

Frequent *p16^{INK4A}/CDKN2A* alterations in chemically induced Syrian golden hamster pancreatic tumors

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The *p16^{INK4A}/CDKN2A* (*p16*) tumor suppressor gene is known to be inactivated in up to 98% of human pancreatic cancer specimens. Chemically induced pancreatic tumors in Syrian golden hamsters have been demonstrated to share many morphological and biological similarities with human pancreatic tumors and represent a potentially suitable model for the evaluation of therapies targeting *p16*. The purpose of this study was to evaluate primary hamster pancreatic tumor specimens for potentially inactivating *p16* alterations. Tumors were induced with *N*-nitroso-bis-(2-oxopropyl) amine, followed by two cycles of augmentation pressure, and were harvested on day 100. Foci of tumor cells were identified by light microscopy after staining with hematoxylin and eosin, and corresponding tumor tissues were excised for DNA extraction. The techniques of multiplex real-time PCR, direct sequencing and methylation-specific PCR were used to evaluate 30 tumor specimens for homozygous deletions, mutations and aberrant methylation of 5' CpG islands, respectively. Homozygous deletions were identified in 11 of 30 (36.7%) specimens, mutations were identified in four of 30 (13.3%) specimens, and aberrant methylation of 5' CpG islands was found in 14 of 30 (46.7%) specimens. The overall frequency of *p16* alterations was 93.3% (28 of 30 specimens) and the majority of changes (83.3%) were noted to be secondary to methylation or homozygous deletion. The four mutations significantly impaired cyclin-dependent kinase 4 inhibitory activity, and two resulted in perturbation of the global structure of P16 protein. These findings indicate that *p16* inactivation is a common event in chemically induced hamster tumors, and that this animal model is appropriate for comparative studies evaluating pancreatic cancer therapeutic strategies targeting *p16*.

Introduction

Pancreatic cancer is currently the fifth most common cancer causing death in the US (1). Surgical resection offers the only hope for long-term survival, but the results are suboptimal and most patients are not candidates due to aggressive local tumor invasion or the presence of metastatic disease at the time of presentation. Despite advances in preoperative imaging, surgical technique, perioperative management and adjuvant chemotherapy and radiation, the 5-year survival rate is only 4% (2). Novel therapeutic strategies are desperately needed and this has been the focus of a NIH progress review group (3). Numerous studies evaluating *p16* tumor suppressor gene alterations in human pancreatic tumor specimens indicate that *p16* may provide such a target (4–8).

The *p16* gene product binds to cyclin-dependent kinase 4 (CDK4) and inhibits retinoblastoma susceptible gene product (Rb)-phosphorylating activity. This prevents the release of active transcription factors, E2Fs, from incompetent Rb/E2F complexes and results in cell cycle arrest at the G₁/S transition point. Abrogation of the *Rb/p16* tumor suppressive pathway appears to contribute to unregulated cell growth, and inactivating alterations of the gene have been commonly identified in a number of human malignancies (reviewed in refs 9 and 10). Described mechanisms of inactivation include homozygous deletion, mutation and aberrant methylation of 5' CpG islands. Inactivating alterations of *p16* have been identified in up to 98% of human pancreatic adenocarcinoma specimens (8). Transfection of wild-type *p16* into the human pancreatic cancer cell line MIAPaCa-2 results in cell cycle arrest, further supporting the hypothesis that *p16* inactivation plays an important role in human pancreatic adenocarcinoma tumorigenesis (11).

Syrian golden hamster (SGH) pancreatic tumors, induced by *N*-nitroso-bis-(2-oxopropyl)amine (BOP) have been demonstrated to be a useful model for the study of human pancreatic cancer for a number of reasons (12). These include the relative ease of tumor induction, high tumor induction rate and short tumor latency. Biological similarities include a ductal phenotype on histopathological analysis, the propensity for aggressive local invasion and metastatic growth, and global effects resulting in cachexia (13–17). Genetic similarities include frequent (78%–95%) and early *k-ras* mutations (13,14,16). Previous reports from our laboratory demonstrate that hamster P16 protein is structurally and functionally similar to the human P16 homolog (18). Furthermore, homozygous deletion of *p16* has been identified in two SGH pancreatic cancer cell lines (H2T and KL5B) (19). These preliminary findings suggest that *p16* tumor suppressor gene inactivation may play a role in the development of chemically induced hamster pancreatic tumors. The purpose of the current study was to evaluate chemically induced primary pancreatic tumors for potentially inactivating *p16* alterations.

