Deletion and Mutation Analyses of the P16/MTS-1 Tumor Suppressor Gene in Human Ductal Pancreatic Cancer Reveals a Higher Frequency of Abnormalities in Tumor-derived Cell Lines Than in Primary Ductal Adenocarcinomas

Lingyi Huang, Tamra L. Goodrow, Shi-Yu Zhang, Andres J. P. Klein-Szanto, Hong Chang, and Bruce A. Ruggeri

INTRODUCTION

Pancreatic ductal adenocarcinoma is the fourth leading cause of cancer death in men and the fifth leading cause in women in the United States, with nearly coincident incidence and mortality rates (1). Although relatively little is known regarding the underlying molecular pathology of pancreatic ductal adenocarcinoma, several salient characteristics have been identified. Among these are activating mutations predominantly in codon 12 of the K-ras oncogene in >90% of ductal pancreatic adenocarcinomas (reviewed in Refs. 2 and 3) and predominantly transition-type missense mutations and microdeletions within highly conserved regions of the p53 tumor suppressor gene in ~50% of pancreatic cancer cases (4-7). Similarly, evidence, albeit limited, has been presented for the involvement of the putative tumor suppressor gene DCC in the metastatic progression of pancreatic carcinoma (8, 9).

The central role of cyclin-cdk3 complexes and their respective inhibitors in the modulation of cell cycle progression has become widely recognized (reviewed in Refs. 10 and 11). Recently, a putative tumor suppressor gene, p16/MTS-1, located on chromosome 9p21 was cloned by several laboratories (12-14) and was found to encode a cell cycle-related protein that binds specifically to cyclin D-ckd-4 complexes and inhibits cdk-4-mediated phosphorylation of several growth-regulatory proteins. Among the species regulated in this manner are RB-1 and the RB-1-related proteins p107 and p130 (12). Initial studies (12-14) revealed a high frequency of homozygous deletions in the p16/MTS-1 gene in a variety of human tumor-derived cell lines, including those from lung, kidney, breast, and ovarian cancers, as well as osteosarcomas, leukemias, and melanomas, the latter containing missense mutations in 40% of the cell lines examined (13). In particular, a high frequency of homozygous deletions in p16/MTS-1 has been observed in primary glioblastomas, anaplastic astrocytomas, and glioma cell lines (15, 16), with CDK4 gene amplification observed commonly in tumors not showing a loss of p16/MTS-1 alleles (17, 18). Despite these findings, controversy exists regarding the significance of deletions and/or mutations in the p16/MTS-1 gene in primary tumors versus cultured cell lines (see Refs. 19 and 20). Recent investigations of p16/MTS-1 gene alterations in primary carcinomas of the lung, bladder, kidney, brain, breast, and head and neck (19-23) revealed a markedly reduced frequency of mutations and deletions in primary tumors compared with tumor-derived cell lines from the same tissue origin. Mutations in p16/MTS-1 have been described in >50% of primary esophageal squamous cell carcinomas (24); however, other investigators (25) have reported a much lower frequency of p16 mutations: 21% (5 of 24) of esophageal squamous carcinomas and 5% (1 of 19) of esophageal adenocarcinomas. In either event, the frequency of p16 alterations is much lower than the 67% reported in esophageal carcinoma cell lines (26). With regard to pancreatic carcinomas, an elevated frequency of p16 gene alterations (41% deletions and 38% mutations) has been reported in pancreatic carcinoma xenografts (27) and tumor-derived cell lines (26, 27).

As part of our ongoing studies on the molecular pathology of ductal pancreatic carcinoma, we examined gene and protein alterations in the RB-1 tumor suppressor gene and cyclin D1/PRAD-1 proto-oncogene in a large series of pancreatic carcinomas and cell lines and found these genes and proteins to be altered relatively infrequently in ductal pancreatic cancer (28). These findings prompted us to examine additional regulators of RB-1 and cyclin D1/PRAD-1 function, i.e., the cdk-4 inhibitor p16/MTS-1. In this report, we describe deletion and mutation analyses of the p16/MTS-1 gene in 30 microdissected primary ductal pancreatic carcinomas and 18 pancreatic carcinoma-derived cell lines. Furthermore, we have examined expression of p16/MTS-1 in a subset of carcinoma-derived cell lines by immunoblotting. Our findings suggest that, although p16/MTS-1 may play a

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3 The abbreviations used are: cdk, cyclin-dependent kinase; TBE, Tris-borate EDTA; SSCP, single-strand conformation polymorphism.
role in the pathobiology of pancreatic cancer as suggested by others (27), inactivation of this putative tumor suppressor gene occurs more frequently in tumor-derived cell lines than in primary pancreatic ductal carcinomas.

