number of copies of DNA (Panel A) and number of amplicons (Panel B) among the four types of melanoma ($P<0.001$ by the Kruskal–Wallis test). Boxes are outlined by the first and third quartiles, with the median as a horizontal line inside. Whiskers are drawn as 1.5 times the distance between the first and third quartiles. Points outside the whiskers are considered outliers. The notches indicate the 95 percent confidence interval for the median. Panel C shows the unsupervised hierarchical clustering of melanomas on skin with chronic sun-induced damage and melanomas on skin without chronic sun-induced damage. Genomic clones are displayed in vertical orientation according to their genomic position from chromosomes 1 to 22. The p arms of chromosomes are indicated in light blue (odd chromosomes) and yellow (even chromosomes), and the q arms are indicated in dark blue (odd chromosomes) and green (even chromosomes). The *CCND1* locus was frequently gained in the right branch of the tree (arrowhead), which was enriched for specimens from skin with chronic sun-induced damage ($P=0.001$ by the Kruskal–Wallis test). Ten samples from skin without chronic sun-induced damage were incorrectly grouped with samples from skin with such damage, and 10 samples from skin with chronic sun-induced damage were incorrectly grouped with samples from skin without such damage. There was a significant association between groups and cluster assignment ($P<0.001$ by Fisher's exact test).

on chromosome 12q14, was also subject to recurrent focal amplifications (Fig. 4B). *CDK4* amplifications were more common in acral and mucosal melanomas than in the other two groups ($P=0.005$ by the Kruskal–Wallis test). None of the 11 specimens with *CDK4* amplifications had mutations in either *BRAF* or *N-RAS* or had amplification of *CCND1* (Fig. 4D). The strong complementarity of the genetic interactions among *CDK4*, *CCND1*, *BRAF*, and *N-RAS* implicate *CDK4* and *CCND1* as independent oncogenes in melanoma.

The CDKN2A protein (also commonly known as p16) is a melanoma-susceptibility factor and the main negative regulator of the CDK4–CCND1 complex, representing a crucial gatekeeper at the $G_1$–S checkpoint.[32] We found deletions of the *CDKN2A* locus in 50 percent of all melanomas, making it the most commonly lost genomic region (Fig. 4G). In 10 percent of our specimens, comparative genomic hybridization showed a homozygous deletion of the *CDKN2A* locus paralleled by a complete loss of expression of CDKN2A protein on immunohisto-

