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Homozygous deletions within human chromosome band 9p21 in melanoma

(cutaneous malignant melanoma/loss of heterozygosity/chromosome 9 rearrangements/α- and β-interferon genes/tumor-suppressor genes)

JANE W. FOUNTAIN*†‡, MARIA KARAYIORGOU*†, MARC S. ERNSTOFF§, JOHN M. KIRKWOOD¶, DANIEL R. VLOCK¶, LINDA TITUS-ERNSTOFF§, BRIGITTE BOUCHARD‖, SETALURI VIJAYASARADHI‖, ALAN N. HOUGHTON‖, JILL LAHTI**, VINCENT J. KIDD**, DAVID E. HOUSMAN*†, AND NICHOLAS C. DRACOPOLI†,††

*Center for Cancer Research, †Department of Biology, and ††Center for Genome Research, Massachusetts Institute of Technology, Cambridge, MA 02139; §Dartmouth–Hitchcock Medical Center, Dartmouth University, Lebanon, NH 03756; ¶Pittsburgh Cancer Institute, Division of Medical Oncology, University of Pittsburgh, Pittsburgh, PA 15213; ‖Immunology Program and Department of Medicine, The Memorial Sloan–Kettering Cancer Center, New York, NY 10021; and **Department of Tumor Cell Biology, St. Jude Children's Research Hospital, Memphis, TN 38105

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ABSTRACT **Genetic studies have implicated the early involvement of a gene on chromosome arm 9p in the development of cutaneous melanoma. We have performed loss-of-heterozygosity studies to confirm these original findings and identify the most frequently rearranged or deleted region of 9p. Eight markers were analyzed, including (from 9pter to proximal 9q) *D9S33*, the β-interferon (*IFNB1*) locus, the α-interferon (*IFNA*) gene cluster, *D9S126*, *D9S3*, *D9S19*, the glycoprotein 4β-galactosyltransferase (*GGTB2*) gene, and the argininosuccinate synthetase pseudogene 3 (*ASSP3*). Two or more of these loci were found to be hemizygously reduced in 12 of 14 (86%) informative metastatic melanoma tumor and cell line DNAs, and homozygous deletions of the marker *D9S126* were observed in 2 of 20 (10%) melanoma cell lines. These findings have resulted in the identification of a small critical region of 2–3 megabases on 9p21 in which a putative melanoma tumor-suppressor gene appears likely to reside. Several 9p candidate genes, including *IFNB1*, the *IFNA* gene cluster, *GGTB2*, and the tyrosinase-related protein (*TYRP*) locus, have all been eliminated as potential targets because they are located outside of the homozygously deleted regions.**

Cytogenetic and molecular studies suggest that several critical genes are important in the progression of cutaneous melanoma (CM) (for review, see ref. 1). One focus is on the p arm of chromosome 9, where four independent reports support the existence of a tumor-suppressor locus which is mutated during the early stages of melanoma tumor development (2–5). Overall, rearrangements of this chromosome occur in nearly half of all melanomas, with the most frequently targeted region extending from 9pter to 9q13 (1). The α-interferon (*IFNA*) gene cluster and β-interferon (*IFNB1*) locus are located within this region and are viewed as potential candidates for involvement in CM because they have growth-inhibitory properties and, as therapeutic agents, have caused the regression of metastatic melanoma (6–8). In addition, interferon-like molecules have been detected in the basal layer of the epidermis, suggesting that they may contribute to the control of normal epidermal growth or regeneration (9).

The glycoprotein 4β-galactosyltransferase (*GGTB2*) locus and the tyrosinase-related protein (*TYRP*) gene are also candidates for involvement in CM and reside within this region of 9p. The enzyme encoded by *GGTB2* plays a role in glycosylation, and mutations in this protein could adversely affect cell adhesion and/or cell growth (10, 11). This gene has been mapped to 9p13–p21 (6). *TYRP* is the human homolog of the mouse brown locus (12, 13) and a member of the tyrosinase-related gene family (14). *TYRP* encodes a transmembrane melanosomal glycoprotein, gp75, which was originally identified in melanoma cells by using autoantibodies from an affected patient (15, 16). The gp75 antigen is expressed exclusively in melanocytic cells (17) and already has an established role in the development of melanoma (18). The *TYRP* locus was localized initially to 9p13–pter (19) and more recently to 9p23 (20).

