ANTICANCER RESEARCH *41*: 2441-2449 (2021)
doi:10.21873/anticanres.15019

MiR-3663-3p Inhibits the Progression of Gastric Cancer Through the CCND1 Pathway

KOJI TAKAO, HIROTAKA KONISHI, DAIKI MATSUBARA, KATSUTOSHI SHODA, TOMOHIRO ARITA, HIROKI SHIMIZU, SHUHEI KOMATSU, ATSUSHI SHIOZAKI, YUSUKE YAMAMOTO, TAKESHI KUBOTA, HITOSHI FUJIWARA, KAZUMA OKAMOTO and EIGO OTSUJI

Division of Digestive Surgery, Department of Surgery, Kyoto Prefectural University of Medicine, Kyoto, Japan

Abstract. *Background/Aim: The regulation of gene expression by miRNAs plays an important role in cancer progression. Here, we investigated the role of miR-3663-3p in gastric cancer. Patients and Methods: The relationship between miR-3663-3p expression, clinicopathological features and prognosis were retrospectively analyzed in 80 gastric cancer patients. Results: miR-3663-3p expression was significantly lower in gastric cancer tissue than adjacent non-cancerous tissue (p=0.002). Recurrence free survival was significantly lower in patients with low miR-3663-3p expression (p=0.016). Low miR-3663-3p expression was also an independent predictive factor for recurrence (p=0.029). Overexpression of miR-3663-3p in gastric cancer cell lines significantly suppressed cell proliferation, migration/ invasion, and induced G0/G1 arrest (p<0.01). Furthermore, overexpression of miR-3663-3p decreased Cyclin D1 mRNA and protein, and reduced the phosphorylation of Retinoblastoma (Rb) protein. Conclusion: miR-3663-3p is a clinically useful predictor of gastric cancer recurrence. It exhibits tumor suppressive features through limited entry into the cell cycle in a Cyclin D1-Rb dependent manner.*

Gastric cancer (GC) is the fifth most common cancer and third leading cause of cancer related death worldwide, with more than 1,000,000 new cases and about 783,000 deaths in 2018 (1). The mortality associated with GC is caused by metastases to distant organs, lymph nodes, or abdominal disseminations. The overall survival of patients with GC has been improved due to early detection, popularization of radical surgery, and advances in chemotherapy (2). Despite advances in GC treatment, the prognosis of advanced gastric cancer remains poor and the mortality high (3), therefore, identification of valuable diagnostic markers and development of effective adjuvant therapies are significant.

Recently, novel non-coding RNAs, including microRNAs (miRNAs), which are endogenous short non-coding RNAs of 20-24 nucleotides, have been discovered with the advent of next generation sequencing techniques (4). miRNAs are important in normal development, differentiation, growth control, and pathogenesis of many diseases, including cancer (5). The primary function of miRNA is the suppression of target messenger RNA (mRNA) expression (6), and abnormally expressed miRNAs act as tumor suppressive or oncogenic molecules, depending on the function of their target genes (7-11).

Despite the wealth of information on miRNA-dependent processes, the function of many newly reported miRNAs is still unknown. We have previously found abnormal miR-3663-3p levels in blood samples from patients with GC as a published data, with only one study reporting on the function of miR-3663-3p (12). We, therefore, focused on the minimally characterized miRNA, miR-3663-3p, as its role in GC had not been previously explored. In the present study, we analyzed the clinical significance of miR-3663-3p expression in GC tissue, and examined its functions in GC cell lines.

Patients and Methods

Clinical characteristics of gastric cancer patients. Eighty patients with primary GC who underwent radical gastrectomy between 2014 and 2017 at the hospital of Kyoto Prefectural University of Medicine were registered in the present study. A database containing the clinicopathological features and prognoses of these patients was retrospectively reviewed. The median length of the follow-up period for censored cases was 41.8 months (range=26.2-51.6 months). The clinical features were diagnosed using i) gastrography, ii) upper gastrointestinal endoscopy, and iii) computed tomography (CT) prior to operation. The pathological features were evaluated using the 15th edition of Japanese Classification of Gastric Cancer. Patient follow up appointments, roughly 5 years postoperatively, were done in an outpatient clinic using upper gastrointestinal endoscopy and computed tomography to evaluate a possible recurrence and confirm the prognosis.

Correspondence to: Hirotaka Konishi, Division of Digestive Surgery, Department of Surgery, Kyoto Prefectural University of Medicine, 465 Kajii-cho, Kamigyo-ku, 6028566, Kyoto, Japan. Tel: +81 752515527, Fax: +81 752515522, e-mail: h-koni7@koto.kpu-m.ac.jp

Key Words: Gastric cancer, miR-3663-3p, CCND1, biomarker.

All patients provided a written informed consent for their treatments and participation in the present study. The research was conducted in accordance with the principles of the Helsinki declaration. Ethical approval was granted by the Faculty of Science Ethics Committee at Kyoto Prefectural University of Medicine (ERB-C-67-4).

Tissue sample collection. Cancer and non-cancerous tissues were collected from frozen gastric tissue from 80 patients with GC who had undergone gastrectomy. All GC samples were histologically diagnosed as a primary gastric adenocarcinoma.

GC cell lines and cell culture. The human GC cell lines (MKN7, MKN28, MKN45 MKN74, NUGC4, and HGC27) and non-cancer cell lines (Met-5A and WI-38) were used in the present study. MKN7, MKN45, and MKN74 were purchased from RIKEN BioResource Center (Tsukuba, Japan). MKN28 was purchased from the Japanese Collection of Research Bioresources Cell Bank (Osaka, Japan). HGC27 was obtained from Cell Lines Service (Eppelheim, Germany). The human normal mesothelial cell line MeT-5A was purchased from ATCC (Manassas, VA, USA). The human normal fibroblast cell line WI-38 was purchased from the Japanese Collection of Research Bioresources Cell Bank (Osaka, Japan).

HGC27 cells were cultured in Dulbecco's Modified Eagle Medium (Nacalai Tesque, Kyoto, Japan). WI-38 cells were cultured in Basal medium Eagle (Thermo Fisher Scientific, Waltham, MA, USA). The other cell lines were cultured in Roswell Park Memorial Institute 1640 medium (Nacalai Tesque). All media was supplemented with 10% fetal bovine serum (Corning, New York, NY, USA). All cell lines were cultured in a humidified incubator with 5% of CO_2 at 37˚C.

