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MicroRNA profiles following telmisartan treatment in pancreatic ductal adenocarcinoma cells

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Original Article

MicroRNA profiles following telmisartan treatment in pancreatic ductal adenocarcinoma cells

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ABSTRACT

Background: Pancreatic ductal adenocarcinoma (PDAC) is the most devastating of all cancers with an extremely poor prognosis. It has few effective and reliable therapeutic strategies. Telmisartan, a widely used antihypertensive drug, is an angiotensin II type 1 (AT1) receptor blocker (ARB). Telmisartan inhibits cancer cell proliferation, but the underlying mechanisms in PDAC, remain unknown.

Material and Methods: In the present study, we evaluated the effects of telmisartan on human PDAC cell proliferation *in vitro*. We assessed the effects of telmisartan on human PDAC cells using the cell lines PK-1 and PANC-1.

Results: Telmisartan inhibited the proliferation of these cells via blockade of the G0 to G1 cell cycle transition. This was accompanied by a strong decrease in cyclin D1. Telmisartan was also shown by receptor tyrosine kinase and angiogenesis arrays to reduce the phosphorylation of epidermal growth factor receptor (EGFR), and miRNA expression was markedly altered by telmisartan in PK-1 cells.

Conclusion: In conclusion, telmisartan inhibits human PDAC cell proliferation by inducing cell cycle arrest. Furthermore, telmisartan significantly altered miRNA expression *in vitro*. Taken together, our study demonstrated the therapeutic potential of telmisartan and provides molecular mechanistic insights into its anti-tumor effect on PDAC cells.

KEY WORDS: Angiotensin II type 1 receptor blocker, cell cycle arrest, microRNA, pancreatic ductal adenocarcinoma, telmisartan

INTRODUCTION

Pancreatic ductal adenocarcinoma (PDAC) is the eighth leading cause of cancer death in men and the seventh leading cause in women, worldwide.[1] AQ4 Pancreatic cancer has a rapid disease progression, which is accompanied by the absence of specific symptoms and largely precludes early diagnosis and curative treatment.[2,3] Therefore, the vast majority of patients are not diagnosed until after their cancer has metastasized. Surgery is the only curative treatment, but most patients present at an advanced stage because of late diagnosis, and only a minority (10%–20%) are amenable to surgical intervention.[4]

Angiotensin II type 1 (AT1) receptor blockers (ARBs) are widely used as an antihypertensive drug. Several types of cancer cells have recently been reported to express AT1 receptors (AT1Rs), such as hepatocellular carcinoma,[5-7] and several ARBs were shown to inhibit the angiogenesis of cancer cells expressing AT1Rs. Angiotensin II is associated with cancer progression, and ARBs inhibit tumor growth by antagonizing AT1Rs.[8-10] ARBs inhibit the growth of breast,[11] endometrial,[12] and gastric adenocarcinoma cells[13] *in vitro* and *in vivo*, but some studies revealed increased risk of cancer with ARBs.[14] Conversely, ARB treatment of hypertensive patients was associated with lower cancer incidence and mortality rate.[15,16]

Telmisartan is a widely prescribed ARB for the treatment of hypertension, heart failure, and chronic kidney diseases. It was shown to inhibit proliferation by inducing apoptosis in urological,[17,18] gynecologic,[12] and colorectal malignancies.[19] In addition, telmisartan induced cell cycle arrest in hematologic and nonhematologic malignancies.[20,21] A recent study revealed an association between telmisartan and inhibition of esophageal adenocarcinoma cell proliferation

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via cell cycle, growth factors, angiogenesis-related cytokines, and microRNA (miRNA) regulation.[21] However, little is known about whether telmisartan inhibits PDAC cell proliferation.

In this study, we evaluated the antiproliferative effect of telmisartan in pancreatic cancer cells and the mechanism of action. Our results revealed that telmisartan inhibited PDAC cell proliferation and induced cell cycle arrest and miRNA expression.

MATERIALS AND METHODS

Drugs, chemicals, and regents

Telmisartan and valsartan were purchased from Tokyo Chemical Industry Co. (Tokyo, Japan). Irbesartan and losartan were purchased from Wako Pure Chemical Industries (Osaka, Japan). Telmisartan was prepared as a 10 mM stock solution in dimethyl sulfoxide (DMSO). Irbesartan and valsartan were prepared as 100 mM stock solutions in DMSO, and losartan was prepared as a 100 mM stock solution in H_2O. The stock solutions were stored at −20°C.

