

Activating Somatic *FGFR2* Mutations in Breast Cancer

Nadine Reintjes^{1,2,3}, Yun Li^{1,2,3}, Alexandra Becker⁴, Edyta Rohmann^{1,2,3}, Rita Schmutzler⁴, Bernd Wollnik^{1,2,3*}

1 Institute of Human Genetics, University of Cologne, Cologne, Germany, **2** Center for Molecular Medicine Cologne (CMMC), University of Cologne, Cologne, Germany, **3** Cologne Excellence Cluster on Cellular Stress Responses in Aging-Associated Diseases (CECAD), University of Cologne, Cologne, Germany, **4** Department of Molecular Gynecology and Oncology, Gynecology and Obstetrics Clinic, University of Cologne, Cologne, Germany

Abstract

It is known that *FGFR2* gene variations confer a risk for breast cancer. *FGFR2* and *FGF10*, the main ligand of *FGFR2*, are both overexpressed in 5–10% of breast tumors. In our study, we sequenced the most important coding regions of *FGFR2* in somatic tumor tissue of 140 sporadic breast cancer patients and performed MLPA analysis to detect copy number variations in *FGFR2* and *FGF10*. We identified one somatic heterozygous missense mutation, p.K660N (c.1980G>C), within the tyrosine kinase domain of *FGFR2* in tumor tissue of a sporadic breast cancer patient, which is likely mediated by the *FGFR2*-IIIb isoform. The presence of wild type and mutated alleles in equal quantities suggests that the mutation has driven clonal amplification of mutant cells. We have analyzed the tyrosine kinase activity of p.K660N and another recently described somatic breast cancer mutation in *FGFR2*, p.R203C, after expression in HEK293 cells and demonstrated that the intrinsic tyrosine kinase activity of both mutant proteins is strongly increased resulting in elevated phosphorylation and activity of downstream effectors. To our knowledge, this is the first report of functional analysis of somatic breast cancer mutations in *FGFR2* providing evidence for the activating nature of *FGFR2*-mediated signalling in the pathogenesis of breast cancer.

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* E-mail: bwollnik@uni-koeln.de

Introduction

Genome-wide association studies identified variants in the fibroblast growth factor receptor 2 (*FGFR2*) tumor suppressor gene as a genetic risk factor for breast cancer susceptibility. The most strongly associated variants were located in intron 2 of the *FGFR2* gene [1,2]. Intron 2 of *FGFR2* shows a high degree of conservation in mammals and contains several putative transcription factor binding sites, some of which lie in close proximity to the relevant variants. Therefore, it is speculated that the association with breast cancer is mediated through the regulation of *FGFR2* expression [2]. The expression and amplification of *FGFR2* has long been known to be elevated in 5–10% of breast tumors [3]. Somatic missense mutations of *FGFR2* that are likely to be implicated in cancer development have also been demonstrated in primary tumors and cell lines of multiple tumor types [4,5].

Other genome-wide association studies of breast cancer predisposition identified variants on chromosome 5p12 some 274–317 kb distal to the fibroblast growth factor 10 (*FGF10*) that confer a risk, preferentially for estrogen receptor-positive breast tumors. *FGF10* is amplified in approximately 10% of breast cancers, and possibly the observed risk variants influence *FGF10* expression [6].

Mouse models of mammary carcinogenesis have long established the FGF signalling pathway as a major contributor to tumorigenesis [7], and a mouse mammary tumor virus (MMTV) insertional mutagenesis screen for genes involved in breast cancer has identified both *FGFR2* and *FGF10* [8]. However, as yet, little is

known about the mechanism by which *FGFR2* and *FGF10* mutations act as risk factors in predisposition to breast cancer [9].

The receptor tyrosine kinase *FGFR2* is one of four fibroblast growth factor receptors designated *FGFR1-4* that activate FGF signalling upon trans-autophosphorylation of the receptor dimers. The activation of receptor tyrosine kinase signalling is one of the mechanisms underlying tumor development and growth.

The FGF system consists of at least 22 distinct FGFs which have been identified in a variety of organisms from nematode and drosophila to mouse and human. Although FGFs vary in size from 17 to 34 kDa, all members of the family share a conserved sequence of 120 amino acids that show 16–65% sequence identity [10]. The binding of FGFs to FGFRs in the presence of heparin sulphate glycosaminoglycan induces receptor dimerization and activation of the protein tyrosine kinase domain [11]. Tyrosine autophosphorylation and the recruitment of a complement of downstream signalling molecules result in the stimulation of various signalling cascades that play critical roles in various cellular processes [12]. FGFs fulfill versatile functions throughout the human life cycle commencing at germ cell maturation [13,14], continuing throughout embryonic development [15,16,17] and into adulthood [18]. During embryogenesis, FGFs are essential in morphogenesis by regulating cell proliferation, differentiation and cell migration [19,20]. In the adult, FGFs continue to regulate tissue homeostasis but are also involved in the control of the nervous system, in tissue repair, wound healing, cholesterol

