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Evidence that P12, a specific variant of P16^{INK4A}, plays a suppressive role in human pancreatic carcinogenesis

Ming J. Poi [a], Thomas J. Knobloch [b,c], Chunhua Yuan [d], Ming-Daw Tsai [e], Christopher M. Weghorst [b,c], Junan Li [b,c,*]

[a] Department of Pharmacy, The Arthur G. James Cancer Hospital and Richard J. Solove Research Institute, The Ohio State University, Columbus, OH 43210, United States
[b] Comprehensive Cancer Center, The Ohio State University, Columbus, OH 43210, United States
[c] Division of Environmental Health Sciences, College of Public Health, The Ohio State University, Columbus, OH 43210, United States
[d] Campus Chemical Instrument Center, The Ohio State University, Columbus, OH 43210, United States
[e] Genomics Research Center and Institute of Biological Chemistry, Academia Sinica, Taiwan

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ABSTRACT

The *INK4a-ARF* locus plays a central role in the development of pancreatic tumors as evidenced by the fact that up to 98% of pancreatic tumor specimens harbored genetic alterations at the *INK4a-ARF* locus. Interestingly, in addition to the well-known P16^{INK4A} (P16) and P14ARF tumor suppressors, the *INK4a-ARF* locus in pancreas encodes another protein, P12, whose structure, function, and contributions to pancreatic carcinogenesis remain to be elucidated. In the current study, we demonstrated that over-expression of *p12* in human pancreatic cancer cells led to cell arrest at the G1 phase and such cell cycle arrest was related to down-regulation of a number of oncogenes, such as *c-Jun*, *Fos*, and *SEI1*. Furthermore, unlike P16, P12 did not retain any cyclin-dependent kinase 4 (CDK4)-inhibitory activity. Instead, P12 exhibited a transactivating activity not found in P16. We also examined the genetic status of *p12* in a cohort of 40 pancreatic tumor specimens and found that *p12* alteration was prevalent in pancreatic tumors with an incidence of 70% (28/40). These results support that P12 is a tumor suppressive protein distinct from P16, and its genetic inactivation is associated with pancreatic carcinogenesis.

1. Introduction

The *INK4a-ARF* locus plays a central role in tumor suppression as reflected by the fact that a significant proportion (~50%) of all human cancers harbored an inactivated *INK4a-ARF* locus [1,2]. As shown in Fig. 1A, the *INK4a-ARF* locus (*CDKN2A*) encodes two proteins, P16^{INK4A} (hereafter, P16) and P14ARF [1–4]. *p16* and *p14ARF* are transcribed from distinctive first exons (exon 1α and exon 1β, respectively) spliced onto common exons 2 and 3 in alternative reading frames. P16 specifically inhibits cyclin-dependent kinases (CDK) 4/6-mediated phosphorylation of the tumor suppressor pRb (the retinoblastoma susceptible gene product) as well as the subsequent E2F-mediated transcription of genes required for entry into S phase; P14ARF functions to prevent the degradation of the tumor suppressor P53 through interacting with MDM2. Due to its prevalence in human cancers, genetic inactivation of *p16* as well as *p14ARF* has been recognized as the primary cause of a variety of neoplasia, including pancreatic cancer [5,6].

However, recent studies have demonstrated that there are some unique features of the *INK4a-ARF* locus in pancreas [7,8]. First, while both *p16* and *p14ARF* are expressed at low levels in most of human tissues, *p16* is highly expressed in pancreas [7]. Conversely, *p14ARF* expression is not detectable in pancreas, indicating that P14ARF is transcriptionally silenced through unknown mechanisms in pancreas [8]. Secondly, an alternatively spliced *INK4a* transcript, termed *p12*, is expressed in the pancreas at high levels [7]. In the *p12* transcript (Fig. 1A), an additional 274 bp on intron 1, contiguous with the 3′ end of exon 1α, is included in the normal exon 1α sequence followed by exons 2 and 3. An in-frame stop codon in the intron 1-derived sequence results in P12, a polypeptide of 116 residues with an N-terminus (52 residues) identical to P16 and a novel C-terminus (64 residues) (Fig. 1B). It has been reported that over-expression of *p12* in Panc-1 (a pancreatic cancer cell line) and C33A (a cervical cancer cell line) results in cell cycle arrest at G1 and G2 phases in a pRB-independent manner [7]. However, knowledge about the biochemical properties of P12 and its potential contributions to pancreatic carcinogenesis remains limited. Here we present our studies on biochemical characterization of

* Corresponding author at: Division of Environmental Health Sciences, College of Public Health, The Ohio State University, Columbus, OH 43210, United States. Fax: +1 614 292 4053.
E-mail address: li.225@osu.edu (J. Li).

