Article type: original article – revision

Title:

Tumor Heterogeneity of *Fibroblast Growth Factor Receptor 3 (FGFR3)* Mutations in Invasive Bladder Cancer: Implications for Peri-Operative anti-FGFR3 Treatment

Authors list:
D. Pouessel[1,2]*, Y. Neuzillet[3]*, L.S. Mertens[3], M.S. van der Heijden[4], J. de Jong[5], J. Sanders[5,6], D. Peters[6], K. Leroy[7], A. Manceau[7], P. Maille[8], P. Soyeux[1], A. Moktefi[8], F. Semprez[1], D. Vordos[9], A. de la Taille[1,9], C.D. Hurst[10], D.C. Tomlinson[10], P. Harnden[10], P.J. Bostrom[11,19], T. Mirtti[12], S. Horenblas[3], Y. Loriot[13], N. Houédé[14], C. Chevreau[15], P. Beuzeboc[16], S.F. Shariat[17,19], A.I. Sagalowsky[17], R. Ashfaq[18], M. Burger[20], M.A.S. Jewett[21], A.R. Zlotta[21,22], A. Broeks[6], B. Bapat[23], M.A. Knowles[10], Y.Lotan[17], T.H. van der Kwast[24], S. Culine[2,25], Y. Allory[1,8,26#], B.W.G. van Rhijn[3,20-23#]

*shared first authorship, #shared senior authorship.

Affiliation address:
[1]Inserm U955, Hôpital Henri Mondor, APHP, Team 7 Translational Research of Genito-Urinary Oncogenesis, Créteil, France
[2]Hôpital Saint-Louis, AP-HP, Department of Medical Oncology, Paris, France
Departments of [3]Surgical Oncology (Urology), [4]Medical Oncology, [5]Pathology & [6]Molecular Pathology, Netherlands Cancer Institute, Antoni van Leeuwenhoek Hospital, Amsterdam, The Netherlands
[7]Tissue Biobank Unit, [8]Department of Pathology & [9]Urology, Hôpital Henri Mondor, APHP, Créteil, France
[10]Leeds Institute of Cancer and Pathology, St James's University Hospital, Leeds, United Kingdom
Departments of [11]Urology & [12]Pathology, University of Turku, Finland
[13] Department of Cancer Medicine and INSERM U981, Gustave Roussy, Cancer Campus, Grand Paris, Villejuif, France
[14]Institut Bergonié, Oncological Medicine, Bordeaux, France
[15]Institut Claudius Régaud, Oncological Medicine, Toulouse, France
[16]Institut Curie, Oncological Medicine, Paris, France
Departments of [17]Urology & [18]Pathology, University of Texas, Southwestern Medical center, Dallas, TX, USA
[19]Department of Urology, Medical University of Vienna, Vienna General Hospital, Vienna, Austria
[20]Department of Urology, Caritas St. Josef Medical Centre, University of Regensburg, Regensburg, Germany
Department of [21]Surgical Oncology (Urology) & [24]Pathology, University Health Network, Princess Margaret Cancer Centre, University of Toronto, Toronto, Canada
Departments of [22]Surgery (Urology) & [23]Cancer Genetics, Samuel Lunenfeld Research Institute, Mount Sinai Hospital, University of Toronto, Canada
[25]Paris 7 University, Paris, France, [26]Université Paris Est, UPEC, F-94000 Créteil, France

Running title: Tumor heterogeneity of *FGFR3* mutations in bladder cancer
Word count: Abstract 250; Body Text 2346; Figure 1x; Tables 3x; References 22.
Conflicts of Interest for this manuscript: None

Correspondence to:
Dr. Bas W.G. van Rhijn
Dept. of Surgical Oncology (Urology)
Netherlands Cancer Institute - Antoni van Leeuwenhoek Hospital
Plesmanlaan 121
1066 CX Amsterdam - The Netherlands
Tel: +31-205122553
Email: b.v.rhijn@nki.nl

ABSTRACT

Background: FGFR3 is an actionable target in bladder cancer. Preclinical studies show that anti-FGFR3 treatment slows down tumor-growth suggesting that this tyrosine kinase receptor is a candidate for personalized bladder cancer treatment, particularly in patients with mutated *FGFR3*. We addressed tumor heterogeneity in a large multi-center, multi-lab study, as this may have significant impact on therapeutic response.

Patients and methods: We evaluated possible *FGFR3* heterogeneity by the PCR-SNaPshot method in the superficial and deep compartments of tumors obtained by trans-urethral resection (TUR, *n*=61) and in radical cystectomy (RC, *n*=614) specimens and corresponding cancer-positive lymph nodes (LN+, *n*=201).

