A Germline DNA Polymorphism Enhances Alternative Splicing of the KLF6 Tumor Suppressor Gene and Is Associated with Increased Prostate Cancer Risk


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Abstract

Prostate cancer is a leading and increasingly prevalent cause of cancer death in men. Whereas family history of disease is one of the strongest prostate cancer risk factors and suggests a hereditary component, the predisposing genetic factors remain unknown. We first showed that KLF6 is a tumor suppressor somatically inactivated in prostate cancer and since then, its functional loss has been further established in prostate cancer cell lines and other human cancers. Wild-type KLF6, but not patient-derived mutants, suppresses cell growth through p53-independent transactivation of p21. Here we show that a germline KLF6 single nucleotide polymorphism, confirmed in a tri-institutional study of 3,411 men, is significantly associated with an increased relative risk of prostate cancer in men, regardless of family history of disease. This prostate cancer–associated allele generates a novel functional Skp4 DNA binding site and increases transcription of three alternatively spliced KLF6 isoforms. The KLF6 variant proteins KLF6-SV1 and KLF6-SV2 are mislocalized to the cytoplasm, antagonize wtKLF6 function, leading to decreased p21 expression and increased cell growth, and are up-regulated in tumor versus normal prostatic tissue. Thus, these results are the first to identify a novel mechanism of self-encoded tumor suppressor gene inactivation and link a relatively common single nucleotide polymorphism to both regulation of alternative splicing and an increased risk in a major human cancer. (Cancer Res 2005; 65(4): 1213-22)

Introduction

Prostate cancer is among the most prevalent neoplasms worldwide and is the second leading cause of male cancer–related death in the United States. Incidence is expected to double among men over the age of 65 in the next 25 years (1). Despite progress in identifying the molecular pathways involved in other major cancers (2, 3), few candidate prostate cancer–associated genes have emerged (4). One approach to identify prostate cancer genes has focused on the use of linkage studies in hereditary prostate cancer families. Three notable candidate genes have however been identified: HPC2/ELAC2 (5), RNASEL (6), and MSR1 (7); yet, their effect has been limited at best to only a small subset of familial cases and susceptibility alleles are rare in the general population (8). Nonetheless, epidemiologic and molecular studies suggest that relatively common inherited genetic risks do exist and that prostate cancer, and indeed all common cancers and complex diseases, arise from the combined interplay of many genes, each having its own modest effect. Thus, targeted analysis of candidate genes having maximal biological plausibility represents another approach to identify these high-frequency, low-penetration prostate cancer genes and their germline sequence variants.

KLF6, a Kruppel-like zinc finger transcription factor, is a tumor suppressor gene inactivated by allelic loss and somatic mutation in men with sporadic prostate cancer (9, 10). Recently, KLF6 gene mutations have also been identified in other human cancers, including colorectal cancer (11), malignant glioma (12), and nasopharyngeal carcinoma (13). Additional inactivating mechanisms include transcriptional silencing by promoter hypermethylation in esophageal cancer cell lines (14) and down-regulation of KLF6 mRNA levels in both primary lung cancer samples (15, 16) and in prostate cancer cell lines (10), whereas expression of KLF6 has been shown to revert the tumorigenic phenotype in glioblastoma cell lines in culture and in vivo (17). Furthermore, KLF6 has been shown to inhibit a number of key oncogenic signaling pathways (17), including the cyclin-dependent kinase complex CDK4-cyclinD1 (18) and c-jun (19). Especially intriguing are the most recent associations of KLF6 expression status with predictive outcomes in both prostate (20) and lung cancers (15). Combined, these studies highlight a growing number of tumors in which KLF6 is functionally inactivated and elucidate molecular mechanisms by which KLF6 functions as a tumor suppressor gene, raising the possibility of a generalized role in cancer pathogenesis. As part of our overall investigations into the molecular basis of KLF6-related tumorigenesis, we investigated the possibility that inherited KLF6 mutations or polymorphisms exist and may be associated with increased prostate cancer risk.

Note: The authors disclose that they have no competing financial interests.

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**Materials and Methods**

**Study Population.** The overall study population analyzed was selected from the Johns Hopkins University (JHU), Mayo Clinic (Mayo), and Fred Hutchinson Cancer Research Center prostate cancer registries, which have been previously described, along with their respective population controls (21–23). All samples were collected and analyzed under appropriate Institutional Review Board approval for each institution. The JHU prostate cancer registry comprised 142 families affected with hereditary prostate cancer. Families with at least three first-degree relatives with prostate cancer were ascertained and studied at the Brady Urology Institute at Johns Hopkins Hospital. Families were ascertained from three sources. Sixty-eight families were ascertained through referrals generated in response to a letter by one of us (P.C.W.) to 8,000 urologists throughout the United States. The second source, from which 37 families were identified, was family history records of patients seen at Johns Hopkins Hospital for prostate cancer treatment. The remaining families came from the respondents to articles, which seemed in various lay publications, describing studies of families with hereditary prostate cancer. Diagnosis was verified by medical records for each affected man studied. Age at diagnosis of prostate cancer was confirmed either through medical records or through two other independent sources. The mean age at diagnosis was 64.3 years. For the case subjects in these families, 84% of the families are White and 8.8% are Black.