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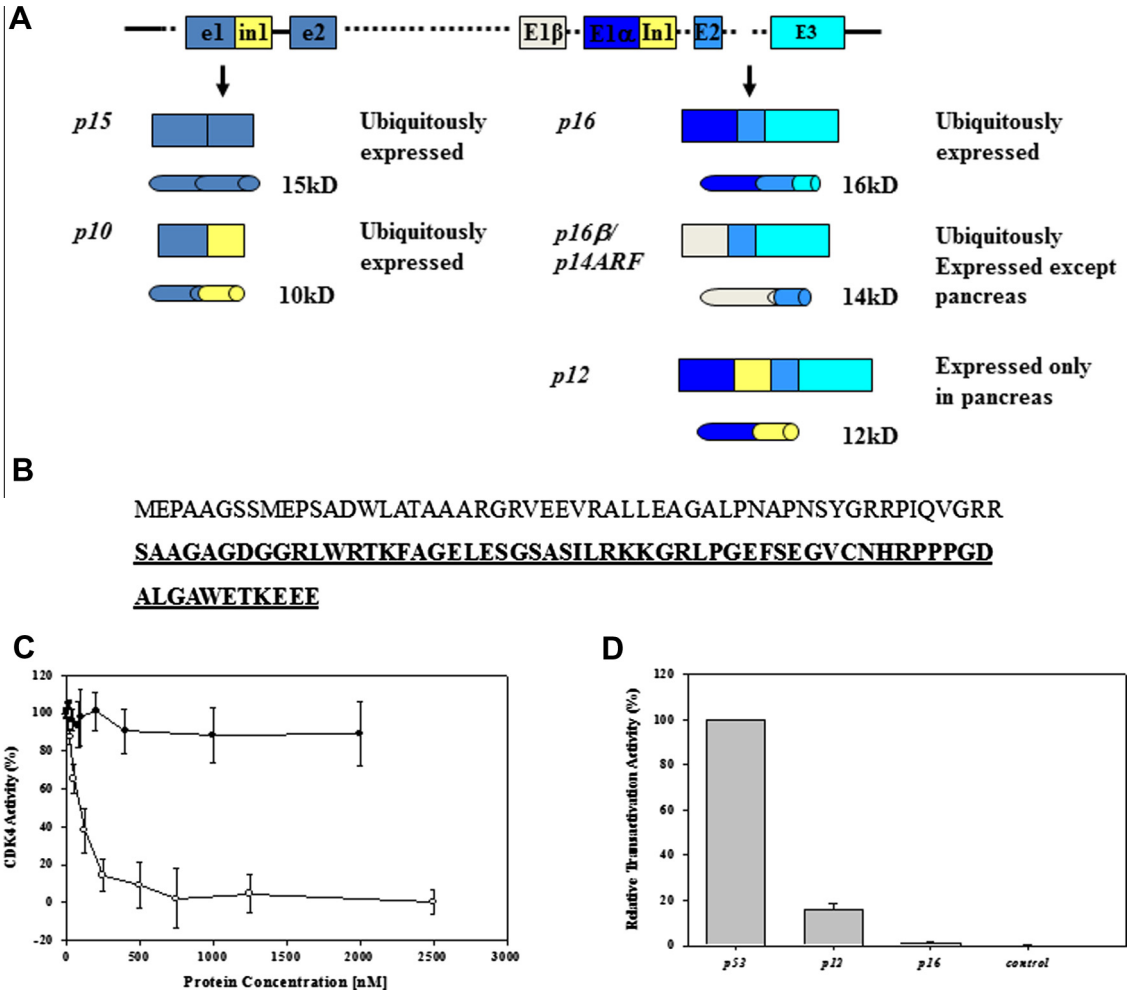

