Original Article

Metformin alleviates bevacizumab-induced vascular endothelial injury by up-regulating GDF15 and activating the PI3K/AKT/FOXO/PPARγ signaling pathway

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Background: Previous studies have reported that the combination of metformin and bevacizumab exhibit favorable efficacy in the treatment of cancer patients, and metformin possesses effects on relieving vascular injury in multiple diseases. Nonetheless, the effect of metformin in alleviating bevacizumab-induced vascular injury remains unknown. Therefore, the present study aimed to investigate the impact of metformin on apoptosis, vascular endothelial injury marker expressions, and inflammation in human umbilical vein endothelial cells (HUVECs), as well as its possible molecular mechanism.

Methods: HUVECs were treated with bevacizumab, metformin or both, and subsequently treated with growth differentiation factor 15 (GDF15) overexpression plasmid, negative control (NC) plasmid, GDF15 small interfering ribonucleic acid (siRNA), NC siRNA, and the phosphoinositide 3-kinase (PI3K) inhibitor LY294002, respectively. After treatment, apoptosis, levels of endothelial injury biomarkers and the potential downstream proteins were detected.

Results: Bevacizumab increased the levels of apoptosis, vascular endothelial injury marker expressions and pro-inflammatory cytokine expressions in HUVECs, while metformin alleviated these effects in bevacizumab-treated HUVECs. Furthermore, GDF15 overexpression reduced the apoptosis, vascular endothelial injury marker expressions, pro-inflammatory cytokine expressions, and activated the PI3K/protein kinase B (AKT)/forkhead box O (FOXO)/peroxisome proliferator-activated receptor γ (PPARγ) signaling pathway in bevacizumab-treated HUVECs. Subsequently, GDF15 siRNA reduced the effects of metformin on the bevacizumab-induced vascular endothelial injury (as described above) in HUEVCs. Lastly, the PI3K inhibitor exhibited similar effects to those of GDF15 siRNA in bevacizumab-treated HUVECs.

Conclusions: Metformin protected against bevacizumab-induced vascular endothelial injury via activation of GDF15 and the PI3K/AKT/FOXO/PPARγ signaling pathway.

Keywords: Vascular endothelial injury; bevacizumab; metformin; GDF15; PI3K/AKT/FOXO/PPARγ signaling pathway

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Introduction

Despite the considerable treatment progress, cancer is still one of the leading contributors to global mortality. Factors responsible for this not only include the disease itself, but also others such as complications in cancer patients (1). Cancer patients could be accompanied by numerous complications, of which vascular diseases, such as cardiovascular diseases, heart failure, and stroke, are the most harmful (2-4). The majority of vascular endothelial diseases occur during treatment with angiogenesis inhibitors; these agents have been increasingly reported to lead to vascular injury, which is mainly caused by damage to vascular endothelial function (5,6). This situation has led to an increase in research for a combination therapy containing both anti-angiogenic target drugs and drugs that protect against vascular damage.

Bevacizumab, an anti-angiogenic agent broadly applied in cancer therapy, is a monoclonal antibody that specifically targets the combination of vascular endothelial growth factor A (VEGF-A) and its receptors (7,8). Bevacizumab is approved and used in multiple cancers; most commonly cervical cancer, glioblastoma, ovarian cancer, etc., with acceptable efficacy (9-11). Nevertheless, the clinical application of bevacizumab remains an issue, mostly due to bevacizumab-related complications, especially vascular-related diseases (hypertension, venous thromboembolism, etc.) (12,13). As a targeted drug often used in combination with other anti-tumor therapeutics, it is possible that the addition of other drugs could reduce the injury to vessels induced by bevacizumab. Hence, there is a pressing need for research with this aim.

Metformin has long been the first-line therapeutic agent for type 2 diabetes. However, researchers in the field of oncology have been particularly intrigued by its anti-tumor effect, which has been discovered in recent years (14). A previous randomized controlled study reported that the combined use of metformin with bevacizumab, paclitaxel, and carboplatin shows better survival outcomes in advanced non-small cell lung cancer patients (15). Metformin also exhibits an anti-vascular injury effect in many other diseases via multiple pathways, such as repressing inflammation caused by lipopolysaccharides (LPS) in vascular smooth muscle cells (VSMCs) by modulating peroxisome proliferator activated receptor gamma (PPARγ) (16). It is also reported that metformin alleviates vascular endothelial injury in sepsis or atherosclerosis (17,18). However, whether the use of metformin is beneficial to the prevention or reduction of bevacizumab-induced vascular endothelial injury in cancer patients has yet to be explored. Besides, numerous studies have disclosed the critical pathways that involve in the regulation of vascular endothelial injury, such as the signal transducer and activator of transcription 3 pathway, the phosphoinositide 3-kinase (PI3K)/protein kinase B (AKT)/forkhead box O (FOXO) pathway, transforming growth factor-beta1/Smad pathway, etc. (19-21).

Thus, the aim of the present study is to investigate the impact of metformin on apoptosis, vascular endothelial injury marker expressions, and inflammation in human umbilical vein endothelial cells (HUVECs), as well as its possible molecular mechanism. We present the following article in accordance with the MDAR reporting checklist (available at https://dx.doi.org/10.21037/atm-21-4764).

Methods

Reagents

HUVECs (Procell, China; cat.no.: CL-0122; within 3–6 passages) were cultured in Roswell Park Memorial Institute (RPMI)-1640 medium (Hyclone, USA) containing 10% fetal bovine serum (FBS) (Tianhang, China). Bevacizumab was obtained from Sinopharm Group Le-Ren-Tang Medicines Co., Ltd. (Sinopharm, China). Metformin and LY294002 were obtained from Med Chem Express Co., Ltd. (MCE, China). The Annexin V-fluorescein isothiocyanate isomer (FITC) Apoptosis Detection Kit was purchased from Beijing Solarbio Science & Technology Co., Ltd. (Solarbio, China). Trizol reagent was acquired from Thermo Fisher Scientific Inc. (Thermo, USA). HiFiScript genomic deoxyribonucleic acid (DNA) Removal complementary DNA Synthesis Kit and ChemoHS quantitative polymerase chain reaction (qPCR) Mix (None ROX) were purchased from Beijing ComWin Biotech Co., Ltd. (CWBIO, China) and Monad Biotech Co., Ltd. (Monad, China), respectively. E-selectin (CD62E) (KE00169), endothelial-1 (ET-1) (E-CL-H0064), interleukin (IL)-6 (KE00139), thrombomodulin (E-EL-H0166), tumor necrosis factor (TNF)-α (KE00068), and von Willebrand factor (vWF) (E-EL-H2168) enzyme linked immunosorbent assay (ELISA) kits were obtained from Proteintech Group, Inc. (Proteintech, China) and Elabscience Biotechnology Co., Ltd. (Elabscience, China), respectively. The GDF15 overexpression plasmid, negative control (NC) overexpression plasmid, GDF15 small interference ribonucleic acid (siRNA) and NC siRNA were acquired from Shanghai GenePharma Co. Ltd. (GenePharma, China). Lipofectamine™ 2000

Transfection Reagent was purchased from Thermo Fisher Scientific (China) Co., Ltd. (Thermo, China). (Radio immunoprecipitation assay) RIPA buffer was obtained from Beijing Solarbio Science & Technology Co., Ltd. (Solarbio, China). The Western Blotting Luminol Reagent was bought from ZSGB-Bio Co., Ltd. (ZSbio, China).

Bevacizumab and metformin treatment

For bevacizumab treatment, the HUVECs were incubated with 0.25 mg/mL bevacizumab (22) for 48 hours (h) at 37 ℃. For metformin treatment, the HUVECs were cultured with 50 μM metformin (23) for 48 h at 37 ℃. For bevacizumab combined with metformin treatment, the HUVECs were incubated with 50 μM metformin plus 0.25 mg/mL bevacizumab for 48 h. HUVECs that were cultured normally served as the controls. Following incubation, the cells and supernatant were collected for further detection.