Trypan blue was purchased from Sigma-Aldrich (St Louis, MO, USA), RPMI-1640 medium was from Gibco, (NY, USA), FBS was from Wako Pure Chemical Industries (Osaka, Japan), and penicillin–streptomycin was from Invitrogen (Tokyo, Japan). Other reagents include the cell counting kit-8 (CCK-8; Dojindo Laboratories, Kumamoto, Japan), cell cycle phase determination kit (Cayman Chemical, Ann Arbor, MI, USA), and protease inhibitor cocktail (Pro-Prep, complete protease inhibitor mixture; iNtRON Biotechnology, Seongnam, Korea).

Primary antibodies used for western blot analyses were obtained from the following sources. β-actin antibody was obtained from Sigma-Aldrich (St. Louis, MO, USA). Cyclin D1 antibodies were obtained from Thermo Fisher Scientific. Cyclin E, Cdk2, Cdk4, and Cdk6 antibodies were obtained from Santa Cruz Biotechnology (Santa Cruz, CA, USA). HRP-linked anti-mouse and anti-rabbit IgG secondary antibodies were obtained from Cell Signaling Technology, Danvers, MA, USA.

Cell lines and culture

The human PDAC cell lines PK-1 and PANC-1 were provided by the Japanese Cancer Research Resources Bank (Osaka, Japan). All cell lines were maintained at 37°C with 5% CO_2 in RPMI-1640 medium supplemented with 10% fetal bovine serum (FBS), 20 U/ml penicillin, and 100 μg/ml streptomycin.

Cell proliferation assays

The cell proliferation assay was performed as described previously.[21] Briefly, cell proliferation was assayed using the CCK-8 kit as per the manufacturer's instructions. Moreover, these assays were conducted in the PK-1 and PANC-1 cells. Then, 5.0×10^3 cells of each experiment group were equally seeded on 96-well plate and were treated as indicated for 48 h before cell proliferation assay. The absorbance was measured at 450 nm using a microplate reader.

Cell invasion/migration assays

PK-1 cells' invasion and migration were measured using CytoSelect 96-well cell invasion assay (basement membrane, colorimetric format; Cell Biolabs, San Diego, CA, USA) according to the manufacturer's instructions. Cells were treated with 0, 10, 50, 100 μM telmisartan and incubated for 48 h at 37°C in 5% CO_2. Invasive cells on the bottom of the membrane were stained and analyzed using a microplate reader.

Cell migration was measured using a CytoSelect Assay (Cell Biolabs, San Diego, CA, USA). Cells were treated with 0, 10, 50, and 100 μM telmisartan and incubated for 24 h at 37°C in 5% CO_2. Cells in the serum-free medium were dispensed inside the insert, while serum medium was placed into the well. Migratory cells traveled through the pores in the direction of the serum medium in the well and could be stained or measured on a plate reader. CyQUANT GR Dye solution was then added to the cells, and fluorescence was measured at 480/520 nm on a fluorescence plate reader. The migratory cells were quantified by measuring their absorbance and plotted against treatment concentrations to determine the effect of telmisartan on cell migration.

Cell cycle analyses

Cell cycle analyses were performed in accordance with our previously described methods.[21] Briefly, PK-1 and PANC-1 cells were collected and seeded into 100-mm culture dishes at 1.0×10^6 per dish and then maintained for 24 h. Next, cells were treated with 100 μM telmisartan for 24–48 h. Cell cycle was determined by flow cytometry using Cytomics FC 500 flow cytometer (Beckman Coulter, Indianapolis, IN, USA), according to the manufacturer's protocol. The percentage of cells was analyzed using Kaluza software (Beckman Coulter, Indianapolis, IN, USA). All experiments were performed in triplicate.

Western blotting analysis

Gel electrophoresis and western blotting were performed as previously described.[21] Briefly, proteins were subjected to sodium dodecyl sulfate polyacrylamide gel electrophoresis and then transferred to nitrocellulose membranes. After blocking in 5% dried skimmed milk in 0.05% Tween-20/Tris-buffered saline (TBS) buffer, the membranes were incubated with primary antibodies followed by peroxidase-conjugated secondary antibodies in 5% dried skimmed milk in 0.05% Tween-20/TBS buffer. Proteins were visualized on X-ray film using an enhanced chemiluminescence detection system (PerkinElmer Co., Waltham, MA, USA).

