ANTICANCER RESEARCH *41*: 4821-4836 (2021)
doi:10.21873/anticanres.15297

Impact of Oncogenic Targets Controlled by Tumor-Suppressive *miR-30a-5p* in Pancreatic Ductal Adenocarcinoma

PRAMOD NEPAL[1], YUTO HOZAKA[1], TAKAKO TANAKA[1], MASUMI WADA[1], SHUNICHI ASAI[2], CHIKASHI MINEMURA[2], TETSUYA IDICHI[1], TAKAAKI ARIGAMI[1], HIROSHI KURAHARA[1], NAOHIKO SEKI[2] and TAKAO OHTSUKA[1]

[1]*Department of Digestive Surgery, Breast and Thyroid Surgery, Graduate School of Medical and Dental Sciences, Kagoshima University, Kagoshima, Japan;*
[2]*Department of Functional Genomics, Chiba University Graduate School of Medicine, Chiba, Japan*

Abstract. *Background/Aim: Our recent miRNA analyses revealed that miR-30a-5p has tumor-suppressive activity in pancreatic ductal adenocarcinoma (PDAC). Herein, we sought to identify tumor-suppressive genes controlled by miR-30a-5p, emphasizing on genes that are closely involved in the molecular pathogenesis of PDAC. We uncovered several novel findings regarding the pathogenesis of this disease. Materials and Methods: In silico analyses were used to identify the putative target genes of miR-30a-5p and assess their expression levels. Direct regulation of RRM2 by miR-30a-5p and its oncogenic functions were evaluated in PDAC cell lines. Overexpression of RRM2 was demonstrated in clinical samples. Results: A total of 24 putative targets were identified by in silico database analysis. High expression of 4 genes (CBFB, RRM2, AHNAK, and DCBLD1) was significantly associated with shorter survival of patients with PDAC. Functional assays demonstrated that knockdown of RRM2 attenuated the malignant phenotype of PDAC cells. Conclusion: The miR-30a-5p/RRM2 axis facilitated the malignant transformation of PDAC cells.*

Pancreatic ductal adenocarcinoma (PDAC) is an extremely aggressive cancer with the highest mortality rate among various human cancers (1, 2). The 5-year survival rate for patients with PDAC is around 8%, and approximately 459,000 people were diagnosed with PDAC and 432,000 died in 2018 (3). A notable feature of PDAC is that patients have few symptoms in the early stages of disease. By the time symptoms appear, many patients have reached an advanced stage in which there is invasion of surrounding tissues or distant metastasis (4, 5). The prognosis for advanced cases (surgically unresectable cases) is extremely poor (most patients die within a year) (5).

Cancer cell development and progression are linked to the accumulation of genetic mutations, epigenetic dysregulation and aberrant activation of oncogenic signaling (6, 7). Driver mutations are only a small fraction of the genetic alterations identified in patients. The remaining genomic changes likely contribute to oncogenic transformation (8). There are three types of RAS proteins, KRAS, NRAS and HRAS. Mutations in these genes are frequently detected in human cancers, and they are closely involved in human oncogenesis (9). KRAS mutations are detected in most patients with PDAC. Thus, KRAS mutations are driver mutations in this disease (10). Controlling the activation of KRAS-mediated oncogenic signaling is an important issue, but therapeutic drugs that control KRAS have not yet been developed (11, 12).

A vast number of studies indicate that non-coding RNAs (ncRNAs) are involved in a wide range of biological processes, *e.g.*, cell proliferation, apoptosis, development, the epithelial-to-mesenchymal transition (EMT) and chromatin modelling (13-15). In human cancer, aberrant expression of ncRNAs can contribute to malignant transformation, metastasis and drug resistance (16). However, the biological roles and functions of non-coding RNAs are mostly uncharacterized.

MicroRNAs (miRNAs) are small ncRNAs that control the expression of RNA transcripts in both normal and disease cells in a sequence-dependent manner (17). A single miRNA can influence numerous transcripts of protein coding and non-coding genes, accounting for approximately 60% of cellular RNAs (18). During the development of cancer cells, the abnormal expression of microRNAs may disrupt RNA networks. There is currently a consensus that aberrantly expressed miRNAs contribute to cancer cell development, progression, EMT, metastasis, and drug resistance (14, 18).

Correspondence to: Naohiko Seki, Ph.D., Associate Professor of Functional Genomics, Department of Functional Genomics, Chiba University Graduate School of Medicine, Chiba 260-8670, Japan. Tel: +81 432262971, Fax: +81 432273442, e-mail: naoseki@faculty.chiba-u.jp

Key Words: microRNA, *miR-30a-5p*, *RRM2*, pancreatic ductal adenocarcinoma, tumor-suppressor.

Based on a PDAC miRNA signature obtained by RNA sequencing, we have identified tumor-suppressive miRNAs and their oncogenic targets that are closely associated with PDAC pathogenesis. There are multiple miRNAs with the same mature sequence, and they are grouped in "families", *e.g.*, the *miR-29*-family, *miR-30*-family, *miR-200*-family *etc*. Focusing on the *miR-30*-family, we have been searching for oncogenic genes that are controlled in pancreatic cancer. Our recent study showed that both strands of pre-*miR-30a* (*miR-30a-5p*: the guide strand and *miR-30a-3p*: the passenger strand) were down-regulated in PDAC tissues. Ectopic expression assays demonstrated that both strands of pre-*miR-30a* had tumor-suppressive roles, suggesting these miRNAs controlled oncogenic genes in PDAC cells (19).

The aim of the present study was to identify genes controlled by tumor-suppressive *miR-30a-5p,* genes that are tightly associated with PDAC molecular pathogenesis. Herein, we found 4 genes (*CBFB*, *RRM2*, *AHNAK*, and *DCBLD1*) that significantly predicted abbreviated survival of PDAC patients. We focused on *RRM2*, and investigated the functional significance of this gene in PDAC oncogenesis.

Materials and Methods

Clinical specimens and PDAC cell lines. All the patients in this study provided written prior informed consent and approval. The present study was approved by the Bioethics Committee of Kagoshima University (Kagoshima, Japan; approval no. 160038 28-65, date of approval: 4 September 2016). We utilized the PDAC clinical samples of two patients who underwent surgery at Kagoshima University Hospital from 2012 to 2014. The clinical samples were staged according to the American Joint Committee on Cancer/Union Internationale Contre le Cancer (UICC) TNM classification.

The two PDAC cell lines used in this study, SW1990 and PANC-1, were purchased from the American Type Culture Collection (Manassas, VA, USA) and RIKEN Cell Bank (Tsukuba, Ibaraki, Japan), respectively. Both PANC-1 and SW1990 were maintained in RPMI-1640 medium with 10% fetal bovine serum in a humidified atmosphere of 5% CO_2 and 95% air at 37˚C. Further details are available in our previous study (19).

RNA extraction and quantitative real-time reverse transcription polymerase chain reaction (qRT-PCR). Total RNA was extracted from frozen cell lines. We previously described our methods for extracting RNA from the cell lines, and methods used for qRT-PCR (19-21). TaqMan probes and primers used in this study were obtained from Applied Biosystems (Waltham, MA, USA).

Transfection of PDAC Cells with Mature miRNAs, small-interfering RNAs (siRNAs), and plasmid vectors. We have described the methods for transfection of miRNAs, siRNAs and plasmid vectors into PDAC cells previously (19-21). The pre-miR miRNA precursors were obtained from Applied Biosystems (Waltham, MA, USA) and stealth RNAi siRNA of RRM2 was obtained from Invitrogen (Waltham, MA, USA).

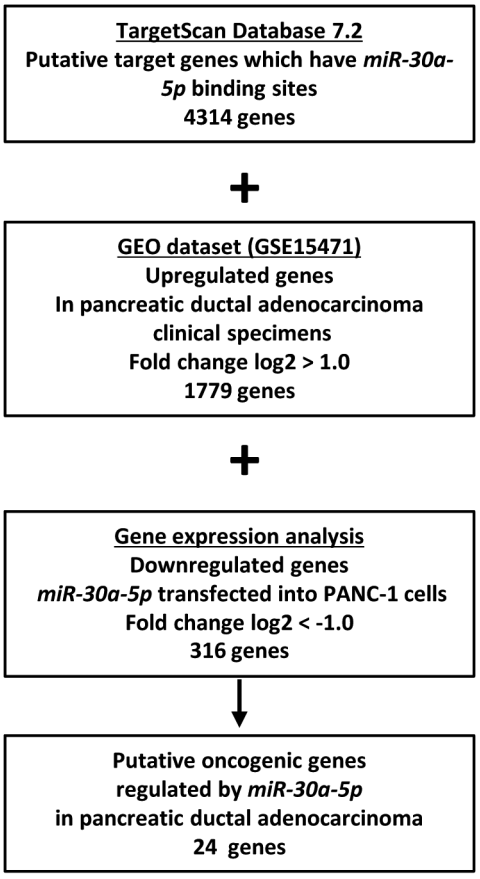

Figure 1. *Flowchart summarizing the search for oncogenic targets of miR-30a-5p regulation in PDAC cells. To identify miR-30a-5p target genes in PDAC cells, we screened putative targets using the TargetScan database and two types of gene expression profiles, GSE15471 (expression of PDAC clinical specimens) and GSE155659 (miR-30a-5p transfected PANC-1 cells). Finally, a total of 24 putative targets were identified as candidate genes regulated by miR-30a-5p in PDAC cells.*

Cell proliferation, migration, and invasion assays in PDAC cells. The methods for functional assays of cancer cells (proliferation, migration and invasion) were described in our previous studies (19-21). PDAC cells were transfected with 10 nM miRNAs or siRNAs. For invasion and migration assays, SW1990 cells at 1.2×10^5 and PANC-1 cells at 1.0×10^5 were transfected in 6-well plates. After 72 h, SW1990 and PANC-1 cells were adjusted to 2.5×10^5 and were added into each chamber. The cells on the lower surface were counted for analysis after 48 h. All experiments were performed in triplicate. XTT assays were used to assess cell proliferation (19-21).

Identification of the miR-30a-5p targets in PDAC. To identify the putative target genes of *miR-30a-5p*, we used the strategy shown in Figure 1. We used a combination of genome-wide gene expression and *in silico* analyses as described before (19-21). TargetScanHuman ver.7.2 (http://www.targetscan.org/vert_72/) was used to find the target genes regulated by *miR-30a-5p*. Our microarray data (from *miR-30a-*

Table I. *Candidates of miR-30a-5p targets in PDAC cells*.

