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Localization of Multiple Melanoma Tumor–Suppressor Genes on Chromosome 11 by Use of Homozygosity Mapping-of-Deletions Analysis

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Loss-of-heterozygosity (LOH) studies have implicated one or more chromosome 11 tumor-suppressor gene(s) in the development of cutaneous melanoma as well as a variety of other forms of human cancer. In the present study, we have identified multiple independent critical regions on this chromosome by use of homozygosity mapping of deletions (HOMOD) analysis. This method of analysis involved the use of highly polymorphic microsatellite markers and statistics to identify regions of hemizygous deletion in unmatched melanoma cell line DNAs. Regions of loss were defined by the presence of an extended region of homozygosity (ERH) at ⩾5 adjacent markers and having a statistical probability of ⩽.001. Significant ERHs were similar in nature to deletions identified by LOH analyses performed on uncultured melanomas, although a higher frequency of loss (24 [60%] of 40 vs. 16 [34%] of 47) was observed in the cell lines. Overall, six small regions of overlapping deletions (SROs) were identified on chromosome 11 flanked by the markers *D11S1338/D11S907* (11p13-15.5 [SRO1]), *D11S1344/D11S11385* (11p11.2 [SRO2]), *D11S917/D11S1886* (11q21-22.3 [SRO3]), *D11S927/D11S4094* (11q23 [SRO4]), *AFM210ve3/D11S990* (11q24 [SRO5]), and *D11S1351/D11S4123* (11q24-25 [SRO6]). We propose that HOMOD analysis can be used as an adjunct to LOH analysis in the localization of tumor-suppressor genes.

Introduction

Cytogenetic, molecular, and biological evidence all support the existence of a melanoma tumor–suppressor gene(s) on chromosome 11 (Fountain 1998). Deletions of this chromosome have been identified in 26%–58% of metastatic melanomas and are also associated with advanced tumor stage, younger age at presentation, poorer prognosis, and metastasis to the brain (Trent et al. 1990; Morse et al. 1992; Tomlinson et al. 1993, 1996; Herbst et al. 1995; Walker et al. 1995). Predominantly, large deletions of ⩾44 cM (Dib et al. 1996) have been identified on 11q22-25 (Herbst et al. 1995; Tomlinson et al. 1996), although additional loss-of-heterozygosity (LOH) findings suggest that another melanoma gene(s) may reside on 11p or proximal 11q (Tomlinson et al. 1996). Findings from suppression-of-tumorigenicity studies also support the existence of an 11q melanoma tumor–suppressor gene(s) (Robertson et al. 1996), and, recently, we have narrowed the location of this gene(s) through the characterization of melanoma hybrids containing fragments of 11q (Robertson et al. 1999). During the course of this effort, we identified a previously unsuspected region of deletion on 11q in a parental melanoma cell line (UACC 903) that was genotypically homozygous at all microsatellite loci ($N = 72$) distal to *D11S2000* on 11q22.3-23.1. This discovery influenced us to use our other unmatched melanoma cell line DNAs to further narrow regions of hemizygous deletion on chromosome 11.

Deletions or rearrangements of chromosome 11 have also been frequently detected in many other cancers, including those that originate in the breast (Hampton et al. 1994*a;* Gudmundsson et al. 1995; Negrini et al. 1995; Tomlinson et al. 1995; Winqvist et al. 1995), ovary (Foulkes et al. 1993; Davis et al. 1996; Gabra et al. 1996), cervix (Hampton et al. 1994*b;* Bethwaite et al. 1995), lung (Rasio et al. 1995*b;* Iizuka et al. 1995), kidney (Call et al. 1990), bladder (Shaw and Knowles, 1995), colon (Keldysh et al. 1993; Connolly et al. 1999), prostate (Dahiya et al. 1997; Kawana et al. 1997), nasopharynx (Hui et al. 1996), oral cavity (Uzawa et al. 1996), and endocrine-associated tissues (Lubensky et al. 1996; Tahara et al. 1996). Although several familial-predisposition loci, such as the genes for multiple endocrine neoplasia type 1 (*MEN1;* Chandrasekharappa et al. 1997) and ataxia telangiectasia (*ATM;* Savitsky

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et al. 1995), have been localized and identified on this chromosome, deletions detected in many sporadic cancers do not consistently or exclusively target these genes. Progress in narrowing the location of a novel chromosome 11 tumor-suppressor gene(s) has therefore primarily relied on extended LOH analyses (Negrini et al. 1995; Davis et al. 1996; Gabra et al. 1996; Koreth et al. 1997; Laake et al. 1997; Monaco et al. 1997; Wang and Evans 1997; Wang et al. 1998; Herbst et al. 1999). As a whole, these studies suggest that two or more "multiple" tumor-suppressor genes reside on 11q22-25.

To date, only one homozygous deletion has been identified (in a lung-cancer cell line) on 11q23 (Wang and Evans 1997). Given the total number of tumor DNAs and microsatellite markers screened, the frequency of homozygous deletions observed on this chromosome is extremely low. Although this factor has undoubtedly slowed the identification of an 11q tumor-suppressor gene, it may also provide a clue as to how the activity of this gene(s) is modulated during tumor development. In this regard, results from suppression-of-tumorigenicity studies performed on melanoma (UACC 903; Robertson et al. 1996, 1999) and cervical cancer (HeLa; Misra and Srivatsan 1989; Horikawa et al. 1995) both support the existence of a tumor-suppressor gene on 11q13-23 and suggest that this gene behaves in a dosage-dependent manner. It is possible, therefore, that a single "hit" or haploinsufficiency of a gene on 11q may be all that is required to provide an evolving tumor cell with a growth advantage. The 11q13-23 region is also the home of two maternally imprinted genes, *PGL1* and *PGL2,* which are predisposition loci for the benign head-and-neck-tumor syndrome known as "nonchromaffin paragangliomas" (Baysal et al. 1997*a*). Whether these two loci are targets during the development of any malignant cancers, including melanoma, remains to be seen. Like regions on 11p (e.g., the location of the *H19, IGF2,* and *CDKN1C* genes on 11p15.5), certain regions on 11q may also contain neighboring genes that are imprinted in the germline and contribute to carcinogenesis (Rainer et al. 1993; Biran et al. 1994; Matsouka et al. 1996; Reid et al. 1997). Inactivation of any one of these genes would also require only a single somatic event. The potential existence of a dosage-dependent or imprinted melanoma tumor–suppressor gene(s) on 11q further influenced us to develop a mapping strategy that did not rely on the identification of homozygous deletions in tumor cell lines.

In the present report, we describe the use of a method, which we have denoted as "homozygosity mapping of deletions" (HOMOD), to identify the location(s) of a tumor-suppressor gene(s) on chromosome 11. This method takes into account the probability that a hemizygous chromosomal deletion exists in an unmatched tumor cell line, on the basis of the number of homozygous genotypes observed at adjacent loci. Depending on the heterozygosity values of the markers used, a statistical probability of $\leq .001$ (generally, ≥ 5 homozygous genotypes at adjacent markers) was presumed to mark a deletion. Only one such significant extended region of homozygosity (ERH) was observed in constitutional DNAs analyzed on our patients with melanoma (1/47 individuals screened with 81 markers on chromosome 11; 3,261 independent genotypes evaluated). Overall, 40 melanoma cell lines and 124 microsatellite markers were used to define the locations of six small overlapping regions of deletion (SROs) on chromosome 11. On a gross level, the SROs defined in the cell lines accurately reflected those observed in LOH studies performed, in parallel, on melanoma and control (constitutional) DNAs from 47 patients, and, in one instance, a very small critical region (SRO2) was narrowed on 11p11.2 by identification of deletions in the cell lines that were subsequently confirmed in the tumors. Overall, our findings suggest that at least six melanoma tumor–suppressor genes reside on chromosome 11: two on 11p and four within the 11q22-25 region frequently deleted en masse in metastatic melanomas and other cancers.

