Multiple Genetic Alterations in Hamster Pancreatic Ductal Adenocarcinomas

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ABSTRACT

Pancreatic ductal adenocarcinomas induced in the Syrian golden hamster (SGH) by N-nitrosobis(2-oxopropyl)amine share many similarities with the human disease, including mutations of the K-ras oncogene. In vitro carcinogenesis studies with immortal SGH pancreatic duct cells indicate that neoplastic transformation in this system can occur without mutational inactivation of p53 suppressor gene. In this study we extend the genetic analysis of the in vivo SGH model to increase the number of cases analyzed for the status of K-ras and to determine further the spectrum of alterations involved; we have studied the status of the p53, DCC, and Rb-1 suppressor genes and the status of the mdm2 oncogene, which can involve p53 indirectly. The partial SGH-coding sequence of DCC was determined. K-ras mutation in the second position of codon 12 was present in 17 of 19 (90%) of tumors. Immunohistochemistry and single strand conformation polymorphism analysis showed no evidence of p53 mutation in 21 tumors. RNase protection assays showed overexpression of mdm2 in 5 of 19 (26%) tumors. Semiquantitative reverse transcription-PCR analysis showed a complete or partial loss of DCC expression in 10 of 19 (53%) neoplasms and of Rb-1 (42%) expression in 8 of 19 tumors when compared to matched controls. Deregluation of these genes appears to be significant in SGH pancreatic carcinogenesis as indicated by their frequencies. However, the fact that 6 tumors showed either only a K-ras mutation or the absence of alterations of the 5 genes analyzed indicates that additional as yet unstudied or unknown genes are also involved in SGH pancreatic duct carcinogenesis.

INTRODUCTION

Molecular analysis of human and experimental animal cancers has established that they arise as a result of the deregulation of intricate mechanisms that control cell growth and differentiation. A major theme emerging from such studies is that mutations of both oncogenes and tumor suppressor genes are required for malignancy, the former being necessary for cell transformation and the latter for expression of the malignant phenotype (1). On the basis of the exponential relationship between the incidence rate of human cancer and age, it is postulated that between three and seven genetic mutations are required for a cancer to develop (2). It was from these perspectives that we undertook the genetic analysis of the SGH model of pancreatic duct adenocarcinoma, which closely resembles its human counterpart at the morphological and biological levels (3, 4) and at the time of this study appears to share a molecular identity as well, through the high frequency of K-ras mutation (5–8). In this study we seek to determine whether other genes, largely tumor suppressor genes, such as the p53 (1, 9, 10), deleted in colon cancer (DCC; Refs. 1, 11, 12), and retinoblastoma susceptibility genes (Rb-1; Refs. 1, 10) that have been implicated in human pancreatic cancer are also involved in the SGH model of the disease. We also studied the status of the murine double minute 2 gene (mdm2) oncogene (1, 13) to establish whether it is involved when the p53 gene is not deregulated mutationally since the protein of amplified and/or overexpressed mdm2 binds p53 protein, inactivates it (14), and thereby cancels the tumor suppressive function of the gene (15).

MATERIALS AND METHODS

Sample Preparation and Reverse Transcription. Sixty-four SGHs (80–100 g; Charles River, Boston, MA) with equal male and female distribution received weekly s.c. injections of 15 mg BOP/kg for 12 weeks. Animals were sacrificed when they appeared either ill or moribund from weeks 16 to 26 following the initiation of treatment. Twenty-one invasive adenocarcinomas >0.4 cm in diameter derived from 17 different animals (animals 1–7 and 9–18) were analyzed. Tumors were bisected, with one-half of the tumor and adjacent grossly uninvolved pancreatic tissue as a matched control frozen immediately and maintained in liquid nitrogen. The remaining second half was fixed in 10% buffered formalin and used for the preparation of 4-μm paraffin sections stained with hematoxylin and eosin. Normal pancreas was obtained from untreated SGH. Cultured cells were derived from tumor xenografts that developed in nude mice from pancreatic ductal cells exposed in vitro to 1.2 mM BOP and selected by deprivation of serum and epidermal growth factor. Total RNA from all samples was obtained with the use of RNAzol (Tel-Test, Friendswood, TX). Ten μg of total RNA were denatured at 70°C for 5 min, then reverse transcribed into single stranded cDNA with the use of oligo(dT)18-24 (Pharmacia, San Diego, CA) and 200 units Moloney murine leukemia virus reverse transcriptase (GIBCO-BRL, Gaithersburg, MD). The reaction was incubated at 37°C for 60 min, stopped at 90°C for 5 min, and quenched on ice for 5 min. The mixture was then purified to remove unreacted deoxynucleotides, oligo(dT), and excess salts and stored at 4°C. Reverse transcription reactions without RNA as template served as negative controls.

