MMP-1 and pro-MMP-10 as potential urinary pharmacodynamic biomarkers of FGFR3-targeted therapy in patients with bladder cancer

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Nonstandard abbreviations used:

shRNA: short hairpin RNA
Translational relevance

Dysregulation of FGFR3 has been implicated in the pathogenesis of bladder cancer, and several investigational agents targeting FGFR3 have been evaluated in early phase clinical studies. To facilitate the clinical development of FGFR3-targeted therapy, we sought to identify potential noninvasive pharmacodynamic biomarkers to gauge drug activities. Combining transcriptional profiling and biochemical analyses, we identified and confirmed two matrix metalloproteinases, MMP-1 and MMP-10, as downstream targets of FGFR3. Inhibiting FGFR3 function with a humanized monoclonal antibody, R3Mab, led to a significant reduction of MMP-1 and MMP-10 in xenograft models in conjunction with attenuated tumor growth. Importantly, in a phase I dose-escalation trial, R3Mab caused an acute reduction of urinary MMP-1 and pro-MMP-10 levels in advanced bladder cancer patients. These data warrant further investigation in larger clinical studies using these potentially actionable noninvasive pharmacodynamic biomarkers to stratify patients with elevated MMP-1 and pro-MMP-10 and potentially to monitor therapeutic response.
Abstract

**Purpose:** The aim of this study was to identify noninvasive pharmacodynamic biomarkers of FGFR3-targeted therapies in bladder cancer to facilitate the clinical development of experimental agent targeting FGFR3.

**Experimental design:** Potential soluble pharmacodynamic biomarkers of FGFR3 were identified using a combination of transcriptional profiling and biochemical analyses in preclinical models. Two matrix metalloproteinases (MMPs), MMP-1 and MMP-10, were selected for further studies in human bladder cancer xenograft models treated with a specific anti-FGFR3 monoclonal antibody, R3Mab. Serum and urinary levels of MMP-1 and MMP-10 were determined in healthy donors and bladder cancer patients. The modulation of MMP-1 and MMP-10 by R3Mab in bladder cancer patients was further evaluated in a phase I dose-escalation study.

**Results:** MMP-1 and MMP-10 mRNA and protein were down-modulated by FGFR3 shRNA and R3Mab in bladder cancer cell lines. FGFR3 signaling promoted the expression and secretion of MMP-1 and pro-MMP-10 in a MEK-dependent fashion. In bladder cancer xenograft models, R3Mab substantially blocked tumor progression and reduced the protein levels of human MMP-1 and pro-MMP-10 in tumor tissues as well as in mouse serum. Furthermore, both MMP-1 and pro-MMP-10 were elevated in the urine of patients with advanced bladder cancer. In a phase I dose-escalation trial, R3Mab administration resulted in an acute reduction of urinary MMP-1 and pro-MMP-10 levels in bladder cancer patients.

**Conclusion:** These findings reveal a critical role of FGFR3 in regulating MMP-1 and pro-MMP-10 expression and secretion, and identify urinary MMP-1 and pro-MMP-10 as potential pharmacodynamic biomarkers for R3Mab in bladder cancer patients.
Introduction

Bladder cancer is the fifth most common cancer worldwide, with an estimated 386,330 new cases and 150,200 deaths in 2008 (1). Despite radical cystectomy and intensive chemotherapy, the 5-year survival rate for patients with localized and distant metastatic bladder cancer is approximately 35% and 5-6%, respectively (2). Thus, novel therapeutic agents are urgently needed to improve patient outcome.

FGFR3 belongs to a family of four structurally and functionally related single-transmembrane receptor tyrosine kinases, which transduce signals from many of the 22 identified FGF polypeptides in humans (3-5). Upon canonical FGF binding in a ternary complex with heparin sulfate proteoglycans, FGFR3 undergoes dimerization, autophosphorylation and activation. This triggers the recruitment of adaptor proteins, such as FGFR substrate 2α (FRS2α), leading to activation of several downstream signaling cascades, including the Ras-Raf-MAPK, PI3K-Akt-mTOR, and phospholipase Cγ (PLCγ) pathways. Dysregulation and activation of FGFR3 through a variety of mechanisms has been identified in a wide spectrum of human cancers, including multiple myeloma (6-8), squamous cell carcinoma of the lung (9, 10), breast cancer (11, 12), glioblastoma (13, 14), testicular cancer (15), and bladder cancer (16).

Specifically, accumulating genetic and functional evidence has established FGFR3 as a potential oncogenic driver and therapeutic target in bladder cancer. About 60 to 70% of non-muscle-invasive low-grade papillary and 11-16% of muscle-invasive bladder tumors contain somatic activating mutations in FGFR3 (17-20), the majority of which are missense substitutions in the extracellular or juxtamembrane domains of FGFR3 that lead to ligand-independent
receptor dimerization and activation. Besides activating mutations, gene fusion between FGFR3 and TACC3 or BAIAP2L1 has been recently identified in bladder cancer cell lines as well as primary tumors, resulting in aberrant regulation and activation of FGFR3 (21, 22). In addition, high levels of wild type FGFR3 is found in more than 40-50% of muscle-invasive and metastatic bladder cancers (19, 23). Moreover, expression of activated FGFR3 transforms NIH-3T3 cells and hematopoietic cells in culture and confers tumorigenicity in vivo (24-26). Importantly, several preclinical studies using genetic and/or pharmacological approaches have demonstrated that inhibition of FGFR3 in bladder cancer suppresses cell proliferation in culture and tumor growth in xenograft and orthotopic models (23, 27-31). Collectively, these data suggest that FGFR3 could be an oncogenic driver in a subset of bladder cancers and hence that targeting this receptor may be therapeutically beneficial. Indeed, several small molecule inhibitors of FGFR have entered into clinical investigations for cancer therapy (32-34). A specific human monoclonal anti-FGFR3 antibody we developed (designated R3Mab or MFGR1877S) has been evaluated in a phase I clinical trial (35).

