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A Novel FGFR3 Splice Variant Preferentially Expressed in African American Prostate Cancer Drives Aggressive Phenotypes and Docetaxel Resistance

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Abstract

Alternative splicing (AS) has been shown to participate in prostate cancer (PCa) development and progression; however, a link between AS and PCa health disparities has been largely unexplored. Here we report on the cloning of a novel splice variant of *FGFR3* that is preferentially expressed in African American (AA) PCa. This novel variant (*FGFR3-S*) omits exon 14, comprising 123 nucleotides that encode the activation loop in the intracellular split kinase domain. Ectopic overexpression of FGFR3-S in European American (EA) PCa cell lines (PC-3 and LNCaP) led to enhanced receptor autophosphorylation and increased activation of the downstream signaling effectors AKT, STAT3, and ribosomal S6 compared to FGFR3-L (retains exon 14). The increased oncogenic signaling imparted by FGFR3-S was associated with a substantial gain in proliferative and anti-apoptotic activities, as well as a modest but significant gain in cell motility. Moreover, the FGFR3-S-conferred proliferative and motility gains were highly resistant to the pan-FGFR small molecule inhibitor dovitinib and the anti-apoptotic gain was insensitive to the cytotoxic drug

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docetaxel, which stands in marked contrast to dovitinib- and docetaxal-sensitive FGFR3-L. In an *in vivo* xenograft model, mice injected with PC-3 cells overexpressing FGFR3-S exhibited significantly increased tumor growth and resistance to dovitinib treatment compared to cells overexpressing FGFR3-L. In agreement with our *in vitro* and *in vivo* findings, a high *FGFR3-S/FGFR3-L* expression ratio in PCa specimens was associated with poor patient prognosis.

Introduction

Prostate cancer (PCa) is the most diagnosed cancer in men in the United States and accounts for over one-fifth of all newly diagnosed cancers in men¹. More than 164,000 new cases are diagnosed each year and PCa is the second leading cause of male-cancer related deaths annually. Well established risk factors of PCa include age, Lynch syndrome, and race/ethnicity. Despite increased screening and overall decreasing mortality rates of PCa, African American (AA) men have significantly higher rates of PCa incidence, high-risk cancer, and mortality². AA men are 1.7 times more likely to be diagnosed with PCa and have a 2.4 times greater mortality rate compared to European American (EA) men³. Even after adjusting for clinical and epidemiological factors, AA men still have significantly increased occurrence and mortality rates, suggesting differences in biology and genetics may be playing a role in this disparate disease burden⁴.

Alternative splicing (AS) is the major mechanism for post-transcriptional regulation of gene expression, mRNA diversity, and protein modification. During AS, introns are typically excised from the precursor mRNA (pre-mRNA) and the remaining exons can be joined together in different combinations to produce multiple unique mature mRNA transcripts from a single gene. It is estimated that over 90% of human genes transcribe pre-mRNAs that undergo AS. Cancer cells are known to “hijack” the AS process to promote the “hallmarks of cancer”⁵ and splice variants can be used as biomarkers and targets for potential therapies. It is now apparent that AS can lead to the generation of signaling proteins with unique properties such as resistance to small molecule inhibition⁶. For example, the androgen receptor (AR) splice variant *AR-V7* (missing exons 4–7 that code for the ligand binding domain) is resistant to common PCa therapies such as anti-androgens (e.g. enzalutamide) and CYP17 inhibitors (e.g. abiraterone)⁷.

While AS has been shown to play a role in PCa development and progression, a link between AS and PCa health disparities has been largely unexplored (but see ⁸⁻¹⁰). We recently uncovered global differential AS (dAS) events involving 2,520 distinct genes in a comparison between AA and EA PCa specimens⁸. Of interest, an additional 1,188 genes exhibited ‘simple’ differences in expression (e.g. gene expression differences in the absence of dAS). Taken together, these two observations suggest that dAS may be playing a more prominent role in PCa disparities. Of the compiled dAS events, a novel AA PCa-enriched *PIK3CD* short variant termed *PIK3CD-S* (missing exon 20) was cloned from AA cell lines and PCa specimens⁸. *PIK3CD-S* encodes a phosphatidylinositol-4,5-bisphosphate 3-kinase catalytic subunit delta (p110δ) isoform that is missing 56 amino acids in the catalytic domain and promotes greater oncogenicity (e.g. increased proliferation and invasion) compared to the alternative isoform encoded by the full-length *PIK3CD-L* variant that

retains exon 20. The protein isoform encoded by *PIK3CD-S* is also resistant to inhibition by the small molecule inhibitor (SMI) idelalisib in both *in vitro* assays and mouse xenograft models⁸. In the same study, a novel splice variant of the *fibroblast growth factor receptor 3* (*FGFR3*), a known proto-oncogene, was likewise identified but not cloned or functionally characterized. This newly discovered splice variant *FGFR3-S* (-S for short variant), which is highly expressed in AA and weakly expressed in EA PCa specimens, appears to be missing 123 nucleotides due to an in-frame exon 14 skipping event. Accordingly, dAS of *FGFR3* may likewise play a critical role in the increased oncogenicity of AA PCa.

