Frequent p16INK4A/CDKN2A alterations in chemically induced Syrian golden hamster pancreatic tumors

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The p16INK4A/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.

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 two to four 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 x-octane and ethanol. Genomic DNA was prepared by digesting the samples in the protease 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′-AGCTGACTCTCTCAGGGAT-3′) and reverse (5′-GATGGAGATACCCTGTGCCTAC-3′) primers (Invitrogen, Carlsbad, CA) were used at a final concentration of 0.2 μM. The dual-labeled fluorogenic probe (5′-FAM-CCGGAGACACCCCTGTTGTAATCC-TAMRA-3′) was used at a final concentration of 0.1 μM. Each reaction was performed in the presence of 200 nM dNTPs, 3 mM MgSO₄, 1.5 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′-ACCCAGGCTTCTATGCTTTC-3′; reverse primer, 5′-CCGGAGATAGGGAGCTAG-GCA-3′; and the probe, 5′-TET-CAACCGGTCTACACGGCTGGAC/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 the homozygous deletion (−/−) 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 as described in Materials and methods. The ΔCt 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 ΔCt_{CMHC} is the net Ct_{CMHC} of a sample deduced by Ct_{CMHC} of POT2 (21). While ΔCt_{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 bisulfite-modified using the CpGenome DNA modification kit (Sorelogicals, Norcross, GA). Primers were designed and the amplifier hamster p16 exon 1 were modified/methylated 5′-GGGTTGGTTTAGGTCCG-3′ (forward)/5′-CTACTCTAATCTGAAA-TACGAGCCG-3′ (reverse) and modified/unmethylated 5′-GGAGTATGATGT-GAGTTTTTGTGATG-3′ (reverse)/5′-TATACCTAAATATACAAA-TACAACCAA-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′-CTACCTATAATCTGAAAA-TACGAGCCG-3′ (forward) and 5′-GATGAGATACCCTGTGCCTAC-3′ (reverse); for exon 2, 5′-CGGGAGGGCTTCTTGAGAAC-3′ (forward) and 5′-GCCCGGCCTCGTGTGATTAT-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 mutant proteins were expressed and purified as wild-type P16 protein.

Protein expression and purification

Hamster p16 cDNA was cloned into a pGEX-6p-1 vector (Amersham Pharmacia) at EcoRI and XhoI 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 was 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 correlation coefficient of 0.998.
p16/CDKN2A changes in hamster pancreatic tumors

### 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

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

<table>
<thead>
<tr>
<th>Sample ID</th>
<th>Exon 1 DNA methylation</th>
<th>p16 status in Exon 2</th>
<th>Point mutation</th>
<th>Sample ID</th>
<th>Exon 1 DNA methylation</th>
<th>p16 status in Exon 2</th>
<th>Point mutation</th>
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<tr>
<td>P1</td>
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<td>P16</td>
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<td>−</td>
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<td>+</td>
<td>+/−</td>
<td>−</td>
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<td>+/−</td>
<td>Gly93FS*</td>
<td>P18</td>
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<td>−/−</td>
<td>−</td>
</tr>
<tr>
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<td>+/−</td>
<td>−</td>
<td>P19</td>
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<td>P30</td>
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</table>

*FS, frame shift.

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**Table II. Structural and functional characterization of hamster P16 mutants**

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

*FS, frame shift.

*G93FS mutant was sensitive to proteases, and the GST-tagged protein was used in the kinase assay.

*Loop2, the relatively loose stretch between ankyrin repeats II and III.

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**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.

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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 Phospholmager and IC\textsubscript{50}, 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% D\textsubscript{2}O. One-dimensional proton nuclear magnetic resonance (NMR) analysis was performed at 20°C on a Bruker DMX-600 spectrometer (23).
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
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 → C and G → T), one was a C → T transition, and one was a 1-base insertion (GGG → 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.

Acknowledgements

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References