All nonhereditary prostate cancer prostate cancer case subjects were recruited from among patients who underwent treatment for prostate cancer at Johns Hopkins Hospital. The diagnosis for all these subjects was confirmed by pathology reports. The mean age at diagnosis for these case subjects was 58.6 years. Family histories were not available. More than 93% of the case subjects are White and 3.2% are Black.

Control subjects were selected from among men who participated in prostate cancer screening programs. After excluding those who had abnormal results of a digital rectal examination (DRE) or abnormal prostate-specific antigen (PSA) levels (>4 ng/mL), 264 Caucasian men were eligible for these studies. The mean age at examination was 58 years. About 5.6% of the eligible control subjects have a brother(s) or father affected with prostate cancer. The affection status of relatives was obtained by interview and review of medical records for each affected man studied. Age at diagnosis of prostate cancer was confirmed either through medical records or through two other independent sources. The mean age at diagnosis was 64.3 years. For the case subjects in these families, 84% of the families are White and 8.8% are Black.

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**Statistical Analysis.** A total of 1,011 invitations were sent to men who initially reported a suspicion of prostate cancer. A total of 917 eligible cases, 753 (82%) participated in the study, 591 provided blood samples for analysis, and of these, 558 DNA samples were genotyped for the KL6F single nucleotide polymorphism (SNP). Population-based controls were selected from the same defined geographic region using random digit dialing. Controls were frequency matched to cases by age (same 5-year group) and recruited evenly throughout the ascertainment period of cases (23). Nine hundred forty-one individuals were eligible as controls, 703 (75%) participated, 538 provided samples for analysis, and 521 were genotyped for these studies.

**Sequence and Genotype Analysis.** Genomic DNA was extracted from peripheral blood samples as previously described (21–23). Initially, DNA from 142 probands from the JHU Familial Prostate Cancer Registry were analyzed by direct sequence analysis of the second exon and intron/exon boundaries using KL6F specific primer combinations as previously described (9). PCR products were directly sequenced in both orientations after purification (QiAquick PCR purification kit, Qiagen, Chatsworth, CA). All sequencing was done either on an ABI Prism 3730 or 3700 automated DNA analyzer and sequence data was analyzed using the Sequencer 4.1 program (Gene Codes Co., Ann Arbor, MI) or Phred/Phrap/Consed (University of Washington).

Direct sequencing or a restriction enzyme based assay was also used to screen samples. Genomic DNA was amplified to generate a 171-bp amplicon using the following KL6F-specific primers: ΔATG forward 5′-CGGGCGACCATGTITATCCTGCCTC-3′ and ΔATG reverse 5′-TTCTGAGGCGTAAA-CAATGACGGG-3′. The PCR product was then either sequenced per standard protocols or digested with BsaI (New England Biolabs, Beverly, MA) using the manufacturer’s recommendations and resultant products were gel electrophoresed on a 1.5% TAE gel for 1 hour at 80 V and then visualized by ethidium bromide staining. In addition, all JHU genotypes were reconfirmed at a second institutional site by BsaI restriction enzyme digest. Genotypes for the Mayo Clinic samples were reconfirmed by pyrosequencing as previously described (21), using the same KL6F primers described above except that one primer was biotinylated. As a control, more than half of all sample results were independently confirmed by a second institution.

To evaluate the possibility of combining the data sets, logistic regression with an interaction term (institution by KL6F carrier status) was done to determine if the odds ratios (OR) were significantly different between the registries (24–26). This analysis was conducted for the entire group, as well as for the subgroups. No interaction was statistically significant and thus the data sets were also analyzed as a combined set.

**Cell Culture and Transient Transfection.** All cell lines were obtained from the American Type Culture Collection (Rockville, MD). All transient transfection was done with LipofectAMINE 2000 reagent according to the manufacturer’s protocol (Life Technologies, Gaithersburg, MD). Stable cell lines were generated by cotransfection of the IVSΔA, WILD TYPE, or LacZ minigene expression constructs with a puromycin expressing plasmid. Transfected cells were selected with 2 μg/mL of puromycin.

