Relative Reciprocity of NRAS and PTEN/MMAC1 Alterations in Cutaneous Melanoma Cell Lines

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Abstract

Both inactivation of the tumor suppressor gene, PTEN/MMAC1, and oncogenic activation of RAS have been described in human cutaneous melanoma. In mice, activation of a RAS-containing pathway is a necessary step in the pathogenesis of murine melanomas. Because PTEN negatively regulates on the downstream effects of phosphatidylinositol-3-kinase (PI3-K), we hypothesized that the loss of PTEN/MMAC1 and the activation of RAS may be largely equivalent because PTEN is a known positive upstream regulator of PI3-K. We expanded our previous survey of PTEN/MMAC1 and RAS mutations and analyzed the RAS status of 53 cutaneous melanoma cell lines, 18 glioma cell lines, and 17 uncurtured cutaneous melanoma metastasis. Overall, 51% of the cell lines had alterations in either PTEN/MMAC1 or RAS. We found 16 cell lines (30%) with alterations in PTEN/MMAC1 and 11 cell lines (21%) with activating NRAS mutations; only 1 cell line had concurrent alterations in both genes. Moreover, glioma cell lines with a high frequency of PTEN/MMAC1 inactivation had no identifiable RAS alterations. Ectopic expression of PTEN in several cutaneous melanoma cell lines suppressed colony formation irrespective of PTEN/MMAC1 status; furthermore, PTEN expression in cell lines carrying activated RAS also suppressed colony formation. The relative reciprocity of PTEN/MMAC1 abrogation and NRAS activation suggests that the two genetic changes, in a subset of cutaneous melanomas, are functionally overlapping.

Introduction

The incidence of cutaneous melanoma has been rising over the past several decades. Although the pathogenetic mechanisms underlying melanoma tumor formation are still largely unknown, several genes have been shown to be targets for mutations in cutaneous melanoma. CDKN2A is the most frequently inactivated tumor suppressor gene in cutaneous melanoma (reviewed in 1), whereas the RAS genes are the most commonly mutated oncogenes described thus far for melanoma (2–7). Using these observations, Chin et al (8) generated a murine model of cutaneous melanoma that reflects the human genetics by melanocyte-specific expression of an oncogenic RAS gene on a CDKN2A-null background. Taken together, the human and murine data suggest that a RAS-dependent pathway is distinct from, and cooperates with, the CDKN2A/retinoblastoma (pRb) pathway in melanoma tumorigenesis. However, the downstream components of the RAS-affected pathway(s) in cutaneous melanoma are unknown.

We and others have recently reported that approximately 30% of cutaneous melanoma cell lines harbor mutations or deletions of the tumor suppressor gene, PTEN/MMAC1 (9, 10). Sequence analysis of the PTEN/MMAC1 gene revealed a dual serine/threonine and tyrosine phosphatase domain (11, 12), whereas biochemical analyses identified a lipid phosphatase function that can dephosphorylate PtdIns(3, 4, 5)P3 (13, 14). In mouse studies, homozygous elimination of PTEN/MMAC1 leads to early embryonic lethality (15–17). Stambolic et al (15) demonstrated that murine embryonic fibroblasts that lack PTEN function have elevated levels of PtdIns(3, 4, 5)P3 and PKB activity, a downstream signal target for PtdIns(3, 4, 5)P3. These biochemical data suggest that one function of PTEN is to negatively regulate the PI3-K/PKB pathway.

Several lines of evidence point to a possible genetic relationship between RAS and PTEN/MMAC1. Malignancies that have high rates of RAS mutations, such as colon cancer (18–21) and pancreatic cancer (22), have low rates of PTEN/MMAC1 alterations (23, 24); on the other hand, gliomas have a high frequency of PTEN/MMAC1 inactivation (11, 12, 25–27) but low rates of RAS mutations (28, 29). Biochemically, the induction of PI3-K activity and intracellular levels of PtdIns(3, 4, 5)P3 is mediated through RAS (30–32), and, thus, PTEN’s impact on this pathway may be affected by the level of RAS activity. We hypothesized that the loss of PTEN [a negative regulator of PtdIns(3, 4, 5)P3 levels] and the activation of RAS (a positive regulator of PI3-K) are functionally and—potentially genetically—equivalent in at least a subset of cutaneous melanomas. We, thus, set out to assess for frequency and type of RAS mutations in our panel of melanoma specimens that have been characterized for PTEN/MMAC1 alterations.

