Aberrantly expressed PLOD1 promotes cancer aggressiveness in bladder cancer: a potential prognostic marker and therapeutic target

Yasutaka Yamada1,2, Mayuko Kato1,2, Takayuki Arai1,2, Hiroki Sanada3, Akifumi Uchida3, Shunsuke Misono3, Shinichi Sakamoto2, Akira Komiya2, Tomohiko Ichikawa2 and Naohiko Seki1

1 Department of Functional Genomics, Chiba University Graduate School of Medicine, Japan
2 Department of Urology, Chiba University Graduate School of Medicine, Japan
3 Department of Pulmonary Medicine, Graduate School of Medical and Dental Sciences, Kagoshima University, Japan

Keywords
bladder cancer; inhibitor; microRNA miR-140-5p; passenger strand; PLOD1

Correspondence
N. Seki, Department of Functional Genomics, Chiba University Graduate School of Medicine, 1-8-1 Inohana Chuo-ku, Chiba 260-8670, Japan
Tel: +81 43 226 2134
E-mail: naoseki@faculty.chiba-u.jp

(Received 9 March 2019, revised 18 May 2019, accepted 5 June 2019, available online 27 June 2019)
doi:10.1002/1878-0261.12532

1. Introduction

Bladder cancer (BC) is the ninth most malignant tumor worldwide. Some BC patients will develop muscle-invasive BC (MIBC), which has a 5-year survival rate of approximately 60% due to metastasis. As such, there is an urgent need for novel therapeutic and diagnostic targets for MIBC. Analysis of novel antitumor microRNA (miRNA)-mediated cancer networks is an effective strategy for exploring therapeutic targets and prognostic markers in cancers. Our previous miRNA analysis revealed that miR-140-5p acts as an antitumor miRNA in BC cells. Here, we investigated miR-140-5p regulation of BC molecular pathogenesis. Procollagen-lysine, 2-oxoglutarate 5-dioxygenase 1 (PLOD1) was found to be directly regulated by miR-140-5p, and aberrant expression of PLOD1 was observed in BC clinical specimens. High PLOD1 expression was significantly associated with a poor prognosis (disease-free survival: \( P = 0.0204 \); overall survival: \( P = 0.000174 \)). Multivariate analysis showed PLOD1 expression to be an independent prognostic factor in BC patients (hazard ratio = 1.51, \( P = 0.0099 \)). Furthermore, downregulation of PLOD1 by siRNAs and a specific inhibitor significantly decreased BC cell aggressiveness. Aberrant expression of PLOD1 was closely associated with BC pathogenesis. In summary, the present study showed that PLOD1 may be a potential prognostic marker and therapeutic target for BC.

Abbreviations
BC, bladder cancer; GEO, Gene Expression Omnibus; miRNA, microRNA; PLOD1, procollagen-lysine, 2-oxoglutarate 5-dioxygenase 1; RISC, RNA-induced silencing complex; TCGA, The Cancer Genome Atlas.

Bladder cancer (BC) is the ninth most malignant tumor worldwide. Some BC patients will develop muscle-invasive BC (MIBC), which has a 5-year survival rate of approximately 60% due to metastasis. As such, there is an urgent need for novel therapeutic and diagnostic targets for MIBC. Analysis of novel antitumor microRNA (miRNA)-mediated cancer networks is an effective strategy for exploring therapeutic targets and prognostic markers in cancers. Our previous miRNA analysis revealed that miR-140-5p acts as an antitumor miRNA in BC cells. Here, we investigated miR-140-5p regulation of BC molecular pathogenesis. Procollagen-lysine, 2-oxoglutarate 5-dioxygenase 1 (PLOD1) was found to be directly regulated by miR-140-5p, and aberrant expression of PLOD1 was observed in BC clinical specimens. High PLOD1 expression was significantly associated with a poor prognosis (disease-free survival: \( P = 0.0204 \); overall survival: \( P = 0.000174 \)). Multivariate analysis showed PLOD1 expression to be an independent prognostic factor in BC patients (hazard ratio = 1.51, \( P = 0.0099 \)). Furthermore, downregulation of PLOD1 by siRNAs and a specific inhibitor significantly decreased BC cell aggressiveness. Aberrant expression of PLOD1 was closely associated with BC pathogenesis. In summary, the present study showed that PLOD1 may be a potential prognostic marker and therapeutic target for BC.

Abbreviations
BC, bladder cancer; GEO, Gene Expression Omnibus; miRNA, microRNA; PLOD1, procollagen-lysine, 2-oxoglutarate 5-dioxygenase 1; RISC, RNA-induced silencing complex; TCGA, The Cancer Genome Atlas.

1. Introduction

Bladder cancer (BC) is the ninth most malignant tumor worldwide, and approximately 430 000 cases were newly diagnosed in 2012 (Antoni et al., 2017). BC is clinically divided into two groups: muscle-invasive BC (MIBC) and non-muscle-invasive BC (NMIBC) (Lemke and Shah, 2018). Patients with the latter have a favorable prognosis (5-year survival rate: approximately 90%) after surgical resection. However, approximately 50% of cases develop intravesical recurrence after surgical resection, and approximately 15–40% of recurrent BC cases are invasive and exhibit distant metastasis (Lemke and Shah, 2018). Although radical cystectomy and cisplatin-based combination chemotherapy are the standard treatments for MIBC, the 5-year survival rate of patients with MIBC is approximately 60% (Chou et al., 2016; Lemke and Shah, 2018). In addition, the survival of patients with distant metastasis is only 15 months due to no
effective treatment options (Abufaraj et al., 2018). Therefore, discovery of novel therapeutic and diagnostic targets is urgently needed.

A vast number of studies have shown that a large number of noncoding RNAs encoded by the human genome are functional and play critical roles in various cellular processes, for example, cell growth, migration, invasion, and apoptosis (Bartel, 2004). MicroRNAs (miRNAs), a class of noncoding RNAs, are endogenous single-stranded RNA molecules comprising 19–22 nucleotides that function as fine-tuners of RNA expression (Bartel, 2009; Goto et al., 2015b; Koshizuka et al., 2017a; Kurozumi et al., 2017). A single miRNA regulates a vast number of RNA transcripts, and a bioinformatics study showed that approximately 60% of protein-coding genes are controlled by miRNAs (Bartel, 2009). Aberrantly expressed miRNAs are closely associated with cancer pathogenesis via disruption of RNA networks within cancer cells (Beermann et al., 2016).

Using the knowledge that a single miRNA controls numerous genes, we sequenced miRNA expression in cancer cells and found that several miRNAs are upregulated in cancer tissues (Goto et al., 2017b). During miRNA biogenesis, the passenger strand of the miRNA duplex is degraded and does not play a role in gene regulation in cells (Mah et al., 2010). Our recent studies revealed that the passenger strand of several miRNAs is up- or downregulated in cancer tissues (Goto et al., 2017b; Koshizuka et al., 2017). During miRNA biogenesis, the passenger strand of the miRNA duplex is degraded and does not play a role in gene regulation in cells (Mah et al., 2010). The latest RNA sequencing-based signatures revealed that the passenger strand of some miRNAs is up- or downregulated in cancer tissues (Goto et al., 2015a, 2017; Miyamoto et al., 2016). Identification of dysregulated miRNAs in cancer cells is the first step, and the latest RNA-seq-based sequencing technology is suitable for producing miRNA signatures. Interestingly, analyses of our RNA-seq-based signatures showed that the passenger strand of some miRNAs is up- or downregulated in cancer tissues (Goto et al., 2017b; Koshizuka et al., 2017b).