MATERIALS AND METHODS

Primary Tumor Samples and Cell Lines. Thirty specimens of histologically confirmed cases of ductal pancreatic carcinoma and 12 cases of normal adjacent pancreata were obtained from the Medical College of Pennsylvania hospitals and through the Cooperative Human Tissue Network, a nonprofit organization sponsored by the NIH. For primary tumor cases, patients received no radiotherapy or chemotherapy prior to pancreatoduodenectomy, and tumor tissues were preselected, following histological evaluation, on the basis of a minimum of 25–35% epithelial cellularity. Tumor sections were microdissected to enrich for neoplastic tissues prior to DNA isolation (29). The pancreatic carcinoma-derived cell lines analyzed in this study, CAPAN-1, CAPAN-2, PANC-1, PANC-89, Colo357, CF-PAC1, QGP-1, FG-2, SW979, SU86-86, HS700T, HS766T, ASPC-1, BXPC-3, T3M4, HPAF, CD-11, and CD-18, were obtained from the American Type Culture Collection (Rockville, MD) and from Dr. S. Batra (Duke University, Chapel Hill, NC). The CD-11 and CD-18 cell lines are differentiated and moderately differentiated subclones, respectively, of HPAF cells. Genomic DNA was isolated from primary carcinomas and cell lines by proteinase K and RNase H digestion and sequential phenol-chloroform extraction as described elsewhere (5).

Deletion Analyses of the p16/MTS-1 Gene. Exon 2 of the p16/MTS-1 gene was analyzed by PCR amplification as described (21) in a reaction mix consisting of 1X PCR buffer (1X PCR buffer = 50 mM KCl, 10 mM Tris-HCl, pH 8.3, 1.5 mM MgCl2, 0.001% gelatin) (Perkin Elmer/Cetus, Norwalk, CT), 50 µM deoxynucleotide triphosphates, 5% DMSO, 1 unit Taq polymerase (Perkin Elmer/Cetus), 1 µM primers HM12S and HM12A (21), and 200 ng template genomic DNA. Primers for the human GAPDH gene were used as internal controls for PCR amplification (21). Samples were subjected to 35 cycles of 94°C for 1 min, 58°C for 1 min, and 72°C for 1 min. To enhance the sensitivity of detection of homozygous deletions of p16/MTS-1 in primary microdissected carcinomas in which contamination with normal DNA may be a problem, multiplex PCR was used as described above, but at 20 and 25 reaction cycles. Reaction products from PCR were electrophoresed on 1.2% agarose-TBE gels containing 0.5 µg/mL ethidium bromide.

SSCP Analyses of Exons 1 and 2 of p16/MTS-1. SSCP analyses for mutations within exons 1 and 2 of the p16/MTS-1 gene were conducted using the reaction conditions detailed above in a 10-µl volume containing 0.1 µCi [α-32P]dCTP/sample. Exon 1 analyses used 3.6% formamide, instead of 5% DMSO, and amplifiers HM11S2 and HM11A as described (21). Samples were subjected to 94°C for 1 min, 56°C for 0.5 min, and 72°C for 0.5 min, for a total of 25 cycles. For SSCP analyses of exon 2, two separate fragments were generated by 35 cycles of PCR, each using primer pairs HM12S1 and HM12A1 and HM12S and HM12A at annealing temperatures of 62°C, 1 min and 56°C, 1 min, respectively. A 10% aliquot of each PCR reaction volume was diluted 10-fold with 0.1% SDS and 10 mM EDTA, and 2 µl of this mixture were diluted in 2 µl Sequenase stop solution (United States Biochemical Corp., Cleveland, OH). Samples were denatured at 95°C for 10 min, placed on ice, and electrophoresed under two separate gel conditions: 6% nondenaturing polyacrylamide with 10% glycerol and 1X TBE buffer, and similarly but with 0.5X TBE buffer. Gels were electrophoresed at 5 W in 1X TBE buffer and 2.5 W in 0.5X TBE buffer for 12–15 h at room temperature. All samples were analyzed a minimum of three times with a separate PCR product to confirm electrophoretic banding abnormalities and to eliminate the possibility of an enzyme-generated artifact.

