1 **High RRN3 Expression Is Associated with Malignant Characteristics and Poor**
2 **Prognosis in Pancreatic Cancer**
3

4 Chingunjav Batbayar[1], Norihiro Ishii[1], Norifumi Harimoto[1], Takehiko Yokobori[2], Hideyuki
5 Saito[3], Dolgormaa Gantumur[1], Navchaa Gombodorj[2], Bilguun Erkhem-Ochir[2], Ryo
6 Muranushi[1], Kouki Hoshino[1], Takahiro Yamanaka[1], Kei Hagiwara[1], Mariko Tsukagoshi[1],
7 Akira Watanabe[1], Kenichiro Araki[1], Yasuo Hosouchi[4], and Ken Shirabe[1]
8
9 [1] Division of Hepatobiliary and Pancreatic Surgery, Department of General Surgical
10 Science, Gunma University Graduate School of Medicine.
11 [2] Division of Integrated Oncology Research, Gunma University Initiative for Advanced
12 Research (GIAR).
13 [3] Division of Gastroenterological Surgery, Department of General Surgical Science,
14 Graduate School of Medicine, Gunma University
15 [4] Department of Surgery and Laparoscopic Surgery, Gunma Prefecture Saiseikai Maebashi
16 Hospital.
17
18 **Corresponding Author:** Norihiro Ishii MD, PhD
19 Division of Hepatobiliary and Pancreatic Surgery, Department of General Surgical Science,
20 Gunma University Graduate School of Medicine, 3-39-22 Showa-Machi, Maebashi,
21 Gunma, 371-8511, Japan.
22 Tel: +81-027-220-8224
23 Fax: +81-027-220-8230

1 E-mail: n.ishii@gunma-u.ac.jp
2 ORCID ID: 0000-0001-6591-2439

3

4 **Keywords:** RRN3, cancer, pancreatic, ribosomes, RNA, tumors, malignancy,

**Abstract**

**Objectives:** Pancreatic cancer has an extremely poor prognosis and is one of the most chemo-resistant cancers. Targeting cancer cell transcriptional complexes may enhance chemotherapy effectiveness. RNA-polymerase I (Pol-I) mediated transcription is an essential initial step for ribosome biogenesis and is related to cancer cell proliferation. RRN3 is a Pol-I specific transcription initiation factor. In this study, we aimed to elucidate the function and clinical significance of RRN3 in pancreatic cancer.

**Methods:** We performed immunohistochemical staining to detect RRN3 protein expression in 96 pancreatic cancer tissues, and analyzed the relationship between RRN3 protein expression, clinicopathological factors, and cancer patient prognosis. Moreover, we evaluated RRN3 function *in vitro* and *in vivo* using proliferation, invasion, and chemo-sensitivity assays in PANC-1 and SW1990 cell lines; with/without depleting RRN3 expression.

**Results:** RRN3 was mainly expressed in cancer cell nuclei. High levels of RRN3 expression were associated with Ki-67 expression and shorter overall survival (OS). Additionally, proliferation and invasion ability were decreased when RRN3 was silenced with siRNA, compared to non-targeting siRNA transfected cells. Chemosensitivity analysis showed that inhibition of RRN3 enhanced sensitivity of pancreatic cancer cell lines to gemcitabine and paclitaxel. Furthermore, RRN3 siRNA-transfected PANC-1 tumors showed significantly reduced tumor volumes compared to the control tumors in a mouse xenograft model.

**Conclusion:** High levels of RRN3 expression are associated with poor prognosis and cancer malignancy, such as proliferation, invasion ability, and chemosensitivity in

1 pancreatic cancer. Therefore, RRN3 targeting may be a promising therapeutic strategy

2 using combination therapy with anticancer drugs to overcome refractory pancreatic cancer.

## Introduction

Pancreatic cancer is one of the most aggressive cancers and is characterized by rapid progression and limited response to conventional therapies [1,2]. Despite progression in the development of multidisciplinary treatments, pancreatic cancer patients have poor overall survival (OS) compared to other malignancies. Therefore, to improve the prognosis of patients with pancreatic cancer, it is crucial to elucidate the mechanisms underlying malignant potential and to develop new therapeutic targets.

Ribosome biogenesis is associated with essential cellular processes such as growth and development; proliferating cells require a continuous supply of new ribosomes for protein biosynthesis [3]. The RNA Polymerase I (Pol I)-mediated transcription from ribosomal DNA into pre-ribosomal RNA is identified as an essential initial step for ribosome biogenesis and is abnormally hyperactive in malignant cells compared to normal cells [4]. Cancer cells use the majority of their energy consumption for ribosome synthesis, and pre-ribosomal RNA transcription has been reported to account for 30-50% of total transcriptional activity.

Interestingly, the FDA approved several therapeutic agents that inhibit abnormally activated ribosome synthesis in tumors as anticancer agents. Rapamycin, an mTOR inhibitor, inhibits ribosomal RNA synthesis by blocking RNA Pol I transcription [5]. In addition, everolimus, an orally administered-rapamycin analog, has shown promising results in treating malignancies such as renal cell carcinoma and neuroendocrine tumor [6,7]. Oxaliplatin and doxorubicin also inhibit ribosomal RNA transcription, suggesting that part of their efficacy may be caused by the inhibitory effect on ribosome synthesis [8]. Additionally, ribosomal RNA synthesis is essential not only in cancer but also in non-

cancer cells, suggesting that it is problematic as a specific cancer therapeutic target. However, it has also been reported that inhibition of pre-ribosomal RNA synthesis induced cell death in tumor cell lines, but non-cancer cells can tolerate this treatment [9,10]. By utilizing this threshold difference between tumors and normal tissues, ribosomal RNA attracts attention as a candidate for cancer-specific therapy [4].

Pre-rRNA synthesis is triggered by the pre-initiation complex at the rDNA promoter. The complex is formed by the UBF/SL-1 (TIF-IB) complex, RNA Pol I, and TIF-IA (RRN3). Therefore, this study focused on the RRN3, which can mediate the interaction between UBF/SL-1 complex and RNA Pol I on the rDNA promoter and is an essential regulator of ribosomal RNA synthesis [11]. Interestingly, RRN3-mediated ribosomal RNA synthesis is downregulated in growth-arrested cells with terminal differentiation, contrary to the proliferating cells such as cancer [12]. RRN3-suppressed breast cancer cells showed the inhibited-proliferation ability, and the RRN3-overexpressed mammary epithelial cells could not form the lumen structure of the 3D mammary epithelial cell culture system and achieved proliferative potency in the aberrant 3D acini [13]. However, the function and expression of RRN3, a critical regulatory transcription factor for ribosomal RNA synthesis, have not been well analyzed in clinical pancreatic cancer samples.

This study aimed to elucidate the function and clinical significance of RRN3 in pancreatic cancer. First, we examined the expression levels of nuclear RRN3 in pancreatic cancer using immunohistochemistry to determine whether the evaluation of nuclear RRN3 expression in tumor tissues can be used as a prognostic biomarker for pancreatic patients. Moreover, we inhibited RRN3 expression in pancreatic cancer cell lines to analyze whether

RRN3 targeting is a promising therapeutic strategy to interfere with tumor aggressiveness and chemoresistance in pancreatic cancer.

## Methods

### Patients and Samples

Ninety-six surgically resected pancreatic cancer samples were obtained from patients who underwent surgical treatment at the Department of Gunma University (Maebashi, Japan) and Saiseikai Maebashi Hospital (Maebashi, Japan) between 2003 and 2017. None of the patients received neoadjuvant chemotherapy or irradiation before the surgery. The tumors were classified according to the seventh tumor-node-metastasis (TNM) classification of the Union for International Cancer Control (UICC) and the seventh General Rules for the Study of Pancreatic Cancer of Japan Pancreas Society [14]. All clinical samples and patient data were analyzed following our institutional guidelines and the Declaration of Helsinki (approved number: HS2020-124). The patients' approval for this retrospective observational research was obtained using an opt-out approach.

### Immunohistochemical Analysis

The resected surgical specimens were fixed with 10% formaldehyde and embedded in paraffin blocks. The blocks were cut into 2-µm-thick sections and mounted on glass slides. The staining protocol was carried according to standard methods, as described previously [15]. The sections were incubated overnight at 4 °C with rabbit anti-RRN3 (HPA049837; dilution, 1:100; Atlas Antibodies); or mouse anti-Ki-67 (M7240; dilution,

1 1:150; Dako; Agilent Technologies, Santa Clara, CA, USA) primary antibodies. Each
2 section was counterstained with Mayer's hematoxylin solution and mounted. The negative
3 control was established by replacing the primary antibody with phosphate-buffered saline
4 (PBS) in 0.1% bovine serum albumin, and no detectable staining was observed. Two
5 independent researchers scanned and evaluated immunohistochemical slides in a blinded
6 manner. The nuclear expression intensity of RRN3 was set as 1, weak; 2, moderate; 3,
7 strong, and with intensity 2 and 3 as positive for RRN3 expression.The positive ratio of
8 nuclear RRN3 staining in cancer cells was scored as follows: 0, no staining; 1+, 1-25%; 2+,
9 26-50%; and 3+, >50%. The cut-off point was defined as follows: scores of 0-1 were
10 considered as low expression, and 2-3 indicated high expression. The Ki-67 positive cell
11 number was counted by examining 1,000 cancer cells among three representative areas.
12
13 **Cell Culture**
14 The human pancreatic cancer cell lines AsPC- 1, BxPC-3, PANC-1, and SUIT-2
15 were obtained from RIKEN BRC Cell Bank (Ibaraki, Japan), and SW-1990 cells were
16 obtained from ATCC (Tokyo, Japan). The cells were cultured in Dulbecco's Modified Eagle
17 Medium (Wako, Osaka, Japan) supplemented with 10% fetal bovine serum (FBS) and 1%
18 penicillin-streptomycin (Thermo Fisher Scientific), and maintained at 37°C in a humidified
19 5% $CO_2$ incubator.
20
21 ***In Vitro* Transfection of RRN3-Specific siRNA**
22 RRN3-specific siRNAs (siRNA 1: GCCUAGAUCUUUUGGUUAAtt,
23 UUAACCAAAAGAUCUAGGCtt, siRNA 2: GAGCUUUCAGUGAAGAAUCtt,