Materials and Methods

Cell lines and DNA. The human melanoma cell lines have been described previously (9). In addition, A375, CHL-1, Malme, and HS597 melanoma cell lines were obtained from American Type Culture Collection (Rockville, MD); the K melanoma cell lines were from Dr. K. Huebner (Kimmel Cancer Center, Philadelphia, PA). DNA from 18 glioma cell lines was provided by Dr. G. Robertson (Ludwig Institute for Cancer Research, San Diego, CA). DNA samples of uncultured metastatic melanomas (33) were obtained from Dr. K. Huebner (Kimmel Cancer Center, Philadelphia, PA).

A pSG5-PTEN plasmid containing PTEN cDNA was obtained from Dr. W. Sellers (Dana-Farber Cancer Institute, Boston, MA). The insert was subcloned into the pIRESpuro vector (Clontech, Palo Alto, CA) and pCDNA3.1neo vector (Invitrogen, Carlsbad, CA).

PCR-SSCP. Primers and conditions for PCR-SSCP analysis of the PTEN/MMAC1 gene have been described previously (9). PCR-SSCP analysis of the RAS genes used the following primer sets: (a) HRAS Exon 1F: 5′-CAGG-GCC-TGAGGACGATG-3′, and HRAS Exon 1R: 5′-TATTTGCTTCA-CAAAATGTTCTCT-3′; (b) HRAS Exon 2F: 5′-TCCTGCAAGATCCTAC-CGG-3′, and HRAS Exon 2R: 5′-GGTGCACCCTGACTGGTGGA-3′; (c) HRAS Exon 3F: 5′-GTTGCAAGAGGCTGAGTCT-3′, and HRAS Exon 3R: 5′-GGTGCACCCTGACTGGTGGA-3′; (d) HRAS Exon 4F: 5′-GAGGATGCTCGTCTCCTC-3′, and HRAS Exon 4R: 5′-GGTGCACCCTGACTGGTGGA-3′.

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3 The abbreviations used are: PtdIns(3, 4, 5)P3, phosphatidylinositol-3,4,5-triphosphates; PI3-K, phosphatidylinositol-3-kinase; PKB, protein kinase B; SSCP, single-strand conformation polymorphism.
KRAS exon 1F: 5'-GGCTCTGCTTGAATTCGTA-3', and KRAS exon 1R: 5'-GATCAGGCTACATATGTCG-3'; (d) KRAS exon 2F: 5'-TTCTCACTAGTGAAGTCTG-3' and KRAS exon 2R: 5'-CACACGAAAGCCCTCAGCA-3'; (e) NRAS exon 1F: 5'-CAGGTTCCTGTGGTGAAATGGACTGAG-3', and NRAS exon 1R: 5'-CTACCAGGGCTACTACCGTATG-3'; and (f) NRAS exon 2F: 5'-GTTTATAGGTTGAAACCTG-3', and NRAS exon 2R: 5'-ATACACAGGAGGCCTCCTG-3'.

Amplification was carried out in 10-μl reaction mixtures containing 1 μl of DNA, 2 μCi [α-32P]dCTP (NEB, Boston, MA), and 1 nM each primer under standard conditions. The samples were denatured at 95°C for 5 min, annealed for 30 s using a touchdown protocol (62°C for 2 cycles, 60°C for 2 cycles, 59°C for 2 cycles, 58°C for 2 cycles, 57°C for 3 cycles, 56°C for 3 cycles, and 55°C for 15 cycles), extended at 72°C for 10 min. The reactions were stopped with four volumes of stop buffer (95% formamide, 20 mM EDTA, 0.05% bromphenol blue, and 0.05% xylene cyanol). Samples were denatured at 95°C for 5 min, chilled on ice immediately for 5 min, and loaded directly onto a 0.5× MDE gel (FMC BioProducts, Rockland, ME), with and without glycerol, in 0.6× Tris-Borate EDTA (TBE) buffer. Fragments were subjected to electrophoresis at 4 W overnight at room temperature. After electrophoresis, the gels were dried and exposed to autoradiographic film without a screen for 12–24 h.

DNA samples showing mobility shifts were then prepared by PCR under the same condition, separated on agarose gel, purified using QIAquick kit (Qiagen, Inc., Santa Clarita, CA), and directly sequenced using AmpliCycle sequencing kit (Perkin Elmer, Foster City, CA) or submitted to the Massachussets General Hospital Sequencing Core Facility for automated sequencing.

