

The Involvement of Hepatocyte Growth Factor-MET-Matrix Metalloproteinase 1 Signaling in Bladder Cancer Invasiveness and Proliferation. Effect of the MET Inhibitor, Cabozantinib (XL184), on Bladder Cancer Cells

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OBJECTIVES	To clarify the invasive mechanisms of muscle-invasive bladder cancer (BCa) would be useful for the determination of appropriate treatment strategies. We previously showed that hepatocyte growth factor (HGF)-MET signaling is correlated with invasiveness of BCa cells. Here, we investigated the effects of the MET inhibitor, cabozantinib (XL184), on BCa cells.
METHODS	We first conducted Western blot analysis to investigate MET expression in BCa cell lines. Next, we examined the effect of cabozantinib on their proliferation and invasive abilities using MTT and Matrigel invasion assays, respectively. Invasion assays were performed using the xCELLigence system. Additionally, to investigate the biological function of HGF-MET signaling, we analyzed gene expression profiles and performed real-time polymerase chain reaction analyses of 5637 cells that were cultivated with or without HGF stimulation, with or without cabozantinib.
RESULTS	MET was highly expressed in 4 of 5 BCa cell lines, and 5637 and T24 cells showed especially high protein expression of MET. Cabozantinib suppressed cell proliferation and invasion (cell index; mock, 1.49 vs HGF, 2.26 vs HGF + XL184, 1.47, $P < .05$). Gene expression profile analysis indicated that matrix metalloproteinase 1 (MMP1) was significantly elevated at the mRNA level with addition of HGF. Moreover, cabozantinib suppressed HGF-induced MMP1 expression in 5637 T24 cells.
CONCLUSIONS	These data indicate that cabozantinib suppressed MMP1 expression by blocking HGF-MET signaling and that HGF-MET-MMP1 signaling is involved in the invasiveness and proliferation of BCa cells. These results suggest that cabozantinib might prove useful for future treatment of muscle-invasive BCa. UROLOGY ■■■: 1.e1–1.e7, 2016. © 2016 Elsevier Inc.

Muscle-invasive bladder cancer (MIBC) has an unfavorable prognosis, with a 5-year survival rate of less than 50% and common progression to metastasis. There have been no advances in the treatment of

MIBC for many years, and new therapies need to be developed.

Urothelial carcinoma (UC) begins in the urothelial lining of the bladder wall and arises and progresses along 2 distinct pathways.¹ One pathway results in low-grade and non-invasive UC, which harbors frequent mutations in FGFR3.² The other pathway results in high-grade and invasive UC, which has frequent defects in the p53 gene.³ In addition, members of the erbB family, vascular endothelial growth factor (VEGF), nuclear factor κB, Akt, phosphatase and tensin homolog deleted from chromosome 10 (PTEN), and cyclooxygenase-2, are also implicated in the progression of bladder cancer (BCa).⁴

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Based on our previous studies, we focused our present investigation on the involvement of hepatocyte growth factor (HGF) in BCa invasion.

HGF, also known as scatter factor, is an epithelial cell mitogen that regulates cell proliferation, migration, survival, tumor angiogenesis, and invasiveness.⁵ HGF is known to be a pleiotropic cytokine that acts on epithelial cells in several organs.⁶ Other studies have suggested that HGF plays an important role as a paracrine factor in the invasiveness and metastasis of oral squamous cell carcinoma, and that an elevated HGF serum level is a predictive marker for metastasis in patients with oral squamous cell carcinoma.⁷ In a previous study, we demonstrated that elevated serum HGF levels were related to tumor aggressiveness in BCa.⁸ We also showed the effects of cross talk between mesenchyme and epithelium on HGF expression by mesenchymal cells.⁸

The cell surface receptor tyrosine kinase for HGF, known as MET, is widely present in cells of epithelial origin. HGF-MET signaling is frequently implicated in cancer, driving tumor invasiveness and metastasis.⁹

To determine the role of HGF-MET signaling in BCa cells, and to investigate if inhibition of such signaling might be useful for BCa treatment, in this study, we focused on the MET inhibitor, cabozantinib, which is a potent inhibitor of several tyrosine kinase receptors including MET, VEGFR-2, Axl, Flt3, Kit, Tie2, and Ret.^{10,11} Preclinical studies show that cabozantinib can block tumor growth and invasion in breast, lung, prostate, and pancreatic cancer-derived cells.^{10,12,13}

The aim of the present study was to determine if HGF-MET signaling is involved in the invasiveness and proliferation of BCa cells, and to investigate the effects of the MET inhibitor, cabozantinib (XL184), on BCa cells.