During miRNA biogenesis, the passenger strand of the miRNA duplex is degraded and does not play a role in gene regulation in cells (Mah et al., 2010). The latest RNA sequencing-based signatures revealed that the passenger strand of some miRNAs is up- or downregulated in cancer tissues (Goto et al., 2015a, 2017; Miyamoto et al., 2016). Identification of dysregulated miRNAs in cancer cells is the first step, and the latest RNA-seq-based sequencing technology is suitable for producing miRNA signatures. Interestingly, analyses of our RNA-seq-based signatures showed that the passenger strand of some miRNAs is up- or downregulated in cancer tissues (Goto et al., 2017b; Koshizuka et al., 2017b). During miRNA biogenesis, the passenger strand of the miRNA duplex is degraded and does not play a role in gene regulation in cells (Mah et al., 2010). The latest RNA sequencing-based signatures revealed that the passenger strand of some miRNAs is up- or downregulated in cancer tissues (Goto et al., 2015a, 2017; Miyamoto et al., 2016). Identification of dysregulated miRNAs in cancer cells is the first step, and the latest RNA-seq-based sequencing technology is suitable for producing miRNA signatures. Interestingly, analyses of our RNA-seq-based signatures showed that the passenger strand of some miRNAs is up- or downregulated in cancer tissues (Goto et al., 2017b; Koshizuka et al., 2017b).

2. Materials and methods

2.1. Clinical specimen collection and cell culture

We obtained 15 BC tissues and normal adjacent tissues from patients undergoing total cystectomy at Chiba University Hospital between 2014 and 2015 (Table S1). All patients provided informed written consent forms, and the study protocol was approved by the Institutional Review Board of Chiba University (number: 484). The study methodologies conformed to the standards set by the Declaration of Helsinki. We used the human BC cell lines T24 and BOY. These cell lines were cultured in RPMI 1640 Medium supplemented with 10% fetal bovine serum (HyClone, Logan, UT, USA) as described previously (Yamada et al., 2018d).

2.2. Transfection of mature miRNAs, siRNAs, and plasmid vectors

We used the following agents in this study: the precursor sequences of hsa-miR-140-5p and hsa-miR-140-3p (assay IDs: PM10205 and PM12503, respectively; Applied Biosystems, Foster City, CA, USA), negative control miRNA (miR-control) (assay ID: AM 17111; Applied Biosystems), and PLOD1-specific siRNA (si-PLOD1) (Stealth Select RNAi siRNA, P/N: HSS108122 and HSS108123; Invitrogen, Carlsbad, CA, USA). A plasmid vector containing PLOD1 was provided by OriGene (cat. no. SC119956; Rockville, MD, USA). Transfection of the agents into cells was performed using previously described procedures (Yamada et al., 2018c). miRNAs and siRNAs were incubated with 10 nM Lipofectamine RNAiMax transfection reagent (Invitrogen) diluted in Opti-MEM (Invitrogen). Plasmid vectors were incubated with Lipofectamine 3000 reagent (Invitrogen) in Opti-MEM (Invitrogen) for forward transfection.

2.3. PLOD1 inhibitor studies

We used 2,2′-dipyridyl (07-5990; Sigma-Aldrich, St. Louis, MO, USA), previously reported to be a small-molecule PLOD1 inhibitor, to inhibit PLOD1 in in vitro assays (Jover et al., 2018).

2.4. Quantitative reverse transcription–polymerase chain reaction (qRT-PCR)

TaqMan probes and primers specific to PLOD1 (P/N: Hs00609363_m1; Applied Biosystems), which are assay-on-demand gene expression products, were used to
analyze PLOD1 expression. miR-140-5p (P/N:001187; Applied Biosystems) and miR-140-3p (P/N:002234; Applied Biosystems) expression was analyzed by qRT-PCR. mRNA and miRNA expression levels were normalized to those of GUSB (P/N: Hs99999908_m1; Applied Biosystems) and RNU48 (assay ID: 001006; Applied Biosystems). PCR quantification was performed as described previously (Yamada et al., 2018d).

2.5. Cell proliferation, migration, and invasion assays
Cell proliferation was evaluated by the XTT assay using the Cell Proliferation Kit II (Sigma-Aldrich). Cell migration was assessed by wound healing assays, and invasion was determined using modified Boyden chambers containing Matrigel-coated Transwell membrane filter inserts.

2.6. Cell-cycle assay
Bladder cancer cells were transiently transfected with either the transfection reagent only as the control or the 2,2’-dipyridyl, PLOD1 inhibitor, in six-well tissue culture plates. Cells were harvested by trypsinization 72 h after transfection. For cell-cycle analysis, cells were stained with propidium iodide using the Cycletest Plus DNA Reagent Kit (BD Biosciences, Bedford, MA, USA) according to the manufacturer’s instructions and examined using the CyAn ADP Analyzer (Beckman Coulter, Brea, CA, USA). The percentages of cells in the G0/G1, S, and G2/M phases were calculated and compared. Experiments were performed in triplicate (Matsushita et al., 2015).

2.7. Apoptosis assays
Apoptotic cells were detected using the FITC Annexin V Apoptosis Detection Kit (BD Biosciences) according to the manufacturer’s instructions and the BD FACScélesta Flow Cytometer (BD Biosciences). Cells were identified as viable, dead, or early or late apoptotic cells, and the percentages of apoptotic cells under each experimental condition were compared. Anti-poly (ADP-ribose) polymerase (PARP) (J9542; Cell Signaling Technology, Danvers, MA, USA) was evaluated as a marker of apoptosis in this study (Idichi et al., 2018).

2.8. Western blotting
Western blotting was performed using a polyclonal anti-PLOD1 antibody (1:1000 dilution; SAB1301577; Sigma-Aldrich) and an anti-glyceraldehyde 3-phosphate dehydrogenase (GAPDH) antibody (1:10 000 dilution; ab8245; Abcam, Cambridge, UK) as a control (Fukumoto et al., 2014, 2015).

2.9. miR-140-5p and miR-140-3p localization within the RNA-induced silencing complex (RISC) using Ago2 immunoprecipitation
T24 cells were transfected with 10 nM miRNA by reverse transfection. After 72 h, immunoprecipitation of the RISC was performed using the Ago2 miRNA isolation kit (Wako, Osaka, Japan). The expression levels of miR-140-5p and miR-140-3p in the immunoprecipitates were analyzed by qRT-PCR. miRNA expression levels were normalized to that of miR-26a (P/N: 000405; Applied Biosystems), which was not affected by miR-140-5p or miR-140-3p transfection.

2.10. Identification of candidate target genes regulated by miR-140
To identify candidate target genes regulated by miR-140-5p and miR-140-3p, we used a combination of in silico and genome-wide gene expression analyses. Genes potentially regulated by miRNAs in a sequence-dependent manner are listed in the TargetScan database (release 7.2) (http://www.targetscan.org/vert_70/). Genes upregulated in BC were identified from a publicly available dataset in the Gene Expression Omnibus (GEO; accession number: GSE31684), and we narrowed down the list of candidate genes. Gene expression was also analyzed by our own oligonucleotide microarray analyses (Human GE 60K; Agilent Technologies), the data of which were deposited into the GEO (on June 14, 2018; http://www.ncbi.nlm.nih.gov/geo/) under accession number GSE115800.

2.11. Dual-luciferase reporter assay
The wild-type sequence of the PLOD1 3’-untranslated region (UTR) was inserted between the SgfI and PmeI restriction sites of the 3’-UTR of the hRluc gene within the psiCHECK-2 vector (C8021; Promega, Madison, WI, USA). We also generated PLOD1 3’-UTR sequences containing deletions in the miR-140-5p target sites (positions 43–49 and 725–731) for insertion into the psiCHECK-2 vector as described above. The psiCHECK-2 vector was used as a cloning vector for the synthesized DNA sequences.

2.12. Immunohistochemistry
Immunohistochemistry procedures were performed according to a previously described method. Clinical
tissue sections were incubated overnight at 4 °C with an anti-PLOD1 antibody diluted 1:10 (SAB1301577; Sigma-Aldrich).

2.13. Analysis of genes downstream of PLOD1

To investigate PLOD1-regulated pathways in BC cells, we assessed gene expression changes in T24 and BOY cells transfected with the PLOD1 inhibitor. Microarray analysis was performed to obtain expression profiles in these cells, and the microarray data were deposited into the GEO (on December 4, 2018; accession number: GSE123318).