**Wild-type KL6F and IVSΔA Minigene Constructs.** To amplify the 6.2 kb KL6F genomic locus including ∼100 bp of the 5′ untranslated region and 300 bp of 3′ untranslated region, 200 ng of human genomic DNA were

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**Cancer Research**

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amplified using the following set of primers: KLF6-1F TTGCAGT-CAGTCCGGTGTTTG and KLF6-4R1 GGTGCTATGCCGCTTCTTACAGGAC using the EXPAND Long Template PCR system (Roche, Nutley, NJ). PCR was done according to manufacturer's suggestions. The resultant 6.2-kb PCR product was purified (Quagen) and then subcloned into the TOPO TA expression vector (Invitrogen, San Diego, CA). The primers used for mutagenesis were as follows: KLF6ΔA-F GTATGGCAATCACATGCCTTCTCTGGTT and KLF6ΔA-R AAC-CAGAGAAGGACATGTGATTGCCATGAC. All intron/exon boundaries and 5' untranslated region and 3' untranslated region regions of the constructs used in this study were sequenced in both orientations before use.

**KLF6, p21 Promoter, and SRp40 Constructs.** Full-length KLF6 cDNA was cloned into EcoRI and XhoI sites of pCI-neo (9). KLF6SV1 and SV2 expression vectors were generated by subcloning the appropriate full-length cDNA into the EcoRI site of the pCI-neo expression vector. WtKLF6, KLF6SV1, and KLF6SV2 constructs were generated by cloning the appropriate cDNA into the EcoRI site of the FLAG expression vector pCDNA3 (Invitrogen). The wild-type (wt) and mutant p21 promoter constructs were previously described (9). The SRp40, ASE, and pCGT7 constructs (generous gifts from Dr. A. Krainer, Cold Spring Harbor Laboratory, Cold Spring Harbor, NY) were previously described (27).

**pSUPER Plasmid Construction and Transfection.** The pSUPER-siSV1 and pSUPER-siSV2 plasmids used to down-regulate KLF6v1 and KLF6v2 expression were constructed similarly as described using this pSUPER vector (ref. 28; generously provided by R. Agami, Division of Tumor Biology, The Netherlands Cancer Institute, Amsterdam, The Netherlands). To insert the targeting sequence, DNA oligos were designed and cloned into the BglII/HindIII sites of the pSuper vector.

**Western Blot Analysis.** BPH1 or 293T cells grown in 12-well dishes were transfected with 0.5 μg per well of either the IVS ΔA, wt, or LacZ minigene expression vectors. Cell extracts for Western blotting were harvested in radioimmunoprecipitation assay buffer 24 hours following transfection (Santa Cruz Biotechnology standard protocol, Santa Cruz, CA). Equal amounts of protein (30 μg) as determined by the Bio-Rad DC Protein quantification assay were loaded and separated by PAGE and transferred to nitrocellulose membranes. Western blotting was done using rabbit polyclonal antibodies to KLF6 / Zf 9, p21 (R-173 and H-164, respectively; Santa Cruz Biotechnology), a goat polyclonal antibody to actin (SC-1615), and a T7 monoclonal antibody (Novagen).

**Densitometric Analysis.** Enhanced chemiluminescent images of immunoblots were analyzed by scanning densitometry and quantified with a BIOQUANT NOVA imaging system. Values were expressed as fold change relative to control and normalized to actin.

**Figure 1.** Structure and cellular localization of KLF6 and its splice variants. A, RT-PCR of 293T cells transfected with the full-length wt KLF6 minigene construct reveals that the KLF6 gene is alternatively spliced. B, genomic organization, splice site sequences, and mRNA structures for wt and KLF6 splice variants. Nuclear localization signal (NLS). PCR primer binding sites for qRT-PCR. C, immunocytochemistry of 293 human embryonic kidney cells expressing wtKLF6, SV1, or SV2 proteins. 293HEK cells were transfected with FLAG tagged wKLF6, KLF6SV1, or KLF6SV2 expression constructs. FLAG immunostaining revealed that wKLF6 localizes to the nucleus, whereas the KLF6SV1 and SV2 proteins are predominantly cytoplasmic (arrows).
Analysis of Proliferation. Proliferation was determined by estimating 3H-thymidine incorporation. BPH1 stable cell lines containing either the IVS D A, wt, LacZ minigene expression vectors were plated at a density of 50,000 cells per well in 12-well dishes. Forty-eight hours after plating, 1 µCi/ mL 3H-thymidine (Amersham, Arlington Heights, IL) was added. After 2 hours, cells were washed four times with ice-cold PBS and fixed in methanol for 30 minutes at 4°C. After methanol removal and cell drying, cells were solubilized in 0.25% sodium hydroxide/0.25% SDS. After neutralization with hydrochloric acid (1 N), disintegrations per minute were estimated by liquid scintillation counting.

Luciferase Transactivation. Luciferase transactivation assays were done 24 hours after transfection into 293T cells plated at 100,000 cells per well in 12-well dishes with DNA containing 1.5 µg p21 promoter constructs and either 1.5 µg pCI-neo-KLF6, pCI-neo-KLF6SV1, or pCI-neo-KLF6SV2. The TK promoter- Renilla Luciferase construct (Promega, Madison, WI; 2 ng) was used to normalize each transfection experiment. Proteins from the cells were then extracted and luminescence quantified using the Dual-Luciferase system (Promega). All experiments were done in triplicate.