Abbreviations: BOP, *N*-nitroso-bis-(2-oxopropyl) amine; CDK4, cyclin-dependent kinase 4; Ct, cycle threshold; GST, glutathione S-transferase; NMR, nuclear magnetic resonance; Rb, retinoblastoma susceptible gene product; SGH, Syrian golden hamster.

Materials and methods

Tumor induction and harvesting

Thirty female SGH (Nihon SLC, Shizuoka, Japan) were treated with s.c. injection of *N*-nitroso-bis-(2-oxopropyl)amine (70 mg/kg), and then exposed to two cycles of augmentation pressure as described (20). Animals were killed on day 100 and the pancreas were immediately harvested and fixed in 10% formalin in phosphate-buffered saline (pH 7.4, PBS).

Tissue preparation and DNA extraction

Following resection, the tissues were paraffin-embedded according to routine protocol. Two serial sections (5 and 10 mm) were cut from each tissue block and applied to slides without coverslips. The 5 mm section from each specimen was stained with hematoxylin and eosin (H&E). Foci of tumor cells were identified by light microscopy and corresponding tissue was excised from the 10 mm section using #10 scalpel blades for DNA extraction. A new scalpel blade was used for each sample to avoid cross-contamination. The tissues were placed into 1.7 ml tubes and deparaffinized with *n*-octane and ethanol. Genomic DNA was prepared by digesting the samples in the proteinase K/Tween 20 solution [0.5 mg/ml proteinase K, 50 mM Tris, 1 mM EDTA, and 0.5% Tween 20 (pH 8.5)] for 48 h at 55°C. The amount of DNA extracted from the samples ranged from 0.5 to 2.0 mg as determined by spectrophotometry.

Real-time PCR to detect homozygous deletions in *p16* exon 2

Amplification reactions were carried out using the Cepheid Smart Cycler. Forward (5'-ACTGCGATGTCG TACAGTATCTA-3') and reverse (5'-AGCCCTCAGATTTCAACTCTT-3') primers (Invitrogen, Carlsbad, CA) were used at a final concentration of 0.2 μM. The dual-labeled fluorogenic probe (5'-FAM-TAACTTCTGCCAGACACCCCA-TAMRA-3') was used at the final concentration of 0.1 μM. Each reaction was performed in the presence of 200 μM dNTPs, 3 mM MgSO₄, 1.25 U of *Taq* polymerase (Stratagene, La Jolla, CA), 1× additive (1 mg/ml bovine serum albumin, 750 mM Trehalose and 1% Tween 20), and 2.0 μl of genomic DNA (containing ~5–30 ng DNA). The sample was subjected to 96°C for 1 min, followed by 55 cycles of 30 s at 95°C, 30 s at 60°C, 30 s at 72°C. A final extension step was performed at 72°C for 5 min. A housekeeping gene, α -cardiac myosin heavy chain (α -CMHC), was co-amplified as an internal control. The primers and fluorogenic probe for the internal control are: forward primer, 5'-ACCCAGGG CTTCATGCGTAT-3'; reverse primer, 5'-CCGAGATAGGG AGCTAG-GCA-3'; and the probe, 5'-TET-CAAGCGTCTACCAGCGACTCCAGG-TAMRA-3'. All experiments were performed in duplicate.