Entrez GeneID	Gene symbol	Gene name	Total binding sites	Representative miRNA	PANC1 miR-30a-5p transfectant log2 FC<-1	GSE15471 (FClog2>)	TCGA Oncolnc OS *p*-Value (in 5 years)	Expression in PAAD cancer tissues (*p*-Value) GEPIA2
865	*CBFB*	Core-binding factor, beta subunit	2	hsa-miR-30e-5p	–1.2186823	1.15552854	0.0086	<0.01
6241	*RRM2*	Ribonucleotide reductase M2	1	hsa-miR-30a-5p	–1.0669783	1.1663941	0.0127	<0.01
79026	*AHNAK*	AHNAK nucleoprotein	1	hsa-miR-30c-5p	–1.0647298	1.0858593	0.0179	<0.01
285761	*DCBLD1*	Discoidin, CUB and LCCL domain containing 1	2	hsa-miR-30b-5p	–1.12899	1.73605133	0.0452	<0.01
114907	*FBXO32*	F-box protein 32	3	hsa-miR-30a-5p	–1.0577307	1.90713641	0.0688	<0.01
23333	*DPY19L1*	dpy-19-like 1 (C. elegans)	3	hsa-miR-30e-5p	–1.2512587	1.51232384	0.0796	<0.01
4907	*NT5E*	5'-nucleotidase, ecto (CD73)	2	hsa-miR-30-5p	–2.2615404	1.57594843	0.1164	<0.01
25963	*TMEM87A*	Transmembrane protein 87A	1	hsa-miR-30b-5p	–1.0681242	1.0202431	0.1212	<0.01
55638	*SYBU*	Syntabulin (syntaxin-interacting)	2	hsa-miR-30b-5p	–1.517468	1.34085277	0.2500	<0.01*
10484	*SEC23A*	Sec23 homolog A (S. cerevisiae)	2	hsa-miR-30b-5p	–2.0017452	1.24881661	0.2782	<0.01
5738	*PTGFRN*	prostaglandin F2 receptor inhibitor	3	hsa-miR-30b-5p	–1.8308114	1.40208854	0.3495	<0.01
9644	*SH3PXD2A*	SH3 and PX domains 2A	3	hsa-miR-30b-5p	–1.0122585	1.63137626	0.3623	<0.01
115908	*CTHRC1*	Collagen triple helix repeat containing 1	1	hsa-miR-30a-5p	–1.2798586	4.42892699	0.4671	<0.01
56243	*KIAA1217*	KIAA1217	1	hsa-miR-30b-5p	–1.2816907	1.40839818	0.6087	<0.01
6443	*SGCB*	Sarcoglycan, beta (43kDa dystrophin-associated glycoprotein)	1	hsa-miR-30a-5p	–1.6278781	1.11962311	0.6239	<0.01
8819	*SAP30*	Sin3A-associated protein, 30kDa	2	hsa-miR-30b-5p	–1.7176095	1.09428391	0.6822	<0.01
8417	*STX7*	Syntaxin 7	4	hsa-miR-30e-5p	–1.4063812	1.12234426	0.7001	<0.01
7431	*VIM*	Vimentin	2	hsa-miR-30e-5p	–1.7224925	1.10932325	0.7629	<0.01
5159	*PDGFRB*	Platelet-derived growth factor receptor, beta polypeptide	1	hsa-miR-30e-5p	–1.1151328	1.79906301	0.7749	<0.01
79071	*ELOVL6*	ELOVL fatty acid elongase 6	1	hsa-miR-30b-5p	–1.1026864	1.4875004	0.844	<0.01
4325	*MMP16*	Matrix metallopeptidase 16 (membrane-inserted)	1	hsa-miR-30e-5p	–1.0128733	1.00989799	0.858	N.S
8829	*NRP1*	Neuropilin 1	1	hsa-miR-30b-5p	–1.0871696	1.30218023	0.8906	<0.01
170954	*PPP1R18*	Protein phosphatase 1, regulatory subunit 18	3	hsa-miR-30e-5p	–1.1010675	1.35140377	0.9005	<0.01
54749	*EPDR1*	Ependymin related 1	3	hsa-miR-30e-5p	–1.5695925	1.00831047	0.9820	<0.01

*The expression of *SYBU* was significanlty down-regulated in *PAAD* cancer tissues; N.S- non-significant.

5p transfected cells) were deposited in the Gene Expression Omnibus (GEO) database (GSE155659). To determine the genes up-regulated in PDAC clinical specimens, we obtained expression data from the Gene Expression Omnibus (GEO) database (GSE15471). The expression data of 39 pairs of specimens are contained in this dataset.

Clinical database analysis of miR-30a-5p target genes. The expression level of each putative target gene was extracted and analyzed from the Cancer Genome Atlas (TCGA) – pancreas adenocarcinoma database (TCGA-PAAD) through the GEPIA2 platform [http://gepia2.cancer-pku.cn/#index (accessed on 10 February 2021)] to analyze gene expression levels in 179 tumor samples versus 171 normal PDAC tissue samples. The methods of clinical database analysis of miRNA target genes between normal and PDAC cancer tissues were described in the previous papers (19-21).

Plasmid construction and dual-luciferase reporter assays. Plasmid vectors containing *RRM2* with the wild-type sequences of the *miR-30a-5p* binding sites in the 3'-UTR and without those sequences were prepared. We have described the methods for transfection and dual-luciferase reporter assays in our previous studies (19-21). Dual-Luciferase® Reporter Assay System (Promega) was used following the manufacturer's instructions.

Western blotting and immunohistochemistry. Immunohistochemistry was done on formalin-fixed and paraffin-embedded clinical sections. The procedures for Western blotting and immunohistochemistry were described in our previous studies (19-21). Anti-RRM2 antibody was purchased from Cell Signaling Technology (Danvers, MA, USA) and GAPDH antibody was purchased from Wako (Osaka, Japan).

Statistical analysis. We used Mann–Whitney *U* tests for comparisons between two groups and used one-way analysis of variance and Dunnett's test to compare multiple groups. JMP Pro 14 (SAS Institute Inc., Cary, NC, USA) was used to perform these analyses. All data are presented as the mean±standard error (SE). *p*-Values less than 0.05 were considered significant.

