

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

Goutham Narla,¹ Analisa DiFeo,² Helen L. Reeves,¹ Daniel J. Schaid,³ Jennifer Hirshfeld,² Eldad Hod,¹ Amanda Katz,¹ William B. Isaacs,⁵ Scott Hebbing,⁴ Akira Komiya,⁵ Shannon K. McDonnell,³ Kathleen E. Wiley,⁵ Steven J. Jacobsen,³ Sarah D. Isaacs,⁵ Patrick C. Walsh,⁵ S. Lilly Zheng,¹⁰ Bao-Li Chang,¹⁰ Danielle M. Friedrichsen,⁶ Janet L. Stanford,⁷ Elaine A. Ostrander,⁶ Arul M. Chinnaiyan,⁸ Mark A. Rubin,⁹ Jianfeng Xu,¹⁰ Stephen N. Thibodeau,⁴ Scott L. Friedman,¹ and John A. Martignetti²

Departments of ¹Medicine and ²Human Genetics, Mount Sinai School of Medicine, New York, New York; ³Departments of Health Sciences Research and ⁴Laboratory Medicine and Pathology, Mayo Clinic Foundation, Rochester, Minnesota; ⁵Brady Urological Institute, Johns Hopkins Medical Institution, Baltimore, Maryland; ⁶Divisions of Clinical Human Biology and ⁷Public Health Sciences, Fred Hutchinson Cancer Research Center, Seattle, Washington; ⁸Department of Pathology, University of Michigan Medical School, Ann Arbor, Michigan; ⁹Department of Pathology, Harvard Medical School, Brigham and Women's Hospital, Boston, Massachusetts; and ¹⁰Center for Human Genomics, Wake Forest University School of Medicine, Winston-Salem, North Carolina

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 SRp40 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 wt*KLF6* 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-penetrance 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.

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Requests for reprints: John A. Martignetti, Department of Human Genetics, Mount Sinai School of Medicine, 1425 Madison Avenue, New York, NY 10029. Phone: 212-659-6744; Fax: 212-849-2638; E-mail: john.martignetti@mssm.edu.

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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 for the case subjects in these families; 84% of the families are White and 8.8% are Black.

All nonhereditary prostate cancer case subjects were recruited from among patients who underwent treatment for prostate cancer at the Johns Hopkins Hospital. The diagnosis for all these subjects was confirmed by pathology reports. Mean age at diagnosis for these case subjects was 58.6. 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 of the probands.

The Mayo study population with familial prostate cancer was comprised of 431 men from 177 families, such that each family had a minimum of three affected men with prostate cancer as reported by family history. All men who contributed a DNA sample had their prostate cancer diagnosis verified by review of medical records, and when possible, pathologic confirmation (21). Sporadic prostate cancer patients were selected from respondents to a family history survey, who reported no family history of prostate cancer. To ensure that the sporadic group was similar to the familial group, except for family history, eligible sporadic cases were selected by frequency matching them to the familial index cases according to year of diagnosis, age at diagnosis, and number of brothers. Multiple sporadic cases were identified for each familial case, and these case sets were randomly sampled for recruitment, with a goal of recruiting ~500 men with sporadic prostate cancer. A total of 1,001 invitations were sent to men who initially reported no family history of prostate cancer. The second survey determined that only 740 of these men were eligible (i.e., still no family history of prostate cancer), and of these men, 501 agreed to contribute a blood sample. At the time when assays were done, blood samples were available for 499 men. All but 11 of these men were treated surgically for their prostate cancer.

The control population of 491 men was drawn from a sampling frame of the local population provided by the Rochester Epidemiology Project, from Olmsted County, MN and screened for evidence of prostate cancer by an extensive work-up at baseline and periodic follow-up as previously described (22). This work-up included a DRE and transrectal ultrasound of the prostate, abdominal ultrasound for post-void residual urine volume, measurement of serum PSA levels and creatinine, focused urological physical examination, and cryopreservation of serum for sex hormone assays (22). Any patient with an abnormal DRE, elevated serum PSA level, or suspicious lesion on transrectal ultrasound was evaluated for prostatic malignancy. If the DRE and transrectal ultrasound were unremarkable and

the serum PSA level was elevated (>4.0 ng/mL), a sextant biopsy (three cores from each side) of the prostate was done. An abnormal DRE or transrectal ultrasound result, regardless of the serum PSA level prompted a biopsy of the area in question. In addition, a sextant biopsy of the remaining prostate was done. Those men who were found to be without PC based on this extensive work-up at baseline or at any of the follow-up exams through 1994, with augmentation with random samples from the population accrued over that time, were used as the control population for this study.

The men in the Fred Hutchinson Cancer Research Center study included participants in a population-based case-control study of sporadic prostate cancer risk factors in middle-aged men. The case-control study has been described in detail (23). Cancer patients, representing all grades, stages, and treatments, were ascertained from the Seattle-Puget Sound Surveillance, Epidemiology, and End Results cancer registry. Included were Caucasian men from King County, WA, ages 40 to 64 years, diagnosed with prostate cancer between January 1, 1993 and December 31, 1996. Of the total 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 KLF6 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 KLF6 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 Sequencher 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 KLF6-specific primers: Δ ATG forward 5'-CGGGCAG-CAATGTTATCTGCTCTC-3' and Δ ATG reverse 5'-TTCTGAGGCTGAAA-CATAGCAGGG-3'. The PCR product was then either sequenced per standard protocols or digested with *Bsa*AI (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 *Bsa*AI restriction enzyme digest. Genotypes for the Mayo Clinic samples were reconfirmed by pyrosequencing as previously described (21), using the same KLF6 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 KLF6 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 all 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 Tissue 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 IVS Δ A, WILD TYPE, or LacZ minigene expression constructs with a puromycin expressing plasmid. Transfected cells were selected with 2 μ g/mL of puromycin.