PCR. Oligonucleotide primers were obtained from intronic sequences flanking exons 5–8 of the SGH p53 gene with sequences published previously (16). Five PCR products were obtained and termed p53 E5a, E5b, E6, E7, and E8, with lengths ranging from 132 to 263 bp. A 684-bp cDNA PCR product consisting of the sequence spanning most of the SGH p53 exon 4 through most of exon 8 was amplified with the use of primers at standard conditions described previously (16).

The PCR-based MAMA technique, which allows the detection of mutations at frequencies as low as 1 in 103 gene copies (17), was used to detect K-ras mutations at the second position of codon 12. This was accomplished by PCR amplification of the first exon of K-ras with the use of a modified primer pair developed with an annealing temperature of 50°C to preferentially amplify sequences containing G→A mutations (18). Exon 5A of p53 was amplified as a positive control. A carcinogen-induced pancreatic ductal carcinoma that contains a K-ras codon 12 aspartate mutation (18) served as a positive control for the mutation.

SSCP Analysis. p53 exons 5–8 were amplified with the use of exon-specific primer pairs. The PCR products of each exon were then reassayed with the use of one primer end labeled with [γ-32P]ATP (DuPont New England Nuclear, Wilmington, DE). The labeled products were denatured and electrophoresed through a nondenaturing gel as published previously (16). A SGH transplantable pancreatic ductal carcinoma containing a point mutation in the 60611
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codon 135 and in vitro N-methylnitrosourea-treated SGH pancreatic ductal cells containing a deletion of C in codon 263, both generating band shifts in SSCP analysis, were used as controls for exons 5A and 7, respectively (16).

IHC. Paraffin sections were stained for p53 protein with the use of the mAb DO7 (Novocastra, Newcastle, United Kingdom) and a SGH p53-specific monoclonal antibody, PAbSGH/p53, developed in our laboratory (16) with the avidin-biotin-peroxidase method. Paraffin sections of a pancreatic ductal carcinoma that expressed transfected SV40 T antigen and show an intense nuclear accumulation of p53 protein (19) served as a positive control. Negative controls were performed by omitting the primary antibody.

Partial Cloning of SGH Genes. Published descriptions of both murine and human mdm2 cDNA sequences suggested the possibility of cross-species PCR amplification of the homologous SGH mdm2-coding sequence with the use of the appropriate species primers (13, 20). A 1.4-kb putative SGH mdm2 cDNA was obtained by PCR with the use of SGH pancreatic cDNA and human sense and antisense oligonucleotide primers 5'-TCTGCTATGGCAGATGTTCATGT-CTT and 5'-CTATGGGGAAAATAGTGTAGC-3', respectively (13, 20). A homologous partial SGH DCC cDNA amplified by PCR with the use of the human/rat consensus oligonucleotide primers as reported by Fearon et al. (21) yielded a 233-bp product. A putative 0.9-kb partial SGH G3PDH-coding sequence was amplified with the use of a similar strategy and mouse sense and antisense oligonucleotide primers 5'-GAAGGTCGGTGTGAACGGATTT and 3'-GGCC-3' and 5'-CATGTAGGCCATGAGGTCCACC-3', respectively (Clone-tech, Palo Alto, CA).

All PCR cloning products were cloned into the pGEM-T (Promega, Madison, WI) vector. Sequencing was performed on mixed clones with the use of the Sequenase Ver. 2.0 sequencing kit (United States Biochemical Corp., Cleveland, OH) and [α-32P]dATP (Dupont New England Nuclear) according to the manufacturer's instructions. With the use of an RPA II kit (Ambion), 2.5 or 0.5 μg of total RNA was treated with RNase A/RNase T1, the protected RNA template/antisense probe hybrids were electrophoresed through a denaturing polyacrylamide gel. The intensity of the signal was quantitated by image analysis on a Fujix Bio-Imaging analyzer BAS 2000. Yeast RNA provided by the manufacturer served as a control.

Semiquantitative RT-PCR. Exon connection strategy and sequence information derived from a partial SGH DCC sequence and a nested DCC primer pair (boxed in Fig. 6) from corresponding exons 6 and 7 (22) were used to obtain a 179-bp cDNA PCR product. The SGH Rb-I sense primer (5'-CTCTGTTTGTAGTGTGCTC-3') and antisense primer (5'-AGTCCATTAGTGACACATTCACCA-3') located in exons 8 and 9, respectively (23), were derived from published SGH Rb-1 sequences (24). These primers generated a 201-bp SGH cDNA PCR product. For an internal control and for a control of RNA integrity, a 549-bp SGH G3PDH cDNA segment was amplified with the use of the sense primer (5'-AAAGCTTGTACATCAACGG-GAAGG-3') and antisense primer (5'-GGCCATGTGAGATGACAGGC-3'), in accordance with the SGH G3PDH sequence (Fig. 5). Optimized PCR conditions included denaturation for 1 min at 94°C, annealing for 1 min at 57°C, and extension for 1 min at 72°C for 35 cycles. G3PDH was amplified with the use of identical conditions for 25 cycles.

