Supplemental information

KIAA0495/PDAM is frequently downregulated in oligodendroglial tumors and its knockdown by siRNA induces cisplatin resistance in glioma cells

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Content

Materials and methods

Cell lines

HOG, SKMG-3 and TC620 cells were kindly provided by Dr. Glyn Dawson, University of Chicago, U.S.A., Dr. Joon Uhm, Mayo Clinic, U.S.A., and Dr. Rainer Probstmeier, University of Bonn, Germany, respectively. The A172, U373MG, U87MG lines were obtained from American Type Culture Collection (Manassas, VA), GOS-3 was purchased from German Collection of Microorganisms and Cell Cultures (Braunschweig, Germany), and KG-1-C was obtained from Japanese Collection of Research Bioresources (Osaka, Japan). In addition, gastric tumor cell lines AGS and MKN45 were gifts from Dr. Ka Fai To, the Chinese University of Hong Kong (Hong Kong). The human embryonal kidney 293FT line was purchased from Invitrogen (Hong Kong).

A172, GOS-3, HOG, KG-1-C, LNZ308, TC620, U373MG and 293FT were cultured in Dulbecco's modified Eagle medium (Invitrogen), U87MG and SKMG-3 were grown in alpha-minimal essential medium (Invitrogen), and AGS and MKN45 were cultured in RPMI-1640 medium (Invitrogen). All media were supplemented with 10% fetal bovine serum (Invitrogen). Cell lines were maintained in a humidified atmosphere of 5% CO_2 at 37^{o}C.

Microdissection and reverse transcription-polymerase chain reaction

Approximately 8000 tumor cells per sample were selectively dissected using the PALM Microbeam Systems (Carl Zeiss, Thornwood, NY) according to manufacturer's recommendation. Total RNA was extracted from tumor cells using the PicoPure RNA isolation kit (Molecular Devices, Sunnyvale, CA) and was converted to cDNA using MultiScribe reverse transcriptase (Applied Biosystems, Foster City, CA) and random hexamers as described (Li K et al., Human Path 2009;40:1234-43). PCR was performed in a final volume of 20 μl containing 50 mM KCl, 10 mM Tris-HCl (pH 8.3), 2.5 mM $MgCl_2$, 0.2 mM deoxynucleoside triphosphates, 400 nM of each primer, and 0.25 unit of AmpliTaq Gold DNA polymerase (Applied Biosystems). The cycling conditions included 95°C for 10 min and 28 cycles of 94°C for 30 sec, 60°C-62°C for 30 sec and 72°C for 1 min. PCR products were resolved in 2% agarose gel, stained with ethidium bromide and visualized under UV illumination.

Northern blot analysis

The cDNA of PDAM predicted coding sequence was labeled by random priming in the presence of ^{32}P-deoxycytidine triphosphate (Rediprime II DNA labeling system, GE Healthcare, Hong Kong). A human tissue blot containing poly-A RNA (Origene, Rockville, MD) was hybridized with the radioactively-labeled probe in ULTRAhyb hybridization buffer (Ambion) at 42°C overnight. The blot was then washed two times each in 2X SSC (1X SSC = 0.15 M sodium chloride and 15 mM sodium citrate) and 0.1% sodium dodecyl sulfate for five min at 42°C, and two times each in 0.1 X SSC and 0.1% sodium dodecyl sulfate at 60°C for 20 min. The membrane was then exposed to Kodak X-OMAT AR film.

Antibody production

Rabbit antibody was raised against a synthetic peptide (TTSDLSAREDATPSC), corresponding to amino acid residues 66-79 of PDAM predicted coding sequence (NP_997189), conjugated to keyhole limpet hemocyanin. Antibody titers were monitored by enzyme-linked immunosorbent assay with PDAM peptide conjugated to bovine serum albumin. High-titer sera were subjected to affinity purification on column bound with the PDAM peptide (GenScript, Piscataway, NJ).