RNA extraction and quantitative real time polymerase chain reaction (qRT- PCR). Total RNA was extracted from frozen tissue samples using an AllPrep® DNA/RNA/miRNA universal kit (Qiagen, Hilden, Germany) and from cell lines using a miRNeasy Mini Kit (Qiagen), according to the manufacturer's guidelines. Isolated RNA was stored at –80˚C until use.

miRNA levels were measured by qRT-PCR using a StepOnePlus PCR system (Applied Biosystems, Waltham, USA). A TaqMan MicroRNA Assay (Thermo Fisher Scientific) was used for the detection of has-miR-3663-3p expression (Assay ID: 465775_mat, Thermo Fisher Scientific) and RNU6B expression (Assay ID=001093, Thermo Fisher Scientific). Cycle threshold (Ct) values were calculated using StepOne Software v2.0 (Applied Biosystems).

To determine mRNA levels, a reverse transcription reaction was performed using a High-Capacity cDNA Reverse Transcription Kit (Applied Biosystems). mRNA level was determined by qRT- PCR using a TaqMan Gene Expression Assay (Thermo Fisher Scientific): i) CCND1 (Assay ID: Hs00765553_m1, Thermo Fisher Scientific) and ii) the housekeeping gene ACTB (Hs01060665_g1, Thermo Fisher Scientific). miR-3663-3p and CCND1 expression was normalized using the ΔΔCt method relative to RNU6B and ACTB expression in each sample.

Overexpression of miRNA. A mirVana® miRNA mimic was used for the overexpression of miR-3663-3p (Assay ID: MC20554, miRbase ID: has-miR-3663-3p, MIMAT0028085, Thermo Fisher Scientific). The GC cells lines, MKN74 and NUGC4, were transfected in a 6-well plate at the final miRNA mimic concentration of 3 nM, using Lipofectamine® RNAiMAX Reagent (Thermo Fisher Scientific), according to the manufacturer's instructions. All overexpression experimental results were compared to those in similarly treated cells except for the miRNA mimic (mock). miR-3663-3p overexpression by the mimic was confirmed using qRT-PCR.

Cell proliferation assay. Cell proliferation was evaluated using a WST-8 assay (Cell Count Reagent SF; Nacalai Tesque). GC cells, MKN74 and NUGC4, were seeded on 24-well plate at a density of 1.0×10^4 cells/well and incubated for 24 h, then transfected with a miR-3663-3p mimic. Cell proliferation was measured every 24 h after transfection by measuring the absorbance at 450 nm, using Thermo Scientific Multiskan FC (Thermo Fisher Scientific).

Transwell cell migration or invasion assay. Migration and invasion assays were performed using the BioCoat Matrigel™ invasion Chamber kit (BD Biosciences, San Jose, CA, USA), following the manufacturers guidelines. For the invasion assay, the upper surface (6.4-mm-diameter filters with 8-μm pores) was coated with Matrigel, whereas there was no coating for the migration assay. Media with 10% Fetal Bovine Serum (FBS) was added into the lower chamber. First, GC cells were seeded in a 6-well plate at a density of 1.0×10^5 cells/well, incubated for 24 h and then transfected with miR-3663-3p mimic. Cells were incubated for 48 h following transfection, then detached using Trypsin-EDTA solution (Nacalai Tesque) and reseeded in the upper chamber with serum-free media (MKN74=0.5×10^5 cells/well and NUGC4=1.0×10^5 cells/well). Following incubation, the infiltrative or migratory cells on the duplicate membranes were stained with Diff-Quik stain (Sysmex, Kobe, Japan). The nuclei of stained cells on the lower side of the membrane were counted in five independent fields of view at ×100 magnification for each insert, and the results are shown as a mean±SD. Each assay was performed in triplicate.

Cell cycle analysis. The miRNA mimic-transfected cells were incubated for 48 h after transfection. Then, cells were treated with Triton X-100, and cell nuclei were stained with PI/ RNase staining buffer (Becton-Dickinson Biosciences, San Jose, CA, USA), according to the manufacturer's instructions. The content of DNA was measured using a Becton-Dickinson Accuri C6 Plus Flow Cytometer (BD Biosciences). At least 10,000 cells were counted in each measurement, and the cell cycle distribution was recorded using BD Accuri C6 software.

Western blotting. Cells were lysed by M-PER Mammalian Protein Extraction Reagent (Thermo Fisher Scientific) and centrifuged at 4˚C 12,000 rpm for 5 min. The supernatant was collected, and protein concentration was determined using the Protein Assay Rapid Kit Wako II (Wako, Osaka, Japan). Fifteen ng of protein from the cell lysate was separated using a 12% SDS-PAGE gel, and then transferred onto a PVDF membrane (GE Healthcare, Chicago, IL, USA). The membranes were subsequently probed with the indicated antibodies, and proteins were detected using SuperSignal West Dura Extended Duration Substrate (Thermo Fisher Scientific) and an Amersham imager 680 (GE Healthcare). The following primary antibodies were used: Cyclin D1 (1:1000 dilution, Proteintech, Rosemont, IL, USA), Rb (1:300 dilution, Santa Cruz Biotechnology, Dallas, Texas, USA), phospholyrated Rb (1:1,000 dilution, Cell Signaling Technology, Danvers, MA, USA) and β-Actin (1:2,000 dilution, Sigma-Aldrich, St. Louis, MO, USA). The following secondary, HRP-linked antibodies were used: Anti-mouse IgG antibody for Rb and Anti-rabbit IgG antibody for Cyclin D1 and phospholyrated Rb (Cell Signaling

Technology). Cyclin D1 and phosphorylated Rb protein levels normalized by β-Actin levels are shown in the bar graphs.

