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Genome-wide screen identifies PVT1 as a regulator of Gemcitabine sensitivity in human pancreatic cancer cells

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ABSTRACT

Gemcitabine has been a first-line chemotherapy agent for advanced pancreatic cancer, which is associated with one of the lowest 5 years survival rates among human cancers. Due to our lack of understanding of the genetic determinants of Gemcitabine sensitivity in pancreatic cancer, the therapeutic effectiveness of Gemcitabine chemotherapy is typically unpredictable. Using a genome-wide and *piggyBac* transposon-based genetic screening platform, we identified the PVT1 gene as a regulator of Gemcitabine sensitivity and showed that functional inactivation of the PVT1 gene led to enhanced Gemcitabine sensitivity in human pancreatic cancer ASPC-1 cells. The integration of the *piggyBac* transposon-based vector system into intron 3 of PVT1 was within a common site of oncogenic retroviral insertions and chromosomal translocations. PVT1 is a non-protein encoding gene; the genomic arrangement of PVT1 and its co-amplification with MYC have been implicated in the tumorigenesis of a variety of cancers. The molecular mechanism of PVT1 transcripts in gene regulation remains a puzzle. We demonstrated that overexpression of a full length PVT1 cDNA in the antisense orientation reconstituted enhanced sensitivity to Gemcitabine in naïve ASPC-1 cells, whereas overexpression of a full length PVT1 cDNA in the sense orientation resulted in decreased sensitivity to Gemcitabine. Our results identified PVT1 as a regulator of Gemcitabine sensitivity in pancreatic cancer cells and validated the genome-wide genetic screening approach for the identification of genetic determinants as well as potential biomarkers for the rational design of Gemcitabine chemotherapies for pancreatic cancer.

1. Introduction

Pancreatic cancer is one of the major human cancers with poor clinical prognosis, and the overall 5 years survival rate of patients with pancreatic cancer is less than 5%. In 2010, the estimated number of new cases of pancreatic cancer in the United States was 43,140; it was estimated that 36,800 patients died from this disease. Deaths from pancreatic cancer still rank fourth among cancer-related deaths in the United States [1]. Chemotherapy plays an important role in the overall management of pancreatic cancer, but the inherent resistance of pancreatic cancer to currently available chemotherapeutic agents presents a major challenge. Gemcitabine remains the first line drug for the treatment of advanced pancreatic cancer, either alone or in combination with other chemotherapeutic agents; however, the effectiveness of chemotherapy is highly unpredictable [2–4]. This is partially due to the lack of our understanding of molecular pathogenesis of pancreatic cancer. Therefore, the identification of the genetic determinants of sensitivity to chemotherapeutic agents is imperative for the development of effective treatments and biomarkers for pancreatic cancer [5–8].

Recent studies in cancer genomics of major human cancers, including pancreatic cancer, have revealed complex genetic alternations involved in tumor pathogenesis and progression [9,10]. Comprehensive analyses of the genetic alternations in pancreatic cancer showed several core pathways and regulatory processes altered in 67–100% of the tumors, including K-Ras, TGF-β, small GTPase signaling, controls of G1/S phase transition and DNA damage [11], but their critical roles in pancreatic tumorigenesis and the inherent resistance to Gemcitabine are not well understood. Earlier studies of chemoresistance in pancreatic cancer identified several genes involved in Gemcitabine transport and metabolism [12,13]; recent functional genomics studies have indicated that there are critical regulatory genes that determine susceptibility of pancreatic cancer cells to Gemcitabine. Targeting these regulatory genes in combination with Gemcitabine produced synthetic lethality of pancreatic cancer cells and showed synergistic therapeutic effect [14]. Therefore, identification of these key regulators involved in Gemcitabine susceptibility may

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provide novel therapeutic strategies and biomarkers for pancreatic cancer.