MATERIALS AND METHODS

Cell Lines, Reagent, and Antibodies

The BCa cell lines RT4, 5637, UM-UC-3, J82, and T24 were obtained from the American Type Culture Collection (Manassas, VA). The primary antibodies used for Western blotting were as follows: anti-MET (D1C2) antibody (Catalog #8198; Cell Signaling Technology Japan, Tokyo, Japan); anti-phospho-MET (Tyr1234/1235) (D26) antibody (Catalog #3077; Cell Signaling Technology Japan); VEGF receptor 2 (55B11) antibody (Catalog #; 2479 Cell Signaling Technology Japan); and anti-human β -actin antibody (Catalog A5441; Sigma-Aldrich, St. Louis, MO). Cabozantinib (XL184) was obtained from Selleckchem (Houston, TX).

Microarray Analysis

Total RNA was isolated from cultured cells using the RNeasy kit (Qiagen, Hilden, Germany). Relative purity was determined using an Agilent 2100 Bioanalyzer (Agilent Technologies, Santa Clara, CA). RNA expression was analyzed using the GeneChip Human Gene 1.0 ST Array (Affymetrix, Santa Clara, CA). This microarray chip contains 28,869 oligonucleotide probes for genes of known and unknown function. First-strand cDNA was synthesized from 300 ng of total RNA by using the GeneChip Whole

Transcript Expression Kit (Ambion) according to the manufacturer's instructions. Ten micrograms of cRNA were input into the second-cycle cDNA reaction. The cDNA was then fragmented and end-labeled with the GeneChip Whole Transcript Terminal Labeling Kit (Affymetrix). Approximately 5.5 μ g of fragmented and labeled DNA target was hybridized to the Affymetrix GeneChip Human Gene 1.0 ST Array at 45°C for 17 hours using a GeneChip Hybridization Oven 640 (Affymetrix) according to the manufacturer's recommendation. Hybridized arrays were washed and stained using a GeneChip Fluidics Station 450 and scanned on a GeneChip Scanner 3000 7G (Affymetrix); each array was then used to generate a CEL file.

Quantitative and Semiquantitative Reverse Transcription Polymerase Chain Reaction

Total RNA was extracted from cultured cells using the RNeasy Micro Kit (Qiagen) as previously described.⁸ The appropriate dilutions of each single-stranded cDNA were prepared for subsequent polymerase chain reaction amplification, and reactions were monitored using GAPDH as a quantitative control. The sequences of each primer set are provided in [Supplementary Table S1](#). The primer set for VEGFB analysis (Hs_VEGFB_1_SG QuantiTect Primer Assay: Catalog No. QT00013783) was obtained from Qiagen. Each sample was analyzed in triplicate for each primer pair. The relative expression levels of MMP1, MMP2, MMP3, MMP7, MMP9, VEGFA, VEGFB, and VEGFC (normalized to GAPDH) were calculated using the relative standard curve method and LightCycler Software Ver.3.5 (Roche Diagnostics, Madison, WI).

Western Blot Analysis

Cell lines were lysed using the Mammalian Cell Extraction Kit (BioVision, Mountain View, CA). After centrifugation at 14,000 \times g for 10 minutes, the supernatant was collected as the total protein extract and stored at -80°C. Protein concentration was then measured using a protein assay reagent (Bio-Rad Laboratories, Hercules, CA). Equal amounts of protein were separated by sodium dodecyl sulfate-polyacrylamide gel electrophoresis and electrophoretically transferred to polyvinylidene difluoride membrane. The membrane was blocked with Block Ace (Dainippon Pharmaceutical, Osaka, Japan) for 60 minutes and incubated with primary antibody at 4°C overnight. Blots were developed by chemiluminescence using a horseradish peroxidase-coupled secondary antibody (Invitrogen) and the ECL Plus Kit (GE Healthcare, Piscataway, NJ).