While the advances in developing FGFR3-targeted therapeutic agents are encouraging, identification and validation of accessible, mechanism-based biomarkers to gauge targeted pharmacodynamic modulation is urgently needed to facilitate clinical investigation. In the current study, combining transcriptional profiling and biochemical approaches, we identified MMP-1 and pro-MMP-10 as bona fide FGFR3 downstream targets and the urinary level of these metalloproteinases could serve as potential easily accessible pharmacodynamic biomarkers for FGFR3-targeted therapy in bladder cancer.
Materials and Methods

Cell culture, siRNA transfection, and reagents

Bladder cancer cell lines RT112 which contain FGFR3-TACC3 fusion endogenously (21) were purchased from the German Collection of Microorganisms and Cell Cultures (DSMZ, Germany). RT112 cells stably expressing doxycycline-inducible shRNAs targeting FGFR3 or enhanced green fluorescent protein (EGFP) were described in our previous study (29). Bladder cancer cell line UMUC-14 which contains FGFR3S249C was obtained as described (29). Bladder cancer cell line SW780 which contains FGFR3-BAIAP2L1 fusion (21) was purchased from American Type Culture Collection (ATCC, Manassas, VA). All cells are stored at early passages in a central cell bank at Genentech. Cell lines were authenticated by short tandem repeat and genotyped upon re-expansions, and experiments were carried out with low passage cultures of these stocks. The cells were maintained with Dulbecco’s Modified Eagle Medium (DMEM) supplemented with 10% fetal bovine serum (FBS) (Sigma), 100 U/mL penicillin, 0.1 mg/mL streptomycin and L-glutamine under conditions of 5% CO2 at 37°C.

Rapamycin and PI3K inhibitor LY294002 were obtained from Cell Signaling Technology (Danvers, MA). A potent and selective MEK1/2 inhibitor PD0325901 (Pfizer) was purchased from Synthesis Med Chem (San Diego, CA).

All RNA interference experiments were carried out with ON-TARGETplus siRNAs (25 nM, Dharmaco, Lafayette, CO). Cells were transfected with Lipofectamine RNAiMax (Invitrogen, Carlsbad, CA), and RNA or conditioned medium was collected 48 hr or 72 hr after transfection.
Gene expression array and quantitative reverse transcriptase (qRT)-PCR analyses of mRNA expression level

The microarray studies with RT112 cells expressing doxycycline-inducible shRNAs targeting FGFR3 or EGFP were conducted as described (36), and can be accessed at the Omnibus (GEO) database under the accession number GSE41035. To detect transcripts of MMP-1 and MMP-10, qRT-PCR was performed with pre-designed Taqman gene Expression assays (Applied Biosystems). All reactions were performed at least in triplicates. The relative amount of all mRNAs was calculated using the comparative CT method after normalization to human RPL19.

Protein analyses

Cells were treated as described in the figure legends. For total cell lysates, cells were washed twice with ice-cold PBS and extracted in RIPA buffer (Millipore, Billerica, MA) supplemented with phosphatase inhibitor cocktail PhosSTOP and Complete protease inhibitor cocktail (Roche Applied Science, Indianapolis, IN). For analysis of protein expression in tumor xenografts, lysate from tumor tissues was extracted with lysis buffer (consisting of 150 mM sodium chloride, 20 mM Tris (pH 7.5), 2 mM EDTA, 1% Triton X-100, 10 mM sodium fluoride, supplemented with protease inhibitors and phosphatase inhibitors) by pulverizing the frozen tissues using the FastPrep-24 homogenizer as described by the manufacturer (MP Biomedicals, Irvine, CA).

Protein lysates were analyzed by sodium dodecyl sulfate-polyacrylamide gel electrophoresis (SDS-PAGE) and Western blot. The primary blotting antibody for total FRS2 (sc-8318) was purchased from Santa Cruz Biotechnology (Santa Cruz, CA). The following primary antibodies were purchased from Cell Signaling Technology (Danvers, MA): pFRS2 Y196 (#3864), pMAPK (#9101), total MAPK (#4695), pAKT (#9271), and total AKT (#9272). Anti-
MMP-1 (MAB901) and anti-MMP-10 (MAB910) were purchased from R&D Systems (Minneapolis, MN). The blots were visualized using a chemiluminescent substrate (ECL Plus, Amersham Pharmacia Biotech, Piscataway, NJ).

**Analyses of secreted MMP-1, pro-MMP-10 and MMP-10 in conditioned medium**

Secreted MMPs were measured with the following enzyme-linked immunosorbent assay (ELISA) Kits according to the manufacturers’ instructions: Total human MMP-1 protein (ELH-MMP1) and total human MMP-10 (ELH-MMP10-001) ELISA kits were purchased from RayBiotech (Norcross, GA), and human pro-MMP-10 ELISA kit (DM100) was purchased from R&D Systems (Minneapolis, MN).