Results: We found *FGFR3* mutations in 13/34 (38%) T1 and 8/27 (30%) ≥T2-TUR samples with 100% concordance between superficial and deeper parts in T1-TUR samples. Of eight *FGFR3* mutant ≥T2-TUR samples, only four (50%) displayed the mutation in the deeper part. We found 67/614 (11%) *FGFR3* mutations in RC specimens. *FGFR3* mutation was associated with pN0 ($p<0.001$) at RC. In 10/201 (5%) LN+ a *FGFR3* mutation was found, all concordant with the corresponding RC specimen. In the remaining 191 cases, RC and LN+ were both wild type.

Conclusions: *FGFR3* mutation status seems promising to guide decision making on adjuvant anti-FGFR3 therapy as it appeared homogeneous in RC and LN+. Based on the results of TUR, the deep part of the tumor needs to be assessed if neoadjuvant anti-FGFR3 treatment is considered. We conclude that studies on the heterogeneity of actionable molecular targets should precede clinical trials with these drugs in the peri-operative setting.

Key words: FGFR3, mutations, heterogeneity, bladder, cancer, targeted therapy

Key message:

FGFR3 is a major potential actionable target in urothelial bladder cancer (BC). We found that *FGFR3* mutations appeared conserved in primary BC and corresponding lymph-node metastases. We also showed that the deep part of the tumor needs to be assessed if neoadjuvant anti-FGFR3 treatment is considered. This suggests that personalized anti-FGFR3 therapy may improve BC treatment in the peri-operative setting.

INTRODUCTION

Radical Cystectomy (RC) has been the gold standard for treatment of invasive, non-metastatic, urothelial carcinoma of the bladder (UCB) for more than 50 years. Despite major surgery, five-year survival still only ranges from ±75% in pT2N0 to ±25% in pN+ UCB (1,2). Peri-operative (neoadjuvant and adjuvant) platinum-based combination chemotherapy has only marginally (5-7% overall survival benefit for neoadjuvant chemotherapy) improved patient's prognosis (3-5). Consequently, better systemic treatment is urgently needed to improve clinical outcomes for invasive UCB.

Activating oncogenic mutations of *FGFR3* were identified more than 10 years ago in UCB (6). Interestingly, *FGFR3* mutations were predominantly found in genetically stable UCB with a favorable prognosis (7). Moreover, *FGFR3* and *TP53* mutations rarely coincide and *FGFR3* mutations are, even in advanced UCB, most of the time accompanied by fewer molecular alterations than *FGFR3* wild-type tumors (7-10). This indicates that FGFR3 is also a major potential actionable target in a subgroup of advanced UCB (9-11). Furthermore, preclinical *in-vitro* and *in-vivo* data show that anti-FGFR3 therapy slows down tumor growth, especially in *FGFR3*-mutated tumors (12). However, the heterogeneity of *FGFR3* status within a tumor or a patient has not been adequately addressed and may negatively impact therapeutic response (11).

We report a large multi-center, multi-lab study investigating the heterogeneity of the *FGFR3* mutations in invasive UCB. We analyzed paired samples (superficial and deep compartments of the same lesion) of primary trans-urethral resection (TUR) of 61 patients. We also analyzed paired samples from RC and positive lymph nodes (LN+) of 614 patients who were treated for cN0M0-UCB without prior systemic

chemotherapy and/or radiotherapy. FGFR3 expression was also analyzed by IHC in a subgroup of patients.

MATERIALS AND METHODS

Study populations

Three cohorts of patients with UCB were established to study the heterogeneity of *FGFR3* mutation status in UCB. In total, 10 different hospitals were involved in the treatment of the patients and molecular analyses were done in 4 different laboratories.

1. *Cohort of transurethral resection (TUR):*

To evaluate intra-tumor *FGFR3* mutation heterogeneity, we studied a cohort of 61 patients who underwent a primary TUR for UCB. All tumors were primary UCB. The procedures were performed in 2 hospitals (Toronto; *n*=26 and Leeds; *n*=35) between 1993 and 2006. Mean age at diagnosis was 70,3 years (SD 8,3 years); 15/61 patients were female. All TUR specimens contained muscle as assessed by pathology review (TvdK and PH). For each case, a superficial and deep part of the same tumor specimen were separately dissected from the tissue-block or blank slides for DNA isolation and subsequent *FGFR3* mutation analysis. All DNA-samples of the 61 TURs were analyzed in both labs (Toronto and Leeds) and the results were the same. An additional 4 TUR-cases, in which multiple parts of the same superficial (*n*=3) or invasive (*n*=1) areas were available, were analyzed in Toronto.