Plasmids constructions and transfection

The GDF15 cDNA (NM_004864.4) was amplified and cloned into pcDNA3.1-kana vector to construct the GDF15 overexpression plasmid. The synthesize nonsense DNA fragment was cloned into pcDNA3.1-kana vector to construct the NC plasmid. The GDF15 or NC overexpression plasmids were transfected into HUVECs with the application of Lipofectamine™ 2000 Transfection Reagent according to the manufacturer's instructions, and the OE-GDF15 or OE-NC cells were then generated. Following transfection, the cells were incubated with bevacizumab for 48 h. Subsequently, the cells and supernatant were collected for followed assessment.

siRNA transfection

Lipofectamine™ 2000 Transfection Reagent was adopted to transfect the GDF15 or NC siRNA into the HUVECs. Si-GDF15 and si-NC cells were generated after the transfection. The si-NC and si-GDF15 cells were then treated with bevacizumab alone or bevacizumab plus metformin as described in the "Bevacizumab and metformin treatment" section. Detection of the cells and supernatant was performed after incubation. The target sequences of siRNAs were as follows: GDF15, 5'-AAGACTCCAGATTCCGAGAGT-3'; NC, 5'-AGTGAAACAGTGCAGCTG-3'.

LY294002 treatment

To conduct treatment with LY294002 alone, the HUVECs were treated with 10 μM LY294002 (24) for 48 h. For treatment with LY294002, metformin and bevacizumab, the HUVECs were incubated with 50 μM metformin, 10 μM LY294002, and 0.25 mg/mL bevacizumab for 48 h. Meanwhile, the HUVECs incubating with bevacizumab alone or bevacizumab plus metformin were described in the "Bevacizumab and metformin treatment" subsection.

Apoptosis assessment

The Annexin V-FITC Apoptosis Detection Kit was applied to perform the apoptosis assessment. Briefly, the cells were collected and re-suspended. After incubating with Annexin V-FITC (5 μL) and propidium iodide (PI) (5 μL) in the dark at room temperature for 30 min, the cells were analyzed with a flow cytometer (BD Biosciences, USA).

ELISA

The expressions of CD62E, ET-1, TM, vWF, TNF-α, and IL-6 in the supernatant were respectively evaluated using CD62E, ET-1, TM, vWF, TNF-α, and IL-6 ELISA kits strictly according to the kits' protocols.

Reverse transcription quantitative polymerase chain reaction (RT-qPCR)

Trizol reagent was applied for RNA extraction. After isolation of the RNA, reverse transcription and qPCR were performed using the HiFiScript gDNA Removal cDNA Synthesis Kit (42 ℃ for 15 min, 85 ℃ for 5 min) and ChemoHS qPCR Mix (None ROX) (95 ℃ for 5 min, 1 cycle; 95 ℃ for 10 s, 61 ℃ for 20 s, 40 cycles), respectively. The results were calculated with the $2^{-\Delta\Delta Ct}$ method and the primers are listed in *Table 1*.

Western blot

The cells were harvested and the total protein was extracted using RIPA buffer. After quantification, the thermal denatured protein was separated using 4–20% precast gel (Beyotime, China) and transferred to a polyvinylidene fluoride membrane (Millipore, USA). After incubation with the diluted primaries (4 ℃ overnight) and secondary antibodies (37 ℃ for 1 h), the membrane was incubated with

Table 1 Primers

Gene	Forward primer (5'-3')	Reverse primer (5'-3')
GDF15	TCAGATGCTCCTGGTGTTGC	CTGGTTAGCAGGTCCTCGTAG
PI3K	TCTGTCTCCTCTAAACCCTG	TTCTCCCAATTCAACCAC
BCL-2	TCGCCGAGATGTCCAGC	CCCACCGAACTCAAAGAAGG
Bax	TTTGCTTCAGGGTTTCATC	ACACTCGCTCAGCTTCTTG
PPARγ	ACAGGCCGAGAAGGAGA	CAGCGGGAAGGACTTTATG
β-actin	GGCACCACACCTTCTACAATGA	GGATAGCACAGCCTGGATAGC

Table 2 Antibodies

Antibody	Company/country	Catalog number	Dilution rate
Primary antibody			
Rabbit polyclonal to growth differentiation factor 15 (GDF15)	Abcam [United Kingdom (UK)]	ab211364	1:3,000
Rabbit monoclonal to phosphoinositide 3-kinase (PI3K)	Abcam (UK)	ab32089	1:1,000
Rabbit polyclonal to phosphorylated (p)-protein kinase B (AKT)	Cell Signaling Technology (CST) (USA)	#9271	1:1,000
Rabbit polyclonal to p-forkhead box O 3 (FOXO3)	CST [United States of America (USA)]	#9466	1:1,000
Rabbit polyclonal to peroxisome proliferator-activated receptor γ (PPARγ)	Proteintech (USA)	16643-1-AP	1:800
Rabbit polyclonal to B cell lymphoma-2 (Bcl-2) associated X protein (Bax)	Proteintech (USA)	50599-2-Ig	1:1,000
Rabbit polyclonal to Bcl-2	Proteintech (USA)	12789-1-AP	1:1,000
Mouse monoclonal to β-actin	ZSbio (China)	TA-09	1:2,000
Secondary antibody			
Goat Anti-Rabbit immunoglobulin G (IgG) heavy-chain&light-chain (H&L) horseradish peroxidase (HRP)	ZSbio (China)	ZB-2301	1:10,000
Goat Anti-Mouse IgG H&L (HRP)	ZSbio (China)	ZB-2305	1:10,000

Western Blotting Luminol Reagent to visualize the protein bands. The antibodies are listed in *Table 2*.

Statistical analysis

All experiments were replicated three times with three biological replicants. The data in this study were presented as mean ± standard deviation and analyzed using GraphPad Prism 7.02 (GraphPad Software Inc., USA). One-way analysis of variance (ANOVA) followed by Tukey's multiple comparisons test was used to compare among groups and the unpaired *t* test was used to compare between two groups. P<0.05 was considered statistically significant.

Results

Impact of metformin on bevacizumab-induced apoptosis, vascular endothelial injury markers, and inflammation in HUVECs

Bevacizumab treatment increased cell apoptosis in HUVECs, while treatment with metformin reduced cell apoptosis in bevacizumab-treated HUVECs (P<0.001) (*Figure 1A,1B*). As for the vascular endothelial injury markers, bevacizumab treatment increased the levels of CD62E (*Figure 1C*), ET-1 (*Figure 1D*), TM (*Figure 1E*), and vWF (*Figure 1F*) in HUVECs; however, metformin treatment decreased the levels of these markers (except