Wild-type KLF6 and IVS Δ A Minigene Constructs. To amplify the 6.2 kb KLF6 genomic locus including ~100 bp of the 5' untranslated region and 300 bp of 3' untranslated region, 200 ng of human genomic DNA were

amplified using the following set of primers: KLF6-1F TTGCACTCAGTCCGGTGTGTTG 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 (Qiagen) and then subcloned into the TOPO TA expression vector (Invitrogen, San Diego, CA). The IVS Δ mutation was introduced into the full-length minigene construct by site-directed mutagenesis according to the manufacturer's protocol (Quick-Change, Stratagene, LA Jolla, CA). The primers used for mutagenesis were as follows: KLF6 Δ A-F GTCATGGCAATCACATGCCTTCTCTGGTT and KLF6 Δ A-R AAC-CAGAGAAGGCATGTGATTGCCATGAC. 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 *XbaI* 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, ASF, 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 *BglIII/HindIII* sites of the pSuper vector.

- Si-SV1-F: GATCCCCAGGCTTTTCTCCTTCCCTGGCttcaaga-gaGCCAGGGAAGGAGAAAAGCCTTTTGAAA
- Si-SV1-R: AGCTTTTCCAAAAAGGCTTTTCTCCTTCCCTGGCtctcttgaaGCCAGGGAAGGAGAAAAGCCTGGG
- Si-SV2-F: GATCCCCGCCAGGAGAAAAGCCTTACttcaaga-gaGTAAGGCTTTTCTCCTGGCTTTTGGAAA
- Si-SV2-R: AGCTTTTCCAAAAAGCCAGGAGAAAAGCCT-TACtctcttgaaGTAAGGCTTTTCTCCTGGCGGG
- Luc-F: GATCCCCTACTTCGAAATGTCCGTTTctcaagaga-GAACGGACATTTGAAAGTATTTTGGAAA
- Luc-R: AGCTTTTCCAAAAATACTTCGAAATGTCCGTTTctcttgaaGAACGGACATTTGAAAGTAGGG

Western Blot Analysis. BPH1 or 293T cells grown in 12-well dishes were transfected with 0.5 μ g per well of either the IVS Δ , 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 μ g) 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 / Zf9, 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.