Construction of PDAM expression vectors

The PDAM predicted coding sequence was amplified from cDNA using Phusion HF DNA polymerase (New England Biolabs, Ipwich, MA), and primers 5'-ATGGATCC GCCACCATGTGTCTTTTGTCCAGCTCAGCC-3' and 5'- GTTCTAGATCAAAGTG-CCGCTGGTCGTT-3. In the sense primer, the underlined bases represent a Kozak sequence created upstream of the predicted translational start codon. The DNA fragment was cloned at the BamHI-XbaI restriction sites of pcDNA4/TO vector (Invitrogen) and the resultant plasmid was named pPDAM.

Two chimeric constructs containing green fluorescent protein (GFP) cDNA cloned in-frame upstream (pGFP-PDAM) and downstream (pPDAM-GFP) of PDAM were prepared. The PDAM and GFP cDNA fragments were separately amplified and assembled at AvrII restriction site in pCR2.1, followed by subcloning of the entire chimeric gene at BamHI-XbaI sites of pcDNA4/TO. The GFP cDNA was amplified from pEGFP-N2 (Clontech). Primers used for construction of pPDAM-GFP were 5'-ATGGATCCGCCACCATGTGTC-TTTTGTCCAGCTCAGCC-3', 5'-CTCCTAGGCTGTGCTGGATATCTGCAGAATTGC-

CCAAGTGCCGCTGGTCGTTG-3', 5'-ATCCTAGGAAGCTTAGCGGCCGCATGGTG-AGCAAGGGCGAG-3' and 5'-GTTCTAGATTACTTGTACAGCTCGTCCATGC-3'; whereas primers for preparing pGFP-PDAM were 5'-GTCCTAGGAAGCTTAGCGGCCG-CATGTGTCTTTTGTCCAGCTCAGCC-3', 5'-GTTCTAGATCAAAGTGCCGCTGGTC-GTT-3', 5'-ATGGATCCGCCACCATGGTGAGCAAGGGCGAG-3', and 5'-CTAGGCTG-TGCTGGATATCTGCAGAATTGCCCCTTGTACAGCTCGTCCATGCC-3'.

Fluorescence in situ hybridization (FISH) and microsatellite analysis

The allelic status of chromosomes 1p and 19q was determined either by dual-color FISH or microsatellite analysis as reported (Dong Z et al., Br J Cancer 2004;91:1105-11). Briefly, for dual-colour FISH analysis, formalin-fixed paraffin-embedded tissue sections of 5-μm thickness were deparaffinized, digested in pepsin solution and denatured. DNA probes were labeled by nick translation in the presence of digoxigenin-1-deoxyuridine triphospahte (for target probe) or biotin-16-deoxyuridine triphosphate (for reference probe) (Roche Diagnostics, Hong Kong). The labeled probes were mixed with Cot-1 DNA, denatured and applied onto sections. Hybridization was carried out at 37°C overnight. Sections were washed, treated with either anti-digoxigenin-rhodamine antibody (Sigma-Aldrich) or avidin conjugated-fluorescein isothiocyanate (Vector Laboratories), and counterstained with DAPI. The bacterial artificial chromosome clones used for preparing probes were RP11-62M23 on 1p36.3 (target), RP11-162L13 on 1q25.3-q31.1 (reference), CTD-2571L23 on 19q13.3 (target) and RP11-420K14 on 19p12 (reference). At least 100 non-overlapping tumor nuclei were counted on each section. A sample was considered having deletion of 1p or 19q when more than 60% of counted nuclei exhibited one target signal and two reference signals.

For microsatellite analysis, four loci on chromosome 1p36.12-p36.32 (D1S468 on 1p36.32, D1S1612 on 1p36.23, D1S199 on 1p36.13 and D1S2734 on 1p36.12) and 3 loci on chromosome 19q13.32 (D19S219, D19S112 and D19S412) were interrogated for loss of heterozygosity in paired tumor-blood samples.

Results

Phylogenetic sequence analysis

Phylogenetic comparative analysis reveals that the human PDAM gene is highly conserved in chimpanzee (Fig. S1). The predicted coding sequence of human PDAM shows 99% identity to part of a transcript identified in *Pan troglodytes* (XM_001152021, 1154 bp – 1759 bp). However, the coding sequence of chimpanzee PDAM is predicted further downstream at 1904 bp-2197 bp of the transcript, leading to a predicted product of ~10.2 kDa with amino acid sequence completely different from that of human PDAM. PDAM DNA sequence conservation appears to be confined to the primate infraorder Simiiformes, which comprises the monkeys, apes and humans.