Fig. 1. *p12* gene, P12 protein, and its biochemical properties. (A) The structure of the *INK4a-ARF* locus. Rectangles represent DNA and mRNA, and cylinders represent proteins. e1, e2, in1: exons 1, 2, and intron 1 of $p15^{INK4B}$; E1β, E1α, E2, E3: exons 1β, 1α, 2, 2γ, and 3 of $p16^{INK4A}$; In1: intron 1 of $p16^{INK4A}$; kD, kilo Daltons. Sizes of coding regions and proteins are not in proportion strictly. (B) The amino acid sequence of P12. The novel intron-derived C-terminus is highlighted and underlined. (C) *In vitro* CDK4 inhibition assays. The reaction mixtures included 3 units of CDK4-cyclin D2, 50 ng of GST-pRb379–928, 5 μCi of [γ-32P] ATP, and varying amounts of P12 (•) and P16 (o) proteins. The incorporation of ^{32}P into GST-pRb379-928 was quantitated using a PhosphoImager (Molecular Dynamics). Assays were performed in triplicate. (D) Transactivation assays. *p12* cDNA was cloned into pM GAL4 DNA-binding domain vector in ORF, and pG5-Luc is used as a reporter vector. The empty pM vector and pM-*p53* were used as negative and positive controls, respectively. The relative transactivation activity (%) was defined as the luciferase activity with the target / the luciferase activity with *p53*. Assays were performed in triplicate.

P12 and the prevalence of *p12* alteration in human pancreatic tumor specimens.

2. Materials and methods

2.1. Cell line, cell culture, cell transfection and selection

Both Panc-1 and AsPc-1 cells were purchased from American Type Culture Collection (ATCC; Manassas, VA) and cultured in Advanced DMEM/F12 medium (Invitrogen) containing 5% fetal bovine serum (FBS; Invitrogen) in a 90% relative humidity incubator at 37 °C supplied with 5% CO_2.

pcDNA3.1-*p12* plasmid [7] was transfected into Panc-1 and AsPc-1 cells using Lipofectamine (Invitrogen) according to the manufacturer's recommendation. After selection with G418 (Invitrogene) at 1200 μg/ml for three weeks, five separate clones from each transfection were amplified, and the expression of *p12* was evaluated as described in the following section. Cells from the clone with the highest expression level of *p12* were subjected to flow cytometry analyses as previously described [9]. pcDNA3.1-transfected Panc-1 and AsPc-1 cells were used as control. Each flow cytometry assay was performed in triplicate.

2.2. Quantitatively determining the expression of selected genes in cancer cells

DNA-free RNA was isolated from cells using TRIzol Reagent (Invitrogen) following the manufacturer's instructions. First-strand cDNA synthesis was performed using PowerScript Reverse Transcriptase (BD Sciences) as the manufacturer recommended. Quantitative RT-PCR (qRT-PCR) reactions were carried out using prevalidated TaqMan® gene expression assays (Applied Biosystems) (Supplementary Table 1). The relative expression level of a gene was determined using the comparative Cq method as previously described [10]. Human hypoxanthine phosphoribosyltransferase 1 (*HPRT1*) was the reference gene, and pcDNA3.1-transfect cells were used for normalization. A two-fold increased ($\geqslant 2$) or decreased ($\leqslant 0.5$) value in the relative expression level was considered significant. Each gene was amplified separately, and all experiments were performed in triplicate.

2.3. Expression, purification, and characterization of P12 protein

Human *p12* cDNA gene was cloned into the pGEX-6p-1 vector and expressed as a glutathione-*S*-transferase (GST) -fusion protein in *Escherichia coli* BL21 (DE3) Codon plus cells (Novagen). GST-P12 was purified using reduced Glutathione-Agarose resin (Sigma) and the GST tag was removed using PreScission protease (Amersham) as previously reported [11]. While the structure of P12 was analyzed by NMR and Circular Dichroism (CD) [12], its inhibition to CDK4-mediated phosphorylation of pRb was assessed using an *in vitro* CDK4 activity assay as previously described [11,12]. The IC_{50} value was defined as the concentration of a kinase inhibitor required to achieve 50% of the maximal inhibition of CDK4 [12].