2. *Cohorts of radical cystectomy*

The second (International) cohort included 494 patients treated with radical cystectomy (RC) including a pelvic lymph-node dissection for cN0M0 (staged with at least abdominal CT and chest X-ray) UCB in 4 hospitals in Amsterdam, the Netherlands (*n*=204); Toronto, Canada (*n*=104); Dallas, TX, USA (*n*=132) and Turku, Finland (*n*=54). A previous diagnosis of non-invasive UCB was allowed. Mean age at RC was 65,1 years (SD 10,8 years); 121/494 patients were female. Patients were

treated between 1986 and 2012 by RC without prior neo-adjuvant chemotherapy or pelvic radiation. Of these patients, 83/494 (17%) received adjuvant chemotherapy. Pathology review was done by JdJ, JS (Amsterdam) and TvdK (Toronto, Dallas, Turku). Node samples were available for reliable *FGFR3* analysis in 117/155 pN+ cases. The lab in Amsterdam analyzed the 204 RC-cases from Amsterdam and the 290 RC-cases from Toronto, Dallas and Turku were all analyzed in Toronto.

In the third (French) cohort, 120 cN0M0 UCB patients treated in 5 French hospitals for locally advanced pT3/pT4 (*n*=100) and/or pN+ (*n*=99) UCB were identified. All these patients were treated by RC including a pelvic lymph-node dissection and adjuvant platinum-based chemotherapy between 2000 and 2009 at the Henri Mondor hospital, Créteil (*n*=36); the Gustave Roussy institute, Villejuif (*n*=28); the Curie institute, Paris (*n*=7); the Claudius Regaud institute, Toulouse (*n*=28) and Bergonié institute, Bordeaux (*n*=21). Mean age at RC was 62,1 years (SD 9,1 years); 16/120 patients were female. A previous diagnosis of non-invasive UCB was allowed. None of the patients had prior neo-adjuvant chemotherapy or pelvic radiation. Central pathology review was done by YA. Node samples were available for reliable *FGFR3* analysis in 84/99 pN+ cases. The lab in Créteil analyzed all the RC-cases of the French cohort.

Clinicopathological data collection

The clinico-pathological characteristics, treatment and follow-up data were retrospectively collected. Tumors were staged according to the 2009 TNM classification (13) and graded according to WHO criteria. Local ethics committees and/or translational research boards approved the three experimental protocols and,

if applicable, patients provided written informed consent for central collection of their tissue specimens and clinical data for research purposes.

Tissue (TUR & RC) specimens and DNA extraction

Hematoxylin and eosin slides served as templates for the manual macro-dissection procedure on the formalin fixed, paraffin embedded tissue-block or blank slides. The dissected samples contained a minimum of 70% tumor cells, as assessed by histological examination. DNA was extracted from the tissues according to the manufacturer's protocols using the DNeasy® Tissue Kit in the TUR and international RC cohorts. In the French RC cohort, the Maxwell® 16 FFPE Plus LEV DNA Purification Kit and an automated Maxwell® platform (Promega®) were used for DNA isolation.

FGFR3 mutation analysis

FGFR3 mutation analysis was done using the PCR-SNaPshot method in all labs. Details of this method were reported previously (14,15). Briefly, 3 regions (exons 7, 10 and 15) frequently mutated and representing at least 99% of activating oncogenic *FGFR3* mutations in UCB, were simultaneously amplified by PCR. After removing excess primers and deoxynucleotides, specific SNaPshot primers were annealed to the PCR products, separated by capillary electrophoresis and analyzed in an automatic sequencer (Prism® 3100 genetic analyser). With this PCR-SNaPshot method, a total of 11 known oncogenic *FGFR3* mutations can be detected. The codon numbering refers to the cDNA open reading frame of the FGFR3b isoform expressed in epithelia (6).

FGFR3 expression analysis

FGFR3 expression could be studied with IHC in 357/494 cystectomy specimens and in 72/117 paired RC/LN+ from the International cohort (a subset from Amsterdam, Toronto and Turku). Standard TMA technology was used in both labs (16). The available cases were routinely processed with a monoclonal antibody against FGFR3 (FGFR3 B9, Santa Cruz, CA). Positive and negative controls were included in each run. Slides were assessed by BvR and TvdK (Toronto) and by BVR and JS (Amsterdam). A semi-quantitative scoring system was used: 0, negative; 1, faint/normal; 2, moderate positivity; 3, strong positivity. FGFR3 overexpression was defined by a score of 2 or 3 as previously described (15,17,18).

Statistics

SPSS®, version 20 was used for data documentation and analysis. Chi-square statistics were used to analyze possible associations between *FGFR3* status and pathological variables. Statistical significance was set at $p<0.05$.

