Antitumor effects of metformin via indirect inhibition of protein phosphatase 2A in patients with endometrial cancer
(メトホルミンは子宮体癌組織で間接的に PP2A を抑制し抗腫瘍効果を発揮する)

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ABSTRACT

Objective. Metformin, an antidiabetic drug, inhibits the endometrial cancer cell growth *in vivo* by improving the insulin resistance; however, its mechanism of action is not completely understood. Protein phosphatase 2A (PP2A) is a serine/threonine phosphatase associated with insulin resistance and type 2 diabetes, and its inhibition restores the insulin resistance. This study investigated the antitumor effect of metformin on endometrial cancer with a focus on PP2A.

Material and methods. Metformin (1,500–2,250 mg/day) was preoperatively administered to patients with endometrial cancer for 4 to 6 weeks. Expression of the PP2A regulatory subunits, 4 (PPP2R4) and B (PP2A-B), was evaluated by real-time polymerase chain reaction (RT-PCR) and immunohistochemistry (IHC) using paired specimens obtained before and after metformin treatment. The effect of PPP2R4 inhibition with small interfering RNA was evaluated in the endometrial cancer cell lines HEC265 and HEC1B. *P* values of < .05 were considered statistically significant.

Results. Preoperative metformin treatment significantly reduced the expression of PP2A-B, as determined by IHC, and the mRNA expression of PPP2R4, as determined by PT-PCR, in the patients with endometrial cancer. However, metformin could not directly alter the PPP2R4 mRNA levels in the endometrial cancer cell lines *in vitro*. PPP2R4 knockdown reduced the proliferation and induced the apoptosis by activating caspases 3/7 in HEC265 and HEC1B cells.

Conclusions. Downregulation of the PP2A-B subunit, including PPP2R4, is an important indirect target of metformin. Inhibition of PP2A may be considered a target for the treatment of endometrial cancer patients with insulin resistance.

1.INTRODUCTION

Endometrial cancer (EC) is the most common gynecological malignancy[1,2]. Among the various cancers, EC has the strongest association with obesity. Insulin resistance and type 2 diabetes mellitus caused by obesity are recognized as risk factors of endometrial cancer[2–5]. Improvement of insulin resistance and of abnormal glucose metabolism has been considered a preventive and therapeutic target.

Metformin, an oral biguanide antihyperglycemic drug, is widely prescribed as a first-line therapy against type 2 diabetes mellitus[6]. Besides its effectiveness in diabetes treatment, several population studies have identified additional benefits of metformin, including the metformin-induced decrease in cancer incidence and cancer-related mortality in patients with diabetes. In addition, many *in vitro* studies have shown that metformin exerts antineoplastic effects on various types of cancer cells. However, the metformin concentration used in *in vitro* studies was much higher than the established *in vivo* concentration of orally administered metformin, thus raising questions as to whether such *in vitro* antitumor effects are clinically relevant. As of this point, window of opportunity studies have revealed that the antidiabetic dose of metformin causes growth inhibition in breast cancer and EC *in vivo*. This effect of metformin is likely due to an indirect effect of altering an endocrine metabolic factor; however, the precise mechanism of the anticancer efficacy of metformin has not been elucidated yet. We have previously reported that preoperative metformin treatment significantly reduced the expression of the Ki-67 protein and topoisomerase IIα in EC[7]. This result has been supported by several other reports[8,9] confirming that metformin reduces the tumor proliferation in type 1 EC tissues. However, the mechanism is not clearly understood.

Protein phosphatase 2A (PP2A) is a major serine/threonine phosphatase cells and possessing diverse functions. Activation of PP2A is associated with insulin resistance and type 2 diabetes[10,11], whereas inhibition of PP2A results in enhanced glucose homeostasis and increased insulin sensitivity[12]. PP2A has also been known as a tumor suppressor[13]. Okadaic acid, a potent inhibitor of PP2A, and several endogenous PP2A inhibitors have been shown to promote the malignant growth of human cancers[14,15]. However, other PP2A inhibitors, such as cantharidin and norcatharidin, have been found to repress the invasion of cancer cells and have the ability to induce apoptosis of cancer cells[14,16,17]. In addition, LB-100, a small-molecule inhibitor of PP2A, sensitizes ovarian cancer cells to cisplatin *in vitro* and *in vivo*[18].

In the present study, we investigated the antitumor effect of metformin and its relationship to PP2A in patients with EC. An antidiabetic therapeutic dose of metformin was found to indirectly inhibit the EC cell growth *in vivo* and reduce the PP2A expression. Furthermore, we focused on the PP2A regulatory subunit 4 (PPP2R4), which is required for PP2A regulation[19–22]. We showed that the inhibition of PPP2R4 reduced the EC cell activity.

2.PATIENTS AND METHODS

2.1.Patients

Fourteen patients with endometrioid carcinoma ,who were treated with metformin preoperatively, were included in this study. All the patients were recruited for our previous study, which was registered with the University Hospital Medical Information Network (UMIN) Clinical Trial Registry (UMIN 000004852), and provided re-consent for this

study. The eligibility criteria were those included in the Eastern Cooperative Oncology Group performance status of 0 to 1, as well as normal renal, liver, and cardiac function. The exclusion criteria were as follows: 1) type 2 diabetes requiring medication; 2) a history of metformin use; 3) an abnormal blood coagulation profile and/or a history of thromboembolism; and 4) the presence of mental or life-threatening illnesses.