PCR products for DCC, Rb-I, and G3PDH were cloned and sequenced to confirm their identity. These plasmid DNAs were linearized to synthesize cDNA probes with the use of a PCR labeling technique (25). Aliquots of the labeled probes were electrophoresed through a denaturing polyacrylamide gel, and autoradiography was performed to determine their quality.

Five μl of cDNA aliquots from two matched pancreatic controls (numbers 1 and 17) equal to 1 μg of original total RNA were diluted serially at 0.5 logarithmic intervals. DCC, Rb-I, and G3PDH from diluted templates were amplified by PCR in separate reactions to generate standard curves. Negative controls without template were included in each experiment. The RT-PCR products were electrophoresed through a 2% agarose gel and transferred onto Zeta-Probe membranes (Bio-Rad Laboratories, Hercules, CA) for 3 h. The membranes were prehybridized, then hybridized overnight with denatured radiolabeled probes in a formamide-based hybridization solution at 42°C, and washed in a high stringency buffer. Autoradiographic films were exposed for 20–30 min to G3PDH probed membranes, 60 min to Rb-I probed membranes, and 90 min to DCC probed membranes. The intensity of the signal was quantitated by image analysis on a Fujix Bio-Imaging analyzer. Standard curves of DCC, Rb-I, and G3PDH amplification were constructed from image intensities corresponding to the logarithmic dilution of the cDNA to determine the optimal dilution ranges for subsequent semiquantitative analysis (26). The mRNA expression levels of DCC and Rb-I from samples were normalized by the relative intensity of signals of DCC or Rb-I versus G3PDH as measured by image analysis.

RESULTS

Tumor Histology. Hematoxylin and eosin-stained pancreatic tissue sections showed that except for 1 tumor (number 8) containing <20% neoplastic epithelial cells and a massive lymphoid infiltrate and proliferation of stromal tissue, making it inadequate for study, all the other tumors consisted of 19 well to moderately differentiated invasive pancreatic ductal adenocarcinomas containing sparse to moderate amounts of mucin (Fig. 1A) and 2 multiloculated mucinous adenocarcinomas containing large pools of mucin (Fig. 1B). A total of 21 tumors derived from 17 different animals (animals 1–7 and 9–18) were analyzed. Four animals (animals 10–12 and 16) had two separate tumors appropriate for analysis. These tumors were labeled, e.g., tumors 10-1 and 10-2. Histological analysis of grossly uninvolved pancreatic tissue adjacent to gross tumor showed normal appearing acinar cells, hyperplasia, atypia, and in some instances, carcinoma in situ of ducts in 11 animals (Fig. 1C); chronic pancreatitis and atrophy in 5 animals (Fig. 1D); and pancreatic atrophy and fibrosis in 3 animals (Fig. 1E).

K-ras Mutation. Two hundred ng of tumor DNA were amplified by PCR with the use of the MAMA technique to determine the mutational status of K-ras. Previous studies had shown exclusively the G→A substitution in the second position of codon 12 in BOP-induced SGH pancreatic ductal adenocarcinomas (6–8). An equal amount of template was used to acquire the PCR product of p53 exon 5A (p53 E5A) in a separate reaction. Fig. 2 shows the K-ras/MAMA products and p53 E5A. Nineteen tumors gave consistent p53 E5A amplification to confirm amplification of each template by PCR (tumors 9 and 14 did not amplify). Sixteen of 19 tumors demonstrated unequivocal moderate to strong amplification bands as compared to the positive control (sample D), indicating the K-ras mutation. Although the intensity of the K-ras band in tumor 16-2 was barely detectable, the identically weak JJJ amplification suggested that it did contain a K-ras mutation. Only tumors 5 and 12-1 were negative for the mutation. A G→A mutation of K-ras codon 12 was present in 17 of 19 tumors for an incidence of approximately 90%.

p53 Mutation. SSCP, IHC, and sequencing were performed to determine the status of the p53 tumor suppressor gene in the tumors. SSCP analysis of the PCR products of exons 5–8 from 20 tumors (tumor 14 did not amplify) revealed no abnormal mobilities. Fig. 3 is a representative SSCP autoradiogram of exon 7 PCR products showing no conformational change in tumors 1 through 10-1 in comparison with the positive control (Fig. 3, Lane C). IHC was performed with the use of antibodies PAbSGH/p53 and DO-7, and only two tumors showed a faint scattered nuclear accumulation of p53 in <5% of the neoplastic cells (data not shown); no apparent nuclear staining was present in the remainder of the tumors used for SSCP analysis. In IHC analysis of 42 microscopic invasive carcinomas insufficient for SSCP analysis, 3 showed a faint and scattered pattern of p53 staining. Direct sequencing of a PCR product from p53 cDNA was done to search for possible alterations in exon 4 and showed no sequence changes in tumors 1, 2, and 18. These results suggest that p53 mutations probably are not involved in BOP-induced SGH pancreatic ductal carcinogenesis.