Statistical analysis. Gene expression changes by RT-qPCR were determined using the $2^{-\Delta\Delta Ct}$ method. A Wilcoxon signed-rank test was used to compare the relative expression of miRNA in cancer and non-cancerous tissues. Student's *t*-tests were used for the analyses of proliferation, migration/invasion, and cell cycle assays, as well as the comparison of mRNA expression. A Chi-squared test was used to estimate the relationship between miRNA expression and clinicopathological features. The cut-off value of miR-3663-3p expression was determined with the minimum *p*-Value approach using the recurrence free survival, calculated by the log-rank test. Patients were divided into a "High" or a "Low" expression group using this cut-off value. Survival curves were plotted using the Kaplan–Meier method and compared using a log-rank test. A multivariate survival analysis was performed using the Cox's proportional hazard regression model. All results were analyzed using JMP software, version 13 (SAS Institute, Cary, NC, USA). A *p*-Value<0.05 was considered statistically significant.

Results

Relationship between miR-3663-3p expression and clinicopathological features. miR-3663-3p is a novel miRNA with little information available regarding its expression level and impact on various cancers, including gastric cancer (GC). The clinicopathological features of 80 patients with GC and their miR-3663-3p expression levels are shown in Table I. miR-3663-3p expression was related to pT factor (p=0.049), while no relation with other clinicopathological features was observed. Recurrence was observed in 23 patients, consisting of: i) 8 cases of hematogenous metastasis, ii) 4 cases of lymphatic metastasis, iii) 8 cases of peritoneal dissemination, and iv) 3 cases of other recurrences.

Survival analysis in relation to miR-3663-3p expression. Recurrence free survival (RFS) was significantly lower in the low miR-3663-3p expression group compared to the high expressing group (Figure 1a, p=0.016). Furthermore, cancer specific survival was slightly lower in the same group, but not statistically significant (p=0.086).

Prognostic factors of patients with GC. Using univariate analysis, i) venous invasion (p=0.025), ii) pT factor (p=0.006), iii) pN factor (p=0.001), iv) pStage (p=0.0004), and v) miR-3663-3p expression (p=0.016) were significantly correlated with RFS (Table II). In addition, using multivariate analysis, i) pStage III (HR=3.12, p=0.029) and ii) low miR-3663-3p expression (HR=2.31, p=0.016) were independent predictive factors of RFS.

miR-3663-3p expression in GC tissue and cell lines. miR-3663-3p expression was significantly lower in cancer tissue compared to the adjacent non-cancerous tissue (Figure 1b, p=0.002). We assessed miR-3663-3p expression in 6 GC cell lines and it tended to be lower in 4 cell lines than in the non-cancer cell lines (Figure 1c).

Effect of miR-3663-3p overexpression in GC cell lines. To address the impact of miR-3663-3p levels, we expressed a miR-3663-3p mimic using GC cell lines. miR-3663-3p expression was significantly increased following transfection of MKN74 and NUGC4 cells with the mimic (Figure 2a, p<0.001). The proliferation of MKN74 and NUGC4 cells was significantly reduced by miR-3663-3p overexpression (Figure 2b, p<0.01). The migratory and invasive capacity of the cells

Table I. *Clinicopathological features in relation to miR-3663-3p expression*.

Variables	n=80	High expression (n=47)	Low expression (n=33)	*p*-Value[b]
Age				
<70	37	24	13	0.433
≥70	43	23	20	
Gender				
Female	22	14	8	0.583
Male	58	33	25	
Location[a]				
U	22	10	12	0.139
M L	58	37	21	
Differentiation[a]				
pap/tub	42	23	19	0.446
por/sig/muc	38	24	14	
Tumor size (mm)				
<60	52	32	20	0.491
≥60	28	15	13	
Lymphatic invasion[a]				
Ly0	18	9	9	0.394
Ly1-3	62	38	24	
Venous invasion[a]				
V0	21	13	8	0.732
V1-3	59	34	25	
pT factor[a]				
T1-2	32	23	9	0.049
T3-4	48	24	24	
pN factor[a]				
N0	27	19	8	0.128
N1-3	53	28	25	
pStage[a]				
I-II	49	31	18	0.303
III	31	16	15	
Recurrence				
Presence	23	11	12	0.849
Absence	57	36	21	

[a]According to the Japanese classification of gastric carcinoma, 15th edition (36). [b]p-Values are from Chi-squared test. U: Upper third; M: middle third; L: lower third; pap: Papillary adenocarcinoma; tub: tubular adenocarcinoma; por: poorly differentiated adenocarcinoma; sig: signet-ring cell carcinoma; muc: mucinous adenocarcinoma; Ly: lymphatic invasion; V: venous invasion.