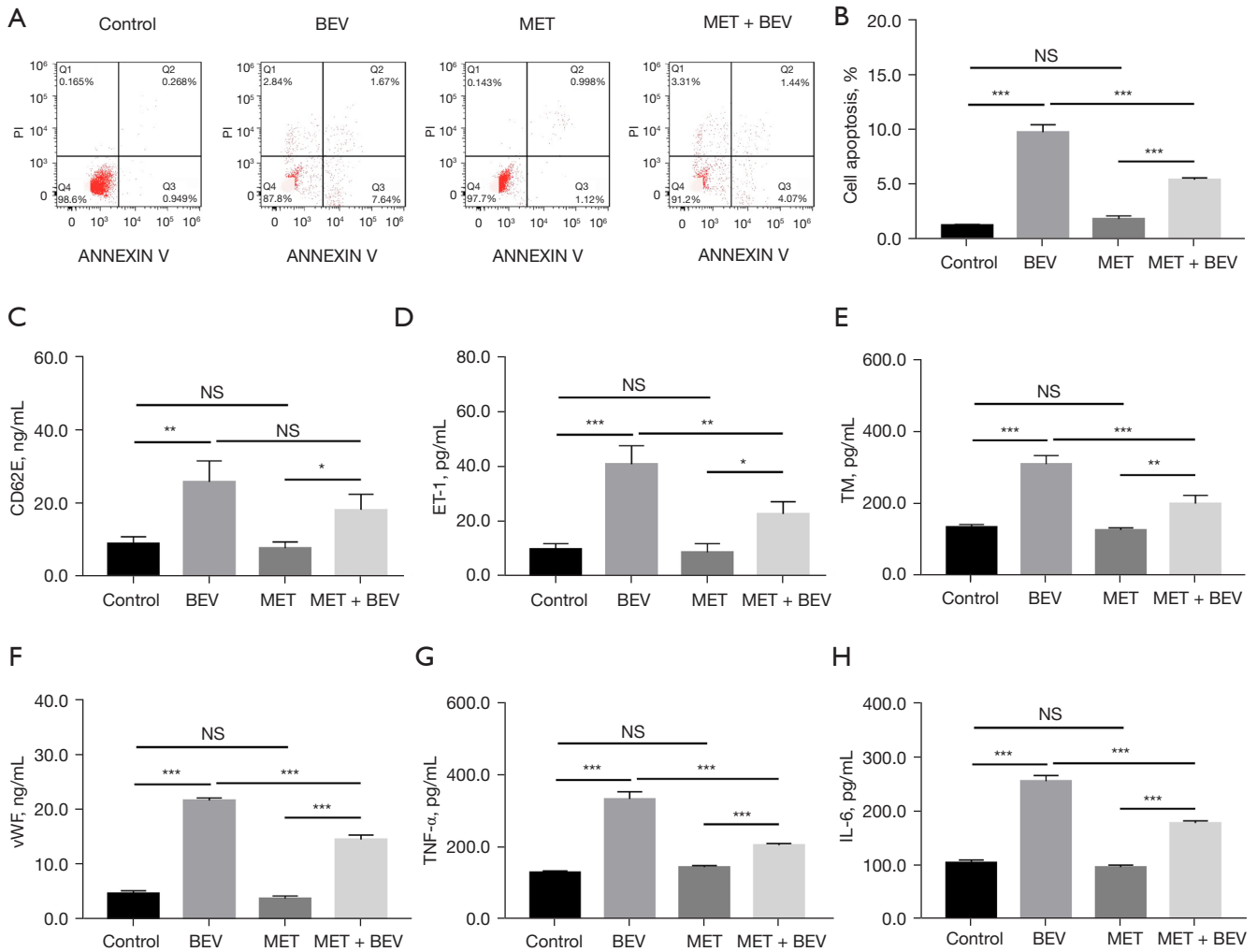

Figure 1 Effect of metformin on bevacizumab-induced apoptosis, vascular endothelial injury markers, and inflammation in HUVECs. Image of flow cytometry of apoptosis (A), cell apoptosis rate (B), CD62E level (C), ET-1 level (D), TM level (E), vWF level (F), TNF-α level (G), and IL-6 level (H) in the control, BEV, MET and MET + BEV groups, respectively. *P<0.05; **P<0.01; ***P<0.001; NS, not significant. HUVECs, human umbilical vein endothelial cells, CD62E, selectin E; ET-1, endothelin-1; TM, thrombomodulin; vWF, von Willebrand factor; TNF-α, tumor necrosis factor-alpha; IL-6, interleukin-6; BEV, bevacizumab; MET, metformin.

for CD62E) in bevacizumab-treated HUVECs (all P<0.05). In addition, bevacizumab also increased the expression of TNF-α and IL-6 in HUVECs; however, metformin reduced TNF-α (*Figure 1G*) and IL-6 (*Figure 1H*) expressions in bevacizumab-treated HUVECs (all P<0.001). The representative images of HUVECs before and after treatment were shown in Figure S1. These results indicated that metformin reduced the bevacizumab-induced apoptosis, vascular endothelial injury markers, and inflammation effects in HUVECs. Subsequently, further experiments revealed that metformin enhanced the GDF15 expression and the activation of the PI3K/AKT/FOXO/PPARγ signaling pathway in bevacizumab-treated HUVECs (*Figure 2A-2F*).

Effect of GDF15 overexpression on apoptosis, vascular endothelial injury markers, and inflammation in bevacizumab-treated HUVECs

GDF15 overexpression reduced cell apoptosis in HUVECs with bevacizumab treatment (P<0.01) (*Figure 3A,3B*). In terms of vascular endothelial injury markers, GDF15