To collect conditioned medium: RT112 and UMUC-14 cells were grown to 90% confluence in DMEM containing 10% FBS, 2 mM glutamine, 100 U/mL penicillin and 100 mg/mL streptomycin. To evaluate the effects of FGFR3 knockdown on MMP-1 and MMP-10 protein expression, cells were cultured in the presence or absence of doxycycline (1 μg/mL) for the indicated time, and conditioned medium was collected. To study the effect of R3Mab on MMP-1 and MMP-10 expression, 10^6 cells/dish were seeded onto 10-cm dishes in complete growth medium and allowed to attach overnight. Then serum-free DMEM containing R3Mab (15 μg/mL) or a control antibody was applied, and supernatants were collected at the indicated time points. The conditioned medium was concentrated 10-fold using Amicon Ultra Centrifugal filters (Millipore, Billerica, MA) for ELISA analysis, and the corresponding cells were lysed in the RIPA buffer (50 mM pH 7.4 Tris-Cl, 1% NP40, 0.25% sodium deoxycholate) with Complete protease inhibitor cocktail (Roche Applied Science, Indianapolis, IN). MMP-1 and MMP-10 amounts were normalized to total cellular proteins.
Xenograft studies and collection of serum for ELISA analyses

All animal studies were conducted in accordance with the Guide for the Care and Use of Laboratory Animals, published by the National Institutes of Health (NIH Publication 8523, revised 1985). The Institutional Animal Care and Use Committee at Genentech reviewed and approved all animal protocols. Female C.B-17 severe combined immunodeficiency (SCID) mice, 6-10 weeks of age, were purchased from Charles River Laboratory (Hollister, CA). Female athymic nude mice were obtained from Harlan Sprague Dawley (Livermore, CA). Mice were maintained under specific pathogen-free conditions. Female C.B-17 SCID or athymic nude mice were inoculated subcutaneously with 7 x 10^6 RT112 cells in HBSS/matrigel (1:1 v/v, BD Biosciences) or 5 x 10^6 UMUC-14 cells in HBSS (0.1 to 0.2 mL/mouse), respectively. Mice with tumors reaching a mean volume of 150 to 200 mm³ were randomly grouped into groups of 12, and were treated with vehicle (PBS) or R3Mab (0.3 - 100 mg/kg) via weekly intraperitoneal injections. Body weights and caliper measurements were taken twice weekly. The tumor volume (mm³) was calculated using the following formula: length × width² × 0.5. Log₂ (tumor volume) growth traces were fitted to each treatment group with restricted cubic splines for the fixed time effect in each group. Fitting was done as previously described (37) via a linear mixed effects model, using the R package "nlme," version 3.1–108 in R version 2.15.2 (R Development Core Team 2012; R Foundation for Statistical Computing; Vienna, Austria). Plotting was performed using Excel 14.3.2 (Microsoft Corporation, Redmond, WA).

To analyze MMP-1 and MMP-10 levels in tumor tissues and serum, mice with tumors reaching a mean volume of 150 to 200 mm³ were randomly grouped into cohorts of 10, and were
treated with a control human IgG1 or R3Mab (30 mg/kg) on days 1, 4, and 7. Tumors and mouse serum were collected at indicated time points and kept frozen. Human MMP-1 and pro-MMP-10 level in mouse serum were determined using the ELISA assays as described above.

Clinical study design

The phase I MFG4991g trial (35) was an open-label dose-escalation study to evaluate the safety, tolerability, and pharmacokinetics of R3Mab (designated MFGR1877S) in patients with solid tumors. The trial consisted of a dose escalation of single-agent R3Mab administered intravenously at 2, 4, 8, 15, and 30 mg/kg once every 28 days. Serum and urine samples were collected at designated time points, frozen in liquid nitrogen, and shipped to Genentech, Inc. for measurement of pharmacodynamic biomarkers of R3Mab activity.

ELISA analyses of MMPs in human serum and urine

The levels of a panel of MMPs were measured by ELISA in serum and urine samples from normal healthy volunteers, procured bladder cancer patients, and patients enrolled in the phase I MFG4991g clinical trial. To this end, the human MMP 3-Plex (i.e. MMP-1, MMP-3, and MMP-9) (Mesoscale Discovery, Gaithersburg, MD) and human pro-MMP-10 immunoassay (R&D Systems, Minneapolis, MN) kits were utilized according to the manufacturer’s directions. For the human pro-MMP-10 immunoassay kit, serum samples were diluted 2-fold in the appropriate assay diluent. For the human MMP 3-Plex kit, serum samples were diluted 10-fold. For all MMP kits, urine was used undiluted. As a reference for quantification, a standard curve was used for each kit according to the manufacturer’s directions. Mann-Whitney tests were used for comparison between two groups. A value of p < 0.05 was considered statistically significant.
Results