MTT Assay

T24 cells were seeded into 96-well plates at a density of 1.0×10^4 cells (100 μ L) per well and were maintained in medium containing 10% fetal bovine serum. After 24-hour incubation, cell proliferation was evaluated using the Cell Counting Kit-8 (Dojindo, Kumamoto, Japan).

In Vitro Invasiveness Assay

The invasiveness assays were performed using CIM-16 plates with 8- μ m pore membranes (Roche). Each well was coated with 20 μ L of 10% Matrigel, which was allowed to gel at 37°C, under 5% CO₂ for 4 hours. Each well of the bottom chamber was then filled with 160 μ L of medium containing 10% fetal bovine serum, and the top and bottom portions of the CIM-16 plate were assembled. Thirty microliters of serum-free medium were added to each well of the top chamber, and the assembled CIM-16 plate was allowed to equilibrate for 1 hour at 37°C, 5% CO₂. For seeding,

cells were rinsed with phosphate-buffered saline, trypsinized for 3 minutes, centrifuged at $280 \times g$ for 5 minutes, and washed with serum-free medium before resuspension in serum-free medium. Cells (4×10^4 cells/well) were seeded onto the top chamber of the CIM-16 plate, and the plate was pre-incubated for 30 minutes at room temperature. The plate was then placed into the xCELLigence system for data collection. The xCELLigence software was set to collect impedance data (reported as cell index) at least once per hour.

Statistical Analysis

Statistical significance was calculated using Student *t* test to evaluate cell proliferation, cell invasion, or gene expression. A difference of $P < .05$ was considered statistically significant.

RESULTS

MET Expression in BCa Cells

We first examined the protein expression of MET in BCa cell lines by Western blot analysis using an antibody specifically directed against human MET. We found high MET protein levels in 4 of the 5 BCa cell lines tested; RT4 cells (papilloma) did not show MET protein expression. The 5637 and T24 cell lines displayed much higher protein expression of MET than did the other cell lines (Fig. 1A).

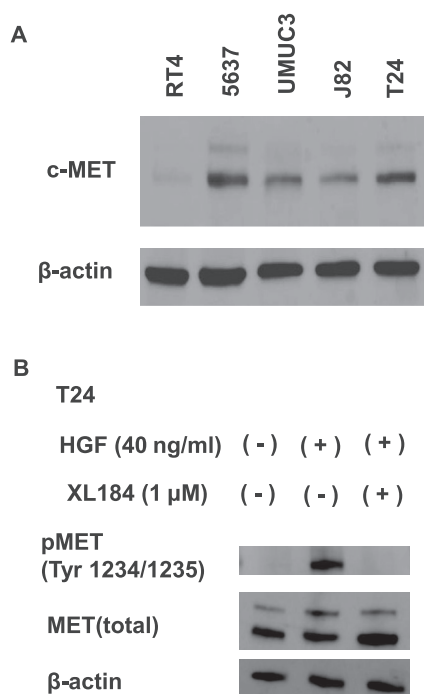


Figure 1. MET protein expression in bladder cancer (BCa) cell lines and inhibition of hepatocyte growth factor (HGF)-induced MET phosphorylation by cabozantinib (XL184). Western blot analysis of (A) expression of the MET protein in the BCa cell lines RT4, 5637, UMUC3, J82, and T24, and (B) the phosphorylation of MET on HGF (40 ng/ml) stimulation with or without cabozantinib (XL184) (1 μ M). The results presented in A and B are representative results of 3 independent experiments.