Immunohistochemistry (IHC) was performed on samples from 14 patients and RNA was isolated from samples collected from six patients. As an IHC control, EC specimens embedded in paraffin blocks were retrospectively collected from 10 patients with endometrioid carcinoma, who underwent surgery at the Chiba University Hospital but did not receive metformin. We also used normal endometria of six women who agreed to participate as a control of PPP2R4 mRNA expression in this study. The six women with normal endometria had no medical history of EC and no obesity.

Detailed characteristics of the patients are shown in Table 1.

2.2.Study Design

The primary objective of the present study was to evaluate the relationship between PP2A regulation and the growth inhibitory effect of metformin in EC tissues. The secondary objective was to evaluate the effect of PPP2R4 inhibition on EC cells *in vitro*. Metformin (initial dose, 750 mg/day; increased weekly up to 1,500 or 2,250 mg/day) was administered for 4–6 weeks until the day of scheduled surgery. Tissue specimens were obtained via endometrial curettage at the time of initial diagnosis (before treatment) and hysterectomy (after treatment). Changes in PP2A expression were determined by IHC and real-time polymerase chain reaction (PCR) using paired endometrial tissue specimens.

2.3.Cell Lines, and Cell Culture and reagents

Type 1 EC model cell lines, HEC265 and HEC-1B, were cultured in Dulbecco's modified Eagle's medium (Gibco, Life Technologies Corporation, Carlsbad, CA, USA) containing 4.5 g/L glucose, 10% fetal bovine serum (FBS; Sigma–Aldrich, St. Louis, MO, USA), 100 U/mL penicillin, and 100 μg/mL streptomycin at 37 °C in an atmosphere of 5% CO_2. The HEC265 and HEC-1B cell lines were purchased from the JCRB Cell Bank (Osaka, Japan). Antibodies for PP2A subunit B (PP2A-B; #4953), phosphotyrosyl phosphatase activator/PPP2R4 (#3330), phospho-p44/42 mitogen-activated protein kinase (MAPK) [extracellular signal-regulated kinase (ERK1/2), Thr202/Tyr204; #4370], p44/42 MAPK (ERK1/2; #9102), phospho-protein kinase B (AKT, Thr308; #2965), AKT (#4691), and β-actin (#4967) were all purchased from Cell Signaling Technology, Inc. (Danvers, MA, USA). PPP2R4-targeting SMART pool small interfering RNA (siRNA) (siPPP2R4) and non-targeting siRNA (siControl) were purchased from Dharmacon (Lafayette, CO, USA). Metformin was from Sigma–Aldrich Chemistry (Germany).

2.4.Immunohistochemical Analysis

PP2A in EC tissues was evaluated by IHC staining of PP2A-B. Tissue sections (3-μm thick) were briefly microwaved in 10 mM citrate buffer (pH 6.0) and then immunostained for PP2A-B. The EnVision FLEX mini kit (K8000; Dako Denmark A/S) and an autostainer S3400 (Dako Denmark A/S) were used to visualize immunostaining. The tissue sections were incubated with the primary antibody (dilution 1:100) at room temperature for 60 min. Then, the secondary antibody [EnVision FLEX/horseradish peroxidase (HRP); Dako

Denmark A/S] was added, followed by incubation at room temperature for 60 min, and 3,3′ -diaminobenzidine tetrahydrochloride (Dako Denmark A/S) was used as a chromogen. The samples were counterstained with hematoxylin. The stained samples were observed and graded semiquantitatively as negative, weakly positive, moderately positive, and highly positive.

Paired specimens obtained at the time of preoperative biopsy and at surgery were stained for PP2A-B. Expression of PP2A was compared between pre- and postoperative tissues with or without metformin administration. The results of PP2A-B IHC were evaluated using immunoreactivity scores (IRSs)[23,24] calculated as follows: IRS = Staining intensity × percentage of positive cells. The staining intensity was categorized as follows: 0 (negative), 1 (weak), 2 (moderate), and 3 (strong). Positive cells were categorized as follows: 0 (negative), 1 (< 10%), 2 (10–50%), 3 (50–80%), and 4 (80–100%). The maximum IRS = 3 × 4 = 12.

