EUROPEAN UROLOGY 76 (2019) 9–13

available at www.sciencedirect.com
journal homepage: www.europeanurology.com

European Association of Urology

Brief Correspondence

APOBEC-mediated Mutagenesis as a Likely Cause of *FGFR3* S249C Mutation Over-representation in Bladder Cancer

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Article info

Article history:
Accepted March 21, 2019

Associate Editor:
James Catto

Statistical Editor:
Andrew Vickers

Keywords:
Bladder cancer
Upper urinary tract cancer
FGFR3 mutation
APOBEC
Lynch syndrome

Abstract

FGFR3 is one of the most frequently mutated genes in bladder cancer and a driver of an oncogenic dependency. Here we report that only the most common recurrent *FGFR3* mutation, S249C (TCC → TGC), represents an APOBEC-type motif and is probably caused by the APOBEC-mediated mutagenic process, accounting for its over-representation. We observed significant enrichment of the APOBEC mutational signature and overexpression of *AID/APOBEC* gene family members in bladder tumors with S249C compared to tumors with other recurrent *FGFR3* mutations. Analysis of replication fork directionality suggests that the coding strand of *FGFR3* is predominantly replicated as a lagging strand template that could favor the formation of hairpin structures, facilitating mutagenic activity of APOBEC enzymes. In vitro APOBEC deamination assays confirmed S249 as an APOBEC target. We also found that the *FGFR3* S249C mutation was common in three other cancer types with an APOBEC mutational signature, but rare in urothelial tumors without APOBEC mutagenesis and in two diseases probably related to aging.

Patient summary: We propose that APOBEC-mediated mutagenesis can generate clinically relevant driver mutations even within suboptimal motifs, such as in the case of *FGFR3* S249C, one of the most common mutations in bladder cancer. Knowledge about the etiology of this mutation will improve our understanding of the molecular mechanisms of bladder cancer.

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https://doi.org/10.1016/j.eururo.2019.03.032
0302-2838/

FGFR3 is one of the most frequently mutated genes in bladder cancer (BLCA). More than 65% of non-muscle-invasive bladder cancers (NMIBCs) and 15% of muscle-invasive bladder cancers (MIBCs) carry an *FGFR3* mutation driving an oncogenic dependency [1,2]. We reviewed publicly available data for 10 032 bladder tumors (Supplementary Fig. 1, Supplementary Table 1) and identified 56 different *FGFR3* mutations, including 14 recurrent mutations (detected in ≥2 samples, Supplementary Table 2, Fig. 1A). The most common was the S249C mutation (TCC → TGC), representing 62% of all recurrent *FGFR3* mutations. We wondered whether this over-representation of *FGFR3* S249C is associated with some specific mutational processes. Considering all the mutational signatures [3], S249C (TCC → TGC) is most similar to an APOBEC-type mutation (TCN → T[G/T]N, where N = any nucleotide, but most frequently A or T). Of all recurrent *FGFR3* mutations, only S249C has an APOBEC-type motif (Fig. 1A). The APOBEC mutational signature accounts for ~30% and 65% of all mutations in NMIBC and MIBC, respectively [2,4]. Thus, we hypothesized that the *FGFR3* S249C mutation might be caused by the activity of APOBEC enzymes.

We analyzed mutational signatures in NMIBC using RNA sequencing (RNA-seq) data and observed that only the APOBEC-type signature (S3 scores) was significantly higher in tumors with the S249C mutation compared to tumors with other recurrent *FGFR3* mutations (Fig. 1B), while other RNA-seq-derived mutational signatures did not differ between these groups (Supplementary Fig. 2).

