

Novel *FGFR1* mutations in Kallmann syndrome and normosmic idiopathic hypogonadotropic hypogonadism: evidence for the involvement of an alternatively spliced isoform

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Objective: To determine the prevalence of fibroblast growth factor receptor 1 (*FGFR1*) mutations and their predicted functional consequences in patients with idiopathic hypogonadotropic hypogonadism (IHH).

Design: Cross-sectional study.

Setting: Multicentric.

Patient(s): Fifty unrelated patients with IHH (21 with Kallmann syndrome and 29 with normosmic IHH).

Intervention(s): None.

Main Outcome Measure(s): Patients were screened for mutations in *FGFR1*. The functional consequences of mutations were predicted by in silico structural and conservation analysis.

Result(s): Heterozygous *FGFR1* mutations were identified in six (12%) kindreds. These consisted of frameshift mutations (p.Pro33-Alafs*17 and p.Tyr654*) and missense mutations in the signal peptide (p.Trp4Cys), in the D1 extracellular domain (p.Ser96Cys) and in the cytoplasmic tyrosine kinase domain (p.Met719Val). A missense mutation was identified in the alternatively spliced exon 8A (p.Ala353Thr) that exclusively affects the D3 extracellular domain of *FGFR1* isoform IIIb. Structure-based and sequence-based prediction methods and the absence of these variants in 200 normal controls were all consistent with a critical role for the mutations in the activity of the receptor. Oligogenic inheritance (*FGFR1/CHD7/PROKR2*) was found in one patient.

Conclusion(s): Two *FGFR1* isoforms, IIIb and IIIc, result from alternative splicing of exons 8A and 8B, respectively. Loss-of-function of isoform IIIc is a cause of IHH, whereas isoform IIIb is thought to be redundant. Ours is the first report of normosmic IHH associated with a mutation in the alternatively spliced exon 8A and suggests that this disorder can be caused by defects in either of the two alternatively spliced *FGFR1* isoforms. (Fertil Steril® 2015;104:1261–7. ©2015 by American Society for Reproductive Medicine.)

Key Words: Hypogonadotropic hypogonadism, Kallmann syndrome, *FGFR1*, *KAL2*, genetics

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Idiopathic hypogonadotropic hypogonadism (IHH) is defined by complete or partial failure of pubertal development due to the compromised secretion of gonadotropins (FSH and LH) and sex hormones (testosterone [T] and estrogens [E]), in the absence of any hypothalamic-pituitary organic cause (1). Congenital forms of IHH include Kallmann syndrome (KS), which is characterized by gonadotropin deficiency with a defective sense of smell (anosmia or hyposmia), and IHH without olfactory defects (normosmic IHH) (2). In addition, nonreproductive phenotypes, such as midline facial defects, dental agenesis, hearing loss, renal agenesis, synkinesis, and digital bone abnormalities, are commonly observed in patients with IHH (2, 3). About one third of patients with IHH reveal a genetic defect in genes that regulate the embryonic development or migration of GnRH neurons, or the synthesis, secretion, or action of GnRH (4, 5). One of the most frequently implicated genes is the fibroblast growth factor receptor 1 (*FGFR1*, OMIM 136350) gene, which is located at chromosome 8p11.2, and comprises 18 coding exons (6, 7). At present, 219 loss-of-function *FGFR1* mutations have been associated with autosomal dominant forms of KS and normosmic IHH (Supplemental Table 1, available online).

The *FGFR1* protein is a transmembrane receptor that comprises an extracellular region of three immunoglobulin-like domains (D1, D2, and D3), a transmembrane helix, and a cytoplasmic tyrosine kinase domain (8). Alternative splicing of the carboxy-terminal half of D3, through the use of either exon 8A or 8B, generates isoforms *FGFR1-IIIb* or *FGFR1-IIIc*, respectively (9). Although these isoforms have different tissue expression and FGF-binding affinity (10), experimental data suggest that *FGFR1-IIIc* is the dominant isoform that carries out most of the biological functions of the *FGFR1* gene, whereas *IIIb* plays a minor and somewhat redundant role (11).

The aim of this study was to identify and determine the prevalence of *FGFR1* mutations in a cohort of Portuguese patients with KS and normosmic IHH, and to investigate the consequences of these mutations.

