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ORIGINAL RESEARCH

Foretinib Inhibits Cancer Stemness and Gastric Cancer Cell Proliferation by Decreasing CD44 and c-MET Signaling

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Purpose: CD44 isoforms are highly expressed in cancer stem cells, initiating tumor growth and sustaining tumor self-renewal. Among these isoforms, CD44 variant 9 (CD44v9) is overexpressed in chronic inflammation-induced cancer. CD44 and the mesenchymal-to-epithelial transition (MET) receptor tyrosine kinase are coactivated in some gastric cancers (GCs). In this study, we characterized MET and CD44 expression and signaling in human GC cell lines and analyzed differences in the susceptibility of these lines to foretinib.

Patients and Methods: We analyzed cell viability and the rate of apoptotic cells using MTS assays and flow cytometry, respectively. Gene and protein expression were assessed by quantitative reverse-transcription polymerase chain reaction (qRT-PCR) and immunoblotting, respectively.

Results: Foretinib treatment resulted in dose-dependent inhibition of growth in c-MET-amplified MKN45 and SNU620 cells with concomitant induction of apoptosis, but not in c-MET-reduced MKN28 and AGS cells. Foretinib treatment also significantly reduced phosphor-c-MET, phosphor-AKT, beta-catenin, and COX-2 protein expression in MKN45 and SNU620 cells. Interestingly, foretinib significantly reduced CD44, CD44v9, COX-2, OCT3/4, CCND1, c-MYC, VEGFA, and HIF-1a gene expression in CD44 and MET coactivated MKN45 cells and increased CD44s gene expression; in contrast, these drugs were only slightly active against SNU620 cells.

Conclusion: The results of this study indicate that foretinib could be a therapeutic agent for the prevention or treatment of GCs positive for CD44v9 and c-MET.

Keywords: c-MET, CD44v9, foretinib, gastric cancer, OCT3/4

Introduction

The rate of gastric cancer (GC) incidence is high in East Asia, Eastern Europe, and South America.[1–3] Mortality rates have decreased markedly in recent years;[4] however, GC remains the third most common cancer, causing 12% of all cancer-related deaths every year.[5,6] To develop and identify new drug candidates with the aim of reducing GC mortality rates, it is important to determine its tumor characteristics and treatment parameters. The MET proto-oncogene encode the receptor tyrosine kinase (RTK) c-MET. In GC, such activation of MET has been attributed to gene amplification.[7] MET amplification occurred in 2–20% of GC patients, however, only 7% of advanced GC patients overexpressed MET.[8,9] In gastric cancer tissue, MET overexpression has been observed in gastric epithelium harboring dysregulated MET signaling, either as a result of high level focal amplification of the MET

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gene (>12 copies) or via HGF autocrine activation of MET.[10] Previous study MET amplification was not detected in the cases with a gene copy number of <4. These study identified MET amplification at a frequency of 1.5% (4 out of 266 cases) in GC.[11] Many MET tyrosine kinase inhibitors (MET-TKIs) were discontinued in clinical trials for multiple cancer types, including GC, because they were unable to determine the amplification cutoff that was MET for patient enrollment. The issue of how to determine the cutoff value for MET amplification in such cases has not yet been resolved.

HGF/c-MET targeting inhibitors are TKIs (e.g. ATP-competitive MET kinase inhibitors, non-ATP-competitive MET kinase inhibitors and multi-targeted inhibitors of MET and other kinases), anti-HGF neutralizing antibodies and anti-MET neutralizing antibodies used for various cancer which is known to overexpress MET.[12,13] Foretinib, ATP-competitive MET kinase inhibitors, it inhibits HGF-mediated MET phosphorylation, migration and invasion of MET-amplified human gastric cancer cell lines.[14] Foretinib failed to meet its primary end point of an objective response rate >25% in Phase II GC clinical trial. In this study, only 3patients had MET amplification, one of whom had disease stabilization.[15] Other research patients based on germline or acquired MET mutation, germline MET mutations were a strong predictor of response to foretinib compared to acquired MET mutations.[16] In GC cancer, gain-of-function mutation of MET are rare, with MET activation having been attributed mostly to gene amplification.[7,17,18] Previous studies MET gene amplification rather than protein overexpression as a true oncogenic driver and a predictive marker for MET TKIs in GC.[11,19,20] Interestingly though, other study revealed a strong association between p-MET expression and MET amplification in GC.[9] Moreover, c-MET amplification is a frequent molecular abnormality in GC.[21,22]

