Novel MEK1 Mutation Identified by Mutational Analysis of Epidermal Growth Factor Receptor Signaling Pathway Genes in Lung Adenocarcinoma

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Abstract

Genetic lesions affecting a number of kinases and other elements within the epidermal growth factor receptor (EGFR) signaling pathway have been implicated in the pathogenesis of human non–small-cell lung cancer (NSCLC). We performed mutational profiling of a large cohort of lung adenocarcinomas to uncover other potential somatic mutations in genes of this pathway that could contribute to lung tumorigenesis. We have identified in 2 of 207 primary lung tumors a somatic activating mutation in exon 2 of MEK1 (i.e., mitogen-activated protein kinase kinase 1 or MAP2K1) that substitutes aspartic acid for lysine at amino acid 57 (K57N) in the nonkinase portion of the kinase. Neither of these two tumors harbored known mutations in other genes encoding components of the EGFR signaling pathway (i.e., EGFR, HER2, KRAS, PIK3CA, and BRAF). Expression of mutant, but not wild-type, MEK1 leads to constitutive activity of extracellular signal–regulated kinase (ERK)-1/2 in human 293T cells and to growth factor–independent proliferation of murine Ba/F3 cells. A selective MEK inhibitor, AZD6244, inhibits mutant-induced ERK activity in 293T cells and growth of mutant-bearing Ba/F3 cells. We also screened 85 NSCLC cell lines for MEK1 exon 2 mutations; one line (NCI-H1437) harbors a Q56P substitution, a known transformation-competent allele of MEK1 originally identified in rat fibroblasts (6), and engineered mutants (with alterations of the key regulatory serine residues) can transform NIH 3T3 cells (7). MEK1/2 mutants have not yet been reported in lung cancer and may provide a target for effective therapy in a small subset of patients with lung adenocarcinoma. [Cancer Res 2008;68(14):5524–8]

Introduction

Lung cancer is the leading cause of cancer-related deaths worldwide (1). Mutational profiling studies have revealed that at least five genes encoding epidermal growth factor receptor (EGFR) signaling pathway components are mutated in non–small-cell lung cancers (NSCLC). Whereas EGFR and KRAS mutations are detected in ~10% and 20% of tumors, respectively, somatic mutations have also been identified in HER2/ERBB2 (~2%; exons 19 and 20), the lipid kinase PIK3CA (~4%; exons 9 and 20), and the serine/threonine kinase BRAF (~2%; exons 11 and 15; ref. 2). All alterations have been shown to confer gain-of-function properties in comparison with their respective wild-type counterparts. Except for PIK3CA alterations (3), mutations in EGFR, HER2, BRAF, and KRAS rarely occur in the same tumor, suggesting that they may have functionally equivalent roles in lung tumorigenesis. These mutations are found predominantly in tumors with adenocarcinoma histology.

The mitogen-activated protein kinase (MAPK) pathway plays a major role in the EGFR signaling cascade. After activation of EGFR signaling, key downstream steps involve phosphorylation by RAF1 kinase of two distinct serine residues on both MEK1 and MEK2 (4). The MEK proteins share 80% sequence homology and encode dual-specificity kinases of the STE kinase family (homologues of yeast sterile-7, -11, and -20; ref. 5). MEK1/2 subsequently phosphorylate specific threonine and tyrosine residues in the activation loops of ERK1/2.

Altered MEK proteins have been implicated in cancer. A transformation-competent mutant of MEK1 with a Q56P substitution in the nonkinase portion of the kinase has been described in rat fibroblasts (6), and engineered mutants (with alterations of the key regulatory serine residues) can transform NIH 3T3 cells (7). Recently, a human ovarian cancer cell line, ES-2, was found to harbor an activating mutation in MEK1 that substitutes asparagine for aspartic acid at position 67 (D67N; ref. 8). Moreover, small-molecule inhibitors of MEK seem to be promising as antimutator agents (9). However, to our knowledge, somatic mutations in MEK1/2 have not yet been reported in lung cancer (9).