A genetic and physical map of the region surrounding the interferon genes on 9p has recently been created using the markers *D9S33*, *IFNB1*, *IFNA*, *D9S126*, *D9S3*, *D9S19*, and *ASSP3* (21). In the present study, these seven markers, along with the gene *GGTB2*, were analyzed for loss of heterozygosity (LOH) in 12 matched sets of metastatic melanoma and lymphoblastoid cell line DNAs and in three sets of uncultured metastatic melanoma and control DNAs. Eight unrelated melanoma cell lines were also tested for homozygous losses in this region. These analyses have resulted in the identification of a 2- to 3-megabase (Mb) critical region on 9p21.

MATERIALS AND METHODS

Melanoma and Lymphoblastoid Cell Line DNAs. DNAs were isolated from 25 established metastatic melanoma cell lines (7th to 100th passage), as well as from 12 related lymphoblastoid cell lines, by previously described techniques (22).

Melanoma Tumor and Constitutional DNAs. Four metastatic melanomas were surgically excised from three unrelated CM patients at the Pittsburgh Cancer Institute. Adjacent skin tissue was also obtained from two of the three patients, and an Epstein–Barr virus-transformed lymphoblastoid cell line was established from the third. Tumor and skin tissues were initially pulverized under liquid nitrogen and all DNAs (including that from the lymphoblastoid cell line) were extracted by standard methods (23).

Somatic Cell Hybrids. Two somatic cell hybrids, PK-87-9 and 132, were used in this study. PK-87-9 is a hamster somatic cell hybrid which contains a *hisD*-tagged chromosome 9 as its only stable human component (24), while 132 is a reduced somatic cell hybrid retaining most of the p arm of chromosome 9 (25). DNAs from these two hybrids and from

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Abbreviations: CM, cutaneous melanoma; LOH, loss of heterozygosity.

‡To whom reprint requests should be addressed.

Table 1. LOH analysis of 9p and proximal 9q loci comparing control and melanoma genotypes

		Locus									
		D9S33		*IFNB1*		*IFNA*		D9S126	D9S3	D9S19	*GGTB2*	*ASSP3*
		Location (pter to qter)										
		p21-23		p21		p21		p21	p21	p21	p13-21	q13-22
A. Melanoma Cell Lines												
Patient code	Cell line											
CO	CO-B	A1/A2	C1	D1	A2	B2	C2	A1/A2	A1	A2/A5	A1	A1/A2
	CO-Mel	A2	C1	D1	A2	B2	C2	A2	A1	A5	A1	A2
AL	AL-B	A2	C2	D1/D2	A1/A2	B2	C2	A1	A1/A2	A2/A5	A1	A1
	AL-Mel	A2	C2	D1	A1	B2	C2	A1	A2	A2	A1	A1
CZ	CZ-B	A1/A2	C2	D1	A2	B2	C2	A1/A2	A2	A1/A2	A1	A2
	CZ-Mel	A1	C2	D1	A2	B2	C2	A2	A2	A2	A1	A2
EL	EL-B	A1/A2	C1/C2	D1/D2	A2	B2	C1/C2	A1/A2	A2	A2	A1	A1
	EL-Mel	A1	C1	D2	A2	B2	C2	A1	A2	A2	A1	A1
CX	CX-B	A1/A2	C2	D2	A2	B2	C2	A1/A2	A1/A2	A1/A5	A1/A2	A1
	CX-Mel	A1	C2	D2	A2	B2	C2	A1	A1	A1	A1	A1
BD	BD-B	A2	C1/C2	D1/D2	A2	B2	C2	A1/A2	A1	A1/A2	A2	A1
	BD-Mel	A2	C1/C2	D1/D2	A2	B2	C2	A1/A2	A1	A1/A2	A2	A1
DE	DE-B	A1	C2	D1	A1/A2	B2	C2	A1	A1/A2	A2	A2	A1
	DE-Mel	A1	C2	D1	A1	B2	C2	A1	A2	A2	A2	A1
FF	FF-B	A1/A2	C1/C2	D1	A1	B2	C2	A1	A1	A1/A2	A1/A2	A1
	FF-Mel	A2	C1	D1	A1	B2	C2	---	---	A1/A2	A1/A2	A1
FC	FC-B	A1	C2	D1/D2	A1/A2	B1/B2	C2	A2	A1	A1	A1/A2	A1/A2
	FC-Mel	A1	C2	D2	A1	B1	C2	A2	A1	A1	A1	A1/A2
AH	AH-B	A1/A2	C2	D1/D2	A1/A2	B1	C2	A2	A1/A2	A2/A3	A1	A1/A2
	AH-Mel	A2 "R"	C2	D1	A2	B1	C2 "R"	---	A1	A2/A3	A1	A1/A2
AV	AV-B	A1	C1	D2	A2	B2	C2	A2	A1	A1	A1	A2
	AV-Mel	A1	C1	D2	A2	B2	C2	A2	A1	A1	A1	A2
DX	DX-B	A1/A2	C2	D1/D2	A1/A2	B1/B2	C2	A1/A2	A1/A2	A1/A2	A1/A2	A1
	DX-Mel *	A2	C2	D1	A2	B1	C2	A1	A2	A1	A2	A1
B. Melanoma Tumors												
Patient code	Cell Type											
BCO	BCO-SK	A1/A2	C2	D1/D2		ND		A1	A1	ND	A1/A2	A1
	BCO-Mel[a]	ND	C2	D2†		ND		A1	A1	ND	A2	A1
	BCO-Mel[b]	ND	C2	D2†		ND		A1	A1	ND	A2	A1
FNA	FNA-SK	A1/A2	C2	D1/D2		ND		A2	A1	ND	A1/A2	A1
	FNA-Mel	(A1/A2)	C2	(D1/D2)†		ND		A2	A1	ND	(A1/A2)	A1
YSI	YSI-B	A2	C2	D1/D2		ND		A1	A1/A2	ND	A1	A1
	YSI-Mel	A2	C2	D2†		ND		A1	A2†	ND	A1	A1