Sequence Analyses of the p16/MTS-1 Gene. Samples demonstrating reproducible SSCP abnormalities in p16/MTS-1 were subjected to DNA sequence analyses. In addition, to distinguish polymorphism-induced banding abnormalities from those caused by potential somatic mutations, genomic DNA was digested with StuI as described (21). Symmetrically generated PCR products for exon 1 were prepared as described for SSCP in the absence of [α-32P]dCTP, ligated into PCR™II vectors, and subcloned using the TA cloning system (Invitrogen, San Diego, CA). A minimum of five positive clones for each sample was subjected to dye deoxy sequencing using the Sequenase version 2.0 protocol (United States Biochemical) and sense and antisense primers directed to the cloning vector. Symmetrically generated PCR products for exon 2 were subcloned and sequenced similarly. In addition, exon 2 symmetric PCR products were reamplified asymmetrically, purified on Sephadex G-50 columns, and subjected to direct dye deoxy sequencing using [γ-32P]ATP-end-labeled primers HM12S1 and HM12S2 (21) according to previously published methods (30). For confirmation of exon 1 sequences, samples were subcloned and sequenced by a commercial laboratory (Bioserve Biotechnologies, Laurel, MD).

Analysis of p16/MTS-1 Expression in Carcinoma Cell Lines by Immunoblotting. Subconfluent pancreatic carcinoma cell lines were synchronized in G0/G1 by serum deprivation and stimulated into S-phase by serum readdition as detailed (31). Cell extracts of actively growing cell populations were prepared and quantitated as described (31). Fifty and 100 µg protein were electrophoresed on 12% polyacrylamide gels in Laemmli buffer and electroblotted onto nitrocellulose membranes at 120 V for 2 h at 4°C. Membranes were subjected to immunoblotting with antihuman p16 (Santa Cruz Biotechnology, Santa Cruz, CA) at a 1:500 dilution according to the protocol of the manufacturer. Signals were developed using an enhanced chemiluminescent detection system (Amersham, Arlington Heights, IL) and quantified densitometrically (28). A recombinant human p16 protein standard (Santa Cruz) was used as normal expression control for immunoblotting.

RESULTS AND DISCUSSION

In this investigation, a total of 30 microdissected primary ductal pancreatic carcinomas and 18 pancreatic carcinoma-derived cell lines was analyzed for alterations in the cyclin D-cdk4 inhibitor and putative tumor suppressor gene p16/MTS-1. A PCR-based deletion analysis assay revealed homozygous deletions of exon 2 of p16/MTS-1 in five of 18 cell lines, PANC-1, CAPAN-1, MIAPaCa2, SU86-86, and BXPC-3 (Fig. 1). These findings are in agreement with recent reports (26, 27) and were confirmed by the absence of p16/MTS-1 protein expression in actively growing cell cultures of those lines exhibiting homozygous deletions of the p16/MTS-1 gene (see Fig. 2). Deletions of p16 were not detected in any of the remaining carcinoma-derived cell lines, and expression of the p16 protein was of low to moderate intensity (Fig. 2). In contrast, only 3 primary pancreatic carcinomas revealed a homozygous deletion from a total of 30 cases examined. This result was obtained by multiplex PCR both at a standard number of reaction cycles (n = 35) and at a lower number of cycles (n = 20), the latter analysis revealing an additional homozygous deletion in a primary ductal carcinoma. The normal adjacent pancreata from these cases did not reveal p16/MTS-1 abnormalities. This deletion frequency (10%) in primary carcinomas is considerably lower than the