FGFR3 knockdown inhibits MMP-1 and MMP-10 expression and secretion

Using doxycycline-inducible shRNA targeting FGFR3, we previously determined the transcriptional profile of RT112-derived cell lines that express endogenous or depleted levels of FGFR3, using microarray analysis (36). To facilitate identification of serum or urine pharmacodynamic markers of FGFR3-targeted therapeutics, we focused on secreted molecules that were specifically modulated by FGFR3 depletion. Comparing independently established stable cell lines transduced with a doxycycline-inducible control shRNA versus three independent FGFR3 shRNAs, we found that two matrix metalloproteinases, MMP-1 and MMP-10, were among the genes showing the greatest decline upon FGFR3 knockdown (Supplemental Table 1). The transcripts were down-regulated by about 16-fold and 4-fold for MMP-1 and MMP-10, respectively (Supplemental Figure 1). These microarray results were further confirmed using quantitative reverse transcriptase (qRT)-PCR analysis of the mRNA abundance of these two genes (Figure 1A). In addition, the FGFR3-dependent regulation of these genes was also verified in UMUC-14 bladder cancer cells subjected to short-interfering RNA (siRNA)-mediated FGFR3 knockdown (Supplemental Figure 2A), or treated with a specific anti-FGFR3 human monoclonal antibody, R3Mab (Supplemental Figure 2B). Together, these data suggest that FGFR3 signaling promotes transcription of MMP-1 and MMP-10 in bladder cancer cells.

Next we examined the effect of FGFR3 knockdown on the expression and secretion of MMP-1 and MMP-10 proteins. RT112 cells were treated with or without doxycycline for 72 hr to deplete FGFR3 protein, and the conditioned media were collected at specific time points thereafter. FGFR3 shRNAs substantially diminished the secretion of MMP-1 and pro-MMP-10 as early as 24 hr after induction and the decrease was sustained at 48 and 72 hr, whereas the
control shRNA had no effect (Figure 1B). MMP-10 was down-regulated in a similar manner by FGFR3 shRNAs (Supplemental Figure 3). Consistently, pre-treatment with R3Mab also blocked the production and secretion of MMP-1, pro-MMP-10 and MMP-10 proteins into conditioned media by both RT112 and UMUC-14 cells (Figure 1C and Supplemental Figure 4). Together, these results suggest that FGFR3 signaling promotes the expression and secretion of MMP-1 and MMP-10 proteins by these bladder cancer cell lines. Since the ELISA detection of pro-MMP-10 is more sensitive than that of MMP-10, and both pro- and enzymes show similar modulation by FGFR3 inhibition, we focused on pro-MMP-10 protein levels in subsequent studies.

**FGFR3 signaling promotes MMP-1 and MMP-10 expression mainly through MEK**

To study the molecular mechanisms underlying FGFR3 regulation of MMP-1 and MMP-10 expression, we activated FGFR3 signaling in bladder cancer cells with FGF1. We first analyzed the mRNA levels of the two MMPs. FGF1 treatment of RT112 cells induces robust FGFR3 activation in a dose- and time-dependent fashion (36), and higher mRNA levels of both MMP-1 and MMP-10 were detected after 6 hr or 12 hr incubation (Figure 2A). Consistent with these changes, secreted MMP-1 and pro-MMP-10 proteins detected in conditioned media were higher after a 24 hr incubation with FGF1 (Figure 2B), and accumulated further at 48 hr and 72 hr (data not shown).

To determine whether the induction of MMP-1 and MMP-10 by FGF1 depends on specific FGFR3 signaling mediators, we blocked the two major signaling branches downstream of FGFR3 using the PI3K inhibitor Ly294002 and the MEK1/2 inhibitor PD325901. In RT112 bladder cancer cells, each inhibitor blocked FGF1-induced activation of its corresponding target, as assessed by the phosphorylation of AKT or MAPK, respectively (Figure 2C). While PI3K
inhibition elicited minimal effect on the expression and secretion of MMP-1 and MMP-10 proteins, MEK inhibition significantly diminished both basal and FGF1-induced expression of these two proteins (Figure 2D). Thus, FGFR3 promotes MMP-1 and MMP-10 expression in RT112 bladder cancer cells mainly through the MEK-MAPK axis.

**R3Mab blocks FGFR3 signaling and reduces human MMP-1 and MMP-10 protein levels in both bladder tumor xenograft tissues and mouse serum**

To evaluate whether MMP-1 and MMP-10 could serve as potential pharmacodynamic markers for FGFR3-targeted therapeutics, we studied the effect of R3Mab on the expression and secretion of these enzymes in vivo. Two R3Mab-responsive tumor xenograft models, RT112 and UMUC-14, were selected for this study. Weekly intraperitoneal (IP) administration of R3Mab suppressed xenograft growth of these models in a dose-dependent manner (Figure 3A and Supplemental Figure 5). First, we evaluated FGFR3 signaling and MMP-1 and MMP-10 protein levels in tumor xenograft tissues. Compared with the control antibody, R3Mab markedly reduced the phosphorylation and activation of FRS2 and MAPK, two of the essential downstream mediators of FGFR3 signaling (Figure 3B). Concomitantly, both total MMP-1 and MMP-10 protein levels in the tumor lysates were decreased significantly in response to R3Mab (Figure 3B).