Partial Cloning of the SGH mdm2 Gene. Previous reports have shown that the mdm2 gene is amplified and overexpressed in some
Fig. 1. Histopathology of SGH pancreatic duct adenocarcinomas and adjacent normal pancreatic tissue. A, well-differentiated pancreatic ductal adenocarcinomas composed of disorganized and crowded profiles of duct-like glands. B, mucinous variant of pancreatic ductal adenocarcinoma consisting of neoplastic ducts distended with pools of mucin containing exfoliated tumor cells. C, normal appearing pancreatic acini surrounding a large pancreatic duct with a focus of epithelial hyperplasia; the edge of an invasive ductal adenocarcinoma is seen in the right of the photomicrograph. D, chronic pancreatitis characterized by an interstitial lymphocytic infiltrate and focal edema; profiles of ducts with hyperplasia and papillary hyperplasia with cellular atypia are also present. E, pancreatic atrophy with replacement by fat tissue; a hyperplastic duct surrounded by chronic inflammation and early fibrosis is present. A and D, X400; B and C, X100; E, X250.

Fig. 2. MAMA analysis of K-ras mutation (K-rasMut). Analysis for G→A mutations in the second position of K-ras codon 12. Lane M, φX174/HaeIII digest marker, 118-bp band. Lane A, negative control, no template added in the PCR reaction; Lane B, normal DNA from untreated SGH pancreas; Lane C, DNA from treated matched SGH pancreas; Lane D, K-ras codon 12 aspartate-positive control; Lane E, K-ras codon 13 aspartate-positive control; Numbered lanes, tumor samples. Arrowheads, tumors 5 and 12-1 are negative for K-ras mutation.

Fig. 3. SSCP analysis of p53 exon 7. Intense broad band, normal mobility of the PCR product from exon 7. Lane A, negative control, no template in the SSCP reaction; Lane B, normal DNA from untreated hamster pancreas; Lane C, positive control, a sample containing one C deletion in exon 7 with the band shift (arrowhead); Lane D, DNA from treated matched SGH pancreas; Numbered lanes, tumor samples.

composition among the different species and SGH mdm2, the latter being 10 and 8 residues shorter than the human and mouse, respectively, the critical functional domains are conserved highly (13, 31). In the potential nuclear localization signal region, SGH, human, and mouse mdm2 proteins all contain consecutive basic amino acids. In a highly negatively charged acidic domain that resembles the domains present in some transcriptional factors, all three species contain a high percentage of glutamic and aspartic acids. The metal-binding regions of the three proteins all showed conservation of the Cys-X-X-Cys motifs. These similarities strongly imply that the gene cloned is the SGH homologue of the mdm2 gene. A 188-bp SGH segment (Fig. 4B,
Fig. 4. Partial SGH mdm2-coding sequence. A, nucleotide sequence (GenBank accession no. U10982). B, predicted amino acid sequences of SGH, human, mouse, and a portion of rat mdm2-coding sequences are compared. The human, mouse, and rat sequences are shown only where they are not identical to the SGH sequence. *, identical nucleotides; ---, a gap in the sequence that allows for maximum alignment. A, two boxes mark the oligonucleotide sequence used to generate a 188-bp PCR product from SGH cDNA for RPA purposes. /?, regions designated A and the corresponding mouse and human sequences are modified from Refs. 13, 14, 20, and 31.
mdm2 Expression. Using SGH specific riboprobes, we analyzed mdm2 expression in pancreatic tumors and uninvolved matched pancreas from the same animal by RPA. Analyses on 19 pairs showed that mdm2 expression was increased in 5 tumors, for an incidence of 26%. Fig. 7 shows representative examples with two tumors having equal expression and 3 tumors having increased mdm2 expression when compared with their uninvolved contiguous pancreatic tissue controls. Expression of the G3PDH gene in various SGH pancreatic controls was consistent as shown in Fig. 7. Quantitation by image analysis after normalization for G3PDH levels showed that the tumor:control ratio of mdm2 expression was approximately 10, 9, 5, 5, and 13 in tumors 4, 5, 10-1, 10-2, and 18, respectively. A Southern blot was performed on EcoRI restriction enzyme digested genomic DNA with the use of SGH mdm2 and control G3PDH cDNA probes to evaluate the copy number of the mdm2 gene. Four overexpressed tumors, along with 9 tumors that expressed mdm2 at normal levels and 1 “normal” pancreatic control, showed no gene amplification (data not shown). Our data demonstrate that mdm2 is overexpressed in the absence of gene amplification in SGH pancreatic adenocarcinomas.