Here, we report identification of a novel somatic mutation in MEK1 in human lung tumors, identified via mutational profiling of genes encoding EGFR signaling pathway proteins in a large cohort of lung adenocarcinomas (10). We determine the functional consequences of this genetic alteration in two separate cell systems. We also examine a large collection of primary tumors and cell lines for the presence of this and other MEK1 exon 2 mutations.

Materials and Methods

Tissue procurement and mutational profiling. All specimens were obtained with patients’ consent via a protocol approved by the Memorial Sloan-Kettering Cancer Center Institutional Review Board. DNA was extracted from formalin-fixed paraffin-embedded tissue sections using the QIAGEN genomic DNA kit (Valencia, CA). DNA from 207 lung adenocarcinomas and 85 NSCLC cell lines was evaluated for MEK1 mutations by fluorescence polarization-based high-throughput sequencing.

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9 http://www.sanger.ac.uk/genetics/CGP/cosmic
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Sloan-Kettering Cancer Center Institutional Review Board (see ref. 10 for details). Cell lines were obtained from American Type Culture Collection and Deutsche Sammlung von Mikroorganismen und Zellkulturen (DSMZ).

**Mutation verification.** See ref. 10 for details. All mutations were verified by bidirectional sequence analysis of an independent PCR product. Variants were deemed somatic if they were found to be absent in matched normal tissue. A combination of dideoxynucleotide sequencing and/or mass spectrometry–based genotyping (Sequenom) was used to screen additional tumor samples for MEK1 exon 2 mutations. Primers used for MEK1 exon 2 sequencing were 5′-TTTCTTTCCATGATAGGAGT-3′ and 5′-ATCAGTCTT-CCTTCTACCT-3′.

**Expression constructs.** The full-length human MEK1 cDNA cloned into the pDRN-Dual vector was obtained from the Harvard Institute of Proteomics. Specific mutations were generated using a QuikChange Site-Directed Mutagenesis Kit (Stratagene) with the following primers: 5′-TTACCAGAAATCAGAAGGTGG-3′ and 5′-CCACCTCTGATCTGGG-TAA-3′ to create MEK1 K57N, and 5′-GCTGGCGCTAGGGAAGCTTT-3′ and 5′-AACAGTCTCAGACCCACGC-3′ to recreate a stop codon. MEK1 WT and MEK1 K57N cDNAs were cloned into pLP-CMVneo plasmid using the Creator DNA Cloning Kit (Clontech). Expression plasmids encoding mutant BRAF V600E or KRAS G12V were kind gifts from J. Fagin (Memorial Sloan-Kettering Cancer Center, New York, NY) and M. Phillips (New York University, New York, NY), respectively.

The full-length human ERBB4 cDNA was obtained from Origene. The N181S mutation was generated using the QuikChange Kit and primers 5′-GCTGGCGCTAGGGAAGCTTT-3′ and 5′-TGAACTACCACTT-GTTGACAC-3′ to create MEK1 K57N, and 5′-AAAGCTTCCCTAGACGCCAGC-3′ to recreate a stop codon.

**Cell culture.** 293T cells, maintained as per established protocols, were transfected with pLP-CMVneo vector alone, pLP-CMVneo MEK1 WT, pLP-CMV-neo MEK1 K57N, pCMV-XL5 ERBB4 WT, or pCMV6-XL5 ERBB4 N181S using FuGENE 6 Transfection Reagent (Roche Diagnostics).

Ba/F3 cells, maintained as per established protocols, were transfected with expression plasmids using Nucleofector II and the Cell Line Optimization Kit V (Amza Biosystems). Stable clones were then derived by 14-d selection in 1.25 mg/ml G418. To obtain interleukin-3 (IL-3)–independent Ba/F3 clones, IL-3 was removed from the cell culture media. Ten days after the first round of IL-3 withdrawal, ~17% and 4% of cells harboring mutant and wild-type cDNAs, respectively, were viable. These cells were then recultured in the presence of IL-3, and after a second round of IL-3 withdrawal, ~50% and 2% of cells harboring mutant and wild-type cells, respectively, were viable (data not shown). After a third round of IL-3 withdrawal, the majority of cells harboring mutant MEK1 cDNAs survived and were able to proliferate in the absence of IL-3. No cells harboring wild-type MEK1 cDNAs were alive. Comparable results were obtained from an independent transfection of parental Ba/F3 cells. All IL-3–independent clones were verified by direct sequencing to harbor mutant cDNAs.