2.4. In vitro transactivation assays

A mammalian One-Hybrid assay (BD Clontech) was used to evaluate the potential transactivation activity of P12 [13]. Briefly, *p12* cDNA was cloned into pM GAL4 DNA-binding domain vector in the open reading frame (ORF), and pG5-Luc was used as a reporter vector, in which the luciferase gene is under the control of a promoter with GAL4 binding sequences. After transfection using Lipofectamine, the luciferase activity was assayed following the manufacturer's recommendation. The pM vector and pM-*p53* (provided by the manufacturer) were used as negative and positive controls, respectively. Assays were performed in triplicate.

2.5. Analyses of p12 alteration in pancreatic tumor specimens

Literature data about genetic alterations (homozygous deletion, methylation, and mutation) of *p16* in a cohort of 40 pancreatic adenocarcinomas specimens were re-examined for potential *p12* alterations in these specimens [5,14,15]. Since *p16* and *p12* share exon 1α, specimens harboring homozygous deletions of *p16* (exon 1α) were regarded *p12* -/-. In specimens harboring *p16* methylation, such "inactivating" event occurred at position +167 (the ATG of exon 1α, the translation initiation site, is numbered as +1), which is located at the beginning of intron 1, i.e. within the ORF of *p12*; therefore, *p12* was also methylated in these specimens. Additionally, intragenic mutations (point mutations and insertions/deletions of small fragments) in exon 1α impaired both *p12* and *p16*.

3. Results

3.1. P12 inhibited the proliferation of pancreatic cancer cells

To investigate the physiological role(s) of P12 in pancreas, we first introduced exogenous *p12* into two pancreatic carcinoma cell lines, Panc-1 and AsPc-1 to obtain cells stably expressing *p12*. Of note, endogenous *p12* was expressed in AsPc-1 but not in Panc-1 due to the deletion of the entire *INK4a-ARF* locus in the latter [7,10]; over-expression of *p12* in transfected Panc-1 and AsPc-1 was confirmed by qRT-PCR (data not shown). Subsequently, we analyzed *p12*-induced changes in cell cycle distribution of both cell lines using flow cytometry. The results are listed in Table 1. About 55.0% of p12-transfected Panc-1 cells were in the G0/G1 phase. In comparison, only 47.0% of control cells, i.e. vector-transfected Panc-1 cells, are in the G0/G1 phase. Thus, over-expression of *p12* in Panc-1 cells (*p12* −/−) brought about a significant increase of cells in the G0/G1 phase ($p < 0.05$). Similarly, over-expression of *p12* in AsPc-1 cells (*p12* +/+) led to a bigger population of G0/G1 cells (62.11% *vs* 53.71%, $p < 0.05$). Taken together, these results suggest that regardless of the genetic status of *p12* in pancreatic cancer cells, over-expression of exogenous *p12* arrests cell cycle progression at the G1 phase.