p16 gene deletion analysis

The accuracy of the above real-time PCR assay was verified using a series of mixtures of genomic DNA from hamster POT2 cells (+/+) and H2T cells (-/-) at various ratios (POT2:H2T = 100:0, 75:25, 50:50, 25:75, 0:100) (19). Multiplex real-time PCR was performed and the resulting cycle threshold (Ct) values were normalized against those of POT2 using the following equation:

$$\Delta Ct = \Delta Ct_{p16} - \Delta Ct_{\alpha-CMHC}$$

where ΔCt_{p16} is equal to Ct_{p16} of a sample minus Ct_{p16} of POT2, and $\Delta Ct_{\alpha-CMHC}$ is the net $Ct_{\alpha-CMHC}$ of a sample deducted by $Ct_{\alpha-CMHC}$ of POT2 (21). While $\Delta Ct_{\alpha-CMHC}$ is correlated with the difference in total genomic DNA concentrations between the sample and POT2, ΔCt_{p16} is ascribed to the 'total' difference in *p16* gene concentrations between the sample and POT2 (wild-type), including the difference in total genomic DNA concentrations and the difference in the *p16* gene dosage. Therefore, ΔCt reflects the difference in the *p16* gene dosage. When the ΔCt values were plotted against the relative ratio of normal *p16* DNA in the mixtures (in exponential form), a linear graph with a correlation coefficient of 0.998 was obtained, indicating that the relative concentration of *p16* gene can be accurately measured using this technique (22). On the basis of this observation, the *p16* gene dosage in 30 hamster pancreatic tumor specimens was determined using the above real-time PCR assay, and the results were interpreted as follows (20): relative concentration <35%, *p16* homozygous deletion (-/-); relative concentration >35% and <70%, *p16* hemizygous deletion (+/-); and relative concentration >70%, *p16* wild-type (+/+).

Methylation-specific PCR of a CpG island in *p16* exon 1

Genomic DNAs from tumor tissues were bisulfate-modified using the CpGenome DNA modification kit (Serologicals, Norcross, GA). Primers used to amplify hamster *p16* exon 1 were modified/methylated 5'-GCGGTTGTTTAGGGTTCGC-3' (forward)/5'-CTACCTAAATCGAAATACGACCG-3' (reverse) and modified/unmethylated 5'-GGAGTAGTATGAGTTTTTTGTGGAT-3' (forward)/5' TATACCTACCTAAATCAAAA TACAACA-3' (reverse). The PCR mixture contained 1× PCR buffer, 1× enhancer, 1.5 mM MgCl₂, dNTPs (each at 1.25 mM), 1 U of Platinum *Taq*

DNA polymerase (Invitrogen), primers (0.2 mM each) and 10 ml of bisulfide-modified DNA in a final volume of 50 ml. Amplification was performed in a GeneAmp 9700 Thermal Cycler (PE Applied Biosystems) with PCR conditions of 95°C for 2 min followed by 50 cycles of 95°C for 30 s, 54°C for 30 s, 72°C for 45 s, and a final elongation step of 72°C for 5 min. The PCR products were analyzed by electrophoresis on a 2% agarose gel. Fragments amplified from methylated and unmethylated *p16* gene were of 100 and 143 bp, respectively.

Automatic sequencing of *p16* exons 1 and 2

Hamster *p16* exons 1 and 2 were amplified by PCR using the following intron-based primers: for exon 1, 5'-ATGGAGCCCTCT GCGGACG-3' (forward) and 5'-GATGAGATACCCTGTGCCTAC-3' (reverse); for exon 2, 5'-CGGG AGGGCTTCTTGAAAC-3' (forward) and 5'-GCCCCCGCTGGTGTAG-TAT-3' (reverse). Exon 3 was not analyzed as it encodes only a small portion of the C-terminus of P16 protein. The PCR mixture contained 1× PCR buffer, 1.5 mM MgCl₂, dNTPs (each at 1.25 mM), 1.0 mM of each primer, 1 U of *Taq* DNA polymerase (Invitrogen), 1.5× enhancer, and 4 ml of genomic DNA in a final volume of 50 ml. The whole procedure included: 96°C for 2 min (1 cycle); 30 s at 95°C, 30 s at 60°C and 30 s at 72°C (50 cycles); 5 min at 72°C (one cycle). The PCR products were purified using a PCR Product Purification kit (Qiagen), and subsequently sequenced using an ABI 377A automated DNA sequencer. Both strands of the PCR-amplified fragments were sequenced to confirm the mutations.