2.14. Analysis of the clinical significance of PLOD1 expression

We investigated the clinical importance of miRNAs and genes in BC patients using RNA-sequencing data available in The Cancer Genome Atlas (TCGA; https://tcga-data.nci.nih.gov/tcga/). The gene expression and clinical data were obtained from cBioPortal (http://www.bioportal.org/), and provisional data were downloaded on October 5, 2018 (Anaya, 2016; Cerami et al., 2012; Gao et al., 2013).

2.15. Statistical analysis

Statistical comparisons involving two or three variables were performed using the Bonferroni-adjusted Mann–Whitney U-test. Spearman’s rank tests were used to analyze the correlations among gene expression levels. These analyses were conducted using EXPERT STATVIEW software (version 5.0, SAS Institute Inc., Cary, NC, USA). Multivariate analysis of prognostic factors for patient survival was conducted using JMP PRO 13 (SAS Institute Inc.).

3. Results

3.1. Expression of miR-140-5p and miR-140-3p in BC tissues

hsa-miR-140 is located on chromosome 16q22.1 in humans. The mature sequences of miR-140-5p and miR-140-3p are 5'-CAGUGGUUUUACCCUAUGGUAG-3' and 5'-UAACCACAGGUAAGAACCAGCCG-3', respectively. The expression levels of miR-140-5p and miR-140-3p were significantly downregulated in BC tissues compared with adjacent normal tissues (P = 0.0013 and P = 0.0004, respectively; Fig. 1A,B). Moreover, Spearman’s rank test revealed a strong positive correlation between miR-140-5p and miR-140-3p expression levels (R = 0.637, P = 0.0006; Fig. 1C).

3.2. Effect of miR-140-5p and miR-140-3p on the proliferation, migration, and invasion of BC cells

Restoration of miR-140-5p and miR-140-3p significantly suppressed BC cell proliferation, migration, and invasion abilities (Fig. 1D–F).

3.3. Effect of miR-140-5p and miR-140-3p on apoptosis and cell-cycle assays in BOY cells

The percentage of apoptotic cells was significantly increased in miR-140-5p- and miR-140-3p-transfected cells compared with the control cells (Fig. S1A,B). Moreover, transfection of miR-140-5p and miR-140-3p upregulated the level of cleaved PARP (Fig. S1C). In a cell-cycle analysis, the proportion of cells in the G0/G1 phase was significantly higher transfected with miR-140-5p compared with the control cells (Fig. S1D).

3.4. miR-140-5p and miR-140-3p localization within the RISC

We performed immunoprecipitation assays using antibodies targeting Ago2, which plays a pivotal role in the uptake of miRNAs into the RISC. After transfection of T24 cells with miR-140-5p and immunoprecipitation using anti-Ago2 antibodies, miR-140-5p levels in the immunoprecipitates were significantly higher than those in the immunoprecipitates from mock- or miR-control-transfected cells as well as miR-140-3p-transfected cells (P < 0.0001; Fig. S2A). Similarly, after miR-140-3p transfection, substantial levels of miR-140-3p were detected in Ago2 immunoprecipitates compared with the controls (P < 0.0001; Fig. S2B).

3.5. Candidate target genes of miR-140-5p and miR-140-3p in BC cells

We identified genes containing putative target sites for miR-140-5p and miR-140-3p within their 3'-UTR sequence that also showed upregulated expression levels (log2 > 0.5) in BC tissues and downregulated expression levels (log2 < −0.5) in T24 cells transfected with miR-140-5p or miR-140-3p (Fig. 2A). Using this strategy, we identified 31 and 33 genes as candidate target genes of miR-140-5p and miR-140-3p, respectively (Table 1A and 1B). Among these genes, we focused on PLOD1, which was found to be a target of the miR-140-5p passenger strand.
3.6. Clinical significance and expression of PLOD1

Clinical data from BC patients were obtained from TCGA database, and information on survival revealed that patients with high PLOD1 expression had a significantly poorer prognosis compared with patients with low expression (disease-free survival: \( P = 0.0204 \); overall survival: \( P = 0.000174 \); Fig. 2B). High PLOD1 expression was also related to a highly malignant tumor morphology, advanced stage, and metastasis (Fig. S3A). According to multivariate Cox proportional hazards regression, high expression of PLOD1 was an independent predictive factor for overall survival in BC patients (hazard ratio: 1.51; 95% confidence interval: 1.1–2.07, \( P = 0.0099 \)) (Fig. 2B).

PLOD1 mRNA expression levels were significantly upregulated in BC tissues compared with normal adjacent tissues (\( P = 0.0464 \)) (Fig. 2C). Immunostaining of PLOD1 in BC clinical specimens indicated high expression of PLOD1 in cancer lesions compared with adjacent noncancerous tissues at the same staining intensity (Fig. 2C).

In addition, expression levels of PLOD2 and PLOD3 were detected in BC clinical specimens (Fig. S4A,B). Also, immunohistochemical staining showed that overexpressed PLOD2 and PLOD3 were detected in cancer lesions (Fig. S4G,H). Interestingly,
high expression of PLOD2 was significantly associated with poor prognosis of the patients with BC (Fig. S3B). Among PLOD family, expression of PLOD1 was the highest in BC tissues (Fig. S4C). Clinicopathological analysis was performed between PLODs expression and BC (NMIBC or MIBC) clinical specimens. However, no significant association was found in this study (Fig. S4D–F).

3.7. PLOD1 was directly regulated by miR-140-5p

PLOD1 mRNA and protein levels were significantly decreased in T24 and BOY cells following transfection with miR-140-5p compared with mock-transfected cells or those transfected with miR-control (Fig. 2D,E). The TargetScan database indicated the presence of two miR-140-5p binding sites (positions 43–49 and 725–731) within the PLOD1 3′-UTR. We performed luciferase reporter assays using a vector containing these sequences to assess whether miR-140-5p directly regulates PLOD1 expression in a sequence-dependent manner. Cotransfection of miR-140-5p with vectors harboring the PLOD1 3′-UTR deletion constructs significantly decreased luciferase activity compared with the activity levels in mock-transfected and miR-control-transfected cells (Fig. 2F).

3.8. Knockdown and rescue studies of PLOD1

We confirmed that both PLOD1 mRNA and protein expression levels were suppressed by siRNA-mediated PLOD1 knockdown in BC cells (Fig. 3A and B). Transfection of si-PLOD1 suppressed cell proliferation, migration, and invasion activities (Fig 3C–E). The percentage of apoptotic cells was significantly increased in si-PLOD1-transfected cells compared with the control cells (Fig. S5A,B). Moreover, transfection of si-PLOD1 upregulated the level of cleaved PARP (Fig. S5C). In a cell-cycle analysis, the proportion of cells in the G0/G1 phase was significantly

Fig. 2. Clinical significance, expression, and regulation of PLOD1. (A) The strategy used to identify miR-140-5p candidate target genes, represented by a Venn diagram. (B) Clinical significance of PLOD1. (C) PLOD1 mRNA and protein expression in BC tissues. Scale bars of ×100 and ×400 represent 200 and 50 μm, respectively. P-values were calculated using Bonferroni-adjusted Mann–Whitney U-test. (D) PLOD1 mRNA expression levels 48 h after transfection of BC cells with 10 nM miR-140-5p. GAPDH was used as the internal control gene. Error bars are represented as mean ± SD (n = 3). P-values were calculated using Bonferroni-adjusted Mann–Whitney U-test. (E) PLOD1 protein expression 72 h after transfection with 10 nM miR-140-5p. GAPDH was used as the loading control. (F) Dual-luciferase reporter assays using vectors encoding the wild-type PLOD1 3′-UTR sequence containing two putative miR-140-5p target sites and 3′-UTR sequences with deletions of the target sites (Deletion). Normalized data were calculated as the ratio of Renilla/Firefly luciferase activities. Error bars are represented as mean ± SD (n = 3). P-values were calculated using Bonferroni-adjusted Mann–Whitney U-test. *P < 0.0001, **P < 0.005.
Table 1. Candidate target genes of miR-140-5p and miR-140-3p in BC.