Random Homozygous Knockout (RHKO) technology is a genome-wide genetic approach that identifies genes based on their biological functions [15]. The design of RHKO enables the functional inactivation of both alleles of randomly addressed chromosomal genes within populations of mammalian cells using gene search vector cassettes that contain a regulated antisense promoter. This strategy has been used successfully to identify genes whose functional homozygous inactivation leads to reversible tumorigenesis or altered sensitivity to chemotherapeutic agents [16]. We have integrated the RHKO technology with a recently developed mammalian genetic transposon system *piggyBac* [17–21], and established a *piggyBac* based genome-wide mutagenesis platform. Here, using this genetic approach, we performed genome-wide mutagenesis screening in a human pancreatic cancer cell line ASPC-1 and identified PVT1 as a novel regulator of Gemcitabine sensitivity; specifically, functional inactivation of PVT1 resulted in increased sensitivity to Gemcitabine. We further validated the functional roles of PVT1 in naïve ASPC-1 cells. Overexpression of antisense PVT1 resulted in increased sensitivity to Gemcitabine, whereas overexpression of sense PVT1 resulted in decreased sensitivity to Gemcitabine. Our results provide a novel genetic approach for the discovery of genetic determinants of Gemcitabine sensitivity and identify PVT1 as a potential biomarker for the prediction of Gemcitabine sensitivity and a candidate gene target for Gemcitabine combination therapy for pancreatic cancer.

2. Materials and methods

2.1. Plasmid construction

The plasmid pPBGS was constructed from pSB-in-PB (Gift of Dr. Alan Bradley, Sanger Center, UK), a modified tetracycline regulated element, an amino glycoside 3′ phosphotransferase (APH or Neo containing a bacterial promoter) expression cassette under the control of a β-actin promoter and plasmid replication origin (Ori, p15A from pACYC184). The neo expression cassette and Ori were first joined as a Nhe1 fragment and then joined with a modified tetracycline-regulated element, which consisted of 14 tetracycline operator binding sites and a minimum cytomegalovirus (CMV) immediate early promoter. This formed a 3.3 kb BamH1 fragment, which was ligated to a 3.9 Kb PCR fragment from pSB-in-PB (containing *piggyBac* transposon 3′ and 5′ terminal repeats) to form pPBGS (Fig 1A). pCAG-tTA was constructed by replacing a BamH1-Not1 fragment of pCYLo43 (Gift of Dr. Alan Bradley, Sanger Center, UK) with a PCR product which encodes the tetracycline transactivator (tTA). pTet-Luciferase, a tTA mediated luciferase report vector [22], and pLLEXP-1 were gifts of Dr. Limin Li (Peking Union Medical College), and pmPB was a gift of Dr. Alan Bradley, Sanger Center, UK.

2.2. Establishment of tetracycline regulated gene expression system in pancreatic cancer cells

Human pancreatic cancer ASPC-1 cell line was cultured in Dulbecco's modified Eagles medium (DMEM, HyClone) supplemented with 10% heat-inactivated fetal bovine serum, 100 units/ml penicillin and 0.1 mg/ml streptomycin, in a humidified 5% CO_2 incubator at 37 °C. ASPC-1 cells were transfected with pCAG-tTA vector with Fugene 6 transfection reagent (Roche, Germany) according to the manufacturer's instructions, and the transfected cells were selected with culture medium containing 1.5 μg/ml of puromycin (InvivoGen, USA). Individual puromycin resistant clones were isolated and analyzed following 1–2 weeks of selection.