RESULTS

Within the TUR cohort, *FGFR3* mutations were detected in 13/34 T1 and 8/27 ≥T2 UCB, respectively. Comparing paired superficial and deep parts, no discordance was found within the T1-TUR samples (Figure1A), whereas discordance was observed in half of the cases within the ≥T2-TUR samples with only 4/8 *FGFR3* mutations in the invasive area (Figure1B). In another 4 TUR cases (one with mutation), multiple samples from same area (3 multiple superficial, 1 multiple invasive areas) were analyzed as a control experiment. We found no difference among these samples.

Within the RC cohort, *FGFR3* status was known for 614 RC of which 254 (41%) were pN+. Of the 254 LN+ cases, *FGFR3* status was available for 201 (79%) paired RC/LN+ samples. In the 614 cystectomies, 67 (11%) *FGFR3* mutations were detected, of which 54 were pN0 (Table1). Suppl. Table1 shows the distribution of mutations for the International and French RC cohorts, respectively. In suppl. Table2, the types of *FGFR3* mutations, with S249C (67%) as the most frequent one, are listed. Table2 shows the clinico-pathological characteristics of the 13 patients with a *FGFR3* mutation and pN+ UCB. In the 201 paired RC/LN+ samples, the same *FGFR3* mutation was detected in the cystectomy and LN+ specimen (Figure1C). Discordance between the 201 paired samples was not observed (specificity: 100%). The presence of a *FGFR3* mutation was associated with lower pT-stage (p<0.001) and pN0 (p<0.001) at RC (Table1).

Finally, FGFR3 expression was studied with immunohistochemistry (IHC) in 357/614 cystectomy specimens (Table3a). In 280 RC, FGFR3 expression was normal and no mutation was found. We found 70 RC with overexpression of whom 37 had a mutation. In 7 cases, we found a FGFR3 mutation with normal expression

at IHC (Table3a). IHC samples were available for 72/201 paired RC/LN+ cases (Table3b). FGFR3 expression was concordant in 64/72 (89%) cystectomy and LN+ specimens.

DISCUSSION

In metastatic UCB, several targeted therapies have been evaluated as second-line treatment (19) but none of them has made it into the clinical practice so far. Although development of effective inhibitors (including anti-FGFR3 treatment) still is at an early stage, FGFR3 is a very promising actionable target in UCB (9-12,19). Comparable to other malignancies, targeted therapy has shown significant activity in only a minority of UCB-patients (10-12,19). Reasons for this limited activity may include the diverse genomic landscape of UCB (10), the absence of molecular tumor-analysis before test-drug administration (12) and lack of adequate studies addressing intra-tumor/patient heterogeneity of potential actionable targets (11). Considering cN0M0 patients in the peri-operative setting, molecular tumor analysis and heterogeneity assessment are pivotal before administering a drug against an actionable target. To our knowledge, the present study is the first to address tumor-heterogeneity for the peri-operative setting in UCB with TUR and RC/LN+ specimens.

FGFR3 activation mostly occurs via oncogenic mutations (6-12), occasionally by rearrangements (10,20) and also via over-expression by other mechanisms such as copy-number gain (10,15,17). Less is known about *FGFR3* intra-tumor/patient heterogeneity in UCB (21). The main purpose of our multi-center, multi-lab study was to address this heterogeneity for the peri-operative setting of invasive UCB. Previous small, single center, single lab studies have shown an approximately 80% concordance in multiple synchronous and metachronous non-invasive UCB (17,21). Furthermore, recent important preclinical work provided a cellular and genetic basis for this diversity in UCB (22). In our study on TUR samples, we showed that *FGFR3* mutation status may differ between the superficial and invasive part of one tumor. So far, only one previous study reported on *FGFR3* heterogeneity in superficial and

deep invasive parts at TUR (17). Within 18 mutated UCB, 9 had the same mutation in the two compartments, 8 had mutation only in the most superficial area and one had different mutations in the two parts. However, the authors were not sure that samples were from the same lesion in the bladder. In the present TUR-series, the same tumor was analyzed. It was notable that we found 4 cases with a *FGFR3* mutation in the superficial part but not in the deep part of the same ≥T2 tumor. Conversely, we did not observe a difference in *FGFR3* status in 201 RC and LN+ samples of our RC cohort. Therefore, it is likely that, at RC, the deep part of the tumor has been analyzed and that the superficial part was already removed by the preceding TUR. The mutation frequency at RC (11%) also corresponded to the mutation frequency of the deep part of the ≥T2-TUR cohort (15%). The frequency of *FGFR3* mutations (12%) in the TCGA-cohort of 131 high-grade muscle-invasive UCB (mostly cystectomy specimens) was also comparable to our cohort. This implies that the deep part of the tumor at TUR needs to be assessed if neoadjuvant anti-FGFR3 treatment is considered.