The objectives of this study were to clone and functionally characterize a heretofore unreported exon 14-skipped variant of *FGFR3* and assess the molecular consequences of this splice variant on PCa oncogenesis. We demonstrate that *FGFR3-S* promotes increased downstream oncogenic signaling, proliferation, migration, and invasion and decreased caspase activity compared to *FGFR3-L* (splice variant retaining exon 14) *in vitro*. We also establish that the *FGFR3-S* protein isoform displays extensive resistance to the pan-FGFR small molecule inhibitor dovitinib both *in vitro* and when examining tumor growth *in vivo*. Thus, dAS of *FGFR3* is postulated to be a contributing factor in PCa health disparities in AA men.

Materials and Methods

Cell culture.

PC-3 (CRL-1425), LNCaP (CRL-1740), and MDA PCa 2b (CRL-2422) cell lines were obtained from the American Type Tissue Collection (ATCC, Manassas, VA) at the time of this work and authenticated by morphology, karyotyping, and short tandem repeat profiling-based approaches. Cell lines were also validated by ATCC for absence of mycoplasma contamination. PC-3 cells were maintained in Dulbecco's Modified Eagle Medium (DMEM) supplemented with 10% fetal bovine serum (FBS) and 1% penicillin-streptomycin and L-glutamine (Thermo Fisher Scientific, Waltham, MA). LNCaP cells were grown in Roswell Park Memorial Institute (RPMI) medium with 10% FBS and 1% L-glutamine (Thermo Fisher Scientific). MDA PCa 2b cells were maintained in BRFF-HPC1 medium (Athena ES, Baltimore, MD) supplemented with 20% FBS. RC77 T/E cell line¹¹ was maintained in Keratinocyte-SFM medium with epidermal growth factor 1–53 and bovine pituitary extract (Thermo Fisher Scientific) on plates coated with FNC coating mix (Athena ES). All cell lines were maintained at 37°C and 5% CO₂.

Molecular cloning of *FGFR3* variants.

RNA was purified from cell lines using the QIAgen miRNeasy kit (Valencia, CA) and RT-PCR was performed to amplify *FGFR3-L* and *FGFR3-S* using random hexamers (Thermo Fisher Scientific). 5' and 3' rapid amplification of cDNA ends (RACE) was performed as previously described⁸. RACE primers were designed based on the reference sequence of *FGFR3* provided by the National Center for Biotechnology Information (NCBI) and are listed in Supplemental Table S1. RT-PCR products were ligated into pCR2.1-TOPO TA vector (Thermo Fisher Scientific) according to manufacturer's instructions. Three to 4 clones per cell line were sequence verified (Sequetech, Mountain View, CA) and the

consensus sequence of the full-length *FGFR3-S* variant was deposited in GenBank (Accession #MK542707).

Generation of stable cell lines.

FGFR3-L/V5-His tag and *FGFR3-S/V5*-His tag DNA sequences were synthesized by Genscript (Piscataway, NJ), cloned into pcDNA3.1+, and sequence verified. Vectors were individually transfected into PC-3 and LNCaP cell lines using Lipofectamine 3000 (Thermo Fisher Scientific) according to manufacturer's instructions and stable cell lines were generated using G418S selection as previously described⁸. Equal expression of both variants were determined via RT-PCR and western blot.

Kaplan-Meier survival curve analysis.

Variant expression of *FGFR3-S* and *FGFR3-L* were determined using the method of Kim et al. (2011) on prostate (PRAD), colon (COAD) and breast (BRCA) RNA-seq data from The Cancer Genome Atlas (TCGA). Briefly, variants were calculated through minimizing a weighted non-negative least squares problem based on exon expression. The expression values of *FGFR3-S* and *FGFR3-L* in these patients were then used as predictors to fit the Cox-Proportional Hazards (Cox-PH) regression model for relapse free survival. A prognosis index (PI) score was generated for each patient using the Cox proportional hazards model as reported earlier^{8,12,13}. Patients were dichotomized into high- versus low- *FGFR3-S/FGFR3-L* ratio groups. The log-rank p value was calculated to assess statistical significance between Kaplan-Meier curves of high- versus low- *FGFR3-S/FGFR3-L* ratio groups.

Western blots.

Rabbit polyclonal antibodies to AKT, pAKT (Ser473), and pSTAT3 (Tyr705), rabbit monoclonal antibodies to pAKT (Thr308), ribosomal protein S6 (S6), and pS6 (Ser235/236), and mouse monoclonal antibody to STAT3 were purchased from Cell Signaling (Danvers, MA). Mouse monoclonal antibodies to V5 tag and β -actin were purchased from Santa Cruz Biotechnology (Santa Cruz, CA). Rabbit polyclonal antibody to pFGFR3 (Tyr577) was purchased from Invitrogen (Carlsbad, CA). Horseradish peroxidase-conjugated secondary antibodies for rabbit and mouse IgG were purchased from Southern Biotech (Birmingham, AL).