1 GAUUCUUCACUGAAAGCUCtt) and negative-control siRNA
2 ( UACUAUUCGACACGCGAAGtt, CUUCGCGUGUCGAAUAGUAtt) were purchased from
3 Dharmacon GE Healthcare (Buckinghamshire, UK). PANC-1 and SW-1990 cells were
4 suspended at a density of 1.0 X $10^6$ cells in 100 mL Opti-MEM I Reduced Serum Media
5 (Thermo Fisher Scientific) and then mixed with RRN3-specific siRNA, or negative-control
6 siRNA. Transfection was performed using a GUY21 EDIT II electroporator (BEX, Tokyo,
7 Japan), with poring and transfer pulses applied at 150 and 10 V, as previously described
8 [15].
9

10 **Protein Extraction and Western Blotting**

11 According to the manufacturer's protocol, the total protein was extracted from
12 transfected PANC-1 and SW-1990 cells using RIPA Buffer (Wako). Extracted proteins were
13 separated by sodium dodecyl sulfate-polyacrylamide gel electrophoresis with 10% TGX
14 gels (Bio-Rad, Hercules, CA, USA) and transferred to nitrocellulose membranes using the
15 wet transfer method. The membranes were blocked with 5% skim milk and then incubated
16 at 4 °C overnight with anti-RRN3 rabbit polyclonal antibody (ab112052, 1:2000, Abcam),
17 anti-RRN3 (Phospho-Ser 649) rabbit polyclonal antibody (A8433, 1:1000, Assay bio Tech),
18 anti-c-Myc rabbit polyclonal antibody (#9402, 1:1000, Cell Signaling Technology: CST),
19 anti-c-Myc (Phospho-Ser62) rabbit monoclonal antibody (#13748, 1:1000, CST), anti-Akt
20 rabbit monoclonal antibody (#4691, 1;1000, CST), anti- Akt (Phospho-Ser473) rabbit
21 monoclonal antibody (#4060), and anti-β-Actin mouse monoclonal antibody (A5316;
22 1:1000; Sigma, St. Louis, MO, USA). Thereafter, membranes were treated with horseradish

peroxidase-conjugated secondary antibodies. Protein bands on the membrane were detected using ECL Prime Western Blotting Detection Reagent and an Image Quant LAS 4000 (GE Healthcare Life Sciences).

**Invasion Assay**

Cell invasion assay was performed using 24-well Corning BioCoat Matrigel Invasion Chambers (Corning, NY, USA). PANC-1 and SW-1990 cells ($1x10^5$) were seeded with 500 mL of medium in the upper chamber, and the lower chamber was filled with 750 mL of medium containing 10% FBS as a chemoattractant. After 48 h incubation, the cells were fixed and stained with Diff-Quik (Sysmex Corporation, Kobe, Japan). After staining, the cells that had invaded through the pores to the lower membrane were counted by microscope. A total of 10 randomly selected fields were evaluated.

***In Vitro* Proliferation Assay**

PANC-1 and SW-1990 cells were cultured in 96-well culture plates at 2,000 cells/well in a 100 µL medium. Cell viability was analyzed after initial cell seeding using the Cell Counting Kit-8 (Dojindo Laboratories, Kumamoto, Japan). Evaluations were performed after 0, 24, 48, and 72 h. Then, the cell counting solution was added at a concentration of 10 µL/well, and cells were incubated at 37°C for 2 hours. The absorbance was detected at 450 nm with the reference wavelength set at 650 nm using an xMarkTM Microplate Absorbance Spectrophotometer (Bio-Rad).

**Drug Sensitivity Assay**

1 Sensitivity for gemcitabine (Selleck Chemicals, Houston, TX, USA) and paclitaxel
2 (Taiho Pharmaceutical, Tokyo, Japan) was evaluated using siRNA-transfected PANC-1 and
3 SW-1990 cells. The cells were seeded at a density of $1x10^4$ cells/well in 96-well plates with
4 100 μL medium. After 24 h, the cells were treated with various concentrations of
5 gemcitabine (0, 5, 10, 25, and 50 nM) and paclitaxel (0, 0.001, 0.01 and 0.1 μg/mL) for 48
6 h. As described above, cell viability was evaluated using CCK-8 assays (Dojindo
7 Laboratories).
8
9 **Xenograft Mouse Models**
10 Mouse experiments were performed in compliance with the guidelines of the
11 Institute for Laboratory Animal Research at Gunma University, Maebashi, Japan). PANC-1
12 cell suspension ($3x10^6$ cells in 200 μL of PBS) was injected subcutaneously into the
13 bilateral flanks of 8-week-old female NOD-SCID mice (CLEA Japan, Inc, Tokyo, Japan).
14 We randomly divided 12 mice into control siRNA, RRN3 siRNA, control siRNA with
15 gemcitabine, and RRN3 siRNA with gemcitabine (each group contained 3 mice
16 respectively). Xenografted tumors of PANC-1 cells were treated with RRN3 siRNA when
17 the tumor size reached a maximum diameter of 5 mm. *In vivo* silencing of RRN3 was
18 performed as described by Tsukagoshi et al [16]. Mice were anesthetized with 1.5%
19 isoflurane (1.5L/min). A fork-type electrode was inserted into the tumor, scooping from the
20 bottom of the tumor, and then RRN3 siRNA (2000 pmol/100 μL) or control siRNA (2000
21 pmol/100 μL) was injected into each. Immediately, the plate-type electrode was put in
22 contact with the surface of the tumor, and electric pulses were delivered to each tumor
23 using the CUY21EDIT II Next-Generation Electroporator (BEX, Japan). RRN3 siRNA was

1 injected into tumors of the left flanks, and control siRNA was injected into tumors of the
2 right flanks. Tumor diameters were measured every other day and calculated using the
3 following formula: tumor volume = S × S × L/2, where S is the short diameter of the tumor
4 in millimeters and L is the maximum diameter of the tumor in millimeters. Therapy was
5 repeated every five days for three weeks (Day 1, Day 5, Day 10, Day 15, and Day 20), and
6 50mg/kg gemcitabine injected intraperitoneally twice a week (Day 1, Day 4, Day 8, Day
7 11, Day 15, and Day 18) to mice under isoflurane anesthesia. Each group contained three
8 xenografts. To collect the xenografted tumors, mice were deeply anesthetized with 4-5%
9 isoflurane and euthanized by cervical dislocation on Day 21.These xenografted tumor
10 formations were microscopically validated after hematoxylin and eosin staining. All mouse
11 experiments were performed in compliance with the guidelines of the Institute for
12 Laboratory Animal Research at Gunma University, Maebashi, Japan (Approved number:
13 18-024).
14
15 **Statistical Analysis**
16 Statistical significance was analyzed using the Mann–Whitney U test or ANOVA
17 for continuous variables and the Chi-square test or Fisher's exact test for categorical
18 variables. When the results of the ANOVA were significant, Tukey's multiple comparison
19 tests were used to assess differences between each group. Survival curves were calculated
20 using the Kaplan–Meier method. Differences between survival curves were analyzed using
21 the log-rank test. Prognostic factors were examined by univariate and multivariate analyses
22 using Cox's proportional hazard model. Results were considered statistically significant

1 when the relevant *P*-value was <0.05, and all statistical analyses were performed using JMP
2 15 software (SAS Institute, Cary, NC, USA).
3
4 **Results**
5 **Immunohistochemical Expression of RRN3 in Pancreatic Cancer**
6 The expression level of RRN3 in the pancreatic cancer part (T) was slightly higher
7 than those of surrounding stromal cells and the pancreatic acinar cells' part (N) (Figure 1A)
8 of the surgically resected pancreatic cancer samples. In addition, the RRN3 expression was
9 detected at the cellular level in the nucleolus and nucleoplasm of the cancer nucleus
10 compared to the cellular cytoplasm and membrane (Figure 1B). Of the 96 patients with
11 pancreatic cancer, 39 patients with low nuclear RRN3 expression in cancer tissues were
12 defined as the low expression group and 57 patients with high RRN3 as the high expression
13 group (Figures 1C and D).
14
15 **Association between RRN3 Expression and Clinicopathological Characteristics of**
16 **Pancreatic Cancer**
17 The relationships between clinicopathological parameters and RRN3 expression in
18 96 pancreatic cancer samples are presented in Table 1. There were no significant
19 differences in age, sex, histological type, T factor, tumor size, lymph node metastasis,
20 venous invasion, lymphatic invasion, perineural invasion, and recurrence between patients
21 with high and low RRN3 expression. The high expression of nuclear RRN3 was associated
22 with the progression of the Ki-67 positive cells' number as a proliferation marker ($P$ =
23 0.003).