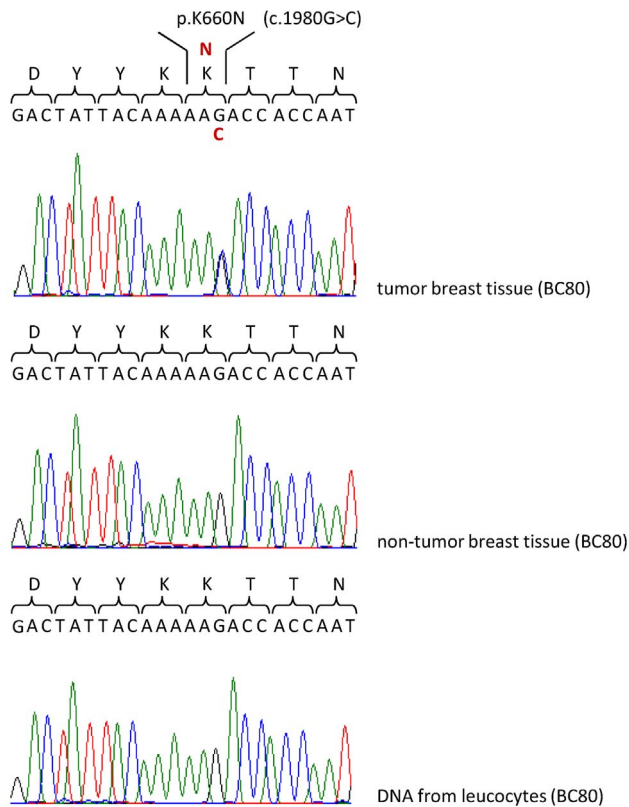


Figure 1. Identified *FGFR2* mutation in tumor tissue. The upper sequence chromatogram shows the heterozygous missense mutation in exon 14 in *FGFR2*, c.1980G>C (p.K660N), found in somatic tumor breast tissue of patient BC80. The middle and lower chromatograms illustrate the normal sequence in non-tumor breast tissue and blood-derived DNA of the same patient.
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metabolism [21], serum phosphate regulation [22] and tumor angiogenesis [12].

FGFRs share 55% to 72% homology at the protein level. Like all receptor tyrosine kinases, FGFRs are composed of an extracellular ligand binding domain, a transmembrane region, and a cytoplasmic region containing a catalytic protein tyrosine kinase core and additional regulatory sequences. The extracellular domain is composed of three immunoglobulin-like domains (designated D1–D3), a stretch of negatively charged amino acids in the linker connecting D1 and D2, termed the acidic box, and a conserved positively charged region in D2 that serves as the binding site for heparin sulphate or heparin [11,23,24,25].

FGF-FGFR specificity is an essential mechanism in the regulation of FGF response and is achieved primarily through alternative splicing in the second half of D3 in FGFRs. Transcripts of *FGFR1*, 2, and 3, but not *FGFR4* are subject to alternative RNA splicing in which exon 7 of the *FGFR* gene encodes a common N-terminal half of D3 (referred to as IIIa) and two different exons 8 code for the C-terminal half of D3 to generate the IIIb and IIIc isoforms, respectively [26,27]. The IIIb isoforms are expressed exclusively in epithelial cells, while the IIIc isoforms are expressed only in mesenchymal cells [28,29,30,31]. Moreover, the IIIb and IIIc isoforms of *FGFR1*, -2 and -3 bind to different complements of FGFs that are expressed exclusively in mesenchymal or epithelial cells, respectively. Mammary epithelial cells express *FGFR2-IIIb*, which binds FGF7 and FGF10, expressed by surrounding mesenchymal cells.

We hypothesized that specific private mutations of an activating nature in *FGFR2* and *FGF10* might influence breast tumor development and growth. To test this hypothesis, we screened sporadic breast cancer patients for mutations in both candidate genes and further investigated identified breast cancer mutations by functional analysis.

Materials and Methods

Patients

DNA isolated from somatic tumor tissue of 140 sporadic breast cancer patients was collected by the Department of Molecular Gynecology and Oncology, Gynecology and Obstetrics Clinic, Cologne, Germany. Once a mutation was identified, DNA from non-tumor tissue and blood-derived DNA from the patient as well as blood-derived DNA from 200 control individuals was tested. Informed written consent for genetic analysis of samples was given by all subjects. Ethical approval for this study was given by the institutional Ethics Committee of the University of Cologne, Germany (07–185, 10/18/2007).