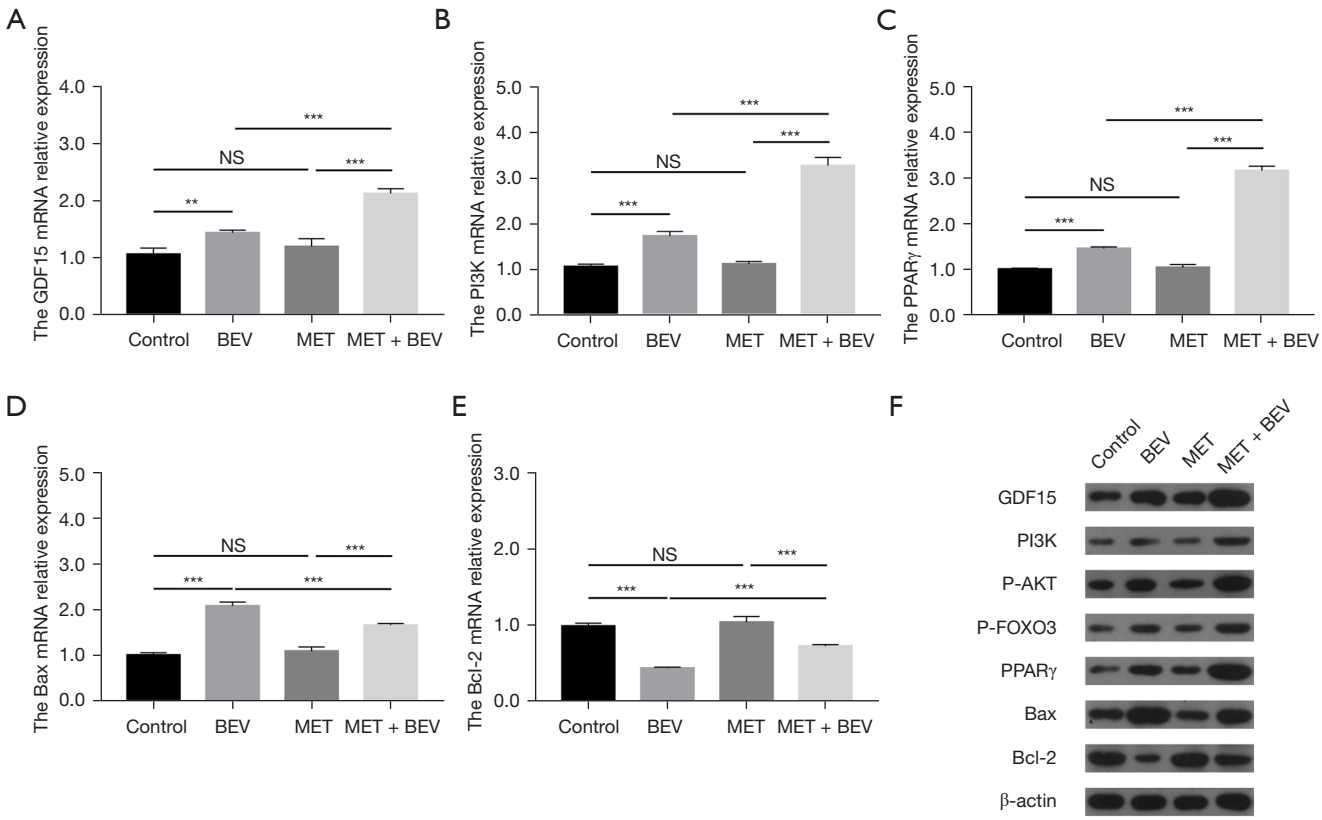

Figure 2 Effect of metformin on GDF15 and PI3K/AKT/FOXO/PPARγ signaling pathway in bevacizumab-treated HUVECs. GDF15 mRNA (A), PI3K mRNA (B), PPARγ mRNA (C), Bax mRNA (D), Bcl-2 mRNA (E) expressions, and protein expressions of GDF15, PI3K, P-AKT, P-FOXO3, PPARγ, Bax, Bcl-2, and β-actin (F) in the control, BEV, MET, and MET + BEV groups, respectively. **P<0.01; ***P<0.001; NS, not significant. GDF15, growth differentiation factor 15; PI3K, phosphatidylinositol 3-kinase; AKT, threonine kinase; FOXO, forkhead box, sub-group O; P-FOXO3, phosphorylated forkhead box, sub-group O3; PPARγ, peroxisome proliferator activated receptor gamma; HUVECs, human umbilical vein endothelial cells; Bax, BCL2 associated X; Bcl-2, B cell lymphoma 2; P-AKT, phosphorylated-threonine kinase; BEV, bevacizumab; MET, metformin.

overexpression inhibited the expressions of CD62E (*Figure 3C*), ET-1 (*Figure 3D*), TM (*Figure 3E*), and vWF (*Figure 3F*) in bevacizumab-treated HUVECs (all P<0.05). As for inflammation, GDF15 overexpression reduced TNF-α (*Figure 3G*) and IL-6 (*Figure 3H*) expressions in bevacizumab-treated HUVECs (all P<0.01). The GDF15 messenger RNA (mRNA) (P<0.001) and protein expressions were up-regulated after plasmids transfection; in bevacizumab-treated HUVECs, GFD15 overexpression elevated the mRNA expressions of *PI3K*, *PPARγ*, and *B cell lymphoma-2* (*Bcl-2*), and decreased the mRNA expression of *Bax* (all P<0.01); it also increased the protein expressions of PI3K, P-AKT, P-FOXO3, PPARγ, and Bcl-2, and reduced the protein expression of Bax in bevacizumab-treated HUVECs (*Figure 4A-4F*). Collectively, GDF15 overexpression inhibited bevacizumab-induced apoptosis, vascular endothelial injury markers, and inflammation in HUVECs.

Impact of GDF15 siRNA on bevacizumab-induced apoptosis, vascular endothelial injury markers, and inflammation in HUVECs

GDF15 siRNA diminished the effect of metformin on mediating apoptosis, as well as the expressions of TM, vWF, TNF-α, and IL-6 (all P<0.01), but not the expressions of CD62E or ET-1 (all P>0.05) in bevacizumab-treated HUVECs (*Figure 5A-5H*). In addition, GDF15 mRNA

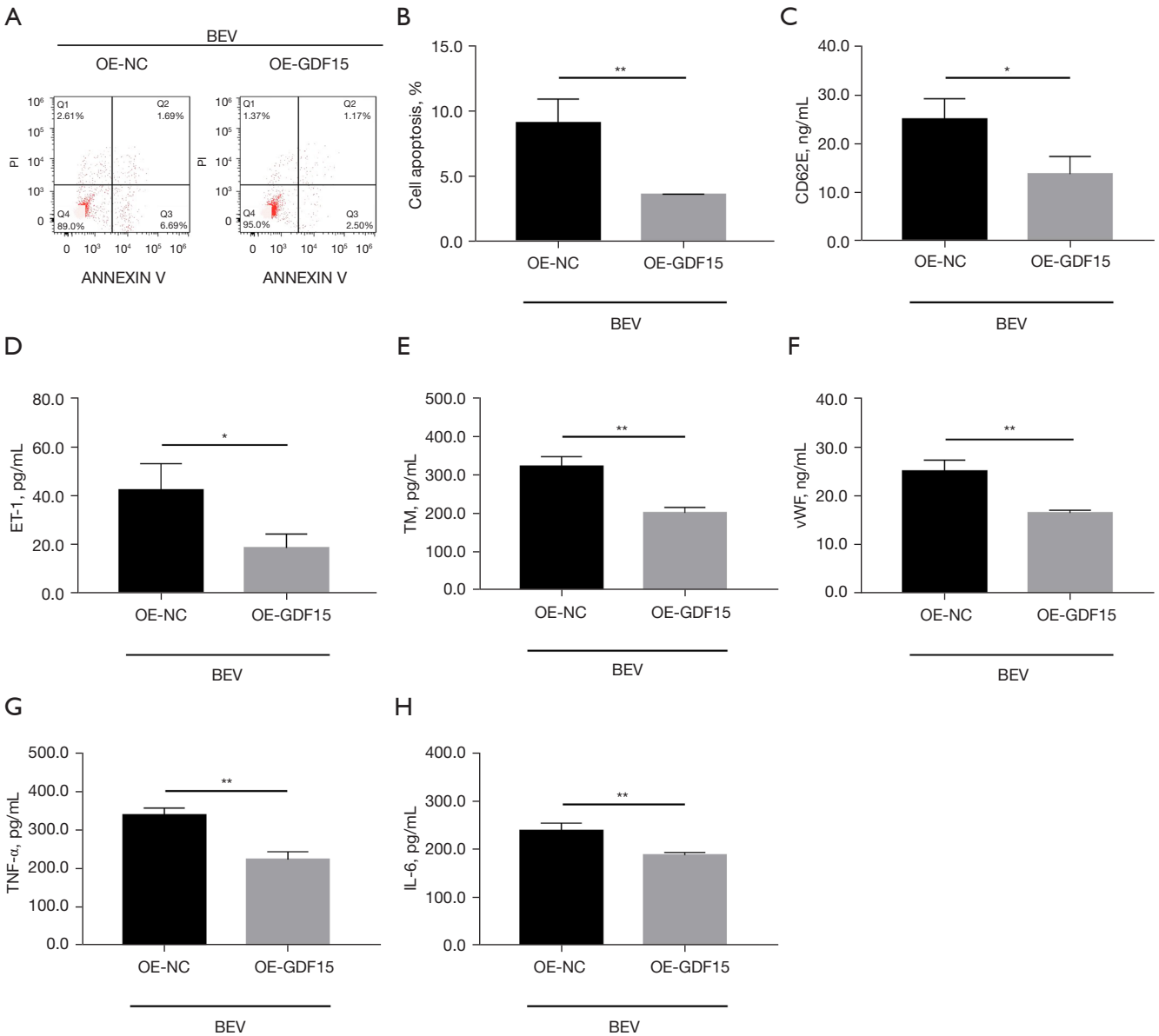

Figure 3 Effect of GDF15 overexpression on apoptosis, vascular endothelial injury markers, and inflammation in bevacizumab-treated HUVECs. Image of flow cytometry of apoptosis (A), cell apoptosis rate (B), CD62E level (C), ET-1 level (D), TM level (E), vWF level (F), TNF-α level (G), and IL-6 level (H) in the OE-NC and OE-GDF15 groups. *P<0.05; **P<0.01. GDF15, growth differentiation factor 15; HUVECs, human umbilical vein endothelial cells; CD62E, selectin E; ET-1, endothelin-1; TM, thrombomodulin; vWF, von Willebrand factor; TNF-α, tumor necrosis factor-alpha; IL-6, interleukin-6; OE, overexpression; NC, negative control; BEV, bevacizumab.

relative expression (all P<0.001) and protein expression was down-regulated post-siRNA transfection; moreover, GDF15 siRNA decreased the effect of metformin on *PI3K*, *PPARγ*, *Bcl-2*, and *Bax* mRNA expressions in bevacizumab-treated HUVECs (all P<0.001); in addition, the protein expressions of PI3K, PPARγ, Bax, Bcl-2, and β-actin exhibited a similar trend as described above, and P-AKT as well as P-FOXO3 protein expressions also displayed similar trends (*Figure 6A-6F*). These results implied that GDF15 inhibition reduced the effects of metformin on bevacizumab-induced apoptosis, vascular endothelial injury markers, inflammation, and PI3K/AKT/FOXO/PPARγ