Cabozantinib (XL184) Inhibits the Phosphorylation of MET

We next assessed whether MET is phosphorylated on HGF stimulation of BCa cells and, if so, if cabozantinib (XL184) inhibits such phosphorylation of MET. Using Western blotting, we detected phosphorylated MET protein in T24 cells on HGF stimulation and found that this phosphorylation was significantly decreased by the addition of cabozantinib (XL184) (Fig. 1B).

Effects of Cabozantinib (XL184) on Cell Growth and Cell Invasion

To assess whether the growth and invasion of invasive BCa cells with or without HGF stimulation might be inhibited by cabozantinib (XL184), we performed MTT and invasion assays. We found that cell growth with or without HGF stimulation was inhibited in a cabozantinib (XL184) dose-dependent manner (Fig. 2A) and that the promotion of cell invasion by HGF stimulation was inhibited by cabozantinib (XL184) (cell index; mock 1.49 vs HGF 2.26 vs HGF + XL184 1.47, $P < .05$) (Fig. 2B).

Genome-wide Analysis of the Influence of HGF on Bladder Tumors

To investigate signals activated downstream of HGF stimulation in BCa cells, we next performed gene expression profiling by cDNA microarray analysis using samples of 5637 cells with or without stimulation by HGF. We identified 26 genes that were upregulated at least 2-fold in 5637 cells in the presence of HGF compared with unstimulated controls. Of these 26 genes, we focused on the matrix metalloproteinase 1-encoding gene *MMP1* as a potential downstream effector of HGF.

Public Database Analysis

According to the Cancer Genome Atlas RNA seq dataset for bladder UC, MET is correlated with MMPs (MMP1, 2, 3, 7, and 9), especially MMP1 (Table 1). This dataset also indicated that MET expression levels of invasive BCa cells were higher than those of noninvasive BCa cells and normal bladder cells.

Cabozantinib (XL184) Inhibited MMP1 Expression Induced by HGF Stimulation

We then examined the mRNA expression of a number of MMPs in the BCa cell lines 5637 and T24 on HGF stimulation. *MMP1* mRNA expression was significantly elevated on HGF stimulation in the 5637 and T24 cell lines. Cabozantinib inhibited *MMP1* mRNA expression induced by HGF stimulation of these BCa cell lines (Fig. 3).

Cabozantinib (XL184) Inhibited VEGFC mRNA Expression Induced by HGF Stimulation

We then examined the mRNA expression of various VEGFs in the BCa cell line T24 on HGF stimulation. *VEGFC* mRNA expression was significantly elevated on HGF stimulation of these cells, and cabozantinib inhibited *VEGFC* mRNA expression induced by HGF stimulations of this BCa cell line (Supplementary Figure S1).