Material and Methods

Melanoma Tumors and Cell Lines

The melanoma tumors and cell lines used in this study were obtained from either the Memorial Sloan-Kettering Cancer Center in New York, NY (designated as "SK-MEL-#"); the Queensland Institute of Medical Research in Brisbane, Australia (designated as "QIMR-#"); or the Massachusetts General Hospital in Boston, Massachusetts (designated as "MGH-MEL-#"). Approvals were obtained from the institutional review boards of these institutions. Lymphoblastoid cell lines or fresh lymphocytes were also paired with each tumor specimen and served as constitutional controls in LOH analyses. All but one of the tumors analyzed in this study were classified as sporadic melanomas. The exception, MGH-MEL-15, has been determined to carry a germline mutation (M53I) within the *CDKN2A* gene (referred to as "M3/6" in Pollock et al. [1998]). Genomic DNAs were isolated directly from fresh or frozen tissues/cells, by use of standard techniques (Ausubel et al. 1994).

Microsatellite-Marker Analyses

Primers for microsatellite-marker assays were purchased through Research Genetics or were synthesized (Gibco BRL) from information available in published reports (Vanagaite et al. 1995) or the Genome Database (GDB). Multiplex PCR reactions (10 μl) were performed on 25–200-ng DNA templates in the presence of 1.0 μCi $\alpha[^{32}P]$-dCTP, 1.5 mM $MgCl_2$, 20 pmol each primer (for

two markers), and 1 U *Taq* polymerase under the following conditions: initial denaturation at 95°C for 3 min; followed by 4 cycles at 94°C for 30 s, 60°C for 30 s, and 72°C for 30 s; then by 26 cycles at 94°C for 30 s, 56°C for 30 s, and 72°C for 30 s; and then by a final extension at 72°C for 5 min. Resultant products were heated to 95°C for 5 min with 10 μl formamide stop dye, were snap cooled on ice, and were then separated on 6% denaturing polyacrylamide gels (Sequagel; National Diagnostics) run at either 5 W overnight or 100 W for 4–6 h at room temperature. Gels were dried and exposed to film for 2–16 hours. Results from all markers were scored independently by two individuals (E.K.G. and J.W.F.), and critical or ambiguous findings were verified in repeat assays.

Results

Identification of Gross Critical Regions on Chromosome 11 in Melanoma Tumors and Cell Lines

Thirty markers were initially chosen to evaluate 47 matched melanoma/control DNAs for LOH on chromosome 11. These markers were concentrated in the 11q22-25 region previously determined to be frequently deleted in melanoma (Herbst et al. 1995; Tomlinson et al. 1996) and within regions that, at the time, were under investigation in our suppression-of-tumorigenicity studies (Robertson et al. 1999). The same markers were also used to screen 40 unrelated melanoma cell lines for ERHs. Sixteen (34%) of 47 melanomas and 20 (50%) of 40 melanoma cell lines were determined to harbor deletions that included one or more of these loci (fig. 1). Deletions in the cell lines were deduced on the basis of marker-heterozygosity values, with calculated probabilities of $\leq$.000032 (~3/100,000) that any set of adjacent homozygous genotypes (N = 7–30) would be observed in normal control DNAs. For example, the cell line SK-MEL-146, which had the smallest ERH, was homozygous at seven adjacent markers on 11p (fig. 1*C*); the probability of observing this number of homozygous genotypes by chance is .41 × .22 × .26 × .20 × .28 × .27 × .09, or 3.2×10^{-5}. By comparison, the available control DNAs analyzed from the 47 patients with melanoma were homozygous at $\leq$4 adjacent markers (calculated probabilities of $\geq$.00136). Additional ERHs that were disregarded in the cell lines also involved $\leq$4 markers and had probabilities similar to those observed in the control DNAs (data not shown).

Most deletions included, extended from, or resided within a region flanked by the markers *D11S2000* and *D11S969* on 11q22-25 (fig. 1*C*). Overall, our results —along with those of others (Herbst et al. 1995; Tomlinson et al. 1996)—suggest that a minimum of three melanoma tumor–suppressor genes exist on chromosome 11. Notably, one (or two) of these genes reside(s) on 11p, which was deleted in 10 (21%) of 47 of the melanoma tumors and in 12 (30%) of 40 of the melanoma cell lines. Overall, the regions targeted on this chromosome were similar in both the melanoma tumors and the cell lines, providing assurance that the deletions delineated in the cell lines were primarily reflective of in vivo events during tumor development.

Four additional factors influenced us to continue to use HOMOD in an effort to further narrow the critical regions on chromosome 11. First, we found that results on the cell lines were often less ambiguous than those generated on the tumors. While evidence of tumor heterogeneity (i.e., an inequality in signal intensities between alleles at a number of markers) was present in both sample sets, the existence of contaminating stromal DNAs had the potential to further confound genotype assignments in the tumors. Second, the higher frequency (1.5-fold) of deletion observed in the cell lines suggested that critical regions might be more rapidly narrowed in these samples. Third, the cell lines provided inexhaustible sources of DNA that could be screened with an unlimited number of microsatellite markers. Fourth, our LOH results for the matched tumor/control DNAs assured us that microsatellite instability (MSI; Boland et al. 1998) was an infrequent event in melanoma. MSI was noted at only 6 (0.5%) of 1,255 total genotypes and in only 4 (8.5%) of 47 melanoma tumors, and occurred only once at a homozygous locus within a region of deletion on chromosome 11 (data not shown). Thus, we expected that few of the ERHs defined in melanoma cell lines would be confounded by MSI.