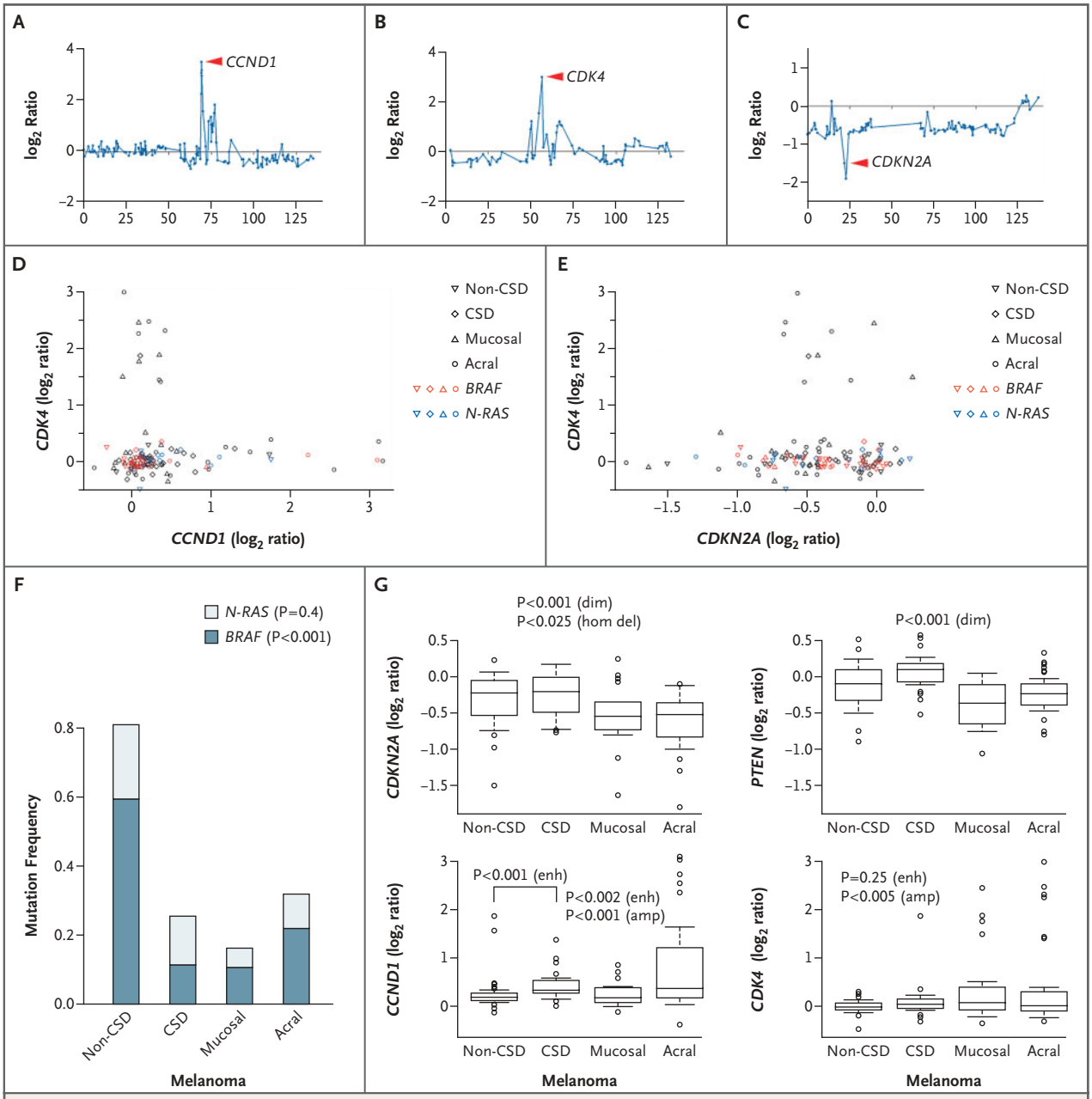



**Figure 4. Genetic Alterations in the Mitogen-Activated Protein Kinase and PI3K Pathways.**

Panels A, B, and C show examples of individual specimens with focal amplification of *CCND1* (Panel A) and *CDK4* (Panel B) and homozygous deletion of *CDKN2A* (Panel C). The y axis represents the mean $\log_2$ ratio for each clone normalized to the genome median $\log_2$ ratio. The x axis represents individual clones ordered with the use of data obtained from the University of California at Santa Cruz Genome Browser (http://genome.ucsc.edu, assembly of July 2003). Panels D and E show interactions between amplification of *CDK4* and *CCND1* and mutations in *BRAF* and *N-RAS* (Panel D) and homozygous deletions of *CDKN2A* and amplifications of *CDK4* (Panel E). The x and y axes show the $\log_2$ ratio for the clone representing the individual genes. Data points labeled in red or blue indicate cases with mutations in the *BRAF* or *N-RAS* genes, respectively. The average $\log_2$ ratio of three highly correlated clones (minimum Pearson's correlation, >0.95) containing the *CCND1* gene were used in Panels D and G. Panel F shows a significant difference in the mutation frequency of *BRAF* but not *N-RAS* among the four groups. Panel G illustrates significant differences in the numbers of copies of *CDKN2A*, *PTEN*, *CCND1,* and *CDK4* among the four types of melanoma. Boxes are outlined by the first and third quartiles, with the median as a horizontal line inside. Whiskers are drawn as 1.5 times the distance between the first and third quartiles. Points outside the whiskers are considered outliers. In Panel G, enh denotes an increased number of copies, dim a reduced number of copies, amp amplification, and hom del homozygous deletion. The y axis indicates the $\log_2$ ratio of the clone representative of the respective gene. CSD denotes chronic sun-induced damage, and non-CSD no chronic sun-induced damage.

chemical analysis (see the Supplementary Appendix). Mucosal and acral melanomas more commonly had losses of the *CDKN2A* locus than did the group of melanomas on skin with chronic sun-induced damage or the group on skin without chronic sun-induced damage ($P<0.001$ by the Kruskal–Wallis test). Homozygous deletions of *CDKN2A* occurred exclusively in samples without *CDK4* amplifications (Fig. 4E). This observation suggests that amplification of *CDK4* obviates the need for deletions of *CDKN2A*. Since the CDKN2A and CDK4 proteins bind each other in a 1:1 ratio,[33] the evolution of melanoma appears to be enhanced by a decrease in the ratio of CDKN2A to CDK4 that loosens checkpoint control for entry into the S phase. We did not note an influence of the tumor thickness on either the frequencies of mutations in *BRAF* or *N-RAS* or on the amplification frequencies of *CCND1* or *CDK4* in the four groups of melanomas.