2.5.RNA Isolation, Reverse Transcription, and Real-Time PCR

Total RNA was extracted from cells using the RNeasy mini kit (Qiagen, Germany). RNA samples were obtained from six patients because only six paired samples were saved. RNA concentrations were determined using a NanoDrop 1000 (Thermo Fisher Scientific, USA). cDNA was synthesized using the Superscript VILO cDNA synthesis kit (Invitrogen, USA) according to the manufacturer's protocol. Absolute transcript levels were quantified using Light Cycler FastStart DNA Master SYBR Green I (Roche, USA) in a Lightcycler 2.0 (Roche, USA). The sense and antisense PCR primers used were as follows: for PPP2R4, 5′ -AGGGTCTCATCCGCATGTAT-3′ and 5′ -CAGGCTCCCGAACTTGAA-3′ ,

respectively; for β-actin, 5′ -CCAACCGCGAGAAGATGA-3′ and 5′ -TCCATCACGATGCCAGTG-3′ , respectively. PCR was performed by initial denaturation at 95 °C for 10 min, followed by 35 cycles of 10 s at 95 °C, 10 s at 60 °C, and 5 s at 72 °C for β-actin or 35 cycles of 10 s at 95 °C, 10 s at 59 °C, and 4 s at 72 °C for PPP2R4. The expression level of PPP2R4 was normalized relative to that of β-actin. The relative quantitative value was obtained by the $2^{-\Delta\Delta Ct}$ method[25].

2.6.siRNA Transfection

The endometrial carcinoma cell lines were transfected with siPPP2R4 or siControl by reverse transfection using Lipofectamine RNAiMAX (Invitrogen, USA). To prepare the siRNA transfection solution for each tube, 20 pmol of siControl or siPPP2R4 was mixed with 50 μL of Opti-MEM reduced-serum medium by gentle pipetting. In parallel, 1.5 μL of Lipofectamine RNAiMAX was mixed with 50 μL of Opti-MEM. The two solutions were mixed by gentle pipetting and incubated for 10–20 min at room temperature to allow siRNA/lipid complexes to form. EC cells were diluted in complete growth medium without antibiotics to 50,000 cells/mL, then gently mixed with 100 μL of the transfection solutions, and plated. The cells were incubated for 24–72 h at 37 °C and then assayed for gene knockdown.

The siRNA sequences used were 5′ -GCAGUUCGCAGCUGAUAGA-3′ , 5′ -UGGAGUGUAUCCUGUUUAU-3′ , 5′ -GAUGAAGACUGGCCCAUUU-3′ , 5′ -CCAACCAGCUGUGGAACAU-3′ (siPPP2R4), and 5′ -UGGUUUACAUGUCGACUAA-3′ (siControl).

2.7.Cell Proliferation Assay

The cells transfected with the siRNAs were seeded at 5,000 cells per well in 96-well plates and incubated in a medium containing 10% FBS. After three days, the WST-8 reagent was added to each well, and the plates were incubated at 37 °C for 1 h. Absorbance was measured at 570 nm using an automated microplate reader (Infinite 200; Tecan, Männedorf, Switzerland).

2.8.Caspase Assays

Caspase activity was measured 72 h after the siRNA transfection using the Caspase-GIO 3/7 assay (Promega, Madison, WI, USA) according to the manufacturer's instructions. Cells (5,000 cells/well) were seeded in 96-well white plates and incubated for 72 h. Then, the caspase-3/7 substrate was added to each well for 1 h at room temperature. Luciferase activity was measured using a microplate luminometer (Infinite 200; Tecan). The luminescence values were divided by the WST-8 absorbance values to correct for the differences in cell numbers and to calculate the luminescence per cell.

2.9.Western Blot Analysis

Cells were lysed in complete lysis-M buffer (Roche Applied Science, Tokyo, Japan) containing the Halt phosphatase inhibitor cocktail (Thermo Fisher Scientific, Inc., Wayne, MI, USA). Lysates (10 μg of protein) were resolved by 10% sodium dodecyl sulfate polyacrylamide gel electrophoresis, and the proteins were transferred to nitrocellulose membranes (GE Healthcare Japan, Tokyo, Japan). The primary antibodies were diluted (1:2,000 for phospho-ERK1/2 and ERK1/2; 1:1,000 for phospho-AKT and AKT; and

1:5,000 for β-actin) and incubated with the membranes overnight at 4 °C. The secondary antibodies [enhanced chemiluminescence (ECL) HRP-conjugated anti-rabbit IgG and anti-mouse IgG; GE Healthcare] were incubated with the membranes at room temperature for 60 min. Signals were detected using the ECL Select western blotting detection kit (GE Healthcare). Signal intensity was quantified using a densitometer (CS Analyzer version 3.0 software; ATTO, Tokyo, Japan) and normalized to the β-actin levels.

2.10.Statistical Analysis

Statistical analysis for the cell proliferation assay was performed using the independent *t*-test. Comparisons between paired values were made using the Wilcoxon signed-rank test. All comparisons were performed using a two-sided test. A P value of $< .05$ was considered statistically significant. All statistical analyses were performed using the SPSS software (version 23; IBM SPSS, Inc., Chicago, IL, USA).

3.Results

3.1.Patients' Characteristics

A total of 14 patients were included in the current study, and patient characteristics are listed in Table 1. The median age was 46 years (range: 28–65 years). Nine patients (64.3%) were insulin-resistant as indicated by the homeostasis model assessment of insulin resistance score of ≥ 2.5 (mean score: 3.54; range: 0.6–8.18). Eight patients (57%) had abnormal glucose tolerance.