Protein expression and purification

Hamster *p16* cDNA was cloned into a pGEX-6p-1 vector (Amersham Pharmacia) at *Eco*RI and *Xho*I restriction sites, and expressed as glutathione *S*-transferase (GST)-fusion protein in *Escherichia coli* BL21 (DE3) as described previously (18). GST-fusion protein was purified by a reduced glutathione-agarose affinity column (Sigma). After incubation with PreScission protease (Amersham Pharmacia) at 4°C for 16 h, the GST tag was removed, and free P16 protein was further purified with a S100 column (Pharmacia) pre-equilibrated with 4 mM HEPES (pH 7.5 at room temperature), 1 mM DTT, and 5 mM EDTA. All *p16* mutants were generated using a Quickchange kit (Stratagene), and mutant proteins were expressed and purified as wild-type P16 protein.

In vitro CDK4 kinase assay

Three units of CDK4-cyclin D2 kinase were incubated with varying amounts of hamster P16 protein at 30°C for 30 min. Then 5 μCi of [γ -³²P]ATP and 50 ng of Rb protein were added into the reaction mixture with a final volume of 15 μl.

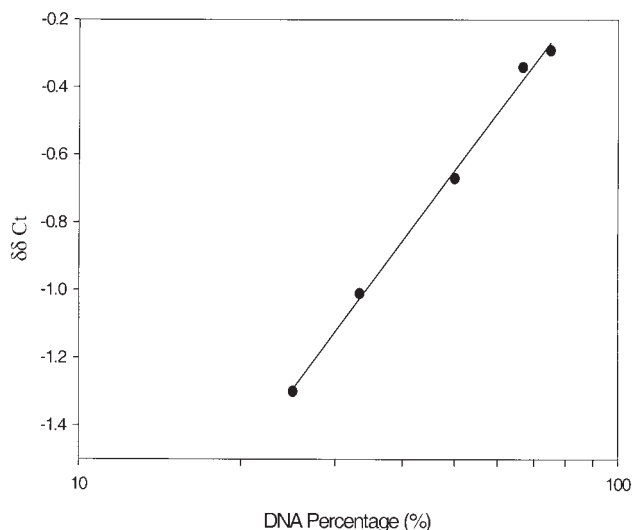


Fig. 1. The standard curve of the real-time assay to detect homozygous deletions in *p16* exon 2. Multiplex real-time PCR for both *p16* and α -CMHC genes were performed with a series of mixtures of genomic DNA from hamster POT2 cells, which are wild-type for *p16*, and H2T cells, which show homozygous deletion of *p16*, at various ratios, and the resulting Ct (cycle threshold) values were normalized as described in Materials and methods. The ΔCt values were plotted against the relative ratio of normal *p16* DNA in the mixtures (in exponential form) to yield a standard curve with a linear correlation coefficient of 0.998.

Table I. Summary of *p16* alterations in chemically induced hamster pancreatic tumors

Sample ID	Exon 1 DNA methylation	<i>p16</i> status in Exon 2	Point mutation	Sample ID	Exon 1 DNA methylation	<i>p16</i> status in Exon 2	Point mutation
P1	+	+/-	-	P16	-	-/-	-
P2	+	+/+	-	P17	+	+/+	-
P3	-	+/-	Gly93FS ^a	P18	-	-/-	-
P4	-	-/-	-	P19	-	-/-	-
P5	-	+/-	Ala92Ser	P20	-	-/-	-
P6	+	+/-	-	P21	+	+/+	-
P7	+	+/-	-	P22	-	+/-	Ala60Pro
P8	-	-/-	-	P23	+	+/-	-
P9	+	+/+	-	P24	-	-/-	-
P10	-	+/+	-	P25	+	+/+	-
P11	+	+/-	-	P26	-	-/-	-
P12	+	+/-	Ala94Val	P27	+	+/-	-
P13	+	+/-	-	P28	-	+/+	-
P14	-	-/-	-	P29	-	-/-	-
P15	-	-/-	-	P30	+	+/-	-

^aFS, frame shift.