| Gene symbol | Gene name                                                      | Entrez gene ID | Cytoband   | GEO expression data fold change (tumor/normal) | miR-140-5p transfection in T24 (Log2 ratio) | Total binding sites | TCGA analysis for OS (high vs low expression: P value) |
|-------------|----------------------------------------------------------------|---------------|------------|-----------------------------------------------|---------------------------------------------|---------------------|------------------------------------------------------|
| (A) miR-140-5p |                                                                 |               |            |                                               |                                             |                     |                                                      |
| CERCAM      | Cerebral endothelial cell adhesion molecule                     | 51148         | hs|9q34.11 | 1.928                                             | −1.801                                      | 1                   | 7.35E-05                                             |
| PLOD1       | Procollagen-lysine, 2-oxoglutarate 5-dioxygenase 1              | 5351          | hs|1p36.22 | 2.150                                             | −1.587                                      | 2                   | 0.000174                                             |
| FADS1       | Fatty acid desaturase 1                                         | 3992          | hs|11q12.2 | 1.741                                             | −1.533                                      | 4                   | 0.000384                                             |
| PAFAH1B2    | Platelet-activating factor acetylhydrolase 1                    | 5049          | hs|11q23.3 | 1.464                                             | −0.595                                      | 1                   | 0.0169                                              |
| PAX6        | Paired box 6                                                   | 5080          | hs|11p13   | 5.729                                             | −0.550                                      | 1                   | 0.0281                                              |
| TNN         | Tenasin C                                                      | 63923         | hs|1q12.5  | 2.521                                             | −0.514                                      | 1                   | 0.0622                                              |
| HDAC7       | Histone deacetylase 7                                          | 51564         | hs|12q13.1 | 1.766                                             | −0.750                                      | 1                   | 0.0858                                              |
| BMP2K       | BMP2-inducible kinase                                          | 55589         | hs|4q21.21 | 2.025                                             | −0.731                                      | 2                   | 0.134                                               |
| PSRC1       | Proline/serine-rich coiled-coil 1                              | 84722         | hs|1p13.3  | 4.470                                             | −0.655                                      | 1                   | 0.157                                               |
| ZNF74       | Zinc finger protein 74                                         | 7625          | hs|1q12.2  | 1.822                                             | −0.508                                      | 1                   | 0.211                                               |
| SOX4        | SRY (sex-determining region Y)-box 4                           | 6659          | hs|6p22.3  | 2.715                                             | −0.816                                      | 1                   | 0.256                                               |
| FRAS1       | Fraser extracellular matrix complex subunit 1                  | 80144         | hs|4q21.21 | 3.262                                             | −1.122                                      | 1                   | 0.297                                               |
| TSC22D2     | TSC22 domain family, member 2                                  | 9819          | hs|3q26.3  | 1.520                                             | −0.918                                      | 1                   | 0.318                                               |
| GIT1        | G protein-coupled receptor kinase interacting ArfGAP 1         | 28964         | hs|17q11.2 | 3.993                                             | −1.293                                      | 1                   | 0.367                                               |
| YES1        | YES proto-oncogene 1, Src family tyrosine kinase               | 7525          | hs|17q22   | 2.736                                             | −1.027                                      | 2                   | 0.401                                               |
| MMD         | Monocyte to macrophage differentiation-associated               | 23531         | hs|17q22   | 2.736                                             | −1.027                                      | 2                   | 0.401                                               |
| SLC6A6      | Solute carrier family 6 (neurotransmitter transporter), member 6| 6533          | hs|3p25.1  | 1.781                                             | −1.153                                      | 2                   | 0.443                                               |
| FEN1        | Flap structure-specific endonuclease 1                         | 2237          | hs|1q12.2  | 4.028                                             | −0.941                                      | 1                   | 0.446                                               |
| RALA        | v-ral simian leukemia viral oncogene homolog A (ras related)    | 5898          | hs|7p14.1  | 1.786                                             | −2.318                                      | 1                   | 0.462                                               |
| TTYH2       | Tweety family member 3                                         | 80727         | hs|7p22.3  | 3.114                                             | −1.493                                      | 2                   | 0.61                                                |
| ZNF710      | Zinc finger protein 71                                         | 374665        | hs|15q26.1 | 1.642                                             | −0.501                                      | 1                   | 0.649                                               |
| TTK         | TTK protein kinase                                             | 7272          | hs|6q14.1  | 43.335                                           | −0.520                                      | 2                   | 0.686                                               |
| BCL2L1      | BCL2-like 1                                                    | 598           | hs|20q11.21| 2.118                                             | −0.689                                      | 1                   | 0.841                                               |
| PTP4A3      | Protein tyrosine phosphatase type IVA, member 3                 | 11156         | hs|8q24.3  | 2.455                                             | −1.177                                      | 1                   | 0.85                                                |
| RABIF       | RAB interacting factor                                         | 5877          | hs|1q21.2  | 1.435                                             | −0.871                                      | 1                   | 0.889                                               |
| WASF1       | WAS protein family, member 1                                   | 8936          | hs|6q21    | 2.068                                             | −0.808                                      | 1                   | 0.895                                               |
| ACSL6       | Acyl-CoA synthetase long-chain family member 6                 | 23305         | hs|5q31.1  | 1.995                                             | −0.505                                      | 2                   | 0.939                                               |
| LMNB1       | Lamin B1                                                       | 4001          | hs|5q32.3  | 10.537                                           | −0.958                                      | 1                   | 0.943                                               |
| C6orf47     | Chromosome 6 open reading frame 47                            | 57827         | hs|6q21.33 | 1.692                                             | −1.018                                      | 1                   | 0.0134*                                              |
| PROX2       | Prospero homeobox 2                                            | 283571        | hs|14q24.3 | 5.328                                             | −0.513                                      | 1                   | No data                                              |
| NTSC1A      | 5'-nucleotidase, cytosolic IA                                  | 84618         | hs|1p34.2  | 5.445                                             | −1.145                                      | 1                   | No data                                              |
| ADAM17      | ADAM metallopeptidase domain 17                                | 6868          | hs|2p25.1  | 2.062                                             | −0.552                                      | 1                   | 0.0033                                               |
| CCDC103     | Coiled-coil domain containing 103                             | 388389        | hs|17q21.31| 2.621                                             | −2.588                                      | 2                   | 0.0471                                               |
Table 1. (Continued).

