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Advances in Brief

Disruption of the *MMAC1/PTEN* Gene by Deletion or Mutation Is a Frequent Event in Malignant Melanoma[1]

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Abstract

The *MMAC1/PTEN* gene, located at 10q23.3, is a candidate tumor suppressor commonly mutated in glioma. We have studied the pattern of deletion, mutation, and expression of *MMAC1/PTEN* in 35 unrelated melanoma cell lines. Nine (26%) of the cell lines showed partial or complete homozygous deletion of the *MMAC1/PTEN* gene, and another six (17%) harbored a mutation in combination with loss of the second allele. Mutations could also be demonstrated in uncultured tumor specimens from which the cell lines had been established, and cell lines derived from two different metastases from one individual carried the same missense mutation. Collectively, these findings suggest that disruption of *MMAC1/PTEN* by allelic loss or mutation may contribute to the pathogenesis or neoplastic evolution in a large proportion of malignant melanomas.

Introduction

Sporadic cutaneous melanoma is a genetically heterogeneous cancer with high mortality (1, 2). Previous karyotypic and LOH[3] studies of primary and metastatic melanomas have indicated that genetic events involving chromosome region 10q22–10qter may be associated with tumor progression (3–6). Recently, the *MMAC1/PTEN* gene was identified as a candidate tumor suppressor at 10q23.3 (7, 8). Approximately 60% of glioma cell lines with LOH in 10q23 harbor mutations and deletions of *MMAC1/PTEN*, and sporadic examples of mutations in tumor specimens from gliomas and prostate, breast, and kidney cancers were reported. Herein, we demonstrate that *MMAC1/PTEN* is deleted or mutated in more than 40% of malignant melanoma cell lines, suggesting that *MMAC1/PTEN* is a critical target in melanoma pathogenesis.

Materials and Methods

Tumors and Cell Lines. Tumor samples were obtained from patients with sporadic melanomas who underwent surgery for primary or metastatic melanoma at the Department of Plastic Surgery, Rigshospitalet (Copenhagen, Denmark). Cell suspensions were prepared from biopsy materials within 2 h of surgical removal. For each tumor, part of the cell suspension was frozen while the remainder was used for the establishment of a long-term cell line, according to a previously described protocol (9). All FM cell lines were used in early passages (passages 3–6). For a number of cell lines, the matched uncultured primary melanoma and metastasis were available as paraffin sections. The cell lines SK-MEL-3, SK-MEL-24, and SK-MEL-31 were obtained from the American Type Culture Collection. Normal cultured melanocytes were purchased from Clonetics.

RT-PCR. RNA was extracted from cell lines using the Purescript RNA Isolation Kit (Gentra). The first strand was synthesized from 1–3 μg of total RNA using an oligo(dT) primer and M-MLV reverse transcriptase (Life Technologies, Inc.). Incubations were performed at 42°C for 50 min. Primers used for amplification of *MMAC1/PTEN* cDNA were MMAC1-1827 (5′-GCTTCTGCCATCTCTCTCCTCC-3′) and MMAC1-1924 (5′-CCTCAGTTTGTGGTCTGCCAG-3′) for the 5′ end and MMAC1-2032 (5′-TTTCATGGTGTTTTATCCCTCTTG-3′) and MMAC1-1774 (5′-AGGTAACGGCTGAGGGAACTC-3′) for the 3′ end. For amplification of a shorter region surrounding exon 3, primer MMAC1-2818 (5′-GTCATCTTCACTTAGCCATTG-3′) was used together with MMAC1-1827. Control primers for amplification of GAPDH cDNA were described previously (10). All PCRs contained 20 mM Tris-HCl (pH 8.4), 50 mM KCl, 2.0 mM $MgCl_2$, 0.005% (w/v) BSA, 0.2 mM cresol red, 12% sucrose, 100 μM each dNTP, 0.6 μM each primer, 100 ng of DNA, and 0.5 units of AmpliTaq DNA polymerase (Perkin-Elmer/Cetus). Amplification was performed with the GeneAmp PCR System 9600 (Perkin-Elmer/Cetus) as follows: 35 cycles of 94°C for 30 s, 60°C for 30 s, and 72°C for 1 min, followed by a final primer extension at 72°C for 5 min.

Detection of Deletions and Mutations. Genomic DNA was isolated from cell lines, cell suspensions, and paraffin sections using the Purescript DNA Isolation Kit (Gentra). The DNA quality was tested by control amplification of the phenylalanine hydroxylase (*PAH*) gene on chromosome 12 (11). Deletion analysis of the *MMAC1/PTEN* gene was performed using the primers and PCR conditions described by Steck *et al.* (7), except for exon 1, for which new primers were designed (see below).