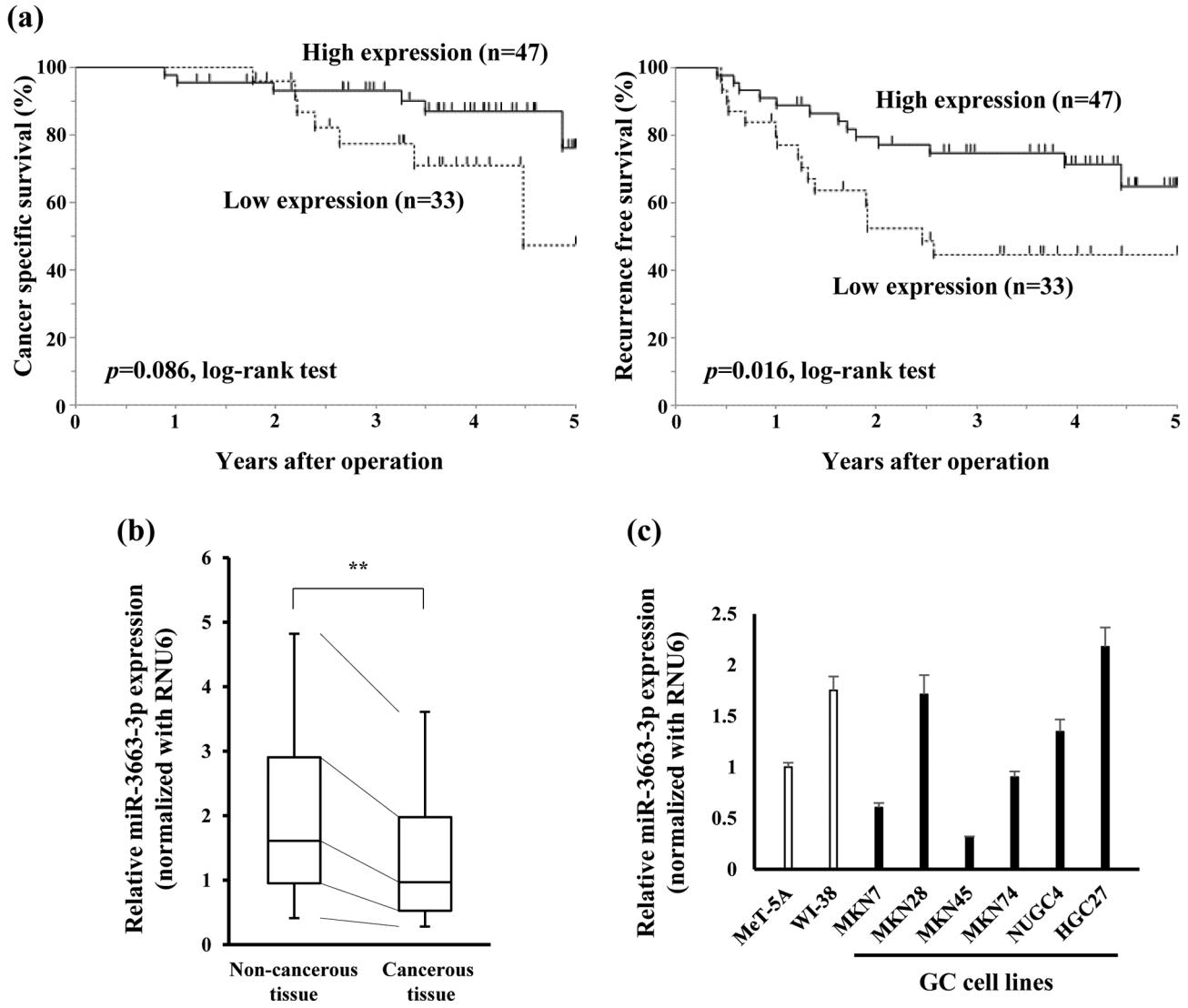

Figure 1. *Survival analysis and miR-3663-3p expression in gastric cancer tissue and cell lines. A) Cancer specific and recurrence free survival in relation to miR-3663-3p expression using the Kaplan–Meier method. Patients were divided into 'High' and 'Low' miR-3663-3p expression groups using a cut-off value determined with the minimum p-value method. B) Relative expression of miR-3663-3p in cancer and non-cancerous tissues (**p<0.01). C) Relative expression of miR-3663-3p in GC and non-cancer cell lines.*

was also suppressed by miR-3663-3p overexpression (Figure 2c, $p<0.001$). Cell cycle analysis using FACS revealed a higher proportion of cells in G0/ G1 phase when miR-3663-3p expression was increased (Figure 2d, $p<0.01$).

Analysis of downstream pathways regulated by miR-3663-3p. We next sought to identify potential mRNA targets of miR-3663-3p. We took a computational approach, using TargetScan Human 7.2, which predicts mRNA targets of specific miRNA. Using TargetScan, it was revealed that the 3'-UTR of *CCND1* gene contains a predicted miR-3663-3p binding sequence (Figure 3a). *CCND1* encodes the cell cycle regulatory protein Cyclin D1, which is involved in cell cycle initiation (13). *CCND1* mRNA expression was significantly reduced following transfection with the miR-3363-3p mimic (Figure 3b, $p<0.001$). Western blot analysis revealed that the levels of Cyclin D1 protein were also reduced by miR-3663-3p overexpression (Figure 3c and d). Furthermore, phosphorylation of Rb protein, a readout of Cyclin D1 activity, was also reduced with no change in Rb protein levels (Figure 3c and d). Taken together, these results show that miR-3663-3p downregulates *CCND1* and reduces the

Table II. *Recurrence free survival analyses*.

Variables	n=80	5-year RFS (%)	Univariate	Multivariate		
			p-Value[b]	HR	95% CI	*p*-Value[c]
Age						
<70	37	67.6	0.263			
≥70	43	60.5				
Gender						
Female	22	68.2	0.822			
Male	58	62.1				
Differentiation[a]						
pap/tub	42	64.3	0.767			
por/sig/muc	38	63.2				
Tumor size (mm)						
<60	52	71.2	0.117			
≥60	28	50.0				
Lymphatic invasion[a]						
Ly0	18	66.7	0.860			
Ly1-3	62	62.9				
Venous invasiona						
V0	21	85.7	0.025	1	0.83-10.47	0.114
V1-3	59	55.9		2.45		
pT factor[a]						
T1-2	32	81.3	0.006	-	-	-
T3-4	48	52.1				
pN factor[a]						
N0	27	88.9	0.001	-	-	-
N1-3	53	50.9				
pStage[a]						
I-II	49	79.6	0.0004	1	1.45-7.12	0.003
III	31	38.7		3.12		
miR-3663-3p expression in cancer tissue						
Low	33	51.5	0.016	2.31	1.09-5.02	0.029
High	47	72.3		1		

[a]According to the Japanese classification of gastric carcinoma (JCGC), 15th Edition (36). [b]*p*-Values in log-rank test. [c]*p*-Values in Cox's proportional hazard model. RFS: Recurrence-free survival; HR: Hazard ratio; CI: confidence interval; pap: papillary adenocarcinoma; tub: tubular adenocarcinoma; por: poorly differentiated adenocarcinoma; sig: signet-ring cell carcinoma; muc: mucinous adenocarcinoma.

phosphorylation of Rb protein (Figure 3e). Cyclin D1 modulates cell cycle entry in GC cells (14), thus we can practically assume that miR-3663-3p-dependent changes in Cyclin D1 levels alter the proliferative capacity.

Discussion

miRNAs play an important role in many normal biological processes and their expression is often de-regulated in diseases, such as cancer. The specific miRNA, miR-3663-3p, has not been characterized as yet. We sought to delineate its role in gastric cancer (GC). Our study identified decreased miR-3663-3p expression in gastric cancer tissue. Overexpression of miR-3663-3p decreased the proliferation, migration, and invasion abilities of GC cells *in vitro*, and identified miR-3663-3p as a target of the cell cycle regulatory factor, Cyclin D1, which gets down-regulated upon miR-3663-3p overexpression. Cyclin D1, along with its cognate cyclin-dependent kinase (CDK), regulates the phosphorylation of Rb family proteins (15). Indeed, miR-3663-3p overexpression led to decreased levels of phosphorylated Rb protein and a higher proportion of G0/G1 cells. To our knowledge, we have provided the first characterization of miR-3663-3p in gastric cancer.