Crosstalk between HGF/c-MET signaling pathway, PI3K/AKT signaling pathway and Wnt/β-catenin signaling pathway has been implicated in numerous cancers.[23,24] The HGF/c-MET interact and cooperate with tyrosine kinases, can stimulate various downstream PI3K/AKT and Wnt/β-catenin signaling pathway in tumor cells. Prominent Wnt/β-catenin target genes include the CD44 and proto-oncogene c-MYC.[25,26] Especially, cell adhesion molecule CD44 promotes stimulates inflammation, M2 macrophages accumulation and ECM deposition.[27] M2 macrophages exert protumoral functions by enhancing invasion and angiogenesis, immunosuppression and drug resistance through high expression of cyclooxygenase-2 (COX-2), VEGF and MMPs.[28] The hypoxic tumor microenvironment induce phenotypic changes by hypoxic-inducible factor 1α (HIF-1α) that promote EMT and the stem-like properties of gastric cancer cells.[29,30] HIF-1α increases CD44 expression levels and the number of CD44-positive cells.[31] HIF-1α regulates CD44 may be associated with the Wnt/β-catenin signaling pathway in gastric cancer.[32] CD44-positive GC cells exhibit the cancer stem cell (CSC) phenotype, which exhibits tumor growth initiation and self-renewal.[34] Other study has demonstrated a positive correlation between NANOG levels and CD44 positive phenotype.[33] CD44 also modulates interaction, movement, and metastatic potential.[35] The standard isoform CD44s is generally expressed in normal epithelial cells; CD44v, the variant isoform, is expressed in epithelial-type carcinomas.[36] CD44v9 is particularly overexpressed in GC caused by chronic inflammation.[37,38] COX-2 stimulates cancer stem cell development in the inflammatory tumor microenvironment[39] and promotes tumor growth by inducing proliferation, invasion, apoptosis inhibition, and angiogenesis induction.[40,41]

In the present study, we evaluated the suppressive effects of the c-MET inhibitor foretinib on GC proliferation, apoptosis and cancer stemness.

Materials and Methods

Cell Culture and Reagents

GC cell lines SNU620, MKN45, MKN28, and AGS were obtained from the Korean Cell Line Bank (Seoul, Korea) and maintained in RPMI1640 supplemented with 10% fetal bovine serum. Cells were cultured at 100% humidity and 5% CO_2 at 37°C. The c-MET inhibitor foretinib (GSK1363089) was purchased from Selleck Chemicals (Houston, TX, USA). The Annexin V-APC/propidium iodide (PI) Apoptosis Detection Kit (Thermo Fisher Scientific, Rockford, IL, USA) was used to determine apoptosis rates.

Growth Inhibition Assays

IC_{50} values for foretinib in SNU620 and MKN45 cells were measured using the MTS assay for foretinib concentrations of 10, 1, 0.1, 0.05, 0.0025, 0.00125, 0.001, 0.0001, 0.00001, and 0.000001 μM for 48 h. On the day of the proliferation assay, the medium was removed, and 200 μL fresh medium was added to each well in 96-well plates, followed by 20 μL of MTS solution. The plates were then incubated at 37°C for 1 h in a humidified environment with 5% CO_2. Absorbance

was read at 490 nm using a microplate reader (Synergy 2 Multi-Mode Microplate Reader; BioTek). IC_{50} values were determined after fitting growth inhibition curves to dose–response curves using the GraphPad Prism software (GraphPad Software Inc., San Diego, CA, USA).

Apoptosis Analysis

SNU620, MKN45, MKN28, and AGS cells seeded into 6-well plates at a density of 5×10^4 cells/mL were treated with foretinib IC_{50} values. Cell death was determined using the Annexin V-APC/PI Apoptosis Detection Kit (Thermo Fisher Scientific) using a CytoFLEX flow cytometer (Beckman Coulter, Brea, CA, USA). Percentages of intact and apoptotic cells were calculated using the CytExpert software (Beckman Coulter).

