Reduced levels of p15INK4b, p16INK4a, p21cip1 and p27kip1 in pancreatic carcinoma

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Abstract. Pancreatic carcinoma is one of the leading causes of cancer mortality worldwide, although the molecular mechanisms of this disease are poorly understood. The aim of this study was to examine the expression of cyclin-dependent kinase inhibitors (CDKIs) and the epigenetic modifications in the promoters of these genes. We also evaluated the correlation between the methylation status of *CDKI* genes and smoking habit in clinical pancreatic carcinoma specimens. Western blotting and real-time PCR were performed to assess CDKI expression. Methylation-specific PCR was carried out to examine the methylation status of the promoters of *CDKI* genes. In this study, we revealed that reduced levels of the CDKI proteins, p15INK4b, p16INK4a, p21cip1 and p27kip1, are a prominent feature of pancreatic carcinoma patients. The DNA hypermethylation of the promoter was observed in 40% (2 of 5) of the *p15INK4b* genes, 60% (3 of 5) of the *p16INK4a* genes and 60% of the *p21cip1* genes, which markedly correlated with their decreased mRNA expression. No hypermethylation was detected in the *p27kip1* gene promoter in 5 pancreatic carcinoma patients with markedly decreased expression of *p27kip1* mRNA, suggesting an alternative mechanism of *p27kip* in these patients. In this study, patients with a smoking habit displayed methylation of 2 *CDKI* genes in their pancreatic carcinoma specimens. We concluded that epigenetic modification via hypermethylation represents a critical mechanism for the inactivation of *CDKI* genes in pancreatic carcinoma.

Introduction

Pancreatic carcinoma is one of the leading causes of cancer mortality worldwide. Pancreatic cancer has been reported to be associated with various environmental and lifestyle risk factors. Although the molecular etiology of pancreatic carcinoma is unclear, age and cigarette smoking are the unequivocal risk factors (1).

Due to the aggressive nature of the disease and the difficulties in diagnosis, the overall 5-year survival rate of pancreatic carcinoma is less than 5% (2,3). Novel approaches for the diagnosis and treatment of pancreatic cancer are necessary to improve the survival rate.

Cancer is a genetic disease where alterations in several genes accumulate and lead to a cancer cell growth advantage. Cell-cycle progression is driven by cyclins and cyclin-dependent kinases (CDKs). The activities of cyclin-CDK complexes are modulated by two classes of CDK inhibitor (CDKI) (4). The INK4 CDKI proteins (p15INK4, p16INK4, p18INK4 and p19INK4) sequester CDKs and inhibit the formation of CDK-cyclin complexes, whereas the Cip/Kip CDKIs (p21Waf1/Cip1, p27Kip1 and p57Kip2) bind to cyclin-CDK complexes (5). p21 (also known as waf1, cip1 or sdi1) is an important cellular checkpoint molecule for the inhibition of a range of cyclin-CDK activities. In our previous study, we demonstrated that association of p21cip1 with CDK2/cyclin E blocks cell-cycle progression at multiple points (6,7). The INK4A locus encodes two unrelated proteins, p14ARF and p16INK4a. p16INK4a is a specific inhibitor of the cyclin D-dependent kinases CDK4 and CDK6 (8) and antagonizes their ability to phosphorylate the retinoblastoma (Rb) family of proteins and so prevent exit from the G1 phase of the cell cycle (9). INK4A is important in mediating the signals that constrain the cell cycle in response to hyperproliferative signals, and, furthermore, are the most frequently inactivated tumor suppressor genes in human cancer (10,11).

Promoter methylation is an alternative form of gene silencing, which relies on epigenetic factors. Previous reports have revealed that aberrant *INK4a* or *Cip/Kip* promoter methylation is a frequent event in human tumors (12). Studies have indicated that suppressed expression by aberrant promoter methylation may be an alternative mechanism for inactivation of the tumor suppressor gene in pancreatic cancer cases (13).