1

2 **Prognostic Significance of Nuclear RRN3 Expression in Patients with Pancreatic**
3 **Cancer**

4 Kaplan–Meier analysis of 96 patients with pancreatic cancer revealed significantly
5 lower overall survival rates in the high RRN3 expression group than in the low RRN3
6 expression group ($P$ = 0.014) (Figure 1E). Our univariate analysis of 96 pancreatic cancer
7 patients identified a high level of nuclear RRN3 expression as a significant prognostic
8 factor associated with poor survival (HR = 2.25, 95% CI = 1.27–4.18, $P$ = 0.0051).
9 Moreover, a multivariate analysis of the four factors identified as significant in the
10 univariate analysis identified high nuclear RRN3 expression as an independent risk factor
11 for poor overall survival (HR = 2.17, 95% CI = 1.21–4.08, $P$ = 0.0086) (Table 2). Among
12 the patients with adjuvant chemotherapy (n=85), the RRN3 high group tended to have poor
13 prognosis compared to the low group, but not significantly (P=0.067). To validate the
14 prognostic significance of RRN3 expression in a larger cohort, we used the TCGA
15 database, which includes the transcriptome data of 179 pancreatic cancer samples with
16 survival information. The validation data were consistent with the data from our cohort,
17 indicating the association between a high level of RRN3 expression in pancreatic cancer
18 samples and poor prognosis in a large cohort ($P$ = 0.023) (Supplementary Figure 1).
19 To analyze the prognostic value of RRN3 in more detail, we evaluated the relationship
20 between nuclear RRN3 expression and post-recurrence survival in our cohort. As a result,
21 the pancreatic cancer patients with high RRN3 had shorter post-recurrence survival than
22 those with low RRN3 ($P$ = 0.049, Figure 1F). Multivariate analysis indicated that high
23 nuclear RRN3 expression in pancreatic cancer tissues was an independent prognostic

1 marker of shorter-post-recurrence survival (HR = 1.71, 95% CI = 1.01–3.03, $P = 0.048$)
2 (Table 3).
3
4 **RRN3-Specific siRNA Inhibited Tumor Cell Proliferation and Enhanced Gemcitabine**
5 **and Paclitaxel Sensitivity *In Vitro***
6 Western blotting was performed to evaluate the expression levels of RRN3 protein
7 in the pancreatic cancer cell lines AsPC-1, SUIT-2, SW-1990, BxPC-3, and PANC-1 and
8 also evaluate the phosphorylation status of RRN3, MYC and AKT respectively (Figure
9 2A), indicating the higher levels of RRN3 in all pancreatic cancer cell lines, except SUIT-2
10 cells. Moreover, cell lines with higher endogenous RRN3 tended to have higher
11 phosphorylated MYC and AKT. Among them, PANC-1 and SW-1990 were selected for
12 subsequent RRN3 knockdown experiments to analyze the functional significance of RRN3
13 in cell proliferation, invasiveness, and chemosensitivity. We used siRNA to knock down the
14 RRN3 expression and Western blotting to confirm the protein suppression (Figure 2B). The
15 RRN3-suppressed cells showed significant decreases mRNA level of pre-rRNA, and in cell
16 proliferation and invasiveness and increased drug-sensitivity against gemcitabine and
17 paclitaxel compared to the control siRNA cells (Figures 2C-E, 3). Moreover, we evaluated
18 the relationship endogenous RRN3 expression and drug sensitivity. As a results, the
19 negative correlation between endogenous RRN3 and anticancer drug sensitivity was
20 observed in consistent with functional analysis of RRN3-siRNA (Supplementary Table 1).
21
22 **RRN3-Specific siRNA Inhibits Tumor Growth and Improves Chemosensitivity in a**
23 **Xenograft Mouse Model**

1 We investigated whether RRN3 suppression could regulate tumor growth and
2 chemosensitivity using a mouse xenograft model. As a result, RRN3 siRNA-transfected
3 PANC-1 tumors showed significantly reduced tumor volumes compared to the control
4 tumors ($P < 0.05$) , and the combination of RRN3-specific siRNA and gemcitabine therapy
5 inhibited tumor growth to the greatest extent (Figure 4).
6
7 **Discussion**
8 This study clarified that high RRN3 expression was associated with poor prognosis
9 in clinical patients with pancreatic cancer, and multivariate analyses demonstrated that
10 nuclear RRN3 accumulation was an independent prognostic factor in pancreatic cancer.
11 Moreover, our experimental data indicated that suppression of RRN3 in pancreatic cancer
12 cell lines was related to a decrease in the synthesis of ribosomal RNA and downregulation
13 of proliferation, invasion ability, and chemo-resistant activity.
14 In this study, the accumulation of nuclear RRN3 in pancreatic cancer tissues was
15 an independent prognostic factor, indicating the potential of using nuclear RRN3 evaluation
16 as a useful prognostic biomarker for pancreatic cancer patients. RRN3 is an essential
17 regulator of ribosomal RNA transcription and ribosome synthesis and is abnormally
18 activated in cancer cells compared to normal cells. Experimentally, RRN3 suppression has
19 been reported to cause inhibition of both ribosomal RNA synthesis and cell proliferation
20 [13]. These findings are consistent with our data regarding the positive correlation of Ki-67
21 and nuclear RRN3 accumulation in clinical pancreatic cancer tissues, and the inhibited-
22 proliferation ability of pancreatic cancer cell lines treated by RRN3 siRNAs. The

1 prognostic value of nuclear RRN3 might reflect the activation of RRN3-mediated
2 ribosomal RNA synthesis, strongly relating to the proliferation ability observed in cancer.
3 RRN3 is a crucial regulator of ribosomal RNA synthesis, which is essential for
4 cancer cell proliferation and is ubiquitously expressed in normal as well as cancerous
5 tissues (Supplementary Figure 2). This paragraph discusses the mechanism of RRN3
6 induction in pancreatic cancers with poor prognosis. RRN3 has been reported to be a
7 downstream target gene of MYC, a transcription factor associated with carcinogenesis and
8 tumor aggressiveness in many carcinomas, including pancreatic cancer. MYC can activate
9 the ribosomal RNA synthesis via activation of RRN3 transcription [17,18]. We could
10 validate this expression relationship between MYC and RRN3 using the TCGA dataset of
11 179 pancreatic cancer patients (Supplementary Figure 3). At the protein regulatory level,
12 RRN3 protein is ubiquitinated and degraded by the proteasome system, and its half-life is
13 reported to be prolonged by AKT, which is a potential therapeutic target in many cancers,
14 including pancreatic cancer [19,20]. The data in this study indicate that RRN3 is highly
15 expressed in pancreatic cancer cells compared to surrounding normal cells and regulates
16 their proliferative potential, invasive potential, and sensitivity to anticancer drugs. These
17 findings suggested that the activation of *MYC* and *AKT*, which are representative vital
18 oncogenes in various cancers, including pancreatic cancer, partially cause the strong *RRN3*
19 induction in the cancer cells compared to the surrounding normal cells without aberrant
20 oncogene activation.
21 Phosphorylation of RRN3 by several oncogenic kinases can regulate RRN3
22 activity and ribosomal RNA synthesis. In addition, cellular stress-induced dysregulation of
23 kinase activity controls the RRN3/ribosomal RNA synthesis axis. For example, AMP-

1 activated protein kinase (AMPK), induced by glucose deprivation stress, represses RRN3
2 activity; and cellular stress, caused by amino acid deficiency in the culture media,
3 suppresses RRN3 activity. These findings suggest that cells need to use more energy for
4 survival in glucose and amino acid deprivation conditions, contrary to a reduced necessity
5 of the high-energy-consuming processes of ribosome synthesis and cellular proliferation
6 mediated by RRN3. Additionally, it has been reported that diabetes mellitus with
7 hyperglycemia and high levels of amino acids in the blood are risk factors for pancreatic
8 cancer pathogenesis [21,22]. These findings suggest that nutritional status and
9 comorbidities may contribute to RRN3 expression and pancreatic carcinogenesis; however,
10 the extent is currently unknown. Therefore, further investigation is needed to clarify the
11 importance of RRN3 in pancreatic carcinogenesis.

12 LKB1, known as serine/threonine kinase 11 (STK11), is frequently lost in sporadic
13 pancreatic adenocarcinoma [23] and can work upstream of the AMPK pathway, which is a
14 critical regulator in cellular energy homeostasis [24]. It has been reported that LKB1 kinase
15 activity is usually required for translocation of RRN3 into the nucleus under stress
16 conditions; however, such stress-induced lung cancer cell death occurred in LKB1-
17 inactivated cells without nuclear RRN3 accumulation, but not in LKB1 wild-type cell with
18 nuclear RRN3 [25]. Interestingly, patients with pancreatic cancer with low LKB1
19 expression were associated with a poor prognosis [26]. Furthermore, LKB1 suppression has
20 been reported to induce mTOR activation, one of the RRN3 activators [27], suggesting the
21 importance of the LKB1/mTOR/RRN3 axis in cancer. We were able to validate the
22 correlation of LKB1, mTOR, and RRN3 expression, which were reanalyzed and validated
23 using transcriptomic data of 179 pancreatic cancer patients in the TCGA dataset