Determination of PDAM gene product

A peptide corresponding to amino acid residues 66-79 of the putative PDAM coding sequence was employed as an immunogen to induce antibody production in rabbits. Immunized sera were then purified on PDAM peptide-conjugated column. To determine the specificity of the anti-PDAM antibody, we created three expression constructs: one plasmid harbored a cDNA corresponding to PDAM predicted coding sequence with an Kozak sequence inserted upstream of the translational start site to facilitate efficient translation (pPDAM), and two chimeric plasmids encoding GFP-tagged PDAM at either ends (pPDAM-GFP and pGFP-PDAM). These plasmids were transfected into 293FT cells and the cell lysates were probed either with anti-PDAM or anti-GFP antibody by Western blotting. Our results demonstrated that in cells transfected with pPDAM a prominent protein of ~24 kDa showed reactivity to the anti-PDAM antibody (Fig. S2). This protein was ~3.4 kDa larger than the expected product (20.6 kDa) of PDAM. Moreover, in cells transfected either with pPDAM-GFP or pGFP-PDAM plasmid, a protein of ~53 kDa or ~52 kDa, respectively, was detected by the anti-PDAM antibody. While the expected sizes for the N-terminal and C-terminal GFP-tagged PDAM were 49.5 kDa and 48.5 kDa, respectively, the fusion proteins detected were about 3.5 kDa larger in size. The source of the extra size observed in PDAM protein and GFP-tagged fusion proteins was unclear. Additionally, the identity of the GFP-tagged PDAM proteins was confirmed by their reactivity to the anti-GFP antibody (Fig. S2). Taken together, these results confirmed the specificity of the anti-PDAM antibody.

To detect endogenous PDAM protein expression, we performed Western blot analysis on a panel of nine cell lines using the anti-PDAM antibody. These cells expressed PDAM transcripts of various abundances, with 293FT showing PDAM mRNA of normal brain level,

the glioma lines exhibiting 2-fold to 10-fold lower level relative to normal brain, and gastric tumor lines AGS and MKN45 displaying no detectable PDAM transcript. Our results revealed that none of the cell lines examined showed any detectable protein in the range of 20 kDa to 24 kDa (Fig. S2), including 293FT expressing PDAM transcript of normal brain level. Moreover, immunofluorescence staining assay showed that fluorescent signals were detectable in 293FT cells transfected with pPDAM, but not in any cell lines without pPDAM transfection (data not shown). Collectively, these findings suggest that the PDAM product may be present at a level undetectable by the methods employed. Alternatively, the PDAM coding sequence may have been predicted incorrectly.

Table S1. Oligonucleotides used in this study.

