1-tert-Butyl-3-[6-(3,5-dimethoxy-phenyl)-2-(4-diethylamino-butylamino)-pyrido[2,3-d]pyrimidin-7-yl]-urea (PD173074), a Selective Tyrosine Kinase Inhibitor of Fibroblast Growth Factor Receptor-3 (FGFR3), Inhibits Cell Proliferation of Bladder Cancer Carrying the FGFR3 Gene Mutation along with Up-Regulation of p27/Kip1 and G₁/G₀ Arrest

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ABSTRACT

Activating mutation of the fibroblast growth factor receptor-3 (FGFR3) gene is known as a key molecular event in both oncogenesis and cell proliferation of low-grade noninvasive human bladder urothelial carcinoma (UC), which is characterized by frequent intravesical recurrence. In this study, we investigated the antitumor potentiality of 1-tert-butyl-3-[6-(3,5-dimethoxy-phenyl)-2-(4-diethylamino-butylamino)-pyrido[2,3-d]pyrimidin-7-yl]-urea (PD173074), a small-molecule FGFR3-selective tyrosine kinase inhibitor (TKI), as a therapeutic modality using eight UC cell lines. In our in vitro cell proliferation assay, PD173074 suppressed cell proliferation remarkably in two cell lines, namely, UM-UC-14 and MGHU3, which expressed mutated FGFR3 protein. In contrast, the other six cell lines expressing wild-type FGFR3 or without FGFR3 expression were resistant to PD173074 treatment. Cell cycle analysis revealed the growth inhibitory effect of PD173074 was associated with arrest at G₁-S transition in a dose-depending manner. Furthermore, we observed an inverse relationship between Ki-67 and p27/Kip1 expression after PD173074 treatment, suggesting that up-regulation of p27 recruited UC cells harboring activating FGFR3 mutations in G₁, that was analogous with the other receptor TKIs acting on the epidermal growth factor receptors. In the mouse xenograft models using subcutaneously transplanted UM-UC-14 and MGHU3, orally administered PD173074 suppressed tumor growth and induced apoptotic changes comparable with the results of our in vitro assay. These findings elucidated the effectiveness of molecular targeted approach for bladder UC harboring FGFR3 mutations and the potential utility to decrease the intravesical recurrence of nonmuscle invasive bladder UC after transurethral surgical resection.
cle invasive diseases (\(\geq pT2\)), requiring further invasive treatments such as radical cystectomy or chemoradiotherapy (Sugano and Kakizoe, 2006). Because cystoscopical examination and repeated surgical resection are distressing to the patients, exploration of novel therapeutic strategy is essential to reduce the frequency of cystoscopy, recurrence rate, and progression rate. Recently, molecular targeted therapy using small molecules or monoclonal antibodies has been developed to improve the clinical outcome in several human cancers (Trudel et al., 2004, 2005; Martínez-Torrecaudrada et al., 2005; Wu, 2005; Bernard-Pierrot et al., 2006; Black et al., 2007).

Fibroblast growth factor receptor (FGFR)-3 belongs to a family of structurally conserved tyrosine kinase receptors (FGFRs 1–4). These receptors play important roles in various biological processes such as embryogenesis, differentiation, angiogenesis, cell proliferation, migration, and apoptosis (Johnson and Williams, 1993). The FGFR3 gene generates two alternative splicing variants, namely, isoforms FGFR3b and FGFR3c, which differ in ligand specificity and tissue distribution. FGFR3b is mainly expressed in the epithelial cells, whereas FGFR3c is a major form in the nonepithelial cells, including chondrocytes. The FGFR3 gene was originally identified as a gene responsible for autosomal dominant skeletal disorders, in which germline-activated FGFR3 mutations seem to exert inhibitory effects by restricting chondrocyte proliferation (Ornitz and Marie, 2002). Recently, somatic mutations of FGFR3 have been found in several malignant and benign tumors, including multiple myeloma, cervical carcinoma, acanthotic skin tumor, urothelial papilloma, and bladder UC. In contrast to the inhibitory effects in bone growth, oncogenic and transforming properties of mutated or overexpressed FGFR3 have been noticed in multiple myeloma (Trudel et al., 2004) and bladder UC (Bernard-Pierrot et al., 2006). In bladder UC, 50 to 70% of low-grade nonmuscle invasive UCs harbor activated FGFR3 mutations, whereas these mutations were detected in only 10 to 20% of high-grade muscle invasive UCs (\(\geq pT2\)) and carcinoma in situ (Sugano and Kakizoe, 2006). These findings support the idea that the activated FGFR3 plays a key role in both oncogenesis and cell proliferation of low-grade noninvasive bladder UCs.