A previous report on miR-3663-3p by Lili Ji *et al*., has shown that miR-3663-3p was tumorigenic and upregulated in papillary thyroid cancer cell lines and tissue (12). The lncRNA, Ribosomal protein L34 antisense RNA 1 (RPL34-AS1), inhibited proliferation and invasion of papillary thyroid cancer cells. RPL34-AS1 competitively binds miR-3663-3p and exerts this function by regulating the miR-3663-3p/ Regulator of G protein signaling 4 (RGS4) axis (12).

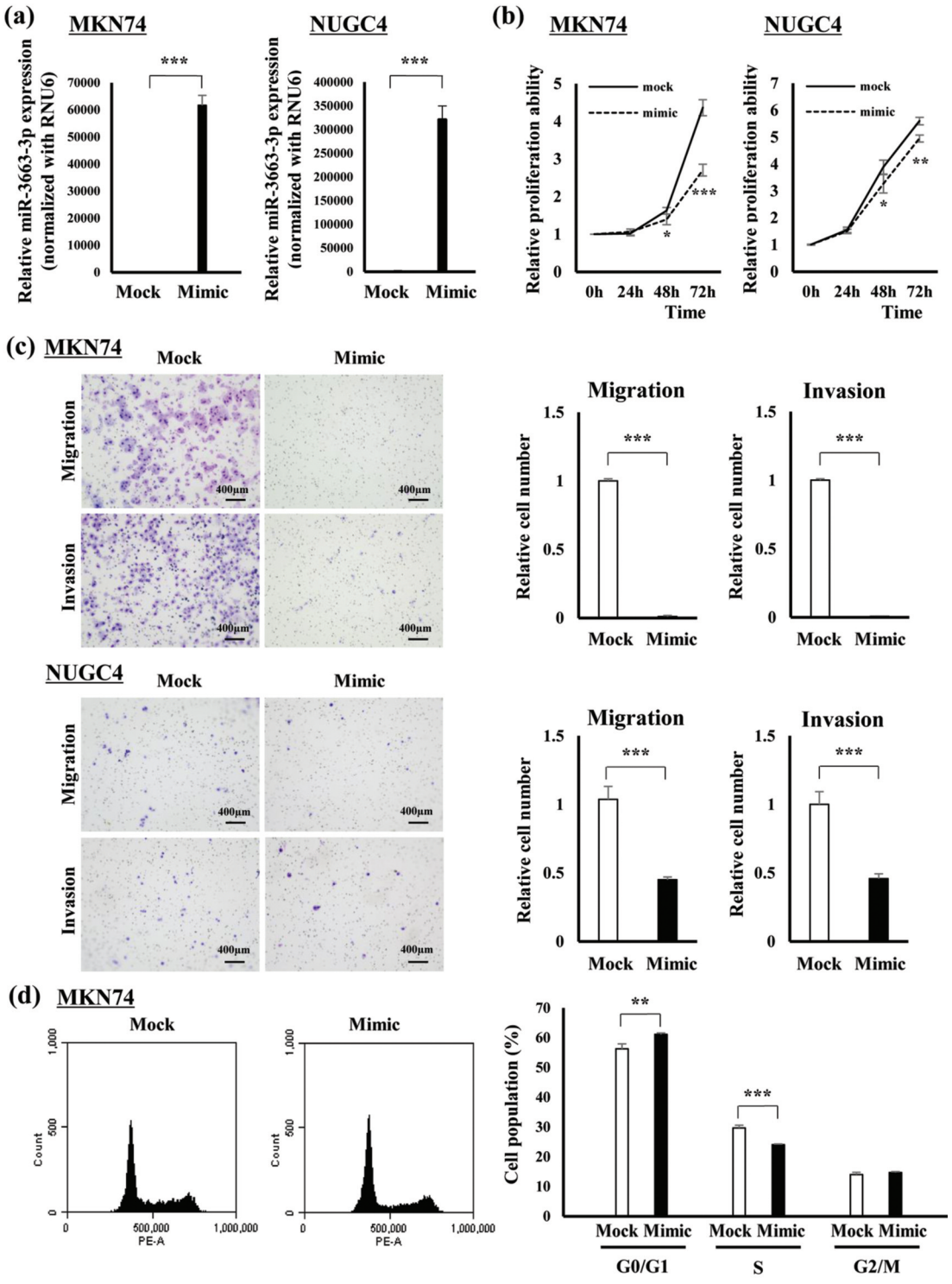

Figure 2. *Effects of miR-3663-3p overexpression in gastric cancer cell lines. A) miR-3663-3p expression measured by qRT- PCR in MKN74 and NUGC4 cells transfected with miR-3663-3p mimic is significantly increased in the bar graphs. The results are shown as a relative value. B) Proliferation assays of MKN74 and NUGC4 cells transfected with miR-3663-3p mimic. The results are shown as a relative value (N=4). C) Representative images of transwell migration and invasion assays of MKN74 and NUGC4 cells overexpressing miR-3663-3p. The cells were stained using crystal violet. The mean number of cells counted in random 5 fields is shown in the bar graphs. The results are shown as a relative value (N=3). D) Cell cycle analysis using Propidium iodide (PI) staining and Fluorescence Activated Cell Sorting (FACS) in MKN74 cells revealed a higher proportion of cells in G0/G1 phase overexpressing miR-3663-3p. The cell proportion in each phase is shown in the bar graph (N=3). *p<0.05; **p<0.01; ***p<0.001.*