We next evaluated the effect of R3Mab on human MMP-1 and MMP-10 protein levels in the circulating blood from xenograft models expressing human bladder tumors. Nude mice with pre-established RT112 tumors were treated with a low or a high dose of R3Mab (3 or 30 mg/kg, respectively), and mouse serum was collected at 48 or 96 hr post-treatment. Human pro-MMP-10 protein levels in mouse serum were reduced in a dose-dependent manner in R3Mab-treated
group at 48 hr and 96 hr, and the effect was more pronounced in the high-dose group at 96 hr (Supplemental Figure 6). We extended the analysis to days 4, 6, and 10 post-treatment. Compared with the control antibody, R3Mab substantially and significantly suppressed the serum levels of human MMP-1 and pro-MMP-10 throughout the time course (Figure 3C, D). A similar reduction of serum levels of these two proteins was induced by R3Mab treatment of mice bearing UMUC-14 tumors (Supplemental Figure 7A, B).

To assess whether the modulation of MMPs by R3Mab correlates with efficacy, we also analyzed pro-MMP-10 levels in three xenograft models whose growth is not affected by R3Mab, namely HT1376, UMUC-4 and SW780 (Supplemental Figure 8A and data not shown). Levels of pro-MMP-10 were too low to be detected in serum from mice bearing UMUC-4 or HT1376 xenograft tumors (data not shown). While pro-MMP-10 was easily detected in mice with SW780 tumors, levels of pro-MMP-10 were not affected upon R3Mab treatment as observed in responsive models (Supplemental Figure 8B).

Together, these data demonstrated that R3Mab-based inhibition of FGFR3 signaling and tumor growth occurs in conjunction with a substantial and sustained reduction of MMP-1 and MMP-10 protein expression and secretion, suggesting that these two metalloproteinases could be explored as potential pharmacodynamic markers of FGFR3 pathway inhibition.

**MMP-1 and pro-MMP-10 proteins are elevated in the urine of bladder cancer patients**

Next we evaluated MMP-1 and pro-MMP-10 protein levels in urine and serum specimens from healthy donors and bladder cancer patients (acquired from commercial sources). In urine samples from healthy donors (n=25), MMP-1 was mostly undetectable or below the lower-limit of quantitation (LLOQ), but pro-MMP-10 was consistently detected above the LLOQ, albeit
within a low and narrow range (Figure 4A). In contrast, both MMP-1 and pro-MMP-10 proteins were detected at significantly higher levels in urine from bladder cancer patients (n=35) (Figure 4A). A different pattern was observed in serum: both metalloproteinases were detected above the LLOQ in all serum samples from the same patients; however, there was no difference in levels detected in healthy donors versus bladder cancer patients (Figure 4B).

Since R3Mab has been evaluated in a phase I dose-escalation clinical trial (MFG4991g) conducted in relapsed or refractory, locally-advanced or metastatic solid tumors (Supplemental Table 2)(35), analogous measurements of MMP1 and pro-MMP-10 were made in clinical pre-treatment urine and serum specimens (n=26). All bladder cancer patients (n=10) enrolled in this study have relapsed or refractory metastatic lesions, and 8/10 patients displayed significantly higher urinary MMP-1 levels, with only two patients having MMP-1 below the LLOQ. In contrast, urinary MMP-1 was undetectable or below the LLOQ in a majority of samples from cancer patients with other solid tumors, including colorectal, squamous cell carcinoma of the head and neck, ovarian, thyroid, esophageal, adrenal, adenoid cystic carcinoma or carcinoid tumor (n=15) (Figure 4A). Similarly, elevated levels of pro-MMP-10 were observed in urine samples from bladder cancer patients relative to patients with other solid tumors (Figure 4A). In agreement with observations in the commercially procured specimens, there was no difference in serum MMP-1 and pro-MMP-10 levels between patients with bladder cancer or other solid tumors (Figure 4B). Together, these data demonstrate that these two metalloproteinases are significantly elevated in bladder cancer patient-derived urine relative to analogous samples from healthy donors or from patients with several other solid tumors. However, we did not observe a correlation between baseline MMP levels and either FGFR3 protein expression level (as
measured retrospectively by immunohistochemistry) or clinical response in the MFG4991g phase 1 trial (38).

**R3Mab reduces urinary MMP-1 and pro-MMP-10 levels in bladder cancer patients**

In order to examine whether MMP-1 and/or pro-MMP-10 could be used as pharmacodynamic biomarkers, patterns of their expression were also evaluated in urine samples from the MFG4991g phase I clinical trial collected predose, or at cycle 1, day 2 (C1D2), cycle 1, day 4 or 5 (C1D4D5) and, in some cases at later time points depending on how long the patient stayed on study and on availability of samples (Figure 5). In all bladder cancer patients (based on 8 evaluable urine sample pairs), exposure to R3Mab reduced MMP-1 levels by >50% (Figure 5A, B). Assessment of pharmacodynamic modulation in non-bladder cancer patients was hampered by the limited number (3 out of 16) of samples having sufficient MMP-1 levels at baseline. While most samples were detectable, only 3 were above the lowest level of quantitation in the assay. However, in the cases in which MMP-1 could be quantified, MMP-1 levels were reduced by >50% (Figure 5A,B).