### ALTERATIONS OF THE PHOSPHATIDYLINOSITOL 3′ KINASE PATHWAY

Several studies have implicated activation of the phosphatidylinositol 3′ kinase (PI3K) pathway as another crucial event in the progression of melanoma. To date, *PTEN*, a negative regulator of the pathway, appears to be the most common somatic target in melanoma, residing in a commonly deleted genomic area on chromosome 10q. In melanoma cell lines, mutations or deletions of *PTEN* occur together with mutations in *BRAF*, but not in *N-RAS*.[34] N-RAS activates both the PI3K pathway and the mitogen-activated protein kinase pathway, whereas BRAF seems to activate only the latter, suggesting that during progression, somatic events activating only one pathway require a second event to activate the other. Our data are consistent with this finding. Specimens with *BRAF* mutations had fewer copies of *PTEN* than specimens with *N-RAS* mutations ($P=0.02$ by the Kruskal–Wallis test). We confirmed that the PI3K pathway had been activated by evaluating 51 specimens with sufficient tissue for immunohistochemical analysis of the expression of phosphorylated Akt. In this subgroup of samples, all 8 specimens with *N-RAS* mutations had increased levels of expression of phosphorylated Akt, and 14 of 17 specimens (82 percent) with *PTEN* loss had increased levels of expression of phosphorylated Akt (see the Supplementary Appendix). The positive correlation between the loss of *PTEN* and mutations in *BRAF* supports the notion that the PI3K pathway is an independent somatic target that is frequently activated in primary melanoma.

## DISCUSSION

Our study shows distinct patterns of genetic alterations in the four groups of primary melanomas as we have defined them. There were differences in both chromosomal aberrations and the frequency of mutations of specific genes, suggesting that these tumors develop by different mechanistic routes in response to different selective influences. For example, acral and mucosal melanomas were uniquely characterized by a much higher frequency of focal amplifications and losses than the group of melanomas that were on skin with chronic sun-induced damage or the group on skin without chronic sun-induced damage, indicating that the first two are mechanistically more similar to each other than to the other types. However, as our data show, selection results in highly divergent sets of chromosomal aberrations in acral and mucosal tumors, findings that support their classification as distinct entities. Because glabrous and mucosal epithelia are morphologically distinct from each other and because solar exposure is unlikely to contribute to melanomagenesis in these sites, we believe that these two groups of melanomas differ primarily as the result of site-specific biologic characteristics. We also found clear genetic differences between the types of melanoma that commonly occur on areas exposed to the sun in light-skinned people. Melanomas on skin without chronic sun-induced damage had frequent mutations in *BRAF* and frequent losses of chromosome 10, whereas melanomas on skin with chronic sun-induced damage had infrequent mutations in *BRAF* and frequent increases in the number of copies of the *CCND1* gene.

Our classification of melanomas related to exposure to ultraviolet light is based on examination of our data and data from previous epidemiologic studies.[14] The previous studies found that various risk factors differed between melanomas on skin with chronic sun-induced damage and melanomas on skin without chronic sun-induced damage and suggested the existence of different developmental paths for these types of tumors. For example, melanomas presenting on sites with chronic exposure to the sun typically occur late in life and are associated with other ultraviolet-light–related neoplasms such as solar keratoses, suggesting that high cumulative doses of ultraviolet light are required for their development. In contrast, melanomas presenting on skin that is intermittently exposed to the sun are typically found in persons who have a larger number of moles but fewer solar keratoses and oc-

cur at a younger age. Moreover, these melanomas and the moles share an important genetic characteristic. In this and a previous study,[16] we have shown that the melanomas on skin that is intermittently exposed to the sun have much more frequent mutations of *BRAF* than do other types of melanoma, and moles have also been shown to have a high incidence of *BRAF* mutations.[35] Thus, we propose that the melanocytes of persons in whom melanomas develop on skin that is intermittently exposed to the sun have an increased susceptibility to ultraviolet exposure that involves a higher probability of acquiring *BRAF* mutations or of proliferating if such mutations occur. Epidemiologic studies and studies in animals indicate that for these people, there may be a window of vulnerability to exposure to ultraviolet light early in life.[2,36,37] These tumors should occur with site-specific incidences related to the patterns and timing of exposure to ultraviolet light and the relative amount of skin at risk. In contrast, patients without susceptible melanocytes would require a sufficiently high cumulative ultraviolet dose to induce melanoma, so that sun-damaged skin would be present and the development would involve mechanisms that do not include *BRAF* mutations. Thus, these persons would have tumors predominantly on sites that are chronically exposed to the sun, such as the face.