Mutation analysis

The protein-coding exons 5, 7–9 and 12–15 of the IIIb isoform (NM_022970.3) and also exon 8 of the IIIc isoform (NM_000141.1) as well as adjacent intronic sequences of the *FGFR2* gene were amplified by PCR using standard conditions (Table S1). PCR fragments were purified and directly sequenced utilizing the corresponding forward or reverse primers with the ABI BigDye Terminator v3.1 Cycle Sequencing Kit and an ABI 3730 DNA Analyzer (Applied Biosystems by Life Technologies, Darmstadt, Germany). The identified mutation was resequenced in independent experiments. Primer design and DNA mutation numbering was given based on cDNA sequence of *FGFR2* GenBank entry NM_022970.3 with 1 corresponding to the A of the ATG translation initiation codon.

Multiplex ligation-dependent probe amplification (MLPA) assay

The MLPA kit for the *FGF10* and *FGFR2* genes (SALSA P231) was purchased from MRC Holland (Amsterdam, The Netherlands). The probe mix contains 34 probes: 6 hybridizing to the *FGF10* gene, 8 to the *FGFR2* gene, and, as controls, 18 to single-copy genes located on other human chromosomes. The assay was conducted according to the instructions by the manufacturer. For the reactions, 100 ng DNA isolated from somatic tumor tissue was used. Amplification products were separated on an ABI PRISM 3100 Avant Genetic Analyzer (Applied Biosystems by Life Technologies, Darmstadt, Germany) using ROX 500 internal size standard (Serac, Bad Homburg, Germany). Data were retrieved by the GeneScan Software 3.7 (Applied Biosystems by Life Technologies, Darmstadt, Germany) and fragment sizes, peak areas and peak heights were determined with Sequence Pilot^{CE} software. Variations in peak area were evaluated by comparison of each sample with four controls always from the same experiment. Deletions were suspected when the peak area was lower than 75% of the controls, duplications were suspected when the peak area was higher than 125% of the controls, and multiple copies were suspected when the peak area was higher than 175% of the controls.

FGFR2 isoform study

RNA was isolated from somatic tumor tissue of 3 sporadic breast cancer patients. First strand cDNA synthesis was carried out by the use of the RevertAid First Strand cDNA Synthesis Kit

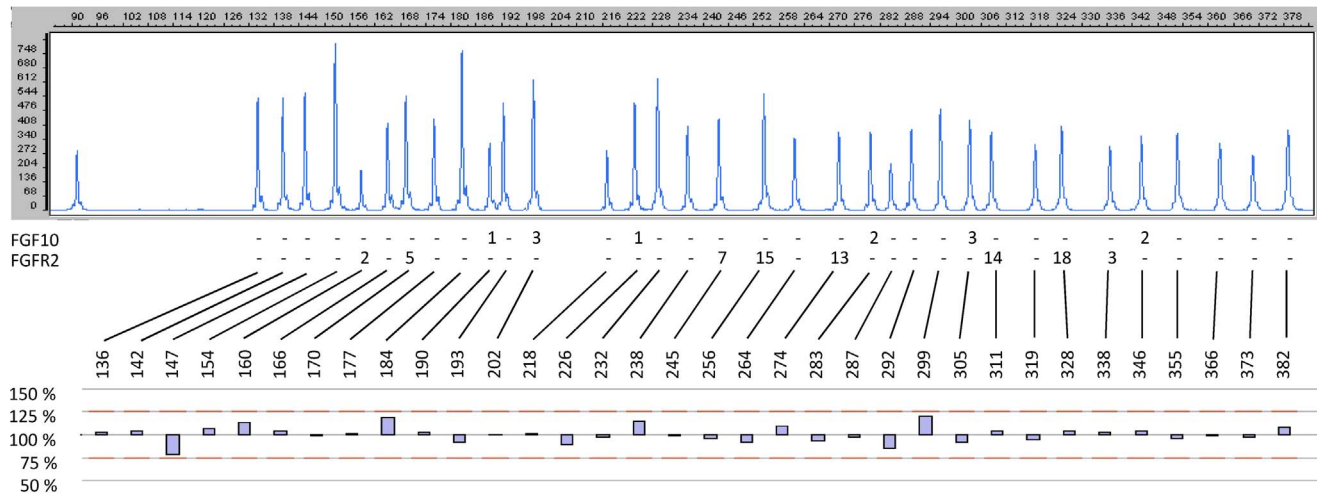


Figure 3. *FGFR2/FGF10* MLPA analysis. Representative MLPA chromatogram and quantification with SequencePilot software. The probe mix contained 34 probes, 6 hybridizing to the *FGF10* gene, 8 to the *FGFR2* gene, and 20 controls hybridizing to single-copy genes located on other chromosomes. Peak areas lower than 75% of the controls are indicative for a deletion, peak area higher than 125% of the controls for a duplication. doi:10.1371/journal.pone.0060264.g003

Cell culture and transfection

HEK293 cells were cultured in Dulbecco's Modified Eagle Medium (DMEM) containing 10% fetal bovine serum (FBS), 0.7 µg/ml amphotericin B, 100 U/ml penicillin and 100 µg/ml streptomycin at 37.8°C in a 5% CO₂/95% air environment.