Cell lysates were collected after incubation of cells with or without growth factor stimulation (20ng/ml FGF2 with 20 μ g/ml heparan sulfate, Sigma, St. Louis, MO), or with or without dovitinib (TKI258) treatment (Selleckchem, Houston, TX) via cell scraper with ice cold PBS. Cell pellets were lysed in 1X cell lysis buffer (Cell Signaling) containing a protease and phosphatase inhibitor (Thermo Fisher Scientific). 150 μ g of lysate was separated using 4–20% precast SDS-PAGE gels (BioRad, Hercules, CA) and transferred to PVDF membranes at 100V for 1hr. Membranes were washed with PBST and blocked for at least 1hr with 5% BSA. Primary antibodies were incubated overnight at 4°C, washed 3X with PBST, and incubated with secondary antibody for at least 1.5hrs at room temperature. After three washes, membranes were incubated with Pierce ECL Western Blotting Substrate (Thermo Fisher Scientific) and exposed to radiography film. Blots were stripped for re-

probing using the *OneMinute* Advance Western Blot Stripping Buffer from GM Biosciences (Frederick, MD).

***In vitro* functional assays.**

Proliferation was assessed using the BrdU Cell Proliferation Assay from Millipore Sigma (Burlington, MA) per manufacturer's instructions. Briefly, 2×10^4 cells were seeded in 100 μ l into 96-well plates. Cells were pretreated with or without dovitinib for 12hrs. Cells were then incubated with BrdU label for 4hrs, fixed and denatured, incubated with anti-BrdU antibody for 1hr, and incubated with peroxidase goat anti-mouse IgG HRP for 1hr. Absorbance at 450–540 wavelengths was measured via SpectraMax MiniMax 300 Imaging Cytometer (Molecular Devices, San Jose, CA).

Migration assays were performed using the Boyden chamber 96-well QCM Chemotaxis Cell Migration Assay (Millipore Sigma) per manufacturer's instructions. Cells were serum starved for 24hrs before 100 μ l of 5×10^4 cells in 0.1% serum medium were seeded into the migration chamber. 150 μ l of 10% serum medium was added to the feeder tray and the appropriate concentration of vehicle or drug was added to both the migration chamber and feeder tray. Cells were incubated at 37°C for 12hrs. The migration chamber plate was incubated in the cell detachment solution for 30min at 37°C, the lysis buffer/dye solution for 15min at room temperature, and read on the SpectraMax MiniMax 300 Imaging Cytometer using a 480/520nm filter set.

For invasion assays, 5×10^4 cells were seeded in the top well of a Matrigel invasion chamber (VWR, Radnor, PA) in 0.1% serum with or without dovitinib. Medium containing 10% serum with or without dovitinib was added to the bottom chamber. After 48hrs, non-invading cells were removed via aspirator and invading cells were fixed and stained with IMEB DIF-QUICK Staining Kit (Thermo Fisher Scientific) per manufacturer's protocol. Invaded cells were counted for each insert under a light microscope.

Apoptotic activity was measured by caspase 3/7-induced luminescence using the Caspase-Glo 3/7 Assay (Promega). PC-3 and LNCaP cells were seeded at 2×10^4 or 1×10^4 , respectively, in 96-well plates and pretreated with or without docetaxel (Selleckchem) for 24hrs. Cells were allowed to equilibrate to room temperature and then incubated with the Caspase-Glo reagent for 1hr. Luminescence was measured via the SpectraMax MiniMax 300 Imaging Cytometer.

***In vivo* xenograft model.**

All experiments were approved by The George Washington University Institutional Animal Care and Use Committee (protocol A272). Male NOD/SCID mice 3–6 weeks of age were purchased from Jackson Laboratory (Bar Harbor, ME). To generate the xenograft model, 2×10^6 PC-3 cells stably overexpressing *FGFR3-L* (PC-3+L) or *FGFR3-S* (PC-3+S) cells were injected subcutaneously into the right hind flank. Once tumors reached a volume of 3mm³, mice were randomized and treated daily *p.o.* with 0.9% saline or 30 mg/kg dovitinib (Selleckchem, Houston, TX). Tumor volume was measured three times a week with calipers. Mice were sacrificed and tumors were dissected after 30 days of treatment.

Immunohistochemistry.

Tissue specimens dissected from mice were paraffin-embedded and mounted by Histoserv, Inc. (Gaithersburg, MD). H&E staining was performed by Histoserv, Inc. Specimens for immunohistochemistry were incubated at 65°C for 30min to remove paraffin. The following washes were then performed in a slide rack: 100% xylene 2X 3min, 1:1 100% xylene:100% ethanol 1X 3min, 100% ethanol 2X 3min, 95% ethanol 1X 3min, 70% ethanol 1X 3min, 50% ethanol 1X 3min, cold running tap water 3min. Slides were immersed in sodium citrate buffer (10mM sodium citrate, 0.05% tween 20, pH6.0) and microwaved for antigen retrieval. Next, slides were blocked with Dual Enzyme Peroxidase (Dako, Carpinteria, CA) and 2.5% BSA. Samples were sequentially incubated with anti-6X His antibody (Abcam, Cambridge, MA) overnight, secondary anti-rabbit and anti-mouse biotinylated IgG (Dako), and streptavidin conjugated to horseradish peroxidase (Dako). Slides were stained with DAB+ chromagen and counterstained with hematoxylin. All images were taken on a Carl Zeiss Cell Observer Spinning Disk Confocal (Thornwood, NY) with a 63X oil immersion objective.