Our study showed that, if a mutated clone progresses in MI-UCB, the *FGFR3* mutation is conserved in the invasive compartment and also in the metastatic node despite the notion that not all the lesions in the RC cohorts were primary (first diagnosis) UCB. We also reported that the *FGFR3* mutation was associated with lower T-stage and pN0 at RC. Others have already reported that *FGFR3* mutations are also in MI-UCB most of the time not accompanied by many other molecular alterations (8,10). Taken together, all these findings suggest that anti-FGFR3 treatment may have significant clinical impact in the peri-operative setting for a relative small subgroup of MI-UCB patients.

FGFR3 expression is another way to explore FGFR3 activity. Turo et al. (18) recently reported a heterogeneity study using FGFR3 expression by IHC without *FGFR3* mutation evaluation. In their cohort, paired RC/LN+ samples were available for IHC analysis in 106/150 pN+-UCB and concordance was found in 79/106 (75%) cases. We here reported IHC-concordance in 64/72 (89%). Previous IHC studies showed that approximately 40% of invasive *FGFR3* wild-type tumors overexpress FGFR3 suggesting an alternative mechanism to activate FGFR3 (10,15,17). In our RC-series, only 10% of wild-type cases showed overexpression (Table3). One of the reasons for this lower percentage might be that we analyzed RC specimens and consequently deeper parts of the tumor than in the previous studies. Nevertheless, we can't exclude that a small subset of patients with wild-type tumors may still benefit from anti-FGFR3 treatment. On the other hand, we showed that *FGFR3* mutation analysis was extremely robust across 4 labs. IHC is likely more prone to observer variability than *FGFR3* mutation analysis making it less appropriate to assess FGFR3 heterogeneity within a tumor or metastases of a patient. Future study should focus on how to combine *FGFR3* mutation, translocation and copy-number status with FGFR3-IHC to guide optimal personalized anti-cancer treatment.

In conclusion, we found that *FGFR3* mutations appeared conserved in primary bladder cancer and corresponding lymph-node metastases. Hence, anti-FGFR3 treatment may have significant clinical impact in the adjuvant setting. We also showed that the deep part of the tumor needs to be assessed if neoadjuvant anti-FGFR3 treatment is considered. Our data on tumor heterogeneity suggest that personalized anti-FGFR3 therapy may improve bladder cancer treatment for a relatively small, well-selected subgroup of invasive UCB patients. Studies on the

heterogeneity of actionable molecular targets should precede clinical trials with these drugs in the peri-operative setting.

ACKNOWLEDGEMENTS

The authors wish to acknowledge all patients who contributed tissue for research. The authors would like to thank Simone Russell, Rati Vajpeyi, Sally Hanna, Roni Sambas and Cynthia Kuk (Toronto) and Floris Groenendijk, Renate de Groot and Chantall Curial (Amsterdam) for help and advice. We thank the Core Facility for Molecular Pathology & Bio-banking of the Netherlands Cancer Institute – Antoni van Leeuwenhoek and the Tissue bank Unit – PRB Mondor for their assistance. We also thank the pathologists who provided tissue blocks for the multicenter French cohort. The authors would like to acknowledge the International Bladder Cancer Network (IBCN) and the Bladder Cancer Advocacy Network (BCAN) for providing the platform to collaborate on this project.

The Regional Ethics Board of the University Health Network, Toronto (02-0515-C and 08-0263-T) gave approval. The Translational Research Board of the Netherlands Cancer Institute – Antoni van Leeuwenhoek hospital (CFMPB 160) approved the study. Approval was obtained from the Leeds East Research Ethics Committee. The Regional Ethics Board of Ile-de-France IX (Comité de protection des Personnes – Ile-de-France IX, Créteil) approved the study (11-052).

Funding Statement:

Financial support was given by the University of Toronto, a grant from the Dutch Cancer Society – KWF Kankerbestrijding, the European Urological Scholarship programme, a programme grant from Cancer Research UK and the French Urological Association. Specific grant numbers are not applicable.

Disclosure Statement:

The authors have declared no conflicts of interest.