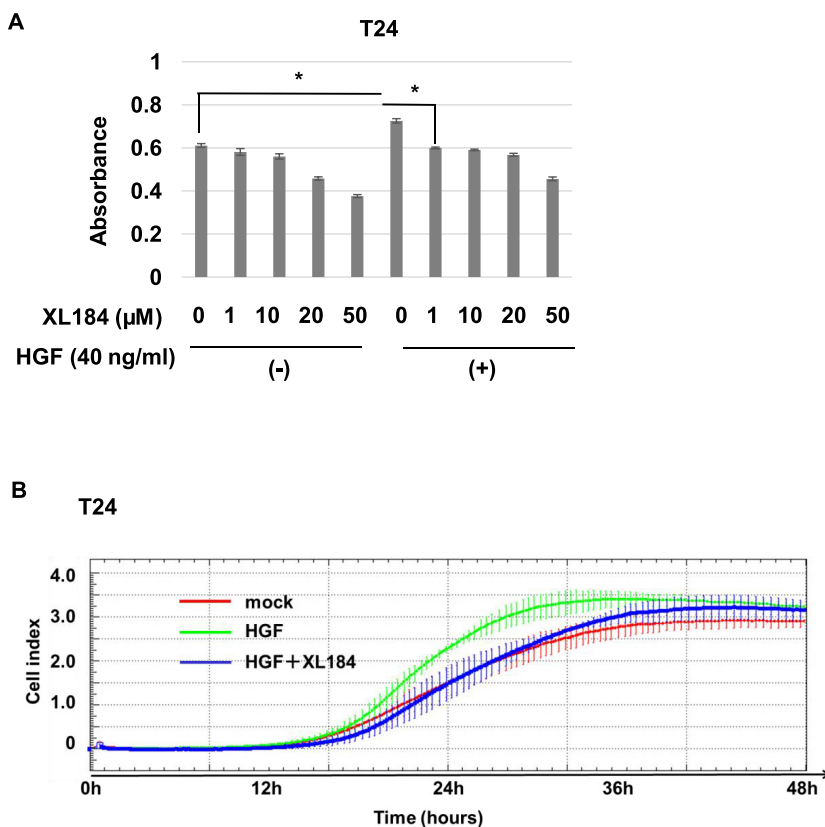


Figure 2. Effect of hepatocyte growth factor (HGF) stimulation or cabozantinib (XL184) on the viability and invasion of the T24 bladder cancer (BCa) cell line. The T24 BCa cell line was stimulated with HGF (40 ng/ml), cabozantinib (XL184) (A: 0, 1, 10, 20, 50 μ M; B: 1 μ M), or both, following which (A) viability of the cells was evaluated using the MTT assay and (B) cell invasion was assayed using Matrigel invasion assays. The results in A and B are presented as means \pm standard error of the mean of 3 independent experiments.

Effect of HGF Stimulation and Cabozantinib (XL184) on VEGFR-2 Protein Expression Levels in BCa Cells

Lastly, we assessed whether the protein level of VEGFR-2 is elevated on HGF stimulation of BCa cells and, if so, if cabozantinib (XL184) inhibits such elevated VEGFR-2 protein level. Western blotting analysis indicated that VEGFR-2 protein expression did not change in T24 cells on HGF stimulation, with or without cabozantinib (XL184) (Supplementary Figure S2).

Comment

We previously reported that elevated serum HGF levels were related to tumor aggressiveness in BCa, and also showed the effects of cross talk between mesenchyme and epithelium on HGF expression by mesenchymal cells.⁸ In the present study, we investigated the biological function of HGF-MET signaling and the effect of cabozantinib on the proliferation and invasion abilities of BCa cell lines. Our study produced 4 key findings. First, high MET protein levels were detected in 4 of 5 BCa cell lines tested, among which 5637 and T24 cells displayed particularly high protein expression of MET; in contrast, the RT4 (papilloma) cell line did not express MET protein. Second, phosphorylation of

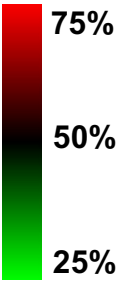
the MET protein was detected, and cell growth and invasiveness were enhanced on HGF stimulation. Third, gene expression profile analysis indicated that expression of *MMP1* mRNA was significantly elevated on HGF addition. Fourth, cabozantinib (XL184) inhibited the phosphorylation of the MET protein, the enhanced cell growth and invasiveness, and the elevated *MMP1* and *VEGFC* mRNA levels that were induced by HGF stimulation.

MMPs are a family of closely related proteolytic enzymes that are involved in the degradation of different components of the extracellular matrix. There is increasing evidence to indicate that individual MMPs have an important role in tumor invasion and tumor spread. Previous studies reported the involvement of *MMP2* and *MMP9* in invasive BCa.^{14,15} In the present study, significant elevation of *MMP1* at the mRNA level was found on addition of HGF to several BCa cell lines; elevation of *MMP2*, *MMP7*, or *MMP9* mRNA was not observed.