Results

Cloning of *FGFR3* variants.

Affymetrix exon array results from a previous study implied the existence of a previously undiscovered exon 14 skipping event in *FGFR3* that may participate in PCa disparities⁸. PCa specimens from EA patients are predicted to predominantly express the full length, or long (-L) variant, containing exons 1–18. AA PCa specimens, however, are predicted to primarily express a novel short (-S) variant missing exon 14 and retaining all other exons. To confirm the existence of this novel *FGFR3* splice variant, we cloned both full-length long (*FGFR3-L*) and short (*FGFR3-S*) variants from EA and AA cell lines using the 5'- and 3'-RACE method. Sequencing of *FGFR3-S* from MDA PCa 2b and RC77 T/E AA cell lines confirmed the in-frame loss of exon 14, while sequencing of *FGFR3-L* variant from EA cell lines PC-3 and LNCaP confirmed the presence of exon 14 (Fig. 1A). No other differences were identified between the two variants. Exon 14 codes for the 41 amino acid activation loop located within the C-terminal intracellular kinase domain of FGFR3 (Fig. 1B). This loop conceals the substrate binding pocket and is involved in shifting the kinase from an inactive to active conformation when stimulated by agonist binding. Thus, we hypothesized that loss of this critical molecular brake may increase FGFR3 signaling and promote a more oncogenic phenotype in AA PCa.

FGFR3-S expression correlates with poor PCa patient prognosis.

To assess the clinical relevance of the *FGFR3-S* variant, prostate, colon, and breast cancer patient expression data from the TCGA database was analyzed for *FGFR3-S/-L* expression ratios in relation to patient survival. A significant decrease in survival ($p < 0.05$) was observed in patients with PCa specimens harboring a high *FGFR3-S/-L* expression ratio ($n=550$) (Fig. 2). While trending towards significance in colon cancer patients, neither colon ($n=328$) nor breast ($n=1,203$) cancer patient survival significantly correlated with the *FGFR3-S/-L* expression ratio. Patient groups were not stratified by race/ethnicity due to insufficient information.

FGFR3-S oncogenic signaling is more active and resistant to dovitinib compared to FGFR3-L.

In order to assess potential differences in oncogenic signaling by FGFR3 isoforms, we generated a panel of stable EA PCa cell lines. PC-3 and LNCaP cells overexpressing FGFR3-S (PC-3+S and LNCaP+S) or FGFR3-L (PC-3+L and LNCaP+L) were treated with vehicle or 20ng/ml FGF2 with 20µg/ml heparin sulfate and subjected to western blot analysis. Equivalent levels of FGFR3 isoform expression were confirmed in each cell line with a V5-tag antibody (Fig. 3A). At baseline, PC-3+S and LNCaP+S cells (collectively referred to as +S cells) exhibited a significant 2.4- to 3.1-fold greater phosphorylation of Tyr577, a residue that is a marker of receptor tyrosine kinase activity¹⁴, compared to PC-3+L and LNCaP+L cells (+L cells) (Fig. 3A, Supplemental Table S2). Moreover, baseline +S cells displayed a significant 1.4- to 4.9-fold greater phosphorylation of downstream signaling proteins AKT (Ser473) and ribosomal protein S6 (S6) (Ser235/236) compared to +L cells. When +L cells were stimulated with FGF2 treatment, phosphorylation of FGFR3, AKT, and S6 was significantly increased by 1.6–2.5 fold over baseline (Fig. 3A, Supplemental Table S2). In contrast, FGF2 stimulation of +S cells did not further increase phosphorylation of FGFR3, AKT, and S6 over baseline (Fig. 3A, Supplemental Table S2). These results suggest that FGFR3-S-mediated downstream signaling at baseline is already maximal since agonist stimulation of FGFR3-S did not further increase signaling.

Next, we analyzed the effect of the SMI dovitinib (TKI258) on downstream signaling. Dovitinib is a multi-kinase inhibitor that targets FGFR1–3, VEGFR1–3, PDGFRβ, c-Kit, and FLT3 at nanomolar levels. In overall agreement with earlier western blot results (Fig. 3A), baseline (e.g. vehicle treatment) phosphorylation of FGFR3, AKT, STAT3, and S6 was significantly higher in +S cells compared to +L cells (Fig. 3B, Supplemental Table S3). Interestingly, we observed no significant differences in baseline phosphorylation of ERK1/2 between +S and +L overexpressing cells (Supplemental Table S3). Upon treatment of +L cells with dovitinib, a statistically significant 50–67% decrease in FGFR3-L autophosphorylation occurred at Tyr577, as well as a 30–60% decrease in phosphorylation of AKT, STAT3, and S6 (Fig. 3B, Supplemental Table S3). In contrast, the phosphorylation status of FGFR3-S, AKT, STAT3, and S6 in +S cells treated with dovitinib were comparable to vehicle treated cells (Fig. 3B, Supplemental Table S3). These data demonstrate that FGFR3-S activity and downstream signaling are resistant to dovitinib treatment.

FGFR3-S confers an oncogenic advantage *in vitro*.