PD173074 is a synthetic compound of the pyrido-[2,3-d] pyrimidine class and a highly selective tyrosine kinase inhibitor (TKI) of FGFR3 (Mohammadi et al., 1998). This compound competes with ATP binding located in the intracellular domain of FGFR3. Reportedly, PD173074 can inhibit the autophosphorylation of FGFR3, with an IC\(_{50}\) value of approximately 5 nM (Trudel et al., 2004), which is lower than that of the other FGFR3-selective TKIs such as SU5402 and CHIR-258 (Trudel et al., 2005; Wu, 2005; Bernard-Pierrot et al., 2006). A previous study demonstrated that the activated mutation of FGFR3 contributes to tumor development of multiple myeloma cells, and a remarkable inhibitory effect was induced by PD173074 in cells expressing the mutated FGFR3 (Trudel et al., 2004). However, there is no report investigating whether PD173074 would be potentially available as a therapeutic approach against bladder UC.

At present, downstream signaling of the mutated FGFR3 in bladder UCs is unclear. p27/Kip1 (hereafter referred to as p27) belongs to a KIP family, which directly inhibits cyclin/cyclin-dependent kinase complex, resulting in growth arrest at the G1-S transition (Sherr, 2000). In the quiescent state (G0 phase), the levels of p27 are generally high. It is suggested that increased activation of receptor tyrosine kinase (such as human epidermal growth factor receptor-2, insulin-like growth factor receptor, and EGFR) is associated with degradation of p27 in the Ras/Raf/mitogen-activated protein kinase kinase or Ras/phosphatidylinositol 3-kinase (PI3K)/Akt/protein kinase B pathway in human cancers (Cheng et al., 1998; Medema et al., 2000; Sherr, 2000; Blain et al., 2003). In this study, we investigated the antitumor potentiality of FGFR3 inhibition by PD173074 as a therapeutic modality for bladder UC in vitro and in vivo, in association with p27 up-regulation and G1/G0 arrest.

## Materials and Methods

### Chemical Compound and Biologic Reagents

A selective TKI, PD173074, was provided by Pfizer (Groton, CT) and dissolved in dimethyl sulfoxide at a concentration of 20 mM. The stock solution was stored at −20°C before use. The chemical structure of PD173074 is described in a previous report (Mohammadi et al., 1998). Acidic fibroblast growth factor (aFGF) and heparin were purchased from R&D Systems (Minneapolis, MN) and Sigma-Aldrich (St. Louis, MO), respectively.

### Cell Lines and Cell Culture

Eight established UC cell lines, namely T24, KU7, UM-UC-2, UM-UC-3, UM-UC-6, UM-UC-14, MGHU3, and J82 (Miyake et al., 2007, 2009), were maintained in RPMI 1640 medium (Nissui, Tokyo, Japan) supplemented with 10% fetal bovine serum (ICN, Aurora, OH) and 1 g/l kanamycin sulfate (Sigma-Aldrich) in a standard humidified incubator at 37°C in 5% CO\(_2\).