Standard Curves and Semiquantitative Analysis. DCC, Rb-1, and G3PDH were amplified from control samples 1 and 17 with the use of cDNA serially diluted at logarithmic intervals. Fig. 8A shows the representative autoradiographic bands derived from the Southern hybridization of PCR products corresponding to the dilution of control sample 1. The membranes then were subjected to image analysis for quantitation. As shown in Fig. 8B, by converting the band intensities generated by the image analyzer to logarithmic values, a linear relationship of template input/amplified products of each gene was determined. Standard curves were constructed from control pancreas 17 that presented similar slopes and correlations (data not shown). According to the standard curves, the concentration for linear amplification of DCC, Rb-1, and G3PDH were 10⁻¹⁵ to 10⁻３⁰, 10⁻²⁰ to 10⁻３⁵, and 10⁻²⁵ to 10⁻⁴⁵ of the original cDNA, respectively. To allow for variability in the concentration of the unknown sample and still remain within the linear slope of the standard curve, we chose a middle point value as the target concentration of the unknown analysis. The optimal concentrations of cDNA input based on corresponding amounts of original RNA were 10⁻２⁰ (0.1 µg) for DCC, 10⁻²⁵ (0.032 µg) for Rb-1, and 10⁻₃⁵ (0.0032 µg) for G3PDH, as indicated by arrows in Fig. 8B. Differences in concentrations up to 30-fold can be distinguished by this semiquantification system.

Fig. 5. Partial nucleotide sequence (GenBank accession no. U10983) of SGH G3PDH open reading frame. Boxes, HindIII restriction enzyme site (AAGCTT). The 160-bp SGH cDNA segment upstream of HindIII with the accompanying 26-bp 5’ mouse oligonucleotide primer and 4 nucleotides from vector (not shown in this figure) was cloned for cDNA sequence upstream of HindIII restriction enzyme site (AAGCTT). The 160-bp SGH G3PDH segment (marked as brackets) present in four of the transcripts was used as a control, the SGH housekeeping gene G3PDH was cloned with the use of this construct.

Fig. 6. Partial cDNA sequence of SGH DCC. A 190-bp cDNA segment between the human/rat consensus primers was amplified, cloned, sequenced, and compared with published human and rat sequences. Boxes, nested primer set generated to amplify a 179-bp SGH-specific segment. Homologies. N, absent nucleotide. Arrow, division of exons 6 and 7. The human and rat sequences were obtained from Ref. 21.

Fig. 7. RPA analysis of mdm2 expression. RPA using antisense probes against SGH mdm2 and G3PDH. The experiments were done in triplicate or quadruplicate. N, pancreatic normal control; T, tumor; numbers, tumor samples.
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**Rb-1 Expression.** RPA analysis of cultured tumor cells designated B2L, B3L, and B1R showed decreased expression by 3.8-, 4.4-, and 3.0-fold as compared to tumor cells B1L. Tumor cells B2R exhibited slightly increased expression by 1.4-fold in comparison with B1L. RNA from these cells was subjected to RT-PCR analysis under the conditions described previously to confirm the accuracy of quantitation. The analysis obtained from RT-PCR revealed that the expression of Rb-1 is decreased in B2L, B3L, and B1R by 2.2-, 3.0-, and 5.0-fold and increased in B2R by 2.3-fold (Fig. 10A). These values were in close agreement with the RPA results.

**DCC Expression.** cDNA from 21 pairs of pancreatic ductal adenocarcinomas and their matched pancreatic controls was utilized for PCR. The Southern blot membrane after autoradiogram was quantified by image analysis. Two replicative experiments showed decreased (at least 80%) or a complete loss of DCC expression in tumors 1, 3, 5–7, 11–2, 13, 17, and 18 as compared with their matched controls. Increased expression (by approximately 200%) was observed in tumor 9. Amplification of DCC in controls 14 and 16 was not successful, while G3PDH amplification was consistent. Otherwise, 7 tumors revealed no apparent differences in DCC expression between the tumors and their matched grossly uninvolved pancreas controls. Fig. 9 demonstrates a Southern blot of representative samples. The upper row shows tumors exhibiting significant decrease or complete loss of DCC expression and consistent expression of the controls. The lower row shows a loss of DCC expression in tumor 18; equal expression in representative tumors 2, 10-1, and 10-2; and increased expression in tumor 9, along with the pairs 14 and 16. In pair 14, amplification of DCC in the control was not successful; therefore, a matched comparison was unavailable. However, the expression level of DCC in tumor 14 was either equal to or slightly higher than all controls. Since comparison of the expression levels of tumors 16-1 and 16-2 with their controls was ambiguous, they were deleted from final analysis. As measured by semiquantitative PCR, the expression levels of DCC mRNA in tumor tissues were <20% of their matched controls in 10 of 19 (53%) sample pairs.

**Fig. 8.** Correlation between band intensities and dilutions of cDNA. A, autoradiograms of membranes hybridized with DCC, Rb-1, and G3PDH probes after RT-PCR of the respective genes at 10^5 intervals. Dashes, data points not utilized in this experiment. B, standard curves for DCC, Rb-1, and G3PDH with the use of logarithmic values converted from the image analysis. The correlation coefficients were 0.998, 0.996, and 0.983 for DCC, Rb-1, and G3PDH, respectively. Arrowheads, dilutions used for semiquantitative RT-PCR.