Table II. Structural and functional characterization of hamster P16 mutants

Sample ID	Hamster mutation	Residue localization	Structure	IC ₅₀ (nM)	Corresponding mutations found in human cancers
P3	G93FS ^a	Ankyrin III	Lost ^b	2000 ± 500 ^b	G101W in familial cancer syndrome, melanoma and pancreas cancers.
P5	A92S	Ankyrin III	Retained	320 ± 55	A100V, A100L and A100P in melanoma; A100P in ovary cancers
P12	A94V	Ankyrin III	Slightly perturbed	970 ± 205	A102E in liver cancers
P22	A60P	Loop 2 ^c	Aggregates	615 ± 124	A68V in melanoma and A68T in squamous cell carcinomas (SCC) of esophagus
	Wild-type		Retained	80 ± 20	

^aFS, frame shift.

^bG93FS mutant was sensitive to proteases, and the GST-tagged protein was used in the kinase assay.

^cLoop2, the relatively loose stretch between ankyrin repeats II and III.

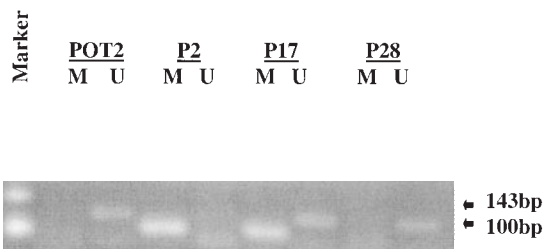


Fig. 2. Analyses of DNA methylation in *p16* exon 1. Three representative examples of methylation-specific PCR for *p16* exon 1 are presented here. The presence of a visible PCR product in Lanes M indicates the presence of methylated genes, and the presence of a visible PCR product in Lanes U indicates the presence of unmethylated genes. Genomic DNA from POT2 cells (with wild-type *p16* genes) was used a control.

After incubation at 30°C for 15 min, the reaction was stopped, and the mixtures were analyzed by SDS-PAGE. The ³²P incorporation into Rb was quantified by a PhosphorImager and IC₅₀, the inhibitory concentration for 50% of maximum inhibition, was determined (18,23).

One-dimensional proton nuclear magnetic resonance analysis

All protein samples were prepared in 4 mM HEPES (pH 7.5 at room temperature), 1 mM DTT and 5 mM EDTA in 10% D₂O. One-dimensional proton nuclear magnetic resonance (NMR) analysis was performed at 20°C on a Bruker DMX-600 spectrometer (23).

Results

p16 homozygous deletion

Several traditional methods such as Southern blot analysis, end-point quantitative PCR and FISH have been used to assess

p16 deletion status in tumor specimens (21,22). The requirements for high tumor to non-tumor cell ratio, laborious post-PCR handling and ‘sufficient’ amount of DNA samples, however, pose major limitations on their application, particularly when they are used to detect homozygous deletions in primary samples. Consequently, we developed an assay to accurately determine *p16* homozygous deletions in hamster pancreatic tumor samples using real-time quantitative fluorescence PCR. This technique allows for the rapid analysis of multiple samples without any post-PCR sampling. Evaluation of wild-type DNA isolated from POT2 cells confirmed that the assay has a dynamic range wide enough for clinical samples (data not shown). *p16* gene dosage was evaluated in samples with known relative *p16* concentrations. A housekeeping gene, *α-CMHC* was used as an internal control. When plotted exponentially (Figure 1), the normalized Ct (ΔCt) values correlated linearly ($r^2 = 0.996$) with known quantities of wild-type DNA (from POT2), indicating validity of the assay. Other investigators have reported similar techniques for the evaluation of human *p16* deletions by multiplex real-time PCR (21,22,24).

