Whole-transcriptome Analysis Reveals Established and Novel Associations with TMPRSS2:ERG Fusion in Prostate Cancer

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Abstract. Background/Aim: Shortcomings of current methods of prostate cancer detection call for improved biomarkers. The transmembrane protease, serine 2:ets-related gene (TMPRSS2:ERG) gene fusion leads to the overexpression of ERG, an E-twenty six (ETS) family transcription factor, and is the most prevalent genetic lesion in prostate cancer, but its clinical utility remains unclear. Materials and Methods: Two radical prostatectomy samples were analysed by next-generation whole-transcriptome sequencing. The chosen samples differed in fusion gene status, as previously determined by reverse transcription polymerase chain reaction (RT-PCR). Results: Next-generation sequencing identified the involvement of novel and previously reported prostate cancer-related transcripts, the WNT signalling pathway, evasion of p53-mediated anti-proliferation and several ETS-regulated pathways in the prostate cancer cases examined. Overexpression of Rho GDP-dissociation inhibitor (RhoGDIB), a gene associated with fusion-positive prostate cancer, was found to elicit spindle-shaped morphology, faster cell migration and increased cell proliferation, phenotypic changes suggestive of cancer progression. Conclusion: The present findings confirm the value of comprehensive sequencing for biomarker development and provide potential avenues of future study.

Prostate cancer is the most prevalent type of cancer affecting Canadian men, yet current non-invasive methods for its detection, such as the digital rectal exam and serum prostate-specific antigen (PSA) measurement, are inadequate – in fact, the U.S. Preventive Services Task Force recently assigned PSA-based screening for prostate cancer a grade D recommendation, which represents the lowest possible confidence in the test’s ability to provide a net clinical benefit (1). This decision was motivated by the fact that these measures cannot distinguish between benign hypertrophy and carcinoma (2). In fact, no existing methods can predict whether a particular instance of the disease is likely to recur after treatment or metastasize (3), so there is a great need for the identification and testing of novel biomarkers by which prostate cancer prognosis may be improved.

In humans, the E-twenty six (ETS) family of transcription factors comprises of 27 members which share a highly conserved DNA-binding motif known as the ETS domain (4). Recently, these proteins were organised into four classes based on differences in their DNA binding profiles (5). They respond to a variety of signals and participate in diverse processes, including development, differentiation, cell cycle control and apoptosis (5), and are therefore also often implicated in disease, including prostate cancer (6).

One particular ETS member, ets-related gene (ERG), participates in the direct transactivation of genes associated with cancer, including vascular endothelial (VE)-cadherin, frizzled-4 and ras homolog J (RHOJ) (7-9). ERG is frequently involved in genomic lesions characteristic of diseases such as Ewing sarcoma, acute myeloid leukemia and prostate cancer (10). Furthermore, ERG overexpression on its own has been shown to be sufficient to transform mouse fibroblast cells in vitro and in vivo (11).

Transmembrane protease, serine 2 (TMPRSS2):ERG gene fusion causes the expression of ERG to become androgen-sensitive, due to a gene re-arrangement event that relocates ERG proximal to the androgen-responsive element of the TMPRSS2 promoter (12). Its occurrence in approximately half of all prostate carcinomas (13) has made it a promising biomarker for the disease. Indeed, its detection in biopsies (14), urine (15) and serum (16) is being actively investigated for diagnostic and prognostic benefit, although there is evidence that contests its predictive ability (17). Improved understanding of the mechanisms by which TMPRSS2:ERG contributes to prostate cancer initiation and progression is therefore necessary to evaluate its potential as a clinically relevant biomarker.
Also of interest is Rho GDP dissociation inhibitor β (RhoGDIB), whose expression has been positively correlated with TMPRSS2:ERG fusion status (18). As Rho activity is strongly implicated in cell motility and metastasis (19), RhoGDIB and its gene product are liable to be important mediators of TMPRSS2:ERG fusion-related prostate cancer.