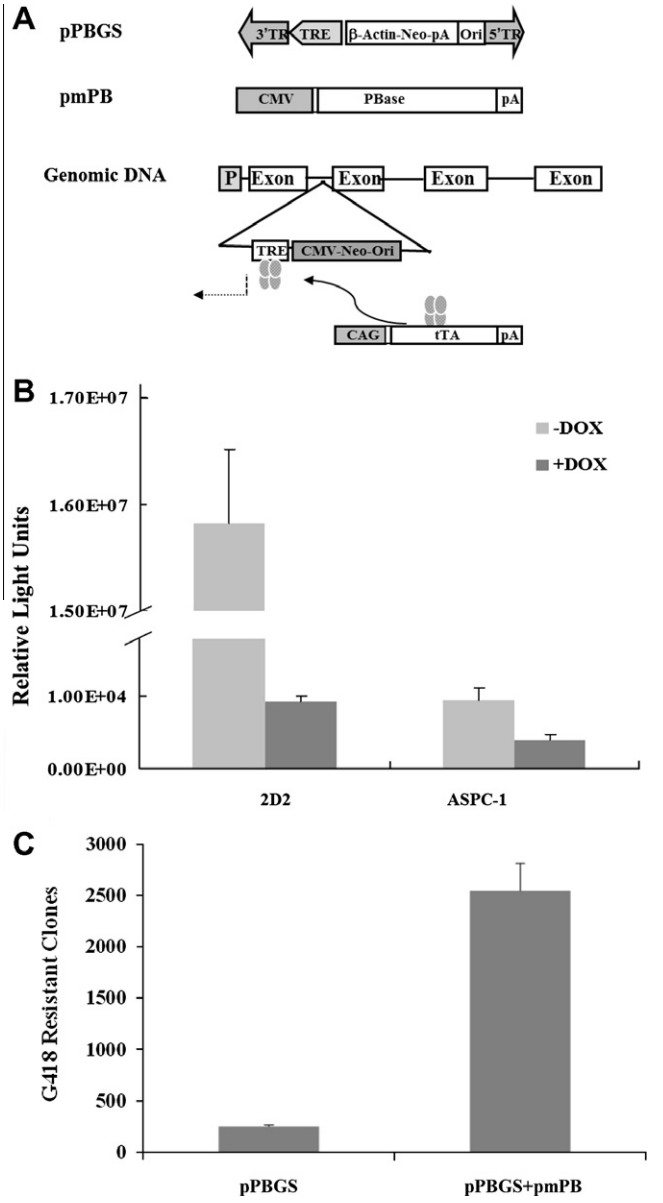

Fig. 1. *piggyBac* transposon-based genome-wide mutagenesis platform. (A) *piggyBac* transposon-based gene search vectors (pPBGS) contain *piggyBac* transposon 3′ terminal repeat (3′TR), 5′ terminal repeat (5′TR), a modified tetracycline regulated element (TRE), a Neo expression cassette and a bacterial plasmid replication origin (Ori); pmPB is an expression vector for *piggyBac* transposase. An example of genomic integration of pPBGS is depicted; antisense transcription initiated at TRE is mediated by a tTA encoded by pCAG-tTA. (B) Tetracycline regulated gene expression in 2D2 and control cells. Gene expression was measured by luciferase reporter activities in the presence or absence of doxycycline (Dox). Representative of three experiments, each assay was performed as triplicate. (C) *piggyBac* transposon mediated genomic integration in 2D2 cells, pPBGS alone and co-transfection of pPBGS and pmPB. Stable integrations of pPBGS were selected as G418-resistant clones. Representative of more than 20 experiments, each assay was performed in triplicate.

2.3. Assays for the expression of luciferase reporter gene

ASPC-1 cells derived from individual puromycin resistant colonies were plated in 96-well plates at 1×10^4 per well 12–24 h prior to transfection; the cells were then transfected with pTet-Luciferase (50 ng/well) with Fugene 6. Transfected cells were cultured