3.2. P12 modulated a number of cancer-related genes in pancreatic cancer cells

We then investigated potential changes in gene expression profiling in Panc-1 and AsPc-1 cells upon *p12* transfection through cDNA microarray analyses (data not shown). Out of those potential *p12*-related genes, a group of cell proliferation-related genes, including *Akt3*, *Aukra*, *Bax*, *Birc5*, *CDK4*, *cyclin D1*, *cyclin E*, *Fos*, *gankyrin*, *HIF1A*, *Jun*, *Mdm2*, *NFκB1*, $p18^{INK4C}$, $p19^{INK4D}$, *p21*, *SEI1*, *TP53*, and *VEFGA*, were quantitatively evaluated at the mRNA level using Taqman® gene expression assays (Supplementary Table 1) [9,10]. As shown in Fig. 2, over-expression of *p12* influenced multiple oncogenes and tumor suppressor genes at the transcription level. These genes can be categorized into three groups. The first group includes *Bax*, *Mdm2*, *c-Jun*, *Fos*, *SEI1*, and *cyclin E*. Upon *p12* transfection, the expression of *Mdm2*, *c-Jun*, *Fos*, *SEI1*, and *cyclin E* oncogenes was significantly down-regulated in both Panc-1 and AsPc-1 cells. Even though products of these five oncogenes function in different signaling pathways, their down-regulation generally disfavors cell proliferation. In parallel, up-regulation of *Bax*, a pro-apoptotic gene, in *p12*-transfected Panc-1 and AsPc-1 cells positively influences apoptosis. The second group consists of $p19^{INK4D}$ (*p19*) and *TP53* (*p53*). These two tumor suppressor genes were transcriptionally up-regulated in *p12*-transfected Panc-1 (Fig. 2A), which favor cell proliferation inhibition and apoptosis, respectively. Conversely, no significant changes in the expression of *p19* and *p53* were observed in *p12*-transfected AsPc-1 cells, indicating that the influence of P12 on *p19* and *p53* varies with pancreatic cell lines (Fig. 2B). This notion is further supported by observed changes in the expression of *Birc5* and *Aukra* (the third group). Upon *p12* transfection, *Birc5* and *Aukra* were expressed at significantly reduced levels in Panc-1 cells, but were transcriptionally up-regulated in AsPc-1 cells. While the underlying molecular mechanisms are yet to be defined, it is assumed that the cell line-dependent impact of P12 on the expression of *p19*, *p53*, *Birc5*, and *Aukra* may be partially attributed to the differences in the genetic background of pancreatic cancer cell lines. For example, since the entire *INK4a-ARF* locus (including $p15^{INK4B}$, *p16*, *p14ARF*, and *p12*) is deleted in Panc-1, elevated levels of *p19* mRNA in *p12*-transfected cells might partially compensate the loss of both P15

Table 1
p12-induced G1 arrest in pancreatic cancer cells.

Cell lines	Transfection vector[#]	Cell cycle distribution (%)		
		G0/G1	S	G2/M
Panc-1 (*p12* −/−)	pcDNA3.1	46.99 ± 1.3	29.38 ± 2.1	24.33 ± 1.4
	pcDNA3.1-*p12*	55.04 ± 2.4[*]	19.80 ± 2.2	25.16 ± 1.7
AsPc-1 (*p12* +/+)	pcDNA3.1	53.71 ± 1.8	26.89 ± 2.3	19.30 ± 1.8
	pcDNA3.1-*p12*	62.11 ± n3.2[*]	22.07 ± 1.5	15.82 ± 2.2

[#] *p12* (or control)-stably expressing cell lines were analyzed. Experiments were performed in triplicate.
[*] $p < 0.05$. Comparisons were between *p12*-transfected cells and the corresponding pcDNA3.1-transfected cells.