RNA and Quantitative Real-time PCR Analysis. Normal, BPH, and prostate cancer RNA samples were collected and extracted as previously described (29). Cell line RNA was extracted using the Rneasy Mini kit (Qiagen). All RNA was treated with DNase (Qiagen). A total of 1 µg of RNA was reverse transcribed per reaction using first strand complementary DNA synthesis with random primers (Promega). Quantitative real-time (qRT-PCR) was done using the following PCR primers on an ABI PRISM 7900HT Sequence Detection System (Applied Biosystems, Foster City, CA): wtKLF6 forward 5’-CGGACGCACACGGAAA-3’ and wtKLF6 reverse 5’-CGGGTTGCTTCTGAGGTGT-3’. Total KLF6 forward 5’-CTGGCGCCTTTGAGAGGT-3’ and Total KLF6 reverse 5’-TCCACAGATCTTCTTGCACTGTC-3’; p21 forward 5’-ACCTCAGGCTGAAAGG-3’ and p21 reverse 5’-CCTCAGGCTTCCAGAGCTG-3’; GPDH forward 5’- GATTGACCCCTTTCATTGAC-3’ and GPDH reverse 5’-GATCTTCTCTTGAAGGATG-3’; Sp60 forward: 5’-CAACAAGGATGCGATGATCTTGC-3’ and Sp60 reverse 5’-GGGACCATTCTGCTTACATTTGAG-3’. All experiments were done in triplicate and normalized to GPDH. To calculate the fold change in KLF6 alternative splicing, the fold change in total KLF6 (wtKLF6 + alternatively spliced KLF6 transcripts) was divided by the fold change in wild-type KLF6 alone.

Immunocytochemistry. 293 human embryonic kidney cells were cultured on growth promoting coverslips (Fisherbrand, Raleigh, NC) in 12-well dishes. At 24 hours, cells were transfected with pCDNA3 FLAG (empty vector), FLAG-wtKLF6, FLAG-SV1, and FLAG-SV2. At 18 hours, cells were washed twice with ice-cold saline, fixed for 10 minutes with ice-cold methanol, and then with ice-cold acetone for 1 minute. Blocking was with 5% bovine serum albumin/PBS for 10 minutes before incubation with primary FLAG monoclonal antibody (1:100) and subsequent detection with Texas Red conjugated anti-mouse secondary antibody (Vector Laboratories, Burlingame, CA).

Table 1.

<table>
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Abbreviation: FHCRC, Fred Hutchinson Cancer Research Center.

*For the FHCRC and combined data, OR is calculated twice: Sporadic versus all controls; Sporadic versus only PSA-tested controls.
Results

Screening for KLF6 Polymorphisms. To begin investigating the possibility that inherited mutations or polymorphisms in the KLF6 tumor suppressor gene are associated with increased prostate cancer risk, we first sought to identify and characterize biologically relevant SNPs. Therefore, we directly sequenced genomic DNA isolated from blood samples from an exploration set of 142 probands from a familial prostate cancer registry. A number of silent, conserved, and intronic KLF6 gene changes were identified by DNA sequence analysis. A relatively common intronic KLF6 gene polymorphism, IVS1 +27 G > A, the IVSΔA allele, was identified as the most frequent polymorphism (Fig. 1B). In total, 26 of 142 probands (18.3%) possessed the IVSΔA variant. Three other variants were also detected in our sample set but not further characterized in this study because their frequencies were quite small by comparison to the IVSΔA variant. The most frequent of these variants (3550 G > A; silent) was only present in 3 of 142 samples (2.1%). The other two variants were present in 2 of 142 samples each (1.4%).

Prostate Cancer Association Studies. Having identified the IVSΔA variant as the KLF6 SNP with the highest minor allele frequency, we next sought to identify a possible association between this SNP and prostate cancer risk. Therefore, we did a sequential series of association studies by genotyping germline DNA from 3,411 geographically diverse men was analyzed. The centers, samples, definitions of hereditary and sporadic cases, control populations, and collection methods have been previously described (21–23). Because the majority of men in the prior studies from these centers were Caucasian, this analysis was limited to only Caucasian men. In total, the samples represented men divided into three groups: 1,253 men with sporadic prostate cancer, 882 men with familial prostate cancer from 294 unrelated families (three men from each family), and 1,276 control men. Genotype frequencies for hereditary cases were determined for two of the study populations. Two statistical approaches were used to evaluate possible prostate cancer associations. First, the frequency of IVSΔA carriers between