The *p16* gene status of the 30 hamster pancreatic tumor specimens was analyzed by the above real-time PCR assay and homozygous deletions were detected in 11 of 30 specimens (36.7%). This frequency is consistent with deletion frequencies found in studies of human pancreatic tumors (4–7).

p16 methylation status

Since information about the promoter of hamster *p16* gene is not available, methylation-specific PCR was performed to

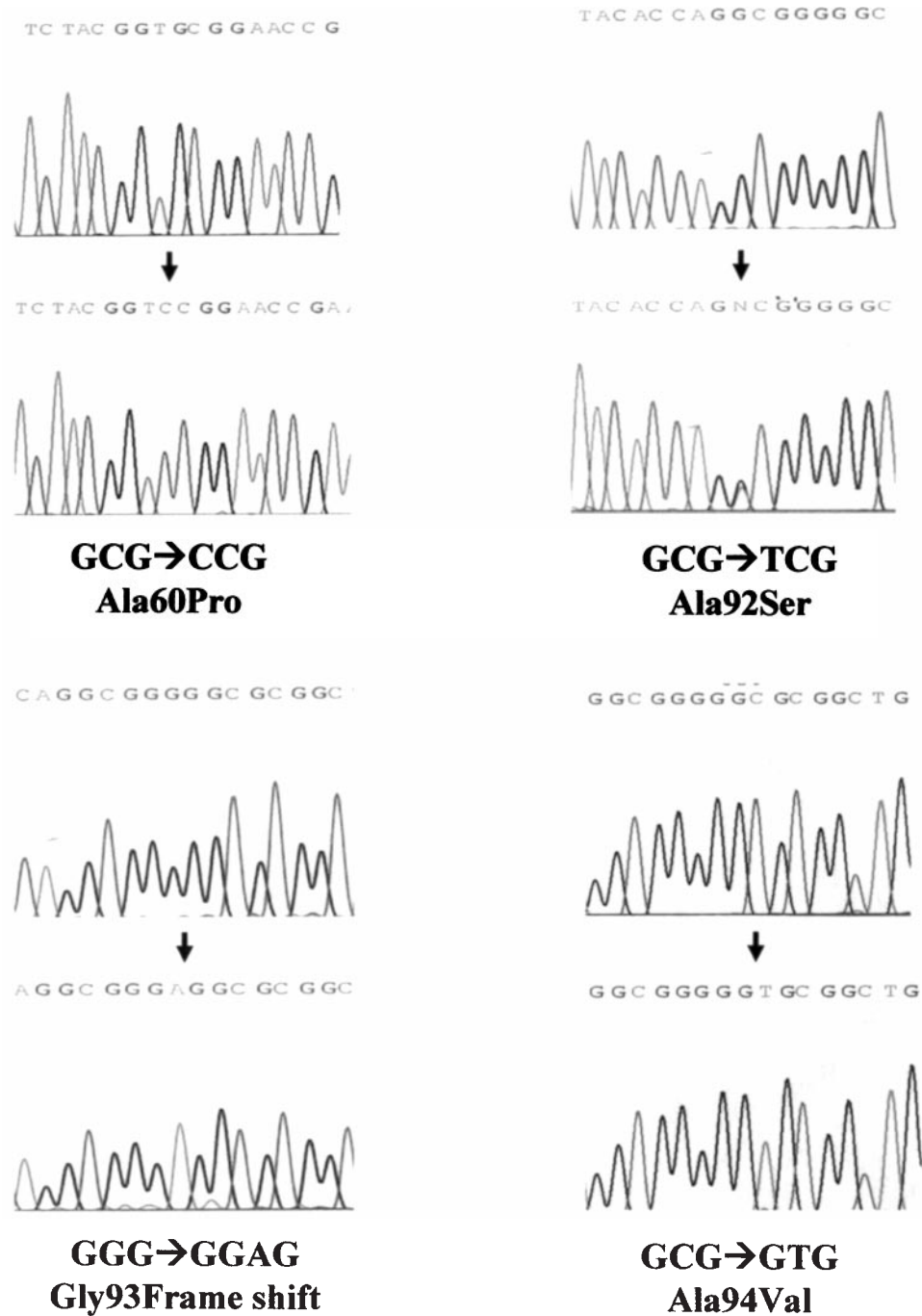


Fig. 3. Automatic sequencing results to determine point mutations in *p16* exons 1 and 2. Hamster *p16* exons 1 and 2 were separately amplified by PCR using intron-based primers, and the PCR products were purified and sequenced using an ABI 377A automated DNA sequencer. While both strands of the PCR-amplified fragments were sequenced, only the results for the sense strand are presented.

target 5' CpG islands in *p16* exon 1. The results are summarized in Table I, and a representative gel is pictured in Figure 2. Aberrant methylation in *p16* exon 1 was identified in 46.7% of specimens (14 of 30), indicating that methylation of 5' CpG islands is a common event in chemically induced hamster pancreatic tumors. Lower frequencies of *p16* methylation have been found in two studies of human pancreatic carcinoma (14 and 21%, respectively) (8), while higher frequencies of *p16* methylation have been found in other forms of human cancer including hepatocellular carcinoma (62.5%), squamous

cell carcinomas of the head and neck (57.7%) and pancreatic endocrine tumors (52%) (25–27). The underlying mechanism for an increased incidence of *p16* gene methylation in chemically induced SGH pancreatic tumors is unclear. One hypothesis is that methylation-associated silencing of *p16* may be a gradual process, during which a subset of cytosine residues within 5' CpG islands become progressively methylated, and tumors at later stages may have more extensive methylation of the 5' CpG islands of *p16* (8). Hence, the observation of different methylation frequencies between hamster and human