In the present study, we reported that reduced levels of p15INK4b, p16INK4a, p21cip1 and p27kip1 CDKI proteins and mRNA are prominent features of pancreatic carcinoma. We observed that promoter hypermethylation of genes was partly correlated with decreased *p15INK4b*, *p16INK4a* and *p21cip1*, but not *p27kip1*, mRNA in the tumors from clinical patients.

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Materials and methods

Study subjects. Five frozen fresh tumor specimens were surgically isolated from patients with pancreatic carcinoma. All of these patients were admitted into the third General Surgery Department, Zhongshan Hospital. Ethical approval for this study from the Zhongshan hospital and agreement by all patients were obtained.

Western blot analysis. The tumor and human normal tissues were prepared in lysis buffer from the MC-CelLytics kit (Shenergy Biocolor, Shanghai, China). The protein content was determined using the Bradford calorimetric assay method (Shenergy Biocolor). The lysate was resolved by 10% polyacrylamide-sodium lauryl sulfate gel electrophoresis and Immobilon-P transfer membrane (Millipore, MA, USA). Antibodies used for detection were p15INK4b (Santa Cruz), p16INK4a (Santa Cruz), p21cip1 (Cell Signaling) and p27kip1 (Santa Cruz). Then, the blot was incubated with a secondary antibody, IRDye 800 conjugated affinity purified anti-mouse or anti-rabbit IgG (Rockland Immunochemicals, Gilbertsville, PA, USA), and detected with an Odyssey Infrared Imaging System (LI-COR Biosciencece, Lincoln, NE, USA).

Real-time RT-PCR analysis. Total RNA was extracted using TRIzol reagent (Invitrogen, Carlsbad, CA, USA) from tumor specimens and normal tissues. RNA was reverse transcribed using a PrimeScript™ RT reagent kit (DRR037A; Takara, Dalian, China). Real-time PCR analysis was performed in a final volume of 25 μl, containing 2 μl of cDNA template, 0.5 μl of each primer (10 μM) and 12.5 μl of a SYBR-Green master mix (2X) according to the instructions of the Real-time PCR kit (Takara, Japan) to evaluate the levels of mRNA expression. The following primers were used: 5'-AAGCTGAGCCCAGGT CTCCTA-3'(forward)and5'-CCACCGTTGGCCGTAAACT-3' (reverse) for p15INK4b; 5'-ACCCTTGTGCCTCGCTCAG-3' (forward) and 5'-GGTCTGCCGCCGTTTTC-3' (reverse) for p21cip1, and published primers for p16INK4a (14) and p27kip1 (15). The average amount of the genes was normalized to the levels of GAPDH (16), an endogenous housekeeping gene.

Methylation analysis. The tumor and normal tissue DNA was extracted by Tissue Genomic Isolation kits (Dingguo Bio-tech, Beijing, China). For methylation-specific PCR (MSP), samples were prepared according to the instructions of the CpGenome™ FAST DNA Modification kit (S7824; Chemicon International, CA, USA). Bisulfite-treated DNA was amplified using MSP primers specific for either methylated or unmethylated DNA using published primers for p15INK4b (17), p16INK4a (17), p21cip1 (18) and p27kip1 (18). PCR was carried out with Ex-Taq Hot Start DNA polymerase (Takara). The annealing temperatures used were 60˚C for p15 U/M, 65/60˚C for p16 U/M, 57˚C for p21 U/M and 55˚C for p27 U/M.

Results

Reduced levels of p15INK4b, p16INK4a, p21cip1 and p27kip1 proteins detected in pancreatic carcinoma. The events leading to pancreatic carcinoma development remain largely unknown.