To seek for mutations in the *MMAC1/PTEN* gene, we developed a method based on DGGE (12). To achieve the highest possible mutation detection rate, we applied a set of stringent criteria, as described previously (13). In particular, we optimized the melting characteristics of all amplification products by means of computer simulations (14) and "GC-clamping" (15) to ensure that the sequence of interest was contained within one low-melting domain. A total of 10 primer sets were developed that allow amplification and DGGE analysis of the nine MMAC1/PTEN-coding exons and their immediately flanking intronic sequences (Table 1). Exon 5 was amplified into two overlapping products. Because of the presence of a 14-bp poly(T) stretch in the acceptor splice site of intron 7 (7), the upstream primer for amplification of exon 8 spanned the exon-intron boundary. The method thus allows scanning of 15 of 16 splice sites and more than 98% of the coding sequence of the *MMAC1/PTEN* gene. PCR was performed as above, using the following cycling parameters: an initial denaturation step of 94°C for 5 min, 38 rounds of thermal cycling [94°C for 20 s, the annealing temperature (Table 1) for 20 s, and 72°C for 20 s], and a final incubation at 72°C for 5 min. For amplification of exon 1, 1.5% DMSO was included in the reaction.

Ten μl of the GC-clamped PCR product were loaded onto a 10% denaturant/6% polyacrylamide-70% denaturant/12% polyacrylamide double-gradient gel as described (16). The gels were run at 160 V for 6 h or overnight at 80 V in 1× TAE buffer kept at a constant temperature of 56°C, stained in 1× TAE buffer containing ethidium bromide (2 μg/ml), and photographed under UV transillumination. For semiquantitative determination of the mutant fraction in a DNA sample, the relative intensities of heteroduplex and homoduplex bands on a denaturing gradient gel were estimated by comparison with standards with known mutant:wild type ratios.

Direct sequencing of double-stranded PCR products was performed with a ^{33}P-end-labeled primer using the ThermoPrime Cycle Sequencing Kit (Amersham), according to the manufacturer's instructions. After electrophoresis, the

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[3] The abbreviations used are: LOH, loss of heterozygosity; DGGE, denaturing gradient gel electrophoresis; RT-PCR, reverse transcriptase-PCR.

Table 1 *Amplification primers for DGGE-based mutation analysis of the MMAC1/PTEN gene*

Exon/primer	Sequence (5′ → 3′)[a]	Product length (bp)	T_m of domain[b] (°C)	Annealing temperature (°C)
1-F[c]	ccgTCCTCCTTTTTCTTCAGCCAC	190	71.2	56
1-R	[GC]-GAAAGGTAAAGAGGAGCAGCC			
2-F	[GC]-TTAGTTTGATTGCTGCATATTTC	222	65.4	54
2-R	cggcgACATCAATATTTGAAATAGAAAAGC			
3-F	TGTTAATGGTGGCTTTTTG	154	64.2	56
3-R	[GC]-GCAAGCATACAAATAAGAAAAC			
4-F	[GC]-TTCCTAAGTGCAAAAGATAAC	187	65.9	56
4-R	TACAGTCTATCGGGTTTAAGT			
5I-F	[GC]-TTTTTTCTTATTCTGAGGTTATC	225	69.1	56
5I-R	TCATTACACCAGTTCGTCC			
5II-F	TCATGTTGCAGCAATTCAC	217	68.4	54
5II-R	[GC]-GAAGAGGAAAGGAAAAACATC			
6-F	[GC]-AGTGAAATAACTATAATGGAACA	272	68.1	54
6-R	GAAGGATGAGAATTTCAAGC			
7-F	cgcgcccgAATACTGGTATGTATTTAACCAT	268	70.5	56
7-R	[GC]-TCTCCCAATGAAAGTAAAGTA			
8-F	gcccgTTTTTAGGACAAAATGTTTCAC	303	66.6	54
8-R	[GC]-CCCACAAAATGTTTAATTTAAC			
9-F	GTTTTCATTTTAAATTTTCTTTC	285	68.3	54
9-R	[GC]-TGGTGTTTTATCCCTCTTG			

[a] Lowercase characters represent nucleotides incorporated into the primer to modulate the melting profile of the amplification product.
[b] Melting temperature of the lower melting domain of the GC-clamped PCR product, as determined by MELT87 (14).
[c] F, forward primer; R, reverse primer; [GC], CGCCCGCCGCGCCCCGCGCCCGTCCCGCCGCCCCCGCCCG.

gel was dried and exposed to a Storage Phosphor Screen (Molecular Dynamics) for 24 h.

