Alterations of the PPP1R3 Gene in Human Cancer

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Abstract

Recently, the PTEN/MMAC1 gene encoding a protein phosphatase (PP) and the PPP2R1B gene encoding a regulatory subunit of PP2A have been identified as being genetically altered in several types of human cancers, indicating that aberrations of intracellular signaling pathways via PPs are involved in human carcinogenesis. Here we report genetic alterations of the PPP1R3 gene located at chromosome 7q31, which encodes regulatory subunit 3 of PP1, in various types of human cancers. Mutations of the PPP1R3 gene were detected in 5 of 33 (15%) non-small cell lung cancer cell lines and 2 of 38 (5%) primary non-small cell lung cancers and were also observed in cell lines derived from a small cell lung cancer, an ovarian cancer, a colorectal cancer, and a gastric cancer. Mutations were widely dispersed in the coding region of the PPP1R3 gene. Three of the 11 detected mutations were nonsense mutations, whereas the remaining ones were missense mutations, most of which caused substitutions of evolutionarily conserved amino acids. These findings suggest that PPP1R3 alteration plays a role in the development of human cancers and that PPP1R3 could act as a tumor suppressor gene.

Introduction

PPIs have important roles in regulating a variety of cellular processes, including metabolism, the cell cycle, and intracellular signaling (1). Recently, the PTEN/MMAC1 gene encoding a dual specificity PP has been identified as a tumor suppressor gene that is inactivated in a variety of human cancers including glioma, prostate cancer, melanoma, endometrial cancer, and lung cancer (1–4). The PTEN gene was also reported as being responsible for Cowden disease and Bannayan-Zonana syndrome, which are autosomal dominant disorders of hamartomatous polyposis syndromes (1). Genetic alterations of the PPP2R1B gene encoding a regulatory subunit of PP2A have been detected in lung and colorectal cancers (5). Therefore, it is highly possible that aberrations of several other PPs are involved in human carcinogenesis.

PPP1R3 is an important regulatory enzyme that relays intracellular signals for cell proliferation, RNA splicing, and glycogen metabolism (6, 7). The PPP1A holoenzyme exists as a dimer consisting of a catalytic subunit and a regulatory subunit. Regulatory subunits modulate physiological functions of PPP1 by regulating enzymatic activity and specificity and/or targeting catalytic subunit(s) to various subcellular structures and substrates. The PPP1R3 gene, which encodes a polypeptide of 1122 amino acids corresponding to the regulatory subunit 3 of PP1, has been mapped to chromosome 7q31.1–q31.2 (Refs. 8 and 9; Fig. 1a). 7q31 is known to be a region that is frequently affected in human cancers. Loss of heterozygosity at 7q31 has been detected in a variety of human cancers, including lung, breast, gastric, colorectal, kidney, and ovarian cancers (10–12). Chromosome 7q deletion is also a common chromosomal alteration in hematological malignancies, and a common region of 7q deletions has been mapped to 7q31 (12, 13). These results indicate the presence of a tumor suppressor gene(s) in the 7q31 region.

In the present study, we performed mutational analysis of the PPP1R3 gene in 104 cancer-derived cell lines of various histological types and in 55 primary lung cancers to define whether PPP1R3 alterations are involved in human carcinogenesis. Mutations of the PPP1R3 gene were detected in the cell lines derived from five NSCLCs, a SCLC, an ovarian cancer, a colorectal cancer, and a gastric cancer. Somatic PPP1R3 mutations were also observed in two cases of primary lung cancers. Three of the detected mutations were nonsense mutations, which resulted in the production of truncated PPP1R3 proteins. This result indicates that PPP1R3 alteration plays a role in the development of a subset of human cancers.