HEK293 cells were transiently transfected with Lipofectamine 2000 (Invitrogen by Life Technologies, Darmstadt, Germany) and incubated in transfection medium for 6 h. This was followed by changing the medium to DMEM containing 10% FBS. After 18 h, cells were washed with PBS and lysed for 5 min on ice in 50 mM HEPES, 150 mM NaCl, 1% TX-100, 10% glycerine plus protease inhibitors.

Additionally, HEK293 cells were transiently transfected using calcium phosphate. This was followed by changing the medium to DMEM devoid of FBS. After starvation overnight, cells were stimulated for 5 min with 25 ng/ml of growth factor FGF1 (R&D Systems, Wiesbaden Nordenstadt, Germany). FGF1 was prepared and used as a stock solution at a concentration of 100 µg/ml with 5 mg/ml heparin. Cells were washed with PBS and lysed for 5 min on ice in 150 mM NaCl, 0.5 mM EDTA, 1% NP40, 20 mM Tris, 1 mM NaVO₄, 10 mM NaF, 10 µM NaMoO₄ and proteinase inhibitors.

Lysates were centrifuged at 16100 g for 10 min at 4°C. The supernatant was designated the soluble fraction. Protein concentrations of soluble cellular fractions were determined using the BCA protein assay kit (Thermo Fisher Scientific, Bonn, Germany).

Immunoprecipitation and immunoblot analysis of protein extracts

Proteins expressed in HEK293 cells transiently transfected with lipofectamine were immunoprecipitated from soluble lysates using protein A/G PLUS agarose and antibody Bek (C-17), rabbit polyclonal IgG (Santa Cruz Biotechnology, Heidelberg, Germany) according to manufacturer's guidelines. Immunoprecipitates were washed in 20 mM HEPES, 150 mM NaCl, 0.1% TX-100 and 5% glycerine. Equal amounts of protein extracts were prepared by addition of NuPAGE LDS Sample Buffer and NuPAGE Sample Reducing Agent and heated at 95°C for 5 min. Samples were loaded on 7% NuPAGE Tris-Acetate Gels (Invitrogen by Life Technologies, Darmstadt, Germany). Proteins were transferred to

nitrocellulose membranes by electrophoresis over night at 12 V at 4°C in NuPAGE Transfer Buffer (Invitrogen by Life Technologies, Darmstadt, Germany). Membranes were blocked with 5% non-fat dry milk in PBS for 1 h at room temperature (RT). Samples of cells transiently transfected with calcium phosphate, also previously prepared by addition of NuPAGE LDS Sample Buffer and NuPAGE Sample Reducing Agent and heated at 95°C for 5 min, were loaded on 4–12% NuPAGE Bis-Tris Gels (Invitrogen by Life Technologies, Darmstadt, Germany). Proteins were transferred to nitrocellulose membranes by electrophoresis for 3 h at 30 V at RT in NuPAGE Transfer Buffer and subsequently membranes were blocked with 5% non-fat dry milk in TBST buffer (TBST buffer: 0.1% Tween 20, 20 mM Tris, 150 mM NaCl) for 1 h at RT. After blocking, all membranes were incubated overnight at 4°C in TBST buffer containing the primary antibodies Bek (C-17) (Santa Cruz Biotechnology, Heidelberg, Germany), p-Tyr (PY99) (Santa Cruz Biotechnology, Heidelberg, Germany), p-FRS2-α (Tyr196) (Cell Signaling Technology by New England Biolabs GmbH, Frankfurt am Main, Germany), p-STAT3 (9E12) (Santa Cruz Biotechnology, Heidelberg, Germany), p-MEK1/2 (S217/221) (Cell Signaling) and Actin (Sigma-Aldrich, München, Germany). This was followed by washing the membranes with TBST buffer and an incubation for 1 h at 4°C with TBST buffer containing the secondary antibodies goat anti-rabbit IgG or goat anti-mouse IgG, conjugated to horseradish peroxidase (Santa Cruz Biotechnology, Heidelberg, Germany). Reactions were revealed after washing with Super-Signal West Pico Chemoluminescent Substrate (Thermo Fisher Scientific, Bonn, Germany).

