

SnoRNA U50 is a candidate tumor-suppressor gene at 6q14.3 with a mutation associated with clinically significant prostate cancer

Xue-Yuan Dong^{1,2}, Carmen Rodriguez³, Peng Guo^{1,2}, Xiaodong Sun^{1,2}, Jeffrey T. Talbot³, Wei Zhou^{1,2,4}, John Petros^{1,2,5,6,7}, Qunna Li^{1,2}, Robert L. Vessella⁸, Adam S. Kibel⁹, Victoria L. Stevens³, Eugenia E. Calle³ and Jin-Tang Dong^{1,2,4,5,*}

¹Winship Cancer Institute and ²Department of Hematology and Oncology, Emory University School of Medicine, 1365 Clifton Road, Atlanta, GA 30322, USA, ³Department of Epidemiology and Surveillance Research, American Cancer Society, 1599 Clifton Road, Atlanta, GA 30329, USA, ⁴Program in Genetics and Molecular Biology, ⁵Department of Urology, ⁶Department of Pathology and Laboratory Medicine, Emory University School of Medicine, 1365 Clifton Road, Atlanta, GA 30322, USA, ⁷Atlanta Veterans Affairs Medical Center, 1670 Clairmont Road, Atlanta, GA 30033, USA, ⁸Department of Urology, University of Washington, Seattle, WA 98195, USA and ⁹Department of Surgery, Washington University School of Medicine, Barnes-Jewish Hospital, St Louis, MO 63110, USA

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Deletion of chromosome 6q14–q22 is common in multiple human cancers including prostate cancer, and chromosome 6 transferred into cancer cells induces senescence and reduces cell growth, tumorigenicity and metastasis, indicating the existence of one or more tumor-suppressor genes in 6q. To identify the 6q tumor-suppressor gene, we first narrowed the common region of deletion to a 2.5 Mb interval at 6q14–15. Of the 11 genes located in this minimal deletion region and expressed in normal prostates, only snoRNA U50 was mutated, demonstrated transcriptional downregulation and inhibited colony formation in prostate cancer cells. The mutation, a homozygous 2 bp (TT) deletion, was found in two of 30 prostate cancer cell lines/xenografts and nine of 89 localized prostate cancers (eleven of 119 or 9% cancers). Two of 89 (2%) patients with prostate cancer also showed the same mutation in their germline DNA, but none of 104 cancer-free control men did. The homozygous deletion abolished U50 function in a colony formation assay. Analysis of 1371 prostate cancer cases and 1371 matched control men from a case–control study nested in a prospective cohort showed that, although a germline heterozygous genotype of the deletion was detected in both patients and controls at similar frequencies, the homozygosity of the deletion was significantly associated with clinically significant prostate cancer (odds ratio 2.9; 95% confidence interval 1.17–7.21). These findings establish snoRNA U50 as a reasonable candidate for the 6q tumor-suppressor gene in prostate cancer and likely in other types of cancers.

INTRODUCTION

Prostate cancer is the most common non-skin cancer in the developed regions of the world. The majority of prostate cancers, however, do not present clinical symptoms during a man's natural life and are considered indolent or clinically insignificant (1,2). With widespread prostate-specific antigen

(PSA) testing, many indolent prostate cancers are unnecessarily detected (3), and as many as seven of eight patients with screen-detected prostate cancer could be unnecessarily treated (4). An important question is which men with prostate cancer should be treated and who should be left for watchful waiting. Prostate cancer is considered a multistep disease resulting from the accumulation of genetic alterations

*To whom correspondence should be addressed at: Winship Cancer Institute, Emory University School of Medicine, Room C4080, 1365 Clifton Road, Atlanta, GA 30322, USA. Tel: +1 4047122568; Fax: +1 4047122571; Email: jdong2@emory.edu

including activation of oncogenes and inactivation of tumor-suppressor genes. Identification and characterization of genetic alterations underlying prostate cancer could help not only in detecting clinically significant prostate cancers but also in understanding prostate cancer biology.