**Fig. 9.** Semiquantitation of DCC expression. Autoradiogram of 13 representative sample pairs obtained in one experiment. N, pancreatic normal control; T, tumors; T1 and T2, two separate tumors induced in each SGH numbers 10 and 16. Note the decreased expression of DCC in tumors 1, 3, 5–7, 13, 17, and 18.

**Fig. 10.** Semiquantitation of Rb-1 expression. A, comparison of RT-PCR to RPA analysis of 5 in vitro BOP-induced tumor cell cultures designated B1L, B2L, B3L, B1R, and B2R. Top two rows, Southern hybridization of the PCR products. The RT-PCR values obtained from image analysis and the RPA values as generated previously are listed for comparison. Arrowheads, increase or decrease of values in other samples relative to B1L. B, rearranged autoradiogram of representative sample pairs obtained from a single experiment. N, matched pancreatic normal control; T, tumors; T1 and T2, tumors 10-1 and 10-2; –, negative control. Note the decreased expression of Rb-1 in tumors 1, 4–6, and 17.
The signals representative of Rb-1 expression in control tissue produced by RT-PCR were not as homogeneous as those for DCC, although the G3PDH signals were consistent. Rb-1 expression in control pancreas could be classified into 3 groups, with the exception of control 16, for which Rb-1 expression was not detected and excluded from further analysis. The first group (intermediate expression) contains 9 controls (e.g., controls 1, 3, 10, 13, and 17; Fig. 10B), the second group (low expression) contains 8 controls (e.g., controls 6 and 14), and the third group (high expression) contains controls 4 and 5.

Using semiquantitative PCR, we found a significant decrease or loss of Rb-1 expression in tumors 1, 4, 5, and 17 when compared with matched controls (Fig. 10B). Although Rb-1 was expressed in controls 6, 11-1, 11-2, and 18, there was a complete absence of expression in the corresponding tumors (see representative pair 6 in Fig. 10B). There was an increase of expression over control levels in tumors 2, 9, 10-1, 13, and 14 (see representative pairs 13 and 14). The remaining 6 tumors exhibited no differences in Rb-1 expression between matched controls and tumors (see representative pairs 3 and 10-2). No test pair showed excessive expression of Rb-1 in both the matched control and tumor due to amplification into the plateau phase. Since we have established that semiquantitative PCR reliably reflects changes in the amount of template, we consider the data as valid. The levels of Rb-1 mRNA were less in tumors than in matched controls in 8 of 19 (42%) sample pairs.

DISCUSSION

Studies by our laboratory and others have established that SGH pancreatic duct adenocarcinomas share a molecular identity with the human disease through the high prevalence of K-ras mutations (5–8, 33), the nature of the mutation (G to A transition), and its localization in the second position of codon 12. Use of the highly sensitive MAMA PCR-based technique to detect K-ras mutations allowed identification of alterations undetectable by oligonucleotide hybridization analysis (17, 18) and increased the prevalence of this mutation in SGH pancreatic duct carcinomas from an average of 80% in previous studies (6–8) to 90% in the present one.

An extensive review of 170 human pancreatic carcinomas surveyed by PCR analysis showed that 44% of the neoplasms contained p53 mutations (9). This incidence is second only to carcinomas of the lung (56%) and colon (50%) and is equivalent to that of carcinomas of the esophagus, skin, and ovary. It is notable that 41% of the p53 mutations in human pancreatic carcinomas are G to A transitions, identical to the most common carcinogen-induced lesion in the K-ras oncogene. The use in this study of IHC, SSCP, and direct sequencing of the highly conserved regions of p53 for analysis of pancreatic duct tumors would identify the most common alterations of p53 that have been encountered in human malignant neoplasms (1, 9). The negative results obtained were in sharp contrast to p53 analysis of human pancreatic carcinomas and corroborate the results obtained from tumors derived from in vitro carcinogenesis of SGH pancreatic duct cells.5 The frequency of p53 mutations in rodent tumors is known to be significantly lower than that in human neoplasms (see review in Ref. 9). In experimental tumors of mouse lung and colon, even when the mutation rates of K-ras and tumor morphology were similar to their human counterparts, p53 alterations were rarely detectable. To further clarify whether a relationship exists between p53 mutation and BOP-induced carcinogenesis of SGH pancreatic ducts, studies using different doses of carcinogen may be necessary as has been suggested for experimental pulmonary and colon carcinomas (9).