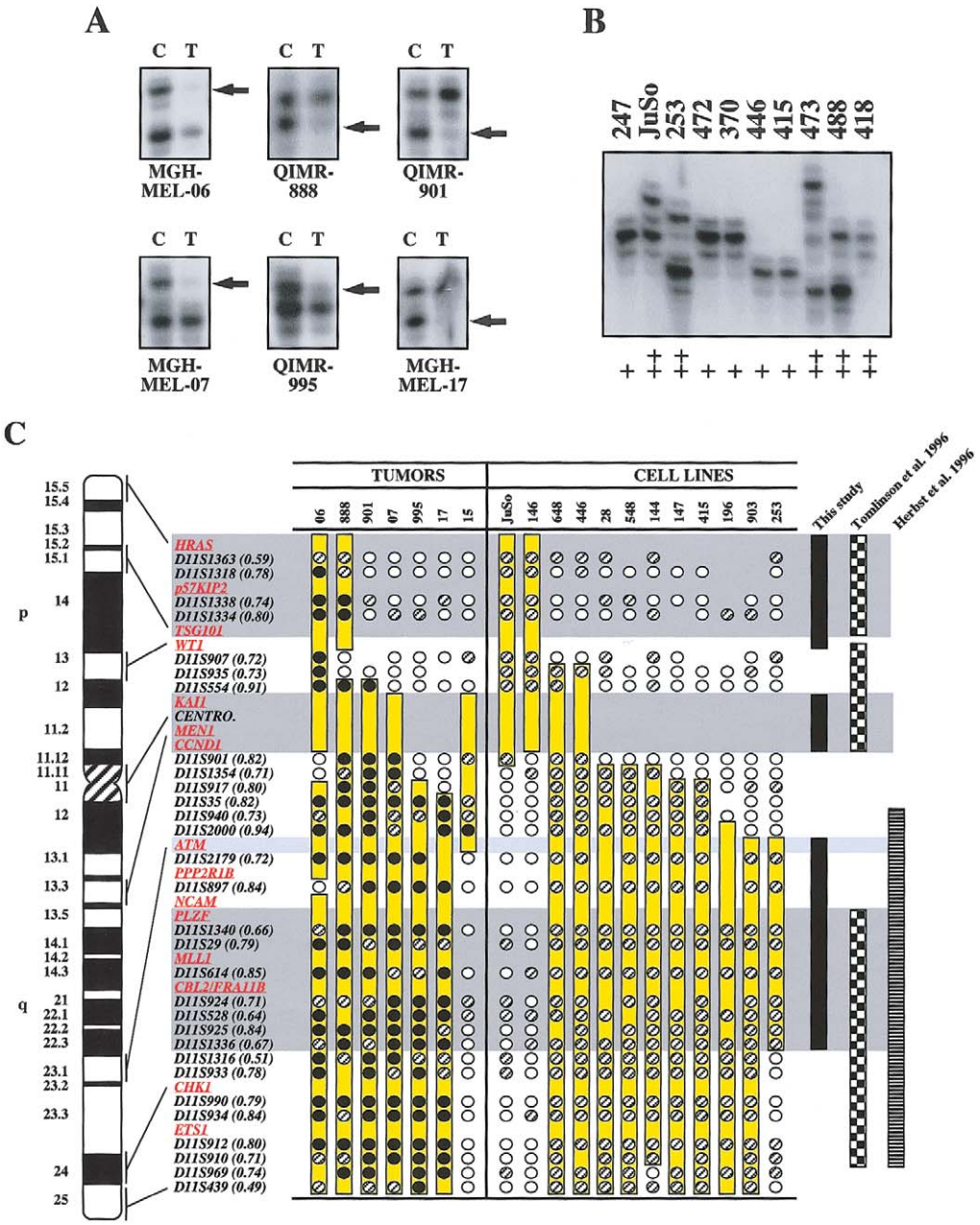
Heterozygosity Values of Markers

We next investigated how the use of heterozygosity values obtained from three different sources (Dib et al. [1996], GDB, and CEPH), versus those calculated on our mixed population of American and Australian patients, would affect the probability values used to establish ERHs in the melanoma cell lines. Overall, we found that (*a*) these values did not differ substantially (e.g., 1.9–2.7-fold for two of the smallest ERHs identified in the melanoma cell lines SK-MEL-146 and QIMR-253 [fig. 1*C*] or $\leq$1.7-fold for the largest ERH identified in the constitutional DNAs) when heterozygosity values from any source were compared and (*b*) heterozygosity values from the GDB were often closest to those obtained on our patients with melanoma. The latter observation led us to use GDB heterozygosity values in our extended HOMOD analyses whenever possible. These comparisons also enabled us to establish the maximum probability of a significant ERH as .001, given the range of values (.00117–.00200) calculated for the largest ERH observed in the control samples. The

value of .001 was also concordant with the (average) probability of observing an ERH that included ⩾5 markers (i.e., $[.25]^5$, or .00098).

Density and Relative Order of Markers

An additional 94 microsatellite markers (for a total of 124 markers) were used to screen the 40 melanoma cell lines for hemizygous deletions or ERHs on chromosome 11. Three of these 94 markers have not previously been described and were designed from high-throughput genomic sequence (htgs) available on two P1-derived artificial chromosomes (PACs; pDJ149k2 and pDJ360p17 [GenBank accession numbers AC001234 and AC001235, respectively]) located on 11q23.1-23.2 (table 1). Most of the markers used were concentrated on 11q and proximal 11p, specifically within the regions immediately flanking the centromere and between 11q22-25. Coverage was especially dense between *D11S2000* and *D11S969*, where markers ($N = 78$, noninclusive) were analyzed at average intervals of 310–380 kb (James et al. 1994; Arai et al. 1996; GDB). Marker order was established using physical-mapping information obtained from radiation hybrids as well as YACs, PACs, and cosmids (James et al. 1994; Vanagaite et al. 1995; Arai et al. 1996; Baysal et al. 1997*b*, 1998; GDB; Whitehead Institute for Biomedical



Research/MIT Center for Genome Research; E. K. Goldberg, J. Salcedo, J. Welch, N. K. Hayward, and J. W. Fountain, unpublished data). In certain instances (e.g., proximal to *D11S2000* and distal to *MLL1*) genetic-mapping information was also used to orient some loci (Dib et al. 1996). The location of the DNA-damage checkpoint–control gene, *CHK1* (Sanchez et al. 1997), relative to microsatellite markers on 11q24-25, was also determined through analyses performed on YACs (fig. 1*C*) (E. K. Goldberg, J. Salcedo, J. Welch, N. K. Hayward, and J. W. Fountain, unpublished data).

Identification of Two Critical Regions on 11p

In total, 27 markers were screened on 11p and the pericentromeric region of 11q, to determine the location of a melanoma tumor–suppressor gene(s) on 11p and/or proximal 11q. Markers were chosen primarily because of their proximity to the centromere and usefulness in distinguishing deletions on 11p from those on 11q. Genotypes were scored as heterozygous or homozygous at each locus in the 40 melanoma cell lines (fig. 2*A*). Overlaps between significant ERHs (probabilities of $\leq .001$, which were calculated primarily with GDB heterozygosity values) revealed two independent SROs on 11p (fig. 2*B* and 2*C*). A subset of markers from this region ($N = 18$) was also used to screen for LOH in the matched melanoma/control DNAs. Both SROs were supported by distinct deletions identified in the melanoma tumors (fig. 2*B* and 2*D*). Whereas SRO1 was estimated to span a large region of 14–16 Mb on 11p13-15.5 (GDB), SRO2 was presumed to span a region of only 200 kb on 11p11.2. The size of the latter SRO was based on the existence of a nonchimeric YAC clone (y962g6) in the CEPH YAC library that was positive for the contiguous markers *D11S436, D11S1344, D11S4174*, and *D11S1385* and only 200 kb in size (Whitehead Institute for Biomedical Research/MIT Center for Genome Research). A known metastasis-suppressor gene, *KAI1* (Dong et al. 1995)—as well as a putative liver tumor–suppressor gene (Coleman et al. 1997)—map within or close to this region.