### Western Blot Analysis

Total protein was extracted using a 1× RIPA buffer containing 20 mM Tris-HCl, pH 7.5, 150 mM NaCl 1 mM Na\(_2\)EDTA, 1 mM EGTA, 1% Nonidet P-40, 1% sodium deoxycholate, 2.5 mM sodium pyrophosphate, 1 mM β-glycerophosphate, 1 mM Na\(_2\)VO\(_4\), and 1 μg/ml leupeptin purchased from Cell Signaling Technology Inc. (Danvers, MA). The protein concentration was quantified with Protein Quantification Kit-Wide Range (Dojindo, Kumamoto, Japan). Twenty micrograms of total protein was diluted by SDS loading buffer with or without 2.5% β-mercaptoethanol and then boiled at 95°C for 5 min and electrophoresed onto 10% SDS-polyacrylamide gels using a Mini-PROTEAN Tetra electrophoresis cell (Bio-Rad Laboratories, Hercules, CA) at 200 V for 35 min. The gels were subjected to transfer onto polyvinylidene difluoride membranes (Hybond-P; GE Healthcare, Little Chalfont, Buckinghamshire, UK) using semidry transfer apparatus (Trans-Blot SD Semi-Dry Transfer Cell; Bio-Rad Laboratories) at 15 V for 45 min. After blocking in Tris-buffered saline containing 5% skimmed milk and 0.1% Tween 20 overnight, the membrane was incubated for 1 h with anti-FGFR3 rabbit polyclonal antibody (clone C-15; Santa Cruz Biotechnology, Inc., Santa Cruz, CA), anti-p27 mouse monoclonal antibody (clone 57; BD Biosciences Transduction Laboratories, Lexington, KY), anti-actin mouse monoclonal antibody (Santa Cruz Biotechnology, Inc.) as an internal loading control, followed by 1 h incubation in horseradish peroxidase-conjugated sheep anti-rabbit IgG or anti-mouse IgG antibody. The bound secondary antibody was detected using an enhanced chemiluminescence kit (ECL Plus Western Blotting Detection system; GE Healthcare).

### Cell Viability Assay

The cells were seeded in a 96-well plate at a density of 2000 cells/well in growth medium and incubated for 24 h. They were treated with increasing concentrations of PD173074. After incubating the plates for 48 h, cell viability assay was performed using Cell Counting Kit-8 (Dojindo) according to the manufacturer’s directions. The viability index was expressed by the relative value to the untreated cells. The data were expressed as mean ± S.D.

### Immunoprecipitation

The cells were seeded at a density of 2 × 10\(^6\) cells in 10-cm dish and incubated in growth medium for 24 h. For

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Miyake et al. 796
serum starvation, the cells were incubated in fetal bovine serum-free medium for 24 h. After treatment with PD173074 at the indicated concentrations for 30 min, the cells were stimulated with 20 ng/ml aFGF and 10 μg/ml heparin for 10 min. The cell extracts were prepared as mentioned above. After preclarification by 1 μg of agarose-conjugated normal rabbit IgG (Santa Cruz Biotechnology, Inc.), 1 mg of total protein was immunoprecipitated with 10 μg of the agarose-conjugated anti-FGFR3 C-15 antibody at 4°C overnight. After the mixture was washed three times by 1× RIPA buffer, the precipitate was boiled with 40 μl of 2× SDS sample buffer for 5 min and centrifuged at 400g for 2 min. The supernatant was used for Western blot analysis using mouse monoclonal anti-phosphotyrosine (clone 4G10; Millipore, Billerica, MA) or mouse monoclonal anti-FGFR3 (clone B-9; Santa Cruz Biotechnology) as the primary antibody. For detecting the membrane phosphotyrosine, Blocking One-P (NakaraiTest, Kyoto, Japan) containing 1 mM Na3VO4 was used as the blocking solution.

**Cell Cycle Analysis.** The cells were grown in growth medium for 24 h. Then, medium was replaced by fresh medium containing PD173074 (0, 5, 25, and 100 nM final concentration). After incubation for 48 h, the cells were washed and trypsinized. The cells were fixed in 70% ice-cold ethanol, followed by incubation in nuclei-staining buffer (PI/RNase staining buffer; BD Biosciences Pharmingen, San Diego, CA) for 30 min according to the manufacturer's recommendations. In total, 1 × 10⁶ cells were subjected to cell cycle analysis using a FACScan flow cytometer (BD Biosciences, San Jose, CA).

**Immunocytochemical Analysis.** The cells were seeded at a density of 50,000 cells/well in a Lab-Tek II 4-well Chamber Slide (Nalge Nunc International, Rochester, NY) and incubated in growth medium for 24 h. They were treated with PD173074 at the indicated concentrations for 48 h. To fix the cells, the slides were immersed in 4% paraformaldehyde solution for 20 min at 4°C. To detect the intranuclear expression of Ki-67 or p27, immunocytochemical staining was performed with Envision (DAKO, Glostrup, Denmark) and incubated in 3,3’-diaminobenzidine chromogen reaction. Then, slides were counterstained with methyl green (Dako Japan) and mounted with mutoal (Muto Chemical, Tokyo, Japan).