To better understand the effects of the short isoform on oncogenesis, we first measured BrdU incorporation as a marker of cell proliferation. PC-3+S and LNCaP+S cells exhibited a significant 2- to 3-fold higher baseline proliferative capacity compared to PC-3+L and LNCaP+L cells, as well as vector control cells (PC-3+vector, LNCaP+vector) (Fig. 4A). We then tested the effects of dovitinib treatment on proliferation. In +L cells, dovitinib dose-dependently inhibited proliferation with IC₅₀ values of $0.90 \pm 0.28 \mu\text{M}$ and $0.39 \pm 0.28 \mu\text{M}$, respectively (Fig. 4B,C; Supplemental Table S4). By comparison and remarkably, +S cells were relatively resistant to dovitinib-mediated inhibition of proliferation with a rightward shift in the dose-response curves and IC₅₀ values of $7.53 \pm 2.23 \mu\text{M}$ and $11.14 \pm 0.83 \mu\text{M}$, respectively.

Next, we employed the Boyden chamber assay to measure migratory activity. Baseline migration was significantly higher in +S cells compared to +L and +vector cells (Fig. 5A). By comparison, +S cells were relatively resistant to dovitinib inhibition, displaying significantly greater migratory capacity compared to +L cells at 4 out of 6 tested inhibitor concentrations (Fig. 5B,C). Moreover, dovitinib exhibited a 4- to 6-fold higher IC_{50} in +S compared to +L cells (Supplemental Table S4). These data support the notion that the short isoform is more oncogenic and relatively more resistant to SMI treatment compared to the long isoform.

Given that +S cells exhibited greater migratory capacity compared to +L cells, we next evaluated the invasive potential in these lines as measured by Matrigel assay at both baseline and with dovitinib treatment. Baseline invasion between +S and +L cells was not significantly different, whereas these cell lines exhibited a significant 1.8- to 2.6-fold greater ability to invade compared to +vector cells (Fig. 5D). The invasive capacity of +S cells appeared to be moderately more resistant to the inhibitory effects of dovitinib compared to +L cells, albeit significant differences in invasion were observed only at inhibitor concentrations of 1 and 10 μ M in the PC-3+S line and 0.1 μ M in the LNCaP+S line (Fig. 5E,F). Notwithstanding, the IC_{50} values of dovitinib in +S cells was approximately 6-times higher compared to +L cells (Supplemental Table S4).

To examine the effects of *FGFR3* splice variants on apoptosis, we treated +vector, +L, and +S cells with docetaxel, a standard chemotherapy treatment for PCa, and measured caspase 3/7 activity via a luminescence assay. At baseline, +S cells exhibited a significant 50–70% decrease in caspase 3/7 activity compared to +L and +vector cells, indicating that FGFR3-S imparts resistance to apoptosis (Fig. 6A). Both +vector and +L cells demonstrated increasing caspase 3/7 activity (i.e. apoptosis) with increasing doses of docetaxel (Fig. 6B,C,E,F). Surprisingly, PC-3+S and LNCaP+S cells were completely resistant to docetaxel-induced apoptosis (Fig. 6D,G). Moreover, the combination of docetaxel and dovitinib was likewise completely ineffective in inducing apoptosis in +S cells (Supplemental Fig. S1).

FGFR3-S is relatively resistant to dovitinib in a xenograft model of tumor growth.

The above findings indicate that the FGFR3-S isoform is more oncogenic and exhibits resistance to SMI and taxane treatment compared to FGFR3-L. Furthermore, FGFR3-S imparts greater proliferative and anti-apoptotic gains while offering no invasive advantage over FGFR3-L *in vitro*. Hence, we employed a xenograft mouse model to investigate the effects of *FGFR3* splice variants on tumor growth *in vivo*. We evaluated tumor growth by subcutaneous injection of 2×10^6 PC-3+S or PC-3+L cells into the right hind flank of 3–6 week old NOD/SCID male mice. When tumors grew to a size of 3mm^3 , 30 days of daily *p.o.* treatment with 0.9% saline or 30mg/kg dovitinib was initiated. Mice injected with PC-3+S cells developed tumors significantly earlier than mice injected with PC-3+L cells, 16.4 ± 0.67 versus 20 ± 0.55 days, respectively (Fig. 7A). Dovitinib treatment significantly reduced the tumor volume in mice injected with PC-3+L compared to animals receiving saline treatment (Fig. 7B,C,D). At day 30, the average PC-3+L tumor volume in mice treated with dovitinib was decreased more than 3-fold compared to mice treated with saline (Fig. 7B,C,D). In contrast, dovitinib treatment had no significant effect on tumor volume in mice

injected with PC-3+S cells compared to matching animals treated with saline. Additionally, the tumor volume in saline-treated mice with PC-3+S cells was 2.4 times greater than tumors in saline-treated mice harboring PC-3+L xenografts at day 30 (Fig. 7B,C,D). All tumor specimens showed positive staining with an anti-His tag antibody via immunohistochemistry, confirming that tumors were derived from PC-3+L or PC-3+S cells (Fig. 7E). H&E staining showed comparable areas of necrosis, neutrophil infiltration, increased abnormal mitotic events, and invasion of tumor cells into surrounding muscle and adipose tissue in all groups (Fig. 7E).

