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Novel potential oncogenic and druggable mutations of FGFRs recur in the kinase domain across cancer types

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ABSTRACT

Fibroblast growth factor receptors (FGFRs) are recurrently altered by single nucleotide variants (SNVs) in many human cancers. The prevalence of SNVs in FGFRs depends on the cancer type. In some tumors, such as the urothelial carcinoma, mutations of FGFRs occur at very high frequency (up to 60%). Many characterized mutations occur in the extracellular or transmembrane domains, while fewer known mutations are found in the kinase domain. In this study, we performed a bioinformatics analysis to identify novel putative cancer driver or therapeutically actionable mutations of the kinase domain of FGFRs. To pinpoint those mutations that may be clinically relevant, we exploited the recurrence of alterations on analogous amino acid residues within the kinase domain (PK_Tyr_Ser-Thr) of different kinases as a predictor of functional impact. By exploiting MutationAligner and LowMACA bioinformatics resources, we highlighted novel uncharacterized mutations of FGFRs which recur in other protein kinases. By revealing unanticipated correspondence with known variants, we were able to infer their functional effects, as alterations clustering on similar residues in analogous proteins have a high probability to elicit similar effects. As FGFRs represent an important class of oncogenes and drug targets, our study opens the way for further studies to validate their driver and/or actionable nature and, in the long term, for a more efficacious application of precision oncology.

1. Introduction

Delivering the proper treatment at the right time to the right person is the goal of precision oncology. Genetic and molecular diagnostics could be exploited to overcome the 'one-size-fits-all' approach of standard medicine and move towards more personalized treatments. However, the 'one-size-fits-one' strategy is far from being economically and practically applicable in routine clinical practice. This is mainly due to the high costs of personalized anti-cancer drugs and to the time-consuming procedures required to characterize the genetic changes of each patient's cancer that could become potential drug targets. Instead, finding patient-specific genetic alterations that recur in a wide number of patients is the challenge of a really applicable precision cancer medicine. Among others, the analysis of cancer-associated mutations at the protein domain (PD)-level could help to this aim [1–3]. PDs are conserved functional units of proteins exploited for protein classification. For example, the database Pfam classifies proteins into protein families based on the presence of PDs in their sequence [4]. Cancer genetic alterations often accumulate in PDs [5]. The analysis of cancer somatic mutations at the PD-level enables the identification of those mutations that cluster on analogous residues across proteins containing a given domain (called mutation hotspots). By doing so, it is possible to reveal correspondence between well-known and unknown mutations and to transfer information about functional consequences and druggability from characterized variants to uncharacterized ones [2]. Also, PD-based methods highlight rare mutations, which may hide potential patient-specific drug targets [3]. In the long term, we think that such an approach will increase the number of candidate patients for a given drug, virtually avoiding the time-consuming characterization of all single patient-specific variants [6]. We recently applied a PD-based strategy to the analysis of VEGFR2/KDR mutations that could play a causative role in cancer and found two novel activating mutations of KDR. Similar to the corresponding mutation in B-Raf, the expression of the activating mutation of KDR may confer to melanoma cells an

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increased sensitivity to kinase inhibitors. Again, similarly to the B-Raf mutation [7], KDR substitution increases the dependence on glutamine over glucose for proliferation in melanoma cells. This novel metabolic vulnerability could become a novel therapeutic target [8,9]. Our previous results confirm the power of mutation analyses at the PD-level.

The family of fibroblast growth factor receptors (FGFRs) consists of 4 transmembrane receptor tyrosine kinases (RTKs), FGFR1-4. FGFRs contain 3 extracellular immunoglobulin (Ig)-like binding domains, a transmembrane domain and an intracellular tyrosine kinase domain. FGFRs activate intracellular signaling pathways that promote cell survival, proliferation and differentiation [10]. FGFRs represent key oncogenes and an important class of drug targets in various types of cancer [11–13]. FGFRs are aberrantly activated in 5–10% of all human cancers *via* single nucleotide variants (SNVs), gene amplifications or gene fusions and the tyrosine kinase inhibitors (TKi) [14] Erdafitinib and Pemigatinib are approved to target mutated FGFRs in urothelial carcinoma and cholangiocarcinoma, respectively [14]. Despite this, the landscape of FGFRs' mutations in cancer that may be therapeutically targeted is far from being fully understood, while it is of particular interest, given the evidence of therapeutic efficacy of FGFR inhibitors in patients with susceptible alterations. A recent study analyzed the gene alterations of FGFR1-3 in more than 250,000 patients revealing numerous novel alterations. In particular, it highlighted 270 SNVs [15] that may hide potential drug targets. Establishing the role of each of these mutations in tumorigenesis and drug response of each of these FGFR variants has the potential to facilitate precision medicine. However, it would require time and tremendous economical efforts. Also, such in-depth characterization may end up in useless information, as many of them might turn out as clinically irrelevant. In this context, PD-based approaches may be suitable for the analysis and prioritization of FGFRs' mutations.

To pinpoint those variants of FGFRs that may be clinically relevant, we analyzed the mutations recurring in the tyrosine kinase domains of FGFRs and many other protein kinases, including other similar RTKs. As a result, we identified novel uncharacterized mutations of FGFR1-4 which recur in other kinases. This revealed the unanticipated correspondence of these variants with known druggable mutations that could be exploited to predict their functional effects and their susceptibility to FGFR inhibitors. Our findings set the basis for a complete understanding of the mutational landscape of FGFRs in cancer. In the long term this will lead to a wider and more feasible application of precision oncology.