The mdm2 oncogene has transforming ability that can be activated by overexpression and/or amplification (13). This gene has been found to be amplified and overexpressed in about 30% of human sarcomas and in 8–10% of gliomas (20, 27–29), and only overexpressed at the mRNA level but not amplified in 50% of human leukemias (30). Immunohistochemical studies have shown overexpression of mdm2 without amplification in some sarcomas (29), indicating that various levels of mdm2 deregulation can play an important role in human tumorigenesis. mdm2 protein can complex p53 protein and modulate its transactivating activity (14, 15, 31). In addition, the mdm2 gene has a p53-responsive element that activates the gene when p53 protein binds to it (34). Together, these mutual but different binding affinities serve to function as a feedback loop that regulates p53 protein activity on the one hand and mdm2 gene expression on the other (35). It has been hypothesized that tumors with amplification of the mdm2 gene rarely have alterations of p53 (27).

The present study demonstrates that 26% of BOP-induced SGH pancreatic duct carcinomas that lack p53 mutation contain high levels of mdm2 mRNA due to overexpression, and basically supports the hypothesis. It is of interest that in more than 50% of the human pancreatic carcinomas, p53 is not mutated, and it might be worthwhile to assess the status of the mdm2 oncogene in such cases.

DCC encodes a membrane-bound protein structurally similar to those of other cell adhesion molecules (21, 36). In contrast to them, DCC is expressed normally at relatively low levels, leading to the suggestion that it may play a role in cell differentiation rather than adhesion. Frequent loss of expression and/or loss of heterozygosity of DCC has been shown in various human epithelial tumors, including pancreatic duct carcinomas (11, 12, 19, 36–41). Due to a generally low expression, detection of DCC in certain human organs is feasible only by RT-PCR (11, 12). This was corroborated in SGH pancreatic tissue in which DCC expression was not detectable by Northern blot analysis or RPA (data not shown). Therefore, we used RT-PCR quantitation to study this gene in SGH pancreatic carcinogenesis. Since we amplified the target gene and the internal G3PDH control in separate reactions and compared across samples, the data can only be considered semiquantitative. Among 19 paired samples, we identified significantly decreased or a complete loss of DCC expression in 10 carcinomas (53%) as compared to adjacent grossly normal pancreas, whereas 8 tumors showed identical or slightly higher expression as compared to controls. Most notable was the overall invariant DCC expression in many of the controls, suggesting that sampling was adequate and allowed interpair comparison. With the use of this comparison, tumor 14 was interpreted to have expression equal to that of controls, while DCC expression in tumors 16-1 and 16-2 was not convincingly lower than that in several control samples (Fig. 9). The discrepancy between the capacity to amplify G3PDH and the failure to amplify DCC from controls 14 and 16 is unclear. Since pancreas controls are composed primarily of acinar cells that produce abundant nucleases and show varying degrees of pancreatitis (which cause acinar cells to leak enzymes), contamination with RNase during tissue preparation could degrade completely the relatively few DCC transcripts, while some of the more abundant G3PDH mRNA would remain and be transcribed and amplified. However, this would not be the case in pancreatic carcinomas, since these are composed largely of duct cells and to a lesser extent stromal cells, both of which phenotypically produce far less RNase than acinar cells. Therefore, the apparent underexpression of DCC in tumors is probably valid. There is minor interpair variation of G3PDH expression that could result from an exaggerated amplification effect of PCR due to minor dilution errors or the larger size of G3PDH amplicons (549 bp) that are more degradable. However, consistent intrapair amplification ensures the validity of G3PDH as an internal control. In human colon carcinomas, high levels of DCC expression have been found in a variant named mucinous adenocarcinomas (36). These tumors are characterized by
the abundant production of mucin, a marker of differentiation. It is noteworthy in this regard that in the present study, in tumors 16-2 and 17, both of which were pancreatic mucinous ductal adenocarcinomas, DCC expression was low; conversely, in tumors 9 and 10, in which DCC expression was high, mucin production was low to nonexistent. Although sample size is admittedly small, these findings suggest that in the SGH tumor model DCC protein may serve a function other than controlling duct differentiation.