REFERENCES

1. Stein JP, Lieskovsky G, Cote R, Groshen S, Feng AC, Boyd S, et al. Radical cystectomy in the treatment of invasive bladder cancer: long-term results in 1,054 patients. J Clin Oncol 2001;19:666-75.
2. Ghoneim MA, Abdel-Latif M, el Mekresh M, Abol-Enein H, Mosbah A, Ashamallah A, et al. Radical cystectomy for carcinoma of the bladder: 2.720 consecutive cases 5 years later. J Urol 2008 ;180:121-7.
3. Griffiths G, Hall R, Sylvester R, Raghavan D, Parmer MK, et al. International phase III trial assessing neoadjuvant cisplatin, methotrexate, and vinblastine chemotherapy for muscle-invasive bladder cancer: long-term results of the BA06 30894 trial. J Clin Oncol 2011; 29:2171-7.
4. Zargar H, Espiritu PN, Fairey AS, Mertens LS, Dinney CP, Mir MC, et al. Multicenter assessment of neoadjuvant chemotherapy for muscle-invasive bladder cancer. Eur Urol 2015;67:241-9.
5. Sternberg CN, Skoneczna I, Kerst JM, Albers P, Fossa SD, Agerbaek M, et al. Immediate versus deferred chemotherapy after radical cystectomy in patients with pT3-pT4 or N+ M0 urothelial carcinoma of the bladder (EORTC 30994): an intergroup, open-label, randomised phase 3 trial. Lancet Oncol 2015;16:76-86.
6. Cappellen D, De Oliveira C, Ricol D, de Medina S, Bourdin J, Sastre-Garau X, et al. Frequent activating mutations of FGFR3 in human bladder and cervix carcinomas. Nat Genet 1999;23:18-20.
7. van Rhijn BW, Vis AN, van der Kwast TH, Kirkels WJ, Radvanyi F, Ooms EC, et al. Molecular grading of urothelial cell carcinoma with fibroblast growth factor receptor 3 and MIB-1 is superior to pathologic grade for the prediction of clinical outcome. J Clin Oncol 2003;21:1912-21.
8. Neuzillet Y, Paoletti X, Ouerhani S, Mongiat-Artus P, Soliman H, de The H, et al. A meta-analysis of the relationship between FGFR3 and TP53 mutations in bladder cancer. PLoS One 2012;7:e48993.
9. Iyer G, Al-Ahmadie H, Schultz N, Hanrahan AJ, Ostrovnaya I, Balar AV, et al. Prevalence and co-occurrence of actionable genomic alterations in high-grade bladder cancer. J Clin Oncol 2013;31:3133-40.
10. Cancer Genome Atlas Research Network: Comprehensive molecular characterization of urothelial bladder carcinoma. Nature 2014;507:315-22.
11. Lerner SP. Targeted therapies for metastatic bladder cancer. J Urol 2015;193:8-9.
12. Mazzola CR, Siddiqui KM, Billia M, Chin J. Dovitinib: rationale, preclinical and early clinical data in urothelial carcinoma of the bladder. Expert Opin Investig Drugs 2014;23:1553-62.
13. Edge S, Byrd D, Compton C. AJCC cancer staging manual, 7th edn. New York , NY: Springer, 2010.
14. van Oers JM, Lurkin I, van Exsel AJ, Nijsen Y, van Rhijn BW, van der Aa MN, et al. A simple and fast method for the simultaneous detection of nine fibroblast growth factor receptor 3 mutations in bladder cancer and voided urine. Clin Cancer Res 2005;11:7743–8.
15. Neuzillet Y, van Rhijn BW, Prigoda NL, Bapat B, Liu L, Bostrom PJ, et al. *FGFR3* mutations, but not FGFR3 expression and FGFR3 copy-number variations, are associated with favourable non-muscle invasive bladder cancer. Virchows Archiv 2014;465:207-13.
16. van Rhijn BW, Catto J, Goebel PJ, Knüchel R, Shariat SF, van der Poel HG, et al. Molecular markers for urothelial bladder cancer prognosis: towards implementation in clinical practice. Urol Oncol 2014;32:1078-87.
17. Tomlinson DC, Baldo O, Harnden P, Knowles MA. FGFR3 protein expression and its relationship to mutation status and prognostic variables in bladder cancer. J Pathol 2007;213:91-8.
18. Turo R, Harnden P, Thygesen H, Fleischmann A, Thalmann GN, Seiler R, et al. FGFR3 expression in primary invasive bladder cancers and matched lymph node metastases. J Urol 2015;193:325-30.
19. Rouanne M, Loriot Y, Lebret T, Soria JC. Novel therapeutic targets in advanced urothelial carcinoma. Crit Rev Oncol Hematol 2015;doi:10.1016/j.critrevonc.2015.10.021:[Epub ahead of print].
20. Williams SV, Hurst CD, Knowles MA. Oncogenic FGFR3 gene fusions in bladder. Hum Mol Genet 2013;22:795-803.

21. Gerlinger M, Catto JW, Orntoft TF, Real FX, Zwarthoff EC, Swanton C. Intratumour heterogeneity in urologic cancers: From molecular evidence to clinical implications. Eur Urol 2015;67:729-37.
22. Van Batavia J, Yamany T, Molotkov A, Dan H, Mansukhani M, Batourina E, et al. Bladder cancers arise from distinct urothelial sub-populations. Nat Cell Biol 2014;16:982-91.