Discussion

While genomics (e.g. global gene expression, mutation screening) and genetic studies (e.g. genome-wide association or GWAS) focusing on PCa disparities have increased over the past 10 years, the RNA splicing landscape has not been adequately examined as a potential mechanism for ancestry-related PCa aggressiveness. Recent work from our laboratory has highlighted genome-wide dAS events numbering in the thousands that occur specifically or preferentially in AA PCa⁸. Less forthcoming has been functionally validated examples of AA-specific/enriched splice variants and corresponding protein isoforms that promote PCa aggressiveness. A handful of examples have been described in the literature, including a resistance phenotype exhibited by PIK3CD-S to SMIs⁸, hnRNPH1-mediated regulation of the AR-V7 isoform⁹, and promotion of stemness in AA PCa cell lines by the MBD2_v2 isoform¹⁰. We now add FGFR3 to this growing list of functionally validated AA-specific/enriched splice variants participating in enhanced oncogenicity.

FGFR3 alterations (mutations, overexpression, gene fusions) have been identified in many cancers, including multiple myeloma, bladder, urothelial, cervical, breast, lung, and prostate¹⁵ (Supplemental Fig. S2). Recent studies have underscored the importance of FGFR signaling in PCa, including AR-dependent and AR-independent PCa^{16,17}. Previously, *FGFR3* mutations have been linked to low-grade PCa. Examination of exons 7, 10, and 15 of *FGFR3* have revealed polymorphisms (F386L), missense mutations (S249C, F384L, F386L, A393E), and frameshift mutations in low-risk or low-grade PCa¹⁸⁻²⁰. Other studies, however, have found no *FGFR3* mutations in PCa specimens when analyzing these hotspot mutation sites^{21,22}.

The roles of FGFR subtypes (FGFR1–4) and their various isoforms encoded by AS variants in PCa development, progression, and/or disparities are currently poorly understood. The extracellular domains of FGFR1–4 consist of three Ig-like loops that are involved in regulating ligand binding. Splice switching of the third Ig-like domain of FGFR1–3, but not in FGFR4, has been reported. Switching of *FGFR2* from the IIIb to IIIc variant has been associated with PCa progression. The FGFR2-IIIb isoform is exclusively expressed in normal prostate epithelial and androgen-sensitive cancerous prostate tissue, while FGFR2-IIIc is predominantly expressed in androgen-insensitive PCa²³. This splice switching event is functionally associated with distinct ligand binding patterns, namely decreased affinity for FGF7 (secreted by stromal cells to promote homeostasis) and increased affinity for FGF8b, as well as distinct functional consequences such as increased epithelial to mesenchymal transition, increased proliferation, decreased differentiation, and decreased apoptosis.

The cloning of the *FGFR3-S* variant represents the first example of an AS event (or more specifically an exon skipping event) involving the tyrosine kinase domain in the FGFR family and in non-receptor tyrosine kinases (nRTKs). Three other splicing events within *FGFR3* have been previously identified, but all take place within the extracellular Ig-like domains: IIIb (inclusion of exon 8, exclusion of exon 9), IIIc (inclusion of exon 9, exclusion of exon 8), and 7–9 variant (skipping of exons 7–9)^{24,25}. To the best of our knowledge, exon skipping events involving the kinase domain have only been identified in one other RTK, RON. The kinase defective RON 170 isoform is missing exon 19 due to a nucleotide polymorphism in intron 18, resulting in a frameshift and a premature stop codon^{26,27}. Skipping of exons 15–19, 16–19, 16–17, 16, 19, and 18–19 corresponding to the kinase domain of RON have been identified, but not functionally characterized^{28,29}.

Prior studies have also highlighted the clinical significance of nRTKs splice alterations in cancer. A splice variant of the BCR-ABL fusion protein, *BCR-ABL35INS*, retains 35 intronic nucleotides, causing a frameshift and pre-mature termination³⁰. This variant encodes an inactive fusion isoform that is associated with poor patient outcome to therapy with imatinib (a TKI used to treat chronic myelogenous leukemia) by mechanisms unknown³¹. In melanoma, a 61 kDa isoform encoded by a *BRAF* splice variant missing exons 4–8 lacks the RAS-binding domain, resulting in constitutive isoform dimerization and kinase activity and resistance to the inhibitor vemurafenib³². A splice variant of *BIM* (*BIM-γ*), encoding a protein that lacks the BH3 domain, has been identified as a mechanism for TKI (e.g. imatinib, gefitinib) resistance in chronic myeloid leukemia^{33–35}. It should be noted that none of these splicing events occur within the tyrosine kinase domain.

The skipping of exon 14 in *FGFR3-S* leads to the loss of the activation loop (A-loop), a 41 amino acid dynamic loop that, in the inactive conformation, covers the substrate binding pocket of the C-terminal intracellular tyrosine kinase domain³⁶. The A-loop contains two key Tyr residues (Tyr647, Tyr648) that, when phosphorylated will: 1) disengage the kinase domain's molecular brake and help stabilize the kinase in the active state; 2) upregulate kinase activity; and 3) expose the docking site for interactions with downstream signaling proteins. Activating mutations in the A-loop, such as K650E, can generate constitutive FGFR3 signaling by stabilizing the A-loop in the active state, even in the absence of phosphorylation of the two key tyrosine residues^{37–39}.