Gene	Accession no	Sense primer	Antisense primer	Annealing temperature (°C)	Product size (bp)
RT-PCR analysis					
AJAP1/SHREW1	NM_018836	5'-GACGCCGTGTGTCTGTTTCA-3'	5'-CCCTTTCGCTGGGTATCCTC-3'	60	300
B2M	NM_004048	5'-GTGCTCGCGCTACTCTCTCT-3'	5'-TGTGCATAAAGTGTAAGTGTATAAGCA-3'	60	444
C1orf174	NM_207356	5'-TGTCCGTGCCAAAACACAGT-3'	5'-GAAGACGCAGGTGGGAGGT-3'	60	223
CCDC27/FLJ32825	NM_152492	5'-TGCCCTGGTACCTCTCAGTC-3'	5'-GCCTTTCAGAAGGCATTTCA-3'	60	146
DFFB	NM_004402	5'-CGGATCCGGAGTTACCTGAG-3'	5'-GCAGCTGTCCATGTCAAAGG-3'	60	219
KIAA0562	NM_014704	5'-CAAATGGTGGAGAGATGTCGAA-3'	5'-GCAACCAAAGCAACCTGATTAT-3'	60	296
LOC100133612	NR_024455	5'-CCATATTTGAAAGGGGCAGAC-3'	5'-GGATAGCACCGGAGAAACG-3'	62	120
LOC284661	NR_027088	5'-TGTGAGATTGGACTTTCGGATA-3'	5'-AGCTGCAAGCAGAACAGCTA-3'	60	148
LOC388588	NM_001163724	5'-AGCCCTCAGGCAAGGTTC-3'	5'-GCCGTCCTCCCACCTACTAT-3'	60	146
LRRC47/KIAA1185	NM_020710	5'-TGTGGATGCAGACGGTGAT-3'	5'-TGGAAGTTGTCCAGAGACTGC-3'	60	244
PDAM	NM_207306	5'-GCTGCTTGCTGTACGTGGTG-3'	5'-CGTGGCTGACACAAACTTGC-3'	60	178
SYBR Green-based RT-PCR					
BAD	NM_032989	5'-CAGTCACCAGCAGGAGCAG-3'	5'-GGTAGGAGCTGTGGCGACT-3'	62	87
BAK1	NM_001188	5'-TTTTCCGCAGCTACGTTTTT-3'	5'-TGCAACATGGTCTGGAACTC-3'	58	193
BBC3/PUMA	NM_001127240	5'-GACGACCTCAACGCACAGTA	5'-CTGGGTAAGGGCAGGAGTC-3'	62	113
BCL2	NM_000633	5'-GAAACTTGACAGAGGATCATGC-3'	5'-TCCAATTCCTTTCGGATCTT-3'	62	123
BCL2L1/BCL-xL	NM_138578	5'-GGTATTGGTGAGTCGGATCG-3'	5'-TGCTGCATTGTTCCCATAGA-3'	60	121
BID	NM_197966	5'-TCATCCATGCCTCAGAAGTG-3'	5'-CTGTCCCTAAGAGAGGGAACC-3'	62	113
CDKN1A/p21	NM_000389	5'-GGCAGACCAGCATGACAGATTT-3'	5'-GGCGGATTAGGGCTTCCTCT-3'	62	74
GAPDH	NM_002046	5'-AGCCGAGCCACATCGCTCA-3'	5'-TGGCAACAATATCCACTTTACCAGAGTT-3'	60	122
MCL1	NM_021960	5'-TAACAAACTGGGGCAGGATT-3'	5'-GTCCCGTTTTGTCCTTACGA-3'	60	140
TP53	NM_001126112	5'-ACAACGTTCTGTCCCCCTTG-3'	5'-TCATCTGGACCTGGGTCTTC-3'	60	100
XIAP	NM_001167	5'-CCTGGCGCGAAAAGGTGG-3'	5'-GCCAGTGTTGATGCTGAAAC-3'	60	184
YBX1	NM_004559	5'-GTCATCGCAACGAAGGTTTT-3'	5'-CATCTTCCTTGGTGTCATTCC-3'	60	91
Bisulfite sequencing					
PDAM	AL136528	5'-TAATTAGGGAAATTTTGTGGGGAGAG-3'	5'-ACTCCCATCTAAAAATCCACACCC-3'	64	327
RNA interference					
siBCL2L1	NM_138578	5'-GGAGAUGCAGGUAUUGGUGTT-3'	5'-CACCAAUACCUGCAUCUCCTT-3'		
siPDAM-1	NM_207306	5'-ACACCAGAGUGGUUCGGGATT-3'	5'-UCCCGAACCACUCUGGUGUTG -3'		
siPDAM-2	NM_207306	5'-UGAUUGUGCGGGUUAGAUATT-3'	5'-UAUCUAACCCGCACAAUCAAC-3'		

Table S1. Oligonucleotides used in this study. (cont'd)

Gene	Accession no	Sense primer	Antisense primer	Annealing temperature (°C)	Product size (bp)
Gene cloning					
PDAM full-length 5' fragment	NM_207306	5’-TTGCGGCCGCAGGGTGTGGATCCCTAGATG-3’	5’-TTAAGCTTCACCTCCAGTCTCTGGTC-3’	58	2135
PDAM full-length 3' fragment	AL136528	5’-TTGCGGCCGCAATCCCAGCTACTTGAGAGG-3’	5’-TTAAGCTTAGAGCCACTTGTCACTCAGA-3’	58	4675
GFP full-length	U57608	5’-TAGCGGCCGCCACCATGGTGAGCAAGGGCGAG-3’	5’-CAAGCTTTTACTTGTACAGCTCGTCCATG-3’	58	741