Identification of Four Critical Regions on 11q

Ninety-seven additional markers (distal to *D11S901* on 11q14; fig. 2*B*) were also screened on the 40 melanoma cell lines to search for smaller ERHs on 11q. Results were compiled and are shown in figure 3. In total, three of the melanoma cell lines (SK-MEL-113, SK-MEL-133, and QIMR-383) originally presumed to be deleted for an entire copy of chromosome 11 were now known to harbor at least two distinct deletions: one (or two) on 11p and one on 11q (figs. 2*B* and 3). Four additional significant ERHs were also identified in the cell lines QIMR-472, QIMR-418, and SK-MEL-13 (fig. 3). These ERHs each spanned 7–10 adjacent loci and had probability values of $\leq 1.38 \times 10^{-4}$ (table 2). Overlaps between the ERHs, along with LOH data generated for the tumors (in the case of SRO3), served to define four distinct critical regions on 11q21-25 that ranged in size from ≤ 1 Mb (SRO5 and SRO6) to 12–14 Mb (SRO3 and SRO4). Notably, no homozygous deletions were identified in any of the 40 melanoma cell lines.

A subset of these 11q markers ($N = 55$) was also used to screen for LOH in the 47 melanoma tumors. Although no definitive deletions were identified, subtle differences between allele intensities were noted in five tumors with markers from within SRO4 (data not shown). As a whole, these tentative deletions centered on the marker *D11S897*. This marker was also scored as being retained in the melanoma SK-MEL-06, which was originally presumed to harbor three independent and extensive deletions on chromosome 11 (fig. 1*C*). Since this tumor sample has previously been determined to have levels of stromal contamination of ~50% (Gonzalgo et al. 1997; see fig. 1*A*), there is the potential that *D11S897* is ac-

Figure 1 Gross deletions on chromosome 11 in melanoma tumors and cell lines. *A,* LOH examples at the locus *D11S35* on 11q22.2. Constitutional (*C*) and matched tumor (*T*) results are shown for six individuals. Tumor designations are shown below each panel, and deleted alleles are indicated (*arrows*). *B,* Microsatellite-marker analysis for 10 melanoma cell line DNAs at *D11S35*. Heterozygous (denoted by two plus signs [++]) and homozygous (denoted by a single plus sign [+]) genotypes are indicated. Abbreviated cell-line designations are provided at the top of the panel. *C,* Subchromosomal deletions identified on chromosome 11 in 47 melanoma tumors and 40 melanoma cell lines screened with a panel of 30 microsatellite markers. Tumors ($N = 7$) or cell lines ($N = 8$) presumed to have lost an entire copy of chromosome 11 (or harboring an ill-defined critical region resulting from tumor heterogeneity or a high level of normal DNA contamination [$N = 2$ tumors]) are not shown. An ideogram of chromosome 11 is provided (*right*) with a list of markers and genes (*underlined* [*red*]). The relative order of markers/genes is shown from 11pter-qter, and the approximate cytogenetic location of certain genes/markers is indicated (James et al. 1994; Rasio et al. 1995*a;* Vanagaite et al. 1995; Chandrasekharappa et al. 1997; Kawana et al. 1997; Li et al. 1997; Reid et al. 1997; Wang et al. 1998). Heterozygosity values are provided in parentheses (GDB; Vanagaite et al. 1995; Dib et al. 1996). Abbreviated tumor or cell-line designations are listed at the top. Deletions in each tumor/cell line are boxed in yellow. Unblackened circles denote a heterozygous genotype; blackened circles, LOH; diagonally striped circles, a homozygous genotype; and absence of a circle, an inconclusive or undetermined result. Three overlapping regions of deletions (*black vertical bars*) were identified in this study and were compared with regions defined in two previous LOH studies performed on melanoma (*checkered bars* [Tomlinson et al. 1996]; *horizontally striped bar* [Herbst et al. 1995]). The existence of at least three critical regions—pter to *D11S1324* (proximal boundary determined by Tomlinson et al. [1996]), *D11S554–D11S901*, and *NCAM–D11S1316* (*dark gray–shaded boxes*)—is supported by two or all three of these independent studies; a fourth region, *D11S2000–D11S2179* (*light gray–shaded box*), may also exist near the *ATM* gene.

Table 1

Microsatellite Markers Developed from DNA Sequence Available on PACs pDJ149k2 and pDJ360p17

Marker	Repeat	Primer Sequence	Size[a] (bp)	Heterozygosity[b]	No. (Frequencies) of Alleles[c]
ZK96.9	$(CA)_{21}$	Forward: 5′-CTGTCAGTGAAATGGCATGG-3′ Reverse: 5′-GCAAACACCTTCCATCACCT-3′	213	.74 (*N* = 38)	9 (.014, .014, .014, .200, .129, .100, .443, .057, and .029)
ZK119	$(CA)_{12}$	Forward: 5′-ATCCAGCCAAGTTATCTCGC-3′ Reverse: 5′-GAACCTGTTGTGCATGTTGG-3′	218	.55 (*N* = 22)	7 (.023, .681, .023, .136, .023, .090, and .023)
ZK21.2	$(CA)_{20}$	Forward: 5′-AACTGAAGAGGTGGGTTGGA-3′ Reverse: 5′-TGCTCTGGAATGAAAGCTCA-3′	216	.87 (*N* = 38)	5 (.105, .237, .395, .066, and .197)

NOTE.—Sequence contigs on pDJ149k2 (179 kb) and pDJ360p17 (169 kb) were obtained from the htgs database (GenBank accession numbers AC001234 and AC001235, respectively). Contigs were screened for di-, tri-, and tetranucleotide repeat units ⩾7. A total of nine such sequences were identified at locations 20401, 21661, 96961, and 119881 in pDJ149k2 and at locations 15601, 21241, 40081, 149461, and 155461 in pDJ360p17. Primers were designed to amplify all nine repeats (Primer3 program; Whitehead Institute for Biomedical Research/MIT Center for Genome Research) and were tested on chromosome 11 YACs and hybrid DNAs (Robertson et al. 1999) to assure their location. Products amplified from the 20401 and 15601 repeats were not specific for loci on chromosome 11. Of the remaining candidate loci, only primers designed for the 96961 (*ZK96.9*), 119881(*ZK119*), and 21241 (*ZK21.2*) repeats produced reliable and easily scored genotypes.

[a] Size of fragment amplified from either pDJ149k2 or pDJ360p17.

[b] Number of independent control genotypes assessed (*N*) is indicated in parentheses.

[c] Number of alleles identified in control lymphocyte/lymphoblastoid DNAs; order of frequencies is presented from smallest to largest allele.

tually homozygously deleted, rather than retained, in SK-MEL-06. Evidence of overlapping deletions within the other SROs on 11q was less convincing (data not shown).