1 (Supplementary Figure 4). In our study, *in vitro* analysis showed that RRN3 suppression
2 was associated with growth inhibition and increased chemosensitivity, suggesting that
3 RRN3 may be a promising therapeutic target in pancreatic cancer. In addition, RRN3-
4 targeted therapy against refractory pancreatic cancer lacking the LKB1 is expected to
5 induce specific and potent cancer cell death without causing substantial side effects on non-
6 cancerous cells ubiquitously expressing LKB1. A summay of molecules surrounding RRN3
7 in this study and the anticipated functions are presented in Supplementary Figure 5.
8 Regulation of phosphorylation status with molecular compounds such as
9 rapamycin (mTOR inhibitor) and AICAR (AMPK activator) can inhibit RRN3 function
10 [28]. In addition, hyperthermia, a classic and promising therapeutic tool for inducing
11 cellular stress, has been reported to induce RRN3 inactivation and suppress ribosomal RNA
12 synthesis [29]. Furthermore, hyperthermia treatment is expected to improve drug delivery
13 of the combination drug and act as a direct antitumor effect against pancreatic cancer
14 [30,31]. However, further studies are needed to analyze the combined efficacy of RRN3
15 targeted therapy by mTOR inhibitors/AMPK activators, and hyperthermia can cure
16 refractory pancreatic cancer via suppression of ribosomal RNA synthesis.
17 This study had several limitations. Firstly, our pancreatic cancer cohort was small,
18 and the clinical data were retrospectively analyzed. Moreover, our study period was long as
19 a result of having to collect enough samples. During this long study period, therapeutic
20 tools for pancreatic cancer advanced further in development and chemotherapy options
21 increased; this may have affected the prognosis. In the future, large-cohort prospective
22 studies are warranted to establish the significance of RRN3 evaluation in pancreatic cancer.
23 Second, the patients with pancreatic cancer were not consecutive cases in our hospitals

because we selected only resectable patients without neoadjuvant therapy for immunohistochemical evaluation. Third, our data suggest that RRN3 is a promising candidate therapeutic target in mouse models of pancreatic cancer; however we were unnable to examine this in multiple cell lines and patient-derived xenograft models. Therefore, our data might not generalize to all patients with pancreatic cancer, including unresectable cases.

## Conclusion

We clarified that a high expression level of nuclear RRN3 in tumor tissues was associated with poor prognosis in patients with pancreatic cancer, suggesting that RRN3 is a prognostic biomarker in this population. In addition, our *in vitro* RRN3 suppression analysis further clarified the role of RRN3 in regulating proliferative potency, invasion ability, and chemosensitivity. Therefore, RRN3 may be a promising molecular target in refractory pancreatic cancer with aggressive phenotypes and chemoresistance.

## Acknowledgements

We would like to thank Ms Yukiko Suto for her excellent assistance and Editage (www.editage.com) for English language editing.

**Funding statement:** None

## Availability of data and materials

The whole data presented in this study are available on request to the corresponding author.

## Authors contributions

N.I., T.Y. and B.C. processed the experimental data, performed the analysis, drafted the manuscript, and designed the figures. N.I., T.Y., N.H. and K.S. planned and supervised the work. N.I., T.Y. and K.S. contributed to interpreting the results and revising the draft manuscript. N.I., T.Y. and B. Ch performed all experiments and data analysis in the revision process. All authors discussed the results and commented on the manuscript. All authors have read and agreed to the published version of manuscript.

## Conflict of interest disclosure

The authors declare that there are no competing interests.

## Ethics approval and consent to participate

All clinical samples and patient data were analyzed following our institutional guidelines and the Declaration of Helsinki (approved number: HS2020-124). The patients' approval for this retrospective observational research was obtained using an opt-out approach.

## Patient consent for publication

The patients' approval for this retrospective observational research and publication was obtained using an opt-out approach.

1 **References**


2 1. Hidalgo M: Pancreatic cancer. *N Engl J Med* 2010;362:1605-1617.

3 2. Siegel RL, Miller KD, Jemal A: Cancer statistics, 2016. *CA Cancer J Clin* 2016;66:7-30.

4 3. Grummt I: Life on a planet of its own: regulation of RNA polymerase I transcription in
5 the nucleolus. *Genes Dev* 2003;17:1691-1702.

6 4. Jin R, Zhou W: TIF-IA: an oncogenic target of pre-ribosomal RNA synthesis. *Biochim*
7 *Biophys Acta* 2016;1866:189-196.

8 5. Mahajan PB: Modulation of transcription of rRNA genes by rapamycin. *Int J*
9 *Immunopharmacol* 1994;16:711-721.

10 6. Houghton PJ: Everolimus. *Clin Cancer Res* 2010;16:1368-1372.

11 7. Yao JC, Fazio N, Singh S, Buzzoni R, Carnaghi C, Wolin E, et al: Everolimus for the
12 treatment of advanced, non-functional neuroendocrine tumours of the lung or
13 gastrointestinal tract (RADIANT-4): a randomised, placebo-controlled, phase 3
14 study. *Lancet* 2016;387:968-977.

15 8. Burger K, Mühl B, Harasim T, Rohrmoser M, Malamoussi A, Orban M, et al:
16 Chemotherapeutic drugs inhibit ribosome biogenesis at various levels. *J Biol Chem*
17 2010;285:12416-12425.

18 9. Drygin D, Lin A, Bliesath J, Ho CB, O'Brien SE, Proffitt C, et al: Targeting RNA
19 polymerase I with an oral small molecule CX-5461 inhibits ribosomal RNA
20 synthesis and solid tumor growth. *Cancer Res* 2011;71:1418-1430.

21 10. Bywater MJ, Poortinga G, Sanij E, Hein N, Peck A, Cullinane C, et al: Inhibition of
22 RNA polymerase I as a therapeutic strategy to promote cancer-specific activation of
23 p53. *Cancer Cell* 2012;22:51-65.

1 11. Stepanchick A, Zhi H, Cavanaugh AH, Rothblum K, Schneider DA, Rothblum LI: DNA
2 binding by the ribosomal DNA transcription factor rrn3 is essential for ribosomal
3 DNA transcription. *J Biol Chem* 2013;288:9135-9144.
4 12. Nguyen le XT, Raval A, Garcia JS, Mitchell BS: Regulation of ribosomal gene
5 expression in cancer. *J Cell Physiol* 2015;230:1181-1188.
6 13. Rossetti S, Wierzbicki AJ, Sacchi N: Mammary epithelial morphogenesis and early
7 breast cancer. Evidence of involvement of basal components of the RNA
8 polymerase I transcription machinery. *Cell Cycle* 2016;15:2515-2526.
9 14. The seventh General Rules for the Study of Pancreatic Cancer. Tokyo: Kanehara & Co.,
10 Ltd., Tokyo, 2016.
11 15. Ishii N, Araki K, Yokobori T, Gantumur D, Yamanaka T, Altan B, et al: Reduced
12 FBXW7 expression in pancreatic cancer correlates with poor prognosis and
13 chemotherapeutic resistance *via* accumulation of MCL1. *Oncotarget*
14 2017;8:112636-112646.
15 16. Tsukagoshi M, Araki K, Yokobori T, Altan B, Suzuki H, Kubo N, et al: Overexpression
16 of karyopherin-alpha2 in cholangiocarcinoma correlates with poor prognosis and
17 gemcitabine sensitivity *via* nuclear translocation of DNA repair proteins.
18 *Oncotarget* 2017;8:42159-42172.
19 17. Wirth M, Mahboobi S, Krämer OH, Schneider G: Concepts to target MYC in pancreatic
20 cancer. *Mol Cancer Ther* 2016;15:1792-1798.
21 18. Poortinga G, Wall M, Sanij E, Siwicki K, Ellul J, Brown D, et al.: c-MYC coordinately
22 regulates ribosomal gene chromatin remodeling and Pol I availability during
23 granulocyte differentiation. *Nucleic Acids Res* 2011;39:3267-3281.

19. Song M, Bode AM, Dong Z, Lee MH: AKT as a therapeutic target for cancer. *Cancer Res* 2019;79:1019-1031.

20. Nguyen le XT, Mitchell BS: Akt activation enhances ribosomal RNA synthesis through casein kinase II and TIF-IA. *Proc Natl Acad Sci U S A* 2013;110:20681-20686.

21. Ben Q, Xu M, Ning X, Liu J, Hong S, Huang W, et al: Diabetes mellitus and risk of pancreatic cancer: A meta-analysis of cohort studies. *Eur J Cancer* 2011;47:1928-1937.

22. Katagiri R, Goto A, Nakagawa T, Nishiumi S, Kobayashi T, Hidaka A, et al: Increased levels of branched-chain amino acid associated With increased risk of pancreatic cancer in a prospective case-control study of a large cohort. *Gastroenterology* 2018;155:1474–1482.e1.

23. Su GH, Hruban RH, Bansal RK, Bova GS, Tang DJ, Shekher MC, et al: Germline and somatic mutations of the STK11/LKB1 Peutz-Jeghers gene in pancreatic and biliary cancers. *Am J Pathol* 1999;154:1835-1840.

24. Forcet C, Billaud M: Dialogue between LKB1 and AMPK: a hot topic at the cellular pole. *Sci STKE* 2007;2007:pe51.

25. Liu F, Jin R, Liu X, Huang H, Wilkinson SC, Zhong D, et al: LKB1 promotes cell survival by modulating TIF-IA-mediated pre-ribosomal RNA synthesis under uridine downregulated conditions. *Oncotarget* 2016;7:2519-2531.

26. Yang JY, Jiang SH, Liu DJ, et al. Decreased LKB1 Predicts Poor Prognosis in Pancreatic Ductal Adenocarcinoma. Sci Rep. Sci. Rep.; 5, 2015:10575.

27. Zhou W, Marcus AI, Vertino PM: Dysregulation of mTOR activity through LKB1 inactivation. *Chin J Cancer* 2013;32:427-433.