3.2.Metformin Reduced PP2A Expression in Endometrial Cancer Tissues

First, we investigated the effect of metformin on the expression of PP2A in EC tissues. The 14 Patients who were treated with metformin preoperatively were evaluated for PP2A-B expression using IHC. The expression of PP2A in the EC tissues was higher than that in the normal endometrium specimens. The mean IRSs of PP2A-B expression level of normal endometrium was 1.5 [95% confidence interval (CI), 0.24–2.76], Preoperative metformin treatment resulted in significantly reduced PP2A expression in 11 patients (78.6%), while the expression did not change in three patients (Fig. 1A, B). The mean IRSs of the PP2A-B expression level was reduced from 10 [95% confidence interval (CI), 8.9–11.1] to 5.6 (95% CI, 3.7–7.6; $P < .01$) (Fig. 1C). To confirm that this finding did not result from a natural change that occurred between the two sampling times, we compared 10 additional EC patients who were not administered metformin. The IRSs of the PP2A-B level varied from 8.2 (95% CI, 5.8–10.6) to 7.2 (95% CI, 5.5-8.9), i.e., no significant changes in the PP2A expression were observed in these cases (Fig. 1D, E).

Additionally, we evaluated the effect of metformin on PPP2R4 mRNA expression in EC Tissues. Using paired samples from six patients (before and after metformin treatment), we examined the changes in the PPP2R4 mRNA expression in EC tissue, caused by the administration of metformin. The PPP2R4 expression in EC tissues was higher than that in the normal endometrium. Metformin administration resulted in significantly reduced PPP2R4 mRNA expression (mean proportional decrease of 31.3%; 95% CI, 13–50; $P = .039$) (Fig. 2).

These results indicate that metformin administration downregulated PPP2R4 expression in patients with endometrial cancer.

3.3. Metformin Does Not Directly Affect PP2A in vitro

To examine whether the metformin effect was direct or indirect, we performed an *in vitro* assay using the EC cell lines (HEC265 and HEC1B) stimulated with metformin. There were no significant changes in the expression of PPP2R4 at any concentration of metformin in both cell lines (Fig. 3). Metformin could not directly reduce the PPP2R4 mRNA expression. Therefore, it was considered that metformin indirectly inhibited the PPP2R4 expression.

3.4. Anticancer Effects of PP2A Inhibition in Endometrial Cancer Cell Lines

To explore the role of PPP2R4 in EC cell lines, we knocked down PPP2R4 using a siRNA. HEC265 and HEC1B cells were selected because they are model cell lines of grade 1–2 endometrial carcinoma. The PPP2R4 knockdown efficiency of the gene-specific siRNA was confirmed by real-time PCR and western blot. PPP2R4 expression in endometrial cancer cell lines (HEC265 and HEC-1B) was inhibited at both protein and mRNA levels by transfection with the PPP2R4-specific siRNA (Fig.4A).

The knockdown of PPP2R4 gene expression with the siRNA significantly reduced the proliferation rates of HEC265 ($P < .01$) and HEC1B ($P < .01$) cells compared with those of the cells transfected with the non-targeting siRNA (Fig. 4B). Additionally, the knockdown of PPP2R4 increased the caspase activity of HEC265 ($P < .01$) and HEC1B ($P < .01$) cells (Fig. 4C), which was consistent with the results of the WST-8 assay. These results suggested that downregulation of PPP2R4 in HEC265 and HEC1B cells inhibited the cell proliferation and induced apoptosis.

PP2A inhibitors such as okadaic acid increase the AKT activity[26], which is one of the reasons why PP2A is considered a tumor suppressor. Therefore, we examined the possibility that a PPP2R4 knockdown is associated with the cancer proliferation via the ERK and AKT regulation in EC cells. However, there were no significant differences between the PPP2R4 knockdown and control cells in the expression of ERK and AKT (Fig. 4D).

4.Discussion

In this study, we found that an antidiabetic dose of metformin could reduce the expression of PP2A in patients with EC and demonstrated that PPP2R4 knockdown led to the inhibition of cell proliferation and to the induction of apoptosis in EC cells. Thus, PP2A inhibition was considered one of the antiproliferative effects of metformin administration.

The reduction of PP2A expression was related to the antiproliferative effect of metformin in the EC patients. However, this *in vivo* antiproliferative effect of metformin was considered to be indirect based on the fact that the *in vivo* metformin concentration was too low to inhibit the cell growth *in vitro*[7]. Hormonal factors such as insulin-like growth factor 1 (IGF-1) and leptin have been shown to be decreased by metformin administration[7] and may potentially be responsible for the reduction of the cancer growth-supporting potential of patient sera. Preclinical data in animal models have also suggested that antitumorigenic effects of metformin may depend on the metabolic composition of the host. Metformin has been found to be more effective in inhibiting tumor growth in obese and insulin-resistant animals than in their lean counterparts in breast and lung cancer models[27,28]. In a mouse breast cancer model, metformin could suppress the

obesity-induced secretion of adipokines as well as breast tumor formation and growth. Metformin also suppressed the secretion of IGF-1, IGF-2, leptin, and the tissue metallopeptidase inhibitor 1 and decreased the lipid accumulation during adipocyte differentiation[29]. Furthermore, our findings showed that metformin did not reduce the PPP2R4 expression in EC cell lines *in vitro*. These results led us to conclude that metformin indirectly reduced the expression of PP2A and subsequently induced the antiproliferative effect in EC patients.