**Immunoblotting.** Cell lysates were examined using established protocols. Total MEK1, phospho-ERK1/2 (Thr202/Tyr204), total ERK1/2, and the kinase domain of MEK1 were assessed by 14-d selection in 1.25 mg/ml G418. To obtain interleukin-3 (IL-3)–independent clones, IL-3 was removed from the cell culture media. Ten days after the first round of IL-3 withdrawal, 17% and 4% of cells harboring mutant and wild-type cDNAs, respectively, were viable. These cells were then recultured in the presence of IL-3, and after a second round of IL-3 withdrawal, ~50% and 2% of cells harboring mutant and wild-type cells, respectively, were viable (data not shown). After a third round of IL-3 withdrawal, the majority of cells harboring mutant MEK1 cDNAs survived and were able to proliferate in the absence of IL-3. No cells harboring wild-type MEK1 cDNAs were alive. Comparable results were obtained from an independent transfection of parental Ba/F3 cells. All IL-3–independent clones were verified by direct sequencing to harbor mutant cDNAs.

**Cellular assays.** To assess cellular proliferation, G418-resistant Ba/F3 cells carrying either MEK1 WT or MEK1 K57N, respectively, were plated in the absence of IL-3 on day 0, along with parental Ba/F3 cells cultured with or without IL-3. Viable cells were counted by trypan blue staining each day for 4 consecutive days. Values reported were calculated as percent of viable cells compared with those obtained from day 0. All assays were done at least two independent times.

To assess cell growth inhibition, cells were seeded in 96-well plates (6 × 10^3–8 × 10^3 per well) in triplicate and treated with different concentrations of AZD6244. Cell growth inhibition was measured at 48 h posttreatment using CellTiter Blue Reagent (Promega). All assays were done at least two independent times.

![Figure 1](https://example.com/image1.png)

**Figure 1.** Identification of a MEK1 K57N mutation in human lung adenocarcinoma. A. reverse sequencing chromatograms display presence of a G→T mutation at position 171 in exon 2 of MEK1 in two tumor samples. This mutation was absent from matched normal control samples. (Forward sequence chromatograms not shown due to space constraints.) B. protein sequence alignment of MEK1 from various species shows that K57 is a highly conserved residue. Numbers indicate amino acid positions. C. K57N is located in between the nuclear export signal (NES) and the kinase domain of MEK1. D. docking domain. Shown are mutations found in patients with cardio-facio-cutaneous (CFC) syndrome.
Results

We analyzed genomic DNA from a total of 261 resected, clinically annotated NSCLC specimens (10). We screened the coding sequences of 39 genes encoding mostly components of the EGFR signaling pathway for somatic mutations via high-throughput dideoxynucleotide sequencing of PCR-amplified gene products. Sequencing of 9MB of tumor sequence identified 239 putative nonsynonymous sequence variations that differed from reference sequences listed in the National Center for Biotechnology Information (RefSeq) database for each respective gene. We previously reported the examination of 22 sequence variations found in RAS family genes and 135 sequence variations localized to exons encoding the protein kinase domains (10). In that study, we identified a total of 37 nonsynonymous somatic mutations, found collectively in EGFR, HER2, KRAS, BRAF, PIK3CA, and FGFR4 (10).

Here, we examined the remaining putative genetic variants occurring in exons encoding domains outside of the kinase regions of their respective kinases. Of 82 putative nonkinase domain sequence variations, representing 69 distinct types of mutations, we confirmed the existence of 37 nonsynonymous somatic mutations, found collectively in EGFR, HER2, KRAS, BRAF, PIK3CA, and FGFR4 (10).