Results

Identification of a heterozygous *FGFR2* mutation

In our study we examined the immunoglobulin-like domains D2 and D3, the transmembrane domain and the tyrosine kinase domain of *FGFR2*, i.e. regions in which the typical mutational hot spots of craniosynostosis syndromes are located, in somatic tumor tissue of sporadic breast cancer patients. We sequenced the protein-coding exons 5 and 7–9 in about 50 patients and exons 12–15 in about 140 patients as well as adjacent intronic sequences and we identified one novel heterozygous missense mutation in

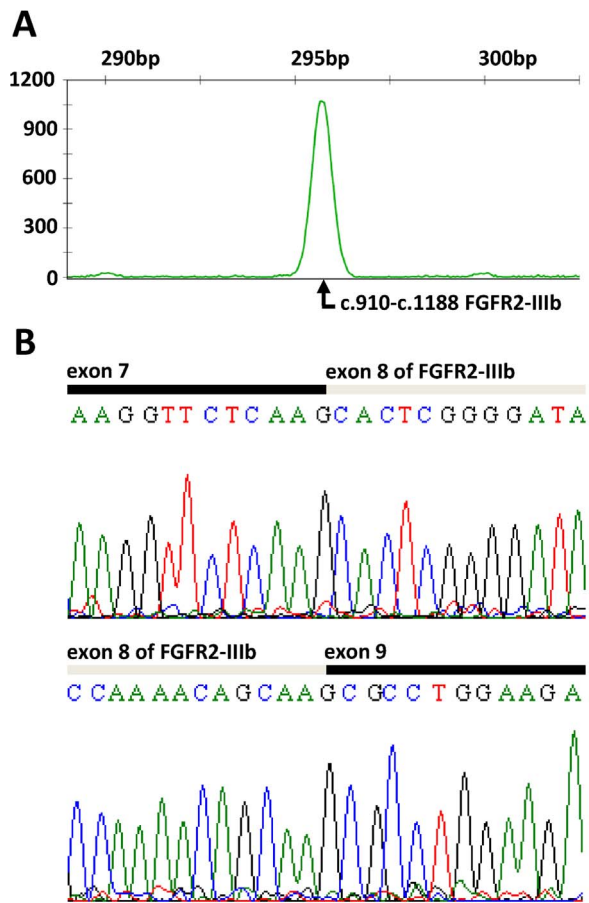


Figure 4. *FGFR2* mRNA isoform expression analysis in tumor tissue of three sporadic breast cancer patients. A) Representative electropherogram of GeneScan analysis. Isoforms *FGFR2-IIIb* and *FGFR2-IIIc* differ in exon 8, resulting in a variation of 3 bp in length of mature mRNA. A PCR fragment of 297 bp for *FGFR2-IIIb* or 294 bp for *FGFR2-IIIc* cDNA spanning exon 8 of both isoforms was amplified by PCR using a fluorescently-labeled primer pair located in exons 7 and 9, which are common in both isoforms. Fragment analysis showing a single sharp peak. **B) Representative sequence electropherogram showing the expression of IIIb isoform.**
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exon 14 of *FGFR2*, c.1980G>C, in tumor tissue of one of them (BC80). This mutation is predicted to lead to a substitution of the lysine at position 660 to asparagine (p.K660N). The mutation was present neither in non-tumor tissue of the same patient nor in blood-derived DNA from the patient or 200 control individuals. In addition, the mutation has not been reported in the Human Gene Mutation Database (HGMD) (URL: <http://www.hgmd.cf.ac.uk/ac/index.php>) or the Exome Variant Server, NHLBI GO Exome Sequencing Project (ESP) (URL: <http://evs.gs.washington.edu/EVS/>). Considering the presence of wt and mutated alleles in equal quantities, the mutation must have either occurred at a very early stage of breast cancer development or expanded via clonal amplification of mutant cells (Fig. 1). Moreover, the lysine at position 660 is strongly conserved between species and all four human *FGFRs*, indicating that this site is of functional importance. Notably, inspection of the structure of the receptor showed that the breast cancer mutation is located in the intracellular tyrosine kinase domain adjacent to the activation loop suggesting that it might affect the tyrosine kinase activity of *FGFR2* (Fig. 2).

In our patient cohort, we detected several single nucleotide polymorphisms, and allele frequencies were consistent with stored frequencies in the HapMap database (www.hapmap.org) (Table S2).

We also performed MLPA analysis to detect copy number variations in *FGFR2* and *FGF10* in breast tissue of 50 sporadic breast cancer patients. However, we did not identify any copy number changes in either of the two genes (Fig. 3).

The *FGFR2-IIIb* isoform is predominantly expressed in tumor tissue of sporadic breast cancer patients

The p.K660N mutation is located in a region common to both *FGFR2-IIIb* and *FGFR2-IIIc* isoforms. To determine whether the identified breast cancer mutation is primarily mediated by the *FGFR2-IIIb* or *FGFR2-IIIc* isoform, we examined the presence of the IIIb and IIIc transcripts in three cDNA samples synthesized from tumor tissue total RNAs of sporadic breast cancer patients. We designed a fluorescently labeled primer pair complementary to the cDNA sequence and flanking the alternative spliced exon 8. Primers were located in exons 7 and 9, which are common in the IIIb and IIIc transcripts, allowing amplification of exon 8 of both isoforms and the adjacent exon boundaries. The PCR product of isoform IIIb was expected to be three base pairs bigger than that of isoform IIIc. Amplification products were evaluated by GeneScan analysis. Notably, the electropherogram of the GeneScan analysis showed a single sharp peak in the case of all three tested cDNA samples suggesting the amplification of only one isoform (Fig. 4A). Subsequent direct sequencing of the products verified that predominantly the transcript of *FGFR2-IIIb* is present in tumor tissue of sporadic breast cancer patients (Fig. 4B).