1 28. Mayer C, Zhao J, Yuan X, Grummt I: mTOR-dependent activation of the transcription
2 factor TIF-IA links rRNA synthesis to nutrient availability. *Genes Dev* 2004;18:423-
3 434.
4 29. Zhao Z, Dammert MA, Hoppe S, Bierhoff H, Grummt I: Heat shock represses rRNA
5 synthesis by inactivation of TIF-IA and lncRNA-dependent changes in nucleosome
6 positioning. *Nucleic Acids Res* 2016;44:8144-8152.
7 30. Ishikawa T, Kokura S, Sakamoto N, Ando T, Imamoto E, Hattori T, et al: Phase II trial
8 of combined regional hyperthermia and gemcitabine for locally advanced or
9 metastatic pancreatic cancer. *Int J Hyperthermia* 2012;28:597-604.
10 31. Miyamoto R, Oda T, Hashimoto S, Kurokawa T, Inagaki Y, Shimomura O, et al:
11 Cetuximab delivery and antitumor effects are enhanced by mild hyperthermia in a
12 xenograft mouse model of pancreatic cancer. *Cancer Sci* 2016;107:514-520.
13
14

1 **Figure legends:**

2

3 **Figure 1.** Immunohistochemical staining of RRN3 in clinical pancreatic cancer tissues.

4 (**A**) Representative immunohistochemical staining of RRN3 in cancerous areas of

5 pancreatic cancer tissues (t) and surrounding non-cancerous areas (n) (original

6 magnification, x100) (scale bar = 100 μm)

7 (**B**) High power view of RRN3 staining in the pancreatic cancer tissues. This figure showed

8 the intracellular localization of RRN3 protein in the pancreatic cancer cells (original

9 magnification, x400) (scale bar = 50 μm).

10 (**C**) Representative section of pancreatic cancer tissue with high levels of nuclear RRN3

11 expression (original magnification, x100) (scale bar = 100 μm).

12 (**D**) Representative section of pancreatic cancer tissue with low levels of nuclear RRN3

13 expression (original magnification, x100) (scale bar = 100 μm).

14 Kaplan–Meier survival curves of pancreatic cancer patients according to nuclear RRN3

15 expression.

16 (E) Kaplan–Meier analysis of overall survival in our cohort of pancreatic cancer patients (n

17 = 96) according to nuclear RRN3 expression.

18 (F) Kaplan–Meier analysis of post-recurrence overall survival (n = 74) according to nuclear

19 RRN3 expression.

20 $^{*}P < 0.05$

21

22 **Figure 2.** Functional analysis of RRN3 in human pancreatic cancer cell lines.

1 (**A**) Protein expression was evaluated in the pancreatic cancer cell lines AsPC-1, SUIT-2,
2 SW-1990, BxPC-3, and PANC-1 by Western blotting. β-actin and histone H3 was used as
3 the loading control.
4 (**B**) RRN3 suppression was evaluated in PANC-1 and SW-1990 cells treated with RRN3
5 siRNAs by western blotting.
6 (**C**) mRNA level of pre-rRNA 5`ETS was evaluated in PANC-1 and SW-1990 cells treated
7 with RRN3 siRNAs by RT-PCR.
8 (**D**) The proliferation of PANC-1 and SW-1990 cells after RRN3 siRNA treatment was
9 evaluated using a Cell Counting Kit-8 kit.
10 (**E**) The invasion assay of PANC-1 and SW-1990 cells after RRN3 siRNA treatment was
11 evaluated. RRN3 siRNA treatment significantly inhibited invasive ability compared with
12 control cells.
13 $^{*}P < 0.05$
14
15 **Figure 3.** Analysis of gemcitabine and paclitaxel sensitivity in RRN3-suppressed
16 pancreatic cancer cells. The chemosensitivity assay was performed using PANC-1 and SW-
17 1990 cells after RRN3 siRNA treatment. The sensitivity of the gemcitabine (A) and
18 paclitaxel (B) was higher in RRN3 suppressed cells compared to control cells.
19 $^{*}P < 0.05$
20
21 **Figure 4.** *In vivo* analysis of RRN3 suppression in a mouse xenograft model.

1 (**A**) RRN3 suppression inhibited tumor growth compared with control siRNA group.
2 Combintation of RRN3 supression and gemcitabine treatment inhibited tumor growth
3 markedly.
4 (**B**) Representative photographs of tumors consisting of PANC-1 cells in each group.
5 (**C**) HE and immunohistochemical staining of RRN3 expression in tumor tissues. The
6 expression of RRN3 was suppressed in PANC-1 cells treated by RRN3 siRNA.
7 $^{*}P < 0.05$
8 HE, Hematoxylin and eosin

1 **Supplementary Figure Legends**

2

3 **Supplementary Figure 1.** Overall survival curve according to RRN3 expression in 179
4 pancreatic cancer samples from the transcriptomic data in the TCGA dataset. Pancreatic
5 cancer patients with high levels of RRN3 expression (n = 89) showed lower survival than
6 those with low levels of RRN3 (n = 89) (log-rank test, $P = 0.023$).

7

8 **Supplementary Figure 2.** RRN3 expression profiles in 55 tissue types and 69 cell lines
9 from different tissue types. These figures were generated by combining the HPA and GTEx
10 transcriptomics datasets using the internal normalization pipeline from The Human Protein
11 Atlas website (https://www.proteinatlas.org). The expression of RRN3 was ubiquitously
12 detected in whole tissues and cell lines, indicating the low tissue specificity of RRN3
13 expression profiles.

14

15 **Supplementary Figure 3.** Relationship between RRN3 and MYC expression in 179
16 pancreatic cancer samples from the transcriptomic data in the TCGA dataset. The
17 expression of RRN3 was positively correlated with that of MYC.

18

19 **Supplementary Figure 4.** Relationship between RRN3, LKB1, and MTOR expression in
20 179 pancreatic cancer samples from the transcriptomic data in the TCGA dataset.
21 Left panel: Expression of *LKB1* was inversely correlated with that of *MTOR*.
22 Middle panel: Expression of *RRN3* was inversely correlated with that of *LKB1*.
23 Right panel: Expression of *RRN3* was positively correlated with that of *MTOR*.

1

2 **Supplementary Figure 5. Summary schema of RRN3 function with surrounding**

3 **molecules.**

**Table 1**: Clinicopathological characteristics according to the RRN3 expression in 96 patients with pancreatic cancer

| Factors | RRN3 expression | | $p$-value |
|---|---|---|---|
| | Low (n = 39) | High (n = 57) | |
| Age (median, range) | 72 (43-87) | 67 (36-84) | 0.059 |
| Sex | | | 0.229 |
| Male | 26 | 31 | |
| Female | 13 | 26 | |
| Histological type | | | 0.186 |
| Well | 7 | 5 | |
| Moderately, Poorly | 32 | 52 | |
| T factor (UICC) | | | 0.297 |
| T1, 2 | 7 | 6 | |
| T3, 4 | 32 | 51 | |
| Tumor size | | | 0.714 |
| ≤40 mm | 31 | 47 | |
| >40 mm | 8 | 10 | |
| Lymph node metastasis | | | 0.549 |
| Absent | 11 | 13 | |
| Present | 28 | 44 | |
| Venous invasion | | | 0.480 |
| Absent | 6 | 6 | |
| Present | 33 | 51 | |
| Lymphatic invasion | | | 0.480 |
| Absent | 6 | 6 | |
| Present | 33 | 51 | |
| Perineural invasion | | | 0.338 |
| Absent | 5 | 4 | |
| Present | 34 | 53 | |
| Reccurence | | | 0.416 |
| Absent | 14 | 16 | |
| Present | 25 | 41 | |
| Ki-67 expression | | | 0.003 |
| Positive cell numbers ( /1000 cells) | 116 ± 22.1 | 343 ± 43.6 | |

Abbreviations: UICC, Union for International Cancer Center

1

1

**Table 2**: Univariate and Multivariate analyses of variables related to overall survival using Cox proportional hazards model

| Variables | Univariate analysis | | | Multivariate analysis | | |
|---|---|---|---|---|---|---|
| | HR | 95%CI | *p*-value | HR | 95%CI | *p*-value |
| Age (<70 vs. ≥70) | 1.17 | 0.65-2.09 | 0.588 | - | - | - |
| Sex (Male vs. Female) | 0.66 | 0.36-1.16 | 0.152 | - | - | - |
| Histological type (Well vs. Moderately, Poorly) | 3.61 | 1.32-14.9 | 0.009* | 1.84 | 0.66-7.70 | 0.275 |
| T factor (UICC) (T1, 2 vs. T3, 4) | 2.33 | 1.01-6.75 | 0.047* | 1.91 | 0.82-5.62 | 0.144 |
| Lymph nodemetastasis (Absent vs. Present) | 1.64 | 0.81-3.77 | 0.175 | - | - | - |
| Venous invasion (v0,1 vs. v2,3) | 1.94 | 0.78-6.46 | 0.166 | - | - | - |
| Lymphatic invasion (ly0,1 vs. ly2,3) | 9.58 | 2.10-169.5 | 0.0008* | 6.48 | 1.36-116.4 | 0.0132* |
| Perineural invasion (ne0,1 vs. ne2,3) | 1.03 | 0.45-2.98 | 0.946 | - | - | - |
| RRN3 (Low vs. High) | 2.25 | 1.27-4.18 | 0.0051* | 2.17 | 1.21-4.08 | 0.0086* |