Fig. 1. PCR-based deletion analysis of exon 2 of p16/MTS-1 gene in primary ductal pancreatic carcinomas and cell lines. Deletion analyses of p16 was conducted on high-molecular-weight genomic DNA at 35 PCR reaction cycles, as described in "Materials and Methods" and Ref. 21. The 244-bp product represents a portion of exon 2 of p16, and the 474-bp product is that of the control GAPDH gene. Lanes 1–5, primary ductal carcinoma samples; lanes 6–12, tumor cell lines. Lanes 1 and 5, 93-04-A221CA and 98635CA tumor samples; lanes 7 and 11, BxPC-3 and MIAPaCa2 cell lines.
observation that 9 (30%) of 27 pancreatic carcinoma xenografts revealed homozygous deletions in the p16/MTS-1 gene (27).

To screen for mutations in the p16/MTS-1 gene, PCR and SSCP analyses and sequence analyses were conducted on genomic DNA from microdissected primary tumors and cell lines. Seventeen (39%) of 18 cell lines and 5 (16%) of 30 primary carcinomas revealed reproducible SSCP abnormalities under two distinct electrophoresis conditions (Fig. 3, A and B). All but one of the samples exhibiting SSCP band shifts were subjected to direct dideoxy sequencing and/or subcloning to confirm the nature of p16/MTS-1 mutations.

Table 1 summarizes the confirmed deletion, SSCP, and DNA sequencing data for the panel of 18 cell lines and 30 primary microdissected pancreatic tumor lesions. Missense mutations, microdeletions, and insertions in the p16/MTS-1 gene were observed in both cell lines and primary tumors (Fig. 4, A–C). In the present study, the overall frequency of p16/MTS-1 alterations (deletions and mutations) observed in pancreatic carcinoma cell lines was 66%, whereas only 27% of primary carcinomas revealed such abnormalities. The frequency and nature of p16/MTS-1 abnormalities observed in pancreatic carcinoma cell lines (Table 1) were comparable to those reported recently (26, 27); an exception being the observation of a 7-bp insertion in codons 11 and 12 of CAPAN-2 cells in contrast to the 6-bp insertion described by others (26, 27). In addition to confirming p16/MTS-1 deletions and mutations in pancreatic carcinoma cell lines described by others (26, 27), we have extended these findings to several additional cell lines and subclones (FG-2, HPAF, CD-11, and CD-18), whereas the remaining pancreatic carcinoma cell lines (PANC-89, Colo 357, CF-PAC-1, QGP-1, SW 979, T3M4, and HS700T) had a wild-type p16/MTS-1 gene and moderate expression of the p16 protein (see Fig. 2).

Our observed frequency of p16/MTS-1 deletions and mutations in primary ductal pancreatic carcinomas is lower than that reported for pancreatic carcinoma xenografts by Caldas et al. (27), although similar types of mutations were confirmed in both studies. The marked desmoplastic reaction characteristic of pancreatic ductal carcinomas can confound molecular analyses of tumor cells due to contamination from surrounding nonneoplastic stromal and inflammatory elements. To circumvent this variable, Caldas et al. (27) analyzed xenograft explants of primary pancreatic carcinomas. We have microdissected our primary carcinomas both to limit contamination from nonneoplastic elements and to enrich for tumor cells (29). Neither approach is completely free of artificial and nonneoplastic contamination influences. Thus, the approach we have taken in this study may underestimate the frequency of p16/MTS-1 alterations in pancreatic carcino-