Some reports have suggested that inhibition of PP2A has the potential as a cancer treatment[14,16,17,30–32]. Thus, LB100 exhibited potential antineoplastic activity, in combination with cisplatin, in an intracranial xenograft model, while cantheridin repressed the invasion of pancreatic cancer cells. This suggests that some inhibitors of PP2A may inhibit the cancer cell growth. siRNA knockdown of PPP2R4 led to the apoptosis and growth inhibition of HeLa cells[19,20] and induced the apoptosis in the HEK 293 and N2a cell lines through a mitochondrial pathway[33]. However, one study reported that both inhibition and overexpression of PPP2R4 induced cell death in human and opossum cells[22]. It is considered that PPP2R4 is necessary for cell homeostasis. In addition to PPP2R4, inhibition of other PP2A subunits is considered the target of cancer treatment. PP2A inhibition by okadaic acid significantly enhanced the response to lapatinib in breast cancer cells via decreased phosphorylation of eukaryotic translation elongation factor 2[34]. Downregulation of the PPP2R5C gene expression might be considered as a new therapeutic target strategy for chronic myeloid leukemia[35]. PP2Ac upregulation showed a poor prognostic impact on the overall survival of hepatocellular carcinoma (HCC) patients, and PP2Ac downregulation was shown to be a potential therapeutic target for

HCC[36,37]. Thus, PP2A inhibition might be considered a target for the treatment of some malignancies.

There is currently a controversy regarding whether PP2A functions as a tumor activator or tumor inhibitor. PP2A has been considered a tumor suppressor based on the fact that activation of PP2A leads to tumor growth inhibition[13]. Okadaic acid, a potent inhibitor of PP2A, and several endogenous inhibitors of PP2A have been shown to promote the malignant growth of human cancer by increasing the expression of ERK and AKT[14,15]. Inhibition of PP2A activity using a small hairpin RNA, leading to the suppression of several B-subunits, induced cell transformation, suggesting that PP2A might act as a tumor suppressor. Functions of PP2A in cancer are diverse, and its effects are different depending on the regulatory subunits involved. The B/56β and B/56γ subunits are known to dephosphorylate ERK[38], while B/55α-dependent PP2A acts as a regulator of AKT signaling[39]. Based on our results, siRNA knockdown of PPP2R4 did not affect the ERK and AKT pathway in EC. Thus, we speculated that PPP2R4 inhibition leads to an antitumor effect rather than to cancer cell proliferation in EC. Extensive molecular analysis is needed to elucidate the mechanism of the antitumor effect of PPP2R4 inhibition in EC.

This study had some limitations. First, we used metformin mainly for grade 1 and 2 EC. Type 1 EC tends to be associated with obesity, insulin resistance, and type 2 diabetes mellitus. Therefore, the frequency of obesity or insulin resistance was inevitably increased in this study and led to a bias. It is not clear whether metformin has antitumor effects in patients with type 2 EC (serous, clear endometrioid carcinoma of grade 3) and in lean patients. Second, PP2A has 92 different holoenzymes[40], but we did not investigate the

other PP2A subunits. To further characterize the function of metformin in relation to PP2A, more data on each holoenzyme are needed.

In conclusion, we found that metformin indirectly downregulated PPP2R4 in EC patients, and PPP2R4 inhibition led to an antitumor effect on EC cells *in vitro*. Our findings provide evidence for the potential mechanism underlying the anticancer effect of metformin. PP2A inhibition might be considered a target for the treatment of EC patients with insulin resistance.

Conflict of interest disclosures

The authors declare no conflicts of interest.

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Table 1. Patient Characteristics

Age (years), median (range)	46 (28–65)
Histology, No. (%)	
Endometrioid carcinoma grade 1	11 (78.6)
Endometrioid carcinoma grade 2	2 (14.3)
Mix carcinoma (Endometrioid grade 1 + serous)	1 (7.1)
Stage[a], No. (%)	
I	9 (60)
II	2 (13)
III	3 (27)
75 g oral glucose tolerance test, No. (%)	
Normal	6 (42.9)
Impaired glucose tolerance	7 (50.0)
DM type	1 (7.1)
BMI, mean (range)	29.8 (18.9–50.2)
≥ 25, No. (%)	11 (73.3)
HOMA-IR, mean (range)	3.54 (0.64–8.18)
≥ 2.5, No. (%)	9 (64.3)

Abbreviations: BMI, body mass index; DM, diabetes mellitus; HOMA-IR, homeostasis model assessment of insulin resistance.

[a] The International Federation of Gynecology and Obstetrics (FIGO) stage.