2. Materials and methods

2.1. MutationAligner

To identify mutation hotspots of the PK_Tyr_Ser-Thr domain and to explore the correspondence among mutations of all different proteins belonging to the PK_Tyr_Ser-Thr protein family [obtained from Pfam database (Pfam PF07714), named Pkinase_Tyr] we used MutationAligner web resource (http://mutationaligner.org/domains/Pkinase_Tyr). MutationAligner aligned the PK_Tyr_Ser-Thr domain of all proteins belonging to PF07714, it retrieved pan-cancer somatic mutations identified from TCGA variant data processed by cBioPortal up to spring 2015, and mapped them on the aligned domain calculating statistical significance as described in [16].

2.2. Low frequency mutations analysis via consensus alignment (LowMACA)

LowMACA (http://www.bioconductor.org/packages/release/bioc/html/LowMACA.html) bioinformatic tool was used to identify the amino acids recurrently mutated in the PK_Tyr_Ser-Thr domain (Pfam PF07714) of a subset of RTKs. To this, KDR, EGFR, PDGFRB, PDGFRA, FLT1,3,4, FGFR1,2,3,4, and MET were selected among all members of Pfam PF07714 and the analysis was run using default settings. LowMACA generated a multiple sequence alignment, retrieved pan-cancer somatic non-synonymous mutation data of selected RTKs from TCGA, COSMIC and cBioPortal databases and summed them all on properly aligned amino acids. A built-in statistical model assessed the statistical significance of mutation hotspots [described in [3]].

2.3. Mutagenesis

pEF_hFGFR1 plasmid encoding wild-type human FGFR1, Mo/mFR3/SV plasmid encoding murine FGFR3 [17] and LTR2HX_hFGFR4 plasmid encoding human FGFR4 [18] were used as templates to generate hFGFR1^{D647N}, mFGFR3^{D641N} and hFGFR4^{G636C} mutants. Point mutations were introduced by QuikChange Lightning Site-directed Mutagenesis Kit (Agilent Technologies) using the following primers: hFGFR1^{D647N} (FOR 5′-GGCCTCGCACGGAACATTCACCACATCG-3′, REV 5′-CGATGTGGTGAATGTTCCGTGCGAGGCC-3′); mFGFR3^{D641N} (FOR 5′-GGCCTGGCTCGAAATGTGCACAACCTGG-3′, REV 5′- CCAGGTTGTGCACATTTCGAGCCAGGCC-3′) and hFGFR4^{G636C} (FOR 5′-GGGCTGGCCCGCTGCGTCCACCACATTGAC-3′, REV 5′- GTCAATGTGGTGGACGCAGCGGGCCAGCCC-3′).

2.4. Cell cultures, transfection and Western blot analyses

Chinese hamster ovary (CHO) cells were grown in Ham's F-12 medium supplemented with 10% fetal calf serum (FCS) and penicillin/streptomycin. CHO cells were transiently transfected with wild-type or mutated FGFR-harboring plasmids using polyethylenimine (PEI 25K™; PolySciences). Total lysates of transfected CHO cells were separated by SDS-PAGE and probed with anti-phospho-FGF Receptor (Tyr 653/654) (Cell Signaling Technology), anti-FGFR1 (Cell Signaling Technology), anti-FGFR3 (Santa Cruz Biotechnology) and anti-FGFR4 (Santa Cruz Biotechnology) antibodies followed by HRP-conjugated secondary antibodies. Chemiluminescent signal was acquired by ChemiDoc™ Imaging System (BioRad).

3. Results and discussion

3.1. A novel mutation of FGFR1 (D647) recurs in the kinase domain of B-Raf, FLT3, KIT, KDR and PDGFRA kinases

In order to search for novel clinically relevant mutations of the kinase domain of FGFRs we re-analyzed the mutation hotspots along the aligned kinase domain of all kinases belonging to the PK_Tyr_Ser-Thr protein family (Pfam: PF07714). To this, we exploited the MutationAligner web resource which analyses non-synonymous somatic mutations in more than 5000 patients across 22 different cancer types [2,16]. MutationAligner retrieved mutation data from TCGA variant data processed by cBioPortal up to spring 2015, and detected 1807 non-synonymous mutations aggregated in 319 positions along the PK_Tyr_Ser-Thr domain (Fig. 1, Table S1). As expected, most of the mutations occurred in highly conserved residues. MutationAligner detected several positions along the PK_Tyr_Ser-Thr domain which harbor clustered mutations. Among these, position 291 of the aligned domain was found to be a statistically significant mutation hotspot, with 415 total mutations. At this position 382 mutations of B-Raf oncogene (V600E/K/R), 14 mutations of FLT3 (D835E/Y/H), 5 mutations of KIT (D816V/Y), 4 mutations of EGFR (L861Q/R), 2 mutations of KDR (D1052N/G), and 1 mutation of FGFR1 (D647N) among others, were aggregated from 10 different cancer types. The residue at this position lies within the activation loop (A-loop) of the kinases, a flexible region of the kinase that upon phosphorylation turns the kinase into its active state thus regulating the enzymatic catalysis. In keeping with its crucial role, its substitution in B-Raf, FLT3, KIT and KDR was previously demonstrated to render the kinases constitutively active [19–23]. Mechanistically, the mutation V600E of B-Raf introduces a negatively charged glutamic acid residue which mimics A-loop phosphorylation,