A

Repeat no.	Sequence (5' to 3')	Coordinate (NM_207306)
1	GGTTGATTGTGCGGGTTAGATAGAGGTCATCAGCCG	2158-2193
2	GGTTGATTGTGTGGGTTAGACAGAGGTCATCAGCCA	2194-2229
3	GGTTGATTGTGTGGGTTAGATAGAGATCATCAGCCT	2230-2265
4	GGTAGATTGTGTGGGTTAGATAGAGGTCATCAGCCA	2266-2301
5	GGTTGATTGTGCGGGTTAGATAGAGATCATCAGCCG	2302-2337
6	GGTTGATTCTACAGGTTAGATAGAGGTCGTCAGCCG	2338-2373
7	GGTTGATTGTGCGGGTTAGATAGAGATCATCAGCCG	2374-2409
8	GGTTGATTGTGCGGGTTAGATAGAGGTCATCAGCTG	2410-2445
9	GGTTGATTGTGTGGGTTAGATAGAGATCATCAGCTG	2446-2481
10	GGTTGATTGTGTGGGTTAGATAGAGGTCGTCAGCTG	2482-2517
11	GGTTGATTGTGCGGGTTAGATAGAGGTCATCAGCCG	2518-2553
12	GGTTGATTCTACAGGTTAGATAGAGGTCATCAGCTG	2554-2589
13	GGTTGATTGTGCGGGTTAGAGATCATCAGCCG----	2590-2621
14	GGTTGATTGTGCGGGTTAGATAGAGGTCATCAGCCG	2622-2657
15	GGTTGATTGTGCGGGTTAGATAGAGATCATCAGCTG	2658-2693
16	GGTTGATTCTGCAGGTTAGATAGAGGTCATCAGCTG	2694-2729
17	GGTTGACTATGTGGGTTAGATAGAGGTCGTCAGCCG	2730-2765
18	GGTTGATTGTGTGGGTTAGACAGAGGTCATCAGCTG	2766-2801
19	GGTTGATTCTGCGGGTTAGATAGAGGTCATCAGCTG	2802-2837
20	GGTTGATTGTGTGGGTTAGATAGAGATCATCAGCTG	2838-2873
21	GGTTGATTGTGTGGGTTAGATAGAGGTCATCAGCTG	2874-2909
22	GGTTGATTGTGTGGGTTAGATAGAGGTCATCAGCTG	2910-2945
23	GGTTGATTGTGTGGGTTAGATAGAGGTCATCAGCTG	2946-2981
24	GGTTGATTGTGTGGGTTAGATAGAGGTCATCAGCTG	2982-3017
25	GGTTGATTGTATGGGTTAGATAGAGGTCATCAGCTG	3018-3053
26	GGTTGATTGTACGGGTTAGATAGAGGTCGTCAGCCG	3054-3089
27	GGTTGATTCCGCAGGTTAGATAGAGGTCGTTAGCTG	3090-3125
Consensus	GGTTGATTGTG[C/T]GGGTTAGATAGAGGTCATCAGC[C/T]G	