**Xenograft Mouse Model and Immunohistochemical Analysis of Tumor Tissues.** Female SCID mice (C.B-17/Icr Cj-scid, C.B-17/Icr Cj-scid, 6 to 8 weeks old) were purchased from Charles River Japan, Inc. (Yokohama, Japan). The mice were maintained under specific pathogen-free conditions and provided with sterile food and water. UM-UC-14 or MGHU3 cells (2 × 10⁶) in 250 μl of RPMI 1640 medium, together with 0.25 ml of Matrigel (BD Biosciences), were injected subcutaneously into the bilateral flanks of each mouse. When the tumors reached 2.0 cm in the longest diameter, the mice were killed for passage of tumor xenografts to other mice. For each cell line, the SCID mice were subcutaneously inoculated with a 3 × 3 × 3-mm tumor fragment developed in the prior xenograft mouse. When the tumor reached 0.5 cm in diameter, the animals were divided randomly into test groups and treatments was initiated (day 0). In the treated groups, 25 mg/kg PD173074 in 400 μl of phosphate-buffered saline were administrated orally twice daily using a disposable soft catheter tube (Fuchigami Co., Kyoto, Japan) during days 0 to 6 and days 14 to 20 for UM-UC-14 xenografts or during days 0 to 6 and days 21 to 27 for MGHU3. In the control group, the mice received vehicle (400 μl of phosphate-buffered saline) at the same schedule. The tumor diameters were measured with calipers, and the tumor volumes were calculated using the formula: [(width)² × length]/2. The mice were killed on day 21 for UM-UC-14 and on day 28 for MGHU3. The tumor sizes and volumes were measured thrice a week. In each xenograft, the relative tumor volume was determined with relative value to that on day 0. In each test group, the data are expressed as mean ± S.E.M. Statistical differences between the relative tumor volumes in the treated and control groups were analyzed using Mann-Whitney U test (statistical tests were two-sided). A P value of less than 0.05 was considered statistically significant.

The resected tumors were fixed by 4% paraformaldehyde solution and embedded in paraffin. The sections (3 μm in thickness) were deparaffinized, followed by antigen retrieval with autoclave treatment. To assess the expression of Ki-67, p27, and cleaved caspase-3, immunohistochemical staining (IHC) was performed as described in the immunocytochemical staining after antigen retrieval by autoclave for 10 min in 0.01 M citrate buffer, pH 6.0. Anti-cleaved caspase-3 rabbit polyclonal antibody (clone 5A1E) was purchased from Cell Signaling Technology Inc.

**Results.**

**Detection of FGFR3 Protein Expression in Bladder Cell Lines.** In a prior report (Miyake et al., 2007), we screened the mutation status of FGFR3 in eight bladder cell lines used in this study by direct sequencing analysis. Our data indicated that UM-UC-14, MGHU3, and J82 harbor a homozygous mutation of S249C (TAT→TGC) in exon 7, a homozygous mutation of Y375C (TAT→TGT) in exon 10, and a heterozygous mutation of K652E (AAG→GAG) in exon 15, respectively. To examine the expression levels of FGFR3 protein in these cell lines, we performed Western blot analysis using anti-FGFR3 antibody (Fig. 1A). The overexpressed FGFR3 was observed in UM-UC-14, and weak to moderate expression was observed in KU7 and MGHU3, whereas the expression was undetectable in the other five cell lines. Reported, the conversion of noncysteine residue to cysteine residue involved in the extracellular Ig2-Ig3 domain (exon 7) and transmembrane domain (exon 10) potentially creates an intermolecular disulfide linkage (Wu, 2005). Western blot was performed simultaneously to determine whether UM-UC-14 and MGHU3 carry a stably dimerized FGFR3 under nonreducing condition (Fig. 1A). The result demonstrated that the S249C mutated FGFR3 expressed in UM-UC-14 formed stable dimers, whereas no stable dimer existed in the Y375C mutated FGFR3 expressed in MGHU3.