Abbreviations: HR, hazard ratio; CI, confidence interval; UICC, Union for International Cancer Center; 0, not observed; 1, slightly observed; 2, moderately observed; 3, highly observed

* $p < 0.05$

2

3

1

**Table 3**: Univariate and Multivariate analyses of variables related to survival after recurrence using Cox proportional hazards model

| Variables | Univariate analysis | | | Multivariate analysis | | |
|---|---|---|---|---|---|---|
| | HR | 95%CI | *p*-value | HR | 95%CI | *p*-value |
| Age (<70 vs. ≥70) | 1.12 | 0.59-2.14 | 0.731 | - | - | - |
| Sex (Male vs. Female) | 0.96 | 0.56-1.62 | 0.901 | - | - | - |
| Histological type (Well vs. Moderately, Poorly) | 2.27 | 0.92-7.54 | 0.076 | | | |
| T factor (UICC) (T1, 2 vs. T3, 4) | 1.71 | 0.62-7.10 | 0.335 | | | |
| Lymph nodemetastasis (Absent vs. Present) | 1.2 | 0.66-2.29 | 0.57 | - | - | - |
| Venous invasion (v0,1 vs. v2,3) | 1.63 | 0.98-2.76 | 0.059 | - | - | - |
| Lymphatic invasion (ly0,1 vs. ly2,3) | 1.42 | 0.84-2.37 | 0.191 | - | - | - |
| Perineural invasion (ne0,1 vs. ne2,3) | 1.99 | 1.13-3.73 | 0.017* | 2.03 | 1.14-3.81 | 0.014* |
| RRN3 (Low vs. High) | 1.67 | 1.02-2.82 | 0.041* | 1.71 | 1.01-3.03 | 0.048* |

Abbreviations: HR, hazard ratio; CI, confidence interval; UICC, Union for International Cancer Center; 0, not observed; 1, slightly observed; 2, moderately observed; 3, highly observed

* $p < 0.05$

2

3

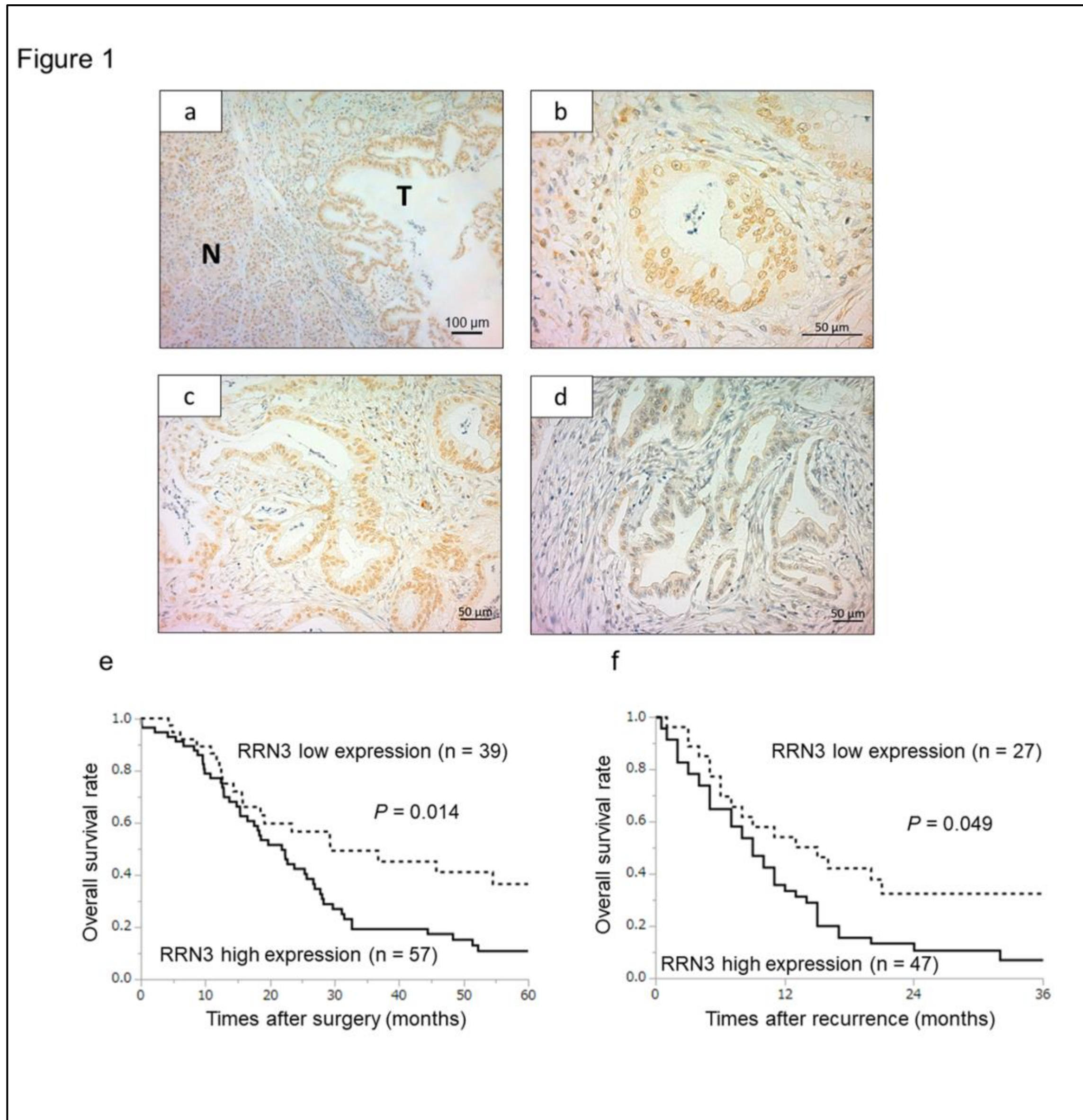
**Supplementary Table 1**. IC 50 of paclitaxel and gemcitabine in pancreatic cancer cell lines

| | IC50 | |
|---|---|---|
| | Paclitaxel (nM) | Gemcitabine (nM) |
| PANC-1 | 24.75 | 26.34 |
| SW-1990 | 23.72 | 21.1 |
| AsPC-1 | 11.93 | 13.36 |
| BxPC-3 | 8.23 | 8.62 |
| SUIT-2 | 5.26 | 6.62 |

1
2

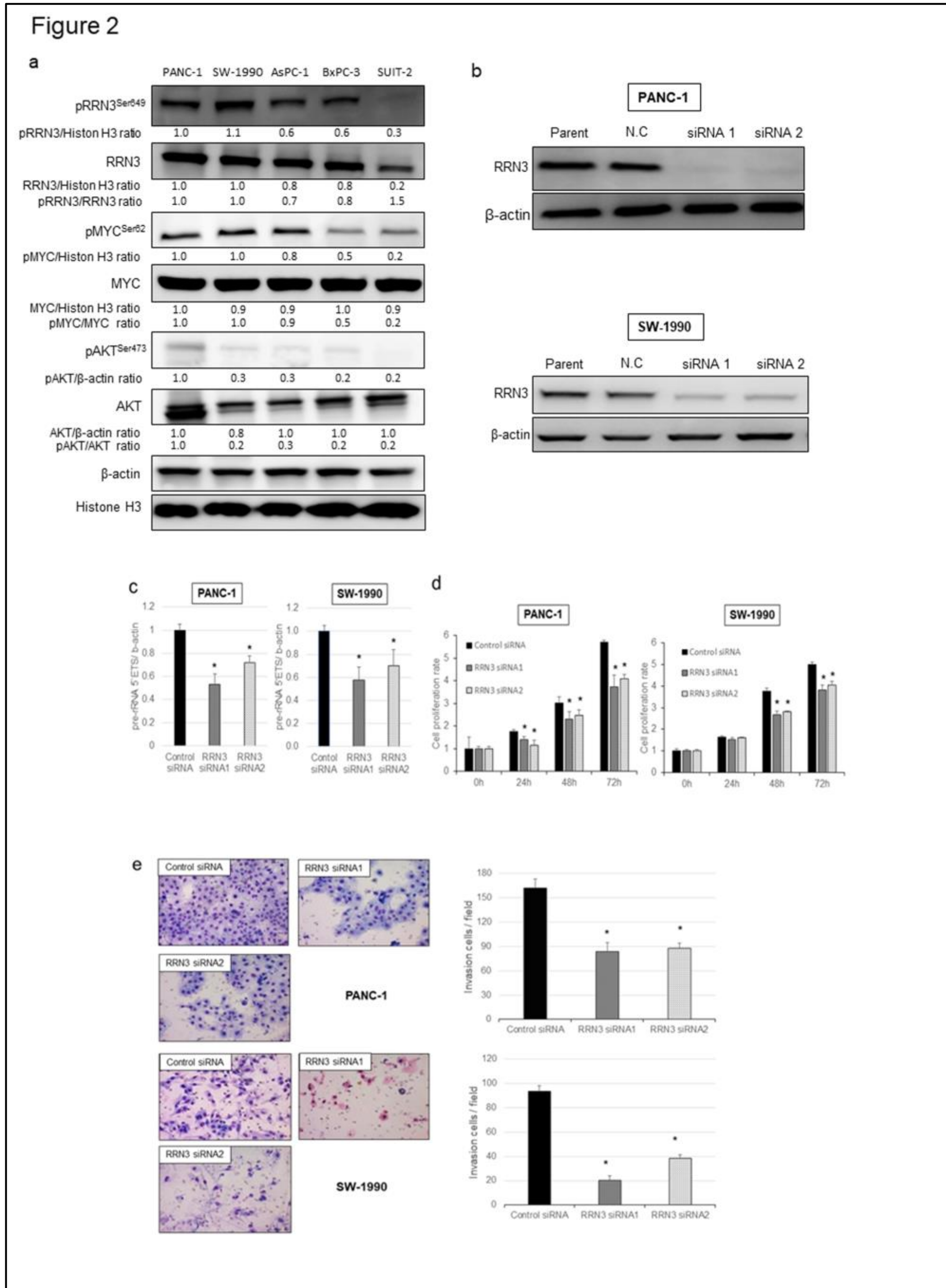
1 Figures.