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**Figure 2.** KLF6 gene is differentially spliced in tumor tissue and can be regulated in cell culture by the IVSΔA variant. A, qRT-PCR was done on six normal prostate tissue samples and 15 prostate cancer specimens using total and wt specific KLF6 primers. The ratio of total KLF6 to wt KLF6 was determined using the PCR primers shown in Fig. 1B and calculated by determining the amount of variant message and then dividing that value by the amount of wtKLF6 message. Prostate tumors had significantly higher splicing ratios ($x = 0.83$), a higher total/wtKLF6 ratio, than normal prostate tissue ($x = 0.41$; $P < 0.01$). B, results of qRT-PCR using total and wt specific KLF6 primers on cells transfected with either the wt or IVSΔA minigene construct. The IVSΔA variant increases alternative splicing of the KLF6 gene by 30% and 50% in BPH1 and 293T cells, respectively, as compared with the wt construct. **, $P < 0.001$ (ANOVA, Bonferroni correction applied, $n = 3$). C, Western blot analysis of BPH1 cells transfected with IVSΔA and wt minigene constructs using a KLF6 polyclonal antibody. The IVSΔA variant results in increased levels of both variant proteins. The ratio of splice variants to wt protein was quantitated by densitometry and increased by ~90%. D, stable expression of the IVSΔA variant in BPH1 results in a 30% increase of KLF6 splicing as measured by qRT-PCR. **, $P < 0.001$ (ANOVA, Bonferroni correction applied, $n = 3$). E, Western blot analysis of BPH1 stable cell lines stably expressing the IVSΔA or wt minigene construct. IVSΔA BPH1 cells express ~60% more variant proteins to wt KLF6 protein as compared with the wt and minigene control.
sporadic cases and controls, was compared using Pearson's $\chi^2$ statistic, and computed ORs and their 95% confidence intervals (95% CI; SAS Institute, Inc., Cary, NC). Second, to compare the IVS\(\Delta A\) carrier frequency between familial cases in the Mayo and JHU data sets (some of which are related to each other in families) and controls, we used a statistical method that accounts for the correlation among related subjects (24–26). Similar methods were used to compare the sporadic and familial cases to each other.

The IVS\(\Delta A\) variant was found to be more frequently present in men with sporadic prostate cancer when data from all three centers were combined and controls were required to minimally have had PSA testing for study inclusion ($P = 0.01$; OR, 1.42; 95% CI, 1.10-1.80; Table 1). Owing to study design differences between

![Figure 3.](image)

**Figure 3.** IVS\(\Delta A\) is associated with increased alternative splicing of the KLF6 gene in prostatic tissues. **A.** qRT-PCR of patient-derived benign prostatic hyperplasia tissue revealed that the presence of IVS\(\Delta A\) variant was associated with a 40% increase in KLF6 splicing ($P < 0.01$). **B.** qRT-PCR of prostate cancer tissues revealed that the IVS\(\Delta A\) variant was associated with 30% and 40% increases in alternative splicing in Gleason 3 + 3 (*, $P < 0.01$; G/G, $n = 3$; G/A, $n = 2$) and Gleason 4 + 4 tumors (**, $P < 0.001$; G/G, $n = 2$; G/A, $n = 2$), respectively.

![Figure 4.](image)

**Figure 4.** The IVS\(\Delta A\) variant creates a functional SRp40 binding site. **A.** Alignment of wt and IVS\(\Delta A\) sequences with the consensus SRp40 sequence. Control experiments with HeLa cells cotransfected with increasing amounts of a SRp40 expression vector and either the IVS\(\Delta A\) variant or wt minigene constructs were done. **B.** qRT-PCR using SRp40-specific real-time primers confirm incremental SRp40 expression. Difference in SRp40 expression between the wt and IVS\(\Delta A\) variant cotransfected with 4 \(\mu\)g of the SRp40 plasmid is not statistically significant. **C.** Western blot analysis using a T7 monoclonal antibody confirmed SRp40 expression. **D.** Incremental SRp40 expression resulted in increased KLF6 alternative splicing only in IVS\(\Delta A\) minigene transfected cells. ***, $P < 0.001$; ***, $P < 0.0001$ (ANOVA, Bonferroni correction applied, $n = 9$).
the three centers, PSA testing and DRE were minimally required for all controls in only two of the studies but not in the third. In the combined group analysis of men in these two centers (JHU and Mayo), the association of allele status and prostate cancer risk was also statistically significant and the magnitude of the relative risk for \( \text{KLF6} \) gene variant carriers was 1.47 \((P = 0.01; \text{95\% CI, 1.08-2.00})\).

A potentially important caveat, requiring further analysis, is that no association between risk and genotype status was present in the third group alone when all their population-based controls, regardless of PSA status were included \((P = 0.57; \text{OR, 0.86; 95\% CI, 0.62 -1.20})\). When data from all three registries were combined without regard to PSA testing in controls from the third study, the association was suggestive but did not reach statistical significance \((P = 0.21; \text{OR, 1.16; 95\% CI, 0.92-1.45; Table 1})\).