**PD173074 Inhibits Cell Proliferation in Bladder Cancer Cell Lines Harboring FGFR3 Mutations.** To evaluate the antitumor potential of PD173074, the growth inhibitory effect against bladder cancer cell lines was assessed by exposure to various concentrations of this compound. Preincubated cells were treated with increasing concentrations of PD173074 in growth medium for 48 h, followed by assessing the cell viability (Fig. 1B). The mutated FGFR3-expressing UM-UC-14 and MGHU3 showed high sensitivity to this compound in a dose-dependent manner, compared with the other six cell lines. In both UM-UC-14 and MGHU3, 50% inhibition was achieved at the concentration of approximately 10 nM. In contrast, KU7 expressing the wild-type FGFR3 was comparatively resistant to this compound.

**PD173074 Inhibits the Phosphorylation of FGFR3.** To confirm whether PD173074 could directly inhibit the phosphorylation of mutated FGFR3, the phosphorylation levels of UM-UC-14 and MGHU3 were analyzed by immunoprecipitation and immunoblotting under various conditions, i.e., with or without serum starvation, aFGF-stimulation and varying concentrations of PD173074 (Fig. 2A). The phosphorylated FGFR3 was detected in the serum-starved UM-UC-14 cells,
FGFR3 in bladder cancers. Phosphorylation was undetectable at the concentration of 100 nM in both cell lines.

**PD173074 Induced Cell Cycle Arrest.** To investigate the effect of PD173074 treatment on cell cycle, we performed cell cycle analysis using UM-UC-14, MGHU3, and KU7 cells. In both UM-UC-14 and MGHU3, ≥25 nM PD173074 induced G1/G0 arrest, whereas, in KU7, there was little effect on the cell cycle (Table 1).

**PD173074 Induced Up-Regulation of p27/Kip1 with Abrogation of Ki-67 Expression.** To examine whether up-regulation of p27 is induced as a result of drug responsiveness in the FGFR3-expressing cell lines, the p27 levels were analyzed by Western blot. The cell lysates were extracted from cells treated with 0, 25, or 100 nM PD173074 for 24 h (Fig. 2A). In the PD173074-sensitive cell lines, UM-UC-14 and MGHU3, increasing expression of p27 protein was observed in a dose-dependent manner. In contrast, little up-regulation of p27 protein was observed in the PD173074-resistant KU7 cells.

To confirm whether PD173074 induces the transition of cancer cells into G0 phase, the cells treated with 0, 25, or 100 nM PD173074 for 48 h were subjected to immunocytochemical analysis of the Ki-67 and p27 expression. In UM-UC-14 and MGHU3, not only the density of cells expressing Ki-67 but also the expression intensity remarkably decreased with an increasing concentration of PD173074 (Fig. 3B). In a sharp contrast to down-regulation of Ki-67, up-regulation of p27 in the nuclei was observed with an increasing concentration as consistent to the result of Western blot analysis (Fig. 3, A and B). In the KU7 cells, there were no significant differences in the Ki-67 and p27 levels between the PD173074-treated cells and the nontreated cells (Fig. 3, A and B). These findings demonstrated that PD173074 induces the transition of proliferating cells into the G0 phase and that the growth inhibitory effect of this compound is mediated by up-regulation of p27 in UM-UC-14 and MGHU3.

**Oral Administration of PD173074 Is Effective for Mutated FGFR3-Expressing Bladder Cancer Cells Developed in Mouse Xenograft Models.** To evaluate the antitumor effect of PD173074 in vivo, xenograft mice bearing UM-UC-14 or MGHU3 cells were prepared as described under Materials and Methods. Xenograft mice were given 25 mg/kg PD173074 orally twice daily and two courses of 7-day

![Fig. 1. Inhibition of cellular proliferation by PD173074 in bladder cancer cell lines expressing mutated FGFR3. A, in eight bladder cancer cell lines, the expression levels of FGFR3 protein was determined by Western blot analysis using anti-FGFR3 and anti-actin as a loading control. Three main bands (97, 125, and 135 kDa) were detected in MGHU3, and a single band (135 kDa) in KU7. UM-UC-14 overexpressed FGFR3, forming a smear consisting of several indistinguishable bands. UM-UC-14 harboring S249C mutation showed the stabilized dimers (arrow) under nonreducing condition. The mutational status of the FGFR3 gene in each cell line is shown below. B, cells were plated in a 96-well plate and treated with the indicated concentrations of PD173074 in growth medium for 48 h. The viability of the untreated cells was defined as 100% (control). The data are expressed as mean ± S.D. in triplicate of repeated experiments.](image1)