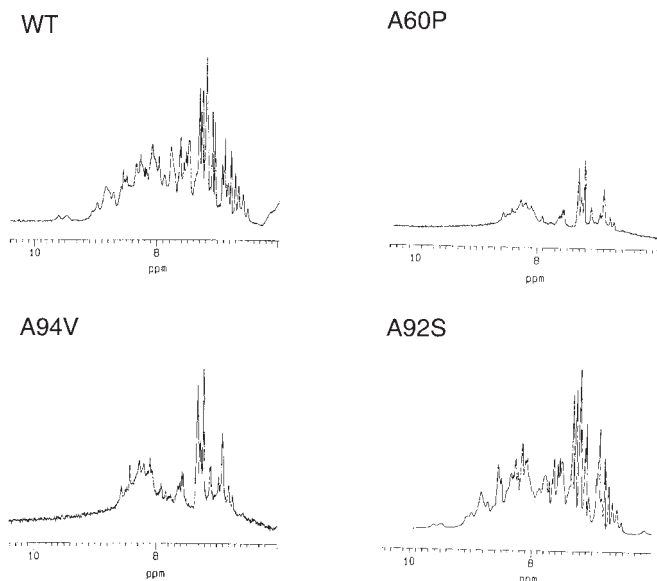


Fig. 4. Comparison of partial one-dimensional proton NMR spectra between wild-type hamster P16 and representative mutants. Recombinant protein samples were prepared in 4 mM HEPES (pH 7.5 at room temperature), 1 mM DTT and 5 mM EDTA in 10% D₂O, and NMR analyses were conducted at 20°C. Only NMR spectra corresponding to aliphatic residues are presented here.

pancreatic tumor specimens may be due to the evaluation of specimens at different stages in tumor development.

p16 mutation analysis

Table I summarizes the point mutations found in all 30 specimens, and Figure 3 depicts the sequencing of two representative mutations. A total of 13.3% of specimens (four of 30) harbored *p16* mutations, resulting in one frame shift, and three missense mutations. These occurred exclusively in *p16* exon 2. Interestingly, all of the observed mutations are located in loop 2 and ankyrin repeat III, regions where most interactions between P16 and CDK4 lie (23,28). Corresponding mutations have been described in human tumor specimens (Table II) (10). Further functional and structural characterization indicated that all four mutations impaired the CDK4-inhibitory ability of P16 by 4–25-fold (Table II). Of the four mutations, two resulted in significant perturbation of the global P16 protein structure as evaluated by NMR spectroscopy (Figure 4). These observations are consistent with previous findings indicating that most *p16* mutations in human cancers affect the function and/or structure of P16 protein (10,23,29).