Increased tyrosine kinase activity of *FGFR2* breast cancer mutant

In order to reveal the molecular mechanism of the p.K660N and another recently described somatic breast cancer mutation in *FGFR2*, p.R203C [32], expression vectors that direct the synthesis of *FGFR2-IIIb* carrying the breast cancer mutations were constructed and tested for their biological activity following transient expression in HEK293 cells. The p.R203C mutation is located in the extracellular Ig domain 2 of the receptor and the arginine at this position is highly conserved between species and all four human *FGFRs* (Fig. 2). The tyrosine kinase activities of *FGFR2-IIIb* carrying breast cancer mutations were compared to those of wt *FGFR2-IIIb*, of a dominant-negative kinase-defective (KD) *FGFR2* mutant (p.K508A), and of a Pfeiffer syndrome gain-of-function *FGFR2* mutant (p.K642R; previously named p.K641R according to another reference sequence) as controls. Lysates from cells expressing wt *FGFR2-IIIb* or one of the various mutants were subjected to immunoprecipitation with anti-*FGFR2* antibody followed by SDS-PAGE and immunoblotting with anti-p-Tyr antibody. The intrinsic tyrosine kinase activities in both mutant proteins (p.R203C and p.K660N) were strongly increased as compared to the tyrosine kinase activity of wt *FGFR2-IIIb* (Fig. 5A). Different degrees of tyrosine autophosphorylation were detected for all *FGFR2-IIIb* mutants with the p.K660N mutant showing the highest tyrosine kinase activity.

Moreover, cells expressing wt and mutant *FGFR2-IIIb* were stimulated with FGF1 and total lysates from unstimulated and FGF1-stimulated cells were analysed. Both breast cancer mutants led to an increased tyrosine phosphorylation of FRS2, a well-characterized *FGFR2* substrate, as revealed by immunoblotting with anti-p-FRS2 antibody after SDS-PAGE. We also showed that MEK1/2 and STAT3 stimulation in response to FGF1 stimula-