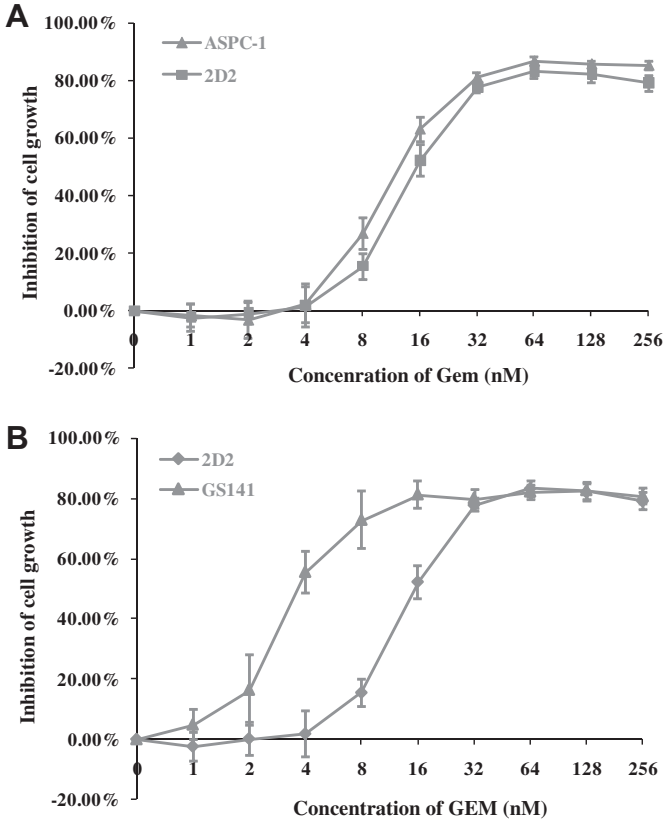

Fig. 2. Identification of Gemcitabine sensitive clone GS141. (A) Gemcitabine dosage responsiveness and IC_{50} of parental ASPC-1 cells and tetracycline regulated 2D2 cells were determined with MTT assays in different concentrations of Gemcitabine (Gem). (B) Identification of Gemcitabine sensitive clone GS141 with 2D2 cells with MTT assays. Representative of three experiments, each assay was performed in triplicate.

in the absence (−) or presence (+) of 2 μg/ml doxycycline (Dox, Sigma–Aldrich) for 48 h. Cultures were lysed with harvest buffer, and then mixed with the luciferase substrate and an assay buffer [23]. Relative light units were measured using a Luminometer (TURNER Modulus, American).

2.4. Generation of piggyBac transposon mediated genome-wide mutagenesis library

ASPC-1-2D2 cells were co-transfected with pPBGS and pmPB plasmids with Fugene 6. Transfected cells were selected with culture medium containing 600 μg/ml of G418 (CALBIO-CHEM) for 4–6 weeks and individual G418 resistant clones were isolated or pooled for further screening of Gemcitabine sensitivity.

2.5. MTT assay

The 3-(4,5-Dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide (MTT) assay kit (Roche, Germany) was used for the detection of Gemcitabine (GEMZAR, Lilly France) sensitivity. Cells were plated at a concentration of 4×10^3 cells/well in a 96-well plate for 12–24 h. Gemcitabine at different concentrations diluted directly from stock solution was added to each well. We performed the MTT assay according to the manufacturer's instructions. Absorbance was measured at 570 nm using a microplate reader (Labsystems Dragon, Wellscan MK3).

2.6. Genomic DNA Splinkerette-PCR and sequence analysis

Genomic DNA was isolated with standard procedure. RNA-free genomic DNA was digested with Sau3AI and ligated with Splinkerette adaptor primer pairs [24]. Splinkerette-PCRs were performed with the following primer sets: Splink-1 5′-CGAAGAGTAACCGT TGCTAGGAGAGACC-3′, and PB3-1 5′-TAAATAAACC TCGATATACAG ACCGATAAA-3′; Splink-2 5′-GTGGCTGAATGAGACTGGTGT CGAC-3′, and PB3-2 5′-ATATACAGACCGATAAAACACATGCGTCAA-3′. PCR products were gel purified and sequenced. DNA sequences were mapped to human genome and analyzed with the UCSC Genome Browser, NCBI human genome resources and Ensemble Genome Browser. Genomic integration of gene search vector in GS141 and 2D2 cells were further analyzed using genomic PCR with following primer sets: PVT1 five-primer integration site, 141-G5 5′-TGGTGG GCCAC AGTGAGTGAGGCCAGADNA-3′ and PB5-1 5′-CAAAATCAGT-GACACTTACC GCATTGACAA-3′; PVT1 three-primer integration site, 141-G3 5′-CTAGCTTT GCAGAGAAAGCCCTAGA G-3′ and PB3-1 5′-TAAATAAACCTCGATATACAGA CCGATAAA-3′; PVT1 control, 141-G5 5′-TGGTGGGCCACAGTGAGTGAGG CCAGA-3′ and 141-G3 5′-CTAGCTTTGCAGAGAAAGCCCTAGAG-3′.

2.7. Expression of human PVT1 full length cDNA in naïve ASPC-1 cells

A BamH1 fragment containing 1900 bp (without 18 bp 3′ polyA tail) of human PVT1 full length cDNA (NCBI reference sequence, NR_003367.1) was synthesized by Blue Heron biotechnology Inc., USA, and the sequence was confirmed. The cDNA was cloned into pLLEXP-1 in both sense and antisense orientations. pLLEXP-1 contains a puromycin resistance gene as a selection marker. pLLEXP-1 vectors carrying sense, antisense PVT1 cDNAs and no insert control were transfected into ASPC-1 cells, respectively. Transfected cells were selected in culture media with 1.5 μg/ml of puromycin for 1–2 weeks; puromycin resistant cells were assayed for their sensitivity for Gemcitabine.