qRT-PCR Analysis

To quantitate mRNA expression, total RNA from each sample was reverse-transcribed into cDNA using the High Capacity cDNA Reverse Transcription Kit (Applied Biosystems, Foster City, CA, USA). qRT-PCR was performed using the Power SYBR Green PCR Master Mix and a LightCycler 96 instrument (Roche Applied Science, Indianapolis, IN, USA). Transcript levels of glyceraldehydes-3-phosphate dehydrogenase (GAPDH) were used for sample normalization. The primer sequences were as follows: c-MET (FW 5′-AAG AGG GCA TTT TGG TTG TG-3′; RW 5′-GAT GAT TCC CTC GGT CAG AA-3′), POU5F1 (OCT3/4, FW 5′-TTC AGC CAA ACG ACC ATC TG-3′; RW 5′-GAA CCA CAC TCG GAC CAC ATC-3′), NANOG (FW 5′-CAC CAG TCC CAA AGG CAA AC-3′; RW 5′-GCC TTC TGC GTC ACA CCA TT-3′), CCND1 (FW 5′-GAT CAA GTG TGA CCC GGA CT-3′; RW 5′-TCC TCC TCT TCC TCC TCC TC-3′), VEGFA (FW 5′-AGG CCA GCA CAT AGG AGA GA-3′; RW 5′-TTT CTT GCG CTT TCG TTT TT-3′), CD44 (FW 5′-AGC ATC GGA TTT GAG ACC TG-3′; RW 5′-GTT GTT TGC TGC ACA GAT GG-3′), CD44s (FW 5′-AAA GGA GCA GCA CTT CAG GA-3′; RW 5′-TGT GTC TTG GTC TCT GGT AGC-3′), CD44v9 (FW 5′-ACC ATC CAA CAA CTT CTA CTC TGA CA-3′; RW 5′-CCT TCA GAA TGA TTT GGG TCT CTT-3′), ECAD (FW 5′-TGG GCC AGG AAA TCA CAT CC-3′; RW 5′-GGC ACC AGT GTC CGG ATT AA-3′), COX-2 (FW 5′-TGA GCA TCT ACG GTT TGC TG-3′; RW 5′-AAC TGC TCA TCA CCC CAT TC-3′), HIF-1α (FW 5′-CCA CCT ATG ACC TGC TTG GT-3′; RW 5′-TAT CCA GGC TGT GTC GAC TG-3′), c-MYC (FW 5′-TCA AGA GGC GAA CAC ACA AC-3′; RW 5′-GGC CTT TTC ATT GTT TTC CA-3′), and GAPDH (FW 5′-TTC ACC ACC ATG GAG AAG GC-3′; RW 5′-GGC ATG GAC TGT GGT CAT GA-3′).

Immunoblot Analysis

Immunoblot analysis was performed using standard procedures. Commercially available primary antibodies were directed against anti-phospho-c-MET (Tyr1234/1235; 1:1000; #3077; Cell Signaling Technology, Danvers, MA, USA), anti-c-MET (1:1000; #4560; Cell Signaling Technology), anti-phospho-AKT (1:1000; #4060; Cell Signaling Technology), anti-AKT (1:1000; #1085-1; Epitomics), anti-β-catenin (1:1000; #610153; BD Biosciences), anti-COX-2 (1:1000; sc1745; Santa Cruz Biotechnology), anti-β-actin (1:1000; sc47778; Santa Cruz Biotechnology), and anti-α-tubulin (1:4000; # 05–829; Millipore).

Statistical Analysis

Data were analyzed using the Prism 5 (GraphPad Software Inc.). Values are means ± standard deviation (S.D.). Statistical significance was determined using one-way analysis of variance (ANOVA); significance was determined at a level of $P < 0.05$.

Results

Determining the Effective Dose of Foretinib in c-MET-Positive Cells

We tested the dose-dependent inhibitory effects of foretinib in SNU620 and MKN45 cells (Figure 1). Cells were treated with different concentrations of foretinib for 48 h, and the optimal dose was determined by evaluating cell viability using MTS assays. Treatment with foretinib decreased cell viability in a dose-dependent manner in c-MET-amplified

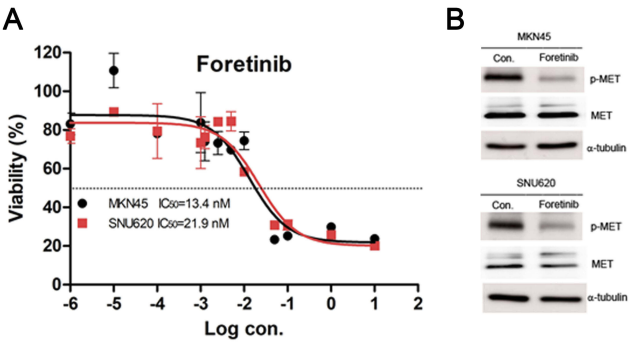

Figure 1 Effect of foretinib on gastric cancer (GC) cells positive for c-MET amplification. (**A**) SNU620 and MKN45 cells were treated with various concentrations of foretinib for 48 h. (**B**) Immunodetection of endogenous c-MET and phosphor c-MET (pY1234/1235) in GC cell lines.