Rb-1 encodes a nuclear protein the phosphorylation status of which is linked closely to the cell cycle (1, 42). Rb-1 mutations that cause the inactivation of its protein were detected in virtually all retinoblastomas and most small cell lung carcinomas (1, 43). Lower frequencies of Rb-1 mutation have been detected in bladder and breast carcinomas (1, 44). However, its role in human pancreatic carcinomas remains unclear. Two reports documented immunohistochemical evidence of Rb-1 underexpression in either 2 of 10 or in 0 of 7 primary pancreatic carcinomas, respectively (10, 41). Thus, it appears premature to draw conclusions on this issue from these data until further studies using larger numbers of human pancreatic carcinomas are done. Since the expression of Rb-1 in normal human tissue is ubiquitous (45), and since IHC is the most convenient way to study Rb-1 gene expression in primary tumors, we attempted to detect Rb-1 expression by IHC with the use of several commercial human Rb-1 antibodies on SGH normal pancreatic and control human tissue sections. Our tests indicated the inapplicability of human antibodies to SGH tissue, although they worked well on human tissue. Since some of the SGH tumor samples were as small as 0.2 cm in diameter for RNA extraction, the RNA was insufficient for Northern blot or RPA analysis. We used semiquantitative PCR for Rb-1 analysis as described above. Comparison of the two assays showed that signals obtained from semiquantitative PCR corresponded favorably to the relative amount of original Rb-1 transcripts. Unlike DCC, Rb-1 expression in SGH control pancreas is nonhomogeneous. Rb-1 transcripts in most controls tended to be close to or lower than that of the controls used in our standard curve, although two controls showed strikingly high expression. The decreased RNA signal obtained for normal controls 2, 14, 16, and 18, as contrasted to the other normal controls for DCC and Rb-1, may be a result of both decreased pancreatic tissue mass due to atrophy and the release of degradative enzymes from the pancreatitis present in these animals. It might also be explained by the presence of excessive stromal cells in the control tissue; however, this is not likely since if it were so, DCC expression would be as irregular as that of Rb-1. It should also be noted that normal pancreas control tissue, although free of gross tumor, has been exposed to BOP for months and may have sustained clonal genetic alterations. Thus, another interpretation draws on the hypothetical influence of BOP on Rb-1 transcription. Variable Rb-1 transcription could also be due to susceptibility differences of each individual animal. Although we were unable to clarify these possibilities or the reason for the elevation of Rb-1 expression in several tumors, we identified 8 of 19 (42%) SGH pancreatic duct adenocarcinomas with Rb-1 underexpression. Considering the proposed function of Rb-1, further analysis of its protein levels and correlation with mRNA levels are necessary for a better understanding of its complicated role in carcinogenesis (43, 45).

The multistep pathogenesis of cancer first postulated and established in experimental carcinogenesis (46, 47) has been corroborated recently and extended significantly through molecular studies of human malignancies (2, 48, 49). In the present study, 13 of 19 tumors that had sufficient material to allow examination by genetic analysis had 2 or more different genetic alterations, distributed as shown (Table 1): 1 tumor had 4, 7 tumors had 3, and 5 tumors had 2 abnormalities. However, 6 tumors showed either solitary K-ras mutation or the absence of any alterations for the 5 genes that were analyzed, suggesting that other genes that remain to be identified are also involved in pancreatic duct carcinogenesis. Incidences of 90% for K-ras, 53% for DCC, 42% for Rb-1, and the absence of p53 mutations except for the cases (26%) in which the mdm2 oncogene was overexpressed are significant features of SGH pancreatic adenocarcinomas. The spectrum of genetic alterations identified in the various neoplasms in the present study allows us to address preliminarily whether an increased number of genetic alterations correlate with either the histological grade or the size of tumors. Tumor 18 with 4 genetic alterations, tumors 1, 6, 11-1 with 3 alterations, and tumor 12-2 with a single change were all graded as well differentiated ductal adenocarcinomas; the single exception was the moderately well differentiated tumor 2 with one genetic alteration. Similarly, no correlation was evident between the number of genes altered and tumor size (Fig. 11). With the exception of the K-ras mutation, which we have shown in a previous study of BOP-induced SGH pancreatic carcinogenesis occurs early in the process with an incidence of 46% in preneoplastic lesions (8), we have no similar information on the approximate timing or sequence, if such exists, of any of the genetic alterations presented in this report. Detailed in situ studies for the detection of gene products or transcripts will be required to time the appearance of gene alterations, particularly DCC and Rb-1 during BOP-induced SGH pancreatic duct carcinogenesis. The multiple genetic alterations, many of which are identical to those in human pancreatic cancer, that were identified in the SGH model emphasize its value for studies in pancreatic carcinogenesis and the experimental development of effective therapies for the disease.

### Table 1 Summary of gene alterations in SGH pancreatic duct adenocarcinomas

<table>
<thead>
<tr>
<th>Alterations Identified</th>
<th>Gene Alterations</th>
<th>Tumor Number</th>
</tr>
</thead>
<tbody>
<tr>
<td>4</td>
<td>K-ras p53 mdm2 DCC Rb-1</td>
<td>18</td>
</tr>
<tr>
<td>3</td>
<td>X X X X</td>
<td>4</td>
</tr>
<tr>
<td>2</td>
<td>X X X X X X</td>
<td>5</td>
</tr>
<tr>
<td>1</td>
<td>X X X X X X</td>
<td>3, 7, 13</td>
</tr>
<tr>
<td>0 or ?</td>
<td>2, 12-2, 15</td>
<td>9, 12-1, 14b</td>
</tr>
</tbody>
</table>

* incidence of gene alteration for K-ras is 90%, p53 is 0%, mdm2 is 26%, DCC is 53% and Rb-1 is 42%

* not analyzed for K-ras mutation

* not analyzed for mdm2 expression

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**REFERENCES**


GENETIC ALTERATIONS IN HAMSTER PANCREATIC CARCINOMAS


Multiple Genetic Alterations in Hamster Pancreatic Ductal Adenocarcinomas

Kuo-Wei Chang, Sergio Laconi, Kathy A. Mangold, et al.


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