In the present study, cabozantinib potently inhibited HGF-induced signaling of BCa cell lines. However, cabozantinib is a potent inhibitor not only of MET but also of several other tyrosine kinase receptors including VEGFR-2, Axl, Flt3, Kit, Tie2, and Ret. In addition to HGF, fibroblast growth factors and VEGFs have also been reported

Table 1. The Cancer Genome Atlas RNA seq dataset for Bladder Urothelial Carcinoma (BLCA)**Tumor / Normal Ratio**

Case ID	MET	MMP1	MMP2	MMP3	MMP7	MMP9
TCGA-BL-A13J	9.3181	98.805	0.9407	0.0914	N	5.9053
TCGA-BT-A20U	9.3165	29.256	5.1098	12.271	178.25	6.6752
TCGA-CU-A0YN	7.8027	15463	0.5858	4.8845	183.11	152.8
TCGA-BT-A20Q	6.8053	102.87		0.5944	N	N
TCGA-BT-A20N	4.8482	45.097	0.2802	0.0614	4.3342	0.2594
TCGA-BT-A20R	4.4593	1.7445	1.9424	0.0016	3259	192.66
TCGA-BT-A2LB	3.0294	114.97	2.730	55.262	134.82	1.0361
TCGA-K4-A5RI	2.1747	19.135	0.2457		36.347	0.0493
TCGA-GD-A3OQ		62.15	0.4094	4.0081	1.0235	23.971
TCGA-GD-A3OP		3.4263	1.6012	3.5512	1.0328	0.9219
TCGA-CU-A0YR	1.1896	725.38	0.3914	145.3	1.8968	3.7826
TCGA-GC-A3BM	1.0953	5.8018	0.1771	0.0818	0.0955	
TCGA-BT-A2LA	0.8017	0.1951	0.3091	0.0439	0.0007	6.9308
TCGA-GC-A3WC	0.6955	10.047	0.2398	3.9068	0.0184	3.4333
TCGA-GD-A2C5	0.6677	0.0646	0.8255	75.723	0.6988	0.5252
TCGA-GC-A6I3	0.6307	0.0597	0.4058	0.2293	0.031	1.0181
TCGA-K4-A3WV	0.6304	3.266	0.5698	34.74	0.9888	27.358
TCGA-BT-A20W	0.3669		0.0728	0.6599	0.2231	
TCGA-K4-A54R	0.2878		1.4546	0.2133	0.1005	17.366



as growth factors for BCa progression. In particular, VEGF is frequently overexpressed in urothelial cell carcinoma of the bladder, and this overexpression has been shown to correlate with poor prognosis in several studies.¹⁶⁻¹⁸ Furthermore, in this study, we found that cabozantinib (XL184) inhibited VEGFC mRNA expression induced by HGF stimulation but that VEGFR-2 protein expression was not affected by HGF stimulation with or without cabozantinib (XL184). These data suggested that cabozantinib (XL184) has potential inhibitory effects on tumor growth through inhabitation of the VEGFC-VEGFR-2 signaling pathway. We previously reported that the serum VEGF level significantly correlates with muscular invasiveness in BCa, that VEGF promotes tumor proliferation and invasion through VEGFR-2, and that VEGF-targeted therapy may be effective in treating invasive BCa.¹⁹ Therefore, cabozantinib inhibition of not only HGF-MET signaling as indicated in the present report, but also of HGF-mediated VEGF signaling as suggested by our present and previous reports, indicates that cabozantinib treatment will be effective for invasive BCa.

The clinical use of cabozantinib is currently under investigation. Advanced metastatic UC or non-transitional cell carcinomas of the bladder, urethra, ureter, or renal pelvis, including but not limited to squamous cell, neuroendocrine, and adenocarcinoma (including urachal and sarcomatoid), were enrolled in a Health Insurance Portability

and Accountability Act-compliant, open-label, prospective, single-arm, phase II clinical trial of cabozantinib that was approved by the National Cancer Institute's institutional review board. Although the details of the therapeutic effect have not been reported, side effects that are similar to those found with other tyrosine kinase inhibitors (TKIs) have been found.^{20,21} Cabozantinib is positioned as a second-line therapy in renal cell carcinoma²² and will soon be approved for the treatment of BCa.

In this study, it was shown that HGF-MET-MMP1 signaling was involved in BCa cell growth and invasion, downstream of HGF-MET signaling and cabozantinib suppressed MMP1 expression by blocking HGF-MET signaling. A limitation of our study is the lack of data regarding normal urothelial cells. Further study will be required to more fully elucidate the mechanism of the anticancer and side effects associated with cabozantinib use.