SNU620 and MKN45 cells (n = 3) (Figure 1). Non-linear regression analysis revealed foretinib IC_{50} values of 13.4 nM for MKN45 cells and 21.9 nM for SNU620 cells.

Effects of Foretinib on Cell Apoptosis

To evaluate the effects of foretinib on cell death in SNU620, MKN45, MKN28, and AGS cells, apoptosis was examined by staining with Annexin V-APC/PI, followed by flow cytometry (Figure 2). Cells were stained with Annexin V-APC and PI, which assess early apoptotic and late apoptotic, and necrotic cell populations, respectively. Foretinib showed the best cell death rates in SNU620 and MKN45 cells, whereas apoptosis was seldom observed in MKN28 and AGS cells (Figure 2A and B), with apoptotic cell

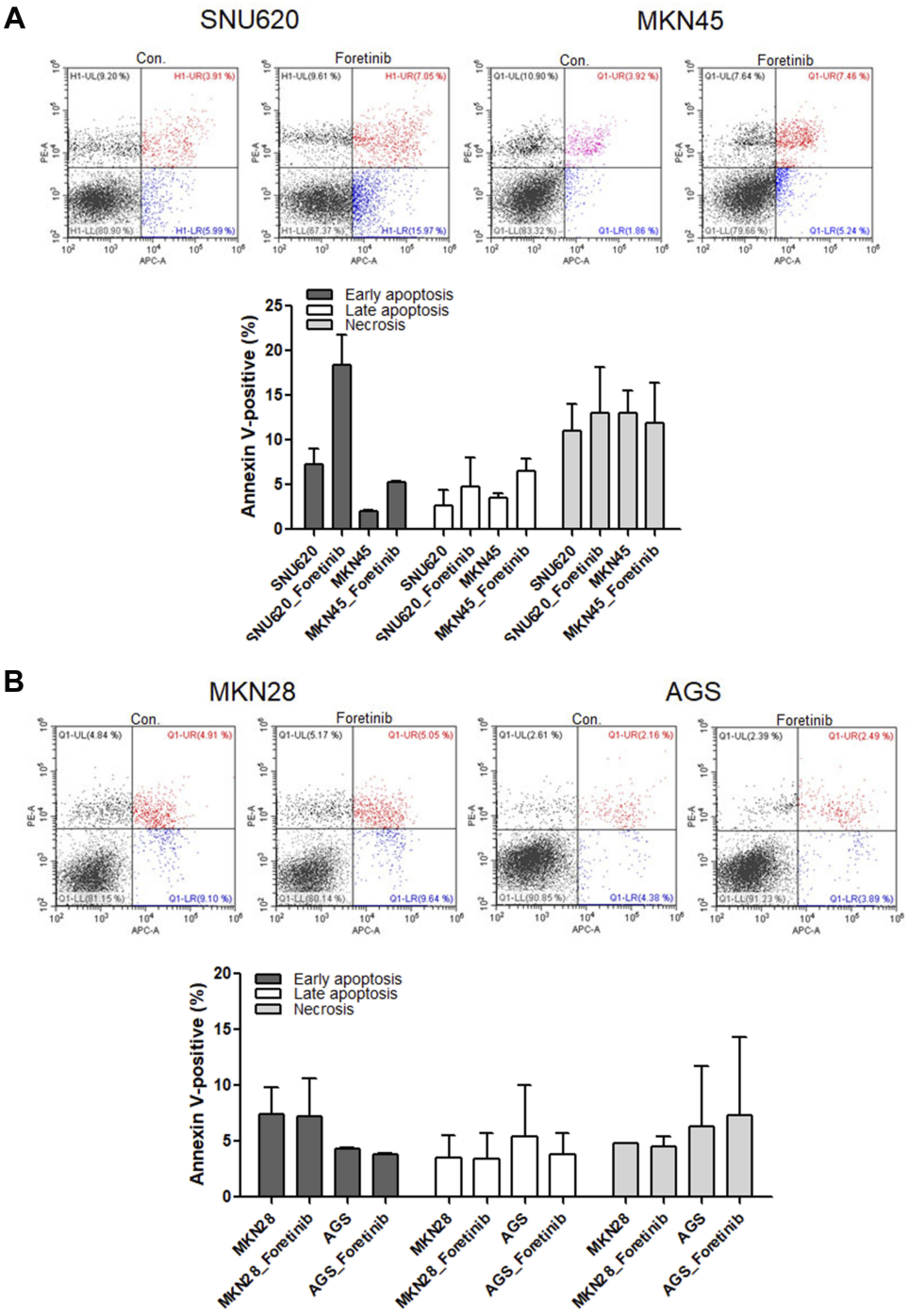

Figure 2 Apoptotic activity of foretinib in (**A**) c-MET-positive SNU620 and MKN45 cells and (**B**) c-MET-negative MKN28 and AGS cells. Flow cytometric assay of GC cells treated with 30 nM foretinib for 48 h. Data are means ± S.D.