In this study, the Applied Biosystems SOLiD next-generation sequencing system was used to assess the prostate cancer relevance of TMPRSS2:ERG fusion by analysing total mRNA transcription from two radical prostatectomy samples (20). The chosen samples were alike in their aggressiveness but differed in fusion gene status. SOLiD sequencing yields data comparable to those obtained through oligonucleotide microarrays and has the advantage of being better-able to identify novel transcripts, sequence variations and splicing events (21). Individual and comparative analysis of our selected transcriptomes is expected to yield insights into transcriptional events that contribute to prostate cancer, which may be applicable to biomarker development. In addition, in vitro examination of a potential ERG target gene, RhoGDIB, was performed to validate one such transcriptional point of interest.

Materials and Methods

RNA samples. Out of 139 radical prostatectomy samples that had previously been determined to differ in fusion gene status by reverse transcription polymerase chain reaction (RT-PCR) (20) and analysed by Illumina’s 502-gene Human Cancer Panel (18), two were selected for whole-transcriptome analysis. A sample of tissue was selected for RNA extraction, as previously described (20). Briefly, specimens were snap-frozen in liquid nitrogen upon collection, quarried, and sectioned into 5-μm slices. Haematoxylin and eosin-stained sections were analysed by the pathologist, who marked regions of tumour from which RNA was extracted using Trizol Reagent (Invitrogen Canada, Burlington, ON, Canada). RNA concentration and integrity were determined via Nanodrop 1000 (Nanodrop, Wilmington, DE, USA) spectrophotometry and Agilent 2100 Bioanalyzer with the RNA6000 Nano chip (Agilent Technologies Canada, Pickering, ON, Canada), respectively, according to manufacturers’ instructions. Poly(A) RNA was then selectively enriched from the total RNA using the MicroPoly(A) Purist kit (Life Technologies, Carlsbad, CA, USA). This enrichment was performed in two rounds to minimize rRNA contamination in the poly(A) RNA fraction. The absence of 18S and 28S rRNA was confirmed using the Agilent 2100 Bioanalyzer with the RNA 6000 Pico kit.

SOLiD system whole-transcriptome sequencing. Whole-transcriptome next-generations sequencing (NGS) was performed according to the manufacturer’s instructions on an Applied Biosystems SOLiD System (Life Technologies). Briefly, Poly(A) RNA from two samples was fragmented using RNase III, ligated to P1 and P2 adaptors and reverse-transcribed into a cDNA library. This library was then size-selected using the Agencourt Ampure XP reagent (Beckman Coulter, Indianapolis, IN, USA) and amplified by PCR with barcoded primers using the SOLiD RNA Barcoding Kit (Applied Biosystems). The concentrations of transcripts were quantified in triplicate using the SOLiD Library TaqMan Quantitation Kit (Applied Biosystems). Resultant barcoded cDNA libraries were pooled together in equal concentrations, driven onto beads to generate bead clones by emulsion PCR, then deposited on a glass slide. Paired-end (50 bp + 35 bp) sequencing was conducted using the SOLiD analyzer.

Sequencing data analysis. Resultant data were filtered for colourspace quality, depleted of non-mRNA reads and mapped to the NCBI B36 human genome assembly using the Geospiza software package (Geospiza, Seattle, WA, USA; www.geospiza.com). Secondary analysis normalised read counts to reads per million (RPM), identified sequence variations and matched data with Ref Seq ID. Genesifter software version 4.1 (www.genesifter.net) was used for statistical analysis in terms of relative expression, alone or organised into gene ontologies and Kyoto Encyclopedia of Genes and Genomes (KEGG)-curated pathways.