HOMOD Probabilities with Increasing Marker Density

By the end of our analyses on chromosome 11, the 47 matched melanoma/control DNAs had been screened with 81 independent microsatellite markers. Of these markers, 73 were analyzed in common on the 40 melanoma cell line DNAs, whereas the remaining 8 markers (*D11S922, D11S860, D11S861, D11S1883, D11S1889, D11S1365, D11S1345,* and *D11S1328*) were scored exclusively on the tumors. Results obtained on the constitutional DNAs from patients with melanoma provided us with the opportunity to reassess the probability of an ERH, given a significant increase in marker density (from 30 to 81 markers). While the markers were now separated by average distances of ~1.7 Mb, in certain regions (e.g., between *D11S2000* and *D11S1316;* fig. 1*C*), they were more closely spaced at intervals of 580–650 kb (James et al. 1994; GDB). Overall, a total of 3,261 independent genotypes were scored and, on average, 40 genotypes were evaluated at each locus. Although the number of ERHs observed increased with increasing marker density, the probability of a constitutional ERH of ⩾5 markers remained remarkably consistent with what was previously observed using the original 30 markers (i.e., ⩾.00135 vs. ⩾.00136). One exception, however, was identified in an individual (matched tumor/control pair MGH-MEL-13) whose constitutional (lymphocyte-derived) DNA was homozygous at seven adjacent loci, spanning a distance of ~2 Mb (GDB) between markers *D11S1336* and *D11S2090*. This ERH overlapped an ERH identified in an unrelated melanoma cell line (SK-MEL-13) and is located between SRO4 and SRO5 on 11q23-24 (fig. 3).

MSI

The frequency of MSI in the 47 matched melanoma/control DNAs was also reevaluated after analysis with 81 markers. A total of 25 cases of MSI were identified (data not shown). This frequency of MSI (25 [.83%] of 3,011 tumor genotypes evaluated) was, again, consistent with the frequency of MSI (.5%) observed with the original 30 markers. Of a total of 47 tumors, in only one (QIMR-976) was MSI identified at multiple loci (*N* = 9); the remaining examples (*N* = 16) were unique to each tumor and were almost exclusive for each locus. MSI was detected twice, at only 4 (*D11S1354, D11S1787, D11S1792,* and *D11S2090/AFM210ve3*) of the 81 loci. Of the 25 cases of MSI, 16 were identified at heterozygous loci (2 of which were also scored as reduced), whereas the remaining 9 cases were homozygous. Almost all of the cases of MSI (24 [96%] of 25) involved the addition or deletion of one (19 [76%] of 25) or two (5 [20%] of 25) dinucleotide repeat(s). These findings, as well as the lack of evidence of MSI in cell lines known to be extensively deleted on either 11p or 11q, further assured us that the genotype assessments made on the melanoma cell lines were accurate.

Discussion

In the present report, we have refined a molecular approach for the localization of novel tumor-suppressor genes on the basis of the identification of *hemizygous*

Table 2

HOMOD Probabilities for ERHs on 11q

	HOMOD Probabilities for			
Cell Line	SRO3	SRO4	SRO5	SRO6
QIMR-253	…	9.72×10^{-24} [a]	…	…
QIMR-472	*.00112*	…	…	3.38×10^{-6}
QIMR-418	…	…	3.24×10^{-6} [b]	
SK-MEL-13	2.11×10^{-6}	…	1.38×10^{-4} [a]	…

Note.—Probabilities were calculated using heterozygosity values provided in figure 3. Only ERHs with significant (≤.001) or borderline (*italics*) probabilities are shown.

[a] The findings from the present study—as well as those from GDB and GenBank database searches—indicated that the *D11S4090/NCAM* (within SRO4) and *D11S2090/AFM210ve3* (just proximal to SRO5) primers recognize the same locus or are in strong linkage disequilibrium with each other. Each of these marker sets was considered as one locus in marker counts and probability calculations.

[b] This ERH extends through (and serves to define) both SRO5 and SRO6.

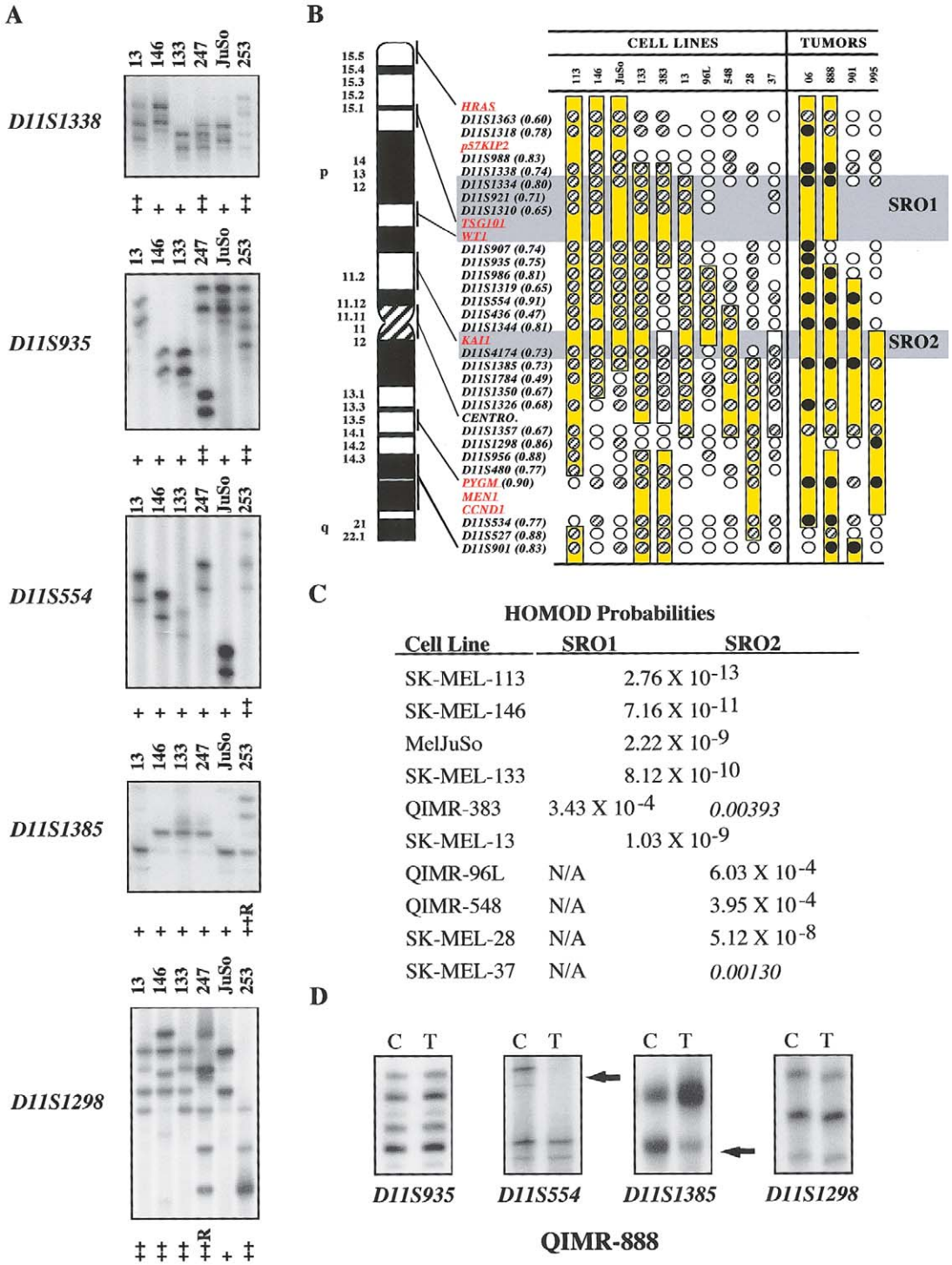
deletions in unmatched tumor cell lines by use of highly polymorphic microsatellite markers and statistics. We have designated this approach as "homozygosity mapping of deletions," or "HOMOD," because it is similar in concept to the homozygosity-mapping methods used to identify recessive disease genes in isolated populations (Sheffield et al. 1995). This approach further extends the usefulness of tumor cell lines in gene-cloning projects, by allowing them to serve as critical reagents in both hemizygous—as well as homozygous—deletion screens. Since these projects routinely involve efforts to identify homozygous deletions in tumor cell lines, findings akin to those described in the present study may exist in many laboratories but may remain largely unscrutinized. While the screening of tumor cell lines for confined hemizygous deletions with microsatellite markers has been performed retrospectively—or after the identification of a critical tumor-suppressor gene (Liu et al. 1995; Castellano et al. 1997)—we believe this is the first time it has been used to assist in the mapping of a novel tumor-suppressor gene(s).