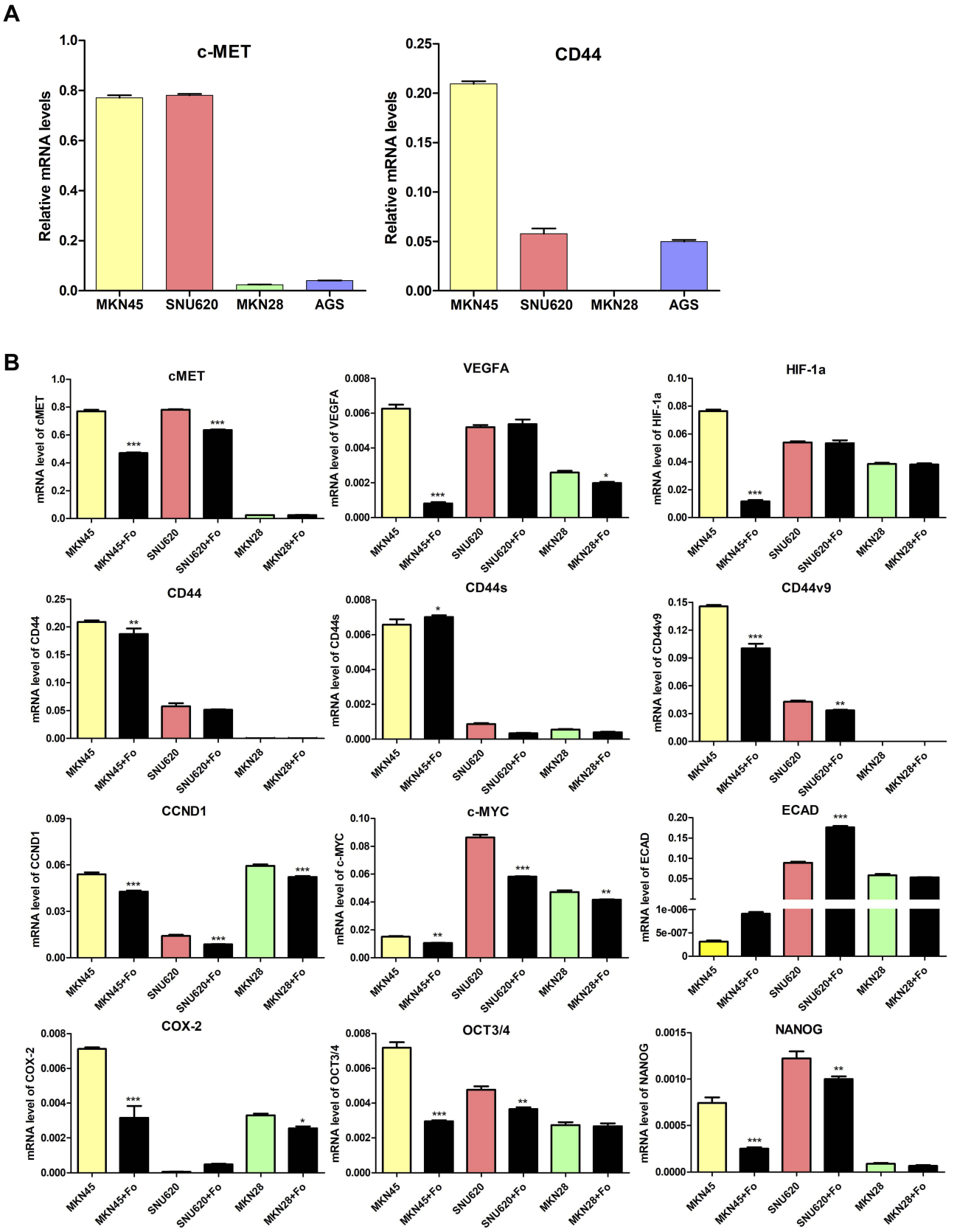

Figure 3 Effect of foretinib on carcinogenesis-related genes in GC cells. (**A**) c-MET and CD44 gene expression in gastric cancer cells and (**B**) mRNA levels of c-MET, HIF-1a, VEGFA, CD44, CD44s, CD44v9, CCND1, COX-2, and ECAD in MKN45, SNU620, MKN28, and AGS cells were determined by quantitative reverse-transcription polymerase chain reaction (qRT-PCR) analysis after treatment with 30 nM foretinib for 48 h. Data are means ± S.D. *P < 0.05; **P < 0.01; ***P < 0.001 (one-way analysis of variance [ANOVA]).

percentages of 23.02 and 12.7%, respectively, after exposure to foretinib for 48 h (Figure 2A). SNU620 and MKN45 were high-c-MET expressors, whereas others such as MKN28 and AGS belonged to the low-c-MET expressor subtype. Notably, MKN45 cells were a high-CD44 expressor subtype (Figure 3A).

Foretinib Inhibits c-MET Activation and Cancer Stemness in GC Cells

To examine the inhibitory effects of foretinib on GC cells (high-c-MET/high-CD44 [MKN45], high-c-MET/low-CD44 [SNU620], and low-c-MET/low-CD44 [MKN28]), oncogenic pathways were examined by analyzing gene and protein expression. Following treatment with foretinib, levels of c-MET, HIF-1α, VEGFA, CD44, CD44v9, CCND1, c-MYC, COX-2, and OCT3/4 mRNA decreased in MKN45 cells, whereas CD44s expression increased. In contrast, these drugs were only slightly active against SNU620 cells (Figure 3B). However, phosphor-c-MET, phosphor-AKT, β-catenin, and COX-2 protein expression decreased in MKN45 and SNU620 cells (Figure 4).

Discussion