Figure legends

Figure 1. Preoperative metformin administration decreased immunostaining of PP2A in endometrial cancer tissues. Representative images of changes in immunostaining are shown for paired specimens obtained before (A) and after (B) metformin treatment. Changes in immunoreactive scores, which were calculated by multiplying the intensity of staining by the percentage of positive cells, are shown for each pair; differences were evaluated using the Wilcoxon signed-rank test (C). Representative images of changes in immunostaining are shown for paired specimens obtained from preoperative biopsy (D) and hysterectomy (E) from patients who did not receive metformin treatment during the study period. Pre, sampling at the time of diagnosis in each group and before the initiation of metformin treatment in the metformin treatment group; Post, sampling immediately before the operation in each group and after metformin treatment in the metformin treatment group.

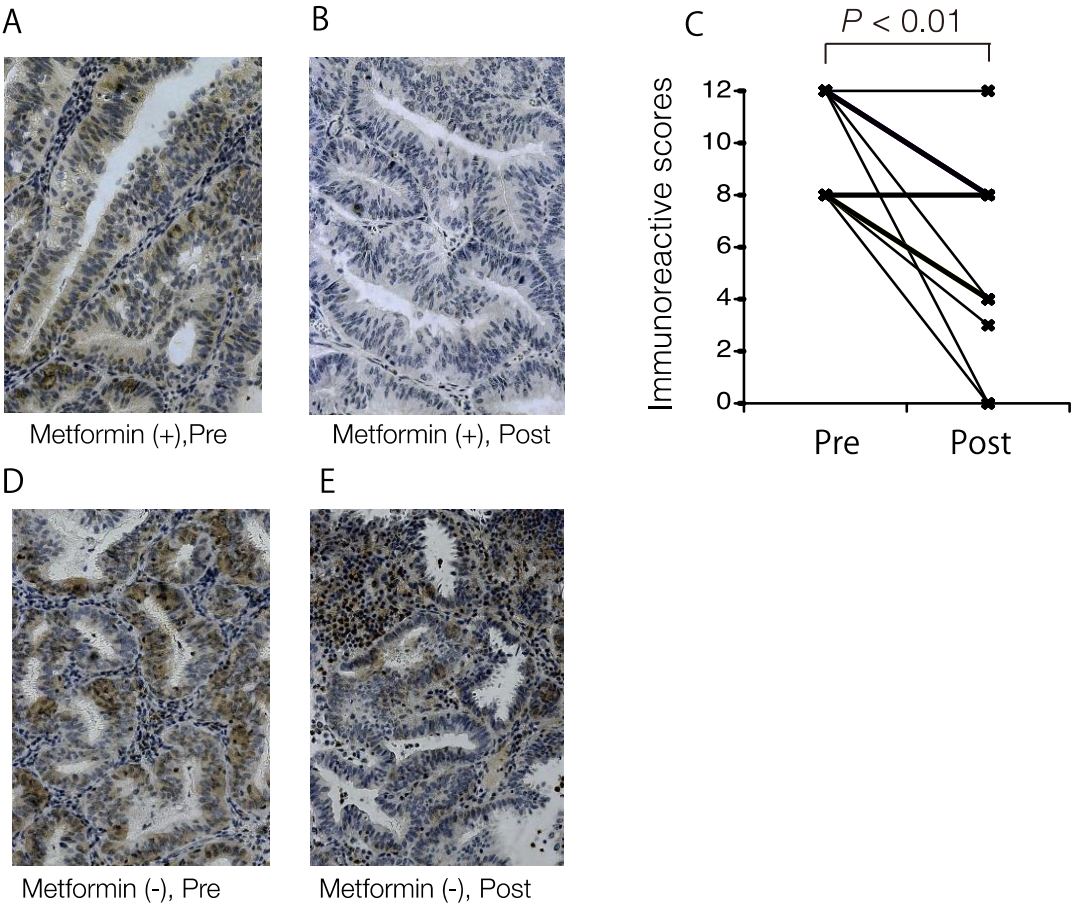

A
B
C
P < 0.01
Immunoreactive scores
12
10
8
6
4
2
0
Pre
Post
Metformin (+),Pre
Metformin (+), Post
D
E
Metformin (-), Pre
Metformin (-), Post

Figure 2. Preoperative metformin administration significantly reduced the PPP2R4 mRNA expression in endometrial cancer tissues. The PPP2R4 expression in endometrial cancer tissues was evaluated by RT–PCR, and the values were normalized to those of β-actin. The intensity of PPP2R4 mRNA expression was based on that of the normal endometrium (n = 6). Pre, before the initiation of metformin treatment; Post, after metformin treatment.