causing B-Raf to adopt an active configuration [24]. Also, it has been proposed that the V600E mutation destabilizes the inactive state, while stabilizing the active state through the formation of a salt bridge between E600 and K507 [25]. Residue D835 of FLT3 is critical in maintaining the inactive state of the receptor. In wild-type FLT3, D835 may stabilize the autoinhibited state by forming interactions with the α-helix, including S838 residue [26]. Mutations at this position lead to increased phosphorylation probably through the destabilization of the auto-inhibited conformation [20,27]. A similar mechanism explains the activating nature of the equivalent mutations (D816V/H) in c-KIT receptor [28]. In mutant c-KIT^{D816V} the side chain of arginine 815 is flipped from its position in the auto-inhibited conformation. Also, D816 stabilizes the small positively charge α-helical dipole through its negative charge. This leads to constitutive phosphotransferase activity [21,29,30]. On these bases, we anticipate that the mutation D647N of FGFR may similarly destabilize the inactive conformation of the A-loop, leading to constitutive phosphorylation of the receptor. These considerations gain great importance as regards the sensitivity to TKi. Inhibitors of tyrosine kinases are classified into 4 classes, including covalent inhibitors and non-covalent type I, II and III drugs (also known as 'allosteric inhibitors' as they bind out of the active site). Type I inhibitors bind the so call 'DFG-in' active state of kinases where the tripeptide DFG (residues 639-641 in FGFR1) is buried in a hydrophobic pocket in the groove between the two lobes of the kinase. Instead, type II drugs bind the so called 'DFG-out' inactive state, locking kinases in the inactive conformation [31]. On these bases, the stabilization of the active (DFG-in) state by mutation, as for mutations at position 291 of the PK_Tyr_Ser-Thr domain, maintains (or increases) the sensitivity to type I TKi, while leading to the unfavorable binding of type II inhibitors. Accordingly, B-Raf (V600E) exhibits an increased sensitivity to type I vemurafenib (PLX4032). Similarly, preclinical evidence showed that the EGFR (L861Q) mutation increases the sensitivity to type I gefitinib and erlotinib [22,32], while a retrospective study reported a moderate clinical response to TKIs [33]. On the other hand, cells expressing KIT (D816V) are resistant to type II imatinib [34]. Our findings revealed unanticipated correspondence among these mutations, and strengthened the hypothesis that, in addition to functional effects, also the therapeutic actionability of mutations clustering at similar positions within PDs may be similar. By highlighting the correspondence between the uncharacterized mutation of FGFR1 at residue D647 and all those notorious druggable variants, our data anticipate that mutated FGFR1 (D647N) may display increased sensitivity to type I inhibitors while showing little sensitivity to type II TKi.

3.2. *Novel mutations of FGFRs recurring in the kinase domain of a subset of RTKs*

We next restricted our analysis to a subset of PK_Tyr_Ser-Thr domain-containing RTKs, that share closer evolutionary and functional relationships with FGFRs. To this, we analyzed the pan-cancer somatic non-synonymous mutations of FGFR1,2,3,4, KDR, EGFR, PDGFRB, PDGFRA, FLT1,3,4, and MET receptors *via* Low frequency Mutations Analysis *via* Consensus Alignment (LowMACA) software [3]. First, LowMACA aligned the kinase domains of the RTKs under investigation (Fig. 2). Then, by retrieving mutational data from cBioPortal, it aggregated all mutations on the consensus sequence and generated a unique mutation profile representative of all RTKs (Fig. 3A). Fifteen significant (p-value <0.05) mutation hotspots were detected along the aligned domain (Fig. 3B, orange columns). P-values were then corrected to obtain q-values by using Benjamini-Hochberg procedure for multiple testing correction. Only 6 mutation hotspots had a q-value <0.05 and thus were considered statistically significant (Fig. 3B, red asterisks). Of note, significant mutation hotspots occurred in highly conserved regions (Fig. 3B–D). The Protter style plot shows the position of mutation