Analysis of phosphorylated receptor tyrosine kinases and angiogenesis-related protein profiles using an antibody array

The human phosphorylated receptor tyrosine kinase (p-RTK) and angiogenesis antibody array was performed as described previously.[21] A Human p-RTK and Human Angiogenesis Antibody Array (R&D Systems, Minneapolis, MN, USA) was

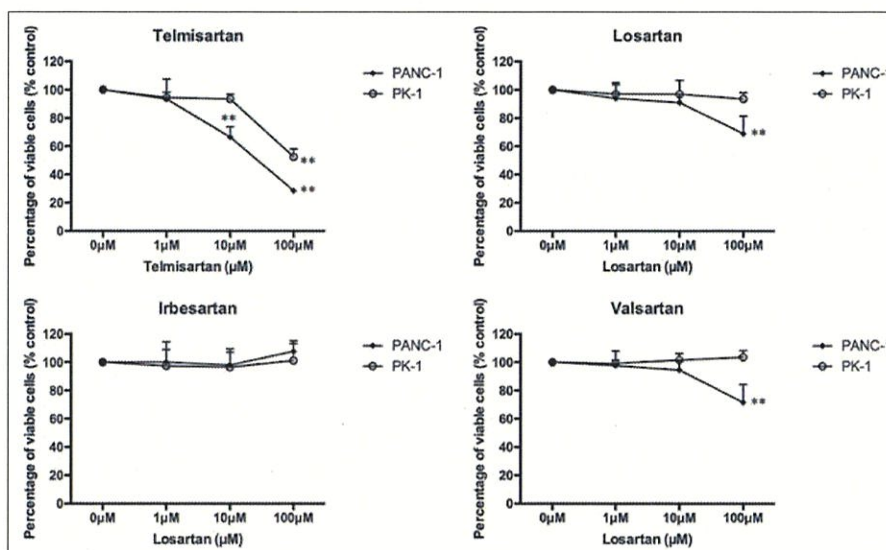

Figure 1: The effects of the angiotensin II type 1 receptor blockers telmisartan, irbesartan, losartan, and valsartan on the proliferation of pancreatic ductal adenocarcinoma cell lines *in vitro*. Telmisartan suppresses the proliferation of pancreatic ductal adenocarcinoma cells. PK-1 and PANC-1 cells were seeded in 96-well plate. After 24 h, angiotensin II type 1 receptor blocker (telmisartan, irbesartan, losartan, and valsartan; 1, 10, and 100 μM) or vehicle was added to the culture medium; 24 h later, the cells were evaluated with cell counting kit-8 assays. Cell viability was assayed daily from 0 to 48 h. The viability of the angiotensin II type 1 receptor blocker-treated cells was significantly different from that of the control cells (**$P < 0.01$)

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Table 1: Statistical results and chromosomal locations of miRNAs in PK-1 cells treated with and without telmisartan

miRNA	Fold change (treated/untreated)	*P*	Chromosomal localization
Upregulated			
hsa-miR-6088	2.30	0.0064	19q13.32
hsa-miR-205-3p	2.12	0.0022	1q32.2
hsa-miR-210-3p	1.98	0.0049	11p15.5
hsa-miR-7150	1.72	0.0152	9q33.3
hsa-miR-100-5p	1.69	0.0050	11q24.1
hsa-miR-99a-5p	1.67	0.0063	21q21.1
hsa-miR-125b-5p	1.65	0.0050	11q24.1
hsa-miR-203a-3p	1.59	0.0022	14q32.33
hsa-miR-19a-3p	1.57	0.0048	13q31.3
miRNA	**Fold change (treated/untreated)**	***P***	**Chromosomal localization**
Downregulated			
hsa-miR-934	0.49	0.0022	Xq26.3
hsa-miR-194-5p	0.53	0.0050	1q41
hsa-miR-192-5p	0.58	0.0080	11q13.1
hsa-miR-196a-5p	0.60	0.0022	17q21.32
hsa-miR-542-3p	0.62	0.0450	Xq26.3
hsa-miR-34a-5p	0.63	0.0022	1p36.22
hsa-miR-769-5p	0.65	0.0022	19q13.32
hsa-miR-93-3p	0.65	0.0043	7q22.1

FC: >1.5, FC<0.67, P<0.05. FC: Fold change

used as per the manufacturer's protocol. This method is a dot-based assay that enables detecting and comparing 49 human p-RTKs and 55 angiogenesis-specific cytokines. Each array membrane was exposed to the X-ray film using a chemiluminescence detection system (PerkinElmer Co.).