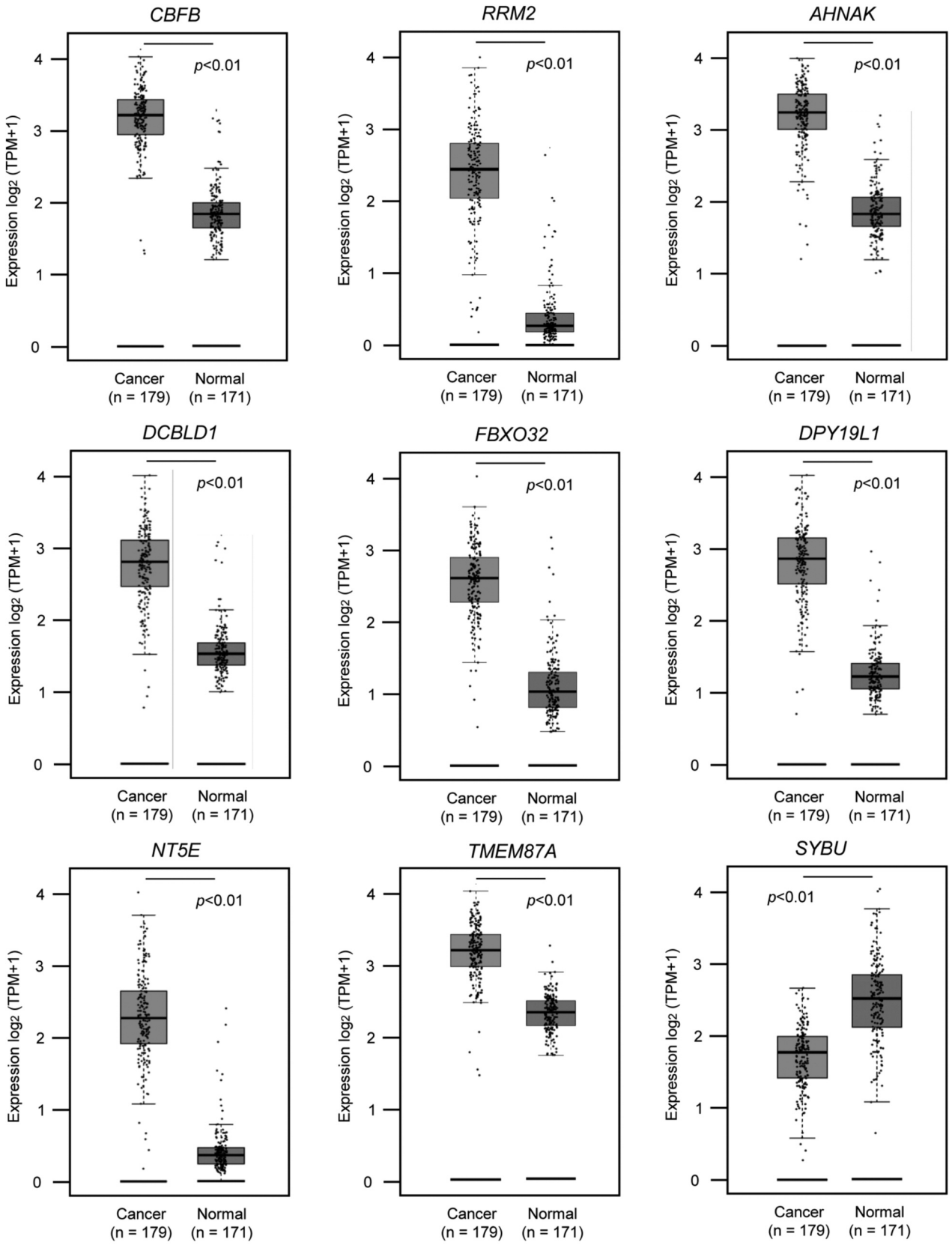

Figure 2. *Continued*

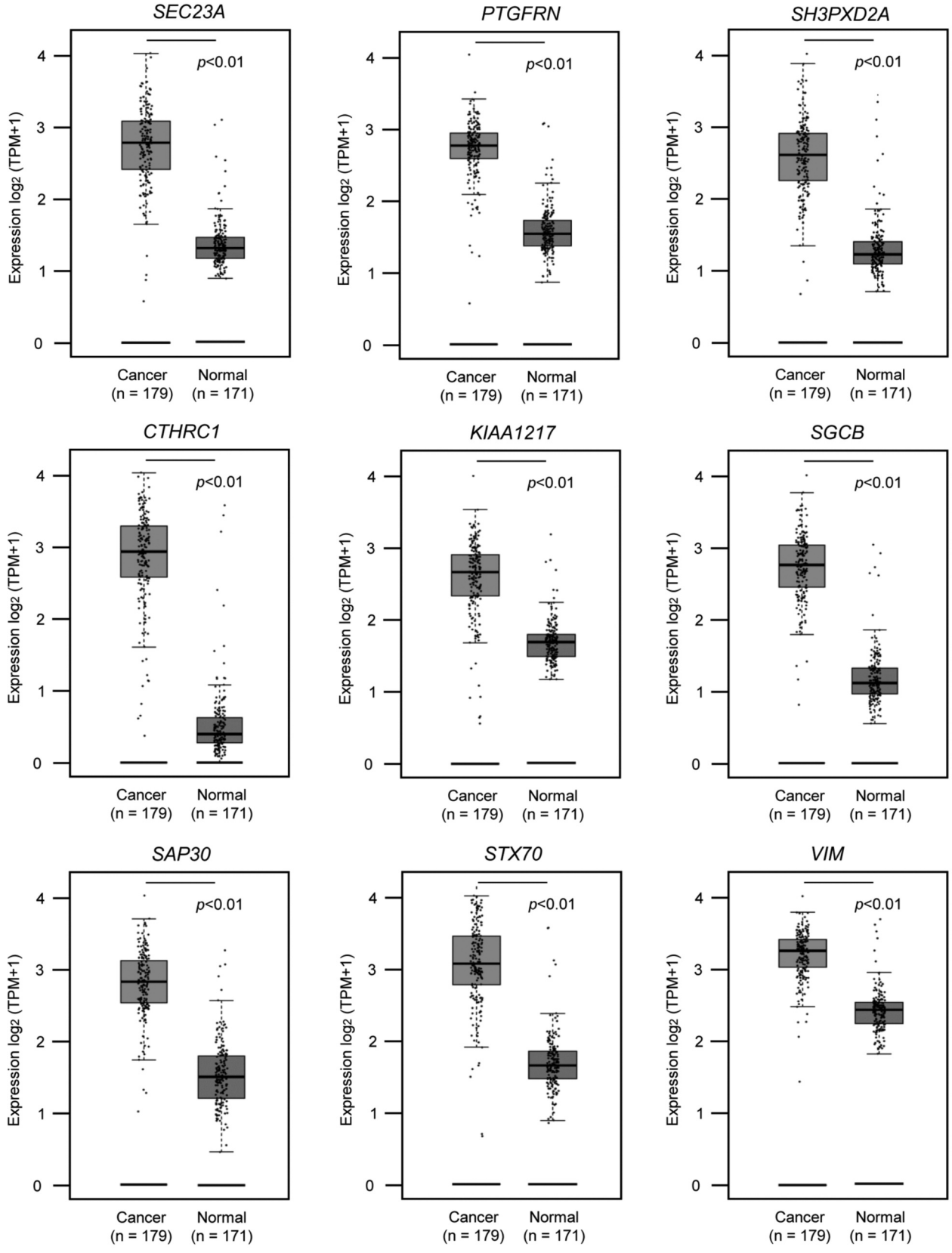

Figure 2. *Continued*

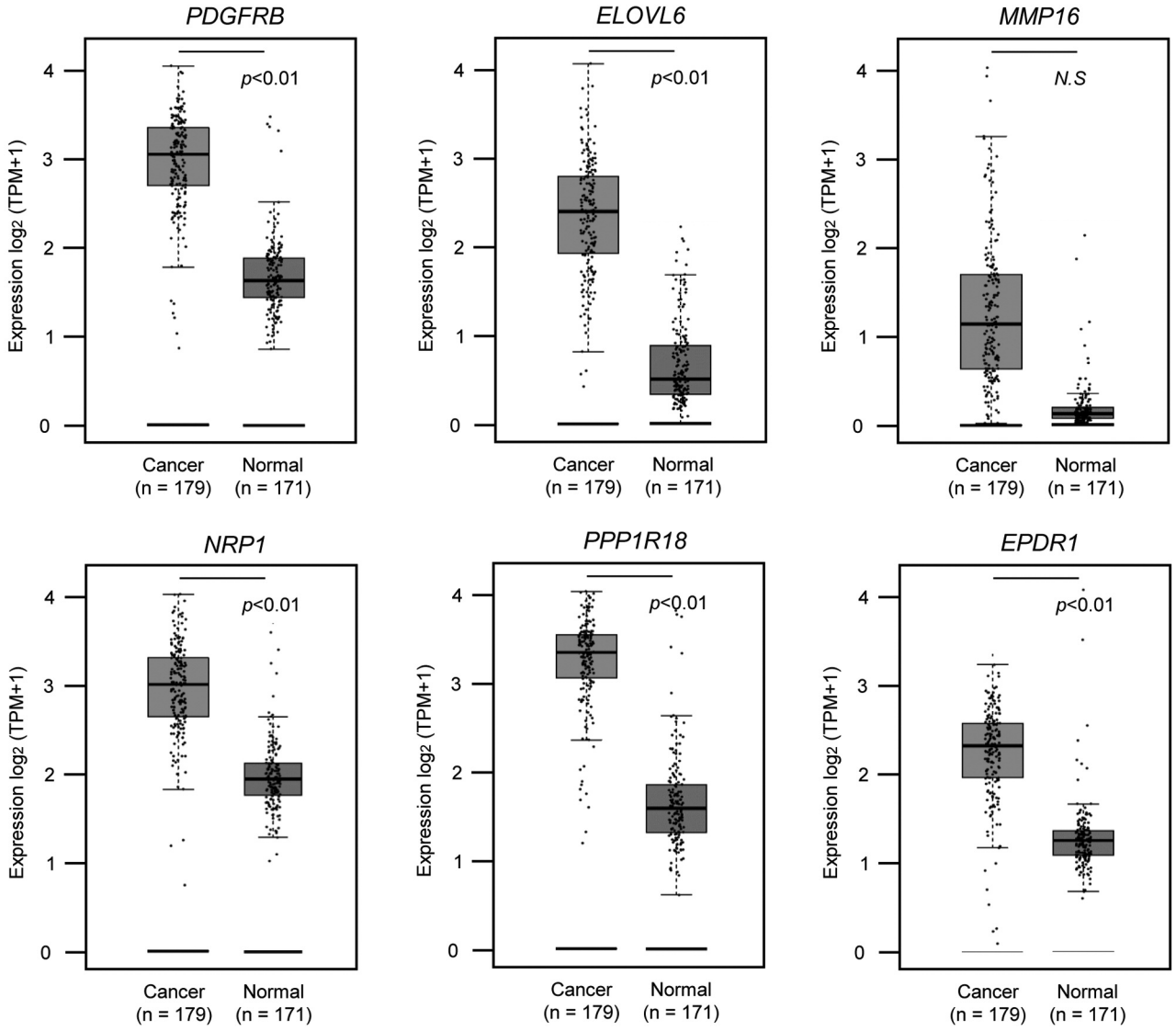

Figure 2. *Expression levels of miR-30a-5p target genes by GEPIA2 database analysis. Confirmation of expression levels of 24 target genes by miR-30a-5p regulation. Expression data of PDAC tissues (n=179) and normal pancreatic tissues (n=171) were obtained from a TCGA-PAAD cohort and analyzed by the GEPIA2 platform.*

Results

To identify the putative oncogenic targets of *miR-30a-5p* in PDAC cells, we used the TargetScanHuman database (release 7.2) and two types of genome-wide gene expression analysis data, *miR-30a-5p*-transfected into PANC-1 cells, and up-regulated genes in PDAC clinical specimens. Our strategy for identifying *miR-30a-5p* target genes is shown in Figure 1. Our strategy successfully identified a total of 24 putative oncogenic targets subject to *miR-30a-5p* regulation in PDAC cells (Table I).

We validated the expression levels of those 24 target genes using TCGA-PAAD database through the GEPIA2 platform. Expression of 22 genes was up-regulated in PDAC tissues (n=179) compared to normal tissues (n=171) (Figure 2).

To determine the clinical relevance, clinicopathological analysis of these target genes was performed using TCGA-PAAD datasets. The expression levels of four out of 24 target genes (*CBFB*, *AHNAK*, *RRM2* and *DCBLD1*) had significant impact on the prognosis of PDAC patients (Figure 3; $p<0.05$) where the higher levels of expression predicted

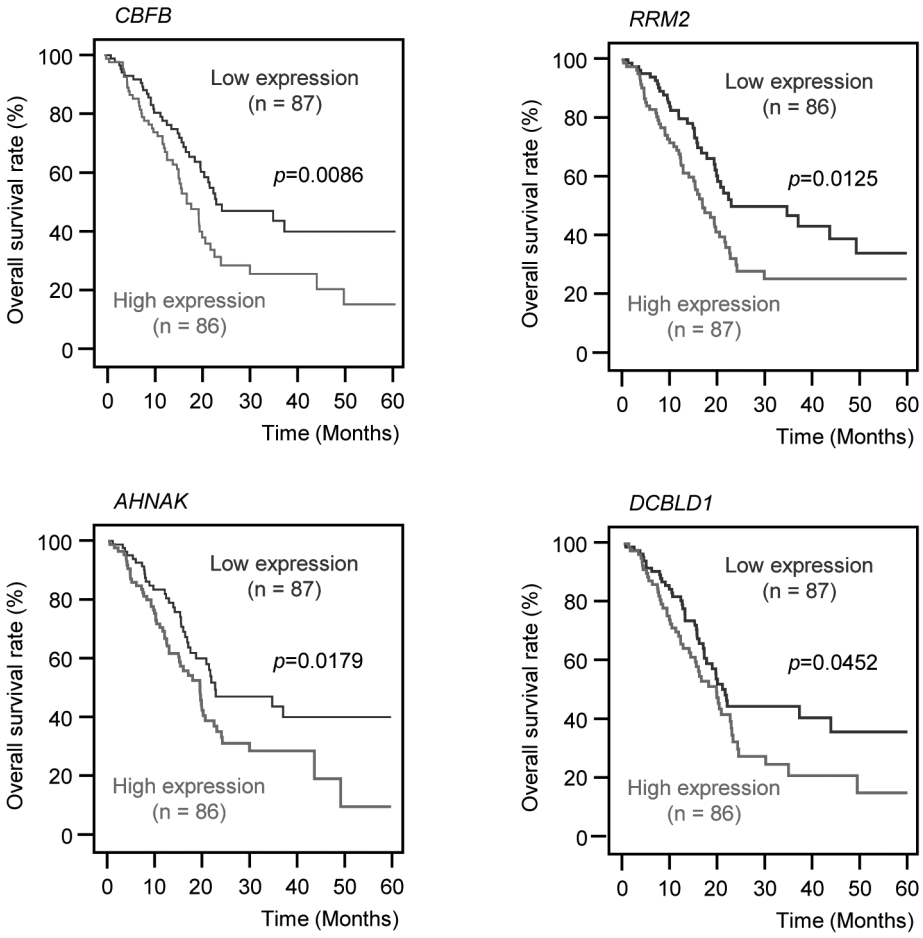

Figure 3. *Clinical significance of miR-30a-5p target genes in TCGA-PAAD database. Based on TCGA-PAAD database, we investigated whether the expression of target genes affects the prognosis of patients with pancreatic cancer. Among 24 putative target genes, high expression of 4 genes (CBFB, AHNAK, RRM2 and DCBLD1) was significantly associated with poor prognosis in patients with PDAC (p<0.05). Kaplan–Meier curves for 5-year overall survivals of 4 genes are shown. Patients were divided into high and low groups (relative to median expression) according to miRNA expression. The grey line shows the high expression group, and the black line shows the low expression group.*

poorer prognoses. Kaplan–Meier curves of the 5-year overall survival for each gene are presented (Figure 3).

Next, we performed multivariate analyses with four prognostic determinants: tumor stage, pathological stage, LN stage, and gene expression. Multivariate analysis revealed that the expression levels of three genes (*CBFB*, *AHNAK* and *RRM2*) were independent prognostic factors in PDAC ($p<0.05$; Figure 4).

In this study, we focused on *RRM2* (ribonucleotide reductase regulatory subunit M2). To investigate the functional significance of *RRM2* in PDAC cells, we asked whether the expression of *RRM2* was regulated directly by *miR-30a-5p* in PDAC cells. The expression levels of *RRM2* (both protein and mRNA expression) were significantly down-regulated following transfection with *miR-30a-5p* (Figure 5A and B, respectively).