Results

Initial PCR amplification of genomic DNA using the intronic primers given by Steck *et al.* (7) revealed partial or complete homozygous deletion of the *MMAC1/PTEN* gene in 9 of 35 melanoma cell lines (Fig. 1*a*). One of the cell lines (FM60) carried a deletion spanning the entire gene, one line (SK-MEL-24) showed deletion of a single exon, and the remainder showed partial deletions involving either the 5′ end or the 3′ end of the gene (Fig. 2). The homozygously deleted cell lines showed no signal when examined for *MMAC1/PTEN* transcripts by RT-PCR analysis (Fig. 1*b*).

We next determined the exon-intron boundary sequences for all nine exons of the *MMAC1/PTEN* gene. This allowed us to develop a method combining PCR and DGGE (12) to search for potential point

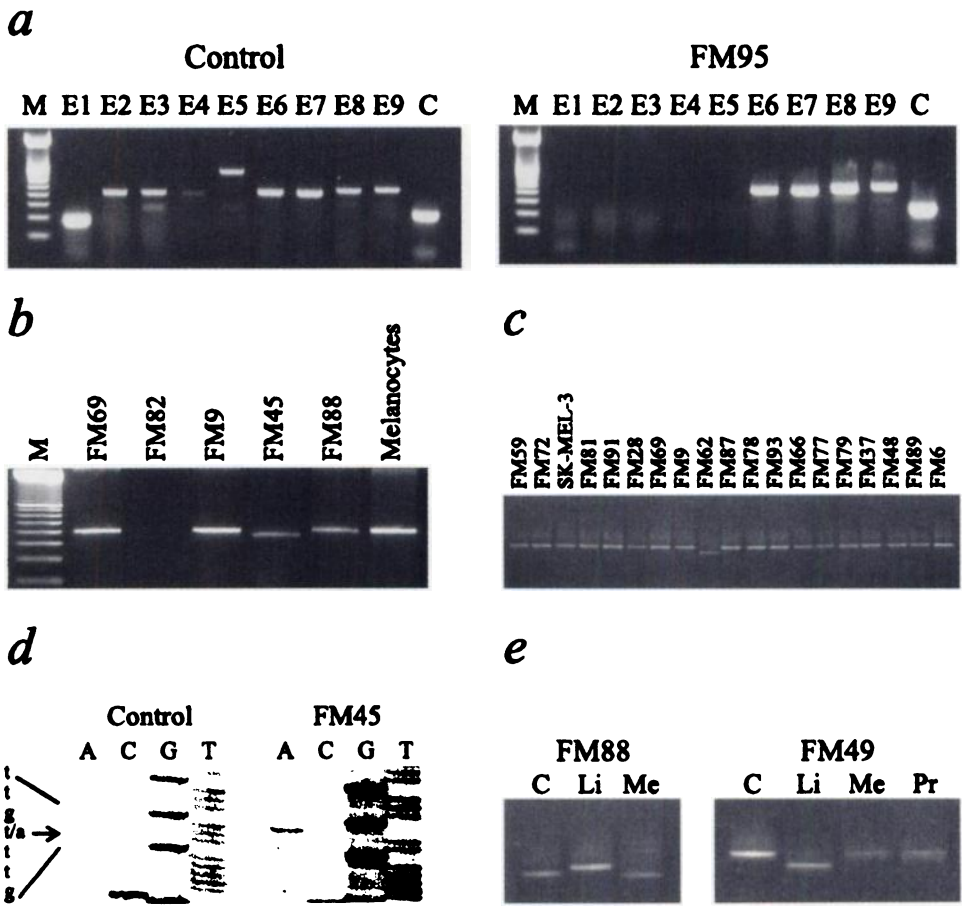

Fig. 1. Nucleic acid analysis of deletion, mutation, and expression of *MMAC1/PTEN* in malignant melanoma. *a*, deletion analysis in melanoma cell line FM95 by genomic PCR amplification of *MMAC1/PTEN* exons 1–9 (*Lanes E1–E9*). *Lane C*, exon 12 of the control (*PAH*) gene; *Lane M*, 100-bp ladder. *b*, RT-PCR analysis of *MMAC1/PTEN* expression in normal melanocytes and melanoma cell lines. Primers MMAC1/PTEN-1827 and MMAC1/PTEN-2818 were used to amplify a 397-bp region in the 5′ end of the *MMAC1/PTEN* cDNA. No amplification is seen for FM82, which carries a homozygous deletion. Aberrant RNA splicing is revealed by a shorter PCR product in FM45, which carries a mutation in the acceptor splice site of intron 2. *c*, mutation detection in the 3′ end of exon 5 (using primers 5II-F and 5II-R) of the *MMAC1/PTEN* gene by PCR/DGGE. Note the presence of a mutant band and the absence of a wild-type band in FM62. *d*, sequence of the mutation found in intron 2 of the *MMAC1/PTEN* gene in cell line FM45. The sequence reads in the sense direction from bottom to top. *e*, DGGE-based mutation analysis in uncultured tumor specimens. *Left*, *MMAC1/PTEN* exon 5 (mutation in FM88: G127E); *right*, *MMAC1/PTEN* exon 8 (mutation in FM49: 949 del 18 bases). *Lanes C*, control DNA; *Lanes Li*, cell line; *Lanes Me*, metastasis; *Lane Pr*, primary melanoma. Weak heteroduplex bands reveal the presence of the G127E mutation in low abundance in the uncultured metastasis related to FM88. In contrast, mutant sequence cannot be detected in metastasis and primary melanoma related to FM49.

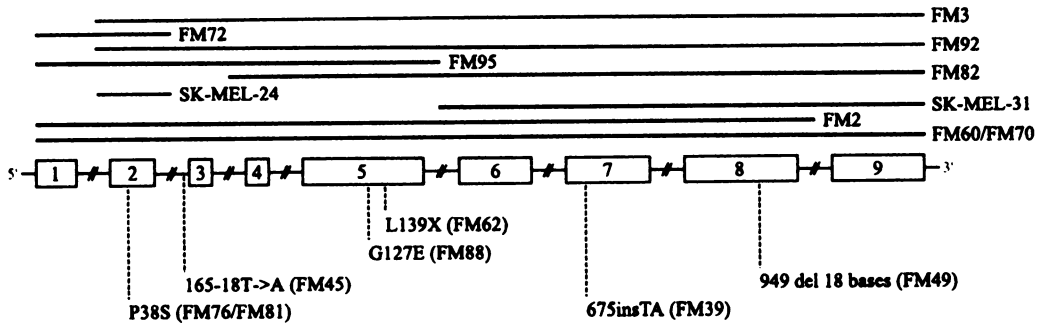