Although we have clearly established that there are genetic differences among these four groups of melanomas, our classification requires validation and refinement. Three important questions come immediately to mind. First, in distinguishing between acral and mucosal melanomas, we have emphasized differences in the affected site and morphologic differences in skin but not the limited exposure to ultraviolet light that acral sites may receive. Second, for sites subject to substantial exposure to ultraviolet light, we have emphasized the importance of histologic signs of sun-induced damage and have not considered the possibility proposed by Whiteman et al.[14] that there could be differences in susceptibility between melanocytes found on the trunk, arms, or legs and those found on the face. Third, we discounted the histologic tumor type because we found that tumors on acral sites have similar genetic aberrations regardless of whether they are histologically acral lentiginous or superficial spreading melanomas and that nodular melanomas clustered with the groups we defined on the basis of site and did not have the features of a separate entity. Future epidemiologic and genetic studies will be required to identify specific genetic or epigenetic factors associated with these variables.

Knowledge of the genetic differences among melanomas could be valuable in the design of therapeutic strategies. Our results lead us to make a prediction. The group of tumors on skin without chronic sun-induced damage, which represent the most common type of melanoma, frequently had a mutation in *BRAF* together with a loss of *PTEN* or mutations in *N-RAS* alone. Thus, they would be expected to be responsive to therapeutic interventions targeting the RAS–RAF–ERK and PI3K pathways. In contrast, the majority of melanomas in the other three groups did not have mutations in *BRAF* or *N-RAS* but instead had increased numbers of copies of the downstream gene *CCND1* or *CDK4*. Thus, these three groups of melanomas would be less likely to respond to therapeutic interventions that target upstream components of the mitogen-activated protein kinase pathway including BRAF, such as sorafenib.[38]

Our study provides genetic support for the existence of distinct molecular pathways to melanoma, each with a unique relationship to exposure to ultraviolet light. This finding should affect the design of future studies involving the treatment and prevention of melanoma and suggests the existence of as-yet-unidentified susceptibility factors.

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## REFERENCES

1. Marks R. Epidemiology of melanoma. Clin Exp Dermatol 2000;25:459-63.
2. Whiteman DC, Whiteman CA, Green AC. Childhood sun exposure as a risk factor for melanoma: a systematic review of epidemiologic studies. Cancer Causes Control 2001;12:69-82.
3. MacKie RM, Aitchison T. Severe sunburn and subsequent risk of primary cutaneous malignant-melanoma in Scotland. Br J Cancer 1982;46:955-60.
4. Beral V, Robinson N. The relationship of malignant melanoma, basal and squamous skin cancers to indoor and outdoor work. Br J Cancer 1981;44:886-91.
5. Vagero D, Ringback G, Kiviranta H. Melanoma and other tumors of the skin among office, other indoor and outdoor workers in Sweden 1961-1979. Br J Cancer 1986;53:507-12.
6. Albino AP, Nanus DM, Mentle IR, et al. Analysis of ras oncogenes in malignant melanoma and precursor lesions: correlation of point mutations with differentiation phenotype. Oncogene 1989;4:1363-74.
7. Davies H, Bignell GR, Cox C, et al. Mu-