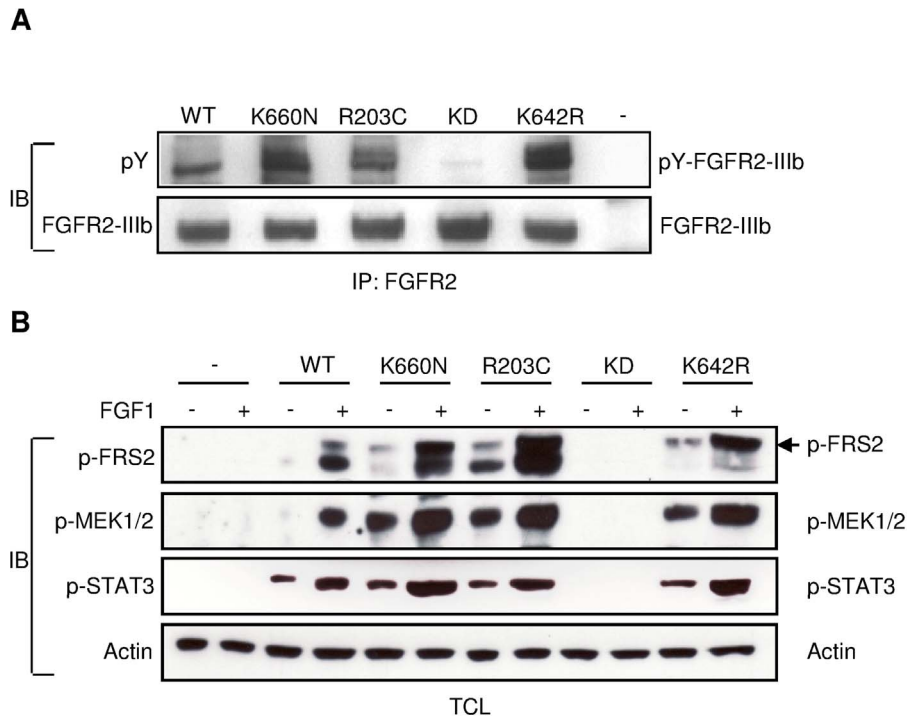


Figure 5. Activating *FGFR2* breast cancer mutations. **A)** Representative Western blot showing increased tyrosine kinase activities of *FGFR2*-IIIb breast cancer mutants compared to tyrosine kinase activities of wt *FGFR2*-IIIb (WT), of a kinase defective (KD) *FGFR2*-IIIb mutant, and of a Pfeiffer syndrome gain-of-function mutant (K642R, previously named K641R according to another reference sequence) as controls. HEK293 cells were transiently transfected using lipofectamine with pRK5 vectors containing cDNA coding for wt *FGFR2*-IIIb or *FGFR2*-IIIb variants containing the indicated amino acid substitution. Untransfected cells served as negative control (-). Lysates from cells were subjected to immunoprecipitation with anti-*FGFR2* antibodies (Bek(C-17)) followed by SDS-PAGE and immunoblotting (IB) with anti-*FGFR2* or anti-p-Tyr antibodies (PY99). **B)** Representative Western blots showing increased substrate phosphorylation by *FGFR2*-IIIb breast cancer mutants compared to substrate phosphorylation by wt *FGFR2*-IIIb (WT), a kinase defective (KD) *FGFR2*-IIIb mutant, and a Pfeiffer syndrome gain-of-function mutant (K642R, previously named K641R according to another reference sequence) as controls. For transient transfection calcium phosphate was used. Untransfected cells served as negative control (-). HEK293 cells expressing *FGFR2*-IIIb as well as untransfected cells were stimulated with FGF1. Lysates of unstimulated or FGF1-stimulated cells were subjected to SDS-PAGE followed by immunoblotting with anti-p-FRS2, anti-p-MEK1/2, anti-p-STAT3 and anti- β -Actin as loading control. doi:10.1371/journal.pone.0060264.g005

tion is increased in HEK293 cells expressing the *FGFR2*-IIIb breast cancer mutants. In addition, results indicated that the responses of downstream effectors are most likely ligand-independent.

On the basis of these experiments, we conclude that the identified somatic breast cancer mutations are of an activating nature resulting in altered FGF-signalling.

Discussion

The aim of our study was to gain new insights into the role of private somatic mutations in *FGFR2* and *FGF10* in breast tumor development. Here, we identified a somatic heterozygous missense mutation in exon 14 of *FGFR2*, c.1980G>C, that is predicted to lead to a substitution of a highly conserved lysine in the tyrosine kinase domain at position 660 to asparagine (p.K660N). We could clearly show that *FGFR2*-IIIb is predominantly expressed in tumor tissue of sporadic breast cancer patients, strongly suggesting that the p.K660N mutation is primarily mediated by this isoform. This finding is important for functional analysis of *FGFR2* breast cancer mutations. Moreover, there has already been strong evidence that the isoform IIIb and not isoform IIIc of *FGFR2* is associated with breast cancer development, since *FGF10*, the main ligand of *FGFR2*-IIIb and not *FGFR2*-IIIc, is amplified in approximately 10% of breast cancers and genome-wide association studies identified variants near *FGF10* as a genetic risk factor

for breast cancer susceptibility, possibly influencing *FGF10* expression [6]. In order to unravel the molecular mechanism of the p.K660N and another recently described somatic breast cancer mutation in *FGFR2*, p.R203C, we compared the tyrosine kinase activities of breast cancer mutants to that of wt *FGFR2*. Our results clearly show that the intrinsic tyrosine kinase activity of both breast cancer mutant proteins is strongly increased. Furthermore, both breast cancer mutations implicated an increased tyrosine phosphorylation of the critical *FGFR2* substrate FRS2 and an increased MEK1/2 and STAT3 activation in response to FGF1 stimulation leading to an accelerated cell signalling. To our knowledge this is the first report of a functional analysis of somatic breast cancer mutations in *FGFR2* providing evidence for the activating nature of identified mutations. We also performed MLPA analysis to detect copy number variations in *FGFR2* and *FGF10* in sporadic breast cancer patients but did not identify any copy number changes in either of the two genes. We therefore speculate that those types of mutations do not play any important role in breast tumor development.

Signalling pathways activated by FGFs and FGFRs have been identified in multicellular organisms from *Caenorhabditis elegans* to vertebrates. It is now well established that the FGFR family of receptor tyrosine kinases and their numerous ligands play crucial roles in many developmental and physiological processes and that a variety of diseases are caused by aberrant signalling induced by FGFs or FGFRs [10,11]. In humans, both loss- and gain-of-

function heterozygous mutations have been described. Several human skeletal dysplasias are caused by gain-of-function mutations in *FGFR1*, *FGFR2* and *FGFR3*. Activating mutations located in the extracellular ligand binding domain were found in *FGFR1* and *FGFR2* associated with Pfeiffer, Crouzon, Jackson-Weiss and Apert syndromes and in the *FGFR2* kinase domain associated with Pfeiffer and Crouzon syndromes. Likewise, activating mutations in *FGFR3* are found in the transmembrane and the tyrosine kinase domains e.g. in achondroplasia, thanatophoric dysplasia type I (TDI) and type II (TDII) [12,33,34,35]. Several of the *FGFRs* have been implicated in cancer through chromosomal translocations, activating mutations and aberrant splicing. Analyses of mutations from protein kinase screens performed in several cancer types further implicate the FGF signalling pathway in tumorigenesis [5]. The molecular consequences of these mutations are complex and may affect receptor activity, receptor stability, as well as receptor localization.