Fig. 2. Schematic representation of the *MMAC1/PTEN* gene and genetic alterations identified in melanoma cell lines. ——, extent of deletions involving entire exons; ⋮, localization of *MMAC1/PTEN* mutations.

mutations and small deletions and insertions in cell lines that retained the *MMAC1/PTEN* gene (Fig. 1*c*). Systematic scanning of all nine exons for mutations led to the identification of sequence alterations in six cell lines. In all cases, the absence of wild-type sequence was demonstrated both by DGGE (Fig. 1*c*) and sequencing (Fig. 1*d*), suggesting that the second allele had been deleted. The identified mutations include two missense variants, one nonsense mutation, one 2-bp frameshift insertion, one 18-bp inframe deletion, and one splice variant (Table 2). The latter mutation involves a T→A transition in intron 2, 18 bases from exon 3 (Fig. 1*d*). RT-PCR analysis (Fig. 1*b*) and sequence analysis showed that this mutation causes the skipping of exon 3, probably by disrupting the polypyrimidine tract (17) of the acceptor splice site of intron 2.

For the six cell lines carrying an *MMAC1/PTEN* mutation, uncultured tissue specimens from the matched primary melanoma and/or the subsequent metastasis were available as paraffin-embedded sections or as cell suspensions (Table 2). Using DGGE, which detects mutant fractions down to 5%, we could demonstrate the presence of the relevant mutation in DNA isolated from the three metastasis cell suspensions but not in DNA isolated from paraffin blocks containing the primary tumors and the remaining metastases (Fig. 1*e* and Table 2). Notably, quantitative estimation of mutant and wild-type bands on the denaturing gradient gel showed that the mutant fraction in two of the metastasis cell suspensions was less than 20% (data not shown).

To further investigate the *in vivo* role of *MMAC1/PTEN* mutations, we analyzed two cases in which tumor cell lines had been established from two different metastases, biopsied at 6-month intervals from one individual patient. In each case, identical *MMAC1/PTEN* gene defects were identified in the two metastatic cell lines; a large deletion spanning the entire gene (cell lines FM60 and FM70) and a missense mutation, P38S (cell lines FM76 and FM81). Although a large gene deletion may be a relatively common and unspecific event, the identification of the same point mutation in two different metastases from one patient must be considered unambiguous evidence of its occurrence *in vivo*.