MATERIALS AND METHODS

Subjects

This cross-sectional study comprised 50 unrelated Portuguese patients with IHH (43 men and 7 women), 21 with KS and 29 with normosmic IHH, recruited by Portuguese clinical endocrine centers from 2010 to 2014. Inclusion criteria were male and female patients with IHH, with failure to enter spontaneous puberty by the age of 18 years, or with medically induced puberty at a younger age, or with documented anosmia. Patients with a history of an acquired cause of hypopituitarism were excluded from the study. Whenever possible, unaffected family members were also studied. The control population consisted of 200 Portuguese unrelated volunteers who were recruited among blood donors. Written informed consent was obtained from all subjects and the study was approved by the local research ethics committee (Faculty of Health Sciences, University of Beira Interior; CE-FCS-2012-012).

Genetic Studies

Genomic DNA was extracted from peripheral blood leukocytes using previously described methods (12). Patients were screened for mutations in *FGFR1* by polymerase chain reaction (PCR) amplification of the 18 coding exons and exon-intron boundaries, and bidirectional sequencing using a CEQ DTCS sequencing kit (Beckman Coulter) and an automated capillary DNA sequencer (GenomeLab TM GeXP, Genetic Analysis System; Beckman Coulter). Primer sequences were previously described by Sato et al. (13), except for primers for exons 14 and 15 that were described by Albuisson et al. (14). Heterozygous frameshift mutations were confirmed by cloning of the PCR products using pGEM-T Easy Vector Systems (Promega Corporation), followed by DNA sequencing of each allele. Mutations were confirmed in patients and excluded in a panel of 200 healthy volunteers (400 alleles) using sequence-specific restriction enzymes. The mutation in exon 14 did not create or eliminate any restriction enzyme recognition site, therefore a restriction site was introduced on the mutated allele using a modified PCR forward primer (5'-GACATTCACCACATCGACTATTA-3') (modified nucleotide underlined). The same occurred for the mutation in exon 2, thereby a restriction site was introduced on the wild-type allele using a modified PCR forward primer (5'-AGAACTGGGATGTGGAACTG-3') (modified nucleotide underlined). Mutation nomenclature followed standard guidelines (15) and was based on the complementary DNA (cDNA) reference sequence for the *FGFR1-IIIc* isoform (GenBank accession NM_023110.2) or the *FGFR1-IIIb* isoform (GenBank accession FJ809917) (in the case of mutation in the alternatively spliced exon 8A). Patients with identified *FGFR1* mutations were screened for digenic/oligogenic mutations by sequencing additional genes related to the hypothalamic-pituitary-gonadal axis (*KAL1*, *GNRH1*, *GNRHR*, *FGF8*, *PROK2*, *PROKR2*, *KISS1R*, *TAC3*, *TACR3*, *NELF*, and *CHD7*) (all primer sequences and PCR conditions are available upon request).

In Silico Structural and Conservation Analysis

The functional consequences of the observed missense mutations were predicted by the use of different bioinformatic tools: SIFT (16), Provean (17), PolyPhen 2.0 (18), and Mutation Taster (19). The mapping of the mutations onto the known *FGFR1* crystal structure was carried out using the PyMol Molecular Graphics System (20). The conservation analysis of mutated amino acids across species was performed using the Mutation Taster software (19). Mutations that resulted in frameshifts did not lead to any other further studies, as they could be considered pathogenic due to their highly disruptive effect on protein structure or expression.

RESULTS

FGFR1 Mutations and Associated Clinical Characteristics

Sequence analysis of the entire coding region of *FGFR1*, including exon-intron boundary regions, revealed six novel heterozygous mutations: two frameshift (c.95dupA

[p.Pro33Alafs*17] and c.1961dupA [p.Tyr654*]) and four missense mutations (c.12G>T [p.Trp4Cys], c.287C>G [p.Ser96Cys], c.1057G>A [p.Ala353Thr], and c.2155A>G [p.Met719Val]) (Fig. 1). Mutation p.Ala353Thr occurred in the alternatively spliced exon 8A, which exclusively affected the FGFR1-IIIb isoform. These variants were not found in any of the online databases, including the ExAC database (21), and were not detected in a panel of 200 normal Portuguese controls (400 alleles). The clinical characteristics of patients with identified *FGFR1* mutations are summarized in Table 1.