| Gene symbol | Gene name | Entrez gene ID | Cytoband | GEO expression data fold change (tumor/normal) | miR-140-5p transfection in T24 (Log2 ratio) | Total binding sites | TCGA analysis for OS (high vs low expression: P value) |
|-------------|-----------|----------------|----------|-----------------------------------------------|-------------------------------------------|--------------------|---------------------------------------------------|
| PLXNA4      | Plexin A4 | 91584 hs| 7q32.3 | 2.195                                         | -0.639                                    | 1                 | 0.0487                                           |
| THPO        | Thrombopoietin | 7066 hs| 3q27.1 | 3.383                                         | -0.615                                    | 1                 | 0.0611                                           |
| NRA4A3      | Nuclear receptor subfamily 4, group A, member 3 | 8013 hs| 9q22.33 | 5.420                                         | -0.693                                    | 1                 | 0.0904                                           |
| AEN         | Apoptosis-enhancing nuclease | 64782 hs| 15q26.1 | 3.713                                         | -0.602                                    | 1                 | 0.101                                            |
| THPO        | Thrombopoietin | 28988 hs| 7p13 | 2.543                                         | -0.585                                    | 3                 | 0.132                                            |
| GABRB2      | Gamma-aminobutyric acid (GABA) A receptor, beta 2 | 2561 hs| 5q34 | 1.944                                         | -0.641                                    | 1                 | 0.143                                            |
| FAM53B      | Family with sequence similarity 53, member B | 9679 hs| 10q26.13 | 1.880                                         | -0.669                                    | 4                 | 0.165                                            |
| COL7A1      | Collagen, type VII, alpha 1 | 1294 hs| 3p21.31 | 2.370                                         | -0.831                                    | 1                 | 0.211                                            |
| SRPA        | Signal-regulatory protein alpha | 140885 hs| 20p13 | 1.573                                         | -0.509                                    | 1                 | 0.236                                            |
| ABCA12      | ATP-binding cassette, subfamily A (ABC1), member 12 | 26154 hs| 2q35 | 13.439                                        | -0.505                                    | 4                 | 0.132                                            |
| KCNK17      | Potassium channel, two-pore domain subfamily K, member 17 | 89822 hs| 6p21.3 | 1.633                                         | -0.683                                    | 1                 | 0.332                                            |
| KCTD16      | Potassium channel tetramerization domain containing 16 | 57528 hs| 5q31.3 | 2.808                                         | -0.702                                    | 2                 | 0.431                                            |
| DAND5       | DAN domain family member 5, BMP antagonist | 199699 hs| 1p35.2 | 2.449                                         | -0.529                                    | 1                 | 0.481                                            |
| KIF5A       | Kinesin family member 5A | 3798 hs| 12q13.3 | 2.610                                         | -0.691                                    | 4                 | 0.592                                            |
| NUDT18      | Nudix (nucleoside diphosphate linked moiety X-type motif) | 79873 hs| 8p21.3 | 2.690                                         | -0.657                                    | 1                 | 0.653                                            |
| SLC17A9     | Solute carrier family 17 (vesicular nucleotide transporter), member 9 | 63910 hs| 20q13.33 | 1.976                                        | -1.701                                    | 4                 | 0.737                                            |
| HMGCS1      | 3-hydroxy-3-methylglutaryl-CoA synthase 1 (soluble) | 3157 hs| 5p12 | 1.971                                         | -1.187                                    | 2                 | 0.808                                            |
| SNX22       | Sorting nexin 22 | 79856 hs| 15q22.31 | 2.226                                        | -0.672                                    | 2                 | 0.861                                            |
| WDR55       | WD repeat domain 55 | 54863 hs| 5p31.3 | 1.736                                         | -0.646                                    | 1                 | 0.942                                            |
| SRCN1       | SRC kinase signaling inhibitor 1 | 80725 hs| 17q12 | 3.306                                         | -0.685                                    | 3                 | 0.000248*                                        |
| BAI2        | Brain-specific angiogenesis inhibitor 2 | 576 hs| 3p21.3 | 1.864                                         | -0.724                                    | 1                 | No data                                          |
| VGLL2       | Vestigial-like family member 2 | 245866 hs| 6q22.1 | 2.171                                         | -0.670                                    | 1                 | No data                                          |
| NOL4        | Nucleolar protein 4 | 8715 hs| 1q21.2 | 2.633                                         | -1.014                                    | 1                 | No data                                          |
| MOBP        | Myelin-associated oligodendrocyte basic protein | 4336 hs| 3p22.1 | 2.795                                         | -0.676                                    | 1                 | No data                                          |
| CYLC1       | Cyclicin, basic protein of sperm head cytoskeleton 1 | 1538 hs| Xq21.1 | 3.014                                         | -0.616                                    | 1                 | No data                                          |
| ELAVL3      | ELAV-like neuron-specific RNA-binding protein 3 | 1995 hs| 19p13.2 | 3.171                                         | -0.656                                    | 1                 | No data                                          |
| SCN1A       | Sodium channel, voltage-gated, type I alpha subunit | 6323 hs| 2q24.3 | 3.609                                         | -0.674                                    | 1                 | No data                                          |
| PAX7        | Paired box 7 | 5081 hs| 1p36.13 | 3.899                                         | -0.653                                    | 1                 | No data                                          |
| KCNK10      | Potassium channel, two-pore domain subfamily K, member 10 | 54207 hs| 14q31.3 | 3.933                                         | -0.679                                    | 1                 | No data                                          |
| SVOP        | SV2-related protein homolog (rat) | 55530 hs| 12q24.11 | 4.259                                         | -0.670                                    | 1                 | No data                                          |
| CAMKV       | CaM kinase-like vesicle-associated | 79012 hs| 3p21.31 | 4.507                                         | -1.615                                    | 4                 | No data                                          |

*aPoor prognosis in patients with low expression.*
higher, transfected with si-PLOD1_2 compared with the control cells, although G2/M phase was significantly elevated in si-PLOD1_1 transfection (Fig. S5D).

In addition, we performed a PLOD1 rescue study in T24 cells to validate whether oncogenic pathways regulated by PLOD1/miR-140-5p are crucial for BC development. PLOD1 and miR-140-5p transfection restored PLOD1 protein expression (Fig. 3F). Functional assays demonstrated that BC cell migration and invasion were significantly recovered by PLOD1 and miR-140-5p transfection compared with miR-140-5p alone (Fig. 3G–I).

3.9. Functional analysis of a PLOD1 inhibitor

After transfection of the PLOD1 inhibitor 2,2’-dipyrididyl into BC cells, cell proliferation was suppressed in a dose-dependent manner (Fig. 4A). The IC50 of 2,2’-dipyrididyl was 82.8 μM in BOY cells and 37.1 μM in T24 cells. Cell migration and invasion were also decreased in a dose-dependent manner in cells transfected with the inhibitor (Fig. S6). In addition, the percentage of apoptotic cells was increased in PLOD1 inhibitor-transfected cells compared with the control cells (Fig. 4B). Moreover, transfection of PLOD1 inhibitor upregulated the level of cleaved PARP (Fig. 4B). In a cell-cycle analysis, the proportion of cells in the G0/G1 phase was significantly higher in BC cells transfected with the PLOD1 inhibitor compared with the control cells (Fig. 4C). In addition, we confirmed that the inhibitor suppressed the mRNA and protein levels of PLOD1 in a dose-dependent manner (Fig. S7). Apoptosis and cell-cycle experiments gave similar results in BOY cells (Fig. S8).
3.10. Genes affected by the PLOD1 inhibitor

PLOD1 acts as lysyl hydroxylases that catalyze hydroxylation of collagen lysines, and it works under the following conditions, extracellular matrix maturation and remodeling. In order to explore the functional significance of PLOD1 on tumor progression, we examined the PLOD1-mediated downstream genes and pathways. As shown in the Venn diagram in Fig. S9, 1518 genes were considerably downregulated after transfection of the PLOD1 inhibitor in BOY and T24 cells. In a KEGG analysis of these genes, we identified 39 pathways enriched among the PLOD1-affected genes, including pathways related to cell cycle and apoptosis (Table 2).

4. Discussion

RNA sequencing is a suitable technology for creating miRNA expression signatures in cancer cells. Analyses of our miRNA signatures in cancers revealed that the passenger strand of some miRNA duplexes is functional in cancer cells by targeting cancer-related genes (Arai et al., 2018b; Goto et al., 2017; Matsushita et al., 2015; Sugawara et al., 2018; Yamada et al., 2018a, 2018b). This makes it possible to identify novel cancer pathways based on aberrantly expressed passenger strand miRNAs.