CONCLUSION

These data indicate that cabozantinib suppressed MMP1 expression by blocking HGF-MET signaling, and that HGF-MET-MMP1 signaling is involved in the invasiveness and proliferation of BCa cells. These results suggest that cabozantinib might prove useful for future treatment of MIBC.

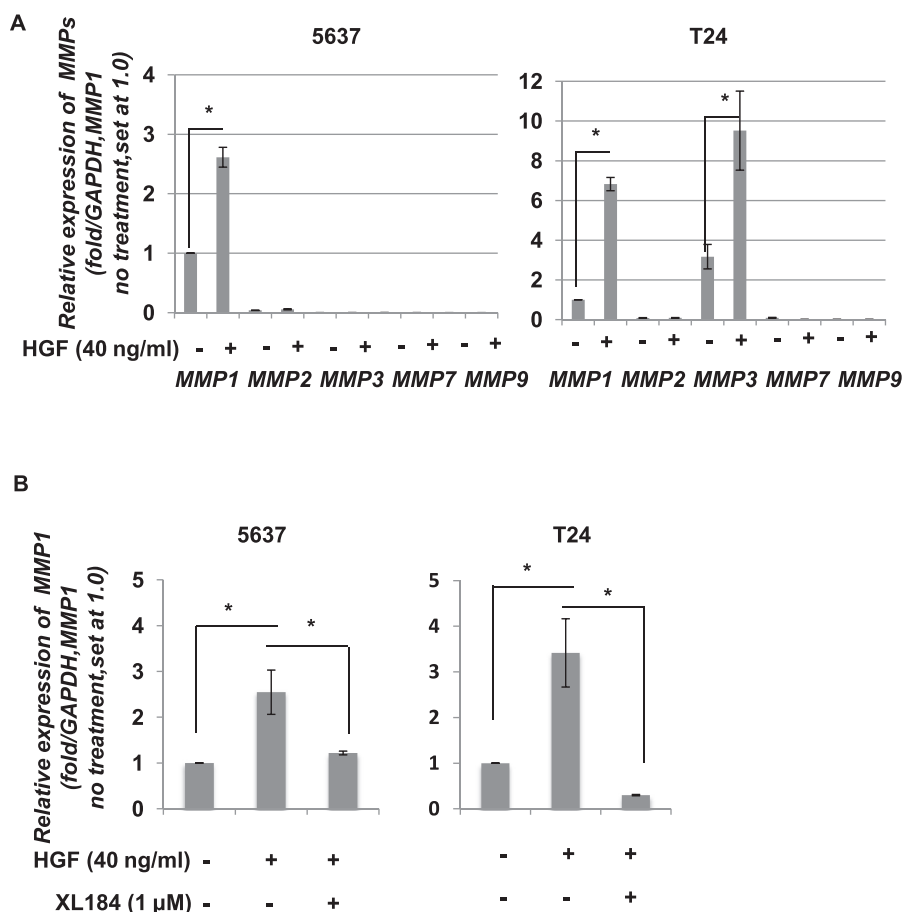


Figure 3. Effect of hepatocyte growth factor (HGF) stimulation with or without cabozantinib (XL184) on MMP mRNA expression in bladder cancer (BCa) cell lines. **(A)** The mRNA expression of MMPs in the BCa cell lines 5637 and T24 on HGF stimulation. **(B)** The BCa cell lines 5637 and T24 were stimulated with control or with HGF (40 ng/mL) with or without cabozantinib (XL184) (1 μM), followed by analysis of the mRNA expression of the indicated MMPs. Cabozantinib (XL184) inhibited the mRNA expression of MMP1. The results in **A** and **B** are presented as means ± standard error of the mean of 3 independent experiments.

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APPENDIX

SUPPLEMENTARY DATA

Supplementary data associated with this article can be found, in the online version, at <http://dx.doi.org/10.1016/j.urology.2016.12.006>.