Carcinogenesis is complex process whereby malignant transformation occurs through genome instability and inflammation.[42] Several studies have reported that COX-2 activation may be involved in inflammation-mediated CSC proliferation and differentiation.[43,44] Many solid tumors possess CSCs, which share several characteristics with stem cells.[45–47] Presently, CD44 is considered the most useful marker of gastric CSCs and a number of other solid tumors.[34,48–51] CD44 is well known as a downstream target of Wnt/ß-catenin signaling.[25] CD44 affect ß-catenin phosphorylation and nuclear accumulation in myeloid leukemia cells.[52] Other study reveals that CD44 regulates Wnt signaling at the level of membrane receptors.[53] In addition, CD44 controls signaling through interactions with TRKs, including c-Met and VEGFR2.[54] Therefore, we examined CD44 downregulated drugs in MKN45 cells, which have a high expression of CD44 among c-MET-amplified cell lines. In a previous study, CD44 inhibitor drugs were screened using qRT-PCR analysis of MKN45 cells with CSCs to determine their suitability as therapeutic agents in GC treatment. Among the six c-MET inhibitor drugs tested, only foretinib downregulated the CD44 gene; therefore, foretinib was selected for further study.

Protein kinase mutations, overexpression, and dysregulation play an important role in the pathogenesis of many diseases including cancer.[55–57] Therefore, this enzyme family (e.g. ALK, EGFR, ERBBs, VEGFR2, FGFR, PDGFR, c-MET, etc.) has become one of the important drug targets during the past 20 years.[58] Foretinib is multi-kinase inhibitor targeting to c-MET and VEGFR2.[14] In the present study, foretinib showed higher inhibition rates but lower apoptosis rates in MKN45 cells than in SNU620 cells. CD44-positive GC cells showed increased resistance for chemotherapy-induced cell death.[34] Alternative splicing results in several CD44 isoforms with diverse functions.