We applied HOMOD analysis to a region of the genome—namely, chromosome 11—that is known to be deleted in many human cancers, including melanoma. Identification of the location of a melanoma tumor–suppressor gene on chromosome 11 has been especially challenging, given the large size of most deletions and the existence of at least three targets (Herbst et al. 1995; Tomlinson et al. 1996). Conclusively, defining a critical region on this chromosome (especially within 11q22-25) has been historically difficult in other cancers as well, presumably because of these same or similar complications. In such instances, many markers may need to be analyzed to identify or narrow the location of a critical gene. This would potentially preclude the use of microdissected tumor DNAs, which are often limited in quantity. In addition, during the course of the present study, we discovered evidence of tumor heterogeneity on chromosome 11 in both our uncultured melanoma tumors and cell lines (E.K.G. and J.W.F., unpublished data). Heterogeneity, which was readily identified via dosage differences between marker alleles, was observed in more than one-fourth of the cell lines that were scored as being largely retained on chromosome 11. In another unrelated melanoma cell line analyzed along with a matching control, we have also identified a clonal (present in all cells) deletion on 11q as well as a nonclonal (heterogeneous) deletion on 11p (E.K.G. and J.W.F., unpublished data). Thus, there is evidence that heterogeneous populations of tumor cells may coexist in culture. This factor has the potential to even further complicate the localization of tumor-suppressor genes, especially in uncultured tumor specimens in which both tumor heterogeneity and stromal contamination may be present.

Tumor cell lines have been shown, in several contexts, to appropriately mimic their uncultured counterparts (Forozan et al. 1997; Wistuba et al. 1999), and they have been especially critical reagents for the localization and identification of tumor-suppressor genes (Kamb et al. 1994; Steck et al. 1997). They are also useful in biological and biochemical analyses designed to verify or elucidate the function of a suppressor gene once it has been identified (Morin et al. 1997; Robertson et al. 1998; Walker et al. 1999). Knowing what genetic alterations exist within these cell lines has, most recently, made them especially powerful reagents for discriminating between the benefits of anticancer agents (Kohn 1996; Heise et al. 1997). The fact that certain genetic alterations occur at a higher frequency in tumor cell lines (e.g., those involving chromosome 9, as reviewed in Walker et al. [1998]) than in uncultured tumors has also contributed to the more rapid identification of particular tumor-suppressor genes, such as the cyclin-dependent kinase inhibitor 2A (*CDKN2A*) gene (Kamb et

al. 1994). Even given the caveat that not all alterations observed in tumor cell lines may have arisen in vivo (or even if they did, may not target a critical gene), knowledge of the detailed genetic characteristics of these cell lines could be of potential value to ongoing and future studies aimed at the testing of new therapeutics.

Overall, HOMOD analysis enabled us to identify six critical regions on chromosome 11, through the analysis of 40 melanoma cell lines with 124 microsatellite markers. As in our previous *CDKN2A* analyses performed on chromosome 9 (Flores et al. 1996), we discovered that deletions on chromosome 11 occurred at a higher frequency (or were more readily detectable) in melanoma cell lines than in uncultured melanomas. Specif-



ically, ERHs or deletions were confidently identified in 24 (60%) of 40 of our melanoma cell lines, compared with only 16 (34%) of 47 of the tumors. Overlaps between ERHs were then primarily used to establish the boundaries of six SROs (SRO1–SRO6) on chromosome 11. Three of these SROs were ≤1 Mb and suggested that HOMOD analysis, like LOH analysis, could be successful in precisely pinpointing the location of a tumor-suppressor gene. A complete absence of homozygous deletions in these cell lines adds credence to the possibility that a critical gene(s) on this chromosome behaves in a dosage-dependent manner, as implied by the results of previous functional studies (Misra and Srivatsan 1989; Horikawa et al. 1995; Robertson et al. 1996, 1999).

The critical regions or SROs identified in the current study are also supported by findings from two other independent LOH studies of melanoma (Tomlinson et al. 1996; Herbst et al. 1999). In the most recent study by Herbst et al. (1999), two critical regions were put forward as primary targets on 11q23; both are contained in their entireties within SRO4. One of these regions centers on the marker *D11S925,* whereas the other maps between *D11S1347* and *D11S4142*. The results of the second (more limited and older) LOH study performed on melanoma are also broadly supportive of these assignments and specifically implicate the region between *NCAM* and *D11S29* (in SRO4) as being the most-likely location(s) of a melanoma tumor–suppressor gene(s) on 11q (Tomlinson et al. 1996). Our own functional (i.e., suppression-of-tumorigenicity) data also support the localization of a tumor-suppressor gene within SRO4 between markers *D11S1786* and *D11S2077* (Robertson et al. 1999). This same confined region overlaps a region that has been independently identified, via both molecular and biological means, in lung cancer (Iizuka et al. 1995; Murakami et al. 1998; Wang et al. 1999).

Recently, an attractive candidate tumor-suppressor gene—the serine/threonine protein phosphatase subunit locus (*PPP2R1B*)—has been localized on 11q23 and implicated in lung and colon cancer (Wang et al. 1998). Although this gene maps on the proximal edge of SRO4, the upper boundary defined by Herbst et al. (1999) for the location of a critical gene within this region (i.e., *D11S1347*) potentially excludes *PPP2R1B* as a primary target in melanoma. Similarly, the recently identified *PGL1* locus, which encodes the succinate-ubiquinone oxidoreductase subunit D (SDHD) protein, also resides proximal to *D11S1347,* diminishing its attractiveness as a candidate gene for melanoma (Baysal et al. 2000).