Plasmids. A 1234-bp region immediately upstream of the RhoGDIB translation start site was amplified from VCaP genomic DNA using Phusion High-Fidelity DNA Polymerase (New England Biolabs Canada, Pickering, ON, Canada) and cloned using XhoI and BglII restriction sites into a luciferase reporter vector, pGL4.28 (Promega, Madison, WI, USA), to generate pGL-RGDBpro-Luc. A full-length RhoGDIB protein coding region was amplified and cloned using BamHI and EcoRI restriction sites into pCMV-Tag 2B (Agilent) to generate pCMV-RhoGDIB. An expression construct designed to produce the TMPRSS2:ERG fusion protein (22) was cloned using HindIII and XhoI restriction sites into pCMV-Tag 2B, yielding pCMV-ANERG. pCMV-nuRGerg was generated from pCMV-ΔNERG by removal of the 255-bp sequence that codes for the ETS domain. SalI restriction sites were formed at both ends of the target sequence using PfuTurbo DNA polymerase (Agilent) amplification of the pCMV-ANERG template.

Cell culture. HEK 293 cells were purchased from the American Type Culture Collection (ATCC, Manassas, VA, USA) and grown in Dulbecco’s modified Eagle’s medium (DMEM) (Life Technologies), supplemented with 10% fetal bovine serum (FBS); PC3 cells were purchased from ATCC; they and their derivatives were grown in DMEM/F12 (1:1) (1) (Life Technologies) supplemented with 10% FBS (complete medium). PC3-7C were derived from PC3 (prostate bone metastasis cells) via transfection with pCMV-RhoGDIB using the Lipod 293 transfection reagent (SignaGen, Rockville, MD). Importantly, PC3 do not harbour the TMPRSS2:ERG gene fusion (23). Stable selection was performed by culturing cells in complete medium with 300 μg/ml geneticin beginning 48 h post-transfection. Surviving colonies were separately grown in a 96-well plate and assayed for Flag-Rhodib expression by western blots.

Luciferase reporter assay. Two days before assay, 5×10⁴ HEK 293 cells were plated in a 24-well plate and cultured overnight. Cells were then transfected with either 250 ng each PGL-RGDBpro-Luc and pCMV-ΔNERG or pGL-RGDBpro-Luc and pCMV using Lipod 293, as above. Luciferase activity was assayed the following day, using the Luciferase Assay System (Promega), according to the manufacturer’s instructions. Briefly, cells were lysed in 40 μl Passive Lysis Buffer; 20 μl of this lysate were then added to 100 μl Luciferase Assay Reagent. Luciferase activity from the reaction was measured over 10 sec with a Lumat LB 9501 luminometer (Berthold Technologies, Oak Ridge, TN, USA).
Wound-closure assay. One day before assay, 2.4×10^5 PC3 and PC3-7C cells were plated in a 6-well plate and cultured overnight. Cells were then scratched with a 10-μl micropipette tip, rinsed once with complete medium, and cultured in 2 ml complete medium with 6 μl 500 mM hydroxyurea. Images were captured using the AxioVert software (Carl Zeiss Canada, Toronto, ON, Canada) over the subsequent 48 h.

3H-Thymidine assay. PC3 and PC3-7C cells were transiently transfected with pCMV-ΔNERG as described above and used for experiments after 48 h. In a 96-well plate, 5,000 PC3 and PC3-7C cells, with or without pCMV-ΔNERG transfection, were plated in triplicate in 100 μl complete medium and cultured for 24 or 72 h, after which time 10 μl 1 μCi/ml 3H-thymidine in PBS was added to each well. After 6 h, cells were harvested and scintillation counts were performed. Differences were assessed by one-way analysis of variance (ANOVA) and the Tukey’s post-hoc test using GraphPad Prism 5 (GraphPad Software, La Jolla, CA, USA); p<0.05 was deemed significant.

Results

Primary analysis of whole-transcriptome sequencing data. To investigate transcriptional characteristics of TMPRSS2:ERG fusion-dependent and -independent prostate cancer, RNA from the two samples were subjected to whole-transcriptome sequencing using the SOLiD System. Over 50 million reads were obtained from each sample and 75.99% of these were mapped to the NCBI B36 genome assembly; approximately 40% of all reads mapped to exons and introns, while only 7.43% of reads mapped to intergenic sequences (Table I).