We also performed dual-luciferase reporter assays using plasmid vectors carrying partial sequences of the *RRM2* 3'-UTR to confirm whether *miR-30a-5p* could directly bind to *RRM2* in PDAC cells. The plasmid vectors included a "wild-type" *RRM2* 3'-UTR sequence containing the predicted *miR-30a-5p* target site, and a "deletion-type" sequence lacking the target site (Figure 5C). Transfection of *miR-30a-5p* and "wild-type" *RRM2* vectors reduced the luciferase activity in the cells (Figure 5C). On the other hand, transfection with *miR-30a-5p* and the "deletion type" vector did not reduce luciferase activity (Figure 5C). Thus, we showed that *miR-30a-5p* was directly bound to the 3'-UTR region of *RRM2* and suppressed *RRM2* expression in PDAC cells.

Next, we assessed the oncogenic functions of *RRM2* in PDAC cells by performing knockdown assays using siRNAs. The efficiency with which *RRM2* expression was knocked

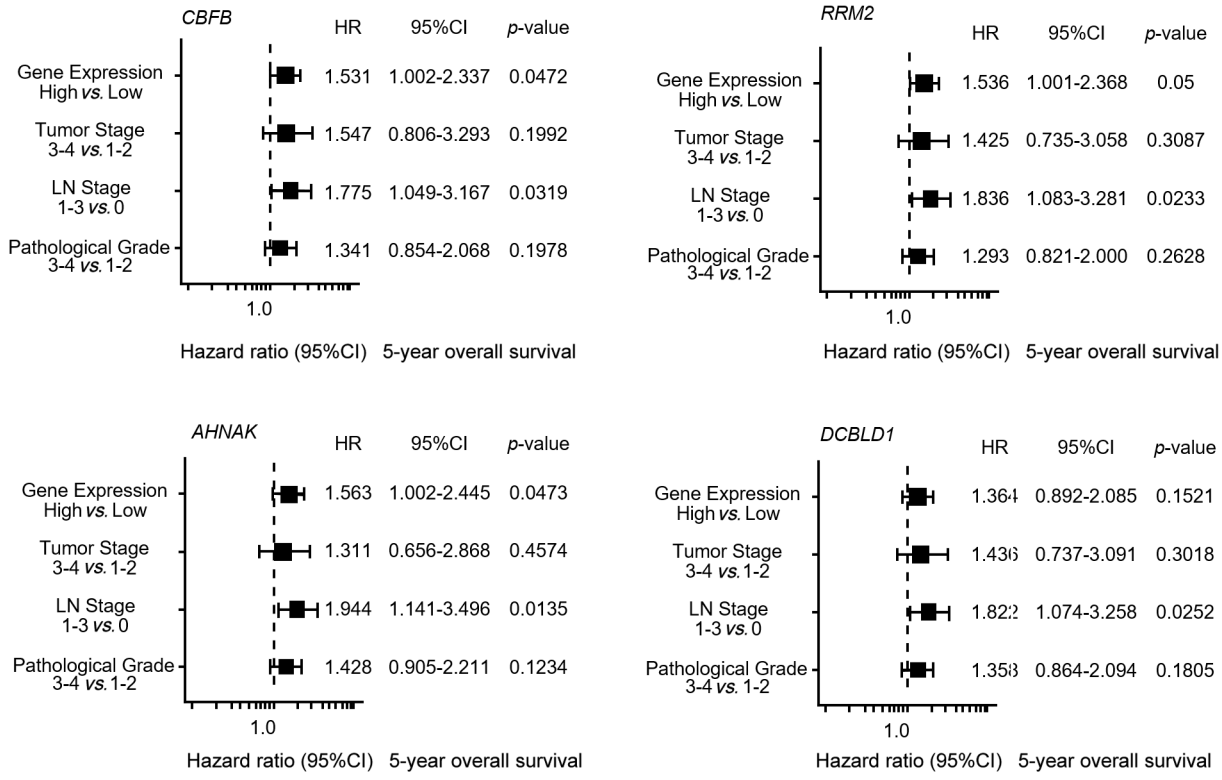

Figure 4. *Multivariate analyses of miR-30a-5p target genes (CBFB, AHNAK, RRM2 and DCBLD1). Forest plot showing multivariate analyses of 4 target genes. The multivariate analysis determined that the expression levels of 3 genes (CBFB, AHNAK, and RRM2) were independent prognostic factors for 5-year overall survival after the adjustment for tumor stage, lymph node metastasis, and pathological stage ($p<0.05$).*

down was evaluated using two types of siRNAs. It was confirmed that both siRNAs significantly reduced the expression levels of RRM2 (both protein and mRNA levels) (Figure 6A and B, respectively). Functional assays showed that siRNA-mediated knockdown of *RRM2* expression attenuated aggressive features of PDAC cells (PANC-1 and SW1990 cell lines), *e.g.*, cell proliferation, migration, and invasive abilities (Figure 6C, D and E, respectively).

We assessed the expression of the *RRM2* in clinical specimens from patients with PDAC using immunohistochemistry. *RRM2* was overexpressed in these cancer tissues as demonstrated by patchy nuclear signals compared to its noncancerous counterparts (Figure 7).

Finally, we investigated the genes differentially expressed by high and low *RRM2* expression groups in a PDAC cohort from TCGA using gene set enrichment analysis (GSEA). A total of 7 gene sets were significantly enriched (FDR *q*-value <0.05) in the high *RRM2* expression groups. The significantly enriched gene sets in the *RRM2* expression groups included "E2F targets", "G2M checkpoint", "MYC targets V2", "DNA repair", oxidative phosphorylation", "Interferon alpha response" and "MYC targets V1" (Figure 8). These results indicate that *RRM2* plays diverse roles as an oncogene in PDAC *via* the cell cycle control pathways.

Discussion

Due to the poor initial symptoms and the high malignancy of the cancer cells, patients with PDAC are in advanced stage at the time of diagnosis (1-5). Therefore, the prognosis of the patients who are not indicated for curative surgical treatment is extremely poor (1-5). The search for early diagnostic markers for PDAC is an important issue and is underway on a global scale using currently developed genomic approaches (22-26).

The current RNA sequencing technology is suitable for the construction of miRNA expression signatures. RNA sequence-based miRNA signatures provide information on dysregulated miRNAs in cancer tissues. Analysis of miRNA signatures has revealed that members of the *miR-30* family are frequently downregulated in several types of cancers, including PDAC (21, 27, 28). In the human genome, the *miR-30* family is composed of 5 species: (*miR-30a*, *miR-30b*, *miR-30c*, *miR-30d* and *miR-30e*). In addition, *miR-30c* is further subdivided into

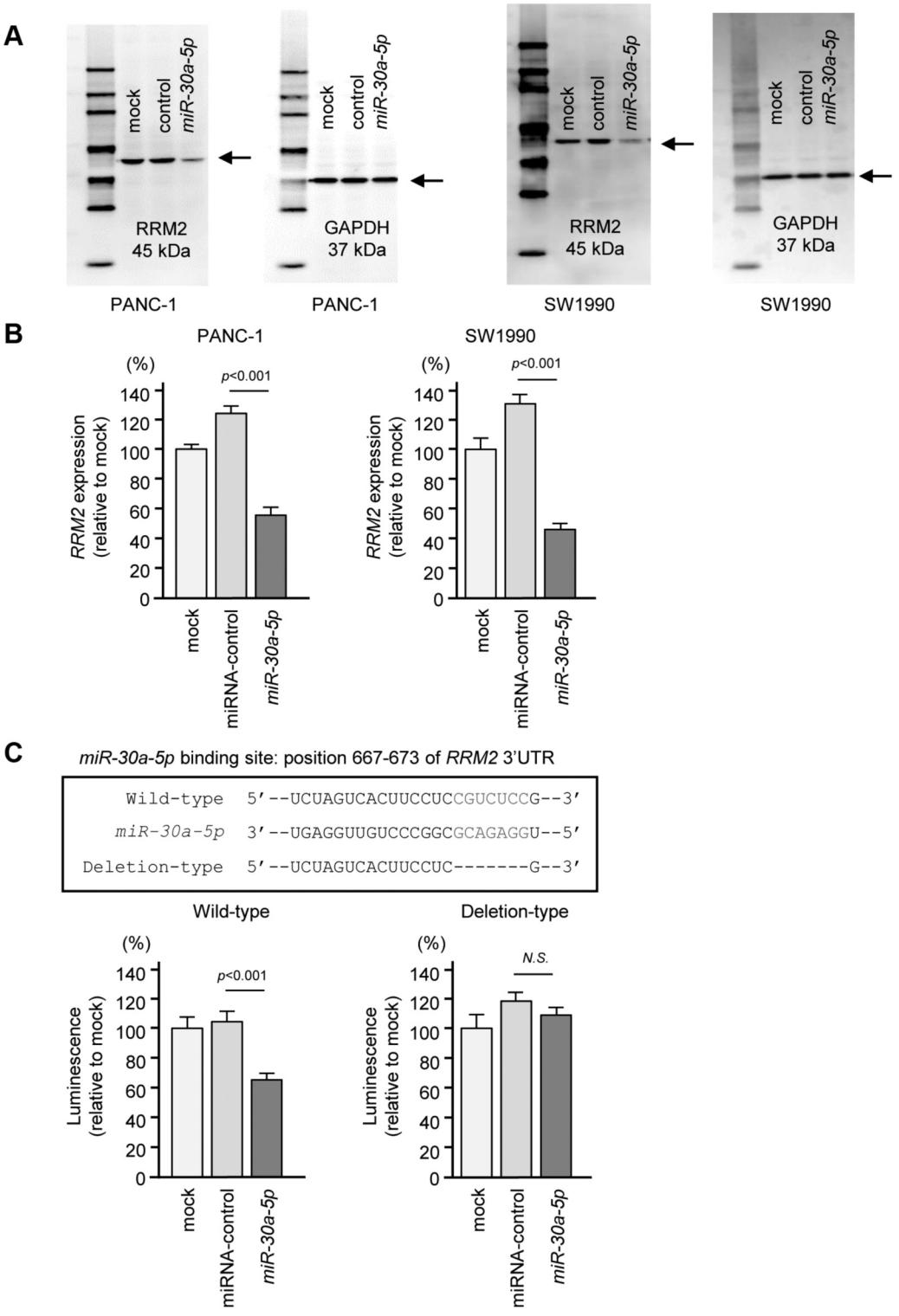

Figure 5. *Direct regulation of RRM2 by miR-30a-5p in PDAC cells. (A) Protein expression levels of RRM2 was significantly reduced by miR-30a-5p transfection into PANC-1 and SW1990 cells (72 h after transfection). Images of whole western blotting gels are shown. GAPDH was used as an internal control. (B) The mRNA expression level of RRM2 was significantly reduced by miR-30a-5p transfection (72 h after transfection). GUSB was used as an internal control. (C) TargetScan database analysis showed that one putative miR-30a-5p binding site was annotated in the 3'-UTR of the RRM2 gene. Dual-luciferase reporter assays showed that luminescence activity was reduced by co-transfection with wild-type vector (containing miR-30a-5p binding sites) and miR-30a-5p in PANC-1 cells. Normalized data were calculated as the ratios of Renilla/firefly luciferase activities.*