Materials and Methods

Cell Lines and Tumor Samples. Thirty-three NSCLC cell lines (A427, A549, Lu65, Lu99, H23, H157, H322, H441, H520, H596, H1155, PC3, PC7, PC9, PC10, PC13, Ma1, Ma2, Ma3, Ma10, Ma12, Ma17, Ma24, Ma25, Ma26, Ma29, Ma31, LC1-Sq, RERF-LCD, RERF-LCOK, RERF-LCMS, ABC1, and EBC1), 14 SCLC cell lines (H69, H82, H209, H526, N417, Lu24, Lu130, Lu134, Lu135, Lu139, Lu140, SBC5, LCMA, and MS18), 7 ovarian cancer cell lines (A2780, 2008, MCF526, OV1063, TKY-nu, and SKOV3), 7 colorectal cancer cell lines (LOVO, DLD1, HCT15, HCT116, KM12, COLO205, and COLO321), 7 gastric cancer cell lines (MKN1, MKN7, OKAJIMA, TMK1, KATOIII, HSC39, and MKN45), 11 renal cancer cell cancer cell lines (RC1, RC2, RC3, RC4, RC5, RC6, KC12, KT1A, KCL1, KP1K1, and KN41), 9 breast cancer cell lines (UACC812, UACC893, BT247, SKBR3, T47D, BT20, ZR75–1, MCF7, and MDA-MB-436), and 16 leukemia/lymphoma cell lines (AML-1, HEL, MOLM1, UT-7, Kasumi-2, CML-BC1, OIHL-1, KG-1, SCC-3, N1, NS9-22, TS9-22, SKW3, and SKW4) were used in this study. Detailed information on these cell lines can be obtained upon request. Fifty-five primary lung tumors (38 NSCLCs and 17 SCLCs) and corresponding noncancerous tissues were obtained from 55 patients with lung cancer who were treated at National Cancer Center Hospital (Tokyo, Japan). The material to be analyzed was selected by a pathologist to ensure that the samples were macroscopically entirely tumorous and chosen from an area devoid of necrotic tissue. Tumors and normal tissues were frozen and stored at −70°C until DNA extraction.

PCR-SSCP Analysis. High molecular weight DNA was prepared from the cell lines, tumors, and adjacent noncancerous tissues as described previously (14). Exons 1–4 of the PPP1R3 gene were amplified for SSCP analysis with eight sets of PCR primers: (a) 1aF (5'-GCCCTCTACTGTCAGTACTG-3') and 1aR (5'-TACCCCTGTTAATCCTGAGGACCT-3') and 1bR (5'-GGGTACACTAATGCTAATG-3') for exon 1; (b) 2F (5'-CTTTAGAGATGTCAGTCCCCAG-3') and 2FR (5'-TCTTCAAATACCTGCTGGAC-3') for exons 2 and 3; (c) 4aF (5'-ATCTACTACAGATAAATTAACCGCCACC-3') and 4aR (5'-GGCTTACCCCATATACCT-3') for exon 4.