Lymphoblastoid (B) or control skin (SK) and melanoma (Mel) genotypes are given on each patient. The loci are presented in order from 9pter to proximal 9q as described by Fountain *et al.* (21). The placement of *GGTB2* relative to *D9S19* is not conclusive, but can tentatively be limited to a region between *D9S3* and *ASSP3* due to the LOH results obtained on patients FF and FC. Allele symbols are from Smith and Simpson (6) or Fountain *et al.* (21). In the case of *GGTB2*, A1 represents the 5.0-kb *Taq* I allele, while A2 indicates the 5.2-kb allele. Homozygous losses are shown (---), and the presence of a rearranged DNA fragment is indicated ("R"). ND, not determined. The critical region deleted or rearranged in CM is shown boxed for patients FF and AH and the cross-hatched area shows the region of overlap of the homozygous deletions present in these two melanoma cell lines.

*Six autologous metastatic melanoma DNAs were analyzed and identical genotypes were detected with all chromosome 9 markers.

†PhosphorImager analysis was used to confirm these genotypes. LOH could not be demonstrated conclusively in FNA-Mel, but both BCO-Mel tumors (a and b, two independent metastases) and YSI-Mel showed an average of 72% and 32% loss of alleles, respectively (see text for further explanation).

the parental hamster cell line CHTG49 were isolated according to Fountain *et al.* (26).

Conventional Southern Blot Analysis. DNA digestions, Southern blotting, hybridizations, and stripping procedures were performed as described (2, 21). Hybridized blots were exposed to Kodak film (XAR-5) for 1–5 days.

DNA Probes. The clones and DNA fragments used as probes have been previously defined, except for those detecting the loci *D9S33* (CRI-L944), *ASSP3* (pAS419), *GGTB2* (hGT2b), and *TYRP* (pGP75-2 and 5-1) (21). In the case of CRI-L944 and pAS419, unique insert fragments of 10–12 kb (*Eco*RI) or 1.5 kb (*Pst* I) were used to detect *Taq* I and *Hin*dIII

polymorphisms, respectively, as described (6). A new *Taq* I polymorphism for *GGTB2* (allele sizes of 5.0 and 5.2 kb) is presented in this study and was detected using the unique 2.5-kb *Eco*RI insert from hGT2b [a 3′ cDNA clone (27)] as a probe. In contrast, no polymorphisms were detected at the *TYRP* locus by using either (*i*) a nonrepetitive 2.3-kb *Eco*RI fragment from the cDNA clone pGP75-2 to probe Southern blots of *Hin*dIII-, *Taq* I-, *Msp* I/*Hpa* II-, and *Eco*RI-digested DNA from three or more unrelated individuals or (*ii*) a 3.0-kb *Eco*RI fragment from the genomic clone 5-1 to probe similar representative *Hin*dIII, *Ban* II, and *Taq* I blots.