Between the two samples, 23,187 genes were identified with at least one read in one sample, representing 88.8% of genes in the database. After filtering-out genes with fewer than 10 mapped reads, 19,625 remained for further analysis. Of these, 5,885 were significantly differentially expressed by more than two-fold between samples, as determined by the Genesifter software, version 4.1 (Figure 1).

ETS, ETS-targeted genes and established prostate cancer-related transcripts were differentially expressed. Many ETS family members are implicated in prostate cancer (6) and in this study, they and several target genes were represented in the group of very highly expressed genes. Ets variant 1 (ETV1) expression was higher in the TMPRSS2:ERG fusion-negative sample while early growth response protein 1 (EGR1) expression was higher in the fusion-positive sample (Table II). RhoGDIB stood out for being expressed 14.16-fold more in the fusion-positive sample (Figure 2A). These findings stress the involvement of ETS pathways in prostate cancer.

Also represented in a survey of genes that were highly expressed (RP >500) in either sample or both were a host of genes and transcripts reportedly associated with prostate cancer, including metastasis-associated lung adenocarcinoma transcript 1 (MALAT1), transmembrane BAX inhibitor motif containing 6 (TMBIM6), cathepsin B (CTSB) and FBJ murine osteosarcoma viral oncogene homolog (FOS) (Table II).

RhoGDIB promoter region contains multiple ETS binding sites (EBS) and responds to ΔN-ERG overexpression. The striking difference in the expression of RhoGDIB (Table II), its previous associations with the TMPRSS2:ERG gene fusion and the presence of multiple putative EBS upstream of the RhoGDIB gene (Figure 2B), motivated us to examine whether RhoGDIB expression is regulated by the ERG protein. To this end, pGL-RGBpro-Luc, which positions the RhoGDIB promoter region upstream of the luc2CP reporter gene (Figure 2C), was co-transfected with either pCMV, pCMV-ANERG or pCMV-mutERG, into HEK 293 cells. Co-transfection with pCMV-ANERG caused an increase in luciferase reporter gene activity relative to pCMV and pCMV-mutERG (Figure 2D), suggesting that the ERG protein is sufficient and the ETS domain is necessary for inducing RhoGDIB expression.

RhoGDIB overexpression increases prostate cancer cell motility and rate of proliferation. To determine the consequences of RhoGDIB overexpression, PC3-7C, a RhoGDIB-overexpressing cell line, was derived by cloning full-length RhoGDIB into pCMV-Tag 2B and stably transfecting the recombinant vector into PC3 cells. PC3 was selected for its TMPRSS2:ERG fusion-negative status and low endogenous RhoGDIB expression (Figure 3A). Visual inspection of PC3-7C cells, cultured alongside parental PC3 cells under identical conditions revealed more spindle-shaped cells in the former (Figure 3B).
As a spindle-shaped morphology is suggestive of an epithelial-to-mesenchymal transition (24), a wound-healing assay was performed to further assess this change in cell behaviour. Monolayers of PC3-7C and parental PC3 cells were scratched and allowed to recover to determine their translational migration ability. Cells were cultured in the presence of hydroxyurea to eliminate differential proliferation from being a confounding variable. PC3 cells required over 30 h, while PC3-7C cells required about 24 h to close gaps of comparable width (Figure 3C), suggesting that RhoGDIB expression contributes positively to cell migration.

In order to determine whether RhoGDIB affected cell proliferation, a $^3$H-thymidine incorporation assay was performed. Cultured PC3-7C and parental PC3 cells were exposed to $^3$H-thymidine for 6 h. Subsequent results showed that PC3-7C cells incorporated much more of the radioactive label and that this effect was amplified by transient transfection with ΔN-ERG (Figure 3D).