Unlike MMP-1, all urine samples had detectable pro-MMP-10 levels for analysis. Pro-MMP-10 was reduced by >50% in half of the bladder cancer patients treated with R3Mab (4/8 evaluable pairs). In contrast, there was no reduction in pro-MMP-10 in urine samples from patients with non-bladder cancer (Figure 5C,D). All of the bladder cancer patients that showed reduced levels of pro-MMP-10 were treated at the highest dose (30 mg/kg). However, two other patients in the highest dose cohort did not show modulation of pro-MMP-10 levels. In contrast, MMP-1 was down-modulated upon antibody treatment in most dose cohorts including the 8, 15, and 30 mg/kg dose cohorts.
A number of MMPs have been postulated as potential diagnostic and/or prognostic biomarkers in bladder cancer, but the role for MMPs as pharmacodynamic biomarkers has largely been unexplored (39). In order to extend our preliminary observations, additional analysis was performed to evaluate two other MMPs, MMP-3 and MMP-9, which also harbor a group 1 promoter element similar to MMP-1 and MMP-10 (40). Statistical analysis (Mann-Whitney test) indicated that MMP-3 (p=0.0251), but not MMP-9 was significantly higher in urine from bladder cancer patients relative to non-bladder cancer patients in the MFG4991g phase I study (Figure 6A). Similar to pro-MMP-10 and MMP-1 data, no significant difference was detected in serum (Figure 6B). Pharmacodynamic monitoring of these MMPs showed that in most but not all cases MMP-3 and MMP-9 were down-modulated by R3Mab similarly to pro-MMP-10 and MMP-1 in patient samples where all four MMPs were detectable (Figure 6C). Together, these data suggested that anti-FGFR3 therapy specifically down-modulated urinary, but not serum, MMP levels in bladder cancer patients. It’s worth noting that similar to observations at baseline, no correlation between pharmacodynamics measures and either FGFR3 expression or clinical response was observed (38).
Discussion

Bladder cancer is the second most common genitourinary tract malignancy, accounting for more than 73,000 new cases each year and about 15,000 deaths in 2012 in the United States alone (2). The receptor tyrosine kinase, FGFR3, has been postulated as an important oncogenic driver and potential therapeutic target in this disease based on multiple lines of evidence, including a high frequency of activating mutations (17-20), aberrant regulation and activation of FGFR3 by gene fusion with TACC3 or BAIAP2L1 (21, 22), overexpression of FGFR3 protein in tumor tissues (19, 23), and a large body of preclinical loss-of-function studies (23, 27-31). Recent progress toward clinical investigation of experimental agents targeting FGFR3 has made it imperative to identify accessible pharmacodynamic biomarkers to gauge target modulation and facilitate clinical studies. Here we report that FGFR3 stimulates MMP-1 and pro-MMP-10 expression and secretion in human bladder cancer cells through the MEK-MAPK pathway. Blocking FGFR3 signaling by a specific anti-FGFR3 human monoclonal antibody, R3Mab, inhibited human tumor xenograft growth in association with a marked reduction in circulating human MMP-1 and pro-MMP-10 levels. Moreover, bladder cancer patients had an increased level of urinary MMP-1 and pro-MMP-10 proteins. Importantly, in a phase I dose-escalation study of R3Mab, inhibition of FGFR3 in relapsed or refractory metastatic bladder cancer patients resulted in an acute reduction of urinary MMP-1 and pro-MMP-10 levels. These results unveil an important role for FGFR3 in regulating MMP-1 and pro-MMP-10 expression and secretion in bladder cancer, and support further evaluation of these mettallaproteinases as potential pharmacodynamic markers in future FGFR3-targeted clinical studies.

In our unbiased expression analysis of genes encoding secreted molecules, we found that upon FGFR3 knockdown in RT112 bladder cancer cells, MMP-1 and pro-MMP-10 were among
the most prominently down-regulated genes. Our studies further demonstrate that ligand-induced FGFR3 activation promotes both mRNA and protein expression of these two metalloproteinases, and acute pharmacological inhibition of canonical FGFR3 signaling markedly diminishes the level of secreted MMP-1 and pro-MMP-10 in the conditioned medium. This inhibitory effect is mainly mediated through the FGFR3-MEK-MAPK pathway, but not the FGFR3-PI3K axis. These results extend earlier studies by others showing that the expression of several MMP genes is regulated by some growth factors and cytokines in bladder cancer. For example, basic FGF is able to stimulate the expression of MMP-2 and MMP-9 in HT1376 cells (41), and EGF elicits the rapid induction of MMP-1 and MMP-10 mRNA in T24 bladder cancer cells through the Stat3/activator protein-1 (AP-1) family of transcription factors (42).

Importantly, in the present study, by selectively blocking FGFR3 function with R3Mab (i.e., a specific anti-FGFR3 monoclonal antibody), we were able to clearly demonstrate that MMP-1 and pro-MMP-10 are *bona fide* downstream targets of FGFR3 signaling in bladder cancer. It is worth noting that the present study does not exclude the possibility that other signaling pathways may also regulate these two MMPs through MAPK or alternative mechanisms in bladder cancers.