FIGURE LEGEND

Legend Figure 1

Figure 1 shows the distributions of *FGFR3* mutations in the superficial and deep compartments of the 61 (34 T1 and 27 ≥T2) patients included in the trans-urethral resection (TUR) cohort and in the 614 radical cystectomy patients with 201 paired cystectomies and metastatic nodes available.

A: The 13 mutated cases in 34 paired T1-TUR samples are displayed. Both parts (superficial and deep) were wild type in 21 cases.

B: The 8 mutated cases in paired ≥T2-TUR samples are displayed. Both parts (superficial and deep) were wild type in 19 cases.

C: The 10 mutated cases in paired cystectomies and metastatic nodes are displayed. The cystectomy and metastatic node were both wild type in 191 cases.

Figure 1A

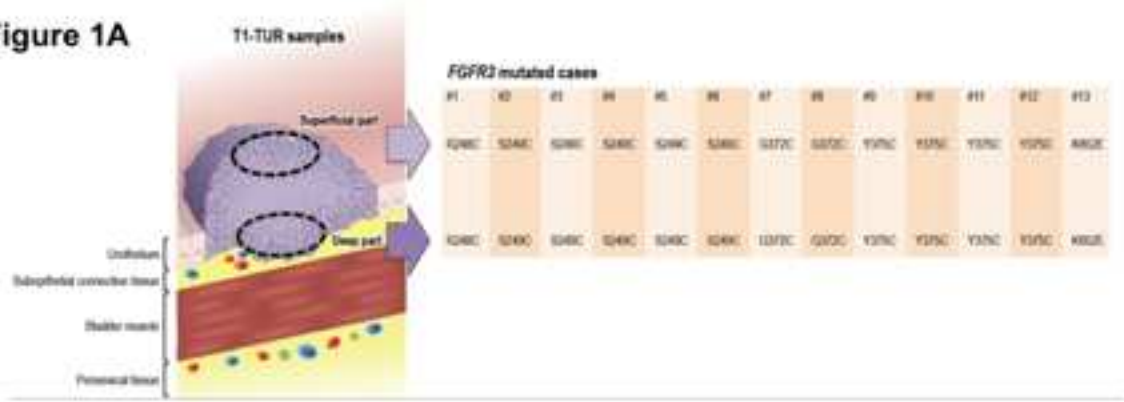
T1-TUR samples
Superficial part
Deep part
Urothelium
Subepithelial connective tissue
Bladder muscle
Perivesical tissue
FGFR3 mutated cases

Figure 1B

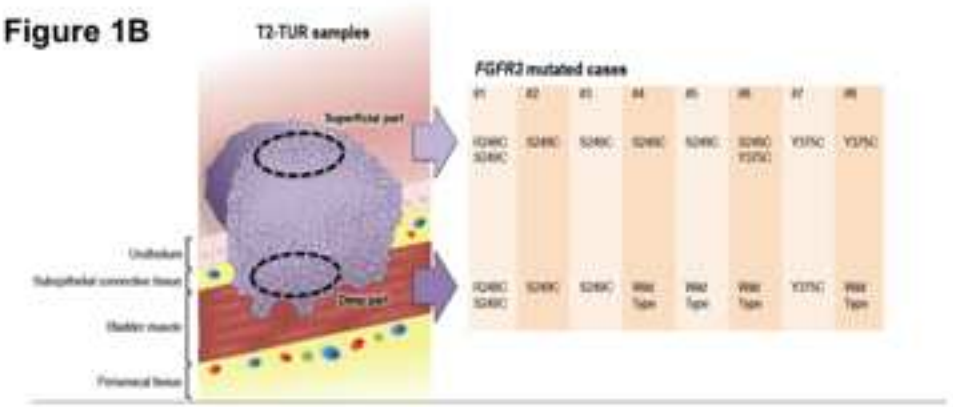
T2-TUR samples
Superficial part
Deep part
Urothelium
Subepithelial connective tissue
Bladder muscle
Perivesical tissue
FGFR3 mutated cases

Figure 1C


FGFR3 mutated cases
Primary Bladder Tumor
Metastatic Lymph Nodes

TABLES

Table 1 – The distribution of samples according to the primary tumors pathologic pT-stage and *FGFR3* mutation status among either N0 or N+ cases in the radical cystectomy cohort. *FGFR3* mutations were associated with lower pT-stage (p<0.001) and pN0 (p<0.001) at radical cystectomy.

		pTa, pT1, pTis	pT2	pT3	pT4	Total
N0	Wild type	47	93	118	48	306
	Mutated	23	11	14	6	54
N+	Wild type	5	46	127	63	241
	Mutated	0	0	6	7	13
Total		75	150	265	124	614

Table 2 - Clinical and pathological characteristics of patients with pN+ UC and a *FGFR3* mutation detected in cystectomy and/or positive lymph node. In 3 cases, the node sample was not available.