In Silico Analysis of *FGFR1* Missense Mutations

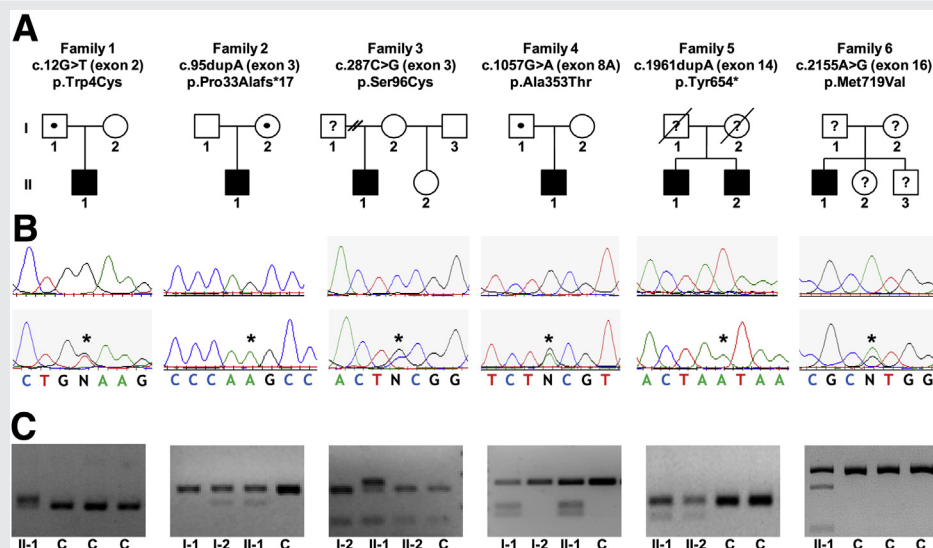
All missense variants in the *FGFR1* gene (p.Trp4Cys, p.Ser96Cys, p.Ala353Thr, and p.Met719Val) were predicted to bring about changes in protein function, with high scores for “damaging,” “deleterious,” “probably damaging,” and “disease causing,” by at least two prediction programs (Supplemental Table 2, available online). Conservation analysis revealed that the p.Trp4Cys, p.Ser96Cys, p.Ala353Thr, and p.Met719Val variants occurred at amino acids that were highly conserved across species (Table 2). The positions of the p.Ser96Cys and p.Met719Val variants were assessed on the crystal structure of the first immunoglobulin-like domain (Protein Data Bank ID: 2CR3) and of the tyrosine kinase domain (Protein Data Bank ID: 3GQI) (22), respectively, of FGFR1 (Supplemental Fig. 1, available online). Residue Ser96 is located within the hydrophobic region of the

immunoglobulin-like domain 1 (D1) playing an important function in D1 folding (Supplemental Fig. 1A). Residue M719 is located in the loop region linking the α -helices, α H and α G, thereby maintaining structural integrity of the tyrosine kinase domain (Supplemental Fig. 1B). Structural analysis of the p.Trp4Cys variant was not possible to assess as it is located within the signal peptide, which is not part of the mature protein. Structural analysis of the p.Ala353Thr variant was not shown as the crystal structure of the immunoglobulin-like domain 3 (D3) of the FGFR1-IIIb isoform is not yet available. The damaging effect of these missense mutations are expected to result from [1] impaired intracellular trafficking of the translated FGFR1 protein (p.Trp4Cys); [2] disruption of D1 folding by disturbing hydrophobic bonds and/or the alteration of the receptor autoinhibition mechanism (p.Ser96Cys); [3] altered D3 ligand binding and specificity (p.Ala353Thr); and [4] decreased tyrosine kinase activity by impairing hydrophobic bonds (p.Met719Val).

Incomplete Penetrance and Oligogenicity

The genetic analysis of available family members showed that in at least three cases (Patients 1, 2, and 4; Fig. 1) the mutation was inherited from an apparently normal parent, thus representing cases of incomplete penetrance. Patients with *FGFR1* mutations were additionally screened for mutations in the most prevalent causative genes and this resulted in the identification of a trigenic mutation in one patient (Patient 1). In

FIGURE 1



Identification of *FGFR1* mutations in affected families. (A) Pedigrees of affected families and mutation nomenclature at the nucleotide and protein level. Filled symbols represent patients with Kallmann syndrome (family 3, 5, and 6) or normosmic idiopathic hypogonadotropic hypogonadism (family 1, 2, and 4); open symbols represent unaffected individuals; open symbols with black dot represent unaffected carriers of the mutation; open symbols with question mark represent untested individuals; squares denote men; circles denote women; and oblique lines through symbols represent deceased individuals. (B) DNA sequence analysis of normal individuals (above) and patients (below). The positions of the mutations are indicated by asterisks. All patients were heterozygous for the mutated nucleotides. In the case of frameshift mutations (families 2 and 5), only the cloned mutated allele is represented. (C) Confirmation of mutations by agarose gel electrophoresis of restriction enzyme-digested fragments (*Bsr*I, *Pfi*MI, *Msp*I, *Acc*I, *As*eI, and *Bst*UI). Lanes correspond to family members represented in (A) and normal controls (C).