Colonies Growth Suppression Assay. On the night before transfection, target cells were plated at 500,000 per well in a 6-well plate with DMEM/10% FCS (no antibiotics). One to two μg of column-purified plasmid (Qiagen, Inc., Santa Clarita, CA) was transfected with Lipofectamine Plus (Life Technologies, Inc., Gaithersburg, MD) using the manufacturer’s protocol. After 48 h, the cells were trypsinized into 100-mm dishes and allowed to settle overnight. The cells were then selected with the appropriate concentrations of G418 or puromycin (Sigma, St. Louis, MO) for 2–4 weeks and stained with Giemsa.

Results

Prevalence of NRAS Alterations in Cell Lines. Using PCR-SSCP, we evaluated 53 cutaneous melanoma cell lines, 17 uncultured cutaneous melanoma metastases, and 18 glioma cell lines for mutations in codons 12/13/61 of NRAS, KRAS, and H-RAS. We found a total of 11 NRAS mutations (11 of 53 or 21%; 1 at codon 12; 10 at codon 61) from our melanoma cell lines and 2 NRAS codon 61 mutations from our 17 uncultured melanoma metastases (2 of 17 or 12%). We did not detect any H-RAS or KRAS mutations in any melanoma samples. Fig. 1 is a representative PCR-SSCP gel delineating multiple NRAS exon 2 (codon 61) fragments with aberrant migration patterns. Fig. 2 shows the sequencing chromatograms for the NRAS codon 12 mutation (Fig. 2A) and the three NRAS Gln61 alterations (Fig. 2B). Table 1 lists all of the NRAS mutations from our cutaneous melanoma cell lines. Two uncultured melanoma metastases had NRAS Glu61Arg mutations.

We found no H-RAS, KRAS, or NRAS codon 12/13/61 mutations in the 18 glioma cell lines (data not shown).

Relative Exclusivity of PTEN/MMAC1 and NRAS Alterations. PTEN/MMAC1 was altered in 16 of our melanoma cell lines (16 of 53 or 30%; Table 1). NRAS was mutated in 1 of 16 of the melanoma cell lines with PTEN/MMAC1 mutations and 10 of 37 of the melanoma cell lines with wild-type PTEN/MMAC1. Overall 27 (51%) of 53 of our cutaneous melanoma cell lines had either PTEN/MMAC1 or NRAS mutations, although only 1 cell line (cell line HS 944) had alterations in both genes. The two uncultured cutaneous melanoma metastases that harbored NRAS codon 61 mutations had wild-type PTEN/MMAC1 (data not shown). Furthermore, Furnari et al. (34) have previously shown that 14 of the 18 glioma cell lines harbor PTEN/MMAC1 alterations.

In our total analysis, 12 of 56 specimens with normal PTEN/MMAC1 harbored NRAS mutations compared with 1 of 32 specimens with aberrant PTEN/MMAC1 (Fisher’s exact test, P = 0.027); thus, the reciprocity of mutations does not appear to be random.

Suppression of Colony Formation by PTEN in the context of PTEN/MMAC1 and RAS Genotypes. We next explored the in vitro colony suppressive function of PTEN in the context of defined genetic backgrounds. Fig. 3A shows colony suppression by PTEN in a PTEN/MMAC1 del/RAS wt background (cell line UACC903), PTEN/MMAC1wt/RASwt background (cell line A375) and PTEN/MMAC1wt/RASdel background (cell line 7520).
contains both mutations of both genes. Our finding of a single cell line that activated may actually be selectively disadvantageous. For instance, activation of RAS and PTEN on PI3-K support this model best. Alternatively, to date, functional data that suggest a reciprocal regulatory effect would be conferred by a second mutation. In other words, in cells setting of a mutation in one of these genes, no selective advantage (35–37), and, if RAS mutated melanomas and that oncogenic activation of the former and inactivation of the latter may be largely equivalent. This relative reciprocity suggests that PTEN/MMAC1 and NRAS status of cutaneous melanoma cell lines shows the divergence of RAS pathways; in this cell line, additional RAS-mediated events may confer an even greater proliferative advantage, prevent RAS-mediated toxicity, or affect the cell at a different stage of tumor progression.