We also analyzed the APOBEC mutation load in MIBC in The Cancer Genome Atlas (TCGA). Even though only 13% of MIBCs had recurrent *FGFR3* mutations (compared to 67% in NMIBC), S249C was found in similar proportions (60%) in MIBC and NMIBC. We were unable to demonstrate a significant association between over-representation of the S249C mutation and the APOBEC mutation load in the much smaller MIBC subset of tumors with recurrent *FGFR3* mutations (n = 52, Supplementary Fig. 3) compared to NMIBC (n = 227). To consider the higher heterogeneity of MIBC compared to NMIBC, we took advantage of the previous stratification of MIBC tumors as APOBEC-high, APOBEC-low, and APOBEC-no [2]. We observed a significantly higher proportion of S249C mutations in tumors with any APOBEC activity (APOBEC-high and -low) compared to APOBEC-no tumors (Fig. 1C). In addition, considering the two groups of tumors with APOBEC activity, the APOBEC mutation load was overall significantly higher in tumors with the S249C mutation than in tumors bearing other recurrent *FGFR3* mutations (Fig. 1D). Thus, it appears that the *FGFR3* S249C mutation is favored in tumors with APOBEC activity; APOBEC-low MIBC and NMIBC may have lower background noise than APOBEC-high tumors, making the S249C enrichment more noticeable in the former compared to the latter.

To identify a possible APOBEC mutagen for the *FGFR3* S249C mutation, we analyzed expression levels of all 11 genes from the *AID*/*APOBEC* gene family (Supplementary Fig. 4). Comparison of tumors with *FGFR3* S249C and those with other recurrent *FGFR3* mutations revealed that only expression of *APOBEC3A* and *APOBEC3H* was significantly different in NMIBC and only expression of *APOBEC3A* and *APOBEC3B* in APOBEC-low MIBC (Supplementary Fig. 5).

APOBEC-mediated mutagenesis preferentially targets lagging DNA strand templates [5], which is consistent with transient excess of single-stranded DNA (ssDNA) during the replication process. The efficiency of APOBEC mutagenesis has also been associated with the propensity of ssDNA to form hairpins, with some APOBEC3 enzymes, such as APOBEC3A, preferentially targeting loops in stem-loop structures [6]. Notably, residue S249 is located in the center of a five-nucleotide ssDNA loop (Fig. 1E). Accordingly, in vitro deamination assays confirmed S249 as a target of APOBEC deamination activity (Fig. 1E). We also performed in silico analysis of genome-wide replication fork directionality data in two cancer cell lines (Fig. 1F) [7]. We conclude that the coding strand of *FGFR3* is replicated predominantly as the lagging strand template, thereby creating an opportunity for ssDNA to form a hairpin and expose S249 to the mutagenic activity of APOBEC enzymes.

Interestingly, dominance of the *FGFR3* S249C mutation was reported in sporadic, low-grade upper-tract urothelial carcinomas (UTUCs) also enriched in APOBEC-signature mutations. By contrast, when associated with Lynch syndrome, an inherited disorder caused by germline mutations in DNA mismatch repair genes, UTUCs lack APOBEC-signature mutations and *FGFR3* S249C but have a high frequency of *FGFR3* R248C, further supporting the link between APOBEC and over-representation of *FGFR3* S249C (Fig. 2A) [8].

We also tested whether the link between APOBEC-mediated mutagenesis and the *FGFR3* S249C mutation exists in other cancers. We reviewed publicly available data (Supplementary Table 1) and catalogued *FGFR3* mutations in some other cancer types, including head and neck cancer (HNSCC), cervical cancer (CESC) and non-small-cell lung cancer (NSCLC) (Fig. 2B–D), in which enrichment of APOBEC-signature mutations has been reported [3]. The *FGFR3* S249C mutation was enriched in all these conditions (Fig. 2B–D). Because the *APOBEC3* genes are interferon-stimulated [9], it is possible that in virally induced cancers, such as HNSCC and CESC, and in BLCA, which may also have infectious etiology, *FGFR3* S249C is generated as a result of APOBEC3 induction in the course of the immune response.

FGFR3 mutations are also detected in benign skin tumors (nevus and seborrheic keratosis) and germline bone disorders (thanatophoric dysplasia). However, in these conditions that have no infectious etiology and have been linked to other causes such as aging [10], R248C (GCG → GTG) is the predominant *FGFR3* mutation (Fig. 2E and F).