2.8. Statistical methods

Error bars was calculated with mean ± S.E.M. Statistical analysis was performed using Student's *t*-test. $P < 0.05$ was considered statistically significance.

3. Results

3.1. Establishment of piggyBac transposon mediated genome-wide gene search platform

To identify potential chromosomal genes that regulate Gemcitabine sensitivity in human pancreatic cancer cells, we constructed *piggyBac* transposon-mediated gene search vectors based on early versions of genome-wide gene search vectors. The *piggyBac* transposon mediated gene search vector (pPBGS) contains a tetracycline regulated promoter that is active in the presence of tTA and the absence of tetracycline (Tet-Off), and a selection marker of aminoglycoside 3′ phosphotransferase (APH or Neo) under the control of human cytomegalovirus (CMV) promoter (Fig. 1A). A tTA expression vector pCAG-Tet was first transfected into human pancreatic cancer ASPC-1 cells, and more than 300 stable transfected and puromycin resistant clones were isolated. Isolated clones were transfected with pTet-Luciferase and were assayed for luciferase reporter activities in the absence and presence of doxycycline (Dox, a semi-synthetic tetracycline). Six ASPC-1 clones had shown more than 300-fold reporter gene regulation with 1–2 μg/ml of Dox; clone 2D2, which showed more than 1000-fold regulations, was selected for this study (Fig. 1B).