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4 The abbreviations used are: PP, protein phosphatase; SCLC, small cell lung cancer; NSCLC, non-small cell lung cancer; SSCP, single-strand conformational polymorphism; RT-PCR, reverse transcription-PCR; GAPDH, glyceraldehyde-3-phosphate dehydrogenase.
Fig. 1. PPP1R3 mutations in human cancers. a, top, schematic representation of the PPP1R3 gene product and positions of mutations in cancer cells. Pink, blue, and purple areas, the putative binding region for PP1 catalytic subunits, the putative glycogen-binding region, and the potential transmembrane region, respectively (8, 20). Red and black arrowheads, the positions of nonsense and missense mutations detected in cancer cells, respectively. Black arrows, the positions of genetic polymorphisms. a, bottom, alignment of amino acid sequences of human and rabbit PPP1R3. Amino acids mutated in human cancers are shaded. Sequence alignment was carried out by Chen et al. (8). Bars and colons, identical and conserved amino acids, respectively. b, PCR-SSCP analysis. Representative results of PCR-SSCP analysis using primer pairs of 2/3F and 2/3R, 1bF and 1bR, and 4eF and 4eR are shown. Shifted bands are indicated by arrows. c, sequence analysis. Nucleotide changes are apparent when compared to unrelated normal DNA samples (H157, Lu140, and SKOV3) or to a matched normal DNA sample (1371T). In the Lu140 cell line, histograms for sequencing in the reverse direction are shown. The mutated nucleotides are underlined.
CTTC-3’), 4bF (5’-TCCAAACAATCTTGACACA-3’) and 4bR (5’-ACACAGCGCTGTTTGGTTCG-3’), 4cF (5’-CCTCTGCAGTGCATCGAGC-3’) and 4cR (5’-AAGAGCAAAGATGACATGGC-3’), 4dF (5’-TGGGAAATAGTACCCAGAC-3’) and 4dR (5’-CAGCTTCTGCTTTCTCAG-3’), and 4eF (5’-GGGAAAATCTTCTAGGCCC-3’) and 4eR (5’-AGAGAGATGATGTGCTGAG-3’) for exon 4. Fifty ng of genomic DNA were suspended in a total volume of 20 µl of PCR buffer containing 10 mM Tris-HCl (pH 9.0), 50 mM KCl, 1.5 mM MgCl2, 300 nM each primer, 200 µM deoxynucleotide triphosphate, 1.5 µCi of [α-32P]dCTP (Amersham Pharmacia Biotech), and 0.5 unit of Taq DNA polymerase (Amersham Pharmacia Biotech). PCR conditions were 60 s at 95°C, 60 s at 55°C or 60°C, and 90 s at 72°C for 35 cycles, followed by 10 min at 72°C. PCR products by 1aF and 1aR primers, 1bF and 1bR primers, 2aF and 2aR primers, 2bF and 2bR primers, and 2cF and 2cR primers, and 4dF and 4dR primers were subjected to SSCP analysis after digestion with restriction enzymes AccI, MspI, AccI, AluI, PstI, HincII, and HinfI, respectively. SSCP analysis was performed in the low pH buffer system that showed improved separation of long mutant fragments (14). Gels were dried and exposed to Kodak XAR films for 4–48 h at ~80°C.

**Sequence Analysis.** PCR products showing different mobilities were purified using a QIA quick-spin purification kit (Qiagen) and sequenced directly in both directions with the Thermo-Sequence dye terminator cycle sequencing pre-mix kits (Amersham Pharmacia Biotech), and the ABI 373S DNA Sequence System (Perkin-Elmer).

**RT-PCR Analysis.** Polyadenylated mRNA was prepared from the cell lines by using the Fast Track mRNA isolation kit (Invitrogen). Polyadenylated mRNA from human normal tissues was purchased from Clontech. Randomly primed cDNAs were reverse-transcribed from 0.5 µg of mRNA by using SuperScriptII reverse transcriptase (Life Technologies, Inc.) according to the manufacturer’s protocol. PCR conditions were 60 s at 95°C, 60 s at 60°C, and 60 s at 72°C for 37 cycles (for PPP1R3) or for 25 cycles (for GAPDH), followed by 10 min at 72°C. A set of primers (5’-TTGTTCTCCTAGGAGCAAGG-3’ and 5’-TCCATCTCTGGAAGCGTACT-3’) was used for the PPP1R3 gene, and another set of primers (5’-AAGGTCTCATCCATGA-CAC-3’ and 5’-CACCTGTTTGCTGTAGCCA-3’) was used for the GAPDH gene. PCR products were electrophoresed on a 3.0% agarose gel and stained with ethidium bromide.