In order to assess whether MMP-1 and pro-MMP-10 could serve as accessible pharmacodynamic biomarkers for FGFR3-targeted therapy, we have evaluated the serum and urinary levels of these two proteins in healthy subjects and in patients with bladder cancer, as well as in patients with various solid cancers enrolled in the MFG4991g phase I study of R3Mab. Compared with healthy controls and patients with non-bladder cancer malignancies, patients with advanced bladder cancer showed significantly elevated urine levels of MMP-1 and pro-MMP-10 protein. By contrast, the concentration of these two proteins in circulating blood
displayed no difference amongst the different groups. This observation is in agreement with several published studies. MMP-1 and MMP-10 mRNA levels have been reported to be significantly higher in bladder cancers compared with normal urothelial tissues (43, 44), and MMP-1 and pro-MMP-10 immunoreactivity is evidently stronger in tumor cells of both superficial and muscle-invasive bladder cancers (45, 46). In addition, bladder cancer patients with an increased urinary MMP-1 protein level are more likely to have an aggressive tumor of advanced stage and grade (47, 48). Although published data on urinary pro-MMP-10 in bladder cancer patients is more limited, a recent study demonstrates that a higher urinary pro-MMP-10 concentration could serve as a potential diagnostic biomarker when used in combination with a panel of six other analytes (44). Taken together, the differential expression pattern between bladder cancer patients and healthy subjects, the relative ease in acquisition of void urine samples, and the robust detection of urinary MMP-1 and pro-MMP-10 levels suggest that these two metalloproteinases could serve as useful and clinically relevant non-invasive biomarkers.

One important finding of our current study is that in preclinical bladder cancer xenograft models, R3Mab treatment inhibited FGFR3 signaling, attenuated tumor growth, and reduced MMP-1 and pro-MMP-10 protein expression in tumor lysates as well as in mouse serum. Consistent with these preclinical studies, our phase I study with 26 patients demonstrated that administration of R3Mab led to an acute reduction of urinary MMP-1 by more than 50% in 8 out of 8 evaluable bladder cancer patients. The evaluation in non-bladder cancer patients was hampered by the fact that very few patients had measurable levels of MMP-1 at baseline. In the only three patients (2 colorectal and 1 head and neck SCC) that were evaluable, MMP-1 was also markedly reduced. It is noteworthy that although a minimum threshold level of MMP-1 is required for monitoring pharmacodynamic modulation, the extent of down-modulation was not
correlated with the baseline values. A similar effect was observed for pro-MMP-10, as R3Mab resulted in a decrease of urinary pro-MMP-10, albeit in only 4 out of 8 bladder cancer patients. Interestingly, no modulation of pro-MMP-10 was detected in non-bladder cancer patients, suggesting that pro-MMP-10 might be more specific for bladder cancer.

Additional MMPs were monitored in the clinical study. Similar to MMP-1 and pro-MMP-10, urinary MMP-3, and to a lesser extent, MMP-9, were also elevated in bladder relative to non-bladder cancer patients. In a majority of patients there was a similar pattern of down-modulation of these MMPs by R3Mab despite some noticeable differences. This observation could be partially due to the fact that these MMPs all have a proximal AP-1 binding site in their promoters (40); conceivably, R3Mab-mediated inhibition of the MEK-MAPK-AP-1 axis may result in decreased transcription of these genes. Indeed, in 4T1 breast cancer cells, the pan-FGFR inhibitor TKI258 suppresses the transcription of MMP-1, -3, -9, and -10 simultaneously (49).

The matrix metalloproteinases degrade extracellular matrix components and activate growth factors, contributing to tumor growth, progression and metastasis (50). Several MMPs have been postulated to be diagnostic or prognostic markers in bladder cancer (39, 50). Our present study demonstrates that FGFR3 signaling directly regulates MMP-1 and pro-MMP-10 expression and secretion in bladder cancer through the MEK-MAPK pathway. Urinary MMP-1 and pro-MMP-10 levels were reduced substantially by R3Mab treatment in both preclinical models and bladder cancer patients. This is the first study, to our knowledge that identifies urinary MMP-1 and pro-MMP-10 as potential pharmacodynamic biomarkers. While we observed clear pharmacodynamic modulation in response to R3Mab administration, there did not appear to be a correlation between modulation and dose or FGFR3 expression level or clinical
benefit (as assessed by time on study or disease progression). The lack of correlation could be
due to small sample size and/or a change in FGFR3 expression levels during disease progression
or prior drug treatment. Although this phase I study was not powered to assess predictive
biomarkers, the data presented here warrant further investigation in larger studies using these
potentially actionable pharmacodynamic biomarkers that can be used in the clinic in a non-
invasive manner to stratify patients with elevated MMP-1 and pro-MMP-10 profiles and
potentially monitor therapeutic response.
Acknowledgements

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References


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Figure legends

**Figure 1** Inhibition of FGFR3 reduces the mRNA and protein expression of MMP-1 and MMP-10 in RT112 bladder cancer cells. (A) Doxycycline-induced knockdown of FGFR3 reduces mRNA levels of MMP-1 and MMP-10. (B) Doxycycline-induced knockdown of FGFR3 reduces total MMP-1 and pro-MMP-10 secreted in the conditioned medium. (C) A specific FGFR3-blocking antibody, R3Mab, reduces total MMP-1, pro-MMP-10 and total MMP-10 secreted in the conditioned medium by RT112 cells.

**Figure 2** FGF1 signaling stimulates MMP-1 and MMP-10 expression through MEK. (A) FGF1 induces MMP-1 and MMP-10 mRNA expression in RT112 cells. (B) FGF1 treatment stimulates MMP-1 and pro-MMP-10 protein expression and secretion into conditioned medium by RT112 cells. (C) Pharmacological inhibition of FGF1 signaling in RT112 cells. RT112 cells were treated with PD325901 (PD901) and Ly294002 (Ly294) at indicated concentrations for 3 hr, followed by FGF1 (25 ng/mL) stimulation for 10 minutes. Cell lysates were subjected to Western blot. (D) MEK inhibitor, but not PI3K inhibitor, blocked FGF1-induced expression and secretion of MMP-1 and pro-MMP-10 by RT112 cells.