We found that the FGFR3 S249C protein has similar potential for transforming NIH-3T3 cells as FGFR3 with the recurrent non-APOBEC-type mutation Y375C (TAT → TGT, 18% of BLCA; Fig. 1A), and FGFR3 with either mutation activates the same transcriptional regulators in BLCA lines, suggesting their comparable functions (Supplementary Fig. 7). Thus, the over-representation of S249C in APOBEC-related cancers is likely due to increased mutation rate caused by APOBEC3 activity rather than increased tumorigenicity of the S249C mutation.

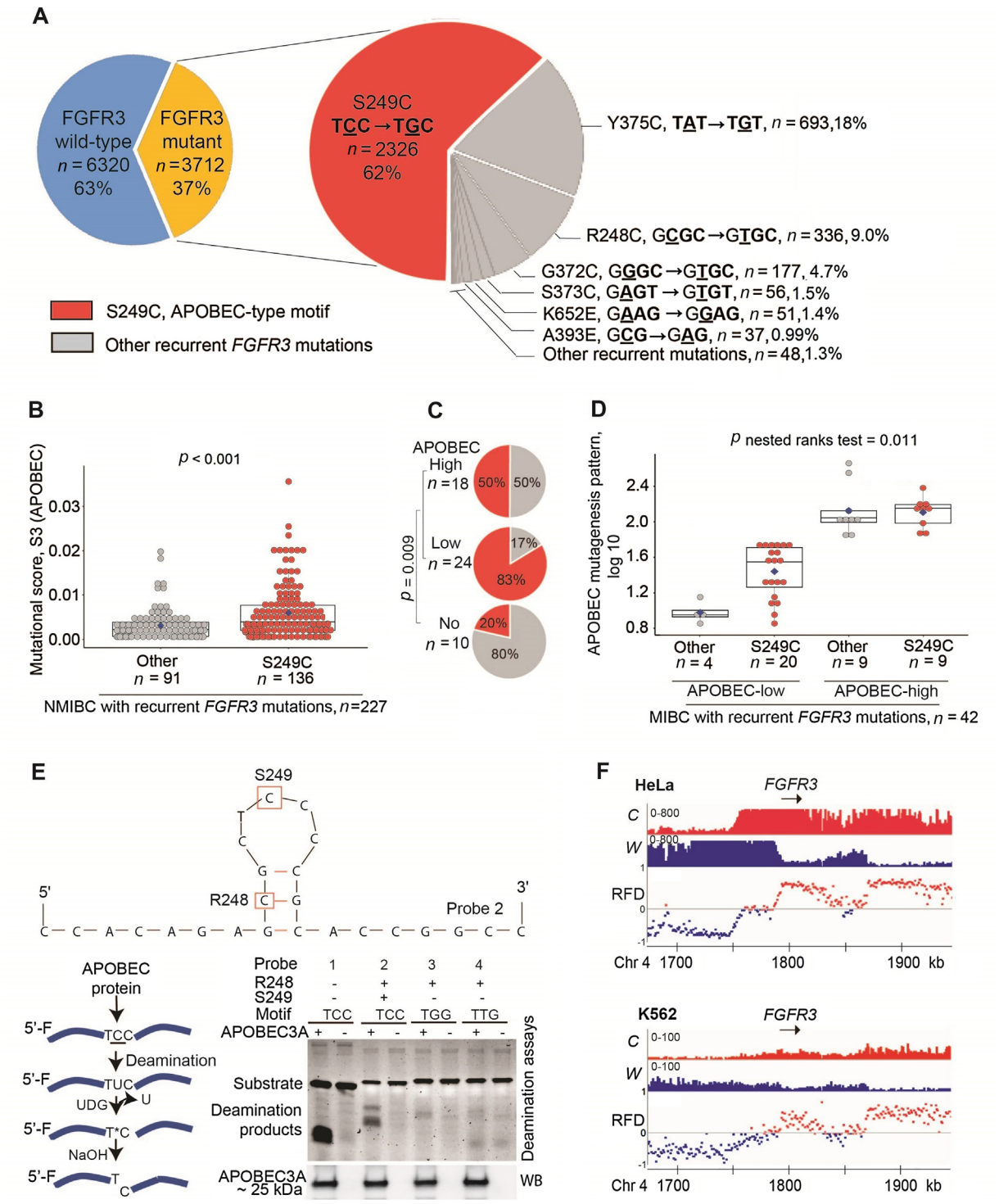

Fig. 1 – The *FGFR3* S249C mutation as a possible outcome of APOBEC-mediated mutagenesis. (A) Rates and distribution of *FGFR3* mutations among 10 032 BLCA patients. Recurrent *FGFR3* mutations observed in at least two BLCA patients are shown, with some patients carrying several *FGFR3* mutations. The mutation numbering corresponds to FGFR3 IIIb as the main isoform in cells of epithelial origin. The FGFR3 IIIb isoform contains two more amino acids than the FGFR3 IIIc isoform. The full list of recurrent *FGFR3* mutations is provided in Supplementary Table 2. The most common recurrent *FGFR3* mutation hotspot, S249 (TCC), is the only motif possibly targeted by APOBEC-mediated mutagenesis. (B) RNA-sequencing-derived APOBEC mutation