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<table>
<thead>
<tr>
<th>Cell line/tumor</th>
<th>Homozygous deletion of p16 gene</th>
<th>SSCP band shift</th>
<th>DNA sequencing alterations</th>
</tr>
</thead>
<tbody>
<tr>
<td>PANC-1</td>
<td>+</td>
<td>N/A</td>
<td>N/Ap</td>
</tr>
<tr>
<td>MIA PACA2</td>
<td>+</td>
<td>N/A</td>
<td>N/Ap</td>
</tr>
<tr>
<td>SU86-86</td>
<td>+</td>
<td>N/A</td>
<td>N/Ap</td>
</tr>
<tr>
<td>BXPC-3</td>
<td>+</td>
<td>N/A</td>
<td>N/Ap</td>
</tr>
<tr>
<td>CAPAN-1</td>
<td>+</td>
<td>N/A</td>
<td>N/Ap</td>
</tr>
<tr>
<td>FG-2</td>
<td>--</td>
<td>Exon 2</td>
<td>cG44, ATG→AG, M→K</td>
</tr>
<tr>
<td>HS7667T</td>
<td>--</td>
<td>Exon 2</td>
<td>Start of intron 2, AGGT→AGGC</td>
</tr>
<tr>
<td>ASPC-1</td>
<td>--</td>
<td>Exon 2</td>
<td>cG69, 2-bp deletion, ACTCTC→ACTCTC</td>
</tr>
<tr>
<td>CAPAN-2</td>
<td>--</td>
<td>Exon 1</td>
<td>cG11→12, 7-bp insertion, CGCCGAC</td>
</tr>
<tr>
<td>HPAF</td>
<td>--</td>
<td>Exon 1</td>
<td>cG26→27, GCG GTG→G TG</td>
</tr>
<tr>
<td>BXPC-3</td>
<td>--</td>
<td>Exon 2</td>
<td>cG38→39, 2-bp deletion, ACTCTC→ACTCTC</td>
</tr>
<tr>
<td>BXPC-3</td>
<td>--</td>
<td>Exon 2</td>
<td>cG49→50, 2-bp deletion, GCC GA→GC G</td>
</tr>
<tr>
<td>HPAF</td>
<td>--</td>
<td>Exon 2</td>
<td>cG43→44, GTC→GTC, V→I</td>
</tr>
<tr>
<td>BXPC-3</td>
<td>--</td>
<td>Exon 2</td>
<td>cG43→44, GTC→GTC, V→I</td>
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<tr>
<td>BXPC-3</td>
<td>--</td>
<td>Exon 2</td>
<td>cG43→44, GTC→GTC, V→I</td>
</tr>
</tbody>
</table>

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1Two additional subclones of HPAF cells, CD-11 and CD-18, showed identical band shifts.
2Homozygous deletion within this tumor sample was detected at 20 PCR reaction cycles, not the standard 35-cycle PCR reaction.
3Deletion of 1 bp in the 5′ portion of exon 2.
4Sample DNA was not available for sequencing.
5Based on PCR-based assay of exon 2 as described in "Materials and Methods".
6Confirmed band shifts under two nondenaturing electrophoresis conditions as described in "Materials and Methods".
7Data shown in result of direct sequencing data confirmed by subcloning in each case.
8Not applicable in samples in which p16 gene is deleted.
9Subcloning and sequencing of this cell line by a commercial laboratory (Bioserve Biotechnology) also revealed a 1-bp deletion (G) in c27 in several subclones.
10Two additional subclones of HPAF cells, CD-11 and CD-18, showed identical band shifts.
mas. However, to avoid possibly underestimating the frequency of homozygous deletions in p16/MTS-1 due to contamination with normal DNA, we carried out multiplex PCR at a lower number of cycles. In contrast, the xenograft approach could overestimate the mutation frequency as a result of additional genetic changes conferring an in vivo selective growth advantage analogous to those described in tumor cell cultures (see Refs. 19 and 20). In addition, the possibility that p16/MTS-1 mutations in primary pancreatic carcinomas were not detected by SSCP and sequence analyses or lie outside of the regions studied (e.g., exon 3 of p16/MTS-1) must be considered. Consequently, differences in the methodological approaches used may account, in part, for the findings obtained in our respective studies.