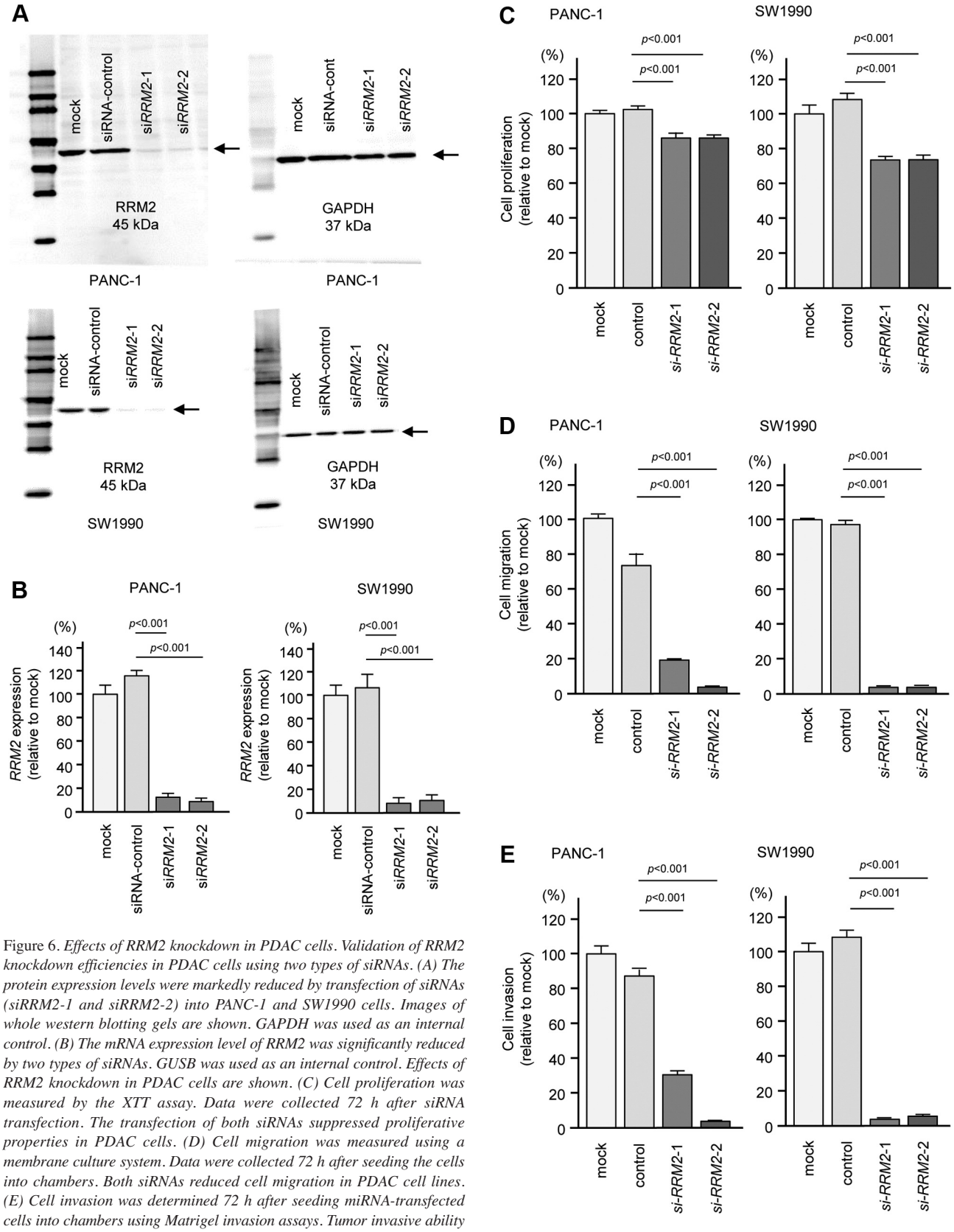

Figure 6. *Effects of RRM2 knockdown in PDAC cells. Validation of RRM2 knockdown efficiencies in PDAC cells using two types of siRNAs. (A) The protein expression levels were markedly reduced by transfection of siRNAs (siRRM2-1 and siRRM2-2) into PANC-1 and SW1990 cells. Images of whole western blotting gels are shown. GAPDH was used as an internal control. (B) The mRNA expression level of RRM2 was significantly reduced by two types of siRNAs. GUSB was used as an internal control. Effects of RRM2 knockdown in PDAC cells are shown. (C) Cell proliferation was measured by the XTT assay. Data were collected 72 h after siRNA transfection. The transfection of both siRNAs suppressed proliferative properties in PDAC cells. (D) Cell migration was measured using a membrane culture system. Data were collected 72 h after seeding the cells into chambers. Both siRNAs reduced cell migration in PDAC cell lines. (E) Cell invasion was determined 72 h after seeding miRNA-transfected cells into chambers using Matrigel invasion assays. Tumor invasive ability was suppressed upon RRM2 knockdown by siRNAs.*

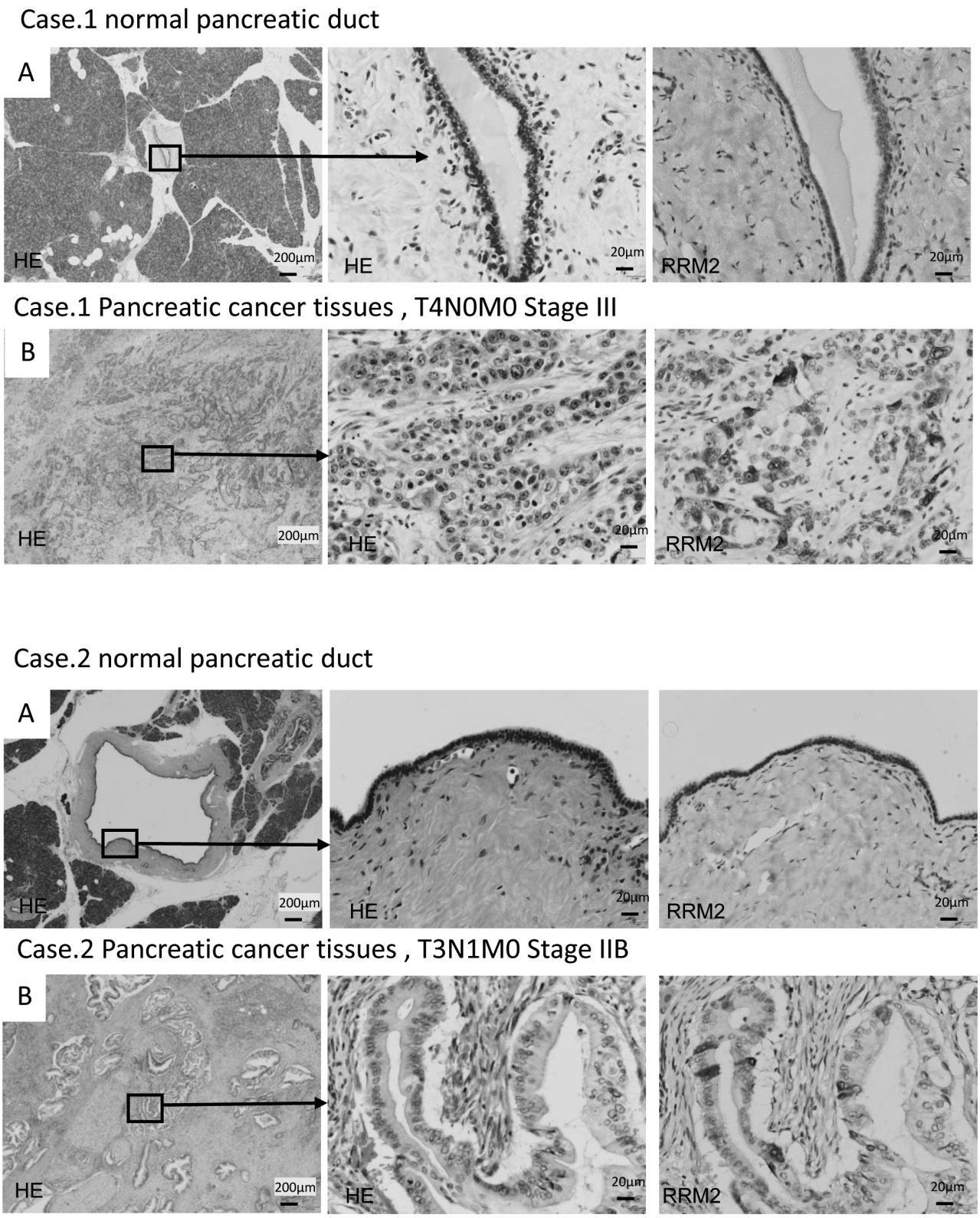

Figure 7. *Overexpression of RRM2 in PDAC clinical samples. RRM2 was overexpressed in these cancer tissues compared to their non-cancerous counterparts as demonstrated by patchy nuclear signals. The representative clinical sections of samples from case 1 (63 years female with T4N0M0 Stage III cancer in pancreas head) and case 2 (66 years female with T3N1M0 Stage IIB tumor in pancreas head).*