score (S3) in 227 NMIBC tumors in relation to recurrent *FGFR3* mutations. *p* value: Mann-Whitney *U* test between two groups. (C) Distribution of recurrent *FGFR3* mutations in 52 TCGA MIBC tumors classified as APOBEC-high, APOBEC-low and APOBEC-no. *p* value: Fisher's exact test for APOBEC-no group versus APOBEC-presenting groups (high and low). (D) APOBEC mutagenesis pattern (log_{10}) in 42 The Cancer Genome Atlas MIBC tumors in relation to recurrent *FGFR3* mutations in APOBEC-high and APOBEC-low groups. Box plots show group medians and 50% of all the values; dots represent individual values and group means. *p* value: nested ranks test between all groups of samples. (E) Predicted secondary structure (Mfold) for *FGFR3* sequence, with R248 and S249 mutation hotspots marked. APOBEC deamination assays showed successful generation of DNA breaks at the cysteine positions only within probe 1 (positive control) and probe 2 in which intact S249 site is located within the single-stranded five-nucleotide loop, but not within probes 3 and 4, which lack the S249 site (negative controls). Additional information and secondary structures for all the probes are provided in Supplementary Fig. 6. (F) Replication fork directionality (RFD) profiles around *FGFR3* gene in HeLa and K562 cell lines determined on