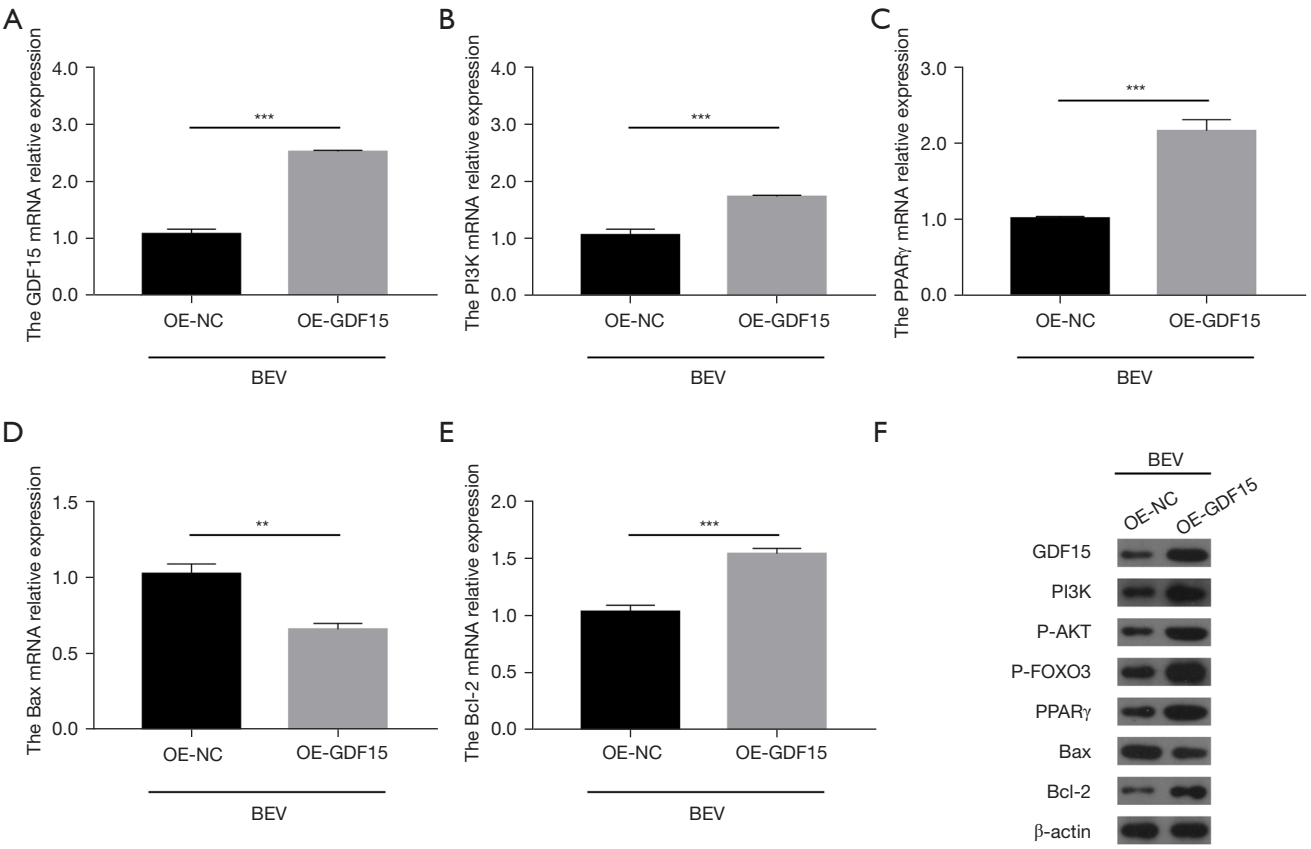

Figure 4 Effect of GDF15 overexpression on the PI3K/AKT/FOXO/PPARγ signaling pathway in bevacizumab-treated HUVECs. GDF15 mRNA (A), PI3K mRNA (B), PPARγ mRNA (C), Bax mRNA (D), Bcl-2 mRNA (E), and protein expressions of GDF15, PI3K, P-AKT, P-FOXO3, PPARγ, Bax, Bcl-2, and β-actin (F) in the OE-NC and OE-GDF15 groups, respectively. **P<0.01; ***P<0.001. GDF15, growth differentiation factor 15; PI3K, phosphatidylinositol 3-kinase; AKT, threonine kinase; FOXO, forkhead box, sub-group O; PPARγ, peroxisome proliferator activated receptor gamma; HUVECs, human umbilical vein endothelial cells; Bax, BCL2 associated X; Bcl-2, B cell lymphoma 2; P-AKT, phosphorylated-threonine kinase; P-FOXO3, phosphorylated-forkhead box, sub-group O3; OE, overexpression; NC, negative control; BEV, bevacizumab.

signaling pathway activation in HUVECs.

Impact of the PI3K inhibitor on bevacizumab-induced apoptosis, vascular endothelial injury markers, and inflammation in HUVECs

The PI3K inhibitor reduced the effects of metformin on ET-1, TM, vWF, TNF-α, and IL-6 expressions in bevacizumab-treated HUVECs (all P<0.05), but did not reduce cell apoptosis or CD62E expression (all P>0.05) (*Figure 7A-7H*).

Additionally, the PI3K inhibitor down-regulated *PI3K*, *PPARγ*, and *Bcl-2* mRNA expressions and up-regulated *Bax* mRNA expression (all P<0.01); however, it did not alter the mRNA expression of *GDF15* (P>0.05) in bevacizumab and metformin-treated HUVECs; as for protein expressions, the PI3K inhibitor decreased PI3K, P-AKT, P-FOXO3, PPARγ, and Bcl-2 protein expressions, increased the protein expression of Bax, and did not alter the protein expression of GDF15 (*Figure 8A-8F*). Together, these results indicated that the PI3K inhibitor diminished the effects of metformin on bevacizumab-induced cell apoptosis, vascular endothelial injury markers, and inflammation in HUVECs.

Discussion

Bevacizumab-induced vascular complications, mostly derived from vascular endothelial injury, have become a major issue