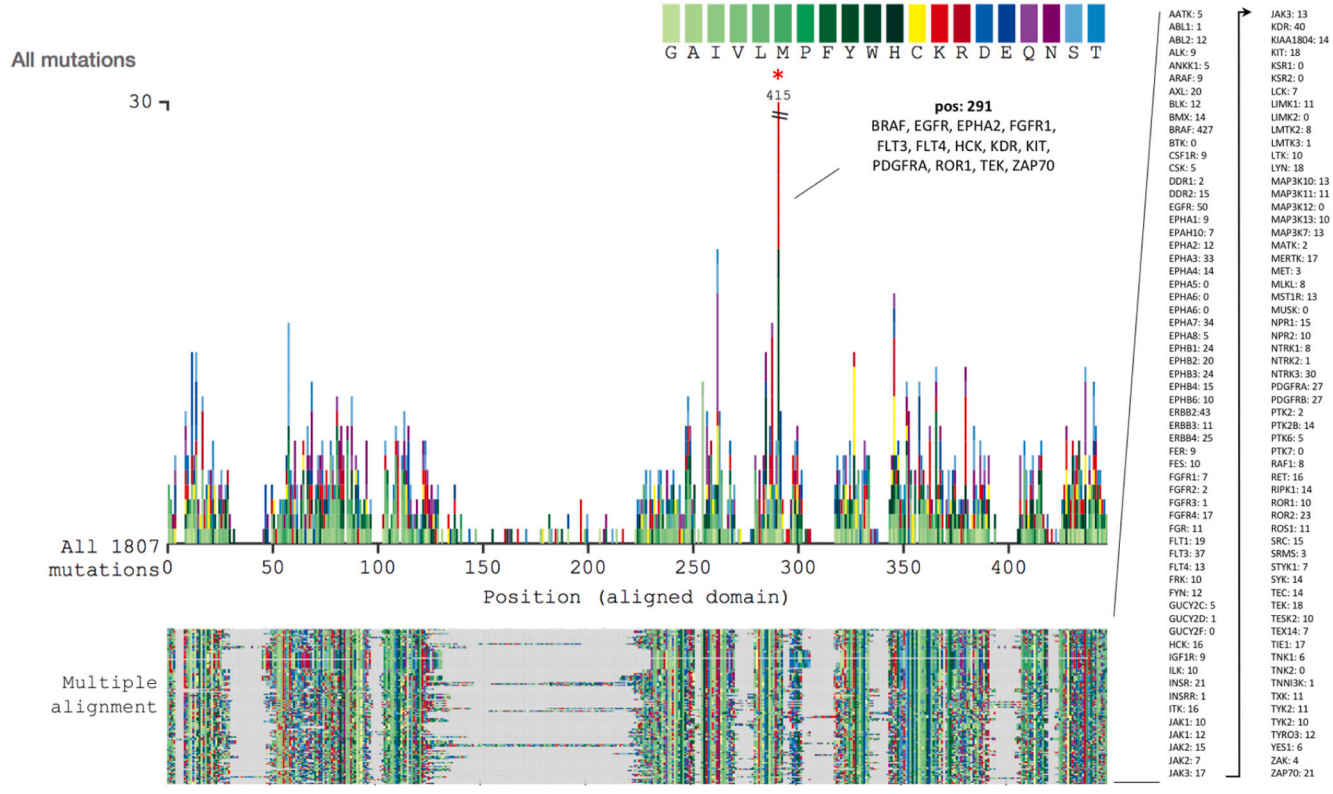

Fig. 1. Mutational profile of the PK_Tyr_Ser-Thr domain analyzed by MutationAligner. A multiple alignment of the PK_Tyr_Ser-Thr domain of all proteins belonging to PF07714 was generated. Mutations were retrieved from TCGA variant data processed by cBioPortal up to spring 2015 and mapped along the aligned domain. A per-position aggregated view of mutated residues (color coded) is provided. The list of all aligned proteins is shown on the right. The only statistically significant mutation hotspot (position 291) is highlighted and all proteins harboring mutations in that position are listed (see Table S1 for the complete mutation list).

Fig. 2. Multiple sequence alignment of the PK_Tyr_Ser-Thr domain of RTKs. The PK_Tyr_Ser-Thr domain of FGFR1,2,3,4, KDR, EGFR, PDGFRB, PDGFRA, FLT1,3,4 and MET proteins was aligned to generate the consensus sequence. Start and end residues are indicated next to each receptor name. Residues with 100% similarity are highlighted in bold.

hotspots along the consensus sequence and their relative p- and q-values (Fig. 4). Despite MutationAligner and LowMACA analyze non-homogenous data and therefore are not comparable, the more numerous significant hotspots found by LowMACA may depend on the higher sequence conservation among RTKs. Indeed, the level of sequence conservation among the proteins under analysis is one of the parameters considered for calculating the statistical significance of mutation hotspots in PD-based mutational analyses. Also, the absence of frequently mutated genes, such as B-Raf, may have enabled other hotspots to reach statistical significance.

The LowMACA approach converged with MutationAligner to identify the position 256 (corresponding to position 291 in MutationAligner numbering) as a statistically significant mutation hotspot (Fig. 3, Table S2). At this position, LowMACA aggregated 33 mutations of 8 different RTKs from 6 cancer types. LowMACA confirmed the correspondence among FGFR1 (D647N), KDR (D1052N), FLT3 (D835E/Y/H), PDGFRA (D842H) and other mutations (Fig. 3 and Table S2). Remarkably, LowMACA detected 2 additional mutations at this position in FGFR2 (D650H) and MET (D1228H) that were not detected by MutationAligner. Again, FGFR2 (D650H) mutation has not been fully characterized so far and we speculate it may activate the kinase similarly to the substitution D647N of FGFR1 and other known corresponding mutations (see above). Experimental validation will be useful to clearly assess the role of such FGFR2 variant as the substitution of D650 with hydrophobic residues (alanine, leucine, valine and isoleucine) increases the kinase activity, while the D650G substitution decreases the enzymatic activity of FGFR2 [35]. Of note, T. Futami *et al* [36] suggested that FGFR1 (D647N) and FGFR2 (D650H) mutations correspond to the activating and oncogenic G636C mutation of FGFR4 found in gastric cancer and to the activating D641N mutation of FGFR3 [37]. The authors also showed that mutated FGFR4 (G636C) is sensitive to the FGFR selective TKi ASP5878 [36]. This data anticipates that also the uncharacterized FGFR1 (D647N) and FGFR2 (D650H) mutations may be sensitive to ASP5878.