Interestingly, the novel p.K660N mutation identified in our study has also been found in endometrial carcinoma [36]. Additionally, germline mutations at the paralogous position have been identified in *FGFR3* associated with three different skeletal syndromes – TDII (p.K650E), severe achondroplasia with developmental delay and acanthosis nigricans (SADDAN) syndrome, and TDI (both due to p.K650M) [37,38,39]. The lysine at position 660 in *FGFR2* and the equivalent lysine at position 650 in *FGFR3* are strongly conserved between species and all four human *FGFRs* and located within the activation loop of the *FGFR2* and *FGFR3* tyrosine kinase domain, indicating that these sites are of functional importance. Amino acid substitutions at this position have been shown to have a dramatic effect on the constitutive activation of *FGFR3*-mediated receptor autophosphorylation [40,41]. The activation loop of *FGFRs* is thought to block substrate binding until FGF binding and receptor dimerization alter its conformation, permitting both autophosphorylation of the receptor and phosphorylation of intermediate signalling molecules [42]. It has been demonstrated that the lysine 650 plays a critical role in stabilizing the *FGFR3* activation loop in an inactive conformation, since different mutations of this residue constitutively activate the tyrosine kinase to varying degrees whereas mutations of adjacent residues have little effect. In addition, mutations of this residue may preclude the need for receptor dimerization at the cell membrane to activate the tyrosine kinase [41,43]. Consistent with this study, we also demonstrated that the p.K660N mutation in *FGFR2* is of an activating nature and effects likely ligand-independent autophosphorylation of the receptor.

In 2005, *Stephens et al* examined the coding sequence of 518 protein kinases, ~1,3 Mb of DNA per sample, in 25 breast cancers. This was the first mutational screen of the full coding sequence of all protein kinases in cancer. A few tumors had numerous somatic mutations with distinctive patterns indicative of either a mutator phenotype or a past exposure. In one breast cancer, *Stephens et al* identified at least a single somatic heterozygous missense mutation in *FGFR* that is predicted to lead to a substitution of a highly conserved arginine in the tyrosine kinase domain at position 203 to cysteine (c.607C>T, p.R203C). Their results indicated that there is no commonly point-mutated and activated protein kinase gene in breast cancer. They suggested that approximately two thirds of the observed mutations are probably

passenger mutations that are not subject to selection and, therefore, occur at a similar frequency throughout the genome [32]. *Stephens et al* did not perform any functional analysis of identified mutations in their study. We now show that the p.R203C mutation in *FGFR2* leads to an increased intrinsic tyrosine kinase activity compared to that of wt *FGFR2*. However, the activating effect of the p.R203C mutation is lower compared to the p.K660N mutation. The arginine at position 203 is located in the extracellular Ig domain 2 of the receptor, which is responsible for heparin and ligand binding. Probably structural molecular changes arising from this amino acid substitution cause an increased ligand affinity of the receptor or ligand-independent activation. The exact mechanism needs to be elucidated.

In summary, we showed that identified somatic missense mutations in *FGFR2* are of an activating nature resulting in altered cell signalling. Our study provides further evidence for the implication of *FGFR2* in tumor development. We suggest that the p.K660N and p.R203C mutations likely have driven clonal amplification of mutant cells.

In 2010, *Goriely and Wilkie* highlighted that paternal age effect (PAE) mutations are an emerging mechanism contributing to the introduction of new disease alleles into the population. PAE mutations mostly encode mutant proteins with gain-of-function properties, are of near-exclusive paternal origin, occur at elevated paternal ages and have an apparent germline mutation rate [44]. Interestingly, PAE mutations have been reported in various craniosynostosis syndromes caused by mutations in *FGFR2* and *FGFR3*. It has been shown that PAE mutations are enriched over time because they confer a selective advantage to the mutant spermatogonial stem cells, leading to their clonal expansion. This process has also been linked to the origin of testicular tumors and it has been suggested that the mechanisms involved in selfish selection are similar to those described for tumorigenesis [14,45].

Furthermore, we propose that the phenotypic outcome of impaired FGF signalling caused by breast cancer mutations in the *FGFR2* gene is further modified by genetic and environmental factors which remain to be discovered. Both mutations influence tumor development by increasing, enhancing and accelerating tumor growth.

Supporting Information

Table S1 Primer for *FGFR2* amplification.
(DOC)

Table S2 Allele frequencies of detected SNPs.
(DOC)

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Author Contributions

Conceived and designed the experiments: NR BW ER YL. Performed the experiments: NR. Analyzed the data: NR. Contributed reagents/materials/analysis tools: AB RS ER. Wrote the paper: NR BW.

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