tations of the BRAF gene in human cancer. Nature 2002;417:949-54.
**8.** Clark WH Jr, From L, Bernardino EA, Mihm MC. The histogenesis and biologic behavior of primary human malignant melanomas of the skin. Cancer Res 1969;29:705-27.
**9.** McGovern VJ, Mihm MC Jr, Bailly C, et al. Classification of malignant melanoma and its histologic reporting. Cancer 1973;32:1446-57.
**10.** Ackerman AB. Malignant melanoma: a unifying concept. Hum Pathol 1980;11:591-5.
**11.** Barnhill RL, Fine JA, Roush GC, Berwick M. Predicting five-year outcome for patients with cutaneous melanoma in a population-based study. Cancer 1996;78:427-32. [Erratum, Cancer 1997;79:423.]
**12.** Balch CM. Cutaneous melanoma: prognosis and treatment results worldwide. Semin Surg Oncol 1992;8:400-14.
**13.** Whiteman DC, Parsons PG, Green AC. p53 Expression and risk factors for cutaneous melanoma: a case-control study. Int J Cancer 1998;77:843-8.
**14.** Whiteman DC, Watt P, Purdie DM, Hughes MC, Hayward NK, Green AC. Melanocytic nevi, solar keratoses, and divergent pathways to cutaneous melanoma. J Natl Cancer Inst 2003;95:806-12.
**15.** Rivers JK. Is there more than one road to melanoma? Lancet 2004;363:728-30.
**16.** Maldonado JL, Fridlyand J, Patel H, et al. Determinants of BRAF mutations in primary melanomas. J Natl Cancer Inst 2003;95:1878-90.
**17.** Bastian BC, Kashani-Sabet M, Hamm H, et al. Gene amplifications characterize acral melanoma and permit the detection of occult tumor cells in the surrounding skin. Cancer Res 2000;60:1968-73.
**18.** Bastian BC, Olshen AB, LeBoit PE, Pinkel D. Classifying melanocytic tumors based on DNA copy number changes. Am J Pathol 2003;163:1765-70.
**19.** van Dijk M, Sprenger S, Rombout P, et al. Distinct chromosomal aberrations in sinonasal mucosal melanoma as detected by comparative genomic hybridization. Genes Chromosomes Cancer 2003;36:151-8.
**20.** Pinkel D, Segraves R, Sudar D, et al. High resolution analysis of DNA copy number variation using comparative genomic hybridization to microarrays. Nat Genet 1998;20:207-11.
**21.** Snijders AM, Nowak N, Segraves R, et al. Assembly of microarrays for genome-wide measurement of DNA copy number. Nat Genet 2001;29:263-4.
**22.** Bastian BC, LeBoit PE, Hamm H, Brocker EB, Pinkel D. Chromosomal gains and losses in primary cutaneous melanomas detected by comparative genomic hybridization. Cancer Res 1998;58:2170-5.
**23.** Snijders AM, Fridlyand J, Mans DA, et al. Shaping of tumor and drug-resistant genomes by instability and selection. Oncogene 2003;22:4370-9.
**24.** Bastian BC, LeBoit PE, Pinkel D. Mutations and copy number increase of HRAS in Spitz nevi with distinctive histopathological features. Am J Pathol 2000;157:967-72.
**25.** Sauter ER, Yeo UC, von Stemm A, et al. Cyclin D1 is a candidate oncogene in cutaneous melanoma. Cancer Res 2002;62:3200-6.
**26.** Fridlyand J, Snijders AM, Pinkel D, Albertson DG, Jain AN. Hidden Markov models approach to the analysis of array CGH data. J Multivariate Anal 2004;90:132-53.
**27.** Fridlyand J, Dimitrov P. Package: aCGH. (Accessed October 21, 2005, at http://www.bioconductor.org/repository/release1.5/package/html/aCGH.html.)
**28.** Cleveland WS. Lowess: a program for smoothing scatterplots by robust locally weighted regression. Am Stat 1981;35:54.
**29.** Westfall PH, Young SS. Resampling-based multiple testing: examples and methods for P-value adjustment. New York: John Wiley, 1993.
**30.** Dudoit S, Fridlyand J, Speed TP. Comparison of discrimination methods for the classification of tumors using gene expression data. J Am Stat Assoc 2002;97:77-87.
**31.** Ikaka R, Gentleman R. R, a language for data analysis and graphics. J Comput Gr Stat 1996;5:229-314.
**32.** Serrano M, Lee HW, Chin L, Cordon-Cardo C, Beach D, DePinho RA. Role of the INK4a locus in tumor suppression and cell mortality. Cell 1996;85:27-37.
**33.** Serrano M, Hannon GJ, Beach D. A new regulatory motif in cell-cycle control causing specific inhibition of cyclin D/CDK4. Nature 1993;366:704-7.
**34.** Tsao H, Zhang X, Fowlkes K, Haluska FG. Relative reciprocity of NRAS and PTEN/MMAC1 alterations in cutaneous melanoma cell lines. Cancer Res 2000;60:1800-4.
**35.** Pollock PM, Harper UL, Hansen KS, et al. High frequency of BRAF mutations in nevi. Nat Genet 2003;33:19-20.
**36.** Noonan FP, Recio JA, Takayama H, et al. Neonatal sunburn and melanoma in mice. Nature 2001;413:271-2.
**37.** Kelly JW, Rivers JK, MacLennan R, Harrison S, Lewis AE, Tate BJ. Sunlight: a major factor associated with the development of melanocytic nevi in Australian schoolchildren. J Am Acad Dermatol 1994;30:40-8.
**38.** Lyons JF, Wilhelm S, Hibner B, Bollag G. Discovery of a novel Raf kinase inhibitor. Endocr Relat Cancer 2001;8:219-25.


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