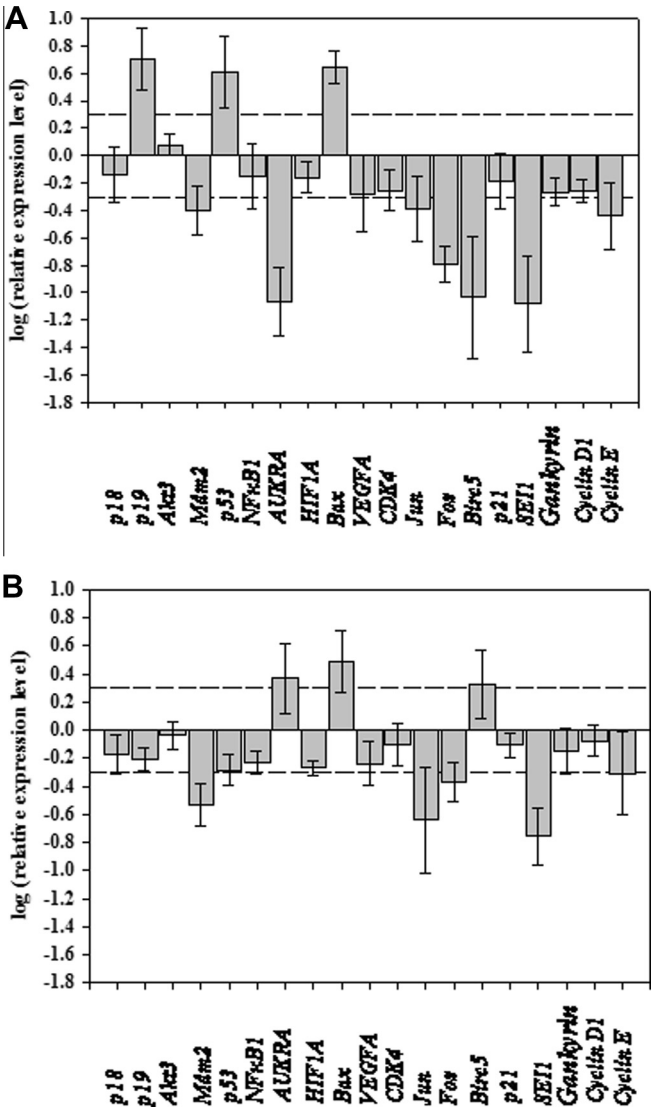

Fig. 2. Quantitative determination of mRNA expression of selected genes in cells. (A) Gene expression in *p12*-transfected Panc-1 cells. pcDNA3.1-transfected Panc-1 cells were used as control for normalization. (B) Gene expression in *p12*-transfected AsPc-1 cells. pcDNA3.1-transfected AsPc-1 cells were used as control for normalization. In both A and B, *HPRT1* was used as an endogenous control. Values (*y*-axis) represent fold-changes (in the log form) calibrated to the expression levels of indicated genes in control cells. Error bars represent standard deviations. Dashed lines represent 2-fold increases (upper) and decreases (lower) in gene expression. Assays were conducted in triplicate.

and P16 due to the well-known functional redundancy among INK4 proteins (specific inhibitors of CDK4 including P15, P16, $P18^{INK4C}$, and P19) in the inhibition of CDK4-mediated phosphorylation of pRb [4,16]. In *p12*-transfected AsPc-1 cells, elevated expression of *p19* was not observed since the cells have intact *p15*. Taken together, our results demonstrate that P12 has the potential to influence multiple cell proliferation or apoptosis-related pathways, thus contributing to pancreatic carcinogenesis. For example, in Panc-1 cells, over-expression of *p12* brought about significant changes in apoptosis (*Bax*, *p53*, and *Mdm2*), pRb-related cell proliferation (*SEI1* and *cyclin E*), and pRb-independent cell proliferation pathways (*c-Jun* and *Fos*). Apparently, these findings are consistent with previous studies showing that over-expression of *p12* in C33A and Panc-1 led to pRb-independent or P53-independent cell arrest at the G1 phase [7].

3.3. P12 did not bind to CDK4 but exhibited a transactivation activity

Since P12 has an N-terminus (residues 1–52) identical to that of P16 (Fig. 1B) [7], one may assume that P12 is able to interact with CDK4 and modulate CDK4-mediated phosphorylation of pRb, thus contributing to cell cycle progression. In regard to this perspective, we evaluated the impact of P12 on CDK4 using an *in vitro* kinase assay as previously described [12]. In a reaction mixture containing CDK4-cyclin D2 holoenzyme, pRb protein, and ^{32}P-ATP, addition of increasing amounts of P12 (up to 2.0 μM) did not bring about any notable change in the kinase activity of CDK4. In comparison, addition of P16 in the reaction mixture significantly eliminated the kinase activity of CDK4 with an IC_{50} value of 75 ± 12 nM, i.e. the activity of CDK4 decreased 50% in the presence of 75 nM P16 (Fig. 1C) [12]. These results indicate that P12 does not function as a CDK4 regulator. This is consistent with the observation that P12 only contains the N-terminal one and half ankyrin repeats of P16 whereas most of contacts with CDK4 are located within the second and third ankyrin repeats of P16 [17]. We also measured the CDK4-inhibitory activity of P16 in the presence of P12, and found that P12 did not influence the inhibition of P16 on CDK4-mediated phosphorylation of pRb (data not shown), suggesting that P12 does not function through blocking the binding of P16 to CDK4. Apparently, a novel function of P12 (in comparison with the function of P16) could underlie the aforementioned *p12*-induced modulation of some pRb-independent genes, such as *Jun*, *Fos*, *Birc5* in pancreatic cancer cells.