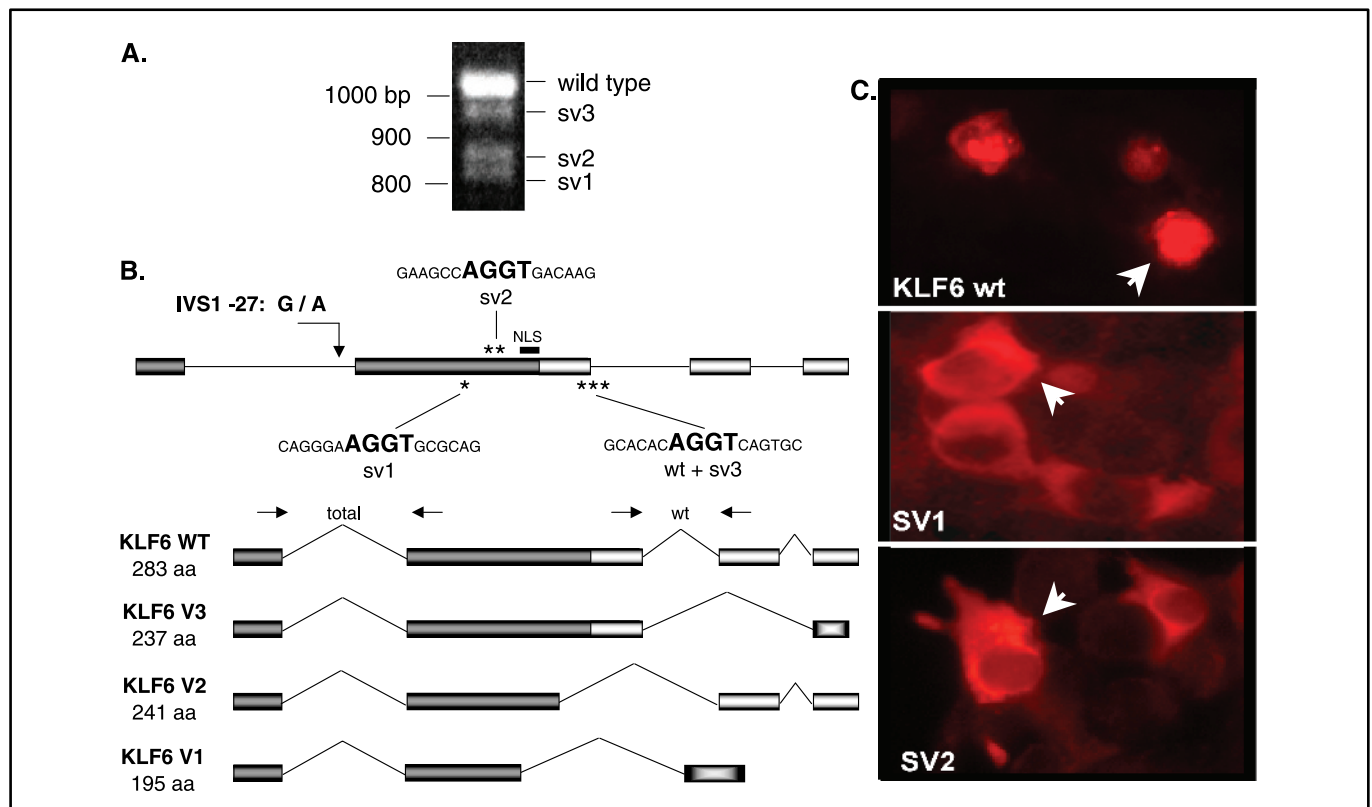


Figure 1. Structure and cellular localization of KLF6 and its splice variants. **A**, RT-PCR of 293T cells transfected with the full-length wt KLF6 minigene construct reveals that the *KLF6* gene is alternatively spliced. **B**, genomic organization, splice site sequences, and mRNA structures for wt 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 wtKLF6, KLF6SV1, or KLF6SV2 expression constructs. FLAG immunostaining revealed that wtKLF6 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 IVSΔA, 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'-CGGACGCACACAGGAGAAAA-3' and wtKLF6 reverse 5'-CGGTGTGCTTTTCGGAAGTG-3'; Total KLF6 forward 5'-CTGCCGTCTCTGGAGGAGT-3' and Total KLF6 reverse 5'-TCCACAGATCTTCTGGCTGTC-3'; p21 forward 5'-ACTCTCAGGGTCGAAAACGG-3' and p21 reverse 5'-CCTCGCGCTCCAGGACTG-3'; GAPDH forward 5'-CAATGACCCCTTCATTGACC-3' and GAPDH reverse 5'-GATCTCGCTCCTGGAAGATG-3'; SRp40 forward: 5'-CCAAGGGATGCA-GATGATGCTG and SRp40 reverse 5'-GGAGCATTCGTCTATCATTTCCA-3'. All experiments were done in triplicate and normalized to GAPDH. 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.

	n	Genotype			P	OR G/G vs A/+ (95% CI)
		G/G	G/A	A/A		
Mayo						
Control	491	434 (88.4)	56 (11.4)	1 (0.2)		
Sporadic	497	421 (84.7)	71 (14.3)	5 (1.0)	0.09	1.37 (0.951-99)
Familial	431	352 (81.7)	77 (17.9)	2 (0.5)	0.02	1.71 (1.13-2.60)
JHU						
Control	264	237 (89.8)	27 (10.2)	0 (0.0)		
Sporadic	198	166 (83.8)	31 (15.7)	1 (0.5)	0.06	1.69 (0.98-2.93)
Familial	451	382 (84.7)	68 (15.1)	1 (0.2)	0.11	1.59 (0.98-2.57)
FHCRC						
Control	521	435 (83.5)	81 (15.6)	5 (1.0)		
Control-PSA*	169	150 (88.8)	19 (11.2)			
Sporadic	558	477 (85.5)	75 (13.4)	6.11		0.86 (0.62-1.20)
Sporadic*						1.34 (0.79-2.28)
Combined						
Mayo/JHU						
Control	755	671 (88.9)	83 (11.0)	1 (0.1)		
Sporadic	695	587 (84.5)	102 (14.7)	6 (0.9)	0.01	1.47 (1.08-2.00)
Familial	882	734 (83.2)	145 (16.4)	3 (0.3)	0.01	1.61 (1.20-2.16)
Combined Mayo/JHU/FHCRC						
Control	1,276	1,106 (86.7)	164 (12.9)	6 (6.05)		
Sporadic	1,253	1,064 (84.9)	189 (15.1)			1.16 (0.92-1.44)
Control-PSA	924	821 (88.9)	103 (11.2)			
Sporadic*	1,253	1,064 (84.9)	189 (15.1)		0.01	1.42 (1.10-1.80)

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 for the presence of this variant from three centers. In total, genomic 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