Chromosomal deletion is a hallmark of tumor-suppressor genes because it can reveal recessive mutations, cause haploinsufficiency or truncate/abolish a gene through loss of heterozygosity, hemizygous deletion or homozygous deletion, respectively. Many chromosomal regions are frequently deleted in human cancer, as demonstrated by various genetic approaches, but the affected genes for most of them are still unknown (5,6). Deletion of chromosome 6 involving q14–q22 is one of the most common deletions in different types of human cancers including prostate cancer (5,6). Functionally, chromosome 6 transferred into cancer cells induces senescence, reduces cell growth, inhibits tumorigenicity and decreases metastatic potential (7–12). These studies indicate the existence of one or more tumor-suppressor genes in 6q, although the critical gene has not been established at present.

In this report, we performed genetic and functional analyses and found that the *U50* snoRNA gene, encoded by an intron, is a reasonable candidate for the 6q tumor-suppressor gene. We also found that a 2 bp germline homozygous deletion of *U50* was associated with clinically significant prostate cancer in a large cohort.

RESULTS

Deletion mapping and expression evaluation of genes from the minimal region of deletion

To identify the 6q14–q22 tumor-suppressor gene(s), we first performed deletion mapping to narrow the most critical region of deletion, following the approach described in our previous study (13). Using 69 sequence-tagged site (STS) markers spanning 6q14–q22 (54.5 Mb), we examined 30 cell lines and xenografts derived from different prostate cancers to detect homozygous and hemizygous deletions by regular and duplex PCR. A homozygous deletion of 3.6 Mb in 6q14–q15 was detected in the LuCaP 73 xenograft (Fig. 1A and D). Hemizygous deletions overlapping with the homozygous deletion were detected in 14 of the 30 (47%) independent prostate cancers (LNCaP, PC-3, CWR21, CWR91, LAPC3, LAPC9, LuCaP 23.1/23.8/23.12, LuCaP 35/35V, LuCaP 41, LuCaP 69, LuCaP 70/70S8, LuCaP 96, LuCaP105 and LuCaP115) (Fig. 1B–D). Although most hemizygous deletions were more extensive than the homozygous deletion, xenografts LuCaP 105 and LAPC3 had hemizygous deletions that narrowed the 3.6 Mb deletion region to 2.5 Mb at 6q14–15, between markers RH118824 and WI-18995 (Fig. 1B–D).

The Entrez gene database (Build 35) at NCBI (<http://www.ncbi.nlm.nih.gov>) and the database of the Human Genome Organization (HUGO) Gene Nomenclature Committee (HGNC) were reviewed. Eleven verified or predicted genes lay within the 2.5 Mb 6q14–15 minimal deletion region: nine protein-coding genes (*LOC389415*, *LOC441163*, *LOC441164*, *LOC441165*, *HTR1E*, *NT5E*, *SNX14*, *SYNCRIP* and *TBX18*), one pseudogene (*LOC401269*) and one gene

(*U50HG*) that hosts two snoRNAs named *U50* and *U50'*. To determine which of these genes is the best candidate(s) for the 6q14–15 tumor-suppressor gene(s), we first examined their expression in a pool of normal prostates, along with 13 other normal tissues (spleen, kidney, stomach, pancreas, uterus, ovary, testis, placenta, thymus, lung, skin, adrenal gland and bone marrow) as positive controls, using the sensitive reverse-transcription PCR (RT–PCR) assay. The expression of hypothetical *LOC389415*, *LOC441163* and *LOC441165* was not detectable in either normal prostates or the 13 additional normal tissues (data not shown). We designed a different pair of primers for each of the three genes, but again no expression was detected by RT–PCR (data not shown). In the latest NCBI databases, these three hypothetical genes have been discontinued. We concluded that the three hypothetical genes do not have real transcripts at least in the 14 tissues examined. On the other hand, *HTR1E* was expressed at different levels in ovary and testis; *SNX14* in thymus, testis, bone marrow and ovary; *TBX18* in bone marrow, thymus, ovary, testis, skin and adrenal gland. However, the expression of *HTR1E*, *SNX14* or *TBX18* was not detectable in normal prostates (data not shown). These results suggest that the genes of *LOC389415*, *LOC441163*, *LOC441165*, *HTR1E*, *SNX14* and *TBX18* most likely do not play a role in the structure and/or function of the prostate. These genes were excluded from further analysis. In addition, the pseudogene *LOC401269* was also excluded from further analysis.