At the same hotspot, LowMACA found the mutation D1228H of MET. This variant is associated with resistance to type I TKi Savolitinib in gastric cancer and Crizotinib in lung cancer [38,39]. This unexpected TKi sensitivity may be related to the peculiar role of D1228 in MET receptor. Indeed, D1228 participates to maintain the residue Y1230 of the A-loop in a conformation accessible to MET inhibitors [40,41]. Even though exceptions can not be excluded, available data prompt to hypothesize a common pattern of sensitivity/resistance to TKi of all analogous mutations at position 256 (291).

LowMACA found 3 additional significant mutation hotspots that aggregated mutations found in FGFRs. These included positions 69, 267 and 306 of the aligned domain (Fig. 3 and Table S2). Position 69

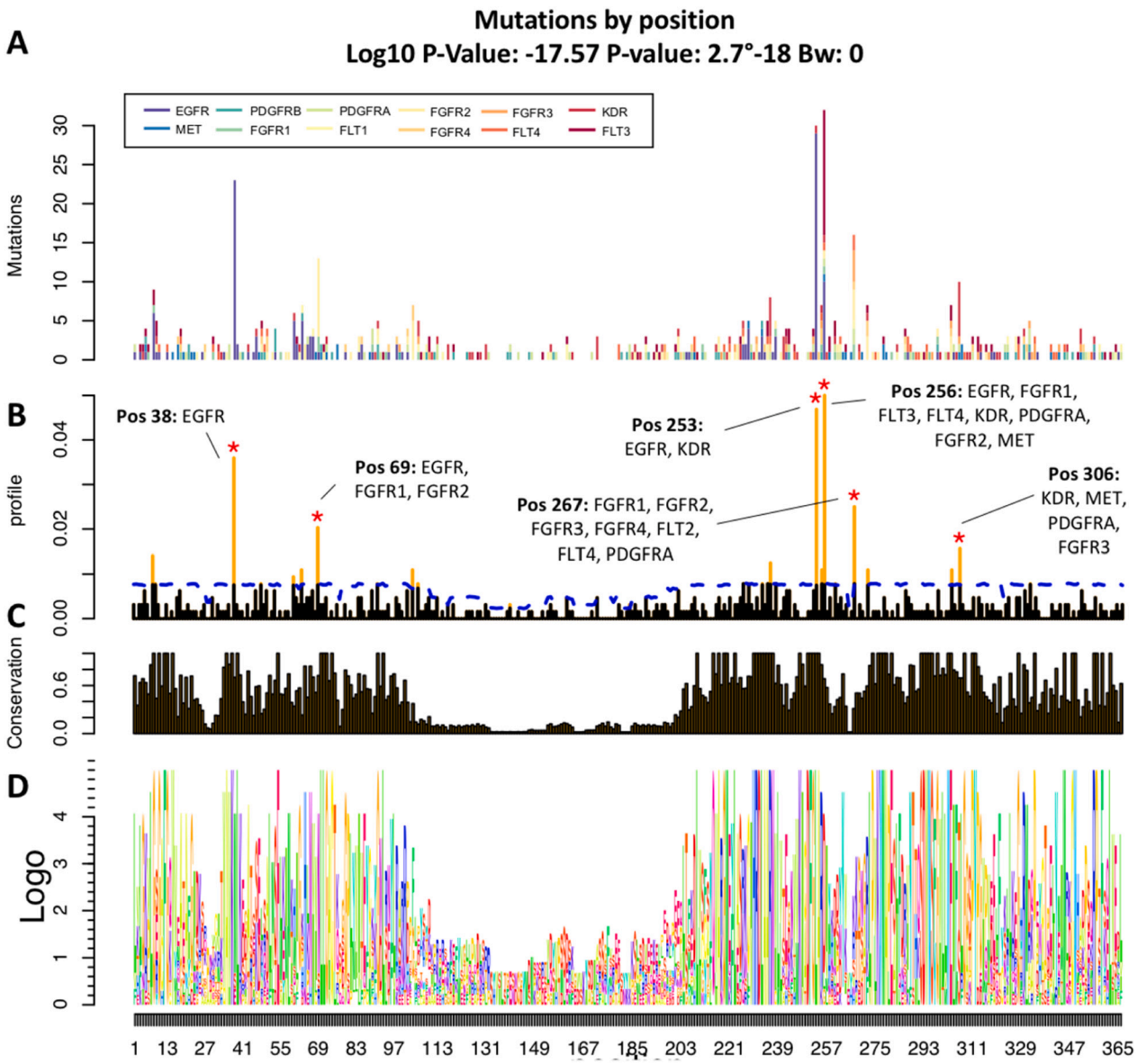

Fig. 3. Mutational profile of the PK_Tyr_Ser-Thr domain of RTKs analyzed by LowMACA. A-B, Mutations detected by LowMACA in the PK_Tyr_Ser-Thr domain of FGFR1,2,3,4, KDR, EGFR, PDGFRB, PDGFRA, FLT1,3,4 and MET receptors were mapped along the consensus sequence (see Fig. 2). A per position aggregated view of all mutations of the different receptors (color coded) is provided in (A). Statistical analyses identified 15 hotspots with a *p*-value <0.05 (orange columns) and 6 significant hotspots (q-value<0.05; indicated by red asterisks). The blue dashed line indicates the statistical significance threshold. For each significant hotspot, the receptors harboring mutations of that specific position are listed (B). C–D, sequence conservation across the different RTKs is shown as conservation score (C) and a sequence logo plot (D). Please see Table S2 for the complete mutation list.