MicroRNA arrays

MiRNA array analysis was performed as described in our previous study.[21] Briefly, total RNA was isolated using a miRNeasy Mini Kit (Qiagen, Hilden, Germany) from PK-1 cells according to the manufacturer's instructions. MiRNA expression analysis was performed using the miRCURY Hy3/Hy5 Power Labeling Kit and human miRNA Oligo chip (v. 21.0; Toray Industries, Tokyo, Japan). The arrays were scanned in a 3D-Gene Scanner 3000 (Toray Industries), and these fluorescence images were analyzed using the 3D-Gene extraction version 1.2 software (Toray Industries).

Statistical analyses

Results were expressed as the mean ± standard deviation. All statistical analyses were performed using GraphPad Prism 6 software (GraphPad Software, La Jolla, CA, USA). The nonparametric Wilcoxon/Mann–Whitney U-test was used to examine the statistical significance between the two groups, while the Kruskal–Wallis test was performed to analyze multiple comparisons. $P < 0.05$ was considered statistically significant.

RESULTS

In vitro telmisartan treatment inhibits the proliferation and invasion of human pancreatic ductal adenocarcinoma cells

We examined the antitumor effects of telmisartan, irbesartan, losartan, and valsartan in two human PDAC cell lines, PK-1 and PANC-1 *in vitro*. Cells were grown in 10% FBS and treated with 0, 1, 10, or 100 μM of the four ARBs (telmisartan, irbesartan, losartan, and valsartan) for 48 h. Telmisartan treatment reduced the proliferation of PK-1 and PANC-1 cells after 48 h