We next examined the potential role of this SNP in men with hereditary prostate cancer. Again, a statistically significant association was seen between the \( \text{IVS}\Delta A \) allele and cancer risk. The magnitude of the relative risk for \( \text{IVS}\Delta A \) carriers was 1.61 \((P = 0.01; \text{OR, 1.61; 95\% CI, 1.20-2.16})\) in the combined analysis from both centers. This association was seen in one of the two registries alone \((P = 0.02; \text{OR, 1.71; 95\% CI, 1.13-2.60})\) whereas a trend towards significance was present in the other group \((P = 0.11; \text{OR, 1.59; 95\% CI, 0.98-2.57; Table 1})\). Interestingly, among these men with a positive family history for prostate cancer in both registries, the carrier frequency was higher among men with an earlier age of diagnosis \((<65\text{ years of age}; P \leq 0.03)\).

\textbf{KLF6 Alternative Splicing.} Thus, the epidemiologic data reinforced the previously identified role of \( \text{KLF6} \) as a tumor suppressor gene in the pathogenesis of prostate cancer \((9, 10)\).

\begin{figure}
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\includegraphics[width=\textwidth]{figure5.png}
\caption{\textbf{IVS}\Delta A variant blunts \text{p21} up-regulation and increases cellular proliferation.} \textbf{A}, endogenous \text{p21} levels in \text{BPH1} cells expressing the minigene constructs. \text{IVS}\Delta A cells have 40\% less \text{p21} mRNA and 60\% less \text{p21} protein than wt cells. **, \( P < 0.001 \) (ANOVA, Bonferroni correction applied, \( n = 4 \)). \textbf{B}, cell proliferation analysis of minigene expressing BPH1 cells. The \text{IVS}\Delta A expressing cells proliferate 30\% more than the wt expressing cells. **, \( P < 0.001 \) (ANOVA, Bonferroni correction applied, \( n = 9 \)). \textbf{C}, \text{KLF6} variant proteins fail to transactivate the \text{p21} promoter and up-regulate endogenous \text{p21}. 293T cells were cotransfected with a \text{p21} reporter plasmid and either \text{wtKLF6}, \text{SV1}, or \text{SV2} expression vectors. All values represent relative luciferase activity. \text{wtKLF6} transactivated the \text{p21} promoter 6-fold (***, \( P < 0.0001 \) (ANOVA, Bonferroni correction applied, \( n = 6 \)), whereas neither \text{KLF6SV1} nor \text{KLF6SV2} resulted in significant transactivation (***, \( P < 0.0001 \) (ANOVA, Bonferroni correction applied, \( n = 9 \))). \text{KLF6SV1} and \text{SV2} proteins fail to up-regulate endogenous \text{p21}. 293T cells were transfected with a \text{wtKLF6}, \text{SV1}, or \text{SV2} expression vector and 24 hours after transfection cells were harvested and Western blot analysis was done using a \text{p21} antibody. \textbf{D}, \text{KLF6SV1} and \text{SV2} proteins fail to suppress cell proliferation. \text{PC3M} cells were transfected with a \text{wtKLF6}, \text{SV1}, or \text{SV2} expression vector and \( ^{3}H \) incorporation was measured (**, \( P < 0.001 \) (ANOVA, Bonferroni correction applied, \( n = 9 \))). Comparisons were made between \text{wtKLF6} and \text{pcI-neo} and variants and \text{wtKLF6}.
\end{figure}
However, the molecular basis by which this specific noncoding germ line sequence variant resulted in increased risk was unclear. We began exploring a potential biological role for the IVSΔA allele in cultured cells by expressing full-length 6.2-kb KLF6 minigene constructs, containing either the wild-type or IVSΔA sequence. Three findings stemming from these experiments suggested that the IVSΔA variant affected a change in wild-type KLF6 tumor suppressor gene expression and function. First, the KLF6 gene is alternatively spliced. Second, a relative overabundance of KLF6 splice variants to wild-type tumor suppressor expression is present in tumor versus normal prostate tissue. Finally, the IVSΔA variant was consistently associated with enhanced KLF6 alternative splicing and variant protein expression both in cell culture and human tissues.

Direct cDNA sequence analysis from both cells transfected with the minigene constructs and from 20 different human tissues, including normal prostate and localized prostate cancer, confirmed the presence of three alternatively spliced KLF6 gene transcripts, KLF6 SV1, SV2, and SV3 (Fig. 1A). These alternatively spliced products, arising from the use of native cryptic splice sites within exon 2 (Fig. 1B), predicted protein isoforms lacking either parts or all of the wild-type activation and/or DNA binding domain. KLF6SV1 and KLF6SV3 are predicted to contain novel 21 and 12 amino acid carboxyl domains, respectively, resulting from out-of-frame splicing of their terminal exons.