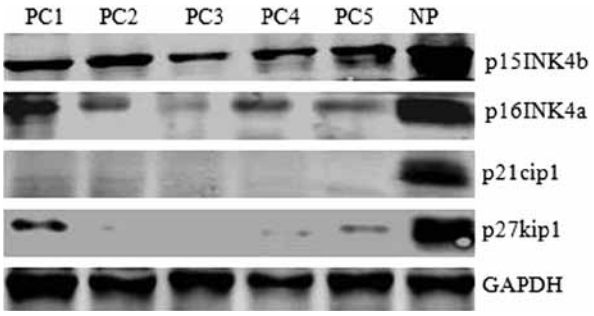

Figure 1. Reduced levels of p15INK4b, p16INK4a, p21cip1 and p27kip1 were detected in pancreatic carcinoma. Western blotting was performed to assess p15INK4b, p16INK4a, p21cip1 and p27kip1 protein levels in pancreatic carcinoma specimens (PC) and normal pancreatic tissue (NP) with GAPDH as the control.

Several studies have shown that the Rb tumor-suppressive pathway is abrogated in almost all studied cases of pancreatic carcinoma, and this disruption is caused exclusively by inactivation of CDKI (19). p15INK4b, p16INK4a, p21cip1 and p27kip1 are considered the most important CDKIs. This led us to ask whether these CDKIs are involved in pancreatic tumor formation. We examined p15INK4b, p16INK4a, p21cip1 and p27kip1 expression in 5 pancreatic carcinoma specimens and controls using western blotting. As shown in Fig. 1, p15INK4b, p16INK4a, p21cip1 and p27kip1 protein levels in 5 pancreatic carcinoma specimens (PC1-5) were much lower than in normal human pancreatic tissue (NP). These data suggest that reduced levels of p15INK4b, p16INK4a, p21cip1 and p27kip1, the negative regulators of cell progression, may be causative of tumor formation in pancreatic carcinoma.

Reduced levels of p15INK4b, p16INK4a, p21cip1 and p27kip1 mRNA expression detected in pancreatic carcinoma. Subsequently, we were interested in whether aberrant mRNA expression levels contributed to these CDKI alterations in pancreatic carcinoma specimens from patients. We evaluated *p15INK4b*, *p16INK4a*, *p21cip1* and *p27kip1* mRNA by real-time RT-PCR analysis to determine whether decreased mRNA accumulation contributed to their protein levels. As shown in Fig. 2, it was preceded by a >3-fold reduction in *p15INK4b* mRNA expression, a >4-fold reduction in *p16INK4a* mRNA expression, a >3-fold reduction in *p21cip1* mRNA expression and a >4-fold reduction in *p27kip1* mRNA expression of PC specimens, as compared to human NP tissue. Our findings suggested that the reduced levels of p15INK4b, p16INK4a, p21cip1 and p27kip1 proteins were likely due to a reduction in their mRNA synthesis, stability or translation in pancreatic carcinoma.

Methylation status of the p15INK4b, p16INK4a, p21cip1 and p27kip1 promoter region in pancreatic carcinoma. To explore the mechanism associated with the transcriptional silencing of the *p15INK4b*, *p16INK4a*, *p21cip1* and *p27kip1* genes, we examined 5 PC and human NP tissue samples to see whether there was methylation alteration in a CpG-rich region of the transcription initiation site of the these genes. The presence or absence of methylation in the promoters of the *p15INK4b*, *p16INK4a*, *p21cip1* and *p27kip1* genes was determined by MSP. MSP distinguishes unmethylated from