PhosphorImager Analysis. Hybridized blots were scanned on a Molecular Dynamics (Sunnyvale, CA) PhosphorImager and a relative decrease in band intensity was calculated as a percentage loss over the remaining allele in the tumor. A difference of 0.5–8.0% was found when alternative allele hybridization signals from control DNA digestions were compared (J.W.F., unpublished results), and a reduction of >25% of one allele relative to the other was considered significant in the tumors.

Polymerase Chain Reaction (PCR) Amplification. Primers from the 3′ end of a *TYRP* cDNA clone (5′-GGATAGTGT-GAAGATCTTTGGCATG-3′ and 5′-ACAGTGGCAAACA-CAGGCAATATCC-3′) (19) were used to amplify a 439-base-pair (bp) product from genomic DNAs. PCRs were performed in a Perkin–Elmer/Cetus thermocycler with 0.2–0.5 μg of DNA in a total volume of 30 μl. Thirty-five cycles of amplification were carried out with *Taq* I polymerase (Cetus or laboratory-prepared) and consisted of 1 min at 94°C, 1 min at 62°C, and 1 min at 72°C. Amplified products were analyzed by electrophoresis in 1.5% agarose gels containing ethidium bromide and 1× TBE (Tris/borate/EDTA buffer; ref. 23).

RESULTS

Twelve matched sets of lymphoblastoid and melanoma cell line DNAs were examined for LOH on 9p and proximal 9q at the polymorphic loci *D9S33*, *IFNB1*, *IFNA*, *D9S126*, *D9S3*, *D9S19*, *GGTB2*, and *ASSP3* (Table 1, part A). Results from these analyses established the importance of two tumors, FF-Mel and AH-Mel, in defining the smallest common region of 9p deleted in melanoma. The Southern blot results obtained on these two tumors are shown in Fig. 1 for the 9p21 loci *IFNA*, *D9S126*, and *D9S3*. Notably, the marker *D9S126* is homozygously deleted in both FF-Mel and AH-Mel, with FF-Mel also being nullizygous for *D9S3* (Fig. 1 *B* and *C*). In addition, an aberrant *IFNA* fragment is present in AH-Mel (arrow in Fig. 1*A*), suggesting that one of the 9p breakpoints in this tumor is located within or near the *IFNA* gene cluster.

Due to the detection of homozygous losses of *D9S126* and *D9S3* in FF-Mel and AH-Mel, eight additional unrelated metastatic melanoma cell line DNAs were analyzed at these two loci without the inclusion of matching lymphoblastoid DNAs. No additional homozygous deletions or rearrangements were detected on blots containing *Taq* I-digested DNAs from these tumors (data not shown).

To confirm the assumption that 9p losses occur *in vivo* during melanoma tumor progression, four uncultured metastatic melanomas were also tested for LOH. A number of the 9p loci were informative here (Table 1, part B) and, in several instances, PhosphorImager analysis was employed to determine the extent of allele loss in these tumors. In the case of BCO, DNAs were isolated from two independent metastatic melanomas and digests from both samples showed clear reduction of the same allele when hybridized with probes that recognize the *IFNB1* and *GGTB2* polymorphisms (Fig. 2). The results from the YSI melanoma were more difficult to interpret by eye, but PhosphorImager analysis of blots hybridized with either an *IFNB1* or a *D9S3* probe revealed a 32% loss of one allele in each case (data not shown). Two independent Southern blots, containing DNAs digested with two different restriction enzymes, were used in these analyses. In retrospect, the high level of normal DNA contamination in YSI-Mel was not surprising, since a blood vessel was noted in this tumor sample prior to DNA isolation. The third uncultured melanoma DNA, FNA-Mel, was also informative at the *IFNB1* locus and subjected to PhosphorImager analysis. Though not considered significant, one allele in this DNA digestion did differ in intensity from the other by 12%