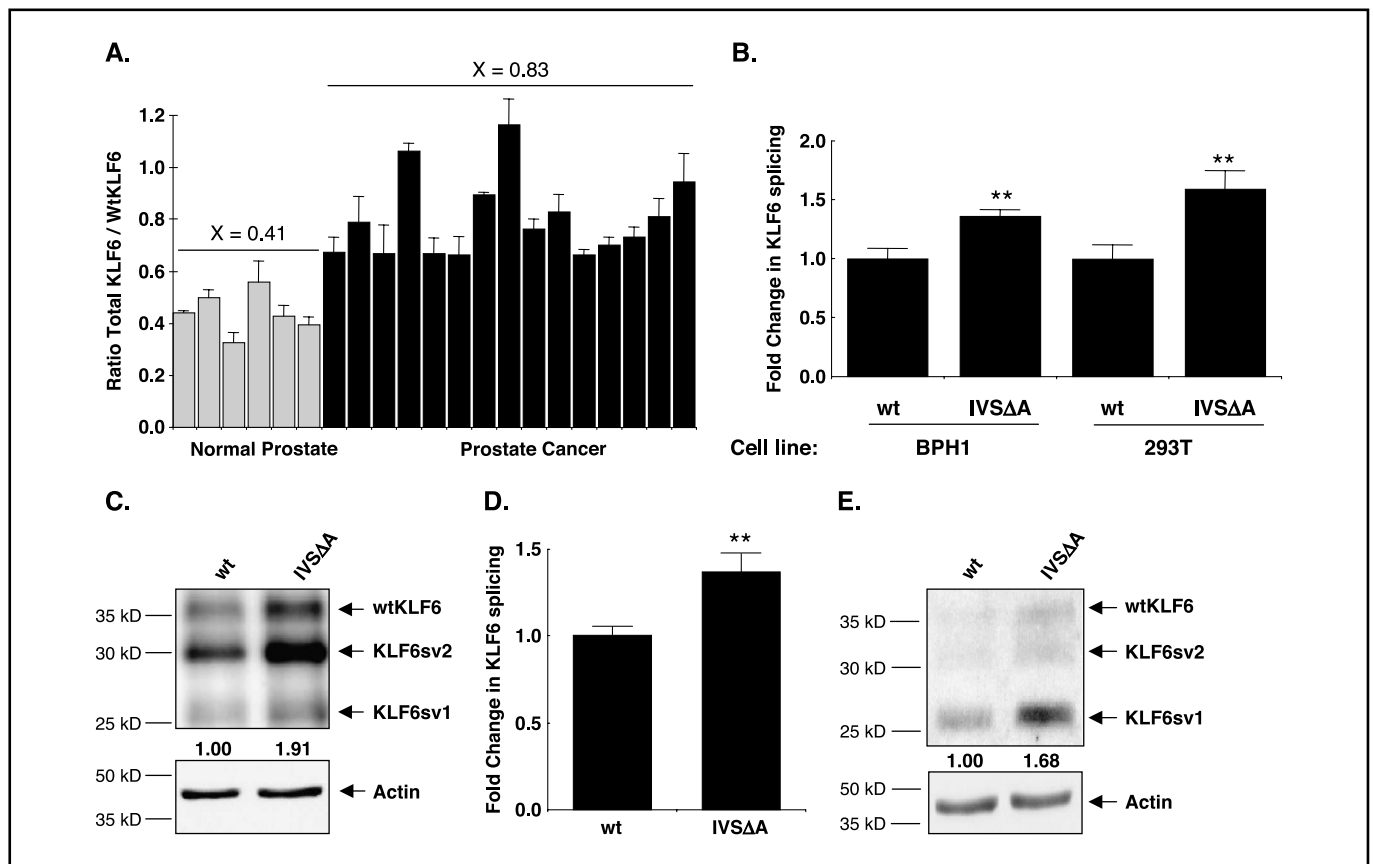


Figure 2. *KLF6* gene is differentially spliced in tumor tissue and can be regulated in cell culture by the *IVSΔA* variant. **A**, qRT-PCR was done on six normal prostate tissue samples and 15 prostate cancer specimens using total and wt specific *KLF6* primers. The ratio of total *KLF6* to wt *KLF6* 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 wt*KLF6* message. Prostate tumors had significantly higher splicing ratios ($x = 0.83$), a higher total/wt*KLF6* 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.

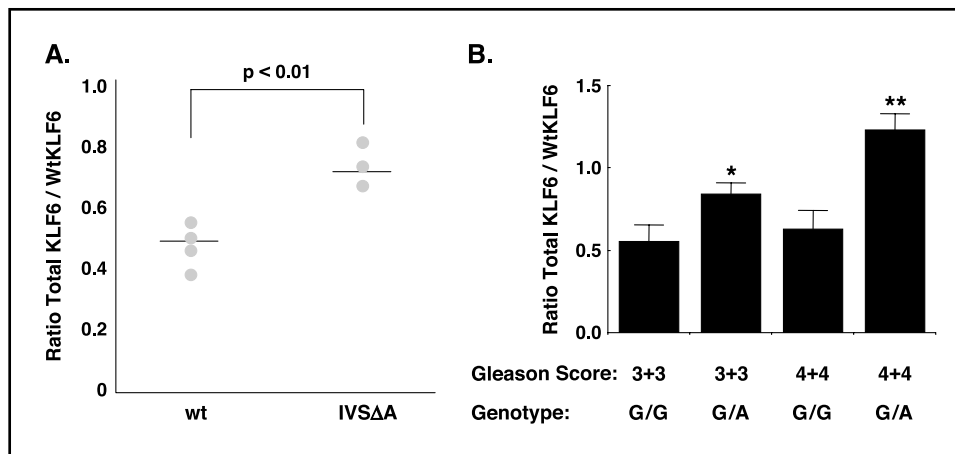


Figure 3. IVSΔA is associated with increased alternative splicing of the *KLF6* gene in prostatic tissues. **A.** qRT-PCR of patient-derived benign prostatic hyperplasia tissue revealed that the presence of IVSΔA variant was associated with ~40% increase in *KLF6* splicing ($P < 0.01$). **B.** qRT-PCR of prostate cancer tissues revealed that the IVSΔA 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.