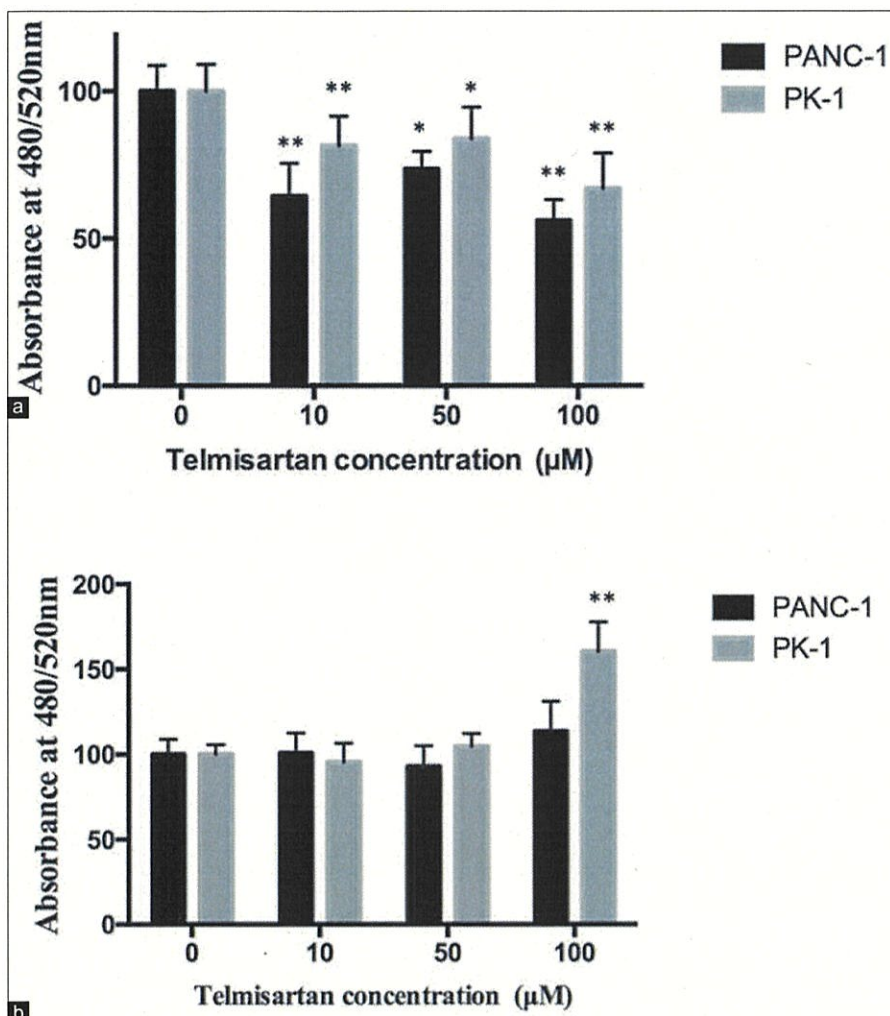

Figure 2: Testing the effects of telmisartan on PK-1 and PANC-1 cells' invasion and migration. (a) We used a transwell invasion assay to measure the invasion of pancreatic ductal adenocarcinoma cells after treatment with various concentrations of telmisartan for 48 h (×100). (b) Transwell migration assays were used to test pancreatic ductal adenocarcinoma cell migration. Pancreatic ductal adenocarcinoma cells seeded in the upper chambers treated with telmisartan for 24 h. Data expressed as means ± standard deviation for six replicates. The absorbance of the angiotensin II type 1 receptor blocker-treated cells was significantly different from that of the control cells (*$P < 0.05$, **$P < 0.01$)

treatment [Figure 1] in a dose-dependent manner. None of the other ARBs (irbesartan, losartan, and valsartan) affected the viability of the both PDAC cell lines [Figure 1].

Next, we used a transwell invasion assay to measure the invasion ability of the PDAC cells. After various concentrations of telmisartan, the number of invasive PDAC cells was significantly lower [Figure 2a]. On the other hand, telmisartan did not reduce the number of PDAC cells 24 h after treatment [Figure 2b]. These results demonstrated that telmisartan strongly inhibits cell proliferation and invasion in the two PDAC cell lines.

Telmisartan induces cell cycle arrest in G_0/G_1 phase and regulates cell cycle-related proteins in pancreatic ductal adenocarcinoma cells

We further investigated the effects of telmisartan on the cell cycle of PK-1 and PANC-1 cells using flow cytometry analysis [Figure 3a]. When PK-1 and PANC-1 cells were incubated with 100 µM telmisartan, the number of cells at S and G_2/M phases increased and the number of cells at G_0/G_1 phases decreased compared with the control group [Figure 3b]. Furthermore, the expression levels of cyclin D1 decreased in the PK-1 and PANC-1 cells, whereas levels of Cdk4 and Cdk6, which are the catalytic units of cyclin D1, decreased in the PK-1 cells 24–48 h after telmisartan treatment [Figure 4]. These results indicate that PK-1 cell growth is suppressed by telmisartan treatment through impaired cell cycle progression.

Association between telmisartan and phosphorylated receptor tyrosine kinases in PK-1 cells

To identify key p-RTKs affected by telmisartan, a human RTK array was applied to PK-1 cells [Figure 5a]. Markedly