B

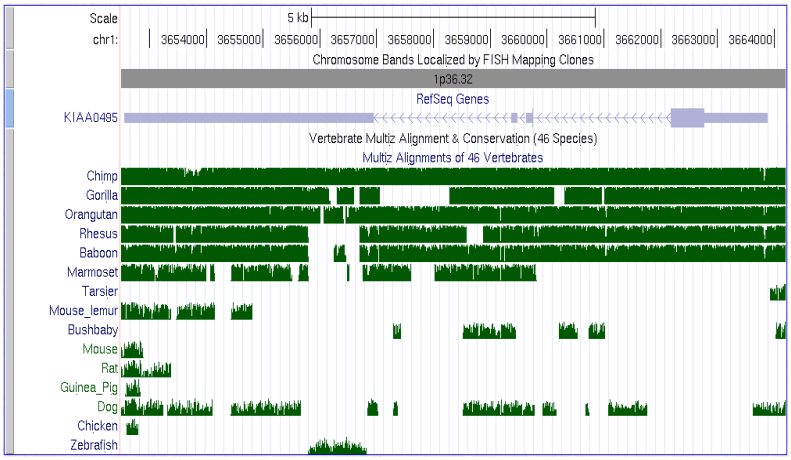

Fig. S1. (A) The unique 36-bp repeat sequences observed in exon 4 of the PDAM gene are shown. Repeat 13, which is located in the center of the repeat region, shows loose base conservation after first 21 bp and has a 4-bp deletion at the 3' end. (B) Phylogenetic comparative analysis reveals that human PDAM is conserved in the primate infraorder Simiiformes (UCSC Genome Browser, http://genome.ucsc.edu/).

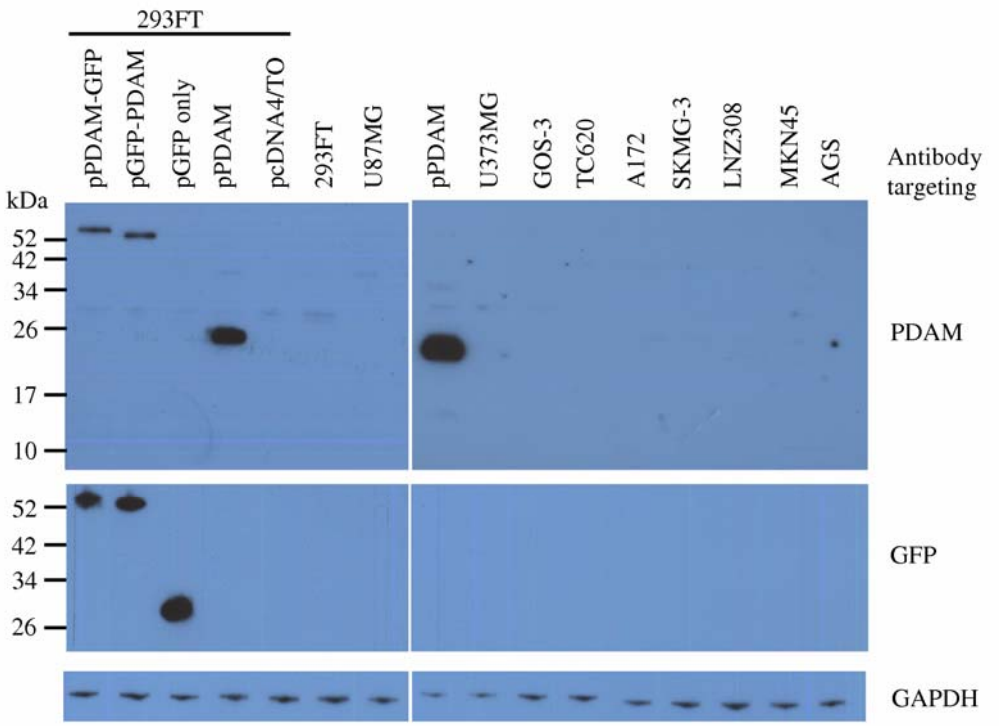

Fig. S2. Detection of endogenous PDAM by Western blot analysis. The specificity of the anti-PDAM antibody is validated by its reactivity to PDAM based on the predicted coding sequence (pPDAM) and GFP-tagged PDAM fusion proteins (pPDAM-GFP and pGFP-PDAM). The latter two proteins are also detected by the anti-GFP antibody. The 293FT cells show PDAM mRNA of normal brain level, the glioma lines exhibit 2-fold to 10-fold lower level relative to normal brain, and gastric tumor lines AGS and MKN45 display no detectable PDAM transcript. None of the cell lines expressing detectable PDAM transcripts show any unique protein bands that are reactive to the anti-PDAM antibody.