Interestingly, a BLAST search (www.ncbi.nlm.org) showed that the C-terminus of P12 shared considerable homology with a zinc finger protein, ZFP276 (*gi|76640280|ref|XP_591505*), especially at ^{86}GRLPGEFSEGVCNHRPPPGD^{105}, which led us to postulate that P12 might retain the ability for DNA binding and activation. To address this premise, we assessed the potential transactivation activity of P12 using an *in vitro* transactivation assay as previously described [13]. Remarkably, P12 exhibited a transactivation activity (about 15% of that of P53, a positive control) in this assay (Fig. 1D). In contrast, the transactivating activity of P16 was negligible. These results suggest that P12 is functionally distinct from P16. While identifying P12-targetted DNA elements by CHIP (chromatin immunoprecipitation) and CHIP-on-chip is on the way, the transactivating ability of P12 may arguably contribute to down- or up-regulation of the aforementioned oncogenes and tumor suppressor genes upon *p12* transfection.

3.4. The p12 gene was frequently altered in human pancreatic tumor specimens

Both *p12* and *p16* are located within the *INK4a-ARF* locus and have identical exon 1α (Fig. 1A), thus any genetic alteration in exon 1α (as well as intron 1) may impair both P16 and P12. To investigate the incidence of *p12* alteration in pancreatic tumor, we analyzed literature data about genetic alterations of *p16* in a cohort of 40 pancreatic adenocarcinomas specimens [4]. As summarized in Table 2, Kern and his colleagues demonstrated that 38 out of these 40 specimens harbored genetic alterations of *p16*, including homozygous deletions (19/40, 47.5%), intragenic mutations (point mutations and insertions/deletions of small fragments) (15/40, 37.5%), and methylation (4/40, 10%) [4]. In the 19 specimens harboring homozygous *p16* deletions, both exons 1α and 2 of *p16* were deleted. Evidently, *p12* was also truncated in these specimens since exon 1α is the first half of *p12* gene. Out of 15 specimens with intragenic mutations, four specimens had mutations in exon 1α, which led to two frameshifts (FS), one missense mutant (G23D) and one nonsense mutant (R50STOP) of P12; another specimen had a deletion of 39 base pairs at 5′UTR including the initiation site before exon 1α, which might affect the expression of *p12*. As for the

Table 2
Alterations of *p16* and *p12* in pancreatic carcinomas.

Pancreatic carcinomas[a]	*p16*[a]	*p12*	Pancreatic carcinomas[a]	*p16*[a]	*p12*
PX13	FS at codon 14	FS at codon 14	PX72	−/−	−/−
PX16	R80Stop	+/+	PX74	−/−	−/−
PX20	W110Stop	+/+	PX75	−/−	−/−
PX21	W110Stop	+/+	PX76	−/−	−/−
PX24	FS at codon 105	+/+	PX86	−/−	−/−
PX27	FS at codon 154	+/+	PX88	−/−	−/−
PX30	H83Y	+/+	PX90	−/−	−/−
PX61	G23D	G23D	PX91	−/−	−/−
PX65	R80Stop	+/+	PX94	−/−	−/−
PX67	R58Stop	+/+	PX102	−/−	−/−
PX68	FS at codon 82	+/+	PX107	−/−	−/−
PX92	5′UTR, no initiation site	No initiation site	PX117	−/−	−/−
PX101	In-frame deletion at codon 34	In-frame deletion at codon 34	PX120	−/−	−/−
PX105	R80Stop	+/+	PX17	M[b]	M[b]
PX122	FS at codon 78	+/+	PX29	M	M
PX19	−/−	−/−	PX45	M	M
PX23	−/−	−/−	PX93	M	M
PX28	−/−	−/−	PX104	M	M
PX55	−/−	−/−	PX66	+/+	+/+
PX56	−/−	−/−	Panc-1	−/−	−/−
PX64	−/−	−/−	AsPc-1	FS at codon 77	+/+

FS, frame shift; M, methylation.
[a] Data from References 5 and 14. Numbering was based on codons.
[b] Methylation of *p16* at position +167 (+1, the transcriptional start site) was investigated [14]. This site is located in intron 1, which is within the ORF of *p12*.

four specimens harboring *p16* methylation, methylation occurred at position +167 (+1, the translation initiation site), which is located within intron 1. Hence, such methylation also impaired the *p12* gene in transcription. Taken together, 28 tumor specimens (70%) harbored *p12* alterations. Such incidence of *p12* alterations in pancreatic tumor specimens could be underestimated since some genetic alterations in intron 1 may impair *p12* while *p16* remains intact. Results from our recent studies support this notion: deletions of intron 1 (i.e. *p12*) were detected in 8 of 20 pancreatic adenocarcinomas specimens. Out of these 8 specimens, 5 specimens harbored deletions in exon 1α, indicating that both *p16* and *p12* genes were truncated in these specimens. Conversely, the rest 3 specimens only had deletions in intron 1 (data not shown).