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TABLE 1

Clinical characteristics of patients with *FGFR1* mutations.

Patient	Sex	Age at diagnosis (y)	Clinical presentation	Olfaction ^a	Associated features	Basal hormone levels	Brain MRI/CT	Family history	Mutation
1	M	19.7	Arrested puberty. Tanner stage 2. Testicular volume 6 mL	Normal	Osteoporosis and osteopenia (lumbar spine T-score -3.1; femoral neck T-score -2.1)	FSH 2.6 mIU/mL; LH 2.0 mIU/mL; total T 0.32 ng/mL (NR 2.60–10.00)	Normal (MRI)	Father had constitutional delayed puberty (>14 y)	Proband: (<i>FGFR1</i> : c.12G>T, p.Trp4Cys) + (<i>CHD7</i> c.3245C>T, p.T1082I) + (<i>PROKR2</i> c.802C>T, p.R268C) Father: (<i>FGFR1</i> c.12G>T, p.Trp4Cys) + (<i>CHD7</i> c.3245C>T, p.T1082I) Mother: (<i>PROKR2</i> c.802C>T, p.R268C) <i>FGFR1</i> : c.95dupA, p.Pro33Alafs*17
2	M	17.4	Delayed puberty. Right cryptorchidism. Tanner stage 1. Left testicular volume 2 mL	Normal	Obsessive–compulsive disorder	FSH 0.31 mIU/mL; LH 0.07 mIU/mL; total T 0.31 ng/mL (NR 2.41–8.27)	Normal (CT)	No	
3	M	15.9	Delayed puberty. Tanner stage 1. Testicular volume 3 mL	Anosmia	History of bilateral cryptorchidism, with right orchiopexy performed at age 2.5 y	FSH 1.2 mIU/mL; LH 0.5 mIU/mL; total T 0.59 ng/mL (NR 2.20–8.00)	Hypoplastic left olfactory sulcus and bulb (MRI)	No	<i>FGFR1</i> : c.287C>G, p.Ser96Cys
4	M	40	Delayed puberty. Tanner stage 3. Testicular volume 2 mL	Normal		FSH 1.3 mIU/mL; LH 0.2 mIU/mL; total T 0.59 ng/mL (NR 2.6–10.0)	Normal (CT)	No	<i>FGFR1</i> (exon 8A): c.1057G>A, p.Ala353Thr
5	M	16	Delayed puberty. Tanner stage 1/2. Testicular volume 2 mL	Hyposmia		FSH 0.53 mIU/mL; LH 0.26 mIU/mL; total T 0.41 ng/mL (NR 2.80–8.00)	Hypoplastic olfactory sulci and agenesis of the olfactory bulbs and tract (MRI)	Brother diagnosed with IHH and anosmia at age 16 y	<i>FGFR1</i> : c.1961dupA, p.Tyr654*
6	M	21	Delayed puberty. Tanner stage 2/3. Testicular volume 3 mL	Hyposmia	History of right cryptorchidism, with orchiopexy performed at age 8 y. Spina bifida occulta. Osteoporosis and osteopenia (lumbar spine T-score -3.6; femoral neck T-score -1.6)	FSH 0.3 mIU/mL; LH 0.1 mIU/mL; total T 0.6 ng/mL (NR 2.7–11.0)	Pituitary microadenoma (MRI) ^b	No	<i>FGFR1</i> : c.2155A>G, p.Met719Val

Note: CT = computerized tomography; IHH = idiopathic hypogonadotropic hypogonadism; MRI = magnetic resonance imaging; NR = normal range.

^a Self-reported.

^b Found to be nonfunctioning in hormone assays.

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TABLE 2

Evolutionary conservation of FGFR1 mutated amino acids (**bold**) across different species.

[illegible]

this patient, two mutations (*FGFR1*, c.12G>T, p.Trp4Cys; and *CHD7*, c.3245C>T, p.T1082I) were inherited from the father who had constitutional delayed puberty, and one mutation (*PROKR2*, c.802C>T, p.R268C) was inherited from the apparently normal mother, thus suggesting an explanation for the phenotypic variability in this family. The *CHD7* variant was absent in the ExAC database (21) and in our tested 400 control alleles. The *PROKR2* variant has a frequency of only 0.005 in the ExAC database (21) and was found in 1 of 400 of our control alleles. The *CHD7* and *PROKR2* amino acid substitutions occurred at conserved positions and were considered pathogenic by all four prediction programs (16–19).