Mutation detection, expression evaluation and functional assay in cancer cells

For the remaining three protein-encoding genes (*LOC441164*, *NT5E* and *SYNCRIP*) and the two snoRNAs, *U50* and *U50'*, hosted in the *U50HG* gene, we conducted three tests to determine which is more likely to be the 6q14–15 tumor-suppressor gene. First, we determined whether any of the genes had mutations in 15 prostate cancer cell lines and xenografts by direct DNA sequencing. Second, we evaluated their expression in a panel of cell lines, xenografts and primary tumors from prostate cancer. Third, we performed colony formation assay to analyze whether any of the genes could alter cell proliferation or survival. In 15 prostate cancer cell lines and xenografts examined, no mutations were detected for the three protein-encoding genes and the snoRNA *U50'*. On the other hand, the snoRNA *U50* showed a homozygous two-base (TT) deletion in a stretch of four thymidines in prostate cancer xenograft LuCaP 96.

Real-time PCR analysis was used to evaluate gene expression in prostate cancer cell lines or xenografts. Compared with normal prostates, the expression of *LOC441164*, *SYNCRIP* and snoRNA *U50'* was not consistently reduced in cancer cells, although one or more cell lines showed lower levels of expression for each of them (data not shown). The expression of *NT5E* and snoRNA *U50* was downregulated in most of the prostate cancer cell lines and xenografts tested (data not shown and Fig. 2C). For snoRNA *U50*, in particular, expression was almost completely absent in the commonly used prostate cancer cell lines 22Rv1, LNCaP and PC-3, as detected by northern blot analysis (Fig. 2B). We also analyzed