2




3

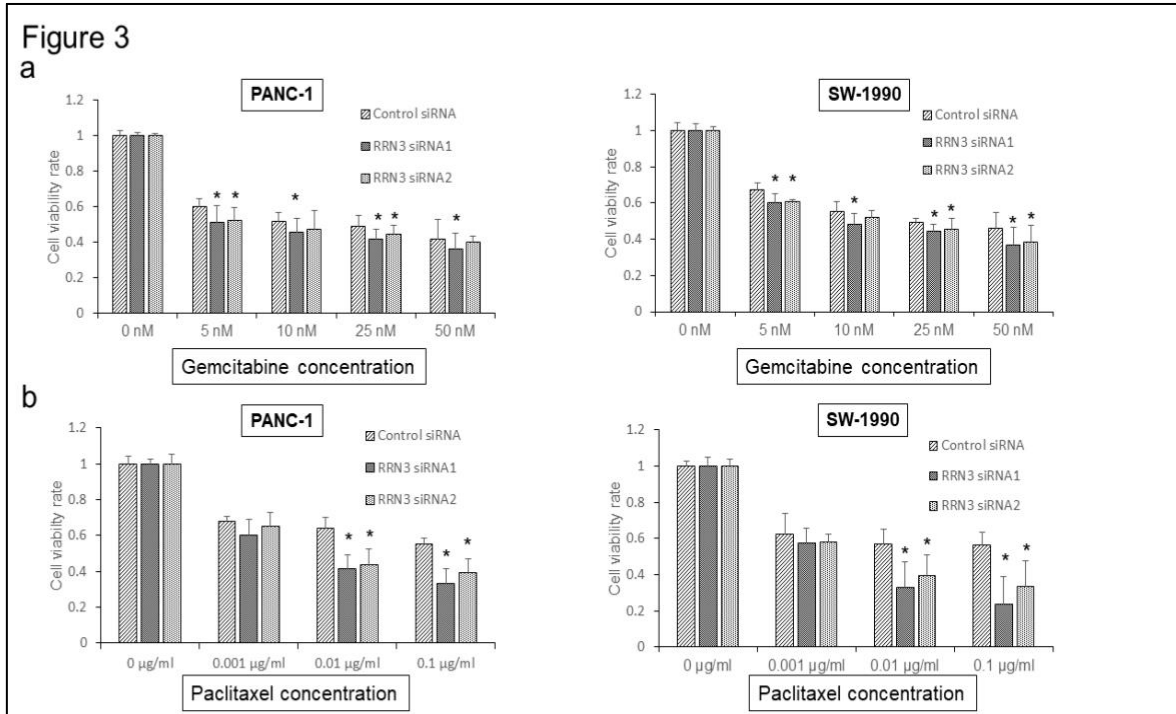
1




2

3

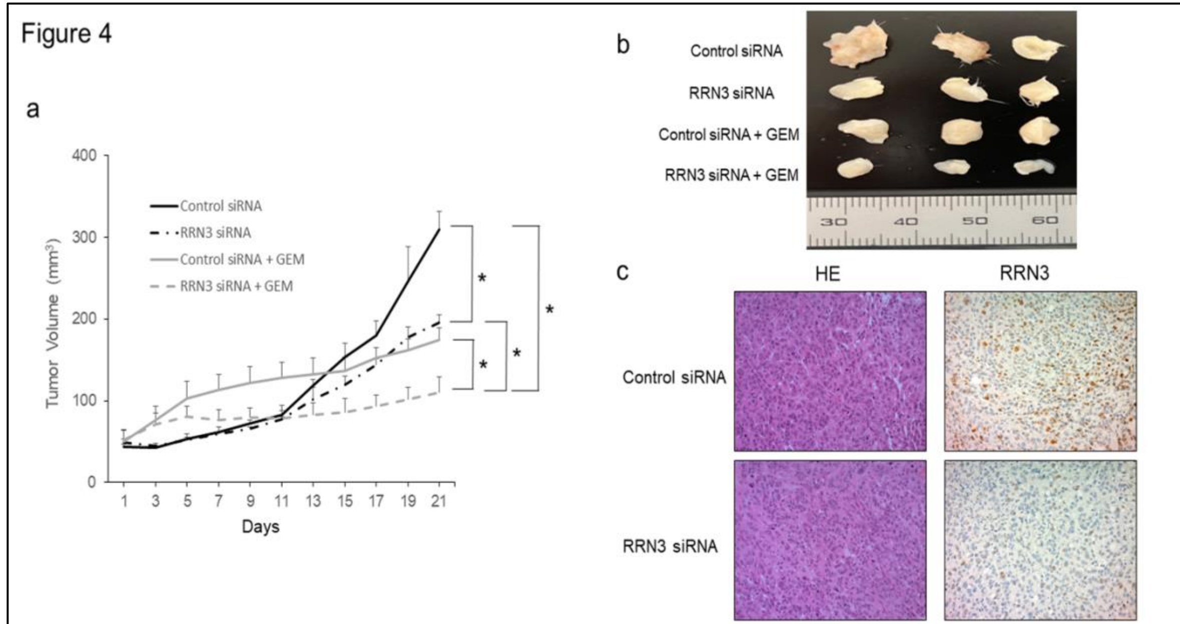
1




2

3

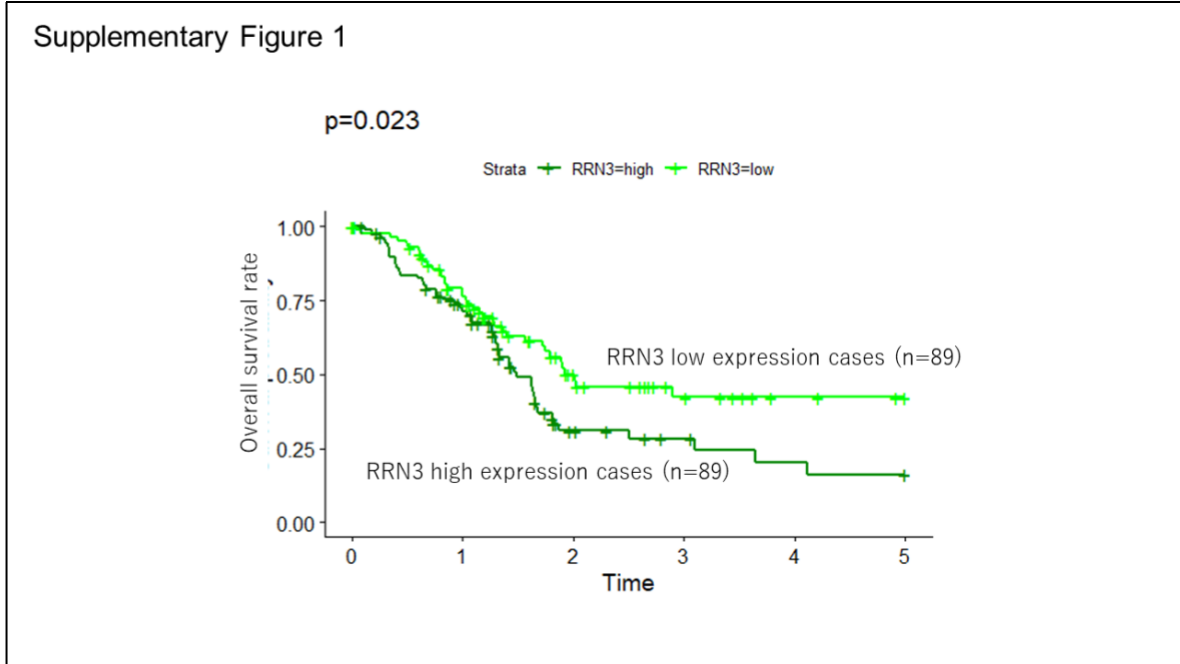
1

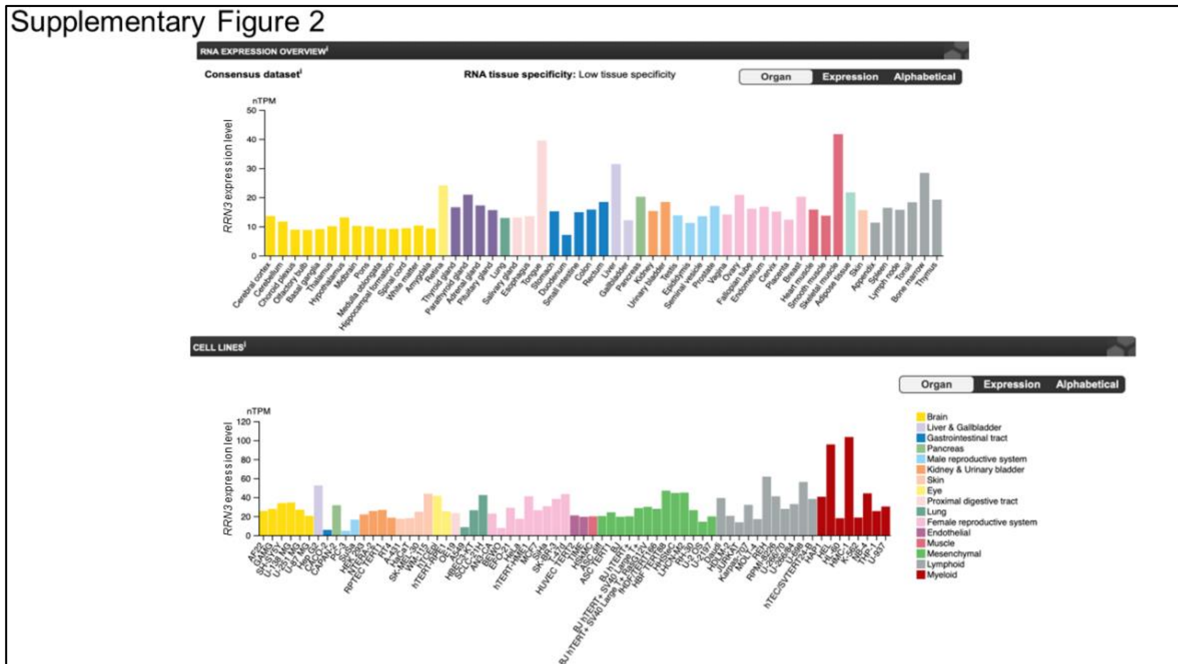