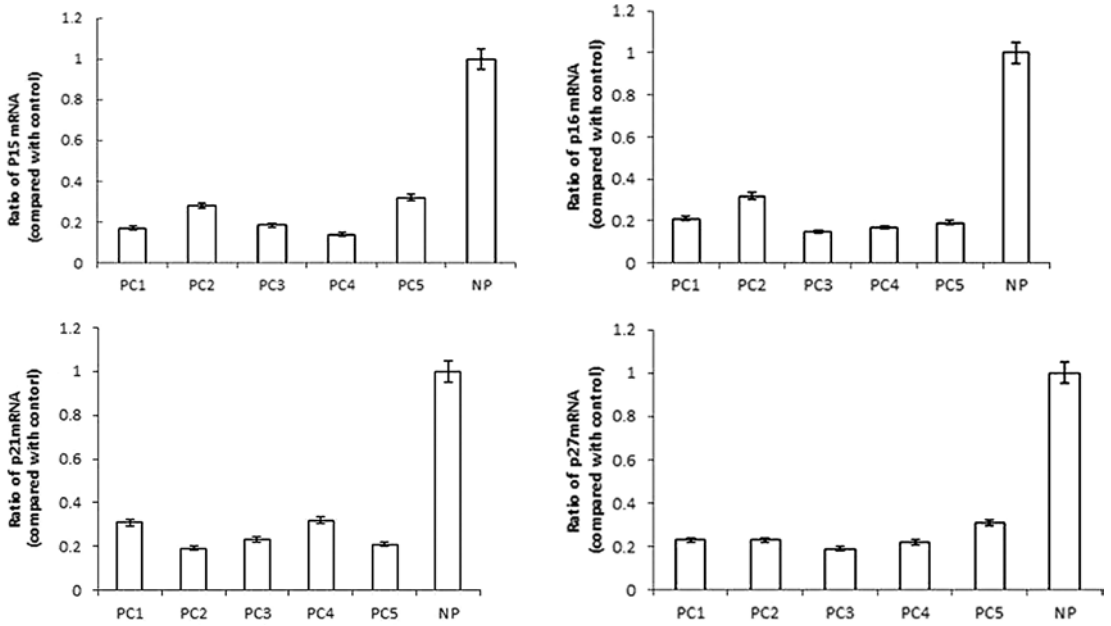

Figure 2. Reduced *p15INK4b*, *p16INK4a*, *p21cip1* and *p27kip1* mRNA expression was detected in pancreatic carcinoma. Real-time PCR was performed to assess *p15INK4b*, *p16INK4a*, *p21cip1* and *p27kip1* mRNA in pancreatic carcinoma specimens (PC) and normal pancreatic tissue (NP) as the control, and normalized to GAPDH expression. The error bars indicate the standard error of the mean of 3 independent experiments.

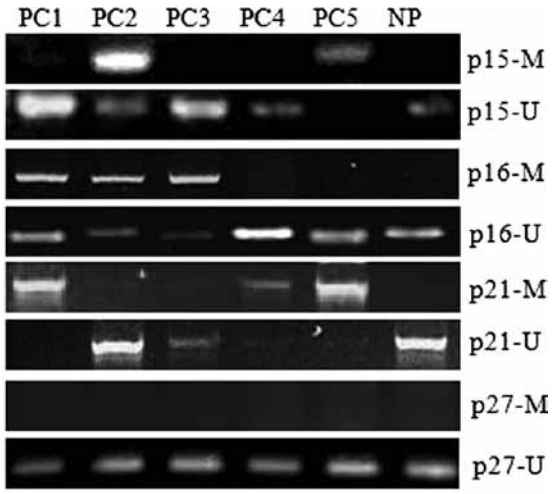

Figure 3. Methylation status of the *p15INK4b*, *p16INK4a*, *p21cip1* and *p27kip1* promoter regions in pancreatic carcinoma. Methylation-specific PCR was carried out. The presence of a PCR product in lanes M indicates the presence of methylated gene promoter and the presence of a product in lanes U indicates the presence of an unmethylated gene promoter in pancreatic carcinoma specimens (PC) and normal pancreatic tissue (NP).

methylated alleles based on sequence changes produced following bisulfite treatment of DNA, which converts unmethylated cytosine to uracil, and subsequent PCR using primers designed for either methylated or unmethylated DNA. As shown in Fig. 3, promoter hypermethylation was detected in PC2 and PC5 samples in the *p15INK4b* gene, PC1, 2 and 3 samples in the *p16INK4a* gene or PC1, 4 and 5 samples in the *p21cip1* gene, while no detectable promoter hypermethylation of the *p27kip1* gene was found in all 5 PC samples. No detectable promoter hypermethylation of the *p15INK4b*, *p16INK4a, p21cip1* and *p27kip1* genes was found in human

Table I. Association between smoking and methylation status.