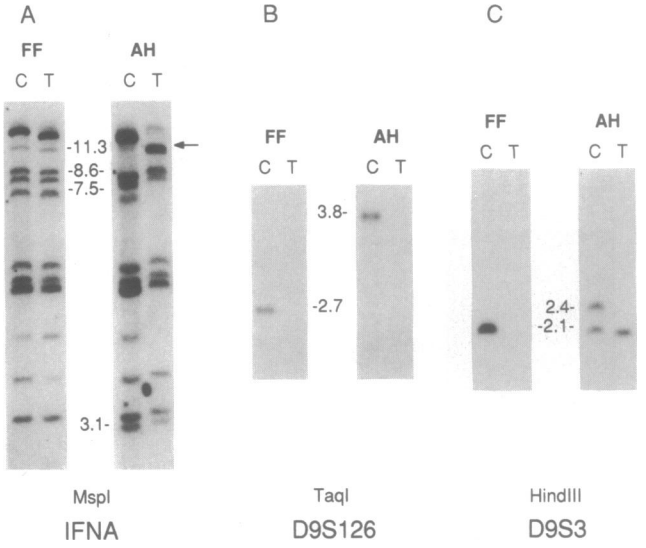

FIG. 1. Loss of restriction fragments in melanoma patients FF and AH. Results from Southern blot hybridizations using probes which recognize the chromosome 9 loci *IFNA*, *D9S126*, and *D9S3* are shown in *A–C*, respectively. The restriction enzyme used in each case is indicated beneath the blots, and polymorphic fragment sizes are given in kilobases. The lymphoblastoid genotypes are shown in lanes C (control) and the melanoma genotypes are shown in lanes T (tumor). In the case of *IFNA* (*A*), a 1:1 ratio of the 8.6-kb allele to the 7.5-kb allele indicates that FF is homozygous at the *IFNA* locus, while a 3:1 ratio of the 8.6-kb to the 7.5-kb fragment in AH indicates that this individual is heterozygous. AH-Mel is reduced at this locus and also harbors a rearrangement of one of the largest *IFNA* fragments (see arrow). The locus *D9S126* (*B*) is constitutionally homozygous for opposite alleles in FF and AH and, in this case, no signal is discernable in either tumor lane. Analogously, FF-Mel also harbors a homozygous deletion of *D9S3* (*C*). The presence of DNA in the nullizygous lanes has been confirmed by hybridizing the same blots with other undeleted chromosome 9 probes (data not shown).

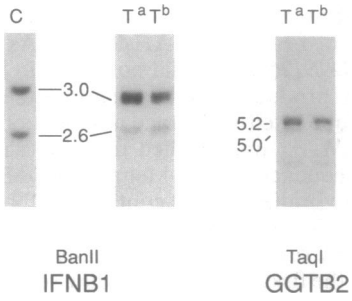

FIG. 2. Southern blot analysis of two uncultured metastatic melanomas from patient BCO. The constitutional (lane C) skin DNA digestion from BCO is shown for *IFNB1* along with digested DNAs from two different metastases (T^a and T^b). Reduction of the 2.6-kb *IFNB1* allele is clear in both tumor DNAs, and PhosphorImager analysis revealed 77% and 67% reduction of this allele in T^a and T^b, respectively. Analogously, reduction of the 5.0-kb *GGTB2* allele is also evident in both of these metastases. Independent hybridization of control DNA confirmed that BCO was informative at this locus (data not shown).