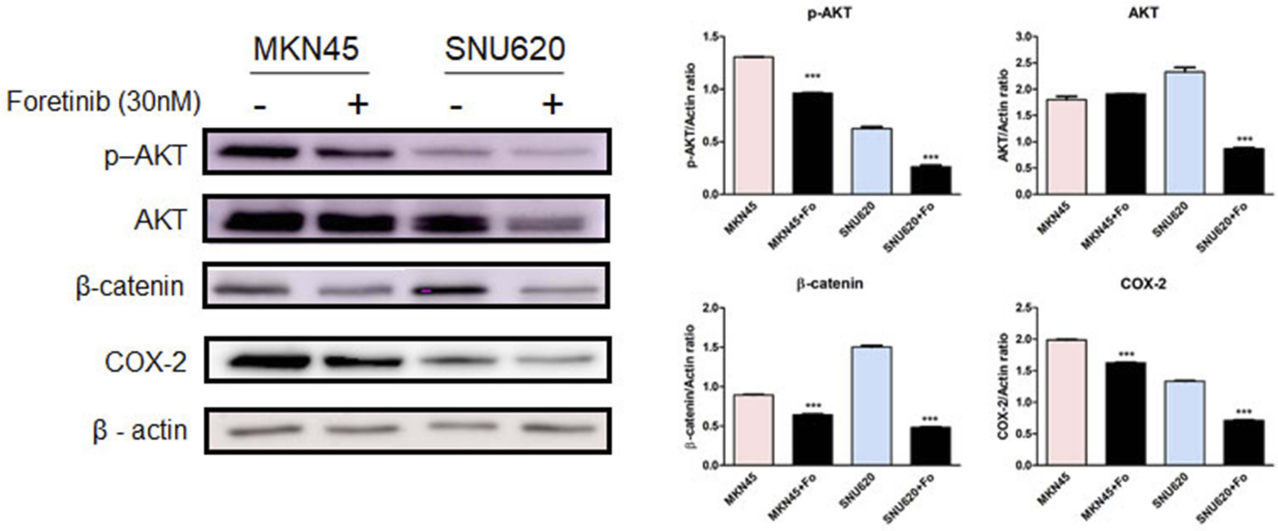

Figure 4 Effect of foretinib on p-AKT, AKT, b-catenin, and COX-2 protein expression in GC cells. Protein levels of p-AKT, AKT, b-catenin, and COX-2 in MKN45 and SNU620 cells were determined by Western blot analysis after treatment with 30 nM foretinib for 48 h. Data are means ± S.D. ***P < 0.001 (one-way ANOVA).

Notably, CD44v9 expression was higher in cancer cells from cancer patients than in normal tissues.[37,38,44] CD44v9 is contained in the v6-10, v7-10, and v8-10 CD44 combinations.[59] Among these, CD44v8-10 acts as a cancer stem cell in cancer development.[60] Stem cell markers include OCT3/4, Nanog, and CD44v9, which are involved in the inflammatory microenvironment during cancer development.[61] When MKN45 cells were treated with foretinib, levels of CD44, CD44v9, OCT3/4, NANOG, and COX-2 genes/proteins decreased. In contrast, those of CD44s increased. Previous study, attenuated ECAD expression, upregulation of β-catenin and enhanced ovarian cancer cell migration.[62] Other study has reported that suppressed EMT through attenuation of AKT phosphorylation and β-catenin.[63] Our results also indicated that foretinib may suppress β-catenin and AKT phosphorylation through increased ECAD expression. Indeed, foretinib inhibited angiogenesis, Wnt/β-catenin signaling, and the PI3K/Akt pathway in this study by inhibiting VEGFA, HIF-1α, c-MET, and AKT phosphorylation, as well as CCND1, c-MYC, and β-catenin in c-MET-positive GC. Our in vitro study strongly supports the clinical evaluation of foretinib, which prevents cancer stemness and c-MET-associated GC.

Conclusion

The results of this study indicate that c-MET and CD44v9 are differentially expressed in GCs and that foretinib exhibits significant inhibitory activity in c-MET- and CD44v9-expressed GC. Our in vitro study strongly supports the clinical evaluation of foretinib, which prevents cancer stemness and c-MET-associated GC.

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Disclosure

The authors report no conflicts of interest in this work.

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