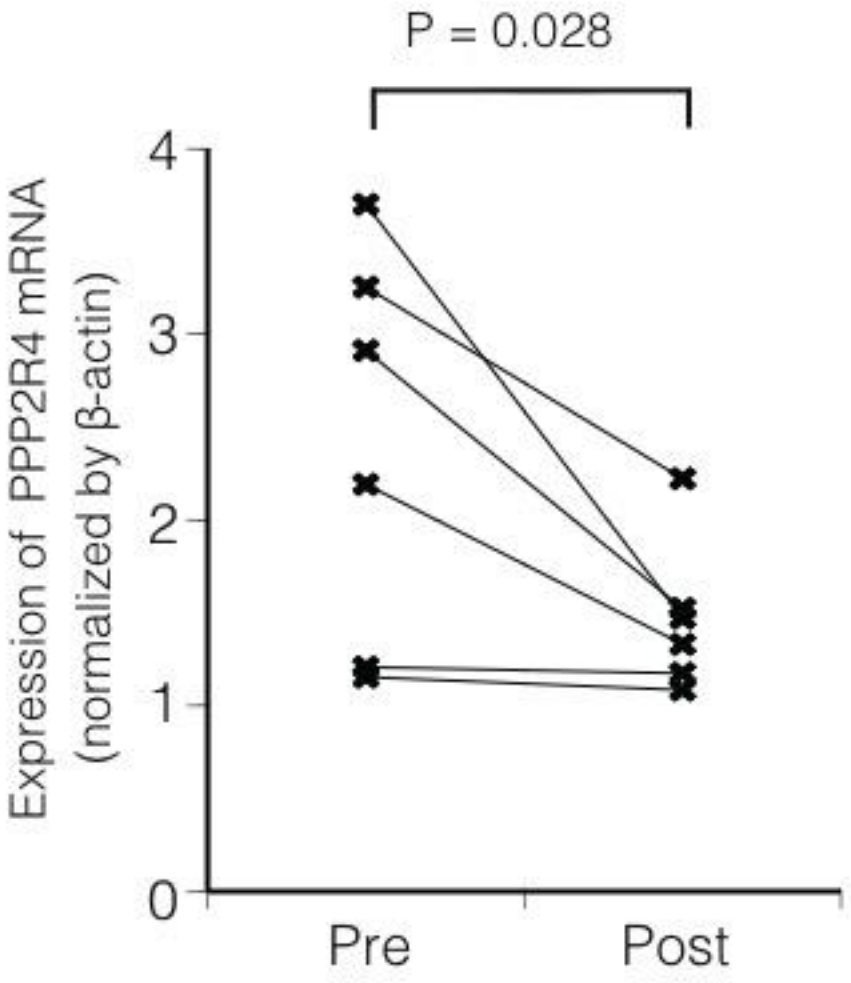


Figure 3. Metformin did not directly alter the PPP2R4 mRNA levels *in vitro*. There were no significant changes in the expression of PPP2R4 at any concentration of metformin in either endometrial cancer cell line. The results are presented as the mean ± standard deviation for at least three independent experiments.