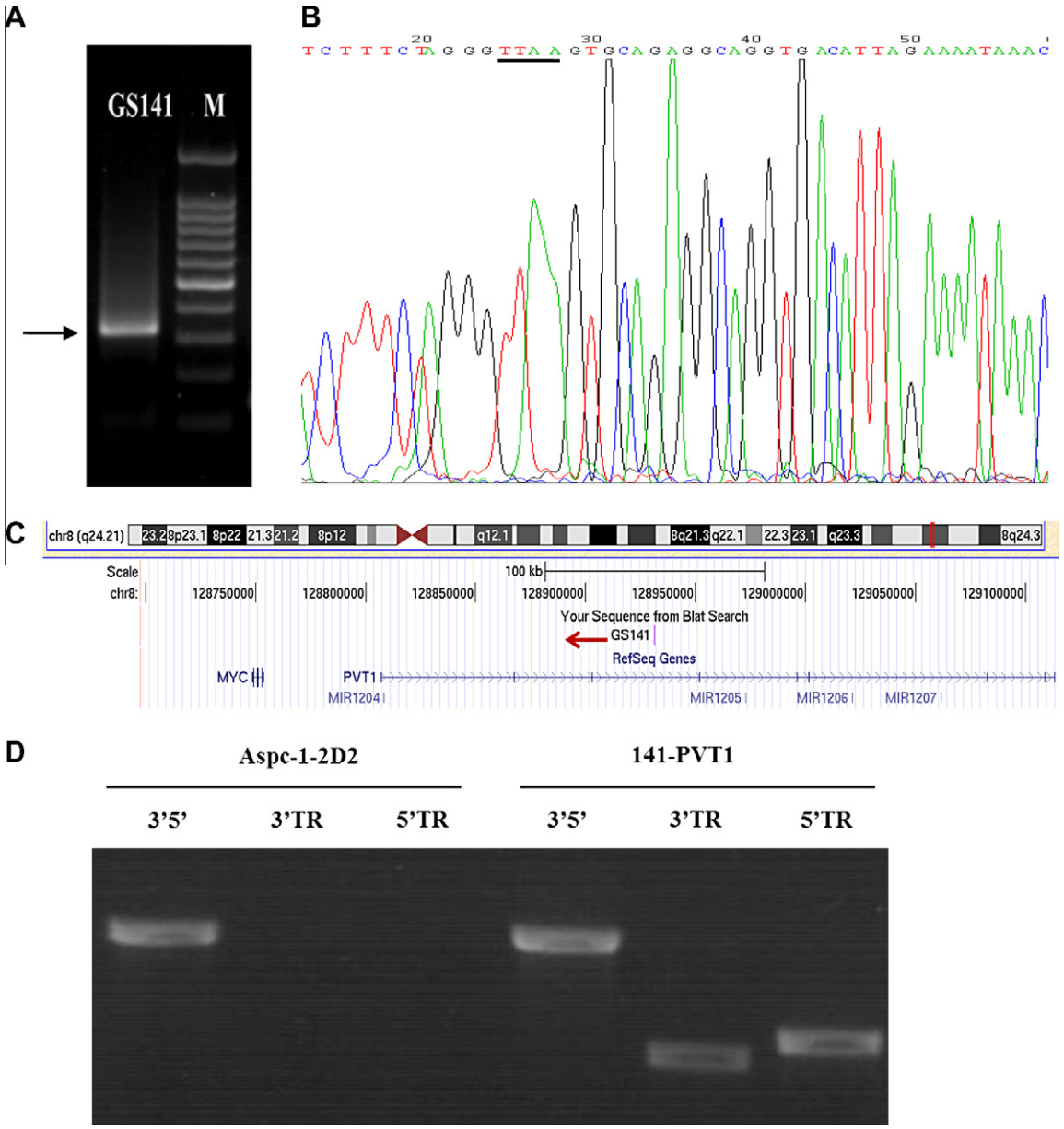

Fig. 3. Molecular cloning and genomic mapping of PVT1. (A) Splinkerette-PCR with genomic DNA isolated from GS141 cells; genomic DNA at 3′TR of pPBGS integration site was amplified as a 300 bp fragment. (B) DNA sequencing analysis showed PVT1 genomic junctions with pPBGS gene search vector; the consensus sequence TTAA of *piggyBac* transposon integration site is shown. (C) Genomic mapping of the integration site in GS141 using the UCSC Genome Browser; the antisense transcription initiation site is indicated by a solid arrow. Locations of MYC, PVT1 and microRNAs are shown. (D) Validation of PVT1 genomic junctions at both ends of the pPBGS integration site, genomic PCRs with specific primer sets produced expected fragments in GS141 cells but not in 2D2 control cells. A specific control PCR product (3′5′) from the uninterrupted PVT1 allele was amplified from both 2D2 and GS141 cells.

The optimal condition for *piggyBac* transposase mediated genome-wide integration of pPBGS in 2D2 cells was determined by co-transfection of pPBGS and a *piggyBac* transposase expression vector (pmPB) with Fugene 6. The optimal pPBGS/pmPB plasmid ratio was obtained at 1:1 with 25 ng pPBGS and 25 ng pmPB DNA for 10^5 2D2 cells, which resulted in an average of 2543 G418 resistant clones (Fig. 1C). High throughput parallel DNA transfections were performed, and a library that contained more than 500,000 G418 resistant clones was constructed for genome-wide screening of genes that regulate Gemcitabine sensitivity.

3.2. Identification of Gemcitabine sensitive clone GS141

We first determined Gemcitabine IC_{50} in human pancreatic cancer ASPC-1 parental cells and clone 2D2 cells using MTT assays, both ASPC-1 and 2D2 cells showed similar Gemcitabine IC_{50} at 25.1 nM (Fig. 2A). Our initial goal was to identify genes whose altered expression could result in increased sensitivity to Gemcitabine; therefore, we performed the library screening using individual G418 resistant clones in replicate MTT assays with and without 25 nM Gemcitabine. Multiple rounds of screening more than 300 clones identified clone GS141, which showed significantly increased sensitivity to Gemcitabine with IC_{50} at 7.6 nM ($P < 0.01$, Fig. 2B); whereas all other clones showed IC_{50} at or above 25 nM.

3.3. Molecular cloning and genome analysis of PVT1

To identify the chromosomal gene that contained the integrated pPBGS, genomic DNA was isolated from GS141 cells; the genomic