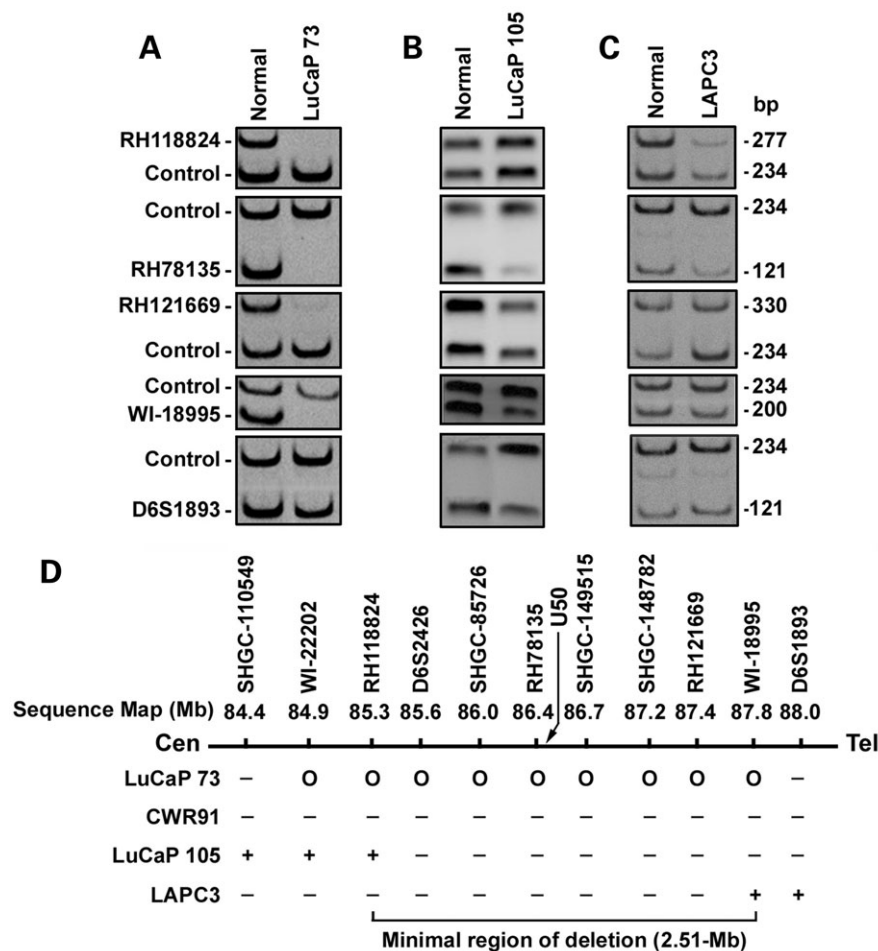


Figure 1. Mapping of the deletion region in 6q14.3–q15 in prostate cancer. (A) Homozygous deletion detected by duplex PCR in xenograft LuCaP 73. (B) and (C) Hemizygous deletions detected by duplex PCR in xenografts LuCaP 105 and LAPC3. Sample names are at the top, markers at the left and sizes (bp) of PCR products at the right. (D) Deletion status for each marker. Normal sample was from a normal human placenta. Definition of the minimal region of deletion at 6q14–15 in prostate cancer. Marker names are at the top, sample names at the left and the minimal region of deletion is marked by a horizontal line at the bottom. The sequence map is indicated for each marker. ‘○’, homozygous deletion; ‘-’, hemizygous deletion; ‘+’, no deletion. The location of *U50* is indicated by an arrow.

U50 expression using real-time PCR analysis in 15 primary prostate cancer specimens. Compared with matched normal cells, *U50* was downregulated in 11 of the 15 cancer specimens, and the downregulation was at least 50% in seven of them (Fig. 2D).

We then performed colony formation assays as described previously (13). Each gene was transfected into a prostate cancer cell line in which reduced levels of expression had been demonstrated: *LOC441164*, *NT5E*, *U50* and *U50'* in the LNCaP cell line, and *SYNCRIP* in the 22Rv1 cell line. The expression of *NT5E* and *U50* was also low in 22Rv1, so this cell line was also used to confirm the findings from LNCaP cells. Each gene was ectopically expressed with empty plasmid as the negative control. Four of the five genes, *LOC441164*, *NT5E*, *SYNCRIP* and snoRNA *U50'*, did not affect colony formation efficiency at all (data not shown). Ectopic expression of the three protein-encoding genes in transfected cells was verified by western blot analysis using an antibody against FLAG, which was attached to the protein (data not shown).

SnoRNA *U50*, on the other hand, significantly reduced colony formation in both 22Rv1 and LNCaP cell lines upon ectopic expression (Fig. 3B and C). Because both *U50* and *U50'* snoRNAs might be simultaneously produced through the splicing of *U50HG* (14), we transfected equal amounts of *U50* and *U50'* into cells for functional analysis. *U50'* did not alter colony formation efficiency, whereas the combination of *U50* and *U50'* still significantly reduced colony formation (Fig. 3D). The expression of snoRNA *U50* in transfected 22Rv1 and LNCaP cells was verified by northern blot assay (Fig. 3A).

Detection of *U50* mutation in prostate cancer samples

On the basis of the results of mutation, expression loss and functional effect on cell proliferation or survival (Figs 2 and 3), *U50* became the primary candidate for the 6q14–15 prostate cancer tumor suppressor. To further evaluate the candidacy of *U50*, we performed PCR combined with a single-strand conformation polymorphism (SSCP) assay,