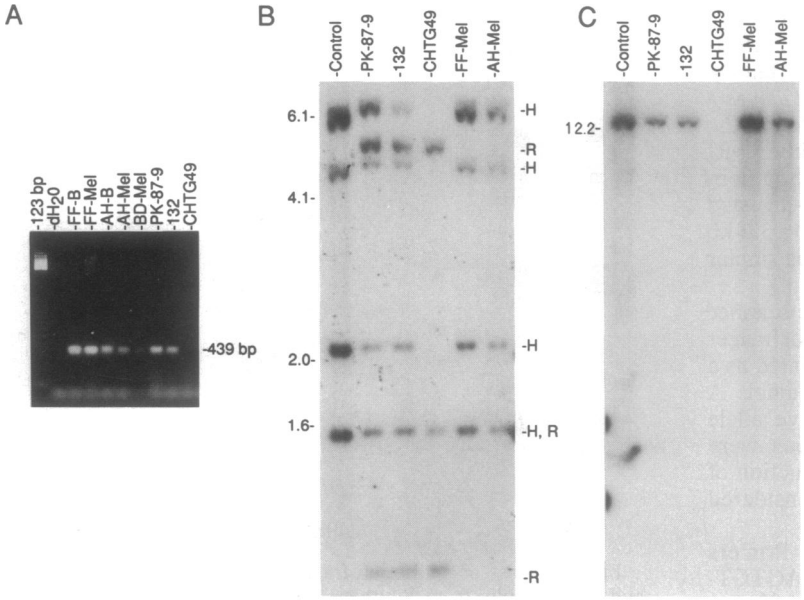

FIG. 3. Analysis of the *TYRP* gene in FF-Mel and AH-Mel. (*A*) Primers from the 3′ end of the *TYRP* coding sequence were used in PCR to amplify the indicated DNA samples. The 123-bp marker from Bethesda Research Laboratories was used as a size marker, and amplification of a 439-bp fragment is seen in all lanes except those containing distilled water (dH_2O) or DNA from the hamster cell line CHTG49. The two hybrids PK-87-9 and 132, which contain all or a portion of human chromosome 9, respectively, were used as positive controls. (*B*) The indicated DNAs were digested with *Eco*RI, blotted, and hybridized with a 2.3-kb cDNA fragment from *TYRP*. Human (H) and hamster (R) bands are designated at right and fragment sizes (kb) are given at left. (*C*) DNAs were digested with *Taq* I and hybridized with a 5′ genomic fragment from *TYRP*. An intact fragment of >12.2 kb is seen in all lanes except the one containing hamster DNA (see text for further explanation).

(see *Materials and Methods*). The loss of chromosome 9 material in FNA-Mel, however, remains inconclusive.

The detection of homozygous deletions in FF-Mel and AH-Mel allowed us to determine whether any 9p candidate tumor-suppressor genes were deleted or rearranged in these two tumors. Since the *IFNB1* locus and the *IFNA* gene cluster were not nullizygous in these tumors, and only one rearrangement of an *IFNA* fragment was detected out of 15 melanomas (Table 1; Fig. 1*A*), it appears unlikely that these genes play a direct role in melanoma tumor progression. Analogously, *GGTB2* has also been eliminated as a candidate gene; this locus is informative and not even reduced in FF-Mel (which presumably harbors the larger of the two homozygous deletions).

The exact location of a fourth 9p locus, *TYRP*, relative to the homozygous deletions was more intensively investigated, since this gene already has an established role in melanoma (15, 18). PCR analysis using primers chosen from the 3′ end of a *TYRP* cDNA clone (19) was initially performed on FF and AH DNA samples (Fig. 3*A*). An amplified product was detected in both of the melanoma DNAs from these two patients (as well as in melanoma DNA from another patient, BD), indicating the absence of any deletions encompassing the entire *TYRP* gene. The somatic cell hybrid DNAs PK-87-9 and 132 were used as positive controls, and the presence of amplified products in these lanes (Fig. 3*A*) also supports the assignment of this locus to 9p (19, 20). In contrast, PCR amplifications of FF-Mel and AH-Mel DNAs using primers derived from *D9S126* clones did not result in the generation of any specific products, establishing that these two DNAs were not contaminated with trace amounts of normal fibroblast DNA (J.W.F., unpublished results). The presence of intact *TYRP* sequences in FF-Mel and AH-Mel was further confirmed by hybridizing blots containing DNA digests from these tumors with both a *TYRP* cDNA and a 5′ genomic probe (Fig. 3 *B* and *C*, respectively). The cDNA clone, pGP75-2, detects all but the first exon of the coding sequence for *TYRP* and the genomic clone, 5-1, spans the first exon and a portion of the first intron. Thus, no gross rearrangements of the entire *TYRP* gene were detected in either FF-Mel or AH-Mel, a result which effectively eliminates it as the putative tumor-suppressor gene deleted in these two tumors.