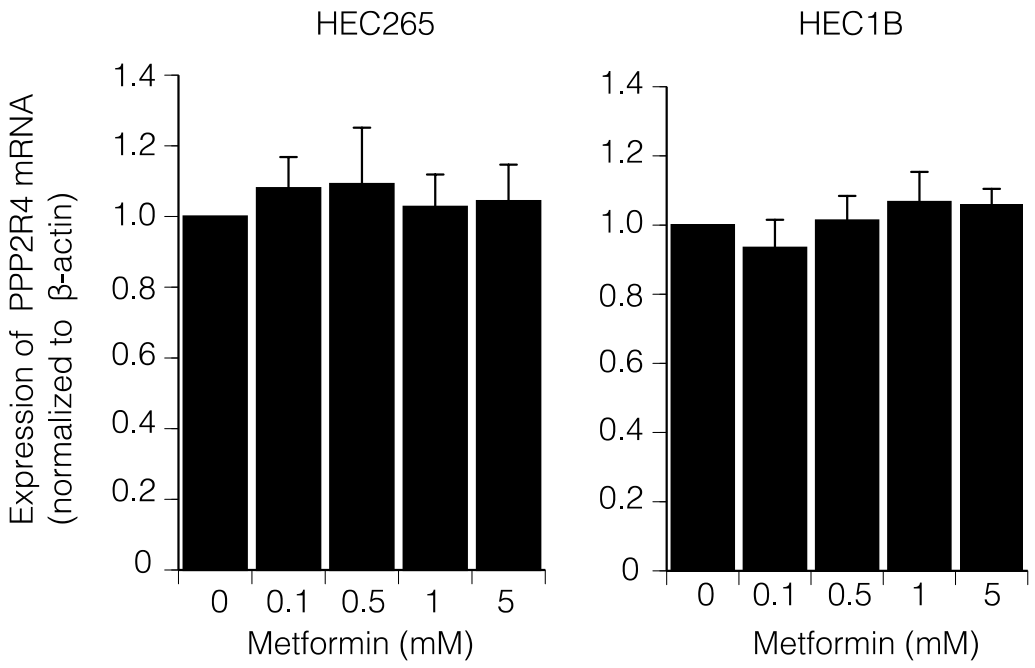
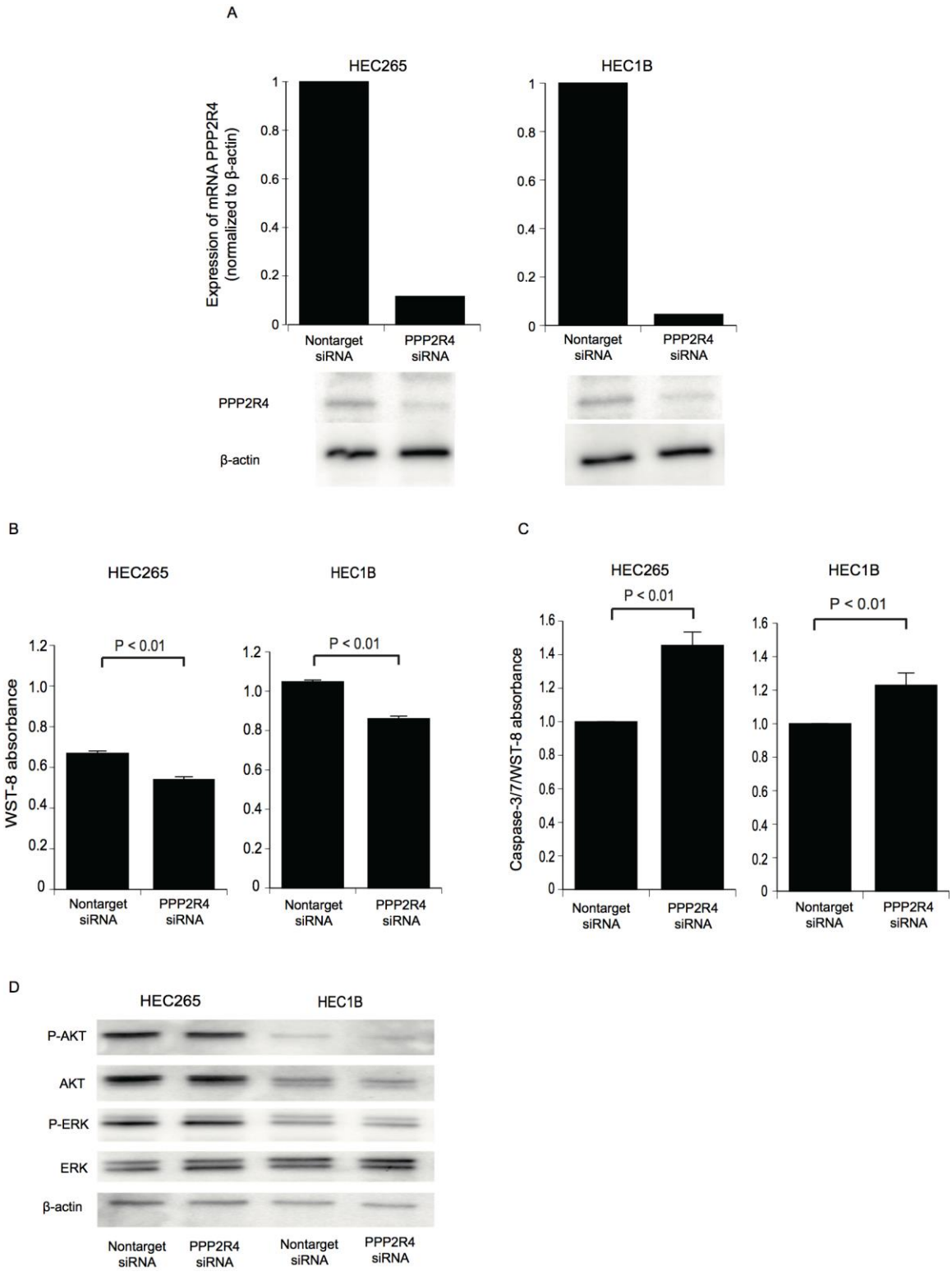


Figure 4. Effects of siRNA knockdown of PPP2R4. (A) RT‑PCR and western blot analysis of cancer cells transfected with PPP2R4-specific siRNA and control siRNA. (B) WST-8 assay using cancer cell lines transfected with the PPP2R4 siRNA and control siRNA. (C) Caspase 3/7 activity in the cancer cell lines transfected with the PPP2R4 siRNA and control siRNA. (D) Western blot analysis of cell signaling changes in endometrial cancer cells transfected with the PPP2R4 siRNA and control siRNA. The expression levels of phospho-extracellular signal-regulated kinase 1/2 (ERK1/2), ERK1/2, phospho-protein kinase B (AKT), and AKT in cancer cells transfected with the siRNAs are shown. Columns and error bars represent the mean ± the standard error of the mean for at least three independent experiments.


A
HEC265
HEC1B
Expression of mRNA PPP2R4 (normalized to β-actin)
1
0.8
0.6
0.4
0.2
0
Nontarget siRNA
PPP2R4 siRNA
PPP2R4
β-actin
B
HEC265
HEC1B
WST-8 absorbance
P < 0.01
1.2
1.0
0.8
0.6
0.4
0.2
0
Nontarget siRNA
PPP2R4 siRNA
C
HEC265
HEC1B
Caspase-3/7/WST-8 absorbance
P < 0.01
1.6
1.4
1.2
1.0
0.8
0.6
0.4
0.2
0
Nontarget siRNA
PPP2R4 siRNA
D
HEC265
HEC1B
P-AKT
AKT
P-ERK
ERK
β-actin
Nontarget siRNA
PPP2R4 siRNA
Nontarget siRNA
PPP2R4 siRNA

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