Because only one-half of our cell lines demonstrate alterations in either gene, other unidentified genes are likely involved in the remaining cell lines. Although the complete pathway integrating RAS and PTEN signaling are still unknown, several identified components are critical in cancer biology. PI3-K is known to be a downstream target of RAS (38, 39), and, recently, Shayesteh et al. (40) found that amplification of the PI-3 K gene, PTEN/MMAC1, may be an important step in the pathogenesis of ovarian cancers. The phospholipid products of PI-3 K, which are substrates of PTEN, activate PKB/C-AKT (41), a protein kinase that, in its constitutively activated form (V-AKT), is a retroviral oncoprotein (42, 43). Two protein substrates of PKB are also involved in cancer: BAD (44), a negative regulator of Bcl-2, and FKHRL1 (45), a member of the human Forkhead family that has been shown to be involved in human malignancies (46, 47).

Functionally, PTEN is able to suppress growth regardless of the endogenous PTEN/MMAC1 status. Li et al. (48) reported similar findings in breast cancer. This is in sharp contrast to glioma cells, in which PTEN is ineffective in the context of a wild-type PTEN/MMAC1 (34). This raises the possibility that other unidentified alterations are potentially upstream of PTEN in cutaneous melanoma and downstream of PTEN in gliomas. In particular, PTEN is able to suppress cell lines with normal PTEN/MMAC1 and activating NRAS mutations. The ability of PTEN to suppress cell lines with mutated RAS is consistent with a function for PTEN downstream of RAS. Along these lines, we found that PTEN is also able to suppress the growth of both NIH3T3 cells and v-RAS-transformed-NIH3T3 cells with equivalent efficacy (data not shown). In our earlier experiments, HS944 (the only cell line with both PTEN/MMAC1 and NRAS alterations) seemed relatively resistant to PTEN suppression. Some of the resistance to growth suppression that is seen in HS944 may reflect the divergence of RAS pathways; in this cell line, additional RAS-mediated events may confer an even greater proliferative advantage, prevent RAS-mediated toxicity, or affect the cell at a different stage of tumor progression.

### Discussion

Our results suggest, for the first time, that RAS and PTEN/MMAC1 may be genetically linked in at least a subset of cutaneous melanomas and that oncogenic activation of the former and inactivation of the latter may be largely equivalent. This relative reciprocity suggests that RAS and PTEN/MMAC1 may lie on a genetic pathway that is commonly abrogated in cutaneous melanoma. Consistent with the melanoma cell line results, the glioma cell lines have a high rate of PTEN/MMAC1 inactivation (14 of 18 or 78%; Ref. 34) but no detectable activating RAS mutations. Two models are consistent with our observations. If the activation of RAS and the loss of PTEN/MMAC1 are equivalent, then in the setting of a mutation in one of these genes, no selective advantage would be conferred by a second mutation. In other words, in cells carrying one mutation, a second mutation would be superfluous. To date, functional data that suggest a reciprocal regulatory effect of RAS and PTEN on PI3-K support this model best. Alternatively, the presence of oncogenic RAS and concurrent PTEN/MMAC1 loss may actually be selectively disadvantageous. For instance, activated RAS can promote apoptosis in certain genetic backgrounds (35–37), and, if RAS activation and PTEN/MMAC loss function cooperatively in this manner, one would not see concomitant mutations of both genes. Our finding of a single cell line that contains both RAS and PTEN/MMAC1 alterations may reflect the
use of the pCDNA3 plasmid instead of the pIRES vector. As can be seen for UACC903, both pCDNA3 and pIRES clearly suppressed growth, but the pIRES vector in our hands seems to be more effective.

Alternatively, the intracellular levels of PtdIns(3, 4, 5)P3 in the presence of both activating RAS and inactivating PTEN/MMAC1 alterations may be higher than the levels resulting from either change alone, and, thus, exogenous expression of PTEN produces a stoichiometrically reduced effect.

In summary, we provide the first genetic evidence in cutaneous melanoma that PTEN/MMAC1 may be a critical component of a RAS-sensitive pathway. Both the human mutational studies and the murine models support the existence of such a pathway. Whether other genes that interact with RAS and PTEN/MMAC1 are also targeted for mutations in melanoma remains to be established. Furthermore, the robust tumor-suppressive effect resulting from restoration of PTEN may have therapeutic implications for cutaneous melanoma.
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