Expression of wingless-integration 1 (WNT) pathway members were higher in the fusion-positive sample. The Genesifter software was used to identify KEGG molecular interaction and reaction pathways in which many genes were differentially expressed between the fusion-positive and fusion-negative samples. In the fusion-positive sample, this led to the discovery of a gene expression profile consistent with increased WNT signalling (Figure 4). Up-regulation of WNT signalling is associated with prostate cancer (25). Genes throughout the signalling cascade were found to be more highly expressed in the fusion-positive sample, with lymphoid...
enhancer-binding factor 1 (\(LEF1\)) being the most striking of these (35.18-fold higher). In concert with these differences, genes of the glycogen synthase kinase (GSK)-3β-inhibiting phosphoinositide 3-kinase (PI3K) pathway were expressed at higher levels, while adenomatous polyposis coli (\(APC\)) itself was expressed at a lower level. In contrast, several frizzled family genes were less highly expressed.

A p53 variant and lower expression of many p53 effectors were observed in the fusion-negative sample. Analysis of the sequencing data revealed the presence of a well-known single-nucleotide polymorphism (SNP) of p53, rs1042522, in the fusion-negative sample only. This SNP represents a C to G substitution that results in arginine instead of proline at residue 72 of the p53 protein and may be associated with prostate cancer (26). Interestingly, a large number of p53 target genes were found to be less strongly expressed in the fusion-negative sample (Table III), including 14-3-3σ (7.31-fold lower) and insulin-like growth factor-binding protein 3 (\(IGFBP3\)) (6.82-fold lower). Insulin-like growth factor 1 (\(IGF1\)) was expressed 2.68-fold higher. Notably, these differences in p53 effector gene expression occur in the absence of a corresponding disparity in p53 expression.

**Discussion**

In this study, we took advantage of the comprehensive nature of whole-transcriptome sequencing to examine the expression of established prostate cancer-related genes and to elucidate novel associations between the disease and oncogenes/tumour suppressor genes. The results provide insights into both \(TMPRSS2:ERG\) fusion-associated and unrelated prostate cancer.

Components of the ETS and \(TMPRSS2:ERG\) gene pathways were found to be dysregulated in this study. First,
Figure 2. Ets-related gene (ERG) positively regulated Rho GDP dissociation inhibitor β (RhoGDIB) expression. A: RhoGDIB expression in the fusion-positive and fusion-negative samples, as measured by next-generation sequencing (NGS). B: The promoter region upstream of RhoGDIB contains many putative E-twenty six (ETS) binding sites (highlighted). C: Structure of pGL-RGDb pro-Luc. D: Luciferase reporter gene activity from HEK 293 cells transfected with pGL-RGDb pro-Luc and co-transfected with pCMV or pCMV-ΔNERG. Data are the mean±SE from triplicate measures and are representative of multiple experiments; *significant differences between fusion-negative and fusion-positive samples by t-test (p=0.0011).
Figure 3. Effects of Rho GDP dissociation inhibitor β (RhoGDIB) overexpression on cell proliferation, motility, and morphology. A: Immunoblot of endogenous RhoGDIB expression in five prostate cell lines. β-Tubulin was used as a loading control. B: Morphology of PC3-7C cells (which stably overexpress RhoGDIB) and parental PC3 cells in cell culture. C: Wound healing assay performed with PC3-7C and parental PC3 cells, cultured in the presence of 1.5 mM hydroxyurea to prevent proliferation. D: 3H-Thymidine incorporation by PC3-7C and parental PC3 cells, with or without ΔN-ERG overexpression. Data are the mean±SE from triplicate measures and are representative of multiple experiments; different lowercase letters indicate significant differences by one-way ANOVA and Tukey’s test (p<0.001).
Figure 4. Diagram representing the expression levels of wingless-integration 1 (WNT) signalling pathway genes in the fusion-positive sample relative to the fusion-negative sample. Higher WNT and phosphoinositide-3-kinase regulatory subunit 5 (PIK3R5) and lower adenomatous polyposis coli (APC) expression are consistent with increased β-catenin signalling in the nucleus. This leads to increased expression of WNT pathway targets such as T-cell factor 7 (TCF7) and lymphoid enhancer-binding factor 1 (LEF1), contributing to proliferation and evasion of apoptosis. Differentially expressed members of gene families are indicated by gene symbol. FZD: Frizzled; GSK: glycogen synthase kinase; PI3K: phosphoinositide 3-kinase; PKB: protein kinase B.
**TMPRSS2** expression was dramatically lower in the fusion-positive sample, an expected consequence of the **TMPRSS2** gene being either deleted or translocated from its promoter during the process of fusion gene formation (12); accordingly, **ERG** expression was higher in that sample. Interestingly, **ETV1**, an ETS gene whose full-length, truncated and fusion transcripts have all been associated with prostate cancer (27), was much more highly expressed in the fusion-negative sample. **ETV1** is, after **ERG**, the second-most frequently overexpressed gene in prostate cancer and its overexpression can be effected by any number of fusions or no fusion at all (10,27). The pattern of **TMPRSS2**, **ERG**, and **ETV1** expression observed here is suggestive of ETS involvement in both samples. Two ETS-targeted genes, **EGR1** and **RhoGDIB**, were highly expressed in the fusion-positive sample. **EGR1** is a target of ETS1 implicated in transformation (28). It positively regulates interleukin 8 expression and enables soft agar growth and Matrigel invasion of prostate cancer cells (29). Consistent with this, in this study, **IL8** was up-regulated by more than four-fold in the fusion-positive sample. Other studies in prostate cell lines have found that **EGR1** is also an intermediary player in proteinase-activated receptor 1 (PAR1)-induced invasion (30) and E2F transcription factor 1 (E2F1)-mediated apoptosis evasion (31). In addition, at least ETS1 and friend leukemia integration 1 transcription factor (FLI-1) were previously shown to bind to EBS within the **EGR1**