![Fig. 2. PD173074 inhibits the phosphorylation of the mutated and wild-type FGFR3. UM-UC-14 and MGHU3 cells with or without serum starvation and aFGF stimulation with or without PD173074 were analyzed for the phosphorylation status of FGFR3. Immunoprecipitates by anti-FGFR3 (C-15) were subjected to Western blot analysis using separate antibodies for anti-PY (4G10) and anti-FGFR3 (B-9) mouse monoclonal antibody as a loading control. Whereas little phosphorylation of FGFR3 was observed in the serum-starved MGHU3 cells. This result was compatible with the notion that the S249C mutation of FGFR3 leads to intensive autophosphorylation of FGFR3 even under serum-free condition. Treatments with PD173074 decreased the phosphorylation level of FGFR3 in a dose-dependent manner, demonstrating that PD173074 could serve as a TKI against](image2)

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* Indicates statistically significant difference relative to the untreated.
treatment were continued with an intermission of 7 or 14 days (Fig. 4A). In the UM-UC-14 xenografts, growth inhibition and regression of the tumor induced by PD173074 was observed for a limited period of treatment (days 0–6 and 14–20). However, tumor regrowth was observed during the withdrawal interval of days 7 to 13. The mean relative tumor volumes in the treated and control groups on day 21 were $0.73 \pm 0.14$ (range, 0.52–1.13) and $2.87 \pm 0.14$ (range, 1.89–5.09), respectively ($P < 0.01$; Fig. 4A). The experimental results in MGHU3 were similar to those in the UM-UC-14 xenografts. After the first 7 days of treatment (days 0–6), approximately 14 days of withdrawal were required for the transition of MGHU3 xenograft tumors into the stably growing phase. The mean relative volumes in the treated and control groups on day 28 were $1.01 \pm 0.39$ (range, 0.42–2.83) and $5.31 \pm 2.06$ (range, 1.78–11.83), respectively ($P < 0.01$; Fig. 4A). No significant body weight loss and appearance change was observed in mice treated by PD173074 compared with controls (Fig. 4B). In UM-UC-14 and MGHU3 cells, tumors from treated mice were significantly smaller and paler than those from control mice (Fig. 4B). These results suggested that bladder cancers carrying the FGFR3 mutation involved in exons 7, 10, or both would be indicated for PD173074 treatment.

In UM-UC-14 xenografts, hematoxylin and eosin (H&E) staining of the resected tumors revealed that PD173074 treatment induced formation of shrunken and circular-shaped cancer cells, decreased nuclear-to-cytoplasmic ratio, and chromatin condensation (Fig. 5, A and E), which were characteristic morphological features of apoptotic cells. In the IHC analysis for FGFR3 expression, a slight increase in the cytomembrane expression levels was detected in the treated tumors compared with the control tumors (data not shown). To investigate the cell proliferation status of the treated and control tumors, the levels of Ki-67 and p27 were analyzed with IHC method. The results showed that PD173074 treatment induced a significant decrease of the intranuclear expression of Ki-67 (Fig. 5, B and F). Inversely, an increase of the intranuclear expression of p27 was observed in the treated tumors (Fig. 5, C and G). These data
were consistent with those observed in vitro. Moreover, the expression of cleaved caspase-3 was observed in the treated tumor, indicating that oral administration of PD173074 could induce apoptosis of tumor cells. The results of IHC analysis in the MGHU3 xenografts were similar to those in the UM-UC-14 xenografts (Fig. 5, I–P).

**Discussion**

In this study, PD173074 treatment resulted in a remarkable growth inhibitory effect in two bladder cancer cell lines, namely, UM-UC-14 and MGHU3, harboring FGFR3 mutations, suggesting that aberrant FGFR3 signaling plays a critical role in the cellular proliferation in bladder cancer cells with FGFR3 mutations affecting either the extracellular Ig2-Ig3 domain or transmembrane domain. FGFR3 is a responsible gene for congenital skeletal disorders, which present a graded spectrum of phenotypic severity depending on distinct missense mutations of FGFR3. Thanatophoric dysplasia, characterized by neonatal lethality and profound dwarfism, derives from FGFR3 mutations identical to those reportedly found in the bladder UCs, including S249C, Y375C, and K652E substitutions.