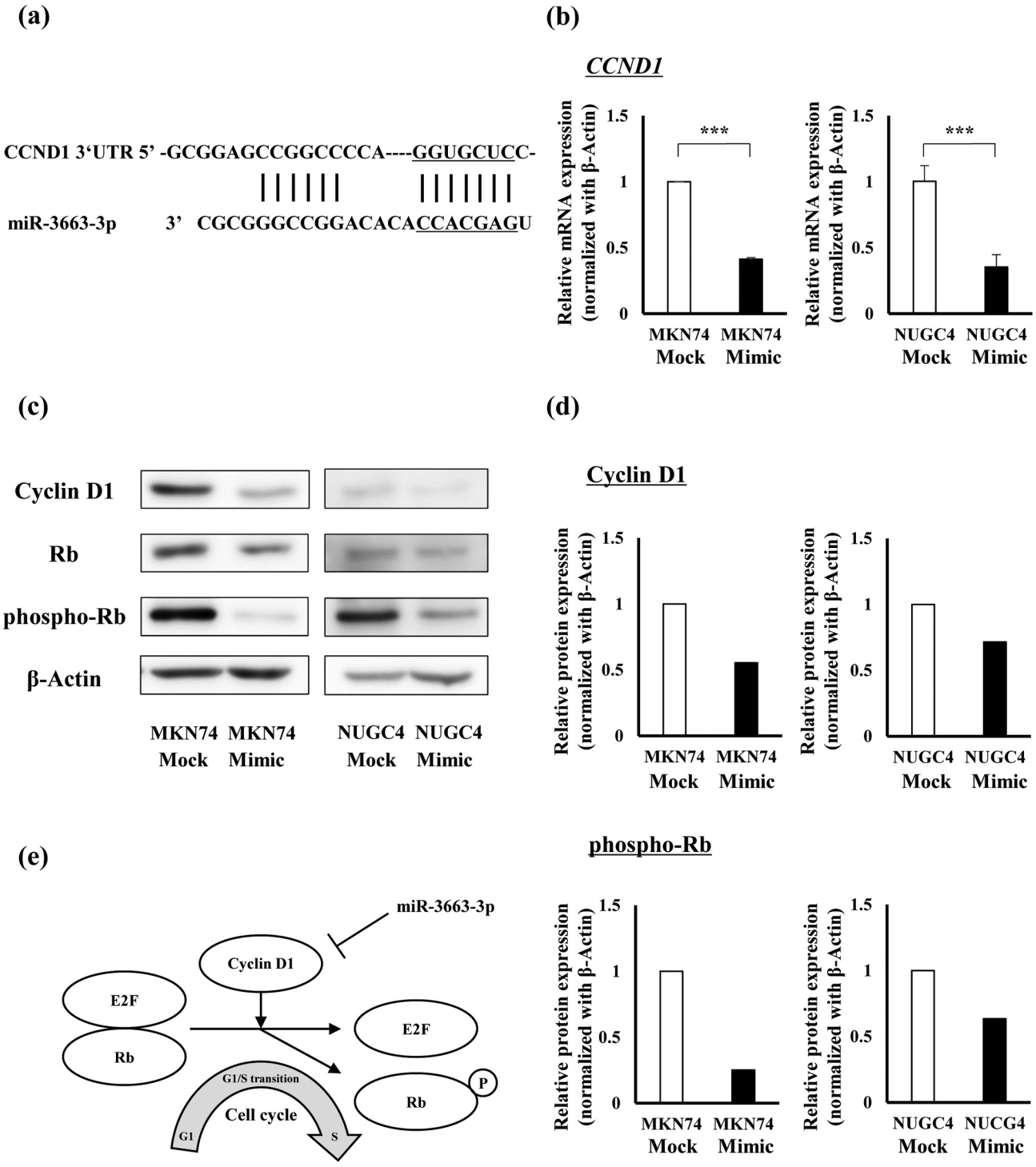

Figure 3. *The levels of cyclin D1 and phosphorylation of Rb protein regulated by miR-3663-3p expression. A) The predicted binding sequence for miR-3663-3p in the 3'-UTR of the CCND1 gene. B) CCND1 mRNA expression measured by qRT-PCR in MKN74 and NUGC4 cells transfected with miR-3663-3p mimic is significantly reduced following transfection with the miR-3363-3p mimic in the bar graphs ($p<0.001$). The results are shown as a relative value C) Expression of Cyclin D1, Rb, and phosphorylated Rb protein in MKN74 and NUGC4 cells overexpressing miR-3663-3p. Protein levels were assessed from total cell lysates by western blot for the proteins indicated. D) Cyclin D1 and phosphorylated Rb protein levels normalized by β-Actin levels are reduced by miR-3663-3p overexpression in the bar graphs. The results were shown as a relative value. E) A model of miR-3663-3p acting on the gastric cancer cell cycle. ***$p<0.001$.*

miRNAs play many diverse roles in diseases including cancer (16, 17), simultaneously promoting or suppressing cancer progression by regulating both tumor suppressive and tumorigenic mRNA molecules, respectively (18). More comprehensive mechanistic analyses of the biological roles of miR-3663-3p and its specific mRNA targets need to be performed to gain an understanding of its role in GC.

Cancer development is a highly orchestrated process requiring transcriptional and post-transcriptional regulation of gene expression by many non-coding RNAs, including miRNAs (19). Furthermore, miRNAs are currently being investigated as potential therapeutic targets in many cancers. and may be playing an important role in cancer diagnosis or even prognosis (20, 21). In this context, miR-3663-3p may represent an important miRNA with regards to its potential use for managing GC, as it seems to be involved in the suppression of this type of tumor.

The transition from G1 to S phase of the cell cycle is crucial for the control of eukaryotic cell proliferation, and abnormal cell cycle progression promotes oncogenesis (22). To this end, the first identified tumor suppressor gene, *Retinoblastoma* gene (Rb), acts as a switch to regulate the initiation of this exact transition (23-25). Overexpression of miR-3663-3p induced cell cycle arrest at the G0/G1 transition. Dysregulation of the Cyclin D1- Rb pathway is one of the most common alterations found in human cancers, including gastric cancer (13, 26), so that it could become a specific prognostic marker (27). Another molecule, miR-218 also negatively regulates cell cycle progression in GC by specifically targeting CDK6 and Cyclin D1 (28). Therefore, it could be assumed that the decreased levels of miR-3663-3p in GC may highlight an important role in the regulation of gastric cancer cell cycle progression.

Studies examining the efficacy of novel miRNA targeting therapies for cancers are currently underway (29, 30). For the realization of these treatments, safe and efficient drug delivery systems (DDS) and reliable tumor suppressive miRNAs are necessary. Regarding DDS, several types of molecules, such as a nano-peptides (31) and collagen derivatives (*e.g.*, atelocollagen) (32) have been described. In addition, several tumor suppressive miRNA molecules have shown their potential as therapeutic agents by limiting tumorigenicity (33-35). Further analysis of miR-3663-3p may shed light on its usefulness in cancer therapeutics or diagnosis in the future.