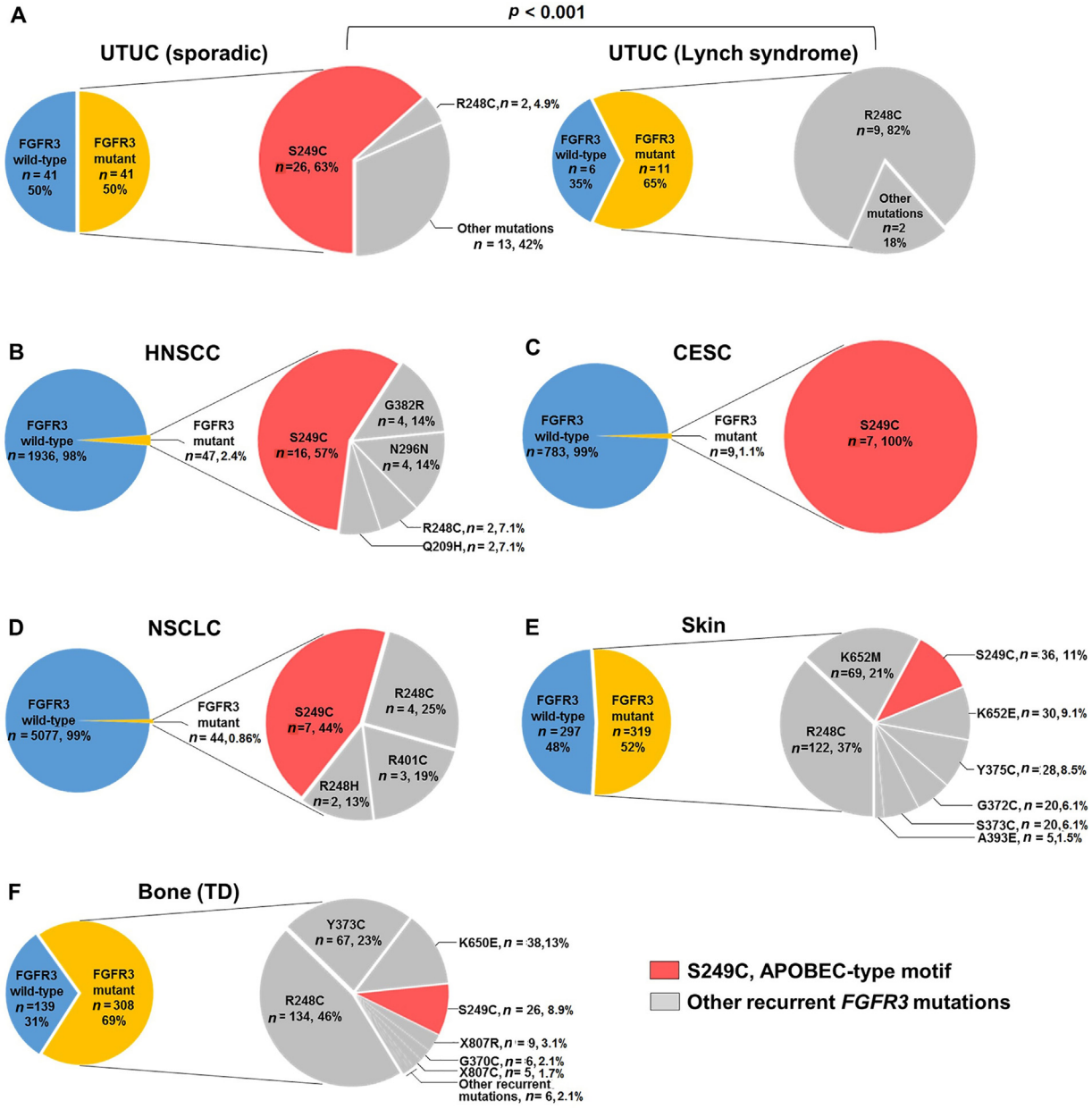

Fig. 2 – *FGFR3* mutation spectrum across several cancer types, benign skin tumors, and bone disorders. (A–F) The rates and distribution of *FGFR3* mutations among patients with sporadic (*n* = 82) and Lynch syndrome-associated (*n* = 17) UTUC, HNSCC (*n* = 1983), CESC (*n* = 792), NSCLC (*n* = 5121), benign skin tumors (*n* = 616), and bone disorders (thanatophoric dysplasia; *n* = 447). Among all recurrent *FGFR3* mutations, only the *FGFR3* S249C mutation motif (TCC) is the possible target of APOBEC-mediated mutagenesis. (A–E) The mutation numbering corresponds to *FGFR3* IIIb as the main isoform in cells of epithelial origin. (F) The mutation numbering corresponds to *FGFR3* IIIc as the main isoform in chondrocytes. The full list of recurrent *FGFR3* mutations with numbering corresponding to both FGFR3 IIIb and IIIc isoforms is provided in Supplementary Table 2. UTUC = upper-tract urothelial carcinoma, HNSCC = head and neck squamous cell carcinoma; CESC = cervical squamous cell carcinoma and endocervical adenocarcinoma; NSCLC = non-small-cell lung cancer; benign skin tumors include seborrhoeic keratosis and epidermal nevus; bone disorders include thanatophoric dysplasia-I (TD-I) and II (TD-II). *FGFR3* mutations found in cancers and benign skin tumors are somatic, those found in bone disorders are germline but identical to somatic mutations in tumors.