**Results.**

**Mutations of the PPP1R3 Gene in Cancer-Derived Cell Lines.** We examined PPP1R3 mutations in 104 cancer-derived cell lines, including those derived from cancers of the lung, colon, breast, stomach, and ovary, and leukemias/lymphomas. The entire coding region of the PPP1R3 gene encompassing four exons was amplified by PCR using eight sets of primers, and the products were subjected to SSCP analysis. SSCP bands with distinct electrophoretic mobilities due to genetic polymorphisms at codons 883 and 905, which have been detected previously (9, 16), were observed in these cell lines (Table 1; Fig. 1a). In addition, two novel polymorphisms at codons 494 and 600 were detected in 50 normal DNA samples (Table 1). Interestingly, nine cell lines (five NSCLCs, one SCLC, one ovarian cancer, one colorectal cancer, and one gastric cancer) showed bands with distinct electrophoretic mobilities (Fig. 1b). Direct sequence analysis of these bands revealed the presence of nucleotide substitutions leading to alterations in the predicted PPP1R3 gene products (Fig. 1c; Table 2). In particular, a NSCLC cell line, H157, and a SCLC cell line, Lu140, had nucleotide substitutions that resulted in the production of truncated PPP1R3 proteins. The remaining seven cell lines had nucleotide substitutions that caused substitutions of amino acids, primarily evolutionarily conserved amino acids (Ref. 8; Fig. 1a; Table 2). These SSCP band patterns were not detected in 50 DNA samples obtained from normal lung tissues; therefore, these variants are likely to be somatic mutations and not genetic polymorphisms. Three cell lines were homozygous for the mutant alleles, whereas the remaining six cell lines were heterozygous for the mutant alleles. Mutations of the PPP1R3 gene were detected in 5 of 33 (15%) NSCLCs, 1 of 14 (7%) SCLCs, 1 of 7 (14%) ovarian cancers, 1 of 7 (14%) colorectal cancers, and 1 of 7 (14%) gastric cancers; mutations were not detected in 11 renal cell carcinomas, 9 breast cancers, and 16 leukemias/lymphomas.

**Mutations of the PPP1R3 Gene in Primary Lung Tumors.** PPP1R3 mutations detected in human cancer cell lines prompt us to examine human primary tumors. Because the incidence of mutations is high, and nonsense mutations were detected in lung cancer cell lines, we examined 55 cases of primary lung cancers (17 SCLCs and 38 NSCLCs) and the adjacent noncancerous tissues of the corresponding patients. Two of 38 (5%) primary NSCLCs showed heterozygous somatic mutations, that is, mobility shifts were detected only in cancerous tissues, and not in the corresponding noncancerous tissues (Fig. 1b). One of the mutations was a nonsense mutation in codon 674, and the other was a missense mutation in codon 1017 (Fig. 1c; Table 2).

<table>
<thead>
<tr>
<th>Name</th>
<th>Type of tumor (subtype)a</th>
<th>Source</th>
<th>Mutation b</th>
<th>Predicted effect</th>
<th>Homozygous/ heterozygous</th>
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<tr>
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<tr>
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<td>T3255A</td>
<td>Leu1062→Thr</td>
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</tr>
<tr>
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<td>NSCLC (AdC)</td>
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<td>C3182A</td>
<td>Gln1062→Lys</td>
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<tr>
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<td>GC</td>
<td>Cell line</td>
<td>A822T</td>
<td>Gly275→Val</td>
<td>Homozygous</td>
</tr>
</tbody>
</table>

a AdC, adenocarcinoma; SgC, squamous cell carcinoma; LCC, large cell carcinoma; OVC, ovarian cancer; CC, colorectal cancer; GC, gastric cancer.

b Location of mutation according to European Molecular Biology Laboratory accession number X78578.

d Downloaded from cancercres.aacrjournals.org on April 20, 2017. © 1999 American Association for Cancer Research.
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2). Thus, PPP1R3 is mutated not only in cultured cell lines but also in primary tumors.

In total, 11 mutations were detected in 104 diverse human cancer cell lines and 55 primary lung cancers. Mutations were widely dispersed and were not clustered in the coding region of the PPP1R3 gene (Fig. 1a).