In this study, we focused on both strands of pre-miR-140 (miR-140-5p and miR-140-3p) and revealed their antitumor functions in BC cells. Previous reports showed that miR-140-3p is downregulated in squamous cell lung cancer and functions as a tumor suppressor by targeting bromodomain containing 9 in vitro and in vivo (Huang et al., 2019). As with miR-140-3p, a tumor-suppressive function of miR-140-5p has been reported in several cancers. miR-140-5p exerted a tumor-suppressive function and enhanced the effect of existing therapeutic drugs in non-small-cell lung cancer.
Aberrant expression of PLOD1 in bladder cancer

Y. Yamada et al.

Table 2. Molecular pathways significantly enriched among the genes affected by PLOD1 inhibitor treatment in BC cells.

| Number of genes | Annotations | P-value |
|-----------------|-------------|---------|
| 18              | (KEGG) 04110: Cell cycle | 2.37E-05 |
| 28              | (KEGG) 05200: Pathways in cancer | 1.90E-04 |
| 13              | (KEGG) 04512: ECM–receptor interaction | 2.36E-04 |
| 15              | (KEGG) 04114: Oocyte meiosis | 3.29E-04 |
| 7               | (KEGG) 00760: Nicotinate and nicotinamide metabolism | 4.73E-04 |
| 13              | (KEGG) 05146: Amebiasis | 1.02E-03 |
| 8               | (KEGG) 00310: Lysine degradation | 4.59E-03 |
| 6               | (KEGG) 00410: Beta-alanine metabolism | 4.74E-03 |
| 15              | (KEGG) 00230: Purine metabolism | 5.32E-03 |
| 6               | (KEGG) 00640: Propanoate metabolism | 1.00E-02 |
| 10              | (KEGG) 04914: Progesterone-mediated oocyte maturation | 1.06E-02 |
| 10              | (KEGG) 04540: Gap junction | 1.06E-02 |
| 6               | (KEGG) 00303: DNA replication | 1.21E-02 |
| 9               | (KEGG) 04070: Phosphatidylinositol signaling system | 1.24E-02 |
| 16              | (KEGG) 04510: Focal adhesion | 1.32E-02 |
| 10              | (KEGG) 04916: Melanosis | 1.84E-02 |
| 9               | (KEGG) 05222: Small-cell lung cancer | 2.10E-02 |
| 14              | (KEGG) 04020: Calcium signaling pathway | 2.26E-02 |
| 11              | (KEGG) 04142: Lysosome | 2.34E-02 |
| 4               | (KEGG) 00670: One carbon pool by folate | 2.51E-02 |
| 9               | (KEGG) 05414: Dilated cardiomyopathy | 2.54E-02 |
| 6               | (KEGG) 01000: Steroid biosynthesis | 2.58E-02 |
| 6               | (KEGG) 00280: Valine, leucine, and isoleucine degradation | 2.68E-02 |
| 6               | (KEGG) 04062: Vasopressin-regulated water reabsorption | 2.68E-02 |
| 14              | (KEGG) 04062: Chemokine signaling pathway | 2.73E-02 |
| 8               | (KEGG) 04146: Peroxisome | 2.83E-02 |
| 6               | (KEGG) 00561: Glycerolipid metabolism | 2.97E-02 |
| 5               | (KEGG) 03410: Base excision repair | 3.06E-02 |
| 11              | (KEGG) 04910: Insulin signaling pathway | 3.08E-02 |
| 8               | (KEGG) 04974: Protein digestion and absorption | 3.08E-02 |
| 6               | (KEGG) 04961: Endocrine and other factor-regulated calcium reabsorption | 3.43E-02 |
| 8               | (KEGG) 04350: TGFB-beta signaling pathway | 3.52E-02 |
| 4               | (KEGG) 03430: Mismatch repair | 3.87E-02 |
| 8               | (KEGG) 04210: Apoptosis | 4.40E-02 |
| 6               | (KEGG) 00590: Arachidonic acid metabolism | 4.46E-02 |
| 10              | (KEGG) 04724: Glutamatergic synapse | 4.46E-02 |
| 4               | (KEGG) 00563: Glycosylphosphatidylinositol (GPI)-anchor biosynthesis | 4.68E-02 |
| 6               | (KEGG) 05217: Basal cell carcinoma | 4.91E-02 |
| 4               | (KEGG) 03440: Homologous recombination | 4.98E-02 |

BC, bladder cancer.

(Flamini et al., 2017). Another report showed that miR-140-5p suppressed cell aggressiveness and suggested that miR-140-5p is a prognostic marker in gastric cancer (Fang et al., 2017). Downregulation of miRNAs was reported to be caused by epigenetic factors such as DNA methylation or histone deacetylation. Previous study showed that suppression of miR-140 expression was influenced by the hypermethylation of the promoter region in breast cancer (Wolfson et al., 2014). Elucidation of the detailed molecular mechanism of downregulation of miR-140-5p and miR-140-3p is also essential in BC cells. These studies indicate that both strands of pre-miR-140 act as critical miRNAs that prevent malignant transformation in cells. To our knowledge, this is the first study to identify a functional role of the miR-140 duplex and its oncogene targets in BC.

Our next focus was to investigate the molecular networks regulated by these miRNAs in BC cells. A total of 31 genes regulated by miR-140-5p and 33 genes regulated by miR-140-3p were identified as putative oncogenic targets in BC cells. Among these targets, the expression levels of eight genes (CERCAM, PLOD1, FADS1, PAFAH1B2, PAX6, ADAM17, CCDC103, and PLXNA4) were closely associated with BC pathogenesis. These genes are promising as therapeutic targets and prognostic markers, and further analysis is necessary to elucidate the molecular pathogenesis of BC. We focused on PLOD1 to investigate its oncogenic functions and clinical significance in BC. PLOD genes encode lysyl hydroxylases, which are crucial for collagen biosynthesis, cross-linking, and deposition (Qi and Xu, 2018). Collagen is a major component of the extracellular matrix (ECM), and collagen cross-linking is related to the stiffness of the ECM, which enhances cancer cell migration, invasion, and focal adhesion (Du et al., 2017; Peinado et al., 2008). The PLOD family consists of PLOD1, PLOD2, and PLOD3. A number of studies have demonstrated that overexpression of PLOD2 and PLOD3 promotes cancer progression and metastasis. Our previous studies showed that aberrant expression of PLOD2 was detected in BC and renal cell carcinoma tissues, and its overexpression enhanced cancer cell malignant transformation (Kurozumi et al., 2016; Miyamoto et al., 2016). We hypothesized that members of the PLOD family member are deeply involved in the molecular pathogenesis of BC. On the other hand, there are not many reports on the role of PLOD1 in cancer (Qi and Xu, 2018). Previous studies showed that aberrant expression of PLOD1 was significantly associated with shorter survival in patients with gastric or colorectal cancer (Wang et al., 2018). Overexpression of PLOD1 was also detected in esophageal squamous cell carcinoma and breast cancer (Gilkes et al., 2013; Li et al., 2017). Mutations in PLOD1 are the cause of PLOD1-related
kyphoscoliotic Ehlers–Danlos syndrome, an autosomal recessive generalized connective tissue disorder (Giunta et al., 2005).

The data from a large number of cohort analyses in TCGA database show that high expression of PLOD1 is significantly associated with a poor prognosis (overall survival: \( P = 0.000174 \), more strongly than are PLOD2 and PLOD3 (OS: \( P = 0.0097 \) and \( P = 0.315 \), respectively) (Fig. S3B,C). Furthermore, multivariate analysis showed that PLOD1 expression was an independent prognostic factor in patients with BC (hazard ratio = 1.51, \( P = 0.0099 \)). Moreover, high expression of PLOD1 was significantly associated with tumor stage and presence of metastasis. Aberrant expression of PLOD1 has been shown to be closely related to the malignant phenotype of BC. Development of a new diagnostic strategy for BC using PLOD1 expression as a marker is desired.

Aberrant expression of PLOD1 was detected in BC clinical specimens, and inhibition of PLOD1 by siRNA-mediated knockdown or treatment with a PLOD1 inhibitor significantly reduced the malignant phenotype of BC cells (e.g., decreases in proliferation, migration, and invasion and an increase in apoptosis). We used 2,2'-dipyridyl, an iron chelator, as an inhibitor of PLOD1 in this study (Bernardes et al., 2018; Jover et al., 2018). Collagen lysyl hydroxylases reportedly depend on Fe2+ binding for stabilization, and 2,2'-dipyridyl prevents prolyl and lysyl hydroxylation (Barsh and Byers, 1981; Guo et al., 2018). A previous report showed that inhibition of PLOD1 and lysyl oxidase suppressed arterial smooth muscle cell calcification via ECM remodeling (Jover et al., 2018). Another study was conducted to investigate the effect of 2,2'-dipyridyl in combination with doxorubicin in breast cancer cells (Bernardes et al., 2018). In this study, we showed that PLOD1 expression and cell proliferation were suppressed after transfection of a PLOD1 inhibitor in a dose-dependent manner. Moreover, the PLOD1 inhibitor induced apoptosis and cell-cycle arrest at the G1-to-S phase transition.