4. Discussion

The association between alterations in the *INK4a-ARF* gene and pancreatic cancer is nearly 100%, the highest of any form of human cancer [14]. Given the fact that *p14ARF* is transcriptionally silenced in normal pancreas [8], the tumor-suppressing activity associated with the *INK4a-ARF* locus in pancreatic tumors has been primarily attributed to P16 in the past decades. However, recent studies have demonstrated that P16 may not be the only or the primary tumor suppressor associated with alterations of the *INK4a-ARF* gene in pancreatic tumors. First, accumulating evidence has shown that in the absence of P16, the tumor-suppressing activities of P15 and $P18^{INK4C}$ (P18) are enhanced accordingly, indicating that P15 and P18 serve as backups of P16 in tumor suppression [18,19]. Secondly, it has been demonstrated that a considerable portion of *p16* missense mutations identified in tumor specimens only led to partial loss of its tumor-suppressing activity [12,20]. Moreover, overexpression of P16 (including wild type and missense mutants) has been frequently found in human cancers [21]. Presumably, elevated expression of missense P16 mutants may compensate the loss in the tumor suppressing activity caused by genetic alterations. From this perspective, the presence of *p12* and its tumor suppressive potential as demonstrated in previous studies and our current study indicate that *p12* may partially contribute to the tumor suppressing activity associated with the *INK4a-ARF* locus in pancreatic carcinogenesis.

One may argue that *p12* is just an abortive splicing form of *p16*, and it may not encode a protein product or the protein product is not functionally important. While previous studies have demonstrated the presence of P12 in pancreas [7] and keratinocytes [22], our NMR and biophysical studies show that P12 itself is structured or at least partially structured in solution. P12 has NOEs between aromatic spins and upfield protons resonating around 0.7 ppm (Supplementary Fig. 1A), indicative of the existence of tertiary or at least secondary structure. In guanidinium hydrochloride (GdnHCl)-induced unfolding (Supplementary Fig. 1B), P12 exhibits a two-state transition, which is similar to most of structured proteins [17]. While P16 has a more compact and stable structure as evidenced by more NOEs in the aromatic-aliphatic region and a higher denaturation energy (the ΔG_d^{water} values for P16 and P12 are 1.97 and 1.02 kcal mol^{-1}, respectively), it is likely that the structure of P12 becomes more stable upon binding to the target DNA element(s) or other proteins. It is also worthwhile to note that there exists an alternatively spliced form of human *p15*, termed as *p10* [23,24]. *p10* contains exon 1 the intron sequence between exons 1 and 2 of *p15* and encodes a protein with the N-terminus of P15 and a novel C-terminus (Fig. 1A). It has been well demonstrated that P10 exhibits a tumor-suppressing activity and is ubiquitously expressed [24]. The parallel between *p10* and *p12* indicates that such alternate splicing could not be an "abortive" molecular event but a common mechanism for gene regulation in the *INK4-ARF* locus. Indeed, it is well known that the *INK4-ARF* locus encodes two distinct tumor suppressors, P16 and P14ARF through alternate splicing [1,2]. Hence, the possibility that the *INK4-ARF* locus encodes the third tumor suppressive protein cannot be ruled out.

In conclusion, we have shown that P12 retains tumor suppressive activities distinct from that of P16 and *p12* is frequently altered in human pancreatic tumors with an incidence of 70%. Our findings strongly suggest that P12 is a potential pancreas-specific tumor suppressor, which could be the primary target or one of the primary targets for genetic alterations in the *INK4a-ARF* locus during pancreatic cancer progression.

Acknowledgments

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Appendix A. Supplementary data

Supplementary data associated with this article can be found, in the online version, at http://dx.doi.org/10.1016/j.bbrc.2013.05.078.

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