The R248C and S249C mutations of FGFR3 involved in exon 7 are associated with ligand-independent activation (Naski et al., 1996; Wu, 2005; Tomlinson et al., 2007b). The Western blot analysis revealed that UM-UC-14 harboring homozygous S249C mutation expressed stably dimerized FGFR3 as expected based on the potential of forming a disulfide bond in the extracellular domain under the nonreducing condition (Fig. 1A). This phenomenon was equally observed in 97-7 bladder cancer cells (Tomlinson et al., 2007b). However, stably dimerized FGFR3 was not detected in MGHU3 expressing the Y375C mutated FGFR3. A previous research demonstrated that mutations affecting the transmembrane domain were likely to enhance mutant receptor dimerization, leading to constitutive ligand-independent activation (Webster and Donoghue, 1996). Alternatively, another report suggested that these mutations disrupt c-Cbl-mediated ubiquitination that serves as a targeting signal for lysosomal degradation and termination of receptor signaling, resulting in the increase of stability of FGFR3 (Cho et al., 2004). The biological characteristic of FGFR3 mutations affecting the transmembrane domain is controversial.

It was surprising that J82 cells carrying the K652E mutation of FGFR3 expressed undetectable levels of FGFR3 protein in the Western blot analysis. Consistently, PD173074 treatment achieved little inhibitory effect against proliferation of J82 cells (Fig. 1A). Reportedly, the frequency of FGFR3 mutations involved at codon 652 in exon 15 are much lower in the bladder UCs compared with those in exons 7 and 10 (Wu, 2005). These findings suggested that this mutation would not play a key role on oncogenesis and development of the bladder UCs. FGFR3 mutations affecting the intracellular kinase domain are known to stimulate the enzymatic activity by stabilizing the noninhibitory conformation of the kinase regulatory loop (Webster et al., 1996; Bernard-Pierrot et al., 2006). In a previous report, the constitutive tyrosine kinase activity of the K652E mutated FGFR3 was at least 100 times greater than that of the wild-type FGFR3, implying that this mutational type has an oncogenic property (Webster et al., 1996). Further studies are required to evaluate the pathogenicity and potential availability of this particular type of FGFR3 mutation involving codon 652 in exon 15 as a therapeutic target for PD173074 in the bladder UCs.

In the bladder UCs, the precise biological effect of FGFR3 mutations and downstream signaling pathways of constitutively phosphorylated FGFR3 have not been elucidated yet. In a recent study (Bernard-Pierrot et al., 2006), the NIH-3T3 cells with stable transfection of the S249C-mutated FGFR3b acquired both high anchorage-independent growth ability on soft agar and high cell proliferation compared with those transfected with the wild-type FGFR3b. In addition, the transfection of the S249C-mutated FGFR3b could induce significant tumorigenicity in the nude mice, whereas little or no tumor development was observed in the cells transfected with the wild-type FGFR3b. However, T24 cells, which harbor a missense mutation of HRAS, did not increase the oncogenic activity by stable transfection of the S249C-mutated FGFR3b, suggesting that the mutated FGFR3 and Ras gene shared a common signaling pathway in the bladder UCs. The idea can be supported by the data of Jebar et al. (2005). They analyzed 98 bladder tumor and 31 bladder cell lines for

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mutations of FGFR3 and three Ras gene members (HRAS, NRAS, and KRAS2), and concluded that FGFR3 and Ras gene mutation coexisted in no cases and no cell lines (Jebat et al., 2005). Likewise, the mutual exclusion of FGFR3 and Ras gene mutations was observed in multiple myeloma cells (Chesi et al., 2001).