Patient	Age	Gender	Histology	Pathological stage	WHO1973 grade	AC	Relapse	Relapse type	Vital status	Follow-up (yr.)	Disease status	*FGFR3* mutation	Mutated samples
Int711	73	M	UC	pT4aN2	3	No	Yes	DM	Dead	1.7	DOD	S249C	T,N
Int1008	44	F	UC+SCC	pT4aN2	3	No	No	-	Alive	0.7	FOD	S249C	T
Int1015	39	M	UC	pT4aN2	3	No	Yes	DM	Alive	1.1	FOD	S249C	T
Int1028	78	F	UC	pT4aN2	2	Yes	No	-	Alive	1.5	FOD	S249C	T,N
Int3097	56	F	UC	pT3bN2	3	No	Yes	DM	Dead	1.2	DOD	R248C	T,N
Int3113	78	M	UC+SCC	pT3aN2	3	No	No	-	Alive	9.4	FOD	S249C	T,N
Int3125	75	M	UC	pT3aN1	3	No	Yes	DM	Dead	1.1	DOD	S249C	T
Int3180	62	F	UC+SCC	pT4bN2	3	No	Yes	DM	Dead	0.4	DOD	S249C	T,N
Int3280	81	F	UC	pT3bN2	3	No	Yes	DM	Dead	1.9	DOD	R248C	T,N
VCA023	77	M	UC	pT3aN1	3	Yes	No	-	Alive	2	FOD	S249C	T,N
VCA045	46	M	UC	pT3bN2	3	Yes	Yes	DM	Dead	3.6	DOD	S249C	T,N
VCA047	56	M	UC	pT4aN1	3	Yes	No	-	Alive	11	FOD	S249C	T,N
VCA090	59	M	UC	pT4aN2	3	Yes	Yes	DM	Dead	2.6	DOD	S249C	T,N

AC: Adjuvant Chemotherapy; M: Male; F: Female; UC: Urothelial Carcinoma; UC+SCC: Urothelial carcinoma with squamous differentiation; T: Tumor; N: Node; DM: Distant Metastasis; FOD: Free of Disease; DOD: Dead of Disease.

Table 3a - FGFR3 expression and *FGFR3* mutation (cystectomy specimens) in a subset of 357/494 cases from the international radical cystectomy cohort.

		FGFR3 expression in cystectomy		Total
		Normal	Over-expression	
FGFR3 mutation in cystectomy	Wild type	280	33	313
	Mutated	7	37	44
Total		287	70	357

Table 3b - FGFR3 expression in cystectomy and corresponding metastatic lymph nodes in a subset of 72/117 pN+ cases from the international radical cystectomy cohort.

		FGFR3 expression in cystectomy		Total
		Normal	Over-expression	
FGFR3 expression in positive node	Normal	57	4	61
	Over-expression	4	7	11
	Total	61	11	72

SUPPLEMENTALS – Online Only

Supplemental Table 1 - Distribution (frequencies) of samples according to the primary tumors pathologic stage and *FGFR3* mutation status among either N0 or N+ cases in the International (A) and French (B) cohorts. Please note that adjuvant chemotherapy was given to all the patients in the French cohort and to 83/494 (17%) patients in the international cohort.

A		pTa, pT1, pTis	pT2	pT3	pT4	Total
N0	Wild type	47	93	106	40	286
	Mutated	23	11	14	5	53
N+	Wild type	2	29	81	34	146
	Mutated	0	0	4	5	9
Total		72	133	205	84	494

B		pTa, pT1, pTis	pT2	pT3	pT4	Total
N0	Wild type	0	0	12	8	20
	Mutated	0	0	0	1	1
N+	Wild type	3	17	46	29	95
	Mutated	0	0	2	2	4
Total		3	17	60	45	120

Supplemental Table 2 - *FGFR3* mutation type in 67 mutated radical cystectomy samples. The *FGFR3* mutations G372C, A393E, K652M, K652T, K652E and K652Q were not detected in this radical cystectomy series. Of note, the types of *FGFR3* mutations in the TUR series (*n*=21) can be derived from Figure 1.

FGFR3 mutation type	Mutations (%)
R248C	9 (13%)
S249C	45 (67%)
S373C	1 (2%)
Y375C	11 (16%)
G382R	1 (2%)
Total:	67 (100%)