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**Figure 5.** Model showing how fusion genes and wingless-integration 1 (WNT) signalling form a positive feedback loop. Overexpression of genes in the WNT pathway mimics WNT signalling, leading to β-catenin stabilization. β-Catenin binds to androgen receptor (AR) and increases its responsiveness to androgens but decreases its responsiveness to inhibitors, favouring AR-mediated transactivation. This, in turn, increases the expression of ets-related gene (ERG) from the TMPRSS2:ERG fusion gene which feeds back into the WNT pathway.
promoter region and promote its transcription (28). Given the similarities between ETS1, FLI-1 and ERG in terms of protein domains and DNA-binding specificity profiles (4), the present findings suggest that ERG also participates in EGR1-associated cell survival and invasion.

Expression of RhoGDIB has been correlated with ETS1 expression (32). TMPRSS2:ERG fusion-positive status (18) and, in this study, ERG overexpression. RhoGDIB is a GDP dissociation inhibitor and, as the term implies, functions as a negative regulator of Rho-family GTPase signalling, either by inhibiting the latter’s exchange of GDP for GTP or by sequestering it when GTP bound, and can affect the activity of RhoA, cell division cycle 24 (CDC24) and Ras-related C3 botulinum toxin substrate 1 (RAC1) (33). The balance between the activity of these GTPases ultimately determines cytoskeletal arrangement and related outcomes, such as invasion ability (19), and indeed, RhoGDIB expression has been paradoxically shown to be stimulatory of breast cancer (34) and inhibitory of lung cancer (35) metastasis. The present study showed that PC3-7C cells, derivatives of PC3 cells stably-overexpressing RhoGDIB, have a spindle-shaped cell morphology. This morphological change is suggestive of cytoskeletal rearrangement and increased cell motility (24) and accordingly, RhoGDIB overexpression also increased the rate of translational cell migration, as determined by wound healing assays. Furthermore, according to 3H-thymidine incorporation assays, PC3-7C cells proliferate more readily than parental PC3 cells, especially with concomitant AN-ERG overexpression. Taken together, these results point to a contribution by RhoGDIB in the development and progression of prostate cancer, particularly under the influence of TMPRSS2:ERG gene fusion.