aggregated 10 mutations of FGFR2 (N549K/H) and 2 mutations of FGFR1 (N546K/D). Position 69 lies within the αC-β4 Loop which acts as a molecular brake inhibiting kinase auto-phosphorylation. Mutations in this region have been shown to disengage the brake, relaxing the kinase towards its active state [42,43]. Accordingly, all the mutations clustering at this position in LowMACA analysis are already known to activate the RTKs. The mutation N549K of FGFR2 found in endometrial cancer activates the receptor [44]. Similarly, FGFR1 (N546K), found in glioblastoma, was postulated to entail the up-regulation of kinase activity from *in silico* studies [45]. Previous studies had already highlighted the correspondence among these mutations and the N540K substitutions of FGFR3 [42,46], which may share a similar mechanism of action. Similarly, the R776H mutation of EGFR, found in lung cancer of non-smoking patients, activates EGFR in the absence of EGF ligand [46,47]. Also in the case of EGFR, mutation of R776 residue leads to the loss of the auto-inhibitory mechanism. Moreover, R776 take contacts with the regulatory C-terminal tail of EGFR which are loss in R776H mutant [47]. As concerns the sensitivity to targeted TKi, the mutation N540K of FGFR3 strongly reduces the sensitivity to FGFR inhibition in cholangiocarcinoma [48]. On the contrary, the EGFR (R776H) variant increases the sensitivity of EGFR to gefitinib *in vitro* [47]. Again, further investigation is necessary to establish the sensitivity to specific TKi of mutations aggregated at this hotspot within the kinase domain.

The mutation hotspot at position 267 aggregated 4 mutations of FGFR2 (K659/N/E) and FGFR3 (K650N/E/T), 2 mutations of FGFR1 (K656E) and 1 mutation of FGFR4 (K645E). Remarkably, FGFR1 (K656E) [49], FGFR2 (K659/N/E), FGFR3 (K650N/E/T) [50,51] and FGFR4 (K645E) [52,53] mutations increase the ligand-independent activity of FGFRs and cause cancer and skeletal disorders [42,50]. Despite this, their therapeutic actionability is unknown. However, the FGFR3 mutation K650N (also known as K652N) was considered an inclusion criteria for phase 1 trial of the FGFR selective TKi ASP5878 in patients

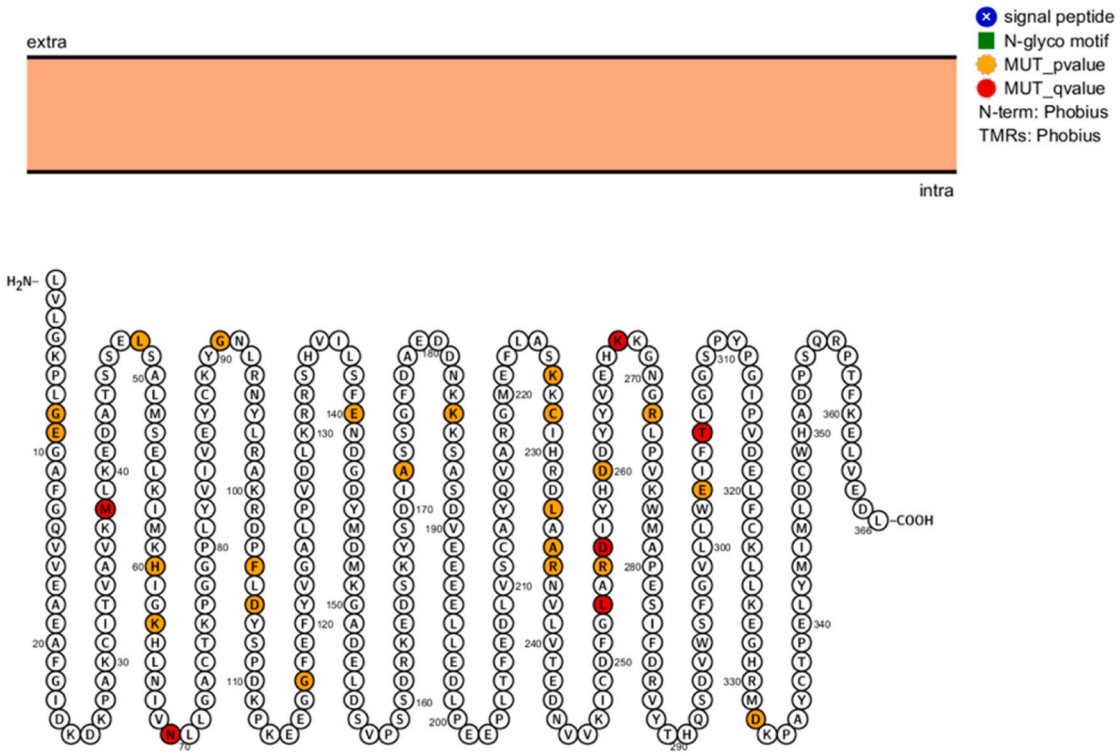

Fig. 4. Protter Style plot. The figure shows a graphical view of the consensus sequence (residues 1–366) of the aligned PK_Tyr_Ser-Thr domains as provided by LowMACA tool. One-letter code is used to indicate amino acid residues. Given the intracellular localization, the domain is depicted below the lipid bi-layer (shown in salmon color) representative of the cell membrane. Significantly mutated positions are highlighted in orange (p-value<0.05) or red (q-value<0.05).