To define potential differences in subcellular localization between wt and variant KLF6 proteins, we generated FLAG fusion expression constructs of wtKLF6 and two of the novel isoforms. Whereas wtKLF6 localized primarily to the cell nucleus, both the KLF6SV1 and KLF6SV2 proteins accumulated predominantly in the cytoplasm (Fig. 1C). This compartmentalization likely reflects the absence of the nuclear localization signal in these variants, which, based on previous analysis of the closely related erythroid Kruppel-like factor, maps to the terminal portion of the activation and DNA binding domains (30).

To assess potential differences in in vivo expression patterns, RNA was isolated from six normal prostate tissue samples and 15 prostate tumors and the amount of wild-type and KLF6 splice variants was determined by qRT-PCR (Fig. 2A). For qRT-PCR, primers were designed to either conserved or unique sequences in the KLF6 transcripts (Fig. 1B) such that either wtKLF6 alone or

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**Figure 6.** KLF6 variants antagonize wtKLF6 ability to up-regulate p21. As a control for siRNA efficacy and specificity, 293T cells were cotransfected with either the pSUPER(−) si-SV1 or si-SV2 plasmid and KLF6 and SV1 or SV2 cDNA expression vectors. **A**, qRT-PCR of 293T transfected cells shows that both the si-SV1 and si-SV2 plasmids result in decreased alternative splicing of the KLF6 gene as compared with a pSUPER-Luc control (***, P < 0.0001; ANOVA, Bonferroni correction applied, n = 6). **B**, Western blot analysis reveals target specific silencing by the pSUPER si-SV1 or si-SV2 plasmids of KLF6 variant proteins. Densitometry results. Wild-type KLF6 and SV2 proteins appear as doublets. **C**, 293T cells cotransfected with the wt full-length minigene construct and either pSUPER plasmids resulted in a significant up-regulation of both p21 mRNA and protein (**, P < 0.001; ***, P < 0.0001; ANOVA, Bonferroni correction applied, n = 3). **D**, silencing of endogenous KLF6 splice forms, SV1 or SV2, in the metastatic prostate cancer cell line PC3M resulted in significant up-regulation of p21 message (**, P < 0.001; ***, P < 0.0001; ANOVA, Bonferroni correction applied, n = 4) and p21 protein. **E**, silencing of endogenous SV1 but not SV2 message in PC3M cells resulted in a 50% decrease in cell proliferation (***, P < 0.0001; ANOVA, Bonferroni correction applied, n = 9).
wt plus alternatively spliced transcripts (total), were amplified. As shown in Fig. 2A, normal prostate expressed twice the relative amount of wt tumor suppressor message to splice products when compared with tumors (P < 0.01).

We then transiently and stably transfected the IVSΔA and wt minigene constructs into a range of cell types and compared the KLF6-related mRNAs and proteins by qRT-PCR and Western blotting, respectively. Regardless of cell line used, the IVSΔA allele resulted in increased alternative splicing by 30% to 50% (Fig. 2B and D; P < 0.0001). The levels of wtKLF6 expression in IVSΔA expressing cells was similar to wild type expressing cells suggesting that the changes in the splicing ratio were secondary to increased alternative splicing and not decreased levels of wtKLF6. Consistent with the RNA data, KLF6SV1 and KLF6SV2 protein was also increased (Fig. 2C and E). The ratio of alternatively spliced KLF6 isoforms to wtKLF6 message and protein was significantly increased by the IVSΔA variant in all cell lines tested.

Next, we directly analyzed the effect of the IVSΔA variant on KLF6 splicing in noncancerous and cancerous prostatic tissues. Tissue from seven men with benign prostatic hypertrophy, a noncancerous condition, was studied. Four samples were homozygous for the wild-type G allele, three were G/A heterozygous. The IVSΔA allele was again associated with an ~40% increase in KLF6 splicing (Fig. 3A). We then haplotyped and determined the splicing ratios of all nine of the 15 originally analyzed prostate tumor samples for which Gleason scores were available (Fig. 3B). As shown, the IVSΔA allele was associated with a 30% increase in splice ratio in tumors graded 3 + 3 and a 40% increase in the higher grade 4 + 4 tumors. Taken together, the cell culture and tissue results suggested that the IVSΔA allele results in increased splicing, whereas an association between increased KLF6 splicing and prostate cancer was noted.