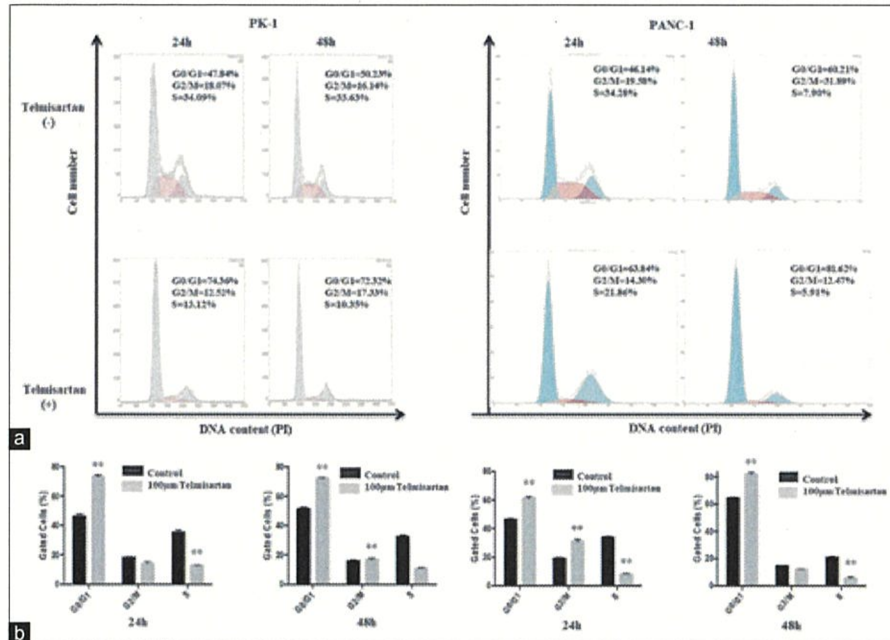

Figure 3: The antiproliferative effects of telmisartan in pancreatic ductal adenocarcinoma cells are mediated via cell cycle arrest. (a) Cell cycle analysis of PK-1 and PANC-1 cells treated with 100 μM telmisartan at 24 and 48 h. (b) Histograms showing the percentage of both cells in G_0/G_1, S, and G_2/M phases (**P* < 0.05, ***P* < 0.01)

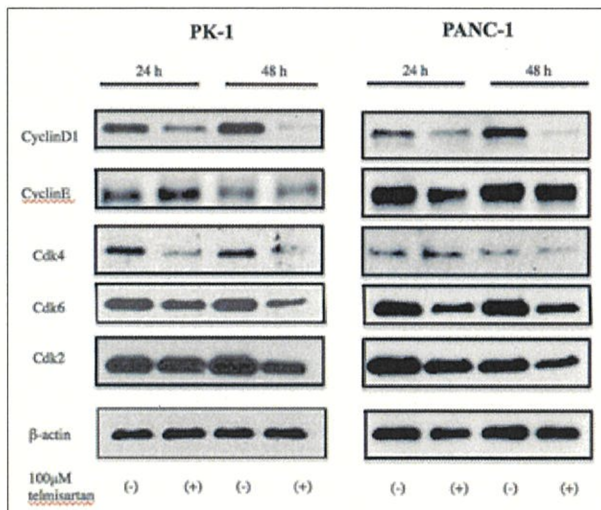

Figure 4: Western blot analysis of cyclin D1, Cdk4, Cdk6, Cdk2, and cyclin E in PK-1 and PANC-1 cells treated with 100 μM telmisartan

decreased level of phosphorylated epidermal growth factor receptor (EGFR) was observed in the telmisartan-treated PK-1 cells compared with controls [Figure 5b]. Quantitative results are shown in Figure 5c.

Association between telmisartan and angiogenesis in PK-1 cells

To identify the angiogenesis factors affected by telmisartan, a human angiogenesis array kit was used to screen PK-1 cells [Figure 6a]. The expression levels of angiogenesis-related protein were not changed by telmisartan treatment in PK-1 cells as detected by the protein array [Figure 6b].

miRNA expression signatures differentiate between telmisartan-treated and -nontreated PK-1 cells

We next examined the expression levels of 2555 miRNAs in the PK-1 cells treated with or without 100 μM telmisartan. After normalization and removing miRNAs with missing values, 36 miRNAs underwent hierarchical clustering [Figure 7]. Of these, 13 were significantly upregulated and the other 23 were downregulated [Table 1].

DISCUSSION

In the present study, telmisartan induced cell cycle arrest at the G_0/G_1 phase by modulating the expression of cell cycle regulatory proteins in the PDAC cells. Our flow cytometric analyses demonstrated that telmisartan significantly induced cell cycle arrest in the PK-1 and PANC-1 cells. However, telmisartan did not increase the proportion of apoptotic cancer cells 48 h after treatment in the PK-1 cells (data not shown), indicating that it inhibited PDAC cell proliferation without inducing apoptosis. These findings were further corroborated by an analysis of cell cycle-related proteins. A substantial reduction was observed in cyclin D1 and Cdks in both cell lines following telmisartan treatment. Cdk4 and Cdk6 complexes with cyclin D1 are required for G_1 phase progression, whereas Cdk2 complexes with cyclin E are required for the G_1–S transition.[22] The expression of various cell cycle-related proteins, including cyclin D1 and Cdks,