No.	Gender	Age (years)	Cigarette smoking	p15[a]	p16[a]	p21[a]
1	M	42	Yes	×	√	√
2	F	62	Yes	√	√	×
3	F	49	No	×	√	×
4	F	60	No	×	×	√
5	M	56	Yes	√	×	√

[a]Methylation status of the gene promoters.

NP tissue samples (Fig. 3). Thus, aberrant DNA promoter hypermethylation of the *p15INK4b*, *p16INK4a* and *p21cip1* genes was thought to play a role in several cases of pancreatic carcinoma that had markedly decreased expression of *p15INK4b*, *p16INK4a* and *p21cip1* mRNA, concomitant with loss of p15INK4b, p16INK4a and p21cip1 proteins, but not p27kip1 protein (Figs. 2 and 3). Thus, we suggest that DNA hypermethylation associated with transcriptional silencing of the *p15INK4b*, *p16INK4a* and *p21cip1* genes may partly contribute to pancreatic carcinoma progression.

Pancreatic carcinoma patients with smoking habits displayed methylation of 2 CDKI genes. According to our results, pancreatic carcinoma patients showed reduced levels of p15, p16, p21 and p27 mRNA or proteins. To investigate the correlation between smoking habit and the methylation status of the promoter region in pancreatic carcinoma, these 5 randomly selected pancreatic carcinomas removed from the patients were further studied. As shown in Table I, the patients having smoked for >15 years (PC1, 2 and 5) displayed methylation of

2 genes in their pancreatic carcinoma specimens. On the other hand, the other 2 pancreatic carcinoma specimens (PC3 and 4), with only 1 methylation region in the *CDKI* genes, occurred in the patients without a cigarette smoking record (Table I). Thus, we suggest that cigarette smoking may be associated with pancreatic tumorigenesis by inducing the methylation of the promoter regions of the *CDKI* genes.

Discussion

In this study, we revealed that the reduced levels of two classes of CDKI protein, including p15INK4b, p16INK4a, p21cip1 and p27kip1, are a prominent feature of pancreatic carcinomas. Moreover, a reduced amount of *p15INK4b*, *p16INK4a*, *p21cip1* and *p27kip1* mRNA expression was found in all of the pancreatic carcinoma samples. We observed that hypermethylation of the *p15INK4b*, *p16INK4a* and *p21cip1* promoters, but not the *p27kip1* promoter, was partly correlated with markedly decreased mRNA expression. Thus, we suggest that hypermethylation associated with transcriptional silencing of the *p15INK4b*, *p16INK4a* and *p21cip1* gene may contribute to the progression of certain pancreatic carcinomas.

Due to the few treatment options for pancreatic carcinoma, understanding of the molecular pathology is a prerequisite for identifying potential molecular targets for drug therapy. CDKIs are negative regulators of cell-cycle progression at the G1-S restriction point. In this study, we showed that the reduced levels of p15INK4b, p16INK4a, p21cip1 and p27kip1 proteins were a prominent feature in the 5 pancreatic carcinoma specimens analyzed (Fig. 1), indicating that the reduced levels of p15INK4b, p16INK4a, p21cip1 and p27kip1, the negative regulators of cell progression, are involved in the tumor formation in pancreatic carcinoma.