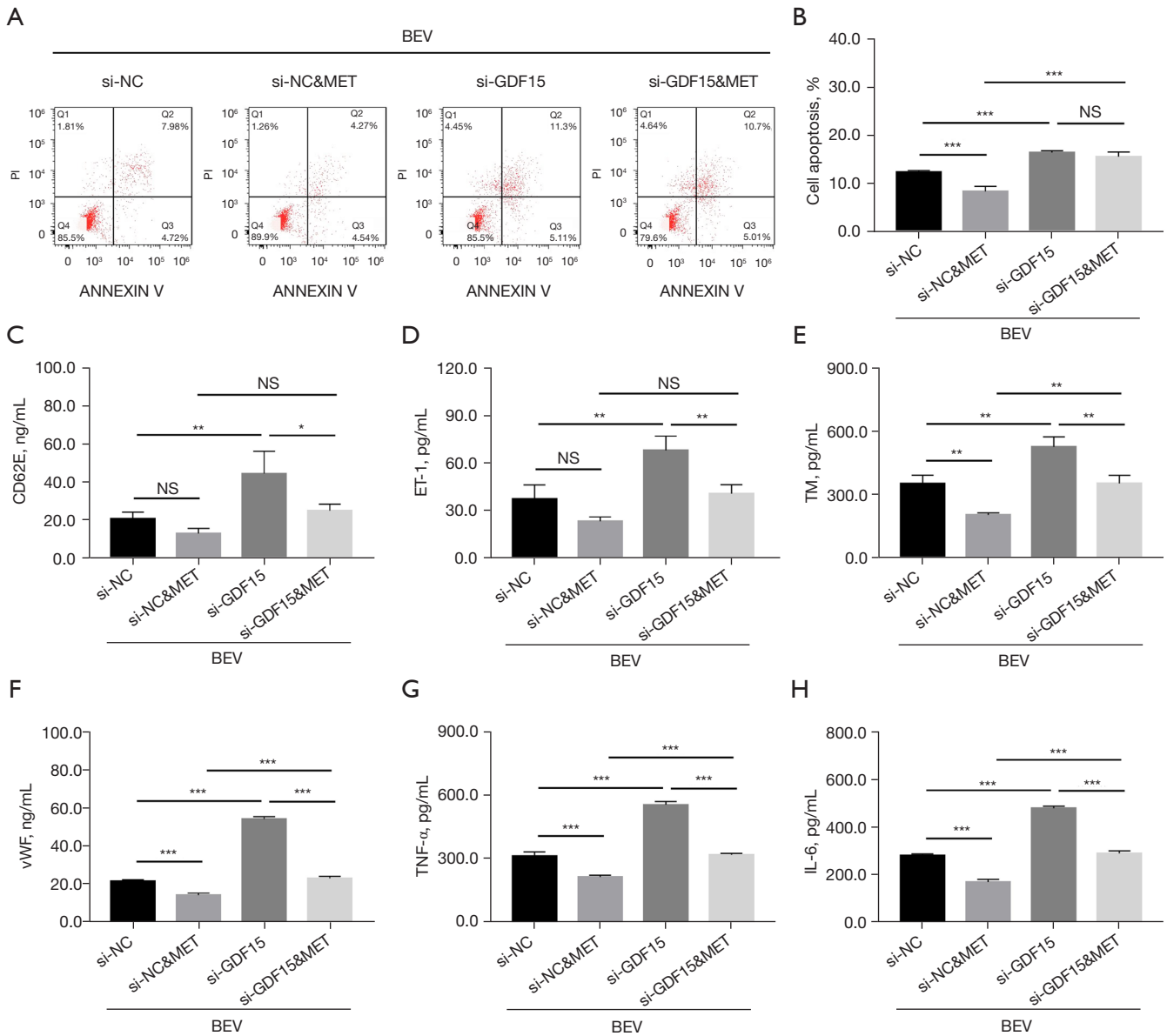

Figure 5 Effect of GDF15 siRNA on apoptosis, vascular endothelial injury markers, and inflammation in bevacizumab and metformin-treated HUVECs. Image of flow cytometry of apoptosis (A), cell apoptosis rate (B), CD62E level (C), ET-1 level (D), TM level (E), vWF level (F), TNF-α level (G), and IL-6 level (H) in the si-NC, si-NC&MET, si-GDF15, and si-GDF15&MET groups, respectively. *P<0.05; **P<0.01; ***P<0.001; NS, not significant. GDF15, growth differentiation factor 15; HUVECs, human umbilical vein endothelial cells; CD62E, selectin E; ET-1, endothelin-1; TM, thrombomodulin; vWF, von Willebrand factor; TNF-α, tumor necrosis factor-alpha; IL-6, interleukin-6; si, siRNA; NC, negative control; MET, metformin; BEV, bevacizumab.

to manage in addition to treating the cancer itself (25). For example, a prior study revealed that in patients with metastatic breast cancer or metastatic colorectal cancer, compared to patients receiving conventional chemotherapy, only patients receiving conventional chemotherapy combined with bevacizumab display cardiovascular and thromboembolic events. In these patients, the number of patients who develop acute myocardial infarction, coronary artery disease, and thromboembolic event are 14.81%, 19.23%, and 17.86%, respectively (26). Additionally, a retrospective cohort study

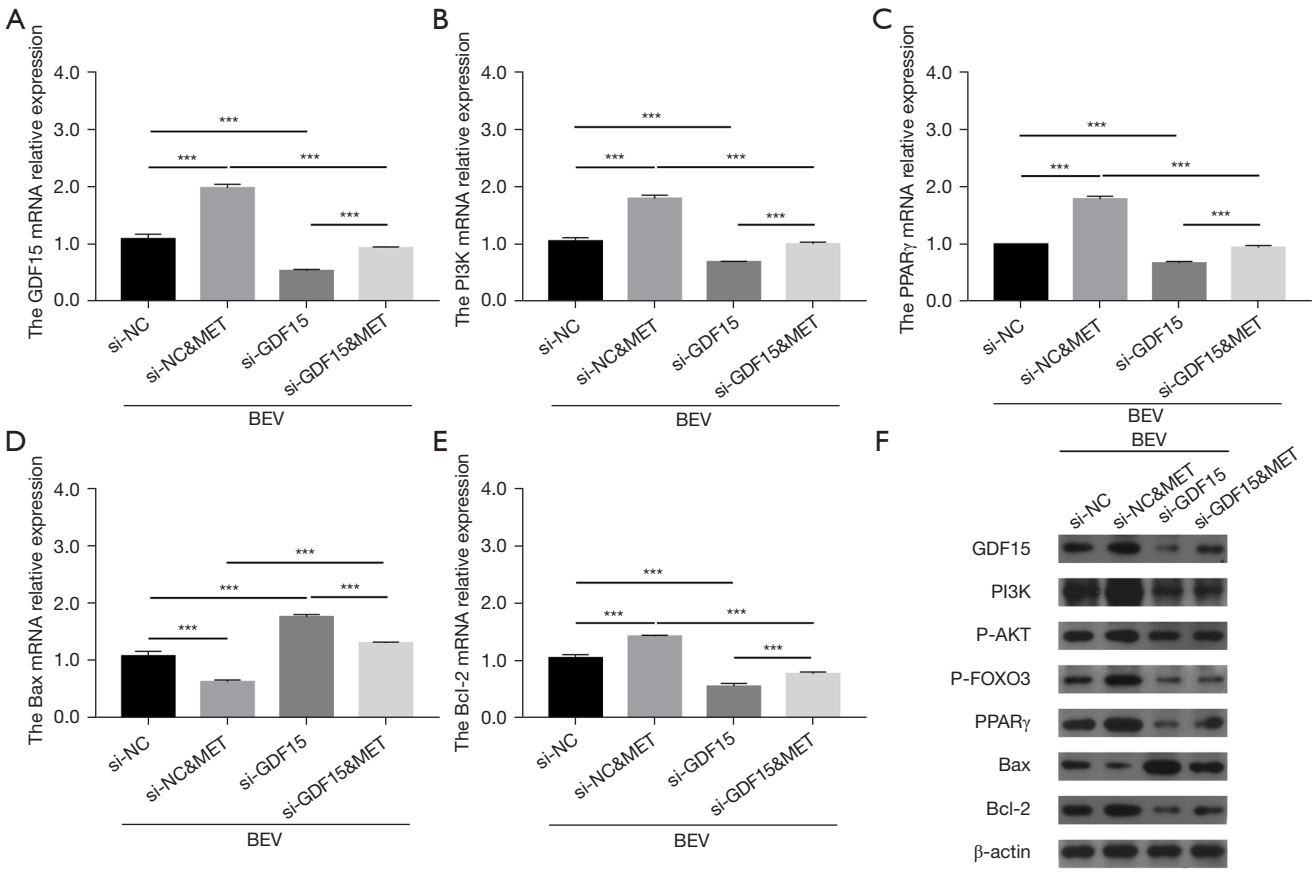

Figure 6 Effect of GDF15 siRNA on the PI3K/AKT/FOXO/PPARγ signaling pathway in bevacizumab and metformin-treated HUVECs. GDF15 mRNA (A), PI3K mRNA (B), PPARγ mRNA (C), Bax mRNA (D), Bcl-2 mRNA (E), and protein expressions of GDF15, PI3K, P-AKT, P-FOXO3, PPARγ, Bax, Bcl-2, and β-actin (F) in the si-NC, si-NC&MET, si-GDF15, si-GDF15&MET groups, respectively. ***P<0.001. GDF15, growth differentiation factor 15; PI3K, phosphatidylinositol 3-kinase; AKT, threonine kinase; FOXO, forkhead box, sub-group O; PPARγ, peroxisome proliferator activated receptor gamma; HUVECs, human umbilical vein endothelial cells; Bax, BCL2 associated X; Bcl-2, B cell lymphoma 2; P-AKT, phosphorylated- threonine kinase; P-FOXO3, phosphorylated-forkhead box, sub-group O3; OE, overexpression; NC, negative control; BEV, bevacizumab.

reported that in cancer patients (multiple types) treated with bevacizumab, 28 out of 230 patients were re-treated in hospital due to major cardiovascular-related events (27). Another study showed that in patient-derived cervical cancer xenograft models, bevacizumab treatment reduces abnormal microvessels but results in worse perfusion and hypoxic damage, suggesting a possible injury to the overall vascular system (28). Nevertheless, there are still no validated solutions to bevacizumab-induced vascular endothelial injury.