The IVSΔA Allele Generates a Functional Srp40 Binding Site. We hypothesized that one possible mechanism influencing alternative splicing, given the IVSΔA allele's proximity to the intron/exon boundary, was by generation or ablation of a splice site recognition sequence. Using the splicing enhancer motif prediction program ESEfinder (31), three overlapping motifs for SR-protein splicing factor binding sites were identified. In the wild-type sequence, high score motifs are present for SF2/ASF (2.61) and Srp55 (2.93). The IVSΔA allele abolishes these binding sites and generates a novel high-scoring Srp40 (4.30) motif (Fig. 4A). We directly tested the functional role of this predicted novel Srp40 site by assessing the effect of incremental expression of Srp40 on alternative splicing in cells transfected with the KLF6 minigenes (Fig. 4B, C, and D). As predicted, Srp40 coexpression resulted in a dose-dependent increase in KLF6 splicing (increased total KLF6/wt ratio) when coexpressed with the IVSΔA, but not the wt minigene (Fig. 4D). Furthermore, coexpression of the SR protein ASF with the IVSΔA mimigene construct did not increase KLF6 alternative splicing (data not shown).

Splice Forms Antagonize wtKLF6 Effects on p21 and Cell Growth. We next explored the impact of these variants on p21 expression and cell growth, since wtKLF6 up-regulates this key cyclin-dependent kinase inhibitor independent of p53 (9). In contrast to cells expressing the wt minigene, the IVSΔA construct was unable to increase p21 RNA and protein levels to the same degree; resulting in ~60% of the wt levels of p21 up-regulation (P < 0.001; Fig. 5A). Moreover, cell proliferation was ~30% greater in cells stably expressing the IVSΔA allele (Fig. 5B). To test the ability of the KLF6 variants to directly up-regulate p21, we cotransfected the wtKLF6, SV1, or SV2 expression plasmids with a p21 promoter reporter construct lacking functional p53 binding sites into 293T cells (9). Unlike wtKLF6, which transactivated the p21 promoter construct 6-fold, neither the KLF6SV1 nor SV2 proteins were transactivating (P < 0.0001; Fig. 5C). In addition, whereas expression of wtKLF6 in 293T cells up-regulated endogenous p21 3-fold, both variant proteins failed to up-regulate p21 (Fig. 5C). Finally, in marked contrast to wtKLF6 (9), neither variant suppressed cell proliferation in the prostate cancer cell line PC3M (Fig. 5D).

To complement the overexpression studies and, more importantly, to directly interrogate the function of the splice variants, we used RNA interference to specifically silence either wt or splice variant expression. These experiments were done in either PC3M cells, which endogenously express wild-type and variant forms of KLF6, or in 293T cells which normally express very low levels of the splice variants (Fig. 6). In each, silencing SV1 and SV2 resulted in up-regulation of p21 mRNA and protein levels (Fig. 6C and D). Of particular interest, silencing of SV1, but not SV2, was associated with ~50% decrease in cell proliferation (Fig. 6E).

Discussion

Taken together, our experimental findings further highlight the role of KLF6 in prostate cancer by defining a novel mechanism of tumor suppressor gene inactivation, through the generation of alternatively spliced products which antagonize wild-type gene function. Moreover, whereas inactivating somatic mutations have been previously identified throughout the KLF6 gene in prostate cancer (9, 10) and other cancers (11–13), this is the first report identifying a relatively common germ line SNP in any known tumor suppressor gene with an increased relative risk of prostate cancer.

Recent findings have suggested that KLF6 expression levels can be used as predictive indicators of prostate (20) and lung cancer outcomes (15). Thus, it will be highly relevant to explore the potential significance of the IVSΔA SNP as a predictive biomarker for lifetime prostate cancer risk in future studies as well as a possible marker for distinct clinicopathologic associations. Nonetheless, the identification of biologically active KLF6 splice variants adds to an increasing appreciation of the mechanisms by which KLF6 inactivation plays a role in human cancer. These studies show that inheritance of a single, common intronic SNP results in increased splice variant production. The novel KLF6 splice variants, in turn, functionally antagonize wtKLF6's growth suppressive properties by affecting expression levels of the cyclin-dependent kinase inhibitor p21. The splice variants may thus possess a dominant-negative function based on their inability to directly transactivate p21, possibly because they lack part or all of the wild-type DNA-binding domain (Fig. 1). Furthermore, confined to the cytoplasm, the variants may compete for transcriptional cofactors and/or lead to wtKLF6 sequestration.

Beyond its effect in cancer biology, these results also highlight the unexpected effects that polymorphisms and alternative splicing can have on major human diseases. The practical consequences of these findings are highly relevant in the genomic era, as large-scale association studies intensify to identify and characterize biologically relevant variations. In this instance, we have shown that a seemingly neutral polymorphism associated with prostate cancer risk is linked to a novel form of tumor suppressor gene inactivation through alternative splicing. Identification and biological characterization of the IVSΔA allele has provided mechanistic insight into a previously unknown pathway relevant to tumorigenesis.
Specifically, these changes in the IVSΔA allele have uncovered the existence of growth-suppressive and growth-promoting forms of KLF6 in normal and cancerous tissues and highlighted a novel paradigm wherein a critical balance between these forms can profoundly influence prostate biology and disease risk.

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References

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