sporadic cases and controls, was compared using Pearson's χ^2 statistic, and computed ORs and their 95% confidence intervals (95% CI; SAS Institute, Inc., Cary, NC). Second, to compare the IVSΔA 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 IVSΔA 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

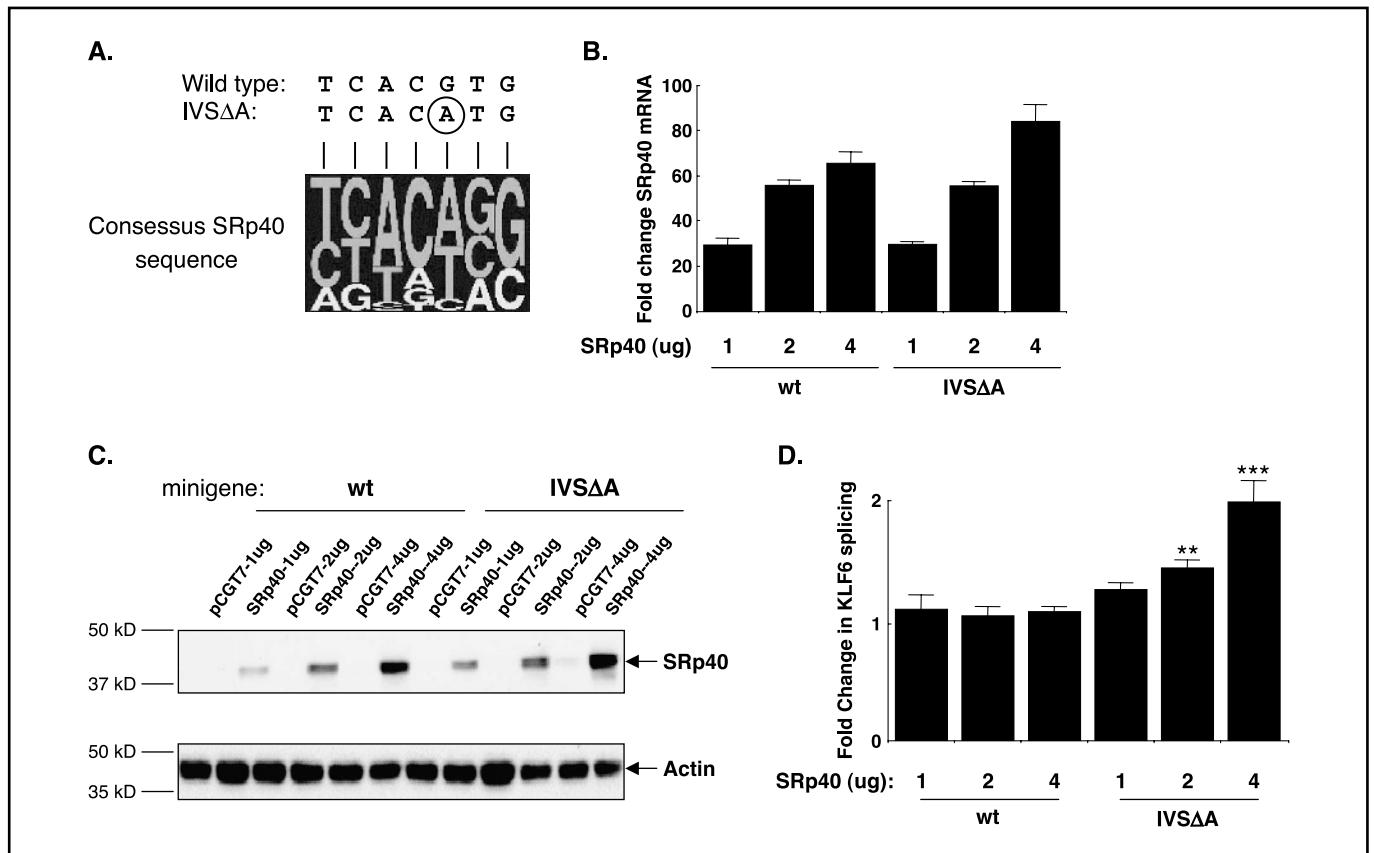


Figure 4. The IVSΔA variant creates a functional SRp40 binding site. **A.** alignment of wt and IVSΔA sequences with the consensus SRp40 sequence. Control experiments with HeLa cells cotransfected with increasing amounts of a SRp40 expression vector and either the IVSΔA variant or wt minigene constructs were done. **B.** qRT-PCR using SRp40-specific real-time primers confirm incremental SRp40 expression. Difference in SRp40 expression between the wt and IVSΔA variant cotransfected with 4 μg 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 IVSΔA 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 *KLF6* gene variant carriers was 1.47 ($P = 0.01$; 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 ($P = 0.57$; 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 ($P = 0.21$; 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 *IVSΔA* allele and cancer risk. The magnitude of the relative risk for *IVSΔA* carriers was 1.61 ($P = 0.01$; 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 ($P = 0.02$; OR, 1.71; 95% CI, 1.13-2.60) whereas a trend towards significance was present in the other group ($P = 0.11$; 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 years of age; $P \leq 0.03$).