Here, we examined the remaining putative genetic variants occurring in exons encoding domains outside of the kinase regions of their respective kinases. Of 82 putative nonkinase domain sequence variations, representing 69 distinct types of mutations, we confirmed the existence of 27 (18 distinct types) on sequencing a second independent PCR product for each respective gene. We identified a total of 37 nonsynonymous somatic mutations, found collectively in EGFR, HER2, KRAS, BRAF, PIK3CA, and FGFR4 (10).

Figure 2. Functional characterization of the MEK1 K57N mutant. A, 293T cells were transiently transfected with expression plasmids encoding various cDNAs, and corresponding lysates from cells maintained in serum were subjected to immunoblotting with the indicated antibodies. Lysates from cells harboring MEK1 cDNAs displayed higher levels of total MEK1 protein compared with control-transfected cells, but only cells transfected with MEK1 cDNAs displayed enhanced phospho-ERK expression, at levels comparable to those induced by mutants BRAF and KRAS. B, Ba/F3 cells were stably transfected with vectors encoding wild-type or MEK1 K57N cDNAs. The resulting cells were then cultured in the absence of IL-3. Numbers of live cells were counted daily for 4 d. Parental Ba/F3 cells grown in the absence or presence of IL-3 serve as controls. C, lysates from IL-3-independent K57N Ba/F3 cells display high levels of pERK and tMEK1 compared with parental Ba/F3 cells grown in the absence of IL-3. Immunoblotting was done with the indicated antibodies.
phosphorylation in 293T cells. Extracts from cells transiently transfected with plasmids encoding wild-type or mutant MEK1 cDNAs displayed higher levels of total MEK1 protein than did control-transfected cells, but only cells transfected with MEK1K57N cDNAs displayed enhanced levels of ERK phosphorylation (Fig. 2A). We observed similar levels of induced ERK phosphorylation in lysates from cells transfected with cDNAs encoding two well-characterized mutants, BRAFV600E and KRASG12V, known to activate the ERK pathway (Fig. 2A).

To characterize additional functional consequences of the K57N change, we generated stable polyclonal populations of Ba/F3 cells expressing wild-type or mutant MEK1. Ba/F3 cells are a murine pro-B cell line that is normally dependent on IL-3 for growth, but they can be rendered IL-3 independent by introduction of transforming tyrosine kinases such as BCR-ABL (11). For some oncogenic proteins, very high levels of mutant kinase are required to derive growth factor independence (12). In two independent assays, we derived Ba/F3 cells harboring cDNAs encoding MEK1K57N that grew in the absence of IL-3. By contrast, we could not derive IL-3–independent Ba/F3 cells expressing the wild-type kinase (Fig. 2B). The growth rate of IL-3–independent Ba/F3 cells expressing mutant MEK1K57N was similar to that of parental Ba/F3 cells cultured in the presence of IL-3 (Fig. 2B). Immunoblotting of lysates from the cells harboring mutant MEK1 revealed very high levels of total MEK1 protein and ERK phosphorylation compared with parental Ba/F3 controls (Fig. 2C). Thus, overexpression of mutant, but not wild-type, MEK1 is able to convert Ba/F3 cells to cytokine-independent growth.

We next assessed whether a small-molecule MEK inhibitor, AZD6244 (13), could affect biological properties induced by the MEK1 mutant. AZD6244 treatment of 293T cells transiently transfected with plasmids encoding MEK1K57N cDNAs readily inhibited the appearance of ERK phosphorylation (Fig. 3A). Moreover, compared with parental Ba/F3 cells growing in IL-3, IL-3–independent MEK1K57N–harboring Ba/F3 cells were more sensitive to the MEK inhibitor (Fig. 3B). Because the administration of IL-3 to cultures of cytokine-independent MEK1K57N–harboring Ba/F3 cells prevented AZD6244-induced growth inhibition, these data suggest that MEK inhibitors may effectively target cancer cells that depend on the activity of mutant MEK1 for survival.