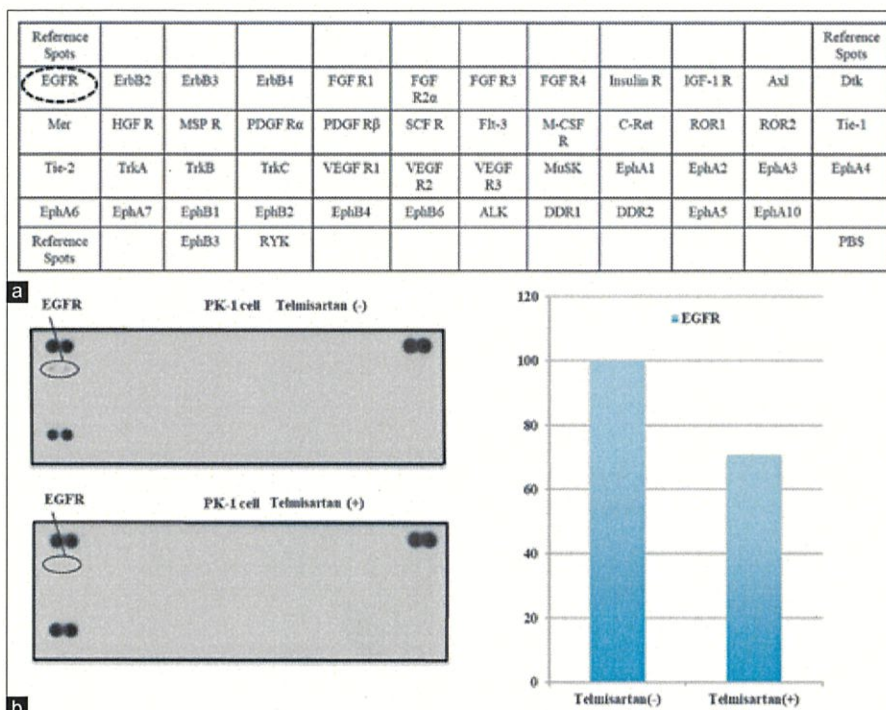

Figure 5: Effects of telmisartan on phosphorylated receptor tyrosine kinase in PK-1 cells. (a) The template indicates the locations of tyrosine kinase antibodies spotted onto a human phosphorylated receptor tyrosine kinase array. (b) Representative expression of various phosphorylated tyrosine kinase receptors in PK-1 cells treated with or without 100 μM telmisartan at 48 h. (c) Densitometry indicated that the ratios of phosphorylated epidermal growth factor receptor spots of telmisartan-treated to -untreated cells was 70.7%, respectively

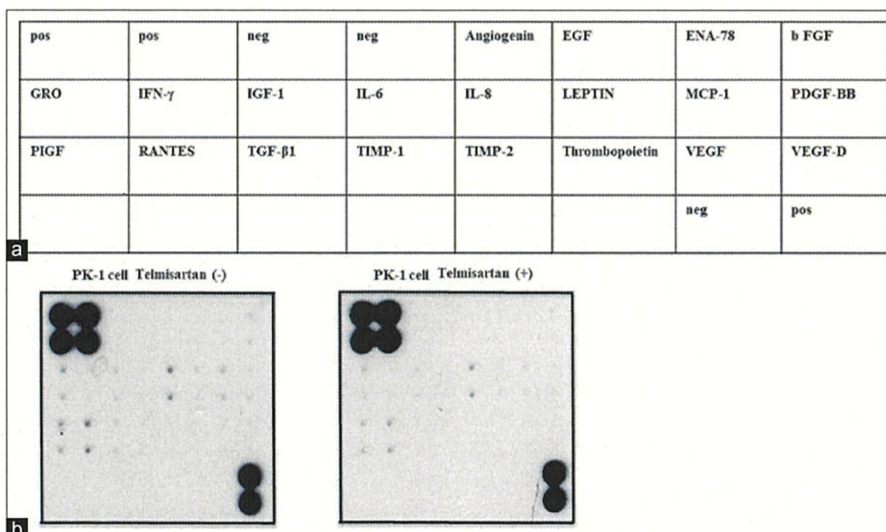

Figure 6: Effects of telmisartan on angiogenesis in PK-1 cells. (a) Template showing the locations of angiogenesis-related proteins spotted onto a human angiogenesis array. (b) Representative expression levels of various angiogenesis-related proteins in PK-1 cells cultured with or without telmisartan

is associated with cancer prognosis.[22,23] Moreover, clinical pathological studies showed that cyclin D1 overexpression correlates with tumor metastasis and prognosis in a series of human cancers.[24,25] These data indicate that major cell cycle regulators such as cyclin D1 are intracellular targets of telmisartan in the human PDAC cell lines. Therefore, inhibition of these molecules could be a promising strategy for controlling PDAC.