A subset of genes that has been previously associated with prostate cancer was also identified by NGS. MALAT1 is transcribed into a non-coding RNA that has been shown to be significantly overexpressed in approximately 25% of prostate cancer samples and, remarkably in one study, in every instance was more highly expressed in a tumour sample than in the matched normal counterpart (36). Bcl-2-associated X protein (BAX) inhibitor-1 (BI-1) is a potent suppressor of physiologically, and pharmacologically-mediated apoptosis (37). BI-1 is up-regulated in human prostate tumours and knockdown of BI-1 is sufficient for inducing apoptosis in a variety of prostate cancer cell lines (38). Cathepsin B, a protease implicated in extracellular matrix degradation and metastasis, is overexpressed in prostate cancer (39). FOS is part of the dimeric activator protein-1 (AP-1) transcription factor, which has been linked to advanced prostate cancer (40). The findings from this study add to the body of research indicating the involvement of these genes in prostate cancer and additionally point to their usefulness as biomarkers, given the high levels at which they were expressed.

Also highly expressed were two prostate cancer biomarkers that are of current interest in the field: α-methylacyl-CoA racemase (AMACR) and prostatic acid phosphatase (PAP) (Table II). AMACR was first identified as being overexpressed in prostate cancer through DNA microarray analysis and found to be very sensitive and specific at detecting prostate cancer in prostate needle biopsies (41). It has since found important applications, when combined with other markers, in detecting small cancerous lesions (42), and in deciding histologically-ambiguous cases (43), but its uses are limited to post-screening biopsy analysis. Here, we found AMACR to be highly expressed only in the fusion-negative sample, highlighting the heterogeneity of prostate cancer in terms of biomarker expression. PAP was the first serum biomarker for prostate cancer. PAP screening has been obsolete since the discovery of PSA (44), but serum levels of PAP have since been found to be predictive of biochemical recurrence (45). This coincides with the present finding of increased PAP expression in the fusion-negative sample, which was derived from a patient who suffered biochemical recurrence.

Organising the transcriptome data according to KEGG pathways uncovered patterns that were indicative of WNT pathway dysregulation. In the canonical WNT pathway, WNT ligand binding inhibits the formation of the GSK-3β/β-catenin/Apc/axin complex, thereby sparing β-catenin from degradation. β-Catenin then accumulates and translocates to the nucleus, where it activates downstream effectors via the T-cell factor (TCF)/lymphoid enhancer-binding factor (LEF) family of transcription factors (46). These effectors include c-MYC, cyclin D1 and survivin, and are responsible for such outcomes as proliferation and apoptosis evasion (47,48). Hence dysregulation of this pathway is a feature of many types of cancer (46). By virtue of their contributions to this pathway, increased expression of WNT, frizzled, TCF7 and LEF1 and decreased APC expression are all generally suggestive of a contributory role for WNT signalling in TMPRSS2:ERG-related prostate cancer. The PI3K pathway also positively modulates WNT signalling by inhibiting GSK-3β, similar to the action of dishevelled protein. Mutations of PI3K itself are very frequent in cancer and aberrations in the PI3K pathways are a feature of all human carcinomas (49). The increased expression in the fusion-positive sample of the PI3K family gene PIK3R5 lends additional credibility to WNT signalling as a mediator of fusion gene-associated prostate cancer. Furthermore, β-catenin, whose protein level is controlled by WNT signalling, directly binds to and acts on androgen receptor (AR). Clinically, androgen deprivation often leads to at least temporary prostate cancer remission (50) and in the laboratory, androgens are necessary for tumour induction. β-Catenin has been shown to have myriad effects on androgen receptor signalling, including modifying its ligand specificity, increasing its sensitivity to androgens and decreasing its responsiveness to inhibitors, such as bicalutamide (51). Given that overexpression of ERG in TMPRSS2:ERG-positive tumours is caused by the placement
of ERG under androgen-sensitive transcription, the up-regulation of WNT pathway components observed in this study alludes to a potential positive feedback loop between gene fusion, WNT pathway up-regulation, increased β-catenin levels and AR modulation (Figure 5). The unexpected decrease in expression of frizzled 3 (FZD3), FZD4 and FZD8 may be indicative of negative feedback regulation in the WNT pathway, although such regulation has only previously been reported to occur via lysosomal degradation of the dishevelled protein (52). In medulloblastoma tissues and cell lines, frizzled receptor expression has been found to be inconsistently up- and down-regulated (53), so perhaps there is a similar need to clarify the significance of frizzled receptor expression in prostate cancer.