In conclusion, we have demonstrated that the *FGFR3* S249C mutation, despite being a less frequent APOBEC motif, is probably caused by APOBEC-mediated mutagenic activity in BLCA and other conditions. Further investigations should explore whether APOBEC mutagenesis alone generates *FGFR3* S249C or other factors are required. Our results pave the way for further studies to explore other APOBEC-induced driver mutations considering broader definition of motifs targeted by the APOBECs.

the basis of mapping of Okazaki fragments to *C* (Crick) and *W* (Watson) DNA strands. Red (blue) RFD profiles mark regions where the Watson (Crick) strands are replicated majority as lagging strand templates. Arrows indicate the position of the *FGFR3* gene (GRCh37_Chr 4: 1795–1811 kb), which is predominantly replicated from the lagging strand template in both cell types. BLCA = bladder cancer; NMIBC = non-muscle-invasive bladder cancer; MIBC = muscle-invasive bladder cancer.

Author contributions: François Radvanyi had full access to all the data in the study and takes responsibility for the integrity of the data and the accuracy of the data analysis.

Study concept and design: Shi, Meng, Dyrskjøt, Prokunina-Olsson, Bernard-Pierrot, Radvanyi.
Acquisition of data: Shi, Chen, Banday, Lamy, Moreno-Vega, Bernard-Pierrot.
Analysis and interpretation of data: Shi, Meng, Banday, Lamy, Yang, Moreno-Vega, Chen, Dyrskjøt, Bernard-Pierrot,Prokunina-Olsson, Radvanyi.
Drafting of the manuscript: Shi, Meng, Prokunina-Olsson, Bernard-Pierrot, Radvanyi.
Critical revision of the manuscript for important intellectual content: Lamy, Chen, Dyrskjøt.
Statistical analysis: Meng, Yang, Banday, Moreno-Vega.
Obtaining funding: Bernard-Pierrot, Radvanyi.
Administrative, technical, or material support: None.
Supervision: Dyrskjøt, Prokunina-Olsson, Bernard-Pierrot, Radvanyi.
Other: None.

Financial disclosures: François Radvanyi certifies that all conflicts of interest, including specific financial interests and relationships and affiliations relevant to the subject matter or materials discussed in the manuscript (eg, employment/affiliation, grants or funding, consultancies, honoraria, stock ownership or options, expert testimony, royalties, or patents filed, received, or pending), are the following: None.

Funding/Support and role of the sponsor: This work was supported by a grant from Ligue Nationale Contre le Cancer (Isabelle Bernard-Pierrot, François Radvanyi, Ming-Jun Shi, Xiang-Yu Meng, Aura Moreno-Vega) as an associated team (Equipe labellisée). Ming-Jun Shi was supported by a scholarship from the China Scholarship Council. Xiang-Yu Meng was supported by a fellowship from AVIESAN ITMO-cancer INSERM. A. Rouf Banday and Ludmila Prokunina-Olsson were supported by the Intramural Research Program of the Division of Cancer Epidemiology and Genetics, National Cancer Institute, USA. Philippe Lamy and Lars Dyrskjøt were supported by Aarhus University, Denmark. The sponsors played no direct role in the study.

Acknowledgments: We would like to acknowledge several colleagues: Jing Liu for improving the R script; Yanish Soorojebally, Florent Dufour, and Elodie Chapeaublanc for data and figure preparation; and Virginia Sanchez-Quiles for manuscript revision. We have used data partly generated by the TCGA Research Network (http://cancergenome.nih.gov/). We acknowledge all members of TCGA working group.

Appendix A. Supplementary data

Supplementary data associated with this article can be found, in the online version, at https://doi.org/10.1016/j.eururo.2019.03.032.

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