FGFR3 dimers, stabilized by transmembrane and intracellular domain interactions have been shown to exist in the absence of ligand, as per the pre-formed RTK model⁴⁰. Receptor dimerization upon binding of FGF2 results in a tight dimerization structure and significant increases in receptor autophosphorylation⁴⁰. Pathogenic *FGFR3* mutations located in the transmembrane region(s) (e.g. A391E) can trap the receptor in a closely packed dimer structure more frequently, thus increasing baseline receptor phosphorylation even in the absence of ligand binding. The immediate downstream signaling ramifications of such an alteration remain unknown. Increased time in the active state decreases the efficacy of inhibition by SMIs, including dovitinib, that preferentially bind the inactive form of FGFRs^{36,39}. Based on our *in vitro* and *in vivo* results, we hypothesize that FGFR3-S “samples” the active conformation more frequently than FGFR3-L, and, as a result, dovitinib has a lower affinity for FGFR3-S. This would explain the decreased sensitivity of the short

isoform to dovitinib treatment compared to the long isoform. It will be of interest to compare FGFR3-L and FGFR3-S in terms of dimer structure, receptor orientation, transmembrane separation, conformation sampling, and monomer versus dimer status in future studies.

Dovitinib is a multi-target RTK SMI that has shown anti-tumor activity in preclinical and clinical models of solid tumors, including PCa⁴¹⁻⁴⁴. While dovitinib failed a critical Phase III trial in metastatic renal cell carcinoma, recent efforts have been taken to identify and select potential responders (e.g. patients with driver FGFR alterations) in future clinical trials. Our work suggests dAS of *FGFR3* may also need to be taken into account when identifying candidates who will or will not benefit from FGFR targeted therapies. Going forward, we posit that FGFR molecular profiling for both mutations and splice variants will be crucial in identifying whether particular FGFR inhibitors will be an appropriate therapeutic choice^{39,45}.

FGFR3-S appears to drive gains in proliferation and anti-apoptosis to a greater extent than invasion. Indeed, western blot analysis revealed significant increased baseline activation of AKT, S6, and STAT3 in FGFR3-S compared to FGFR3-L overexpressing cells. These differences in phosphorylation support the large differences observed in proliferation and caspase 3/7 activity between the two isoforms. Both the PI3K/AKT/S6 and STAT pathways are known to promote proliferation and inhibit apoptosis in PCa cells^{46,47}. Additionally, STAT3 has been shown to promote chemotherapeutic resistance to autophagy⁴⁸. Upstream regulators for both pathways, including RTKs and cytokine receptors, have been demonstrated to promote inflammation and proliferation and suppress apoptosis in PCa⁴⁹. The more modest differences in migration and the total absence of invasive differences imparted by the FGFR3-L and FGFR3-S isoforms correlate with the lack of significant differences in phosphorylation of ERK1/2 when comparing FGFR3-L and FGFR3-S overexpressing cells. The RAS/MAPK pathway is a known contributor to PCa invasion and migration⁵⁰.

Future experiments exploring differences in FGFR3-L and FGFR3-S signaling should focus on cross-talk with AR signaling and/or cytokine receptor signaling (e.g. IL-6 receptor, IL-8 receptor). AR-dependence can be bypassed in PCa cell lines and tumor specimens by elevating FGFR pathway activity¹⁶ and the status of AR signaling in AR-positive PCa cells (such as EA LNCaP and AA MDA PCa 2b) ectopically over-expressing FGFR3-S is currently unknown. The association of IL-6 in inflammation and PCa progression is well characterized and understanding changes in FGFR3-L versus -S signaling in the presence of cytokines will be important for understanding FGFR3-S-promoted oncogenicity. In addition, investigating the efficacy inhibitors that target FGFRs with gatekeeper mutations (e.g. mutations within the ATP binding pocket such as V555M) should provide further insights into SMI resistance exhibited by FGFR3-S.

The FGFR signaling pathway may play an important role in cancer (health disparity) risk, development, progression, and therapeutic response. We have identified a novel *FGFR3* splice variant that may serve as a potential biomarker for cancer health disparities, cancer aggressiveness, and/or SMI resistance. Development of future targeted therapies and

selection of treatments will need to take into account the receptor splice variant expression pattern. This study highlights the consequential role of RNA splicing in cancer and, more specifically, in cancer health disparities.

Supplementary Material

Refer to Web version on PubMed Central for supplementary material.

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Abbreviations:

PCa	prostate cancer
AS	Alternative splicing
FGFR3	fibroblast growth factor receptor 3
AA	African American
EA	European American

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Implications

This work identifies a novel *FGFR3* splice variant and supports the hypothesis that differential AS participates in PCa health disparities.

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Fig 1. Cloning of *FGFR3* splice variants.