2

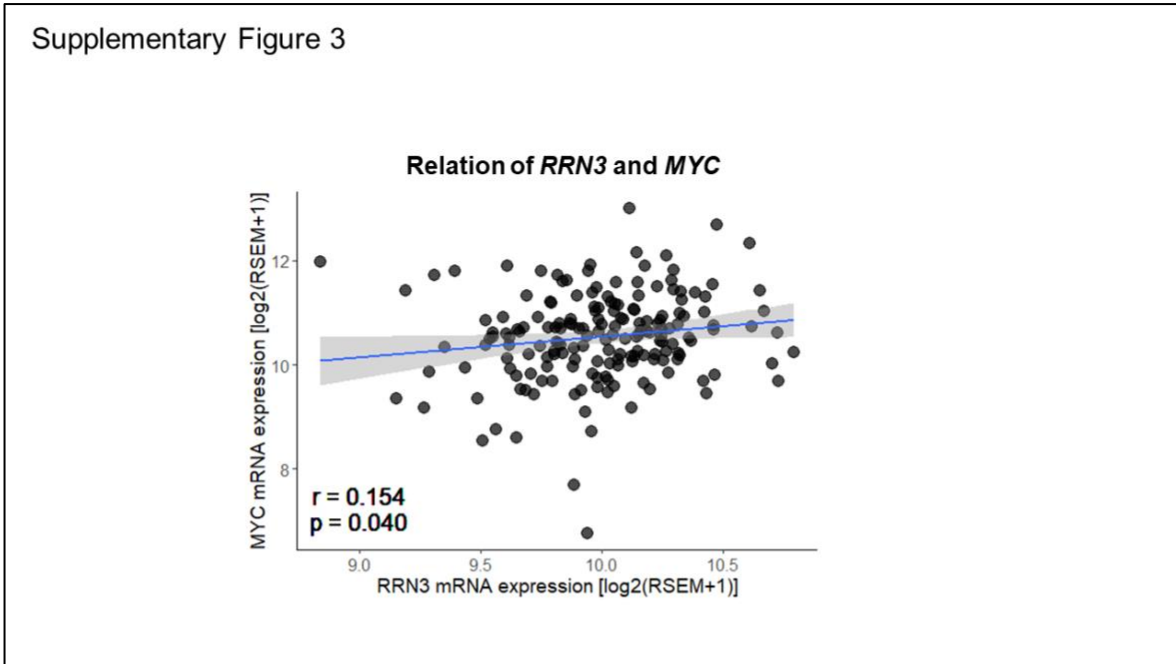
3

Supplementary figures.

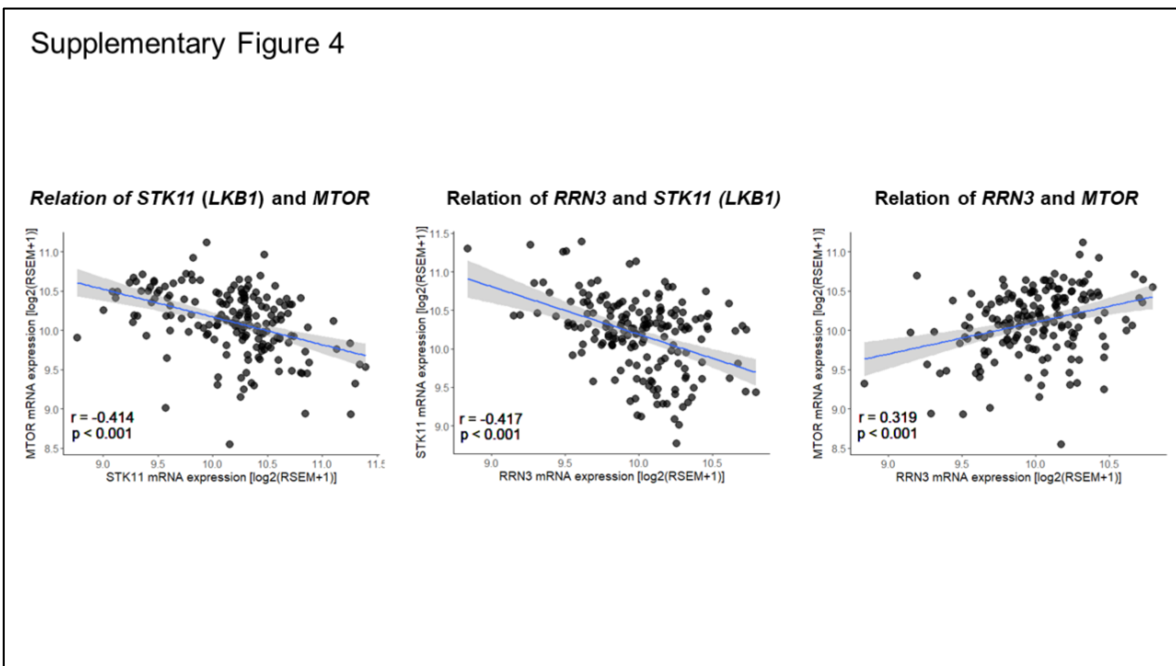


1



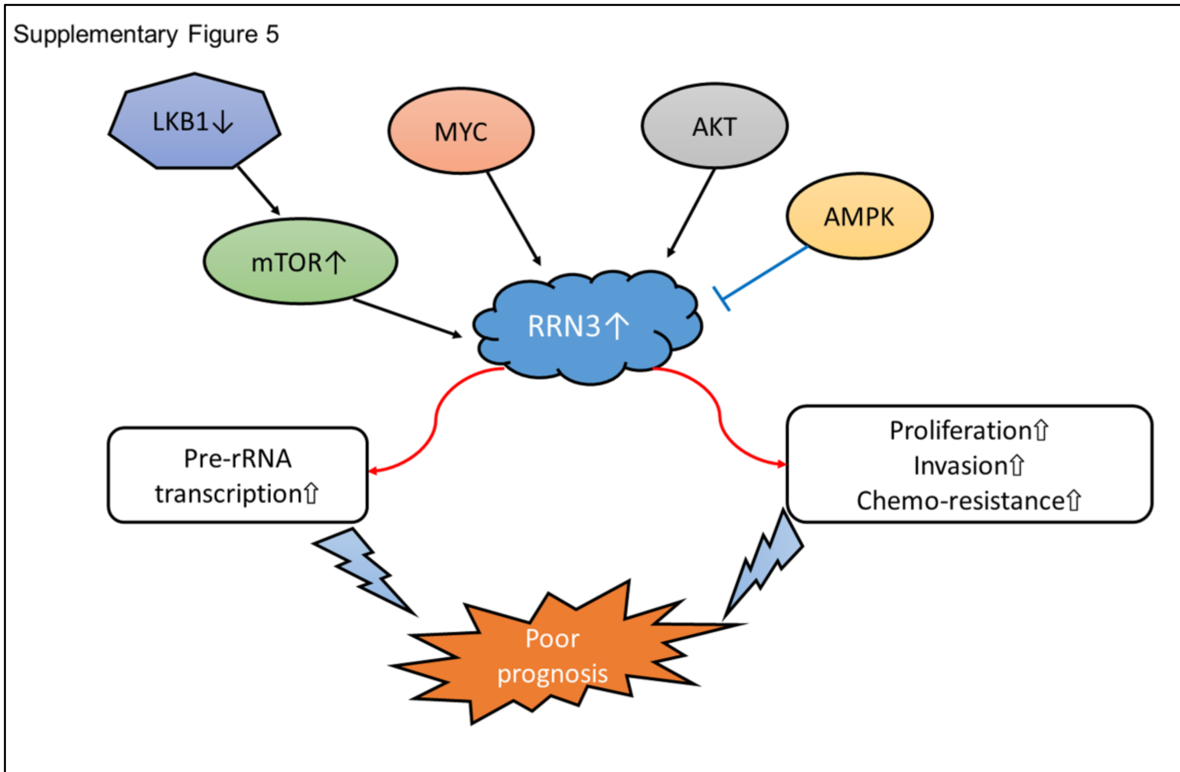

2




3

4

1




2

3