The down-regulation of three known genes located within SRO4—namely, the neural-cell adhesion molecule (*NCAM*), the promyelocytic leukemia zinc finger (*PLZF*), and the myeloid/acute lymphoblastic leukemia (*MLL1*) genes—may also contribute to the development of melanoma and/or a number of the other cancers exhibiting frequent loss on 11q23. There is already evi-

Figure 2 Two distinct SROs defined on 11p by use of HOMOD analysis. *A,* Examples of microsatellite-marker results on 11p and proximal 11q. Abbreviated cell-line designations are provided at the top of each panel for six melanoma cell lines. Heterozygous (denoted by two plus signs [++]) or homozygous (denoted by a single plus sign [+]) genotypes are indicated under each panel. For *D11S1385* and *D11S1298,* examples of microsatellite instability (++R [where R denotes replication error]) are shown on the cell lines QIMR-253 and SK-MEL-247, respectively. *B,* Terminal and interstitial ERHs and regions of LOH defined on 11p in the melanoma cell lines and confirmed in the melanoma/control tumor pairs. Only cell lines with significant ERHs (probabilities ≤.001) or tumors with confined deletions are shown. An ideogram of 11pter-11q22 is provided on the left, along with a list of 27 markers and genes (*underlined* [*red*]); the cytogenetic location of several of these loci is indicated on 11p or 11q. The relative order of the markers was determined using the radiation-hybrid map presented by James et al. (1994) and other physical maps (from GDB and the Whitehead Institute for Biomedical Research/MIT Center for Genome Research) as well as information provided by Kawana et al. (1997) and Coleman et al. (1997) for the placement of markers around the *KAI1* locus. Heterozygosity values for the markers are provided in parentheses, and, with the exception of *D11S1784,* were obtained from either the CEPH database (*D11S1385* only), Research Genetics (*D11S436* only), or GDB (all others). Although the heterozygosity value for *D11S1784* is reported as 0.20 in Dib et al. (1996) and as 0.26 by CEPH, we found this marker to be significantly more polymorphic in our samples. A heterozygosity value of 0.49 (18 of 37) was calculated from control genotypes on our patients with melanoma and is the value we used for determination of the probability of an ERH. Cell-line and tumor designations are provided at the top, and significant ERHs or regions of LOH are boxed in yellow. Other insignificant (in QIMR-383) or borderline (in SK-MEL-37) ERHs identified in the cell lines are indicated by unblackened boxes. Unblackened circles denote a heterozygous genotype; blackened circles, LOH; diagonally striped circles, a homozygous genotype; and absence of a circle, an inconclusive or undetermined result. Two SROs (SRO1 and SRO2 [*gray boxed regions*]) on 11p were defined by overlapping ERHs or regions of LOH in the cell lines and tumors. The putative gene in SRO2 is assumed to span the locus *D11S4174* as a result of the existence of nonoverlapping ERHs in the cell lines QIMR-96L and SK-MEL-28. *C,* Probability values calculated on the 11p ERHs identified in the 10 melanoma cell lines shown in *B*. Heterozygosity values were converted to homozygosity values and were used to determine the probability of each ERH. For example, the smallest significant ERH was identified in QIMR-96L and had a probability of 6.03×10^{-4} (or $.19 \times .35 \times .09 \times .53 \times .19$). As expected, no significant ERHs that included <5 adjacent loci were identified. Even though the ERHs defined in SRO2 in QIMR-383 and SK-MEL-37 spanned five or six loci, respectively, their probability values (*italics*) were not deemed significant. *D,* Noncontiguous deletion on 11p, defined by LOH analysis in the matched control (*C*) and tumor (*T*) pair on patient QIMR-888. In the melanoma obtained from this patient, alleles were deleted (*arrows*) at loci *D11S554* and *D11S1385* but were retained at flanking markers *D11S935* and *D11S1298*. The region of overlapping deletions identified in the tumors (8–11 Mb from *D11S1344* to *D11S1298*; GDB) included SRO2 and served to support the findings from the melanoma cell lines.

dence that disruption or inactivation of these genes positively influences cell growth or, in the case of *NCAM*, invasion and metastasis (Edvardsen et al. 1994; Arakawa et al. 1998; Owens et al. 1998; Shaknovich et al. 1998; Croce 1999; Perl et al. 1999). Therefore, it is possible that loss of this region is associated with the down-regulation or haploinsufficiency of a number of growth inhibitory genes, rather than being ultimately directed at the inactivation of a single target.

Aside from SRO4, which remains a relatively large (14-Mb) region, findings from the present study enabled us to narrow the locations of four other chromosome 11 tumor-suppressor genes implicated in melanoma. This included the refinement of three 11q critical regions recently identified by Herbst et al. (1999), as well as an 11p region originally defined by Tomlinson et al. (1996). The most proximal target on 11q (SRO3) is now limited to a region of 7–8 Mb between 11q22.2-22.3 (GDB), flanked by the markers *D11S35* and *D11S1886*. We have also provided preliminary evidence for the narrowing of two more distal targets on 11q24-25 to regions of ≤1 Mb. It is of note that these two regions (SRO5 and SRO6) each contain an attractive candidate gene (*CHK1* and *ETS1*, respectively). While *CHK1* encodes a protein kinase that controls transition from the G_2 to M phase of the cell cycle (Sanchez et al. 1997), expression of *ETS1* has recently been shown to inhibit