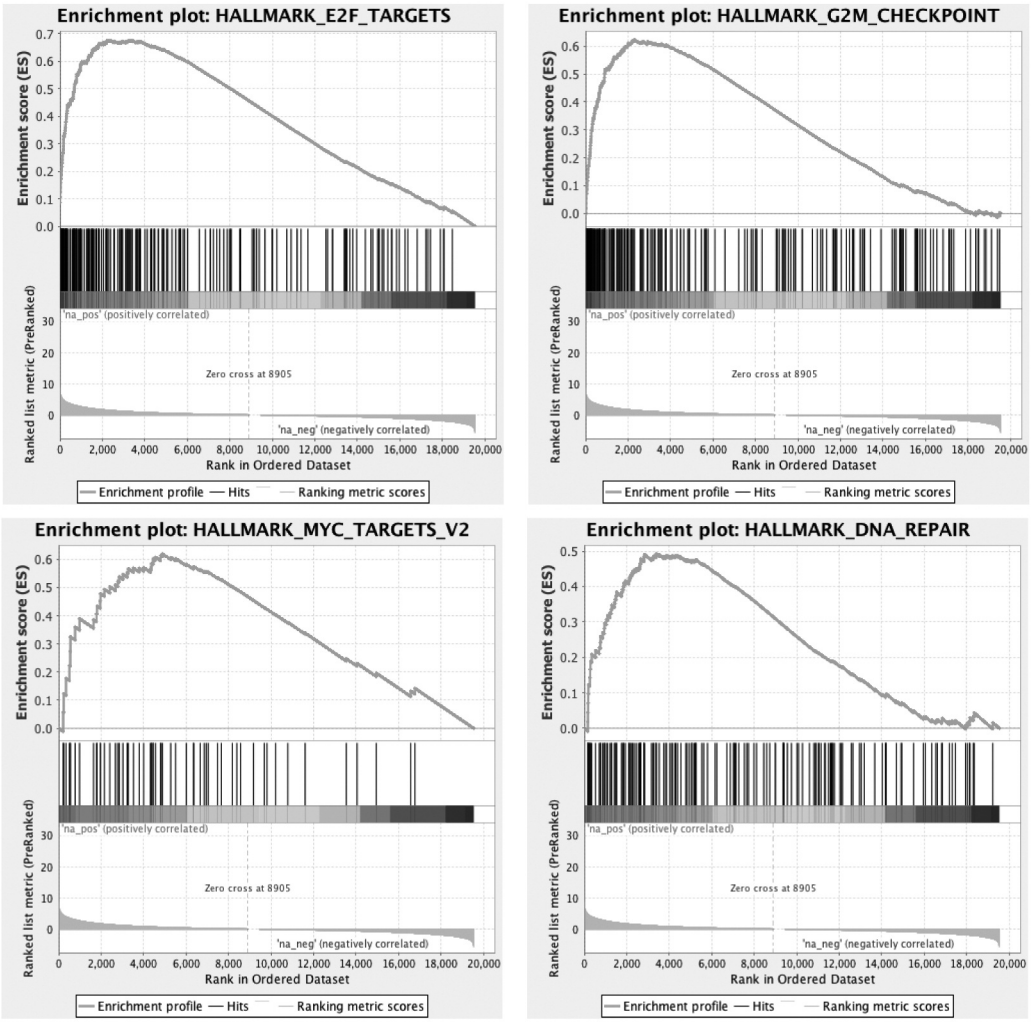

Figure 8. *Continued*

miR-30c-1 and *miR-30c-2*. The *miR-30* family is encoded by six genes located on human chromosomes 1, 6 and 8 (29). The guide strands of the *miR-30* family share the same seed sequence (GUAAACA). Several studies have shown that *miR-30a-5p* has a tumor suppressive function in PDAC cells (19, 30). For example, transfection of *miR-30a-5p* suppressed PDAC cell proliferation, cell-cycle progression and enhanced cancer cell apoptosis *via* targeting *FOXD1*. Moreover, low expression of *miR-30a-5p* is associated with poor prognosis of PDAC patients (31). Other studies showed that *miR-30a-5p* was associated with the gemcitabine response, and its expression directly targeted *SNAI1* (31, 32). Overexpression of Yin-Yang 1 (*YY1*), a zinc-finger transcription factor, has been reported in a wide range of cancers, including PDAC (33, 34). It was found that *YY1* regulated autophagy in PDAC cells, and overexpression of *miR-30a-5p* attenuated the pro-autophagic effects through direct control of *YY1* expression (35). Moreover, our recent study confirmed that both strands of miRNAs derived from pre-*miR-30a* (*miR-30a-5p* and *miR-30a-3p*) acted as tumor-suppressors in PDAC (19). Notably, several genes controlled by *miR-30a-3p* significantly predicted a poorer prognosis of patients with PDAC (19). These findings indicate that the identification of genes controlled by *miR-30a-5p* improves our understanding of the molecular pathogenesis of PDAC.

In this study, we further illuminated the oncogenic properties of *miR-30a-5p* by identifying the genes that it controls in PDAC cells. We successfully identified four genes (*CBFB*, *AHNAK*, *RRM2* and *DCBLD1*) and their significant impact on PDAC patient prognosis. All four genes were overexpressed in PDAC tissues compared to normal tissues. Importantly, upon multivariate analysis, the expression levels of *CBFB*, *AHNAK* and *RRM2* were independent prognostic factors in PDAC.

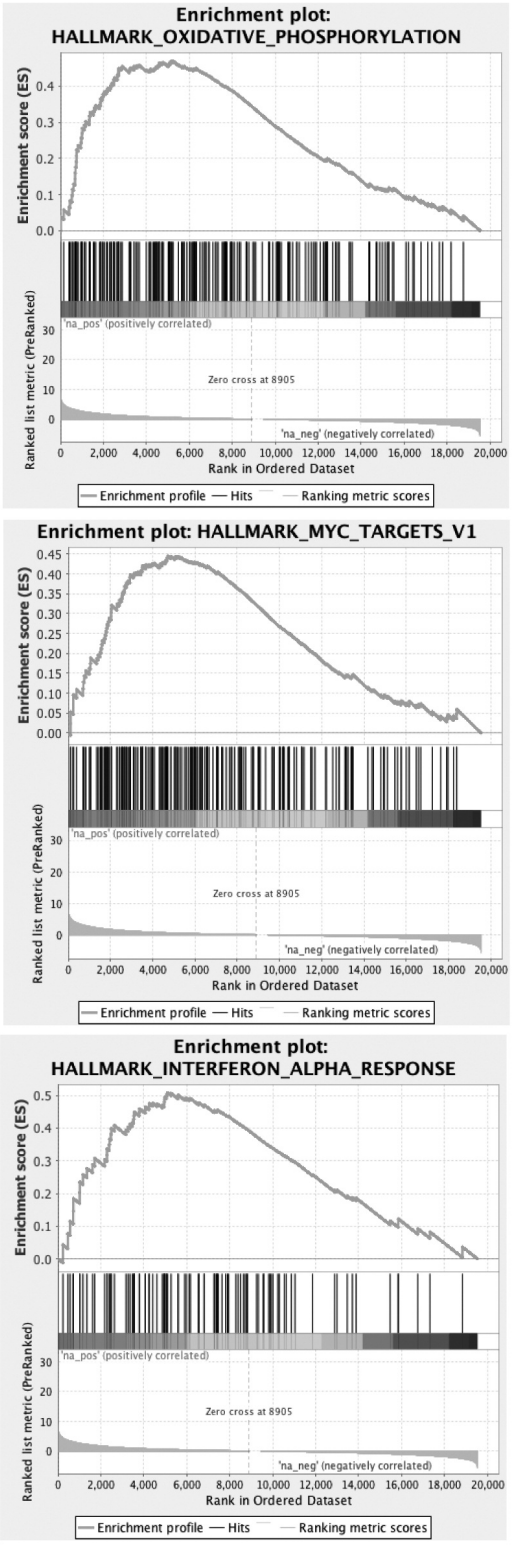

Figure 8. *RRM2-mediated pathways identified by gene set enrichment analysis. The pathways significantly enriched among the differentially expressed genes in the high RRM2 expression group compared with the low expression group according to gene set enrichment analysis.*

The four putative target genes of *miR-30a-5p* regulation (*CBFB*, *AHNAK*, *RRM2* and *DCBLD1*) are reported to have significant roles in cancer. Three of those genes are discussed below. *CBFB* encodes the beta-subunit of Runt domain transcription factor, and forms heterodimers with RUNX proteins (RUNX1, RUNX2 and RUNX3), thereby regulating transcriptional activity (36). The inter-play between CBFB and RUNX proteins has also been described in gastric, hepatocellular and breast carcinomas (37-39). Overexpression of RUNX1 and RUNX2 proteins and their roles in tumor progression has been described in PDAC (36, 40). A previous study showed that silencing of *RUNX2* enhanced gemcitabine sensitivity in PDAC cells through stimulation of *TP53*, *Tap63* and *Tap73*-mediated pathways (36). Another study demonstrated that knockdown of *RUNX1* reduced the invasive ability of PDAC cells (40). Notably, *RUNX1* bound promoter region of *miR-93* and negatively regulated expression of *miR-93* (40). Overexpression of *miR-93* blocked migration and invasiveness abilities of PDAC cells (40).

Likewise, *AHNAK2* is highly expressed in PDAC clinical tissues, and its expression significantly predicted a lower overall survival rate of patients (41). Meta-analysis of PDAC transcriptome data showed that a five-gene classifier (*TMPRSS4*, *AHNAK2*, *POSTN*, *ECT2*, and *SERPINB5*) discriminated PDAC and early precursor lesions from non-malignant tissue (*42*). The function of *AHNAK2* in PDAC is not well understood. Knockdown assay of *AHNAK2* in thyroid carcinoma cells showed that expression of *AHNAK2* contributed to cancer cell metastasis and EMT-process through the Wnt/β-catenin pathway (43). In lung adenocarcinoma cells, overexpression of *AHNAK2* enhanced malignant transformation (*e.g.*, migration, invasion, and EMT) *via* the TGF-β/Smad3 pathway (44).

The DCBLD receptor family consists of two paralogous, DCBLD1 and DCBLD2, amino acid sequences are highly conserved across vertebrates (45). To date, the function of the *DCBLD2* and its relationship with diseases are becoming clear. However, the function of the *DCBLD1* remains uncharacterized (45). A previous study showed that single nucleotide polymorphisms of promoter region of *DCBLD1* was associated with head and neck cancer and lung cancer in never-smoking women (46-49). Analysis of TCGA analysis demonstrated that expression of *DCBLD1* was associated with worse prognosis in the patients with non-small cell lung cancer and invasive breast cancer (50). Notably, high expression of *DCBLD1* was associated with the integrin signaling pathway (50). Expression of these genes is promising as a prognostic marker for patients with PDAC. Furthermore, detailed functional analysis of these genes will lead to the elucidation of the molecular mechanism of malignant transformation of PDAC cells.

In this study, we analyzed the oncogenic roles of *RRM2* in PDAC cells. The RRM2 protein is one of the two subunits of the ribonucleotide reductase complex. This reductase catalyzes the formation of deoxyribonucleotides from ribonucleotides and is thus a key enzyme in DNA synthesis (51). The overexpression and oncogenic roles of *RRM2* have been reported in a wide range of cancers, and high expression of *RRM2* independently predicted the prognosis of several cancers, *e.g.*, lung adenocarcinoma, oral cancer, breast cancer and prostate cancer (51, 52). In pancreatic adenocarcinoma, as in other solid tumors, *RRM2* affects tumor growth and invasiveness. siRNA mediated knockdown of RRM2 effectively suppresses pancreatic tumor growth *in vivo* and *in vitro* (53, 54). Notably, aberrant expression of *RRM2* contributes to chemotherapy-resistance in several types of cancers, including treatment with hydroxyurea, docetaxel, tamoxifen and gemcitabine (55). Previous studies showed that overexpression of *RRM2* induced gemcitabine-resistance in PDAC cells (56). Knockdown of *RRM2* and gemcitabine treatment synergistically inhibited PDAC cell growth and metastasis (57). Furthermore, the sensitivity to gemcitabine treatment can be predicted by measuring RRM2 expression in patients with PDAC and non-small cell lung cancer (58).

In conclusion, we successfully identified a total of 24 oncogenic targets regulated by tumor-suppressive *miR-30a-5p* in PDAC cells. Among these targets, the expression levels of 4 genes (*CBFB*, *RRM2*, *AHNAK*, and *DCBLD1*) significantly predicted shorter survival of PDAC patients. Furthermore, the oncogenic function of *RRM2* in PDAC cells was confirmed, indicating that the *miR-30a-5p*/*RRM2* axis plays a pivotal role in PDAC oncogenesis. Identification of novel tumor-suppressive miRNAs and their regulated oncogenic targets should improve our knowledge of the molecular pathogenesis of PDAC.

Conflicts of Interest

The Authors declare no conflicts of interest.

Authors' Contributions

Conceptualization, N.S.; methodology, N.S.; validation, T.I. and T.A.; formal analysis, Y.H., T.T., N.P., and M.W.; investigation, N.P., Y.H., T.T., M.W., S.A. and C.M.; data curation, T.I., H.K., and T.O.; writing—original draft preparation, N.S. and N.P.; writing—review and editing, H.K., and T.O.; supervision, N.S and T.O.; project administration, N.S.; funding acquisition, M.W., T.I., H.K., N.S., and T.O. All Authors have read and agreed to the published version of the manuscript.