Our study provided a proof of context regarding the importance of miR-3663-3p in GC. Since our study was an initial characterization of miR-3663-3p in GC, there are many open questions to be addressed in the future. Due to the small patient cohort and limitations in sample storage it was not easy to make concrete conclusions about the clinical significance and the role of miR-3663-3p. Furthermore, we have not verified whether *CCND1* is a direct target of miR3663-3p. Although the mRNA and protein levels of CCND1 are decreased by miR3663-3p overexpression and the phosphorylation of Rb protein is decreased, there could be a mediator facilitating the observed effect. Nonetheless, miR-3663-3p expression appears to influence the Cyclin D1-Rb pathway and cell cycle initiation.

Taken together, miR-3663-3p is a clinically useful predictive factor for GC recurrence. Furthermore, our findings shed light on the tumor suppressive role of miR-3663-3p in limiting cell cycle entry by decreasing Cyclin D1 levels. More comprehensive analyses need to be performed to further elucidate the exact role of miR-3663-3p in gastric cancer.

References

1 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

2 Song Z, Wu Y, Yang J, Yang D and Fang X: Progress in the treatment of advanced gastric cancer. Tumour Biol *39(7)*: 1010428317714626, 2017. PMID: 28671042. DOI: 10.1177/1010428317714626

3 Cui Y, Yu S, Zhu M, Cheng X, Yu Y, Tang Z, Wang X, Hou J, Hou Y, Ren D, Mao B, Khalid R and Liu T: Identifying predictive factors of recurrence after radical resection in gastric cancer by RNA Immune-oncology Panel. J Cancer *11(3)*: 638-647, 2020. PMID: 31942187. DOI: 10.7150/jca.38536

4 Kozomara A and Griffiths-Jones S: miRBase: Annotating high confidence microRNAs using deep sequencing data. Nucleic Acids Res *42(Database issue)*: D68-D73, 2014. PMID: 24275495. DOI: 10.1093/nar/gkt1181

5 Jansson MD and Lund AH: MicroRNA and cancer. Mol Oncol *6(6)*: 590-610, 2012. PMID: 23102669. DOI: 10.1016/j.molonc.2012.09.006

6 Baek D, Villén J, Shin C, Camargo FD, Gygi SP and Bartel DP: The impact of microRNAs on protein output. Nature *455(7209)*: 64-71, 2008. PMID: 18668037. DOI: 10.1038/nature07242

7 Ueda T, Volinia S, Okumura H, Shimizu M, Taccioli C, Rossi S, Alder H, Liu CG, Oue N, Yasui W, Yoshida K, Sasaki H, Nomura S, Seto Y, Kaminishi M, Calin GA and Croce CM: Relation between microRNA expression and progression and prognosis of gastric cancer: A microRNA expression analysis. Lancet Oncol *11(2)*: 136-146, 2010. PMID: 20022810. DOI: 10.1016/S1470-2045(09)70343-2

8 Song F, Yang D, Liu B, Guo Y, Zheng H, Li L, Wang T, Yu J, Zhao Y, Niu R, Liang H, Winkler H, Zhang W, Hao X and Chen K: Integrated microRNA network analyses identify a poor-prognosis subtype of gastric cancer characterized by the miR-200 family. Clin Cancer Res *20(4)*: 878-889, 2014. PMID: 24352645. DOI: 10.1158/1078-0432.CCR-13-1844

9 Zhao X, Dou W, He L, Liang S, Tie J, Liu C, Li T, Lu Y, Mo P, Shi Y, Wu K, Nie Y and Fan D: MicroRNA-7 functions as an anti-metastatic microRNA in gastric cancer by targeting insulin-like growth factor-1 receptor. Oncogene *32(11)*: 1363-1372, 2013. PMID: 22614005. DOI: 10.1038/onc.2012.156