Several theories regarding the potential solutions exist, including the combination with metformin, which has been shown to be a protector of vascular functions, especially for cancer patients complicated with diabetes mellitus. For instance, a previous study illustrated that metformin enhances the recovery of peripheral blood flow in patients with peripheral atherosclerosis (29). In addition, another study revealed that the endothelial inflammation caused by high fat can be reduced by treatment with metformin and Abelmoschus esculentus by increasing the microRNA 146a (miR-146a) and miR-155 levels *in vivo* and *in vitro* (30).

According to several previous reports, metformin has been shown to protect the vascular function of cancer patients. One study demonstrated that metformin reduces the processes related tumor progression and enhances chemosensitivity by promoting vessel normalization in metastatic breast cancer *in vivo* and *in vitro* (31). However,

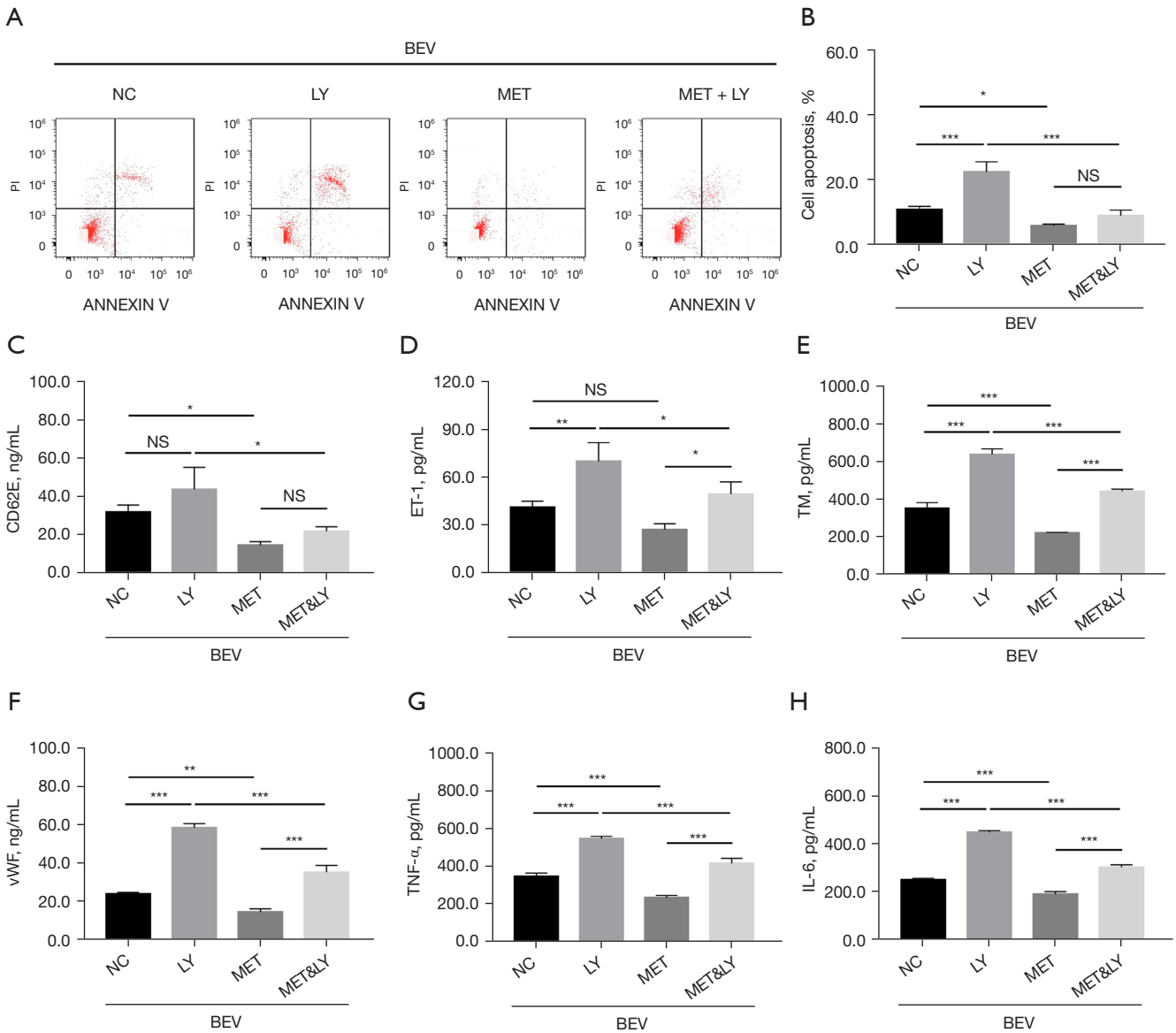

Figure 7 Effect of the PI3K inhibitor on apoptosis, vascular endothelial injury markers, and inflammation in bevacizumab and metformin-treated HUVECs. Image of flow cytometry of apoptosis (A), cell apoptosis rate (B), CD62E level (C), ET-1 level (D), TM level (E), vWF level (F), TNF-α level (G), and IL-6 level (H) in the NC, LY, MET, MET&LY groups, respectively. *P<0.05; **P<0.01; ***P<0.001; NS, not significant. PI3K, phosphatidylinositol 3-kinase; HUVECs, human umbilical vein endothelial cells; CD62E, selectin E; ET-1, endothelin-1; TM, thrombomodulin; vWF, von Willebrand factor; TNF-α, tumor necrosis factor-alpha; IL-6, interleukin-6; NC, negative control; LY, LY294002; MET, metformin; BEV, bevacizumab.

to the best of our knowledge, the potential effect of metformin in relieving bevacizumab-induced vascular endothelial injury was evaluated for the first time in our study. In this study, we found that metformin treatment reduced the effect of bevacizumab in inducing apoptosis, vascular endothelial injury markers, and inflammation in HUVECs. There were some probable explanations for these findings. First, metformin has been elucidated to possess the ability to regulate vascular endothelial function in a protective way, which is achieved through eliminating inflammation and hypoxia damage or promoting angiogenic behaviors, and so on, via regulation of multiple factors