The molecular mechanism of the antitumor effect of the PLOD1 inhibitor in BC cells was evaluated by global gene expression analysis. As a result, genes associated with cell cycle, ECM–receptor interactions, and apoptosis were differentially expressed in cells transfected with the PLOD1 inhibitor, supporting our current data. We focused on several genes (e.g., CCNB1, CCNB2, and SKP2) involved in ‘cell-cycle pathway’. Expression of CCNB2 (cyclin B2) was upregulated in BC tissues, and suppression of its expression significantly inhibited invasive and metastatic abilities (Lei et al., 2016). Our recent study showed that CCNB1 (cyclin B1) was regulated by antitumor miR-223-5p in BC cells and its high expression was closely associated with poor prognosis of the patients with BC by TCGA database analysis (Sugawara et al., 2018). Moreover, overexpression of SKP2 (S-phase kinase-associated protein 2) was significantly related to advanced tumor stage and grade of the patients with BC (Kawakami et al., 2007).

Moreover, we performed rescue experiments by overexpressing PLOD1 and miR-140-5p. The results revealed that PLOD1 can counteract the antitumor effects, in terms of cell migration and invasion, of miR-140-5p in BC cells, indicating that the PLOD1/miR-140-5p axis plays an important role in BC development.

5. Conclusion

Both strands of the miR-140 duplex (miR-140-5p and miR-140-3p) suppressed BC cell malignant transformation. Genes controlled by the miR-140-5p were found to be related to BC pathogenesis. PLOD1 expression was directly regulated by the miR-140-5p in BC cells. Aberrant expression of PLOD1 was closely contributed to BC development. Furthermore, inhibition of PLOD1 expression significantly attenuated to BC cell aggressive phenotypes. PLOD1 might be a novel biomarker and therapeutic target in BC. Further investigation is required for clinical application.

Acknowledgements

The present study was supported by KAKENHI grants 18K16685, 18K16724, 18K16723, 16H05462, and 18K09338.

Conflict of interest

The authors declare no conflict of interest.

Author contributions

YY, NS, and TI designed the whole study and wrote the manuscript. MK, TA, HS, AU, SM, SS, and AK contributed to experimental design and data collection. All authors have agreed with the manuscript and provide their consent for publication.

Data accessibility

Data acquired during the course of this study are available in GEO: GSE115800 and GSE123318.
Aberrant expression of PLOD1 in bladder cancer

Y. Yamada et al.

References

Abufaraj M, Dalbagni G, Daneshmand S, Horenblas S, Kamat AM, Kanzaki R, Zlotta AR and Shariat SF (2018) The role of surgery in metastatic bladder cancer: a systematic review. *Eur Urol* 73, 543–557.

Anaya J (2016) OncoLnc: linking TCGA survival data to mRNAs, miRNAs, and lncRNAs. *PeerJ Comput Sci* 2, e67.

Antoni S, Ferlay J, Soerjomataram I, Znaor A, Jamal A and Bray F (2017) Bladder cancer incidence and mortality: a global overview and recent trends. *Eur Urol* 71, 96–108.

Arai T, Kojima S, Yamada Y, Sugawara S, Kato M, Yamazaki K, Naya Y, Ichikawa T and Seki N (2018a) Pirin: a potential novel therapeutic target for castration-resistant prostate cancer regulated by miR-455-5p. *Mol Oncol* 13, 322–337.

Arai T, Okato A, Yamada Y, Sugawara S, Kurozumi A, Kojima S, Yamazaki K, Naya Y, Ichikawa T and Seki N (2018b) Regulation of NCAPG by miR-99a-3p (passenger strand) inhibits cancer cell aggressiveness and is involved in CRPC. *Cancer Med* 7, 1988–2002.

Barsh GS and Byers PH (1981) Reduced secretion of structurally abnormal type I procollagen in a form of osteogenesis imperfecta. *Proc Natl Acad Sci USA* 78, 5142–5146.

Bartel DP (2004) MicroRNAs: genomics, biogenesis, mechanism, and function. *Cell* 116, 281–297.

Bartel DP (2009) MicroRNAs: target recognition and regulatory functions. *Cell* 136, 215–233.

Beermann J, Piccoli MT, Viereck J and Thum T (2016) Non-coding RNAs in development and disease: background, mechanisms, and therapeutic approaches. *Physiol Rev* 96, 1297–1325.

Bernardes JR, Faria CC, Andrade IS, Ferreira ACF, Carvalho DP, Leitao AC, de Alencar TAM and Fortunato RS (2018) Effect of the Fe(2+) chelation by 2,2’-dipyridyl in the doxorubicin-induced lethality in breast tumor cell lines. *Life Sci* 192, 128–135.

Cerami E, Gao J, Dogrusoz U, Gross BE, Sumer SO, Aksoy BA, Jacobsen A, Byrne CJ, Heuer ML, Larsson E et al. (2012) The cBio cancer genomics portal: an open platform for exploring multidimensional cancer genomics data. *Cancer Discov* 2, 401–404.

Chou R, Selph SS, Buckley DI, Gustafson KS, Griffin JC, Grusing SE and Gore JL (2016) Treatment of muscle-invasive bladder cancer: a systematic review. *Cancer* 122, 842–851.

Du H, Pang M, Hou X, Yuan S and Sun L (2017) PLOD2 in cancer research. *Biomed Pharmacother* 90, 670–676.

Fang Z, Yin S, Sun R, Zhang S, Fu M, Wu Y, Zhang T, Khaliq J and Li Y (2017) miR-140-5p suppresses the proliferation, migration and invasion of gastric cancer by regulating YES1. *Mol Cancer* 16, 139.

Flamini V, Jiang WG and Cui Y (2017) Therapeutic role of MiR-140-5p for the treatment of non-small cell lung cancer. *Anticancer Res* 37, 4319–4327.

Fukumoto I, Hanazawa T, Kinoshita T, Kikkawa N, Koshizuka K, Goto Y, Nishikawa R, Chiyomaru T, Enokida H, Nakagawa M et al. (2015) MicroRNA expression signature of oral squamous cell carcinoma: functional role of microRNA-26a/b in the modulation of novel cancer pathways. *Br J Cancer* 112, 891–900.

Fukumoto I, Kinoshita T, Hanazawa T, Kikkawa N, Chiyomaru T, Enokida H, Yamamoto N, Goto Y, Nishikawa R, Nakagawa M et al. (2014) Identification of tumour suppressive microRNA-451a in hypopharyngeal squamous cell carcinoma based on microRNA expression signature. *Br J Cancer* 111, 386–394.

Gao J, Aksoy BA, Dogrusoz U, Dresdner G, Gross B, Sumer SO, Sun Y, Jacobsen A, Sinha R, Larsson E et al. (2013) Integrative analysis of complex cancer genomics and clinical profiles using the cBioPortal. *Sci Signal* 6, pl1.

Gilkes DM, Bajpai S, Wong CC, Chaturvedi P, Hubbi ME, Wirtz D and Semenza GL (2013) Procollagen lysyl hydroxylase 2 is essential for hypoxia-induced breast cancer metastasis. *Mol Cancer Res* 11, 456–466.

Giunta C, Randolph A and Steinmann B (2005) Mutation analysis of the PLOD1 gene: an efficient multistep approach to the molecular diagnosis of the kyphoscoliotic type of Ehlers-Danlos syndrome (EDS VIA). *Mol Genet Metab* 86, 269–276.

Goto Y, Kojima S, Nishikawa R, Kurozumi A, Kato M, Enokida H, Matsushita R, Yamazaki K, Ishida Y, Nakagawa M et al. (2015a) MicroRNA expression signature of castration-resistant prostate cancer: the microRNA-221/222 cluster functions as a tumour suppressor and disease progression marker. *Br J Cancer* 113, 1055–1065.

Goto Y, Kurozumi A, Arai T, Nohata N, Kojima S, Okato A, Kato M, Yamazaki K, Ishida Y, Naya Y et al. (2017) Impact of novel miR-145-3p regulatory networks on survival in patients with castration-resistant prostate cancer. *Br J Cancer* 117, 409–420.