Additionally, KEGG-assisted analysis drew attention to lower expression of many p53 target genes, particularly those involved in cell cycle arrest and apoptosis, in the fusion-negative sample. The transcription factor p53 is credited with activating DNA repair, promoting senescence and initiating apoptosis upon detection of DNA damage and is therefore a crucial tumour suppressor (54). Decreased expression of p53 targets is therefore consistent with a disease state that is less responsive to antiproliferative signals. A remarkable proportion of the p53-mediated cell cycle genes examined, were specifically involved in pharmacologically-induced arrest in prostate cancer. p21 initiates cell cycle arrest in G2 and mediates growth inhibition in prostate cancer cells in response to treatment with G-1, a potential anticancer agent (55). Similarly, growth arrest and DNA damage 45 (GADD45) causes G1 arrest and is responsible for the antitumor activity of fucoxanthin in liver and prostate cells (56). 14-3-3σ has been shown to be expressed at successively lower levels in normal prostate, prostatic intraepithelial neoplasia and adenocarcinoma (57). These genes for cell-cycle control were all expressed at lower levels in the fusion-negative sample. Several apoptosis-inducing effectors of p53 signalling were also less highly expressed in the fusion-negative sample, again with strong representation from genes mediating drug action in prostate cancer. FAS, caspase-8 and BH3 interacting domain death agonist (BID) positively regulate the caspase cascade and apoptosis. Upon binding FAS ligand, FAS promotes caspase-8 and subsequently BID cleavage. Cleven BID translocates to mitochondria to initiate apoptosis, and this activity was found to be essential to the anticancer action of 2-methoxyestradiol through in vitro and in vivo models of prostate cancer (58). IGFBP3 activates apoptosis pathways independently, by activating the caspase cascade (45), and in concert with its IGF1-sequestering effect, including that as a mediator of 5-fluourouracil activity (59). Interestingly, IGF1 was more highly expressed in the fusion-negative sample, further contributing to decreased apoptosis (60). Transcriptome sequencing revealed that the SNP rs1042522, encoding a P72R variant of p53, was present in the fusion-negative sample. Though automated phylogenetic and structural analysis by PolyPhen-2 (61) predicts this polymorphism to be benign, the 72R form has been reported to be much more efficient at inducing apoptosis (62) but less active at transactivation of target genes (63). The expression profiles of cell-cycle and apoptosis control genes in the fusion-negative sample is consistent with the impaired transactivation ability of the 72R variant of p53. Taken together, these findings suggest that the rs1042522 polymorphism impairs the ability of p53 to act as a tumour suppressor. The involvement of affected downstream genes in mechanisms of anticancer drug action additionally stresses the clinical relevance of this polymorphism. These findings also demonstrate the usefulness of transcriptome sequencing for investigation of gene variants and polymorphisms.

The current study demonstrates the utility of whole-transcriptome sequencing for confirming established associations of genes with disease and for forming new associations of this kind. Further investigation of the genes and pathways identified in this manner will provide insights into mechanisms contributory to prostate cancer and the involvement of TMPRSS2:ERG gene fusion therein. This, in turn, can guide the discovery and evaluation of diagnostic and prognostic biomarkers and therapeutic targets for prostate cancer.

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