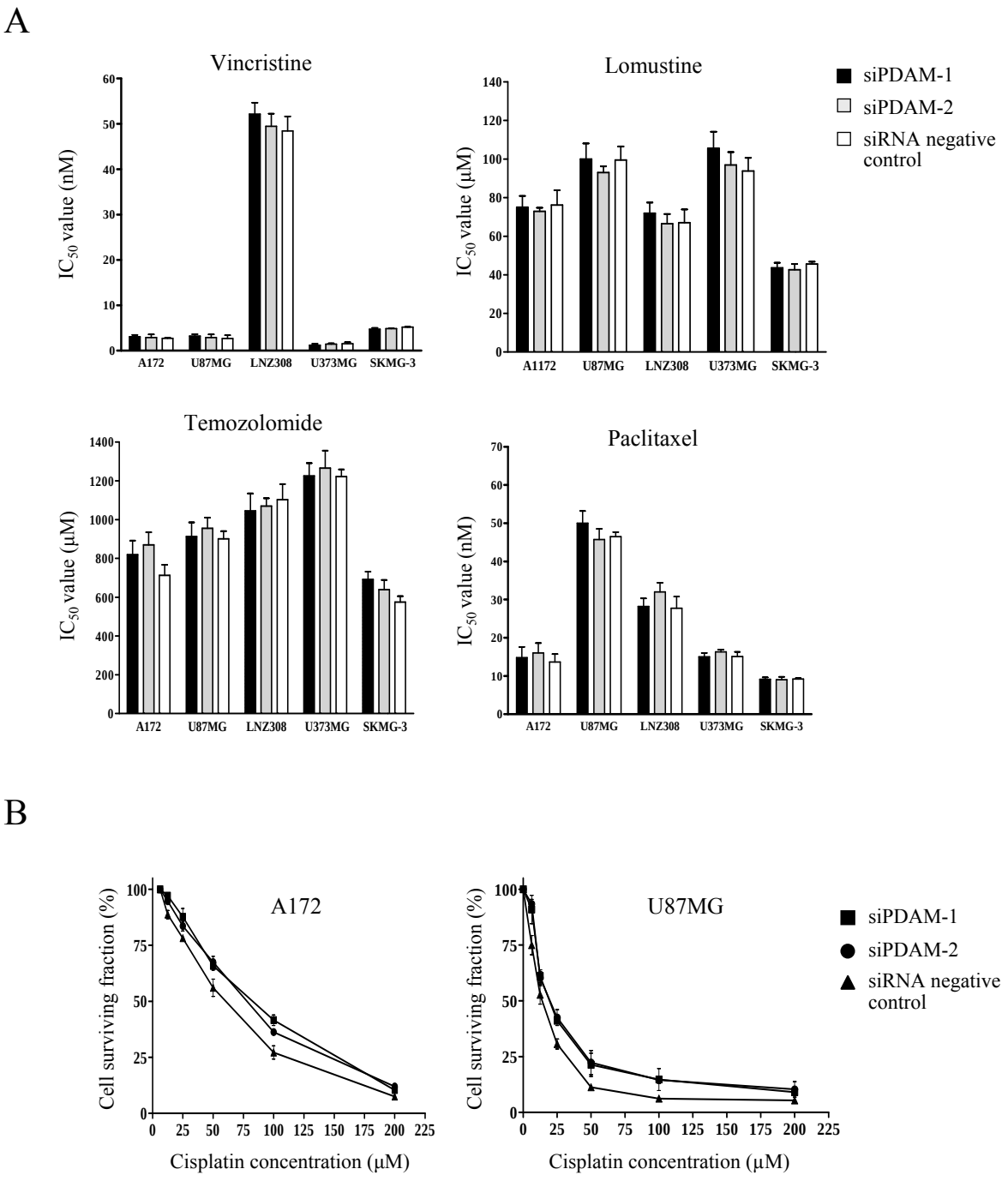

Fig. S3. (A) Drug sensitivity assay reveals that PDAM knockdown has no effects on sensitivity to vincristine, lomustine, temozolomide and paclitaxel in all five glioma lines examined. (B) Cell survival curves indicate increased resistance to cisplatin when PDAM is knocked down in A172 and U87MG cells.