Goto Y, Kurozumi A, Enokida H, Ichikawa T and Seki N (2015b) Functional significance of aberrantly expressed microRNAs in prostate cancer. *Int J Urol* 22, 242–252.

Guo HF, Tsai CL, Terajima M, Tan X, Banerjee P, Miller MD, Liu X, Yu J, Byemerwa J, Alvarado S et al. (2018) Pro-metastatic collagen lysyl hydroxylase dimer assemblies stabilized by Fe(2+) binding. *Nat Commun* 9, 512.

Huang H, Wang Y, Li Q, Fei X, Ma H and Hu R (2019) miR-140-3p functions as a tumor suppressor in squamous cell lung cancer by regulating BRD9. *Cancer Lett* 446, 81–89.
Idichi T, Seki N, Kurahara H, Fukuhisa H, Toda H, Shimonosono M, Yamada Y, Arai T, Kita Y, Kijima Y et al. (2018) Involvement of anti-tumor miR-124-3p and its targets in the pathogenesis of pancreatic ductal adenocarcinoma: direct regulation of ITGA3 and ITGB1 by miR-124-3p. Oncotarget 9, 28849–28865. 

Jover E, Silvente A, Marin F, Martínez-Gonzalez J, Orriols M, Martínez CM, Puche CM, Valdes M, Rodríguez C and Hernandez-Romero D (2018) Inhibition of enzymes involved in collagen cross-linking reduces vascular smooth muscle cell calcification. FASEB J 32, 4459–4469. 

Kawakami K, Enokida H, Tachiwada T, Nishiyama K, Seki N and Nakagawa M (2007) Increased SKP2 and CKS1 gene expression contributes to the progression of human urothelial carcinoma. J Urol 178, 301–307. 

Koshizuka K, Hanazawa T, Fukumoto I, Kikkawa N, Okamoto Y and Seki N (2017a) The microRNA signatures: aberrantly expressed microRNAs in head and neck squamous cell carcinoma. J Hum Genet 62, 3–13. 

Koshizuka K, Nohata N, Hanazawa T, Kikkawa N, Arai T, Okato A, Fukumoto I, Katada K, Okamoto Y and Seki N (2017b) Deep sequencing-based microRNA expression signatures in head and neck squamous cell carcinoma: dual strands of pre-miR-150-3p as antitumor miRNAs. Oncotarget 8, 30288–30304. 

Kurozumi A, Goto Y, Okato A, Ichikawa T and Seki N (2017) Aberrantly expressed microRNAs in bladder cancer and renal cell carcinoma. J Hum Genet 62, 49–56. 

Kurozumi A, Kato M, Goto Y, Matsushita R, Nishikawa R, Okato A, Fukumoto I, Ichikawa T and Seki N (2016) Regulation of the collagen cross-linking enzymes LOXL2 and PLOD2 by tumor-suppressive microRNA-26a/b in renal cell carcinoma. Int J Onkol 48, 1837–1846. 

Lei CY, Wang W, Zhu YT, Fang WY and Tan WL (2016) The decrease of cyclin B2 expression inhibits invasion and metastasis of bladder cancer. Urol Oncol 34, 237.e231-210. 

Lemke EA and Shah AY (2018) Management of advanced bladder cancer: an update. J Adv Pract Oncol 9, 410–416. 

Li L, Wang W, Li X and Gao T (2017) Association of ECRG4 with PLK1, CDK4, PLOD1 and PLOD2 in esophageal squamous cell carcinoma. Am J Transl Res 9, 3741–3748. 

Mah SM, Buske C, Humphries RK and Kuchenbauer F (2010) miRNA*: a passenger stranded in RNA-induced silencing complex? Crit Rev Eukaryot Gene Expr 20, 141–148. 

Matsushita R, Seki N, Chiyomaru T, Inoguchi S, Ishihara T, Goto Y, Nishikawa R, Mataki H, Tatarano S, Itoh H et al. (2015) Tumour-suppressive microRNA-144-5p directly targets CCNE1/2 as potential prognostic markers in bladder cancer. Br J Cancer 113, 282–289. 

Miyamoto K, Seki N, Matsushita R, Yonemori M, Yoshino H, Nakagawa M and Enokida H (2016) Tumour-suppressive miRNA-26a-5p and miR-26b-5p inhibit cell aggressiveness by regulating PLOD2 in bladder cancer. Br J Cancer 115, 354–363. 

Peinado H, Moreno-Bueno G, Hardisson D, Perez-Gomez E, Santos V, Mendiola M, de Diego JI, Nistal M, Quintanilla M, Portillo F et al. (2008) Lysyl oxidase-like-2 as a new poor prognosis marker of squamous cell carcinomas. Can Res 68, 4541–4550. 

Qi Y and Xu R (2018) Roles of PLODs in collagen synthesis and cancer progression. Front Cell Dev Biol 6, 66. 

Sugawara S, Yamada Y, Arai T, Okato A, Idichi T, Kato M, Koshizuka K, Ichikawa T and Seki N (2018) Dual strands of the miR-223 duplex (miR-223-5p and miR-223-3p) inhibit cancer cell aggressiveness: targeted genes are involved in bladder cancer pathogenesis. J Hum Genet 63, 657–668. 

Wang D, Zhang S and Chen F (2018) High expression of PLOD1 drives tumorigenesis and affects clinical outcome in gastrointestinal carcinoma. Genet Test Mol Biomarkers 22, 366–373. 

Wolfson B, Eades G and Zhou Q (2014) Roles of microRNA-140 in stem cell-associated early stage breast cancer. World J Stem Cells 6, 591–597. 

Yamada Y, Arai T, Kojima S, Sugawara S, Kato M, Okato A, Yamazaki K, Naya Y, Ichikawa T and Seki N (2018a) Anti-tumor roles of both strands of the miR-455 duplex: their targets SKA1 and SKA3 are involved in the pathogenesis of renal cell carcinoma. Oncotarget 9, 26638–26658. 

Yamada Y, Arai T, Kojima S, Sugawara S, Kato M, Okato A, Yamazaki K, Naya Y, Ichikawa T and Seki N (2018b) Regulation of antitumor miR-144-5p targets oncogenes: direct regulation of syndecan-3 and its clinical significance. Cancer Sci 109, 2919–2936. 

Yamada Y, Arai T, Sugawara S, Okato A, Kato M, Kojima S, Yamazaki K, Naya Y, Ichikawa T and Seki N (2018c) Impact of novel oncogenic pathways regulated by anti-tumor miR-451a in renal cell carcinoma. Cancer Sci 109, 1239–1253. 

Yamada Y, Sugawara S, Arai T, Kojima S, Kato M, Okato A, Yamazaki K, Naya Y, Ichikawa T and Seki N (2018d) Molecular pathogenesis of renal cell carcinoma: impact of the anti-tumor miR-29 family on gene regulation. Int J Urol 25, 953–965. 

Supporting information

Additional supporting information may be found online in the Supporting Information section at the end of the article.
**Fig. S1.** Effect of miR-140-5p and miR-140-3p on apoptosis and cell-cycle assays in BOY cells.

**Fig. S2.** miR-140-5p and miR-140-3p localization within the RISC.

**Fig. S3.** Clinical database analysis of PLOD1, PLOD2 and PLOD3 expression in BC patients.

**Fig. S4.** Expression analysis of PLOD1, PLOD2 and PLOD3 in BC tissues.

**Fig. S5.** Effect of si-PLOD1 on apoptosis and cell-cycle assays in BOY cells.

**Fig. S6.** Effect of a PLOD1 inhibitor on the migration and invasion of BC cells.

**Fig. S7.** Effect of a PLOD1 inhibitor on PLOD1 expression.

**Fig. S8.** Effect of a PLOD1 inhibitor on apoptosis and cell-cycle assays in BC cells.

**Fig. S9.** Downstream pathways affected by treatment with a PLOD1 inhibitor in BC cells.

**Table S1.** Background characteristics of the BC patients.