DISCUSSION

A total of 219 loss-of-function *FGFR1* mutations have been reported in the literature, which consist of 69.9% missense mutations, 11.9% frameshift deletions or insertions, 9.1% nonsense mutations, 6.8% splice-site mutations, 1.8% gross deletions or rearrangements, and 0.5% in-frame deletions (Supplemental Table 1). Mutations are spread across the coding sequence with no mutational hotspots.

Our study of 50 patients with IHH identified six novel heterozygous *FGFR1* mutations, including a mutation in the alternatively spliced exon 8A of this gene. The frequency of *FGFR1* mutations in KS and in normosmic IHH probands was 14% (3 in 21) and 10% (3 in 29), respectively, which is consistent with results presented in other studies [6, 13, 14, 23–27].

These mutations (two frameshift and four missense mutations) are likely to be pathogenic for the following reasons. Frameshift mutations can be considered highly disruptive mutations as they usually lead to premature stop codons that result in the production of truncated proteins or nonsense-mediated mRNA decay (28). The missense mutations were predicted to be pathogenic using structure-based and sequence-based prediction methods (29). Furthermore, the high degree of conservation of the mutated amino acids across species, and the absence of these variants in ethnically matched controls, are all consistent with a critical role for the four identified missense mutations in the activity of the FGFR1 protein.

Our finding of a patient with an exon 8A mutation (p.Ala353Thr), which selectively disrupts the FGFR1-IIIb isoform, appears to contradict the common assumption of a redundant role for this isoform. Alternative splicing of either exon 8A or exon 8B, generates isoforms FGFR1-IIIb or FGFR1-IIIc, respectively. These two isoforms have been demonstrated to have distinct ligand-binding characteristics and somewhat different expression patterns (30, 31). However, it is the FGFR1-IIIc that is thought to represent the dominant isoform and to perform most of the biological functions of the *FGFR1* gene. Evidence for the relative importance of these isoforms was provided by experiments in genetically engineered mice, in which homozygous mutations in exon 8B resulted in embryonic lethality due to severe developmental defects, whereas homozygous mutations in exon 8A resulted in viable and fertile mice (11). Thus, the mutation affecting exon 8A is unusual and suggests an important role for the FGFR1-IIIb isoform.

At present there has been a single report, by Miura et al. (32), of a mutation in exon 8A (p.Thr358Ile) in a Japanese patient with KS. No other mutation in this exon has since been reported, and this contrasts with mutations in exon 8B, which are present in 19 of 219 families reported in the literature (Supplemental Table 1). In contrast to the patient described by Miura et al. (32), our patient with an exon 8A mutation lacks olfactory defects, and therefore this is the first case of normosmic IHH associated with a selective FGFR1-IIIb defect. Thus, our study provides additional evidence for a role of the FGFR1-IIIb isoform in the pathogenesis of IHH, and indicates the need to include routine sequencing of exon 8A (in addition to exon 8B) in genetic screening of both KS and normosmic IHH in populations of all ethnicities. This is important because researchers occasionally exclude exon 8A from their genetic analysis (23, 33), leading to the risk of missed mutations. It remains to be clarified whether the relative rarity of observed exon 8A mutations is due to lack of appropriate screening of this exon in the DNA sequence analysis, to the absence of phenotypic expression of most mutations in this exon, or to a severe effect of most mutations in this exon that would be incompatible with life.

The genetic studies showed that some of the mutations in our cohort were inherited from apparently normal parents. This observation of incomplete penetrance of mutations has been described before for *FGFR1* and other IHH-associated genes and suggests that other genetic and environmental factors may contribute to the phenotypic variability (34). In addition, we identified a patient with mutations in three different genes. This is in agreement with increasing reports of digenic and oligogenic mutations in IHH, and suggests that genetic screening should be extended to additional genes, even if a mutation has already been identified (35, 36).

A limitation of this study is the lack of in vitro functional studies that would be useful to confirm the physiological effects of the missense mutations, especially for the case of the exon 8A mutation. These could include evaluation of mutant signaling activity and expression levels. However, such assays for FGFR1 are highly laborious and the degree of in vitro functional impairment is not always correlated with phenotypic presentation (24, 37). Nevertheless, for the mutations identified in the present study, there is substantial evidence for their pathogenicity resulting from structure-based and sequence-based prediction methods and from the absence of these variants in normal ethnic-matched controls.