Moreover, a reduced amount of *p15INK4b*, *p16INK4a*, *p21cip1* and *p27kip1* mRNA normal expression was found in 5 pancreatic carcinoma samples (Fig. 2). We observed patients with a markedly decreased >3-fold reduction in *p15INK4b* mRNA, a 4-fold reduction in *p16INK4a* mRNA, a 3-fold reduction in *p21cip1* mRNA and a 4-fold reduction in *p27kip1* mRNA, concomitant with reduced levels of these CDKI proteins in sporadic pancreatic carcinoma (Figs. 1 and 2). The methylation status of the *p15INK4b*, *p16INK4a*, *p21cip1* and *p27kip1* promoter region was detected using MSP. The DNA hypermethylation promoter was found in 40% (2 of 5) of the *p15INK4b* genes, 60% (3 of 5) of the *p16INK4a* genes, 60% (3 of 5) of the *p21cip1* genes and 0% (0 of 5) of the *p27kip1* genes, and markedly correlated with decreased mRNA expression in pancreatic carcinoma patients (Figs. 2 and 3). Moreover, all 5 pancreatic carcinoma patients (100%; 5/5) displayed methylation of 1 or more genes, 60% (3/5) displayed methylation of 2 genes, while control tissue did not display any methylation. It has been shown that aberrant methylation is the most prominent feature of pancreatic carcinoma, causing alterations in the expressions of genes (13). Thus, we demonstrated that the epigenetic modification of *p15INK4b*, *p16INK4a* and *p21cip1* via hypermethylation represents a critical mechanism for, at least in part, the inactivation of these genes in pancreatic carcinoma. In all 5 of our pancreatic carcinoma patients with markedly decreased expression of *p27kip1* mRNA, we were unable to detect hypermethylation in the promoter region, suggesting an alternative mechanism of the *p27kip1* gene in these patients.

Pancreatic carcinoma has been reported to be associated with various environmental and lifestyle risk factors, occupational exposures and medical conditions; however, the only risk factors consistently reported are age and smoking status, and the etiology of the disease remains largely unknown (1). In this study, we analyzed the correlation between smoking status and biological events in 5 randomly selected sporadic pancreatic carcinomas. Indeed, the patients smoking cigarettes for more than 15 years (PC1, 2 and 5) displayed methylation of 2 genes in their pancreatic carcinoma specimens. On the other hand, the other 2 pancreatic carcinoma specimens (PC3 and 4) with only 1 methylation region in the *CDKI* genes were surgically isolated from the patients without a cigarette smoking record (Table I). Thus, we suggest that cigarette smoking may be associated with pancreatic tumorigenesis by inducing the methylation of the promoter regions of the *CDKI* genes.

Our previous data suggested that expression of p21cip1 stops cell growth progression by inactivation of CDK activity, which in turn blocks the cell cycle at the G1 and G2 phases (20,21). Consistent with our previous finding that heightened CDK/cyclin signal transduction concomitant with loss of p27kip1 (22), the present study indicates that the reduced levels of the CDKI protein are a prominent feature of pancreatic carcinoma. These data suggest that the reduced levels of p15INK4b, p16INK4a, p21cip1 and p27kip1 may lead to defects in cell-cycle regulation and confer a selective growth advantage for pancreatic cancer cells. Thus, we showed that reduced levels of p15INK4b, p16INK4a, p21cip1 and p27kip1, the negative regulators of cell progression, may be causative of tumor formation in pancreatic cancer cells.

Taken together, reduced levels of p15INK4b, p16INK4a, p21cip1 and p27kip1 are a fundamental event in tumor formation in the clinical pancreatic carcinoma patients. The CDK inhibitors may interact to mediate signals that are critical growth inhibitors. This growth regulatory circuit would be disrupted, such as in pancreatic carcinoma. Thus, our observations have significant implications for understanding the importance of p15INK4b, p16INK4a, p21cip1 and p27kip1; reduced levels of these CDKI proteins contribute to tumorigenesis in pancreatic carcinoma and developing ways to target aberrantly active parts of this growth regulatory pathway may lead to increased survival.

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