FGFR3 splice variants were cloned by RACE from AA and EA PCa cell lines. A) Portion of consensus sequence of *FGFR3-L* containing exon 14 and *FGFR3-S* with in-frame skipping of exon 14 (123bp). Amino acid sequence of exon 14 is shown in red. B) Schematic of *FGFR3* gene and generation of splice isoforms. Inclusion of exon 14 (top) generates the long isoform (FGFR3-L) containing the activation loop (AL) in the C-terminal kinase domain. Exon skipping of exon 14 results in a shorter isoform (FGFR3-S) that is missing the AL and Tyr647 and Tyr648. Key: Ig-like domains (grey), transmembrane domain (TM) (blue), tyrosine kinase domains (TK) (green), activation loop (AL) (orange), tyrosine residues that are phosphorylated for kinase activation (red flags).

Fig 2. Prostate cancer patients with low *FGFR3-S/-L* ratios have increased survival. Kaplan-Meier survival curves plotting disease-free survival of prostate cancer (n=550), colon cancer (n=328), and breast cancer (n=1,203) patients with high or low *FGFR3-S/-L* expression ratios. RNA-Seq data was obtained from The Cancer Genome Atlas (TCGA; <https://cancergenome.nih.gov/>). Significance determined by the log-rank test, * $p < 0.05$.

A) Western blot analysis of FGFR3, AKT, and S6 phosphorylation in LNCaP and PC-3 cells overexpressing FGFR3-L (+L) or FGFR3-S (+S) with and without FGF2 + heparin sulfate treatment. B) Western blot analysis of FGFR3, AKT, STAT3, and S6 phosphorylation in LNCaP and PC-3 cells overexpressing FGFR3-L (+L) or FGFR3-S (+S) with and without dovitinib treatment. Images are representative of n=3–4 independent experiments. Relative phospho-protein levels were normalized to total protein levels. Values underneath blots indicate fold-change phospho-protein levels relative to +L in the absence of FGF2 + heparin sulfate or dovitinib.

Fig 4. FGFR3-S is relatively resistant to dovitinib-mediated inhibition of proliferation.

A) Baseline proliferation via BrdU assay in EA cell lines PC-3 and LNCaP ectopically overexpressing vector control, FGFR3-L, or FGFR3-S. Relative proliferation in PC-3 (B) and LNCaP (C) overexpressing FGFR3-L and FGFR3-S following treatment with increasing concentrations of dovitinib. Data presented as mean $\pm$ SEM of n=3–4 independent experiments and analyzed by one-way ANOVA with Tukey post-hoc test, *p<0.05 (A) or unpaired t-test (B and C) *p<0.05 (significantly different from corresponding +L isoform).

Fig 5. FGFR3-S is relatively resistant to dovitinib-mediated inhibition of migration and invasion.

A) Baseline migration measured via Boyden chamber assay of PC-3 and LNCaP cells overexpressing +vector, +L, or +S. Relative migration in PC-3 (B) and LNCaP (C) overexpressing FGFR3-L and FGFR3-S following treatment with increasing concentrations of dovitinib. D) Baseline invasion measured via Matrigel assay of PC-3 and LNCaP cells overexpressing +vector, +L, or +S. Invaded cells shown as percent of vehicle-treated PC-3 (E) and LNCaP (F) cells overexpressing FGFR3-L or FGFR3-S following treatment with increasing concentrations of dovitinib. Data presented as mean $\pm$ SEM of n=3–4 independent experiments, and analyzed by one-way ANOVA with Tukey post-hoc test, *p<0.05 (A and D) or unpaired t-test (B,C,E and F), *p<0.05 (significantly different from corresponding +L isoform).

Fig 6. FGFR3-S is resistant to docetaxel-mediated apoptosis.

A) Baseline apoptotic activity was measured via caspase 3/7 luminescence assay in EA cell lines PC-3 and LNCaP ectopically overexpressing vector control, FGFR3-L, or FGFR3-S. Relative caspase 3/7 activity in PC-3 and LNCaP cells overexpressing vector (B,E), FGFR3-L (C,F), or FGFR3-S (D,G) following treatment with increasing concentrations of docetaxel. Data presented as mean $\pm$ SEM of n=3–6 independent experiments, and analyzed by one-way ANOVA with Dunnett post-hoc test, *p<0.05.

Fig 7. FGFR3-S is resistant to dovitinib-mediated inhibition of tumor growth.

A) Number of days for tumor xenografts from subcutaneous injection of PC-3+L or PC-3+S cells to reach 3mm³. Data presented as mean ± SEM of n=20–21 animals, and analyzed by unpaired t-test, *p<0.05. B) Day 30 tumor volumes in dovitinib-treated animals plotted as percent of tumor volumes in saline-treated animals. Data presented as the mean ± SEM of n=10–11 animals and analyzed by unpaired t-test, *p<0.05 or ANOVA, #p<0.05 (significantly different from corresponding saline treatment). C) Volume of PC-3+L or PC-3+S tumor xenografts from day 1–30 of daily *p.o.* treatment of 0.9% saline or 30 mg/kg dovitinib. Treatment commenced when tumor volume reached 3mm³ (Day 1). Data presented as mean ± SEM of n=10–11 animals, and analyzed by one-way ANOVA with Tukey post hoc test. *p<0.05, significantly different from corresponding saline treatment. Representative tumor xenografts (D) and H&E staining and immunohistochemistry with His tagged receptor (brown), hematoxylin (blue), and eosin (red) (E) from each treatment group dissected following day 30 of treatment.