**Figure 3** R3Mab attenuates RT112 tumor growth, blocks FGFR3 signaling and reduces human MMP-1 and MMP-10 levels in xenograft tumor tissues and mouse serum. (A) Effect of R3Mab on pre-established RT112 bladder xenograft tumor growth. R3Mab was administered IP weekly (arrows). n=12 per group. (B) Effect of R3Mab on FGFR3 signaling and MMP levels in tumor xenograft tissues. Mice with pre-established RT112 tumors were grouped into cohorts of 5 for treatment with either a control antibody (Ctrl Ab) or R3Mab (30 mg/kg). Tumors were harvested
at day 7, and lysates extracted from xenograft tumor tissues were subjected to Western blot. (C, D) The kinetics of R3Mab effect on serum levels of human MMP-1 (C) and pro-MMP-10 (D) in mice bearing RT112 xenograft tumors. n=10 per group.

**Figure 4** MMP-1 and pro-MMP-10 are elevated in bladder cancer (BLC) patient urine but not in serum. (A) Levels of MMP-1 and pro-MMP-10 in commercially procured urine samples from bladder cancer patients (BLC) (n=35) relative to healthy donors (n=25), or from the MFG4991g phase I clinical trial, including bladder cancer patients (n=10) and patients with other solid tumor types (non-BLC, n=16). Plotted n values are representative of only those samples that were detectable (≥LOD). The dotted line represents the lower limit of quantitation (LLOQ). (B) Levels of MMP-1 and pro-MMP-10 in serum are equivalent in healthy donors, bladder cancer patients and in non-bladder cancer patients. Note: Only 5 healthy donor serum samples were evaluated.

**Figure 5** MMP-1 and pro-MMP-10 are reduced upon exposure to R3Mab in bladder cancer patients in the MFG4991g phase I study. (A) The overall urinary levels of MMP-1 are reduced by R3Mab in bladder cancer patients but not in non-bladder cancer patients. (B) Relative levels of MMP-1 compared with predose levels. (C) The overall urinary levels of pro-MMP-10 are reduced by R3Mab in bladder cancer patients but not in non-bladder cancer patients. (D) Relative levels of pro-MMP-10 compared with predose levels. C1D2, cycle 1 day 2; C1D4D5, cycle 1, day 4 or day 5.
Figure 6 Levels and modulation of MMP-3 and MMP-9 by R3Mab in the MFG4991g phase I study. (A) Urinary MMP-3 is elevated in bladder cancer patients relative to non-bladder cancer patients in the MFG4991g phase I study. Based on Mann-Whitney test, p=0.0251 for MMP-3, p>0.05 for MMP-9. (B) No corresponding difference of these MMPs was detected in serum. (C) Samples (n=7) with detectable levels of all four MMPs showed variable modulation in response to R3Mab administration. C1D2, cycle 1 day 2; C1D4D5, cycle 1, day 4 or day 5.
Figure 1

A

Relative MMP-1 mRNA (+Dox/Dox) vs. Ctrl(shRNA) vs. R3(shRNA)

B

Total MMP-1 (ng/mg cellular protein) vs. No Dox vs. Dox

C

Total MMP-1 (ng/mg cellular protein) vs. Ctrl Ab vs. R3Mab
Figure 2

A) Relative MMP-1 mRNA level

B) Total MMP-1

C) Western blot for pAKT, AKT, pMAPK, MAPK, and Actin

D) MMP-1 and pro-MMP-1 levels with different treatments

Legend:
- FGF1
- DMSO
- PD901
- Ly294
- + FGF1
- No FGF1

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Figure 3

A

![Graph showing fitted tumor volume over time for different treatment groups.]

B

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pFRS2, Total FRS2, pMAPK, Total MAPK, Total MMP-1, Total MMP-10, Actin

C

Day 4

![Graph showing MMP-1 levels on Day 4.]

Day 6

![Graph showing MMP-1 levels on Day 6.]

Day 10

![Graph showing MMP-1 levels on Day 10.]

D

![Graph showing MMP-10 levels.]

*p<0.0001*

Animal ID: 223, 238, 242, 246, 249, 202, 208, 210, 220, 226

*p<0.0001, p<0.0005, p<0.0001*

Ctrl Ab: Control antibody
R3Mab: R3 monoclonal antibody

Research.
Figure 4

A  Urine

B  Serum

**  **  ***  *
Figure 5

A

B

C

D

MMP-1 (ng/ml)

MMP-1 (% predose)

Pro-MMP-10 (ng/ml)

Pro-MMP-10 (% predose)
Figure 6

A

Urine

B

Serum

C

BLC

non-BLC

MMP-3

MMP-9

MMP values (ng/ml)

MMP values (ng/ml)

MMP values (% predose)

MMP values (% predose)

MMP values (% predose)

MMP values (% predose)
Clinical Cancer Research

MMP-1 and pro-MMP-10 as potential urinary pharmacodynamic biomarkers of FGFR3-targeted therapy in patients with bladder cancer

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