DISCUSSION

The findings from this study support the involvement of a 9p tumor-suppressor gene in the evolution of CM. At least two of seven 9p markers detected hemizygous losses in 12 of 14 (86%) informative metastatic melanoma tumor and cell line DNAs, and 2 of 20 (10%) melanoma cell lines were determined to harbor homozygous deletions of the locus *D9S126*. This frequency of LOH is one of the highest reported for any chromosomal region and well above the background loss of 11–27% detected in metastatic melanoma tumors and cell lines (2, 22, 28). In comparison, LOH values of only 40–60% have been found in melanoma on 1p and 6q, two chromosomal regions presumed to harbor genes involved in the late stages of melanoma tumor progression (refs. 1, 2, 29, and 30; N.C.D., unpublished results). To our knowledge, no homozygous deletions on either 1p or 6q have been reported in CM.

The initial LOH studies performed on 9p involved the analysis of six autologous metastatic melanoma cell lines with the marker *D9S3* (2). All six melanoma DNAs showed a reduction in the same *D9S3* allele, which represented the only identical autosomal change common to these tumors. In the present study, these same melanoma DNAs were reexamined at six additional 9p loci and, as expected, still exhibited identical patterns of LOH (patient DX; Table 1, part A). In addition, two metastases from an unrelated patient showed hemizygous loss of identical alleles at the *D9S33*, *IFNB1*, and *GGTB2* loci (patient BCO; Fig. 2 and Table 1, part B). The combined results from these two studies imply the existence of a 9p tumor-suppressor gene that is mutated in primary tumors, prior to the acquisition of metastatic potential.

The early involvement of a gene on 9p is further supported by a cytogenetic report on CM in which karyotypes from melanoma precursor lesions (dysplastic nevi), primary melanomas, and metastases were compared (3). The only consistent chromosomal aberration noted among these lesions (which presumably represent sequential stages in melanoma development) was the loss or rearrangement of chromosome 9. The most frequently targeted region was noted to be within the vicinity of the *IFNB1* and *IFNA* genes on 9p22–pter.

Preliminary evidence from pulsed-field gel electrophoresis indicates that the two homozygous deletions detected here reside just proximal to the interferons and span 2–3 Mb on 9p21 (M.K. and J.W.F., unpublished results). Overall, homozygous deletions have proven invaluable in the identification of other tumor-suppressor loci, including the retinoblastoma (*RB1*), Wilms tumor (*WT1*), and deleted-in-colorectal-carcinoma (*DCC*) genes (31–33), and, in these cases, a portion or all of the coding sequence of these genes

was found to map within the homozygously deleted regions. We presume the same will be the case for melanoma, where any 9p candidate gene will be deleted or rearranged in one or both of the melanomas that harbor the homozygous deletions of *D9S126* and/or *D9S3*. We have tested four potential 9p tumor-suppressor genes in this study—*IFNB1*, the *IFNA* gene cluster, *GGTB2*, and *TYRP*—and have determined that none of them reside within the overlapping region defined by the homozygous deletions. The direct involvement of any one of these genes would now be feasible only if the homozygous deletions were able to influence gene expression at a distance.

Somatic cell hybrid studies performed on mouse melanoma also support the involvement of an as yet unidentified 9p tumor-suppressor gene in CM. The chromosomal region homologous to 9p, on mouse chromosome 4, has long been implicated in the suppression of melanoma tumorigenicity (34). This finding suggests that the etiology of melanoma is similar between these two species and makes the mouse an especially attractive animal in which to study the effects of this gene.

In addition to mouse melanoma, a number of other human cancers, including leukemia, glioma, and non-small-cell lung carcinoma, also exhibit frequent rearrangements or deletions of 9p (35–43). Specifically, nullizygosity of *IFNB1* and/or *IFNA* has been found in 7–9% of leukemic cells and cell lines (36, 37, 40) and in 7–40% of glial tumors and glioma cell lines (39, 41, 43). However, as in melanoma, some of these deletions have been determined to exclude the interferon genes and extend in a more proximal direction on 9p (36, 43). Ultimately, determining whether or not the same tumor-suppressor gene is targeted in all of these seemingly unrelated forms of cancer will be possible only after the limits of each homozygously deleted region are more clearly defined.

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