Discussion

Chemically induced pancreatic tumors, in SGH, are a well-established model for evaluation of the development and progression of pancreatic adenocarcinomas (18,19). These tumors share many striking morphological and biological similarities with human pancreatic tumors. To our knowledge, BOP-induced SGH pancreatic tumors are the only experimental model that demonstrates a histologically ductal morphology, which is typical of human pancreatic cancers. SGH pancreatic tumors also demonstrate many biological similarities with human tumors including the propensity for aggressive local invasion, metastases and cachexia. Previous studies have demonstrated that there are similar genetic manifestations in

both human and SGH pancreatic tumors, including early *K-ras* alterations (16,30,31), alterations in the *p53* tumor suppressor gene (13,31,32), and expression changes in *DCC* and *Rb-1* genes (33). A recent study indicates that alterations in expression of the fragile histidine triad (*FHIT*) gene occur at greater frequencies in BOP-induced pancreatic tumors (73.3%) than in sporadic human pancreatic adenocarcinomas (50%) (34). Deletion of exons 5 and 8 appears to be a conserved mechanism of *FHIT* inactivation, however, as this finding occurs frequently in both tumor types.

In the current study, *p16* alterations were detected in 93.3% (28 of 30) of chemically induced SGH pancreatic tumor specimens (Table I). A single mechanism of inactivation was found in all but one of 28 specimens (P12 demonstrated both methylation and mutation) noted to harbor *p16* inactivation. This finding suggests that only one form of genetic alteration is necessary for gene silencing. Homozygous deletion and/or CpG island methylation were the most commonly identified mechanisms of inactivation (83.3%) and this is consistent with reported frequencies in human pancreatic cancers. Of the four point mutations found in this study, two were transversions ($G \rightarrow C$ and $G \rightarrow T$), one was a $C \rightarrow T$ transition, and one was a 1-base insertion ($GGG \rightarrow GGAG$) that resulted in a frame shift. Functional analysis of mutated proteins indicates that they are non-functional.

A number of oncogenes and tumor suppressor genes appear to be involved in pancreatic tumorigenesis, and one method of elucidating the role of each gene in neoplastic transformation is to determine when during multistage carcinogenesis a particular gene is activated or inactivated. The SGH model allows for the evaluation of tumors at various stages of progression, including papillary hyperplasias, carcinomas *in situ*, adenocarcinomas and metastatic lesions. Studies using this approach demonstrate that *K-ras* mutation is an early event in BOP-induced hamster pancreatic tumorigenesis (16,30). Further studies evaluating the step-wise progression of *p16* inactivation and its coordination with other genetic alterations may contribute to our understanding of the molecular events involved in pancreatic carcinogenesis. Reintroduction of functional *p16* gene product by adenoviral transfection has been shown to result in decreased tumor cell growth in human pancreatic cancer cell lines (35,36). The SGH model may be useful for evaluating the effects of *p16* gene transfer on tumor growth in an *in vivo* setting. Additionally, other strategies targeting the *p16/Rb/E2F* pathway, such as *E2F*-dependent oncolytic adenoviruses, could be evaluated using this model (37).

In conclusion, *p16* inactivation appears to occur frequently in BOP-induced SGH pancreatic tumors. The rate and mechanisms of inactivation appear to be consistent with those described in human pancreatic tumors. The data extend genetic similarities between human and hamster pancreatic tumors to the *p16/Rb* pathway, and strongly support the hypothesis that the SGH model is appropriate for studies evaluating novel *p16*-targeted therapies for pancreatic cancers. Finally, the SGH model appears to be suitable for studies evaluating the role of *p16* inactivation in pancreatic carcinogenesis.

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