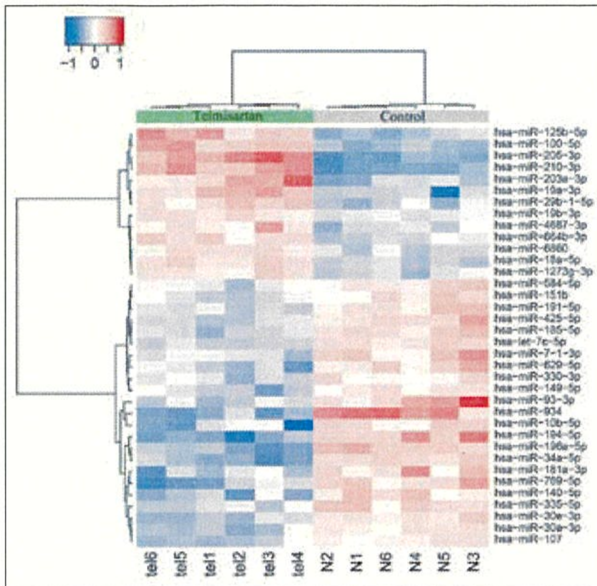

Figure 7: Hierarchical clustering of PK-1 cells cultured with or without telmisartan according to the expression profiles of numerous differentially expressed microRNAs. The analyzed samples are shown in columns, while microRNAs are presented in rows. The microRNA clustering color scale indicates relative microRNA expression levels, with red and blue representing high and low expression levels, respectively

Since the discovery of these proteins, RTKs have been investigated as key regulators of the proliferation, differentiation, and metastasis of cancer cells.[26] The overexpression of EGFR, vascular EGFR, and insulin-like growth factor receptor through mutations or loss-of-pathway regulation is associated with cellular proliferation, anti-apoptosis, and angiogenesis.[27] The EGFR family and their ligands are frequently overexpressed in pancreatic cancer,[28] and EGFR activity correlates with patient prognosis.[29] In the present study, telmisartan reduced the expression levels of phosphorylated EGFR in the PK-1 cells according to the p-RTK array. However, the 20 screened angiogenesis molecules showed no change after treatment with telmisartan in the PDAC lines. Therefore, these results suggest that the antitumor effect of telmisartan may be caused, at least in part, to its reduction of EGFR family phosphorylation, which plays an important role in cancer cell proliferation.

MiRNAs are endogenous mediators of gene expression through their site-specific binding at the 3′ untranslated region or other sites of mRNAs to either degrade their target or inactivate protein synthesis. MiRNAs regulate many biological processes such as cancer cell proliferation, tumor growth, differentiation, apoptosis, and energy metabolism.[30] We identified miRNAs associated with the antitumor effects of telmisartan in the PK-1 cells using miRNA expression arrays. The expression of numerous miRNAs was altered following telmisartan treatment of PK-1 cells, including miR-210-3p which was significantly upregulated in the PK-1 cells treated with telmisartan. miR-210 is recognized as a tumor suppressor through its modulation of tumor growth, apoptosis, and regulation of epithelial-to-mesenchymal transition in PDAC.[31] Upregulation of the miR-210 family has also been reported in pancreatic cancer,[30] while Yang *et al.* found that miR-210-3p suppresses cell proliferation and metastasis in bladder cancer by regulating fibroblast growth factor receptor like-1.[32] Based on previous studies, our data suggest that the altered miRNAs may result from the antitumor effects of telmisartan.

There are some limitations in our current study. First, the inhibition effects of telmisartan on the PK-1 and PANC-1 cells are optimized only for *in vitro* growth and proliferation. Second, miRNA array analysis is a preliminary study *in vitro* model. Third, our *in vitro* study was conducted using a higher dose of telmisartan than that used in human treatments (1–10 μM).[33] As such, it is actually impossible to translate these conditions in clinical setting, especially when the pharmacokinetics of the drug is different in culture setting and in the human body.

CONCLUSION

Our results show that telmisartan inhibits human PDAC cell proliferation by inducing apoptosis and cell cycle arrest through regulating cell cycle-related proteins.

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Nil.

Conflicts of interest

There are no conflicts of interest.

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Author Queries???

AQ1: Kindly check the table formation.

AQ4: Please check whether the original intended sense of the text is retained after all edits.