Acknowledgements

This study was supported by JSPS KAKENHI grant numbers, 19K09077, 20K21633, 20H03753, 21K15597, 21K16426, and 21K09577.

References

1 Siegel RL, Miller KD and Jemal A: Cancer statistics, 2019. CA Cancer J Clin *69(1)*: 7-34, 2019. PMID: 30620402. DOI: 10.3322/caac.21551

2 Rawla P, Sunkara T and Gaduputi V: Epidemiology of pancreatic cancer: global trends, etiology and risk factors. World J Oncol *10(1)*: 10-27, 2019. PMID: 30834048. DOI: 10.14740/wjon1166

3 Bray F, Ferlay J, Soerjomataram I, Siegel RL, Torre LA and Jemal A: Global cancer statistics 2018: GLOBOCAN estimates of incidence and mortality worldwide for 36 cancers in 185 countries. CA Cancer J Clin *68(6)*: 394-424, 2018. PMID: 30207593. DOI: 10.3322/caac.21492

4 Vincent A, Herman J, Schulick R, Hruban RH and Goggins M: Pancreatic cancer. Lancet *378(9791)*: 607-620, 2011. PMID: 21620466. DOI: 10.1016/S0140-6736(10)62307-0

5 Mizrahi JD, Surana R, Valle JW and Shroff RT: Pancreatic cancer. Lancet *395(10242)*: 2008-2020, 2020. PMID: 32593337. DOI: 10.1016/S0140-6736(20)30974-0

6 Guo M, Peng Y, Gao A, Du C and Herman JG: Epigenetic heterogeneity in cancer. Biomark Res *7*: 23, 2019. PMID: 31695915. DOI: 10.1186/s40364-019-0174-y

7 Sever R and Brugge JS: Signal transduction in cancer. Cold Spring Harb Perspect Med *5(4)*: , 2015. PMID: 25833940. DOI: 10.1101/cshperspect.a006098

8 Stratton MR, Campbell PJ and Futreal PA: The cancer genome. Nature *458(7239)*: 719-724, 2009. PMID: 19360079. DOI: 10.1038/nature07943

9 Prior IA, Lewis PD and Mattos C: A comprehensive survey of Ras mutations in cancer. Cancer Res *72(10)*: 2457-2467, 2012. PMID: 22589270. DOI: 10.1158/0008-5472.CAN-11-2612

10 Waters AM and Der CJ: KRAS: The critical driver and therapeutic target for pancreatic cancer. Cold Spring Harb Perspect Med *8(9)*: a031435, 2018. PMID: 29229669. DOI: 10.1101/cshperspect.a031435

11 Liu P, Wang Y and Li X: Targeting the untargetable KRAS in cancer therapy. Acta Pharm Sin B *9(5)*: 871-879, 2019. PMID: 31649840. DOI: 10.1016/j.apsb.2019.03.002

12 Salgia R, Pharaon R, Mambetsariev I, Nam A and Sattler M: The improbable targeted therapy: KRAS as an emerging target in non-small cell lung cancer (NSCLC). Cell Rep Med *2(1)*: 100186, 2021. PMID: 33521700. DOI: 10.1016/j.xcrm.2020.100186

13 Anastasiadou E, Jacob LS and Slack FJ: Non-coding RNA networks in cancer. Nat Rev Cancer *18(1)*: 5-18, 2018. PMID: 29170536. DOI: 10.1038/nrc.2017.99

14 Zaravinos A: The regulatory role of microRNAs in EMT and cancer. J Oncol *2015*: 865816, 2015. PMID: 25883654. DOI: 10.1155/2015/865816

15 Zhang P, Wu W, Chen Q and Chen M: Non-coding RNAs and their integrated networks. J Integr Bioinform *16(3)*: 20190027, 2019. PMID: 31301674. DOI: 10.1515/jib-2019-0027

16 Corrà F, Agnoletto C, Minotti L, Baldassari F and Volinia S: The network of non-coding RNAs in cancer drug resistance. Front Oncol *8*: 327, 2018. PMID: 30211115. DOI: 10.3389/fonc.2018.00327

17 Bartel DP: MicroRNAs: target recognition and regulatory functions. Cell *136(2)*: 215-233, 2009. PMID: 19167326. DOI: 10.1016/j.cell.2009.01.002

18 Chen CZ: MicroRNAs as oncogenes and tumor suppressors. N Engl J Med *353(17)*: 1768-1771, 2005. PMID: 16251533. DOI: 10.1056/NEJMp058190

19 Shimomura H, Okada R, Tanaka T, Hozaka Y, Wada M, Moriya S, Idichi T, Kita Y, Kurahara H, Ohtsuka T and Seki N: Role of miR-30a-3p regulation of oncogenic targets in pancreatic ductal adenocarcinoma pathogenesis. Int J Mol Sci *21(18)*: 6459, 2020. PMID: 32899691. DOI: 10.3390/ijms21186459

20 Hozaka Y, Seki N, Tanaka T, Asai S, Moriya S, Idichi T, Wada M, Tanoue K, Kawasaki Y, Mataki Y, Kurahara H and Ohtsuka T: Molecular pathogenesis and regulation of the *miR-29-3p*-Family: Involvement of *ITGA6* and *ITGB1* in intra-hepatic cholangiocarcinoma. Cancers (Basel) *13(11)*: 2804, 2021. PMID: 34199886. DOI: 10.3390/cancers13112804

21 Tanaka T, Okada R, Hozaka Y, Wada M, Moriya S, Satake S, Idichi T, Kurahara H, Ohtsuka T and Seki N: Molecular pathogenesis of pancreatic ductal adenocarcinoma: Impact of *miR-30c-5p* and *miR-30c-2-3p* regulation on oncogenic genes. Cancers (Basel) *12(10)*: 2731, 2020. PMID: 32977589. DOI: 10.3390/cancers12102731

22 van Huijgevoort NCM, Del Chiaro M, Wolfgang CL, van Hooft JE and Besselink MG: Diagnosis and management of pancreatic cystic neoplasms: current evidence and guidelines. Nat Rev Gastroenterol Hepatol *16(11)*: 676-689, 2019. PMID: 31527862. DOI: 10.1038/s41575-019-0195-x

23 Yang J, Xu R, Wang C, Qiu J, Ren B and You L: Early screening and diagnosis strategies of pancreatic cancer: a comprehensive review. Cancer Commun (Lond), 2021. PMID: 34331845. DOI: 10.1002/cac2.12204

24 O'Neill RS and Stoita A: Biomarkers in the diagnosis of pancreatic cancer: Are we closer to finding the golden ticket? World J Gastroenterol *27(26)*: 4045-4087, 2021. PMID: 34326612. DOI: 10.3748/wjg.v27.i26.4045

25 Kitamura F, Miyata T, Uemura N, Uchihara T, Imai K, Hayashi H, Yamashita YI, Matsusaki K, Ishimoto T and Baba H: Proteomic analysis of malignant ascites from patients with pancreatic ductal adenocarcinoma. Anticancer Res *41(6)*: 2895-2900, 2021. PMID: 34083280. DOI: 10.21873/anticanres.15071

26 Uchinaka EI, Sakabe T, Hanaki T, Tokuyasu N, Sakamoto T, Honjo S, Fujiwara Y and Umekita Y: Cytoplasmic-only expression of maspin predicts unfavorable prognosis in patients with pancreatic ductal adenocarcinoma. Anticancer Res *41(5)*: 2543-2552, 2021. PMID: 33952482. DOI: 10.21873/anticanres.15032

27 Song K, Jiang Y, Zhao Y, Xie Y, Zhou J, Yu W and Wang Q: Members of the miR-30 family inhibit the epithelial-to-mesenchymal transition of non-small-cell lung cancer cells by suppressing XB130 expression levels. Oncol Lett *20(4)*: 68, 2020. PMID: 32863901. DOI: 10.3892/ol.2020.11929

28 Tsukasa K, Ding Q, Miyazaki Y, Matsubara S, Natsugoe S and Takao S: miR-30 family promotes migratory and invasive abilities in CD133(+) pancreatic cancer stem-like cells. Hum Cell *29(3)*: 130-137, 2016. PMID: 26965588. DOI: 10.1007/s13577-016-0137-7

29 Mao L, Liu S, Hu L, Jia L, Wang H, Guo M, Chen C, Liu Y and Xu L: miR-30 Family: A promising regulator in development and disease. Biomed Res Int *2018*: 9623412, 2018. PMID: 30003109. DOI: 10.1155/2018/9623412

30 Zhou L, Jia S, Chen Y, Wang W, Wu Z, Yu W, Zhang M, Ding G and Cao L: The distinct role of CD73 in the progression of pancreatic cancer. J Mol Med (Berl) *97(6)*: 803-815, 2019. PMID: 30927045. DOI: 10.1007/s00109-018-01742-0

31 Zhou L, Jia S, Ding G, Zhang M, Yu W, Wu Z and Cao L: Down-regulation of miR-30a-5p is associated with poor prognosis and promotes chemoresistance of gemcitabine in pancreatic ductal adenocarcinoma. J Cancer *10(21)*: 5031-5040, 2019. PMID: 31602254. DOI: 10.7150/jca.31191

32 Zhang L, Wang Y, Li W, Tsonis PA, Li Z, Xie L and Huang Y: MicroRNA-30a regulation of epithelial-mesenchymal transition in diabetic cataracts through targeting SNAI1. Sci Rep *7(1)*: 1117, 2017. PMID: 28442786. DOI: 10.1038/s41598-017-01320-3

33 Khachigian LM: The Yin and Yang of YY1 in tumor growth and suppression. Int J Cancer *143(3)*: 460-465, 2018. PMID: 29322514. DOI: 10.1002/ijc.31255

34 Chen Q, Zhang JJ, Ge WL, Chen L, Yuan H, Meng LD, Huang XM, Shen P, Miao Y and Jiang KR: YY1 inhibits the migration and invasion of pancreatic ductal adenocarcinoma by downregulating the FER/STAT3/MMP2 signaling pathway. Cancer Lett *463*: 37-49, 2019. PMID: 31404611. DOI: 10.1016/j.canlet.2019.07.019

35 Yang C, Zhang JJ, Peng YP, Zhu Y, Yin LD, Wei JS, Gao WT, Jiang KR and Miao Y: A Yin-Yang 1/miR-30a regulatory circuit modulates autophagy in pancreatic cancer cells. J Transl Med *15(1)*: 211, 2017. PMID: 29052509. DOI: 10.1186/s12967-017-1308-3

36 Ozaki T, Yu M, Yin D, Sun D, Zhu Y, Bu Y and Sang M: Impact of RUNX2 on drug-resistant human pancreatic cancer cells with p53 mutations. BMC Cancer *18(1)*: 309, 2018. PMID: 29558908. DOI: 10.1186/s12885-018-4217-9

37 Sakakura C, Hagiwara A, Miyagawa K, Nakashima S, Yoshikawa T, Kin S, Nakase Y, Ito K, Yamagishi H, Yazumi S, Chiba T and Ito Y: Frequent downregulation of the runt domain transcription factors RUNX1, RUNX3 and their cofactor CBFB in gastric cancer. Int J Cancer *113(2)*: 221-228, 2005. PMID: 15386419. DOI: 10.1002/ijc.20551

38 Miyagawa K, Sakakura C, Nakashima S, Yoshikawa T, Kin S, Nakase Y, Ito K, Yamagishi H, Ida H, Yazumi S, Chiba T, Ito Y and Hagiwara A: Down-regulation of RUNX1, RUNX3 and CBFbeta in hepatocellular carcinomas in an early stage of hepatocarcinogenesis. Anticancer Res *26(5B)*: 3633-3643, 2006. PMID: 17094378.