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

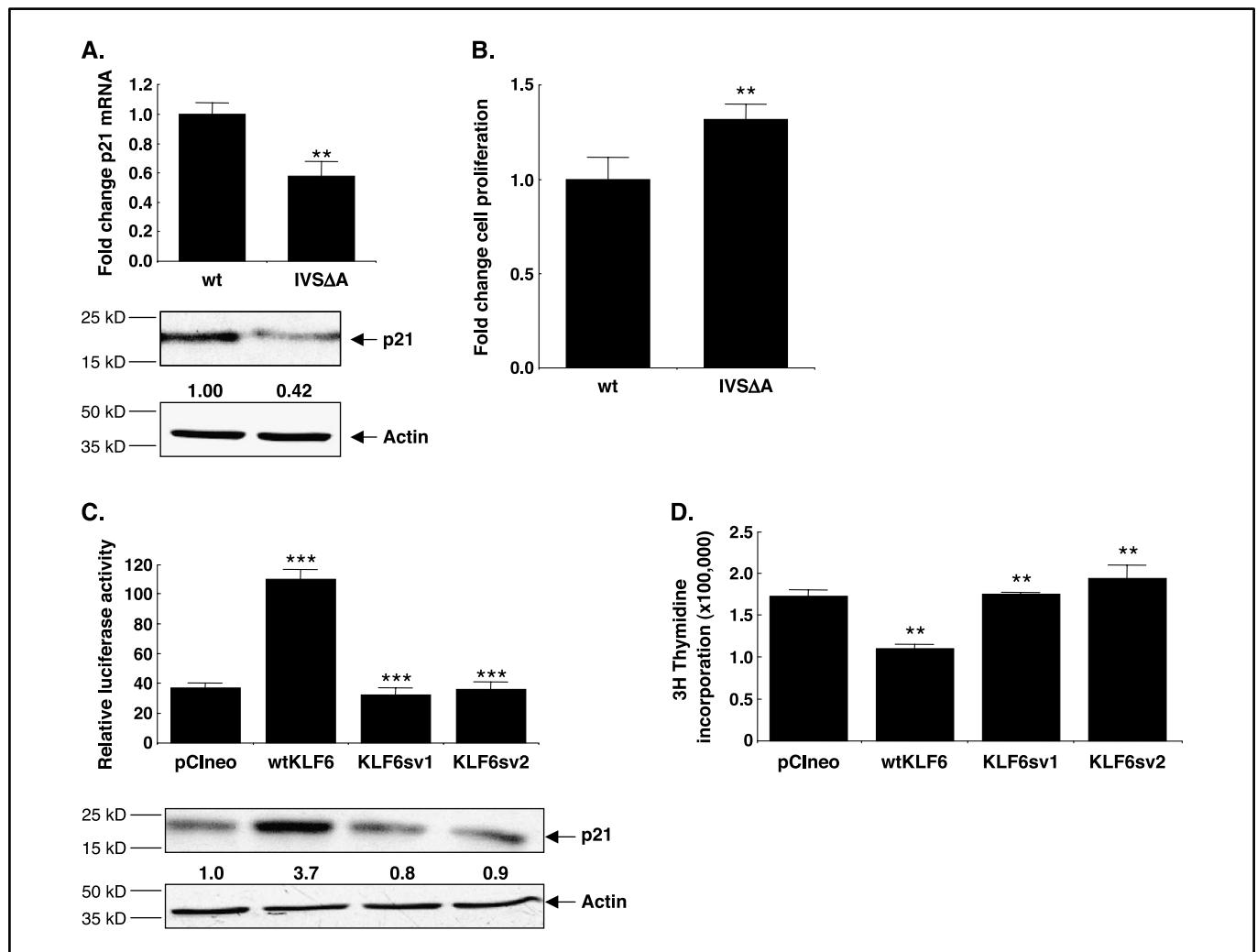


Figure 5. *IVSΔA* variant blunts p21 up-regulation and increases cellular proliferation. **A**, endogenous p21 levels in BPH1 cells expressing the minigene constructs. *IVSΔA* cells have 40% less p21 mRNA and 60% less p21 protein than wt cells. **, $P < 0.001$ (ANOVA, Bonferroni correction applied, $n = 4$). **B**, cell proliferation analysis of minigene expressing BPH1 cells. The *IVSΔA* expressing cells proliferate 30% more than the wt expressing cells. **, $P < 0.001$ (ANOVA, Bonferroni correction applied, $n = 9$). **C**, *KLF6* variant proteins fail to transactivate the p21 promoter and up-regulate endogenous p21. 293T cells were cotransfected with a p21 reporter plasmid and either wtKLF6, SV1, or SV2 expression vectors. All values represent relative luciferase activity. wtKLF6 transactivated the p21 promoter 6-fold (***, $P < 0.0001$; ANOVA, Bonferroni correction applied, $n = 6$), whereas neither KLF6SV1 nor KLF6SV2 resulted in significant transactivation (***, $P < 0.0001$; ANOVA, Bonferroni correction applied, $n = 9$). KLF6SV1 and SV2 proteins fail to up-regulate endogenous p21. 293T cells were transfected with a wtKLF6, SV1, or SV2 expression vector and 24 hours after transfection cells were harvested and Western blot analysis was done using a p21 antibody. **D**, KLF6SV1 and SV2 proteins fail to suppress cell proliferation. PC3M cells were transfected with a wtKLF6, SV1, or SV2 expression vector and ^3H incorporation was measured (**, $P < 0.001$; ANOVA, Bonferroni correction applied, $n = 9$). Comparisons were made between wtKLF6 and pCI-neo and variants and wtKLF6.