10 Liu H, Zhu L, Liu B, Yang L, Meng X, Zhang W, Ma Y and Xiao H: Genome-wide microRNA profiles identify miR-378 as

a serum biomarker for early detection of gastric cancer. Cancer Lett *316(2)*: 196-203, 2012. PMID: 22169097. DOI: 10.1016/j.canlet.2011.10.034
11 Pan HW, Li SC and Tsai KW: MicroRNA dysregulation in gastric cancer. Curr Pharm Des *19(7)*: 1273-1284, 2013. PMID: 23092346. DOI: 10.2174/138161213804805621
12 Ji L, Fan X, Zhou F, Gu J and Deng X: lncRNA RPL34-AS1 inhibits cell proliferation and invasion while promoting apoptosis by competitively binding miR-3663-3p/RGS4 in papillary thyroid cancer. J Cell Physiol *235(4)*: 3669-3678, 2020. PMID: 31587286. DOI: 10.1002/jcp.29256
13 Gao P, Zhou GY, Liu Y, Li JS, Zhen JH and Yuan YP: Alteration of cyclin D1 in gastric carcinoma and its clinicopathologic significance. World J Gastroenterol *10(20)*: 2936-2939, 2004. PMID: 15378768. DOI: 10.3748/wjg.v10.i20.2936
14 Yang K, Hitomi M and Stacey DW: Variations in cyclin D1 levels through the cell cycle determine the proliferative fate of a cell. Cell Div *1*: 32, 2006. PMID: 17176475. DOI: 10.1186/1747-1028-1-32
15 Kato J, Matsushime H, Hiebert SW, Ewen ME and Sherr CJ: Direct binding of cyclin D to the retinoblastoma gene product (pRb) and pRb phosphorylation by the cyclin D-dependent kinase CDK4. Genes Dev *7(3)*: 331-342, 1993. PMID: 8449399. DOI: 10.1101/gad.7.3.331
16 Li PF, Chen SC, Xia T, Jiang XM, Shao YF, Xiao BX and Guo JM: Non-coding RNAs and gastric cancer. World J Gastroenterol *20(18)*: 5411-5419, 2014. PMID: 24833871. DOI: 10.3748/wjg.v20.i18.5411
17 Wang J, Wang Q, Liu H, Hu B, Zhou W and Cheng Y: MicroRNA expression and its implication for the diagnosis and therapeutic strategies of gastric cancer. Cancer Lett *297(2)*: 137-143, 2010. PMID: 20797817. DOI: 10.1016/j.canlet.2010.07.018
18 Svoronos AA, Engelman DM and Slack FJ: OncomiR or tumor suppressor? The duplicity of microRNAs in cancer. Cancer Res *76(13)*: 3666-3670, 2016. PMID: 27325641. DOI: 10.1158/0008-5472.CAN-16-0359
19 Rupaimoole R and Slack FJ: MicroRNA therapeutics: Towards a new era for the management of cancer and other diseases. Nat Rev Drug Discov *16(3)*: 203-222, 2017. PMID: 28209991. DOI: 10.1038/nrd.2016.246
20 Cho WC: MicroRNAs: Potential biomarkers for cancer diagnosis, prognosis and targets for therapy. Int J Biochem Cell Biol *42(8)*: 1273-1281, 2010. PMID: 20026422. DOI: 10.1016/j.biocel.2009.12.014
21 Liu X, Fu Q, Du Y, Yang Y and Cho WC: MicroRNA as regulators of cancer stem cells and chemoresistance in colorectal cancer. Curr Cancer Drug Targets *16(9)*: 738-754, 2016. PMID: 26577538. DOI: 10.2174/1568009616666151118114759
22 Bertoli C, Skotheim JM and de Bruin RA: Control of cell cycle transcription during G1 and S phases. Nat Rev Mol Cell Biol *14(8)*: 518-528, 2013. PMID: 23877564. DOI: 10.1038/nrm3629
23 Friend SH, Bernards R, Rogelj S, Weinberg RA, Rapaport JM, Albert DM and Dryja TP: A human DNA segment with properties of the gene that predisposes to retinoblastoma and osteosarcoma. Nature *323(6089)*: 643-646, 1986. PMID: 2877398. DOI: 10.1038/323643a0
24 Lee WH, Bookstein R, Hong F, Young LJ, Shew JY and Lee EY: Human retinoblastoma susceptibility gene: cloning, identification, and sequence. Science *235(4794)*: 1394-1399, 1987. PMID: 3823889. DOI: 10.1126/science.3823889
25 Lee WH, Shew JY, Hong FD, Sery TW, Donoso LA, Young LJ, Bookstein R and Lee EY: The retinoblastoma susceptibility gene encodes a nuclear phosphoprotein associated with DNA binding activity. Nature *329(6140)*: 642-645, 1987. PMID: 3657987. DOI: 10.1038/329642a0
26 Arici DS, Tuncer E, Ozer H, Simek G and Koyuncu A: Expression of retinoblastoma and cyclin D1 in gastric carcinoma. Neoplasma *56(1)*: 63-67, 2009. PMID: 19152247. DOI: 10.4149/neo_2009_01_63
27 Kishimoto I, Mitomi H, Ohkura Y, Kanazawa H, Fukui N and Watanabe M: Abnormal expression of p16(INK4a), cyclin D1, cyclin-dependent kinase 4 and retinoblastoma protein in gastric carcinomas. J Surg Oncol *98(1)*: 60-66, 2008. PMID: 18484097. DOI: 10.1002/jso.21087
28 Deng M, Zeng C, Lu X, He X, Zhang R, Qiu Q, Zheng G, Jia X, Liu H and He Z: miR-218 suppresses gastric cancer cell cycle progression through the CDK6/Cyclin D1/E2F1 axis in a feedback loop. Cancer Lett *403*: 175-185, 2017. PMID: 28634044. DOI: 10.1016/j.canlet.2017.06.006
29 Hosseinahli N, Aghapour M, Duijf PHG and Baradaran B: Treating cancer with microRNA replacement therapy: A literature review. J Cell Physiol *233(8)*: 5574-5588, 2018. PMID: 29521426. DOI: 10.1002/jcp.26514
30 Bader AG, Brown D and Winkler M: The promise of microRNA replacement therapy. Cancer Res *70(18)*: 7027-7030, 2010. PMID: 20807816. DOI: 10.1158/0008-5472.CAN-10-2010
31 Xiao L, Ma N, He H, Li J, Cheng S, Yang Q, Hou Y, Song F, Jin H, Su X, Dong J, Zuo R, Song X, Duan W and Hou Y: Development of a novel drug targeting delivery system for cervical cancer therapy. Nanotechnology *30(7)*: 075604, 2019. PMID: 30523991. DOI: 10.1088/1361-6528/aaf3f8
32 Yuan Y, Makita N, Cao D, Mihara K, Kadomatsu K and Takei Y: Atelocollagen-mediated intravenous siRNA delivery specific to tumor tissues orthotopically xenografted in prostates of nude mice and its anticancer effects. Nucleic Acid Ther *25(2)*: 85-94, 2015. PMID: 25692652. DOI: 10.1089/nat.2014.0526
33 Ma M, Zhao J, Wu Q, Xiao K, Li S, Zhu H, Liu C, Xie H and Zuo C: MiRNA-545 negatively regulates the oncogenic activity of EMS1 in gastric cancer. Cancer Med *7(6)*: 2452-2462, 2018. PMID: 29733519. DOI: 10.1002/cam4.1520
34 Kalinowski FC, Brown RA, Ganda C, Giles KM, Epis MR, Horsham J and Leedman PJ: microRNA-7: A tumor suppressor miRNA with therapeutic potential. Int J Biochem Cell Biol *54*: 312-317, 2014. PMID: 24907395. DOI: 10.1016/j.biocel.2014.05.040
35 Wang Y, Liu C, Luo M, Zhang Z, Gong J, Li J, You L, Dong L, Su R, Lin H, Ma Y, Wang F, Wang Y, Chen J, Zhang J, Jia H, Kong Y and Yu J: Chemotherapy-induced miRNA-29c/catenin-δ signaling suppresses metastasis in gastric cancer. Cancer Res *75(7)*: 1332-1344, 2015. PMID: 25634213. DOI: 10.1158/0008-5472.CAN-14-0787
36 Japanese Gastric Cancer Association: Japanese Classification of Gastric Carcinoma. 15th ed. Tokyo: Kanehara Shuppan; 2017.

Received February 9, 2021
Revised March 10, 2021
Accepted March 19, 2021