39 Malik N, Yan H, Moshkovich N, Palangat M, Yang H, Sanchez V, Cai Z, Peat TJ, Jiang S, Liu C, Lee M, Mock BA, Yuspa SH, Larson D, Wakefield LM and Huang J: The transcription factor CBFB suppresses breast cancer through orchestrating translation and transcription. Nat Commun *10(1)*: 2071, 2019. PMID: 31061501. DOI: 10.1038/s41467-019-10102-6

40 Cheng Y, Yang H, Sun Y, Zhang H, Yu S, Lu Z and Chen J: RUNX1 promote invasiveness in pancreatic ductal adenocarcinoma through regulating miR-93. Oncotarget *8(59)*: 99567-99579, 2017. PMID: 29245924. DOI: 10.18632/oncotarget.20433

41 Lu D, Wang J, Shi X, Yue B and Hao J: AHNAK2 is a potential prognostic biomarker in patients with PDAC. Oncotarget *8(19)*: 31775-31784, 2017. PMID: 28423668. DOI: 10.18632/oncotarget.15990

42 Bhasin MK, Ndebele K, Bucur O, Yee EU, Otu HH, Plati J, Bullock A, Gu X, Castan E, Zhang P, Najarian R, Muraru MS, Miksad R, Khosravi-Far R and Libermann TA: Meta-analysis of transcriptome data identifies a novel 5-gene pancreatic adenocarcinoma classifier. Oncotarget *7(17)*: 23263-23281, 2016. PMID: 26993610. DOI: 10.18632/oncotarget.8139

43 Lin QY, Qi QL, Hou S, Chen Z, Jiang N, Zhang L and Lin CH: Silencing of AHNAK2 restricts thyroid carcinoma progression by inhibiting the Wnt/β-catenin pathway. Neoplasma, 2021. PMID: 34374294. DOI: 10.4149/neo_2021_210304N276

44 Liu G, Guo Z, Zhang Q, Liu Z and Zhu D: AHNAK2 promotes migration, invasion, and epithelial-mesenchymal transition in lung adenocarcinoma cells via the TGF-β/Smad3 pathway. Onco Targets Ther *13*: 12893-12903, 2020. PMID: 33363388. DOI: 10.2147/OTT.S281517

45 Schmoker AM, Ebert AM and Ballif BA: The DCBLD receptor family: emerging signaling roles in development, homeostasis and disease. Biochem J *476(6)*: 931-950, 2019. PMID: 30902898. DOI: 10.1042/BCJ20190022

46 Lan Q, Hsiung CA, Matsuo K, Hong YC, Seow A, Wang Z, Hosgood HD 3rd, Chen K, Wang JC, Chatterjee N, Hu W, Wong MP, Zheng W, Caporaso N, Park JY, Chen CJ, Kim YH, Kim YT, Landi MT, Shen H, Lawrence C, Burdett L, Yeager M, Yuenger J, Jacobs KB, Chang IS, Mitsudomi T, Kim HN, Chang GC, Bassig BA, Tucker M, Wei F, Yin Z, Wu C, An SJ, Qian B, Lee VH, Lu D, Liu J, Jeon HS, Hsiao CF, Sung JS, Kim JH, Gao YT, Tsai YH, Jung YJ, Guo H, Hu Z, Hutchinson A, Wang WC, Klein R, Chung CC, Oh IJ, Chen KY, Berndt SI, He X, Wu W, Chang J, Zhang XC, Huang MS, Zheng H, Wang J, Zhao X, Li Y, Choi JE, Su WC, Park KH, Sung SW, Shu XO, Chen YM, Liu L, Kang CH, Hu L, Chen CH, Pao W, Kim YC, Yang TY, Xu J, Guan P, Tan W, Su J, Wang CL, Li H, Sihoe AD, Zhao Z, Chen Y, Choi YY, Hung JY, Kim JS, Yoon HI, Cai Q, Lin CC, Park IK, Xu P, Dong J, Kim C, He Q, Perng RP, Kohno T, Kweon SS, Chen CY, Vermeulen R, Wu J, Lim WY, Chen KC, Chow WH, Ji BT, Chan JK, Chu M, Li YJ, Yokota J, Li J, Chen H, Xiang YB, Yu CJ, Kunitoh H, Wu G, Jin L, Lo YL, Shiraishi K, Chen YH, Lin HC, Wu T, Wu YL, Yang PC, Zhou B, Shin MH, Fraumeni JF Jr, Lin D, Chanock SJ and Rothman N: Genome-wide association analysis identifies new lung cancer susceptibility loci in never-smoking women in Asia. Nat Genet *44(12)*: 1330-1335, 2012. PMID: 23143601. DOI: 10.1038/ng.2456

47 Cardin GB, Bernard M, Bahig H, Nguyen-Tan PF, Ballivy O, Filion E, Soulieres D, Philouze P, Ayad T, Guertin L, Bissada E, Rodier F and Christopoulos A: Single nucleotide polymorphism rs6942067 is a risk factor in young and in non-smoking patients with HPV negative head and neck squamous cell carcinoma. Cancers (Basel) *12(1)*: 55, 2019. PMID: 31878157. DOI: 10.3390/cancers12010055

48 Hung RJ, Spitz MR, Houlston RS, Schwartz AG, Field JK, Ying J, Li Y, Han Y, Ji X, Chen W, Wu X, Gorlov IP, Na J, de Andrade M, Liu G, Brhane Y, Diao N, Wenzlaff A, Davies MPA, Liloglou T, Timofeeva M, Muley T, Rennert H, Saliba W, Ryan BM, Bowman E, Barros-Dios JM, Pérez-Ríos M, Morgenstern H, Zienolddiny S, Skaug V, Ugolini D, Bonassi S, van der Heijden EHFM, Tardon A, Bojesen SE, Landi MT, Johansson M, Bickeböller H, Arnold S, Le Marchand L, Melander O, Andrew A, Grankvist K, Caporaso N, Teare MD, Schabath MB, Aldrich MC, Kiemeney LA, Wichmann HE, Lazarus P, Mayordomo J, Neri M, Haugen A, Zhang ZF, Ruano-Raviña A, Brenner H, Harris CC, Orlow I, Rennert G, Risch A, Brennan P, Christiani DC, Amos CI, Yang P and Gorlova OY: Lung cancer risk in never-smokers of European descent is associated with genetic variation in the 5_p15.33 TERT-CLPTM1Ll region. J Thorac Oncol *14(8)*: 1360-1369, 2019. PMID: 31009812. DOI: 10.1016/j.jtho.2019.04.008

49 Yoo SS, Kang HG, Choi JE, Do SK, Lee WK, Choi SH, Lee SY, Lee SY, Lee J, Cha SI, Kim CH, Seok Y, Lee E, Kim MS, Lee JM, Cho HJ, Oh IJ, Kim YC, Cho S, Jheon S, Jung CY, Kim MH, Lee MK and Park JY: Effects of polymorphisms identified in genome-wide association studies of never-smoking females on the prognosis of non-small cell lung cancer. Cancer Genet *212-213*: 8-12, 2017. PMID: 28449811. DOI: 10.1016/j.cancergen.2017.03.003

50 Cardin GB, Bernard M, Rodier F and Christopoulos A: DCBLD1 is associated with the integrin signaling pathway and has prognostic value in non-small cell lung and invasive breast carcinoma. Sci Rep *11(1)*: 12753, 2021. PMID: 34140574. DOI: 10.1038/s41598-021-92090-6

51 Morikawa T, Maeda D, Kume H, Homma Y and Fukayama M: Ribonucleotide reductase M2 subunit is a novel diagnostic marker and a potential therapeutic target in bladder cancer. Histopathology *57(6)*: 885-892, 2010. PMID: 21166702. DOI: 10.1111/j.1365-2559.2010.03725.x

52 Aye Y, Li M, Long MJ and Weiss RS: Ribonucleotide reductase and cancer: biological mechanisms and targeted therapies. Oncogene *34(16)*: 2011-2021, 2015. PMID: 24909171. DOI: 10.1038/onc.2014.155

53 Duxbury MS and Whang EE: RRM2 induces NF-kappaB-dependent MMP-9 activation and enhances cellular invasiveness. Biochem Biophys Res Commun *354(1)*: 190-196, 2007. PMID: 17222798. DOI: 10.1016/j.bbrc.2006.12.177

54 Zheng S, Wang X, Weng YH, Jin X, Ji JL, Guo L, Hu B, Liu N, Cheng Q, Zhang J, Bai H, Yang T, Xia XH, Zhang HY, Gao S and Huang Y: siRNA knockdown of RRM2 effectively suppressed pancreatic tumor growth alone or synergistically with doxorubicin. Mol Ther Nucleic Acids *12*: 805-816, 2018. PMID: 30153565. DOI: 10.1016/j.omtn.2018.08.003

55 Zhan Y, Jiang L, Jin X, Ying S, Wu Z, Wang L, Yu W, Tong J, Zhang L, Lou Y and Qiu Y: Inhibiting RRM2 to enhance the anticancer activity of chemotherapy. Biomed Pharmacother *133*: 110996, 2021. PMID: 33227712. DOI: 10.1016/j.biopha.2020.110996

56 Fisher SB, Patel SH, Bagci P, Kooby DA, El-Rayes BF, Staley CA 3rd, Adsay NV and Maithel SK: An analysis of human equilibrative nucleoside transporter-1, ribonucleoside reductase subunit M1, ribonucleoside reductase subunit M2, and excision repair cross-complementing gene-1 expression in patients with resected pancreas adenocarcinoma: implications for adjuvant treatment. Cancer *119(2)*: 445-453, 2013. PMID: 22569992. DOI: 10.1002/cncr.27619

57 Xia G, Wang H, Song Z, Meng Q, Huang X and Huang X: Gambogic acid sensitizes gemcitabine efficacy in pancreatic cancer by reducing the expression of ribonucleotide reductase subunit-M2 (RRM2). J Exp Clin Cancer Res *36(1)*: 107, 2017. PMID: 28797284. DOI: 10.1186/s13046-017-0579-0

58 Fujita H, Ohuchida K, Mizumoto K, Itaba S, Ito T, Nakata K, Yu J, Kayashima T, Souzaki R, Tajiri T, Manabe T, Ohtsuka T and Tanaka M: Gene expression levels as predictive markers of outcome in pancreatic cancer after gemcitabine-based adjuvant chemotherapy. Neoplasia *12(10)*: 807-817, 2010. PMID: 20927319. DOI: 10.1593/neo.10458

Received August 3, 2021
Revised August 30, 2021
Accepted August 31, 2021