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 effected 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 wt*KLF6* and two of the novel isoforms. Whereas wt*KLF6* 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 wt*KLF6* alone or

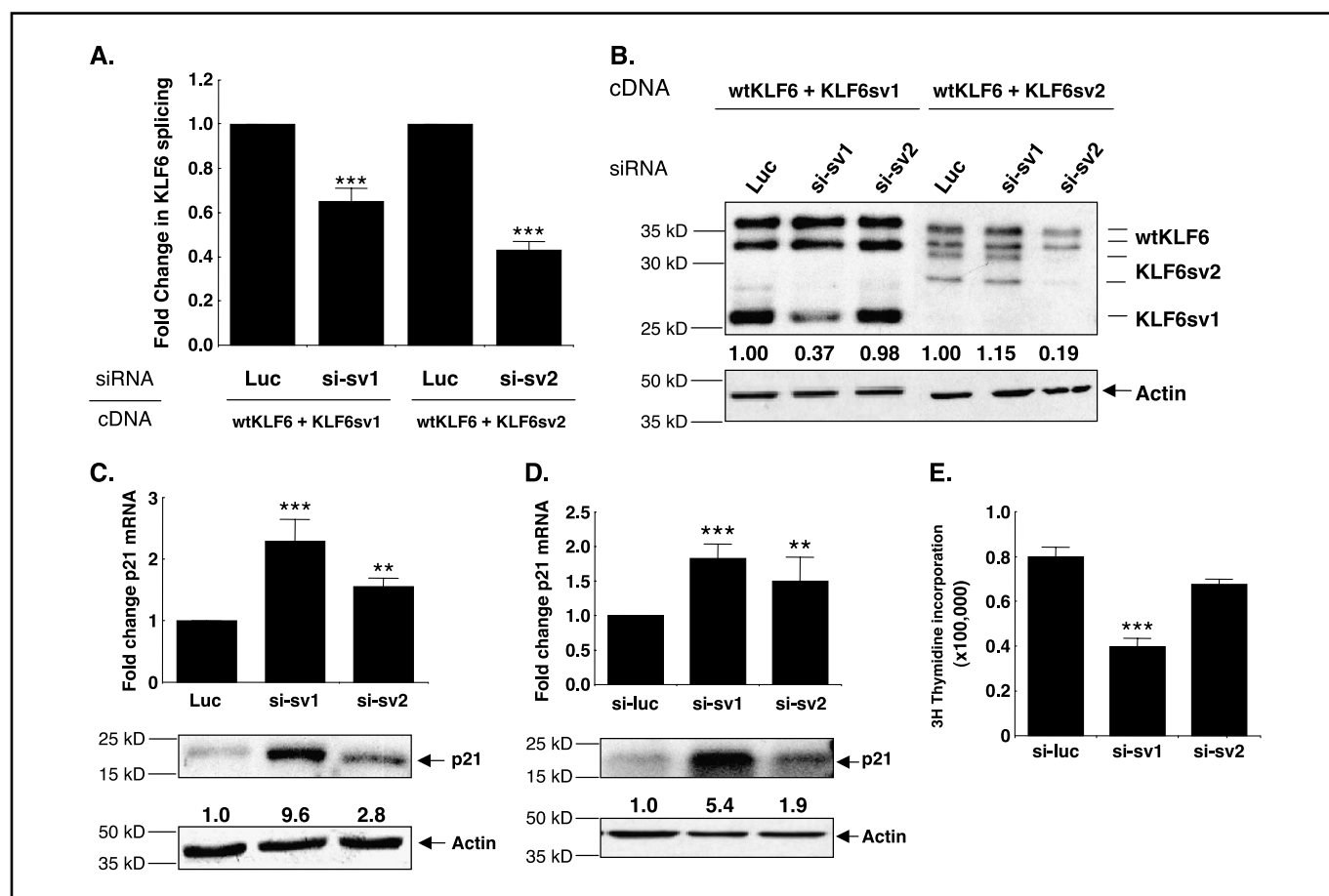


Figure 6. *KLF6* variants antagonize wt*KLF6* ability to up-regulate p21. As a control for siRNA efficacy and specificity, 293T cells were cotransfected with either the pSUPER²⁷ si-SV1 or si-SV2 plasmid and *KLF6* and SV1 or SV2 cDNA expression vectors. **A**, qRT-PCR of 293T transfected cells shows 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; $P < 0.001$). 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 minigene 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 studies of 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