Expression of the PPP1R3 Gene. Expression of the PPP1R3 gene was examined previously by Northern blot hybridization analysis, and the PPP1R3 transcripts were detected in skeletal muscle and in the heart (9). We also performed Northern blot hybridization analysis of various normal tissues, including the heart, brain, placenta, lung, liver, skeletal muscle, kidney, and pancreas, using a PPP1R3 cDNA probe. The PPP1R3 transcripts were detected only in skeletal muscle and the heart, as reported previously (data not shown). Next, we performed RT-PCR analysis against mRNAs from multiple human adult and fetal tissues using a set of primers that amplified a 153-nucleotide cDNA fragment encompassing exons 3 and 4. Expression of the PPP1R3 gene was detected in multiple tissues, including the adult testis, mammary gland, thymus, and bone marrow. PPP1R3 was also expressed in both adult and fetal lung tissue (Fig. 2a). We also examined the expression of the PPP1R3 gene in several lung cancer cell lines. PPP1R3 expression was detected in all cell lines examined, including the ones with mutations of the PPP1R3 gene (Fig. 2b).

Discussion

Mutational analysis of the PPP1R3 gene was performed in a variety of human cancer cell lines and primary lung tumors. Eight sequence variants were detected in nine cancer cell lines including NSCLC, SCLC, ovarian cancer, gastric cancer, and colorectal cancer. Two of the variants were nonsense mutations, whereas the remaining six were missense mutations. These eight variants were not observed in the 50 normal DNAs examined. Therefore, the variants are likely to be somatic mutations rather than genetic polymorphisms, although we cannot exclude the possibility that some of them were rare polymorphisms, because several types of rare polymorphisms were also detected in the present study (Table 1). Somatic mutations, a nonsense mutation and a missense mutation, were confirmed in at least two cases of primary NSCLCs. These results indicate that the PPP1R3 gene is genetically altered in a fraction of diverse human cancers.

The implication of PPP1R3 alterations in human cancer is unclear at present. However, PP1 plays an important role in cell cycle regulation, RNA splicing, and glycogen metabolism and is a target for chemical tumor promoters (6, 7, 17–19). The enzymatic activity of catalytic subunits is controlled by regulatory subunits, including PPP1R3 (6, 7). PPP1R3 protein is reported to be involved in directing the PP1 catalytic subunit(s) to glycogen particles or to the membranes of sarcoplasmic reticulum (6), suggesting that the PPP1R3 protein functions to localize the PP1 catalytic subunit(s) against the substrates. Furthermore, RT-PCR analysis in this study revealed that the PPP1R3 gene is expressed in multiple fetal and adult tissues, suggesting that PPP1R3 protein plays a physiological role in a variety of human tissues. Therefore, PPP1R3 alterations may cause a disturbance of the physiological function of PP1 in a variety of human organs.

It has been speculated that PPs are involved in the suppression of cancer development by antagonizing protein kinases, many of which act as oncoproteins (1, 2). The presence of nonsense mutations as well as the diversity of mutation spots suggests that the PPP1R3 gene acts as a tumor suppressor in human carcinogenesis. However, 8 of 11 (73%) tumors in which PPP1R3 mutations were detected were heterozygous for the mutant alleles and retained the wild-type alleles. Therefore, it is possible that some mutants may act in a dominant negative fashion. Analysis of the expression of mutant and wild-type alleles of the PPP1R3 gene in human cancers is now under way to clarify the mode of action of mutant alleles in human carcinogenesis.

Functional analysis of wild-type and mutant PPP1R3 proteins will also be necessary to understand the biological significance of PPP1R3 alterations in human carcinogenesis.

Until now, two PP genes, PTEN and PPP2R1B, had been shown to be genetically altered in human cancers. Therefore, PPP1R3 is the third PP gene whose alterations have been detected in human cancers. In particular, genetic alterations of all three genes have been detected in human lung cancer (4, 5), and several lung cancer cell lines, including H157, Ma3, and PC10, have alterations of both the PTEN and PPP1R3 genes (4). Therefore, it is likely that aberrations of several distinct pathways via PPs are involved in human carcinogenesis.

Fig. 2. PPP1R3 expression in cancerous and noncancerous cells examined by RT-PCR analysis. a, PPP1R3 expression in human normal organs. b, PPP1R3 expression in human lung cancer cell lines. GAPDH was analyzed to standardize the RNA amount of each sample.

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