A significant involvement of FGFR3 was also observed in multiple myeloma, in which the t(4;14)(p16;q32) translocation occurs frequently and leads to overexpression of FGFR3 from the derivative 14 (Chesi et al., 2001). Both overexpression and mutational activation of FGFR3 are suggested to contribute to tumor development and have the potential of therapeutic target (Trudel et al., 2005). In our work, no bladder cancer cell line overexpressed the wild-type FGFR3. Although only the KU7 cells showed a weak expression of the wild-type FGFR3, there was no remarkable sensitivity to PD173074, implying that cellular proliferation of KU7 was not dependent on the FGFR3 signaling pathway (Fig. 1). In a recent study, Tomlinson et al. (2007a) examined whether FGFR3 mutational status correlated with the FGFR3 protein levels in IHC analysis. Although a significant correlation was shown between the presence of the FGFR3 mutations and the overexpression, 42% of bladder tumors with no detectable FGFR3 mutation showed overexpression of FGFR3. This implies that even the overexpression of the wild-type FGFR3 potentially make up a fraction of nonmutant tumors with transformed phenotype and consequent responsiveness to the FGFR3-targeted therapies. Drug response assay with primary culture of clinical tumor tissues will be required for evaluating whether the patient population with the wild-type FGFR3-overexpressing bladder UC would benefit from PD173074 treatment.

We demonstrated that PD173074 treatment inhibits the cellular proliferation and elicits up-regulation of p27. The levels of p27 are stringently governed by the post-translational modification via the ubiquitin/proteasome pathway (Blain et al., 2003). Previous reports demonstrated that down-regulation or loss of p27 protein expression of the bladder tumor tissues was a significant prognostic indicator of intravesical recurrence or progression in the bladder UCs (Del Pizzo et al., 1999; Sgambato et al., 1999; Lopez-Beltran et al., 2004; Rabbani et al., 2007). These data suggest that the adequate expression of p27 is a key factor for suppressing the potential of development and progression of the bladder UCs. Mitogen-induced activation of receptor tyrosine kinases is followed by activation of the Ras/Raf/mitogen-activated protein kinase kinase or Ras/Raf/PI3K/Akt/protein kinase B signaling pathway, which plays an important role in the regulation of cell cycle (Cheng et al., 1998; Medema et al., 2000; Sherr, 2000; Blain et al., 2003). In human cancer cells, constitutive activation of the Ras-mediated signaling pathway could promote the stabilization of cyclin D1 and degradation of p27 (Sherr, 2000). In the UM-UC-14 and MGHU3 cells, which are sensitive to PD173074, inhibition of FGFR3 phosphorylation resulted in the transition into G0 arrest mediated by the up-regulation of p27 in vitro and in vivo. This finding also supports the assumption that Ras-related signals are downstream effector of mutated FGFR3 signaling in the bladder UCs. Up-regulation of p27 and reversible G1 arrest were induced by inhibition of the EGFR tyrosine kinase in cell lines harboring gene amplification or showing EGFR overexpression (Busse et al., 2000; Ling et al., 2007). They reported that the cells did not reenter S phase with removal of the kinase inhibitor, but treatment of the cells with phosphorothioate p27 oligonucleotides decreased p27 protein in A431 cells and abrogated the quinazoline-mediated G1 arrest. Inhibition of the mitogen-activated protein kinase activity did not induce G1 arrest or increase the levels of p27 but that of PI3K up-regulated p27, and recruited cells in G1, suggesting that the G1 arrest and up-regulation of p27 resulting from EGFR blockade are not due to interruption of mitogen-activated protein kinase, but result from interruption of constitutively active PI3K function. A similar analogy might be true in the case of FGFR3 blockade mediated by PD173074. Mutations of PIK3CA gene (phosphoinositide-3-kinase, catalytic, a polypeptide) were detected in bladder cancer harboring FGFR3 mutations (López-Knowles et al., 2006). Unlike the cases of Ras oncogene, mutations of PIK3CA and FGFR3 genes are not mutually exclusive, but they seem to be compound and work synergistically in bladder carcinogenesis.

In summary, our results suggest that both topical and oral administration of PD173074 are potentially available for the management of bladder tumors carrying the FGFR3 mutation involved in exon 7, exon 10, or both. The major limitation of our work is that the experiments were confined to the analyses using established bladder cancer cell lines. Histoculture drug response assay and clinical trials should be designed for confirming the true clinical benefit of PD173074 treatment and establishing clinico pathological and molecular markers that accurately predict the therapeutic efficacy of this treatment. For example, the expression levels of Ki-67, p27, and FGFR3 protein, presence of FGFR3 mutation and mutational type of FGFR3 are potentially promising predictive markers. We hope that this novel therapeutic approach enables 1) the decrease of intravesical recurrence rate after the initial transurethral resection for nonmuscle invasive diseases and 2) the improvement of the poor prognosis in advanced diseases in the future.

References


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802 Miyake et al.