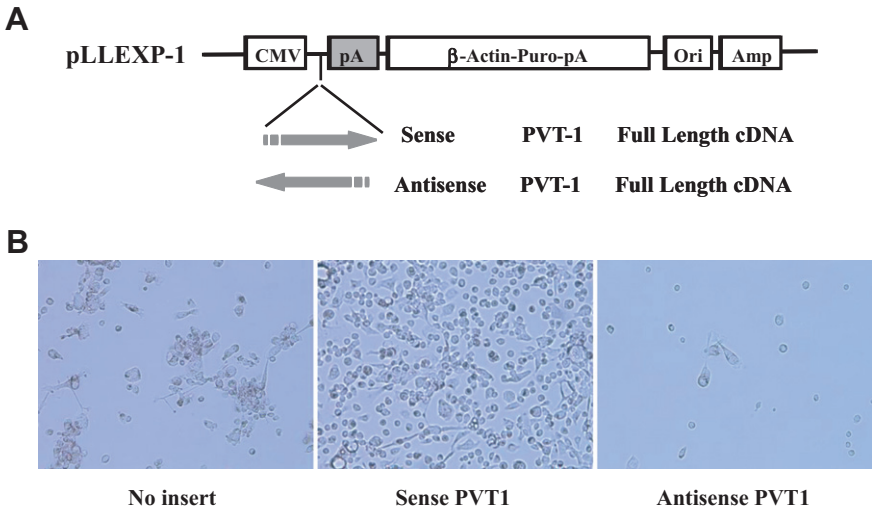

Fig. 4. Overexpression of full length PVT1 cDNA in naïve ASPC-1 cells validated PVT1 as a regulator of Gemcitabine sensitivity. (A) pLLEXP-1 contains an expression cassette for PVT1 cDNAs under control of a CMV promoter and a puromycin selection marker. Full length PVT1 cDNAs were cloned into pLLEXP-1 in both sense and antisense orientation. (B) Naïve ASPC-1 cells were transfected with pLLEXP-1 vectors containing sense and antisense full-length PVT1 cDNAs and no insertion control; each transfection was selected with puromycin, and then treated with 10 nM Gemcitabine. Representative of three experiments, 100×.

DNA fragment at the gene search vector junction was amplified with the splinkerette PCR (spPCR) method. A 300 bp PCR product was obtained and gel purified (Fig. 3A). DNA sequencing of the PCR product showed a 210 bp genomic DNA and 90 bp of 3′ terminal repeats of the integrated pPBGS, which was identified as a consensus sequence TTAA of *piggyBac* transposon integration site (Fig. 3B). A human genome database search with the 210 bp genomic DNA sequences showed a 100% match to human chromosome 8 (128931244–128931453), and the pPBGS integration site was mapped to intron 3 of human PVT1 oncogene (Fig. 3C), which produces a non-protein coding RNA (NCBI Reference Sequence: NR_003367.1). tTA mediated transcription was initiated from pPBGS and read through 3′TR of *piggyBac* transposon into adjacent genomic DNA, and pPBGS integration in GS141 cells produced antisense transcripts to PVT1 and resulted in functional inactivation of the PVT1 gene.

3.4. Functional validation of PVT1 as regulator of Gemcitabine sensitivity in naïve pancreatic cancer ASPC-1 cells

To validate the functional role of PVT1 in Gemcitabine sensitivity, we first performed a series of RT-PCRs to isolate full length human PVT1 cDNA; these RT-PCRs only produced several short isoforms of PVT1 cDNA (<1 Kb), and no full-length cDNA was obtained. We therefore synthesized the 1800 bp (with deletion of the 18 bp polyA tail) full-length PVT1 cDNA based on NCBI Reference Sequence NR_003367.1. The fully assembled 1800 bp PVT1 DNA was validated by sequencing of both strands and cloned into expression vector pLLEXP-1 in both sense and antisense orientations under the control of CMV promoter (Fig. 4A). Sense and antisense PVT1 full length cDNA expression vectors and the control vector without cDNA insert were transfected into naïve ASPC-1 cells; the stably transfected ASPC-1 cells were obtained by selection of puromycin resistance. Pools of puromycin resistance clones from each transfection were treated with Gemcitabine at 10 nM for 9 days. In comparison to ASPC-1 cells transfected with control vector, only a few ASPC-1 cells survived in the transfection group with antisense expression of PVT1; whereas significantly more ASPC-1 cells survived in the transfection group with sense expression of PVT1 (Fig. 4B).