- Jemal AT, Murray T, Samuels A, et al. Cancer statistics. *CA Cancer J Clin* 2002;52:23–47.
- Baselga J, Norton L. Focus on breast cancer. *Cancer Cell* 2002;4:319–22.
- Kinzler KW, Vogelstein B. Lessons from hereditary colorectal cancer. *Cell* 1996;87:159–70.
- Ostrander EA, Stanford JL. Genetics of prostate cancer: too many loci, too few genes. *Am J Hum Genet* 2000;67:1367–75.
- Tavtigian SV, Simard J, Teng DH, et al. A candidate prostate cancer susceptibility gene at chromosome 17p. *Nat Genet* 2001;27:172–80.
- Carpten J, Nupponen N, Isaacs S, et al. Germline mutations in the ribonuclease L gene in families showing linkage with HPC1. *Nat Genet* 2002;30:181–4.
- Xu J, Zheng SL, Komiya A, et al. Germline mutations and sequence variants of the macrophage scavenger receptor 1 gene are associated with prostate cancer risk. *Nat Genet* 2002;32:321–5.
- Schaid DJ. The complex genetic epidemiology of prostate cancer. *Hum Mol Genet* 2004;13:R103–21.
- Narla G, Heath KE, Reeves HL, et al. KLF6, a candidate tumor suppressor gene mutated in prostate cancer. *Science* 2001;294:2563–6.
- Chen C, Hyytinen ER, Sun X, et al. Deletion, mutation, and loss of expression of KLF6 in human prostate cancer. *Am J Pathol* 2003;162:1349–54.
- Reeves HL, Narla G, Ogunbiyi O, et al. Kruppel-like factor 6 (KLF6) is a tumor-suppressor gene frequently inactivated in colorectal cancer. *Gastroenterology* 2004;126:1090–103.
- Jeng YM, Hsu HC. KLF6, a putative tumor suppressor gene, is mutated in astrocytic gliomas. *Int J Cancer* 2003;105:625–9.
- Chen HK, Liu XQ, Lin J, Chen TY, Feng QS, Zeng YX. Mutation analysis of KLF6 gene in human nasopharyngeal carcinomas. *Ai Zheng* 2002;21:1047–50.
- Yamashita K, Upadhyay S, Osada M, et al. Pharmacologic unmasking of epigenetically silenced tumor suppressor genes in esophageal squamous cell carcinoma. *Cancer Cell* 2002;2:485–95.
- Kettunen E, Anttila S, Seppanen JK, et al. Differentially expressed genes in nonsmall cell lung cancer: expression profiling of cancer-related genes in squamous cell lung cancer. *Cancer Genet Cytogenet* 2004;149:98–106.
- Ito G, Uchiyama M, Kondo M, et al. Kruppel-Like Factor 6 (KLF6) is frequently downregulated and induces apoptosis in non-small cell lung cancer cells. *Cancer Res* 2004;11:3838–43.
- Kimmelman AC, Qiao RF, Narla G, et al. Suppression of glioblastoma tumorigenicity by the Kruppel-like transcription factor KLF6. *Oncogene* 2004;29:5077–83.
- Benzeno S, Narla G, Allina J, et al. Cyclin dependent kinase inhibition by the KLF6 tumor suppressor protein through interaction with cyclin D1. *Cancer Res* 2004;64:3885–91.
- Slavin DA, Koritschoner NP, Prieto CC, Lopez-Diaz FJ, Chatton B, Bocco JL. A new role for the Kruppel-like transcription factor KLF6 as an inhibitor of c-Jun proto-oncogene function. *Oncogene* 2004;23:196–205.
- Glinsky GV, Glinskii AB, Stephenson AJ, Hoffman RM, Gerald WL. Gene expression profiling predicts clinical outcome of prostate cancer. *J Clin Invest* 2004;113:913–23.
- Carpten J, Nupponen N, Isaacs S, et al. Germline mutations in the ribonuclease L gene in families showing linkage with HPC1. *Nat Genet* 2002;30:181–4.
- Wang L, McDonnell SK, Elkins DA, et al. Role of HPC2/ELAC2 in hereditary prostate cancer. *Cancer Res* 2001;61:6494–9.
- Stanford JL, Wicklund KG, McKnight B, Daling JR, Brawer MK. Vasectomy and risk of prostate cancer. *Cancer Epidemiol Biomarkers Prev* 1999;8:881–6.
- Slager S, Schaid D. Evaluation of candidate genes in case-control studies: a statistical method to account for related subjects. *Am J Hum Genet* 2001;68:1457–62.
- Weir BS. Genetic Data Analysis II. Methods for discrete population genetic data. Massachusetts: Sinaur Associates, Inc.; 1996.
- Sasieni P. From genotypes to genes: doubling the sample size. *Biometrics* 1997;53:1253–61.
- Liu HX, Zhang M, Krainer AR. Identification of functional exonic splicing enhancer motifs recognized by individual SR proteins. *Genes Dev* 1998;12:1998–2012.
- Brummelkamp TR, Bernards R, Agami R. A system for stable expression of short interfering RNAs in mammalian cells. *Science* 2002;296:550–3.
- Dhanasekaran SM, Barrette TR, Ghosh D, et al. Delineation of prognostic biomarkers in prostate cancer. *Nature* 2001;412:822–6.
- Quadrini KJ, Bieker JJ. Kruppel-like zinc fingers bind to nuclear import proteins and are required for efficient nuclear localization of erythroid Kruppel-like factor. *J Biol Chem* 2002;277:32243–52.
- Cartegni L, Wang J, Zhu Z, Zhang MQ, Krainer AR. ESEfinder: A web resource to identify exonic splicing enhancers. *Nucleic Acids Res* 2003;31:3568–71.