Table 1
Summary of FGFR mutations and relative correspondences detected in this study. Positions along the PK_Tyr_Ser-Thr domain are numbered according to LowMACA numbering.

Position	FGFR1	FGFR2	FGFR3	FGFR4
69	N546K/D	N549K/H	–	–
256	D647N	D650H	–	–
267	K656E	K659N/E	K650N/E/T	K645E
306	–	–	P689S	–

with urothelial carcinoma (Trial ID: NCT01013649). We believe that, together, these variants may become common therapeutic targets across different tumor types.

LowMACA also detected 10 clustered mutations at position 306 of the aligned domain, corresponding to mutations of FGFR3 (P689S), KDR (S1100F), MET (T1278A) and PDGFRA (S890F). The newly identified mutation of FGFR3 (P689S) deserves further attention as the corresponding substitution S1100F of KDR has been shown to be oncogenic in a mouse model although it hampers receptor phosphorylation [54]. To our knowledge, the S1100F substitution of KDR is the only partially characterized variant of this group and no data are available concerning the sensitivity to TKi. Being this residue out of the ATP binding site, we expect that mutations of this position may lack of effects (or exert allosteric effects) on the binding of type I-II TKi.

3.3. The newly identified mutation D647N of FGFR1 increases receptor phosphorylation

To validate the role of mutations clustering at position 256 of the PK_Tyr_Ser-Thr domain as identified by MutationAligner and LowMACA tools, FGFR1,3,4 were mutagenized. Wild type FGFR1,3,4 receptors and the relative mutated $FGFR1^{D647N}$, $FGFR3^{D641N}$ and $FGFR4^{G636C}$ were expressed in CHO cells and the levels of receptor phosphorylation were measured by Western blot. As anticipated by the *in silico* predictions, all 3 FGFR mutants display significantly higher receptor phosphorylation than their wild-type counterparts in the absence of specific ligands (Fig. 5). Consistent with previous data on FGFR3 and FGFR4 mutations [36,37], the newly identified mutation D647N of FGFR1 strongly increases receptor activity, suggesting its potential oncogenic role. These results indicate that we have identified a position within the kinase domain of FGFRs which is of crucial importance for receptor activity and whose mutation may generate constitutively active receptors. Moreover, our findings confirm the previously demonstrated activating nature of analogous mutations on B-Raf, FLT3, KIT and KDR genes [19–23], strengthening the hypothesis that corresponding mutations within protein domains do elicit similar effects.

4. Conclusions

Scattered studies have exploited the correspondence among analogous mutations in different FGFRs to infer the functional consequences of novel uncharacterized variants [42,46]. However, a systematic assessment of the complete landscape of all FGFR mutations in cancer, of their reciprocal correspondence and of the correspondence with mutations of other kinases has not been pursued to date. Such systematic analysis would increase the number of putative driver and/or actionable mutations of FGFRs with incredible benefits for clinical precision oncology. PD-based strategies have the great advantage of pinpointing *in silico* those variants that have a high probability of modifying protein function and being clinically relevant and of highlighting rare variants [6]. These analyses also allow transferring information from known mutations to uncharacterized variants. This is true for the functional effects of mutations. In addition, we speculate that also the therapeutic actionability could be transferred from one mutation to the corresponding ones on different proteins. In this manner, 'common' therapeutic targets can be identified and would allow increasing the number of patient candidates for a given drug. Such a strategy is already applied