In conclusion, our study identified six previously unreported mutations of the *FGFR1* gene, thereby expanding the spectrum of mutations associated with IHH. In addition, we present the first association between normosmic IHH and a mutation that selectively disrupts the FGFR1 IIIb isoform, suggesting that this disorder can be caused by defects in either of the two alternatively spliced FGFR1 isoforms.

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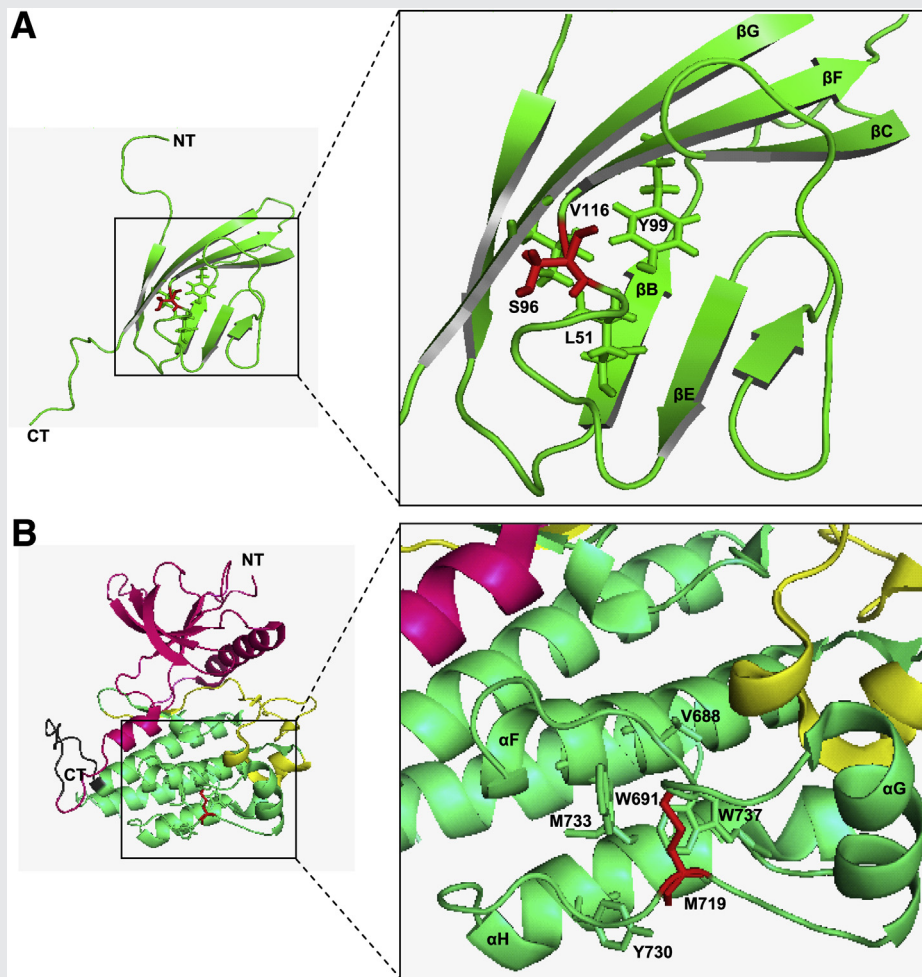
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SUPPLEMENTAL FIGURE 1



Mapping of the S96C and M719V *FGFR1* missense mutations onto the crystal structures of the FGFR1-IIIc protein. **(A)** The mutated S96 residue (red) is mapped on the immunoglobulin-like domain 1 (D1) solution structure (Protein Data Bank ID: 2CR3). D1 is colored in green. The S96 residue points into the hydrophobic nucleus of D1, establishing links with the hydrophobic residues (V116, L51, Y99) and contributing to the tertiary fold of this domain. **(B)** The mutated M719 residue (red) is mapped on the FGFR1 kinase domain (Protein Data Bank ID: 3GQI). The amino-terminal (NT) lobe is in pink. The kinase hinge region is in grey; the activation loop is in yellow; and the carboxy-terminal (CT) lobe is in green. The M719 residue maps to the loop region connecting helices α H and α G, keeping contacts with hydrophobic amino acids from helices α H and α F (M733, W691, V688, W737, M719, Y730).

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