Discussion

The *MMAC1/PTEN* gene is predicted to encode an evolutionarily highly conserved protein with homology to known phosphatases and two cytoskeletal proteins, tensin and auxilin (7, 8). Germ-line mutations of *MMAC1/PTEN* have been identified in patients with Cowden disease, a rare syndrome associated with increased risk for breast, thyroid, and skin cancers (18). Several lines of evidence derived from the present study suggest that the *MMAC1/PTEN* gene is a critical tumor suppressor in the pathogenesis of sporadic malignant melanoma. First, mutation or deletion of both *MMAC1/PTEN* alleles occur in more than 40% of the melanoma cell lines studied here. This figure is compatible with previous LOH studies showing 10q deletion rates of 32–50% (4, 6). Second, the hemizygous sequence alterations are likely to be deleterious, causing protein truncation, aberrant RNA splicing, frameshift, or amino acid substitutions. The two missense variants, P38S and G127E, affect regions that show homology with other proteins; residue 38 is conserved in both auxilin and tensin, and residue 127 is conserved in all protein tyrosine phosphatases and dual-specificity phosphatases (7, 8). Third, sequence alterations were also identified in matched uncultured tumor specimens, excluding the possibility of cell culturing artifacts.

Our results may provide further support of the notion that *MMAC1/PTEN* is involved in later stages of tumorigenesis (7, 8). Mutations identified in tumor cell lines could be demonstrated in most of the uncultured metastases but not in the primary melanomas. This is in agreement with previous LOH studies showing late occurrence of 10q deletions in sequential samples representing different stages of tumor progression (3). It should be noted, however, that the fraction of mutant cells in the uncultured cell suspensions was sometimes very low (less than 20%). This observation may reflect a combination of tumor heterogeneity and the presence of contaminating stroma, which would probably have masked the detection of mutation if less sensitive methods had been used (*e.g.*, direct sequence analysis). Because of these quantitative considerations, we cannot formally exclude the existence of mutations in primary melanomas. Mutation analysis of

Table 2 *MMAC1/PTEN mutations in melanoma cell lines, primary tumors, and metastases*

Mutations are named according to standard nomenclature.

Cell line	Mutation	Exon/intron	Codon	Predicted effect	Primary tumor	Metastasis
FM45	165–18T→A	Intron 2		Skipping of exon 3	MND (par)[a]	ND
FM49	949del 18 bases	Exon 8	317	Val-Leu-Thr-Leu-Thr-Lys-Asn→Asp	MND (par)	MND (par)
FM76/FM81[b]	C112T	Exon 2	38	Pro→Ser	MND (par)	ND
FM39	675insTA	Exon 7	225	Frameshift	ND	MD (susp)
FM62	T416G	Exon 5	139	Leu→Stop	MND (par)	MD (susp)
FM88	G380A	Exon 5	127	Gly→Glu	ND	MD (susp)

[a] MND, mutation not detectable; par, paraffin-embedded material; ND, not done; MD, mutation detectable; susp, cell suspension.
[b] FM76 and FM81 are cell lines derived from two different metastases from the same patient.

multiple sections representing the entire primary tumor may clarify this point.

The recent dramatic progress in unraveling mechanisms that control the mammalian cell cycle has shed new light on the genetic defects that disrupt normal melanocytic differentiation and division and lead to invasive melanoma. Although several chromosomal regions, including 1p, 3p, 6q, 9p, 10q, and 11q, appear to harbor melanoma tumor suppressor genes, the most frequent and consistent changes seem to involve 9p and 10q (5, 6). Among several potential tumor suppressors located to 9p, the *p16* (*CDKN2*) gene at 9p21 represents the strongest candidate because it is mutated or deleted in a large fraction of melanoma cell lines (19–22) and because germ-line *p16* mutations have been implicated as the predisposing factor in 9p-linked melanoma families (23, 24). Most of the cell lines analyzed in this study have previously been shown to harbor defects in *p16* (22). Disruptions of *MMAC1/PTEN* and *p16*, therefore, seem to represent independent tumorigenic pathways. Nevertheless, it is intriguing that the involvement of both p16 and MMAC1/PTEN appears to be common to glioma (7, 25) and melanoma, which share a common developmental origin in that both glia cells and melanocytes are derived from the neural crest.

In summary, our results strongly support the candidacy of *MMAC1/PTEN* as a major tumor suppressor involved in melanoma pathogenesis. To prove or disprove this hypothesis will require extensive studies of the function of MMAC1/PTEN and the biological consequences of *MMAC1/PTEN* mutations.

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