Finally, we screened an additional 114 NSCLCs (including 33 squamous cell carcinomas) for the MEK1K57N change or other mutations in exon 2 of MEK1. No tumors harbored the MEK1K57N mutation. We also examined an additional 85 NSCLC cell lines. Whereas no lines carried the K57N mutation, we did find that NCI-H1437 cells harbor a mutation encoding a Q56P MEK1 variant (data not shown). These cells were originally derived from a male smoker. They do not carry mutations in EGFR, KRAS, BRAF, or...
PK3CA but do display an inferred copy number of up to 3 at the genomic region of MEK1 on chromosome 15.10 H1437 cells also display sensitivity in the nanomolar range to AZD6244 (data not shown).11 We did not detect any other MEK1 exon 2 mutations in 19 primary samples from patients with chronic myelomonocytic leukemia (with wild-type KRAS) or in 54 additional tumor cell lines (14 melanomas, 13 colon carcinomas, 13 breast carcinomas, 6 neuroblastoma/neuroepithelial tumors, 4 prostate carcinomas, and 4 pancreas carcinomas).

Discussion

We report the identification of a novel somatic mutation in MEK1 in human lung adenocarcinoma. The same heterozygous mutation, K57N, was found in 2 of 207 NSCLCs, neither of which harbored mutations in other genes encoding components of the EGFR signaling pathway. Functional characterization of this mutant in vitro indicates that its expression in 293T cells leads to constitutive activation of downstream signaling components (i.e., phospho-ERK activity) and in Ba/F3 cells confers IL-3 independence. Collectively, these results indicate that, in two separate in vitro systems, MEK1K57N mutants display gain-of-function properties compared with wild-type protein.

K57 is located in a region between the nuclear export signal and catalytic domain of MEK1. How the K57N substitution affects the structure of MEK1 is unclear, as the solved crystal structure of the catalytic domain of MEK1. How the K57N substitution affects the structure of MEK1 is unclear, as the solved crystal structure of MEK1. Nonetheless, multiple functional studies involving amino acids in this region of the protein show that it seems to be critical for the regulation of MEK1 activity. For example, in the study that originally identified the Q56R mutation, MEK1Q56R-glutathione-S-transferase fusion proteins were ~170-fold more active than the wild-type counterparts in terms of their ability to phosphorylate recombinant ERK in vitro (6). In a separate study, deletion of a predicted α-helix encompassing residues 32 to 51 resulted in a mutant (ΔN3) with basal activity 45 times greater than that of the wild-type enzyme (7). Similarly, the K57N substitution, shown here, and the D67N substitution (8) both lead to constitutive activation of the MAPK pathway in vitro.

Whereas somatic alterations in MEK1 have not yet been reported in human lung cancers, recent studies have identified germ-line MEK1 mutations in patients with cardiac-facio-cutaneous (CFC) syndrome, a complex developmental disorder involving the heart, face, and skin (15). Mutations in affected individuals include F53S, del59K, P124L/Q, G128V, and Y130C (Fig. IC), the latter two of which occur in the kinase domain (15, 16). The F53S mutant, like the Y130C mutant, is more active than wild type protein in stimulating ERK phosphorylation (15). It is not yet clear if CFC patients are at an increased risk for cancer, but three affected individuals have developed neoplasms (i.e., rhabdomyosarcoma, hepatoblastoma, and acute lymphoblastic leukemia; refs. 17–19).

In future studies, it will be interesting to compare the functional consequences of germ-line versus somatic mutations in MEK1. Notably, germ-line mutations in KRAS and BRAF, associated with Noonan and CFC syndromes, respectively, are also different from their somatic counterparts found in human cancers (15, 20). Finally, we show that the downstream effects induced by the MEK1K57N mutant can be inhibited by treatment with a selective MEK inhibitor. Taken together, our results suggest that mutant MEK1 may provide a target for anticancer therapy in a small subset of patients with lung adenocarcinoma.

Disclosure of Potential Conflicts of Interest

No potential conflicts of interest were disclosed.

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10 K. Michel and R. Thomas, unpublished data.
11 P. Smith (AstraZeneca), personal communication.

References

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