4. Discussion

Human pancreatic cancer is characterized by its intrinsic resistance to chemotherapeutic agents, and this presents a major challenge to the clinical management of pancreatic cancer patients. Although Gemcitabine has been used as the first-line therapeutic agent for pancreatic cancer, there are no reliable biomarkers available for the prediction of therapeutic efficacy and for rational design of individualized therapy. Gemcitabine blocks nucleic acid synthesis during DNA replication and inhibits ribonucleotide reductase, thereby inducing apoptosis. For many years, research efforts have been focused on the mechanisms involved in the development of chemoresistance in pancreatic cancer cells upon treatment with Gemcitabine, which led to identification of several genetic pathways involved in Gemcitabine transport and metabolism. More recently, siRNA library screening in cancer cell lines identified several genes involved in DNA damage and cell cycle checkpoint as Gemcitabine sensitizers, such as ATR and CHK1 [14]. Inhibition of CHK1 may allow tumor cells to escape from checkpoint arrest and to enter mitosis with damaged DNA, which can ultimately induce apoptosis [25]. This may result in the increased sensitivity to Gemcitabine and provides potential gene targets for the design of combination therapies with Gemcitabine.

Using a genome-wide mutagenesis strategy, we identified the PVT1 gene whose functional inactivation resulted in increased sensitivity to Gemcitabine in human pancreatic cancer ASPC-1 cells. It has long been proposed that functional perturbation of PVT1 through DNA rearrangements or amplifications contribute to tumorigenesis [26]. The location of PVT1 near MYC at human chromosome 8q24, which is located 57 kb downstream of MYC and extends more than 200 kb in the telomeric direction from MYC, forms a cluster of MYC-activating chromosomal translocation breakpoints in Burkitt's lymphoma, plasmacytoma, ovarian cancer, breast cancer and lung cancer [27–30]. Co-amplification of human MYC and PVT1 had been shown to correlate with rapid progression of breast cancer and with poor clinical survival in postmenopausal or HER2-positive breast cancer patients [31]. In multiple myeloma, rearrangements of the PVT1 region have been shown to correlate with being refractory to therapy [32]. The functional role of transcripts of PVT1 remains a puzzle. Decades of research have

failed to identify a protein-encoding transcript or even multiple transcripts produced from PVT1 in a variety of cells. A knockout of the Pvt1 locus in mouse was embryonic lethal; this indicated that PVT1 plays a critical role in normal development [33]. Transcription from PVT1 in normal cells produces multiple low-abundance RNAs through a complex pattern of splicing between exons separated by large introns. Abnormal PVT1 RNAs produced in variant Burkitt's lymphomas are abundant chimeric transcripts containing PVT1 and the immunoglobulin light-chain loci [34]. The same cluster of MYC-activating chromosomal translocation is also the area for oncogenic retroviral integrations and is often associated with activation of MYC. In our study, the *piggyBac* gene search vector was inserted into intron 3 of PTV1, and antisense transcription was initiated from the integrated gene search vector. This resulted in functional inactivation of PVT1 transcripts, and potentially functional inactivation of upstream MYC transcripts. Further validation with overexpression of antisense full length PVT1 cDNA in naïve pancreatic cancer ASPC-1 cells has suggested that functional inactivation of PVT1 alone could result in increased sensitivity to Gemcitabine. Although PVT1 has been shown to be a MYC activator, transcriptional regulation of PVT1 is not well understood. The recent discovery of a miRNA cluster (miR1204, miR1205, miR1206, miR1207 and miR1208) within the PVT1 genomic DNA region suggests complex regulation networks within the MYC-PVT1 locus and with other gene networks [35].

Overexpression of sense full-length PVT1 cDNA in naïve pancreatic cancer ASPC-1 cells resulted in decreased sensitivity to Gemcitabine. This is in agreement with early reports that overexpression of PVT1 was associated with the development of resistance to therapeutic agents [33].

In summary, using a *piggyBac* transposon-based genome-wide mutagenesis strategy, we identified the PVT1 gene whose functional inactivation led to the increased sensitivity to Gemcitabine in human pancreatic cancer cells. The PVT1 gene, a non-protein encoding gene, is implicated in the tumorigenesis of a variety of human cancers through DNA rearrangement, co-amplications with MYC and regulation of miRNAs. Further characterization of PVT1 gene, its expression and its regulation may reveal the mechanisms of actions of PVT1 and MYC in tumorigenesis and their roles in regulation of Gemcitabine sensitivity.

Acknowledgments

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