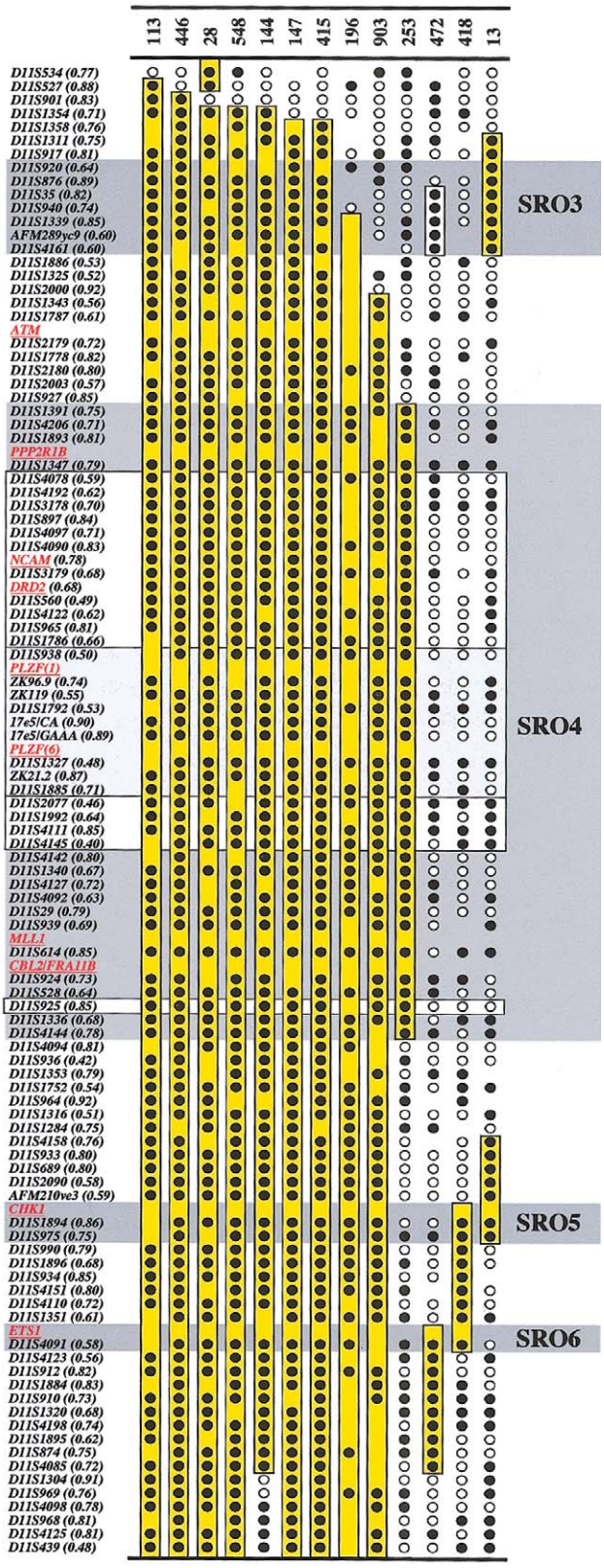


Figure 3 Four distinct SROs defined on 11q13-qter by use of HOMOD analysis. Only melanoma cell lines (*top*) with significant ERHs and breakpoints in this region are shown. Markers ($N = 102$) and genes (*underlined* [*red*]) are listed in order (*left*). In the case of the *PLZF* gene, the locations of the first (*PLZF*[*1*]) and last (*PLZF*[*6*]) exons of this gene are indicated. Heterozygosity values for polymorphic loci are provided in parentheses and come from GDB, with the following exceptions: (1) values for *D11S2000, D11S1787, D11S1778, D11S1391, D11S965*, and *D11S1992* are from the CEPH database; (2) values for *D11S2179, D11S2180*, and *D11S2003* are from Vanagaite et al. (1995); (3) values for *D11S1786, D11S1792, D11S1752*, and *D11S968* are from Dib et al. (1996); (4) values for *17e5/CA* and *17e5/GAAA* are from Baysal et al. (1997*b*); and (5) values for *AFM289yc9, ZK96.9, ZK119, ZK21.2, D11S2077, D11S964, D11S2090, AFM210ve3, D11S1894, D11S1884*, and *D11S439* were calculated, in the present study, from melanoma patient control genotypes. Unblackened circles denote a heterozygous genotype; blackened circles, a homozygous genotype; and absence of a circle, an inconclusive or undetermined result. Significant ERHs (probabilities ≤.001) are boxed in yellow, whereas a borderline ERH (probability .00112) in the cell line QIMR-472 is indicated by an unblackened box. Four SROs (SRO3–SRO6) are shaded in gray. The proximal boundary of SRO3 was established by LOH studies performed on melanoma tumors (figure 1). Two critical regions identified on 11q23 in an independent LOH study performed on melanoma (from *D11S1347–D11S4142* and *D11S528–D11S1345* [a marker just proximal to *D11S1336*]) are indicated by unblackened boxes within SRO4 (Herbst et al. 1999). A region that we recently found to be associated with suppression of tumor formation in the melanoma cell line UACC 903 (abbreviated as "903"; *D11S1786–D11S2077*) is also indicated as a light gray–shaded area within SRO4 (Robertson et al. 1999).

tumor growth as well as induce apoptosis in colon-cancer cells (Huang et al. 1997; Li et al. 1999). Finally, our HOMOD analyses have been successful in precisely pinpointing the location of a fourth tumor-suppressor gene on 11p11.2. This last critical region (SRO2) is supported by 11 independent ERHs/deletions that jointly overlap a common region of ⩽200 kb. The discovery that one of these ERHs exists within a melanoma cell line (MelJuSo; Miele et al. 1996) routinely used in suppression-of-tumorigenicity/metastasis analyses provides us with the immediate opportunity to assess the biological roles of two potential candidate genes within this region—*KAI1* (Dong et al. 1995) and an unrelated liver tumor–suppressor gene (Coleman et al. 1997)—in melanoma tumor growth, invasion, and metastasis.

Although seemingly unrelated to cancer development, our study is also supportive of recent discoveries reported by Broman and Weber (1999). These investigators have determined that large ERHs can exist within the constitutions of both consanguineous and outbred individuals. Through genetic modeling and analyses performed on six CEPH families, their results suggested that up to 20% of all outbred individuals within a population may harbor at least one significant ERH within their genomes. The general design of their study as well as the conclusions drawn from their efforts, including the density of markers screened (one per ~0.5 cM vs. 580–650 kb, respectively) and the size of a significant ERH (⩾5–6 adjacent markers), are remarkably similar to ours. Even though the manner in which significance was calculated differed between the two studies (LOD scores versus probabilities determined via the multiplication of marker-homozygosity values), both revealed that 2%–3% of outbred individuals were constitutionally homozygous for regions spanning 7–9 adjacent markers on chromosome 11 (see the supplemental table in Broman and Weber [1999]). The single significant (constitutional) ERH identified on 11q23.3-24 in the 47 unrelated individuals analyzed in our study had a probability of 4.9×10^{-5}, which differed by ⩾27-fold from that of all other control ERHs (defined by ⩽5 markers with probabilities ⩾.00135) observed in our sample set. Importantly, there was no overwhelming evidence that this particular individual was a product of consanguinity, since he/she was constitutionally heterozygous at 50 (.74) of 68 genotypes evaluated elsewhere on chromosome 11, where the average heterozygosity value for the 81 analyzed markers was 0.73 (Vanagaite et al. 1995; Dib et al. 1996; Baysal et al. 1997*b;* GDB; CEPH; the present study). By use of the methodology of Broman and Weber (along with allele frequencies from CEPH or those calculated from our patient control DNAs; data not shown), this constitutional ERH has now been determined to have a LOD score of 4.45–5.09. This LOD score reaches the minimum significance level established by Broman and Weber (1999), where the smallest ERH had a LOD score of 4.70 and extended across six markers. If our results were extrapolated across the entire genome (of which chromosome 11 represents ~5%), then one might expect to detect a significant ERH in ~40% of individuals within an outbred population. The relationship of these ERHs to disease (e.g., cancer) risk currently remains unknown. Likewise, the molecular origins of these ERHs are not certain and could be attributable to either consanguinity, confined uniparental isodisomy (Martin et al. 1999), or germline deletion. The fact that our constitutional ERH (*D11S1336–D11S2090*) spans a recently identified fragile site on 11q23.3-24 is potentially supportive of either of the latter two possibilities (Tunnacliffe et al. 1999). This region of the genome may be more prone than others to breakage and/or rearrangement without any obvious phenotypic consequence.

Even though the frequency of homozygous segments within human genomes may be higher than was previously suspected, it still does not reach a level of significance that should limit the success of HOMOD. Predictably, the power of HOMOD will also increase with time, as genomic maps become denser and there is less ambiguity in marker order. HOMOD screens could ultimately be performed on DNA microarrays containing single-nucleotide polymorphism (SNP) variants (Lander 1999), as long as a dense set of markers existed that showed minimal evidence of linkage disequilibrium. By screening tumor cell line DNAs with a significant number of available markers, one should be able to rapidly define the locations of most tumor-suppressor genes frequently targeted in human cancers. Once defined, it then becomes a routine exercise to determine (via mutation/methylation/expression studies) the candidacy of genes located within each small region. The true target could then be functionally verified in a cell line already known to be devoid or haploinsufficient for its product. Ultimately, computer databases that would contain comprehensive (total-genome) information on particular cell lines may evolve from such analyses. As our understanding of human cancer continues to increase, these databases could provide us with valuable clues as to the effectiveness of certain therapeutics, given the combination of genetic (and epigenetic) aberrations present in a tumor cell.

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Electronic-Database Information

Accession numbers and URLs for data in this article are as follows:

CEPH, http://www.cephb.fr/
GenBank, http://www.ncbi.nlm.nih.gov/Genbank/index.html (for sequences for PAC clones pDJ149k2 [accession number AC001234] and pDJ360p17 [accession number AC001235])
Genome Database (GDB), http://www.gdb.org/
Research Genetics, Inc., http://www.resgen.com/
Whitehead Institute for Biomedical Research/MIT Center for Genome Research, http://www.genome.wi.mit.edu/

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