The results obtained in this study are largely in agreement with those of previous studies examining a variety of human tumor-derived cell lines (13, 14, 21, 22, 32) in which p16/MTS-1 deletions or mutations were frequent occurrences. Moreover, recent studies of primary carcinomas of the lung, bladder, kidney, brain, breast, and head and neck (19–23), as well as hematopoietic tumors (33), have provided evidence that p16/MTS-1 deletions and mutations are more frequent in cell lines than in primary tumors and are dependent on the tumor type. A notable exception to these observations is the report of mutational inactivation of the p16/MTS-1 gene in ~50% of primary esophageal squamous cell carcinomas by one group (24), but see Ref. 25) and the high frequency of p16/MTS-1 alterations in glioblastomas and anaplastic astrocytomas (15–18). In contrast, p16/MTS-1 genetic alterations appear infrequently in colorectal carcinomas and neuroblastomas (see Ref. 13), as well as in a series of melanoma cases (34). Homozygous loss or mutational inactivation of p16/MTS-1 in tumor cell lines may dysregulate normal cellular growth controls and may confer a selective growth advantage to cells in vitro culture (19–23). Similar hypotheses for genetic alterations following a growth advantage to cells in vitro have been proposed for the loss or inactivation of the p53 gene in a variety of tumor types (35), the amplification of the N-myc gene in neuroblastoma cells (see Ref. 36), and the overexpression of the bcl-2 gene in lymphomas, myeloid cells, prostate carcinomas, and sensory neurons upon growth factor deprivation in vitro (37, 38).

Available evidence suggests that the p53 and p16/MTS-1 proteins are rate-limiting regulators of tumor cell growth converging at pathways of cyclin and cdk inhibition, with p16/MTS-1 acting directly as a specific cyclin D and cdk4 inhibitor (12) and p53 acting indirectly via activation of the transcription of WAF-1/p21, a universal cdk inhibitor (39, 40). One could speculate that the loss or inactivation of one such cdk inhibitor gene could obviate the need for loss or inactivation of the other (see Ref. 21). Alternatively, inactivation of either p53 or p16/MTS-1 could create a selective pressure for the subsequent loss or inactivation of the other, as suggested by Caldas et al. (27) in their studies of pancreatic tumorigenesis. Our ongoing analyses of the p53 gene and protein in a large series of pancreatic ductal carcinomas and metastases lend support to the latter hypothesis, suggesting that alterations in p53 as well as p16/MTS-1 are occurring in specific ductal pancreatic cancers and cell lines, although the frequency of p53 inactivation is higher than that observed for p16/MTS-1 in the present study.4

Our molecular and immunochemical analyses (28) of the RB-1 tumor suppressor gene and protein and the RB-1-associated cell-cycle regulator cyclin D1/PRAD-1 reveal that these growth regulatory genes and proteins are altered infrequently in ductal pancreatic cancers. In this case, the loss or mutational inactivation of p16/MTS-1, a regulator of RB-1 function in cellular growth control (12–14), may obviate the need for the loss or mutational inactivation of RB-1 and cyclin D1/PRAD-1 in this growth-regulatory pathway in a subset of pancreatic carcinomas and cell lines. Alternatively, it has been demonstrated (31) that expression of p16/MTS-1 peaks at the G1-S transition within the cell cycle and is enhanced in cells in which RB-1 is functionally inactive. Transcriptional repression of the p16/MTS-1 promoter by the RB-1 protein has been reported (41), although it remains to be determined whether this regulation is mediated directly by RB-1 or is an indirect effect involving the release of an active RB-1-associated transcriptional factor such as E2F. In support of these observations, a number of recent studies have demonstrated an inverse relationship between loss of a functional RB-1 protein and increased p16/MTS-1 expression in several tumor types (31, 42, 43; see Ref. 44). We cannot state unequivocally that this inverse relationship is supported by the data presently available, because virtually all of our pancreatic carcinoma cell lines had functional RB proteins (28).

Our studies of p16/MTS-1 alterations in human ductal pancreatic carcinomas and cell lines suggest that, although this putative tumor suppressor may play a role in the dysregulated growth of pancreatic cancer, abnormalities in this gene occur more frequently in tumor-derived cell lines than in primary ductal carcinomas. Further studies are required to elucidate the role(s) of p16/MTS-1 in RB-1-related cell growth regulatory pathways and to confirm its putative tumor suppressor function by gene replacement studies in pancreatic tumor cells in which p16/MTS-1 is deleted homozygously or inactivated by mutation.

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4 Manuscript in preparation.
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