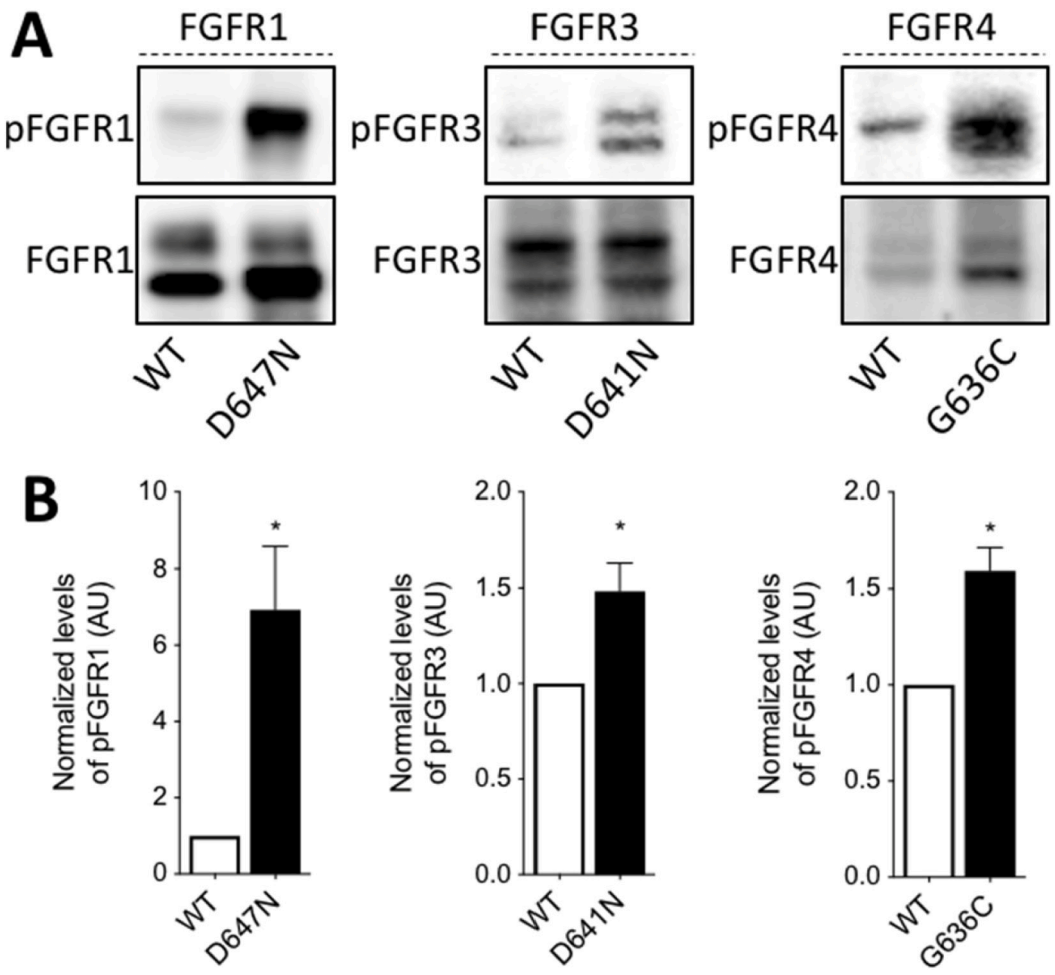

Fig. 5. A, Western blot analysis of phospho-FGFR1,3,4 in total lysates of CHO cells expressing the indicated wild-type or mutated FGFRs. Lysates were analyzed for total FGFR1,3,4 levels as loading control. B, Western blot densitometry of three independent experiments.

to treat adults and pediatric patients with various cancers harboring rare oncogenic fusions of *NTRK1*, *NTRK2* or *NTRK3*. FDA approved the use of a TRK inhibitor for all these "heterogeneous" and rare cases [55].

In the present study, we re-analyzed the mutational data to highlight novel cancer-associated FGFR mutations that have been disregarded (because they are rare events) despite they have the potential of being driver variants. To this, we focused only on the mutations found in the kinase domain of FGFRs and exploited the correspondence with analogous variants in other proteins containing the same kinase domain. By combining the results of 2 different PD-based bioinformatics resources, we pinpointed various novel FGFR mutations (summarized in Table 1) whose mechanism of action is completely (or partially, in some cases) unknown. We inferred their functional impact by considering the correspondence with other well-known variants. In some cases we also predicted their response to tyrosine kinase inhibitors. Further theoretical modelling could help the identification of putative TKi active against the identified mutations. Our *in silico* findings require experimental validation to confirm the oncogenicity of the identified mutations and their therapeutic actionability. As a proof of concept, we selected position 256 of the PK_Tyr_Ser-Thr domain and tested the effects of the corresponding mutations of FGFR1,3,4. *In vitro* experiments demonstrated that all the selected mutations elicit similar effects, providing experimental evidence that mutations clustering on a given hotspot within a PD modify the protein function in a similar manner. This study characterizes for the first time the effects of variants D647N of FGFR1 showing that it increases receptor phosphorylation. Our results also validated the previously known activating nature of analogous mutations D641N of FGFR3 and G636C of FGFR4. These findings strongly suggest that all these variants may be oncogenic and deserve to be investigated further. Overall, our study exploited both *in silico* and *in vitro* analyses to anticipate the role of various uncharacterized FGFR mutations setting the bases for a better understanding of the landscape of FGFR genetic alterations in cancer progression and response to therapeutics.

We found that the mutations clustered on analogous residues display a similar pattern of sensitivity/resistance to TKi. On these bases, we imagine identifying a 'common' inhibitor active against all analogous mutations of a given hotspot on different FGFRs/proteins. This approach will overcome the problematically low 'kinase 1 *vs* kinase 2' specificity typical of TKi (see https://www.discoverx.com/services/drug-discovery-development-services/kinase-profiling/interaction-maps) [31]. Instead it aims at identifying inhibitors that selectively block the activity of mutated kinases (all analogous ones simultaneously) while exerting negligible effects on wild-type enzymes. This 'mutated *vs* wild-type' selectivity has been already achieved for other TKi (*e.g.* vemurafenib [56]). Moreover, our analyses strengthen the hypothesis that therapeutic actionability can be transferred from known mutations to corresponding uncharacterized ones. Once this concept is experimentally corroborated, we speculate that *in silico* identification of a novel mutation corresponding to known variants will no longer require experimental validation before proceeding with patient treatment. In the long term, this will accelerate drug selection and will increase the number of patients candidate for a given drug leading to a wider and more feasible application of precision oncology.

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CRediT authorship contribution statement

Conceptualization, Software, Formal analysis and Data curation: E.G., L.Z., S.M. Investigation: E.G., L.Z, C.G., M.C., C.R. Writing, Reviewing and editing original draft: E.G., S.M. Project administration: S.M.

All authors have approved the revised version of the manuscript.

Declaration of competing interest

Dr. Luca Zammataro has collaborated on this work before his employment at Kiromic Biopharma on Jun 15, 2021. Therefore there are no conflicts of interest to discloseAll other authors declare no Conflict of Interest.

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