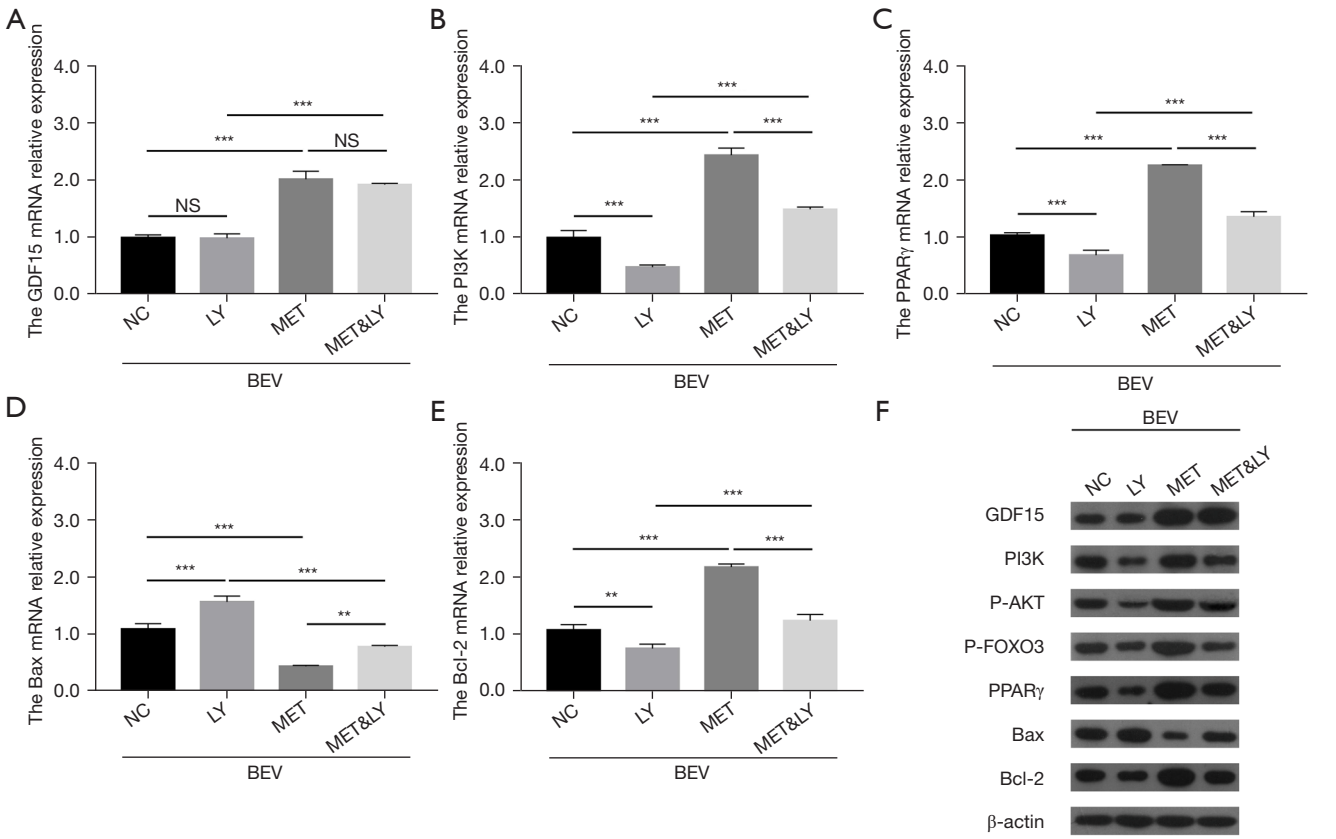

Figure 8 Effect of the PI3K inhibitor on GDF15 and PI3K/AKT/FOXO/PPARγ signaling in bevacizumab and metformin-treated HUVECs. GDF15 mRNA (A), PI3K mRNA (B), PPARγ mRNA (C), Bax mRNA (D), Bcl-2 mRNA (E), and protein expressions of GDF15, PI3K, P-AKT, P-FOXO3, PPARγ, Bax, Bcl-2, and β-actin (F) in the NC, LY, MET and MET&LY groups, respectively. **P<0.01; ***P<0.001; NS, not significant. PI3K, phosphatidylinositol 3-kinase; AKT, threonine kinase; FOXO, forkhead box, sub-group O; PPARγ, peroxisome proliferator activated receptor gamma; Bax, BCL2 associated X; Bcl-2, B cell lymphoma 2; P-AKT, phosphorylated-threonine kinase; P-FOXO3, phosphorylated- forkhead box, sub-group O 3; NC, negative control; LY, LY294002; MET, metformin; BEV, bevacizumab.

(such as (adenosine 5'-monophosphate-activated protein kinase) AMPK, vascular endothelial growth factor receptor (VEGFR), hedgehog pathway, etc.) (17,23,32). Also, as revealed by our further experiments, metformin might also reduce bevacizumab-induced vascular endothelial injury via activation of GDF15 expression and the PI3K/AKT/FOXO/PPARγ signaling pathway in HUVECs. Therefore, our study showed that metformin could reduce bevacizumab-induced vascular endothelial injury in HUVECs.

GDF15 and the PI3K/AKT/FOXO/PPARγ signaling pathway are both involved in the mechanisms through which metformin functions in human body. In terms of GDF15, a previous study revealed that oral metformin elevates GDF15 serum expression, and also decreases food intake, body mass, fasting insulin level, and glucose intake in mice with a high-fat diet; however, these effects are all inhibited in GDF15-null mice (33). A secondary analysis (from a genome-wide association study) showed that up-regulated GDF15 is correlated with decreased risk of coronary artery disease (34). As for the PI3K/AKT/PPARγ signaling pathway, one study elucidated that metformin represses endometrial cancer cell proliferation and migration by mediating the PI3K/AKT/MDM2 signaling pathway (35). In addition, another study revealed that in vascular smooth muscle cells, metformin decreases the inflammatory responses resulting from LPS treatment by modulating Toll-like receptor 4 (TLR4) and PPARγ (16).

Another study demonstrated that metformin treatment alleviates cell senescence and apoptosis by repressing hyperglycaemia sirtuin 1 (SIRT1) decrease and expressions of its downstream targets, including FOXO-1 and p53/p21, in mouse microvascular endothelial cells (36).

Most importantly, GDF15 and the PI3K/AKT/FOXO/PPARγ signaling pathway are also involved in the regulation of vascular endothelial function; although not all in cancer patients, these previous findings still provide valuable information regarding their roles in vascular diseases. For example, one previous study showed that Epac agonist improves endothelial cell survival by enhancing the activation of PI3K/AKT and mitogen-activated protein kinase (MEK)/extracellular signal-regulated kinase (ERK) signaling pathways (37). Additionally, the apolipoprotein M and sphingosine-1-phosphate complex reduces TNF-α-induced endothelial cell injury and inflammation in HUVECs via the PI3K/AKT signaling pathway (38). Another study revealed that PPARγ can interact with ubiquitin protein ligase E3 component N-recognin 5 (UBR5)/ATM interactor (ATMIN), and subsequently enhances the maintenance of homeostasis in endothelial cells (39). An *in vitro* study demonstrated that senescent cell-produced GDF15 promotes the function of vascular progenitor cells in endothelial colony forming cells derived from adult blood (40). Another *in vivo* and *in vitro* study showed that AKT1 increases the recovery of stimuli-induced endothelial-barrier via mediating tight-junction protein turnover, which is modulated by FOXO (41). An interesting previous study revealed that FOXO3 is involved in the regulation of vascular endothelial injury, and our results were consistent with that previous study (42).

Our study found that metformin treatment reduced bevacizumab-induced apoptosis, vascular endothelial injury markers, and inflammation in HUVECs by enhancing GDF15 expression and activating the PI3K/AKT/FOXO/PPARγ signaling pathway. Firstly, these factors, including GDF15 and the PI3K/AKT/FOXO/PPARγ signaling pathway, are all involved in the mechanism of action of metformin. In addition, they also participate in the regulation of endothelial functions; hence, our study indicates that metformin could reduce endothelial damage by regulating GDF15 and PI3K/AKT/FOXO/PPARγ signaling pathway (37-41). To the best of our knowledge, this was the first study assessing the protective role and potential regulatory factors of metformin in bevacizumab-induced vascular endothelial injury in HUVECs. However, more experiments are required to validate our findings, especially regarding the mechanisms identified in this study.

In conclusion, metformin protects against bevacizumab-induced vascular endothelial injury and inflammation via activation of GDF15 and the PI3K/AKT/FOXO/PPARγ signaling pathway.

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Footnote

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