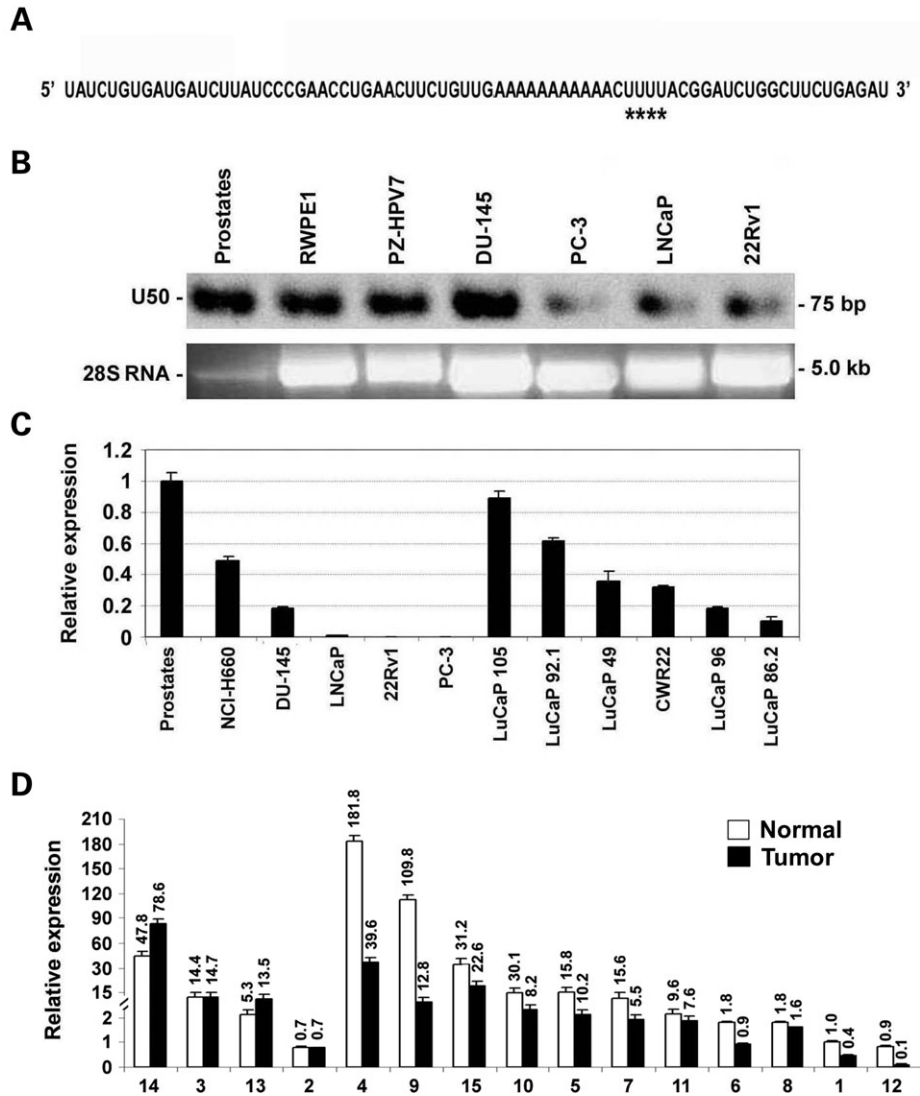


Figure 2. Sequence of snoRNA *U50* (A) and its reduced expression in prostate cancer samples (B)–(D). (A) The nucleotides harboring the deletion are marked by asterisks. (B) Detection of *U50* expression by northern blot analysis. Sample names are shown at the top. The 28S RNA indicates relative amounts of total RNA loaded into each lane. (C) and (D) Detection of *U50* expression by real-time PCR analysis in cell lines and xenografts (C) and 15 localized tumors (D) of prostate cancer. Sample names or case numbers are at the bottom, and *U50* expression in each sample was normalized by that in normal prostates (C) or matched normal tissue from patient 1 (D).

direct DNA sequencing and denaturing polyacrylamide gel electrophoresis to detect mutations in the 30 prostate cancer cell lines and xenografts available. In addition to LuCaP 96, the same homozygous TT deletion was also detected in xenograft LAPC3 (Fig. 4A). Meanwhile, a heterozygous TT deletion was detected in cell line NCI-H660 and xenograft LuCaP 86.2 (Fig. 4A). In addition, a one-base deletion in a stretch of 11 adenines in the neighborhood of the four thymidines in the *U50* genome (Fig. 2A) was detected in two other xenografts, LAPC4 and LuCaP 58. Although it is not clear whether this one-base deletion in the polyA tract affects *U50* function, it is likely that it results from a defective mismatch repair system on the basis of our previous findings that both LAPC4 and LuCaP 58 had microsatellite instability (15) and none of the 89 localized prostate cancers and 104 control samples had the deletion of the polyA tract. Neither did the

1371 men with prostate cancer and 1371 matched control men have this one-base deletion, on the basis of the genotyping results. None of the four samples with a TT deletion had any change in the polyA tract, consistent with the fact that none of them had microsatellite instability (15). We also analyzed *U50'* mutations in the remaining prostate cancer cell lines and xenografts, but found no sequence changes in any of the samples.

To further evaluate the role of *U50* in prostate cancer, we examined 89 grossly dissected primary prostate cancers, with matched non-cancer cells as controls. In total, nine of 89 (10%) tumors showed a homozygous genotype for the TT deletion in tumor cells (Fig. 4B–E). These nine deletions appear to be a combination of more somatic alterations (7/89 or 8%) and less germline polymorphisms (2/89 or 2%). In three of the nine tumors with a homozygous TT deletion,

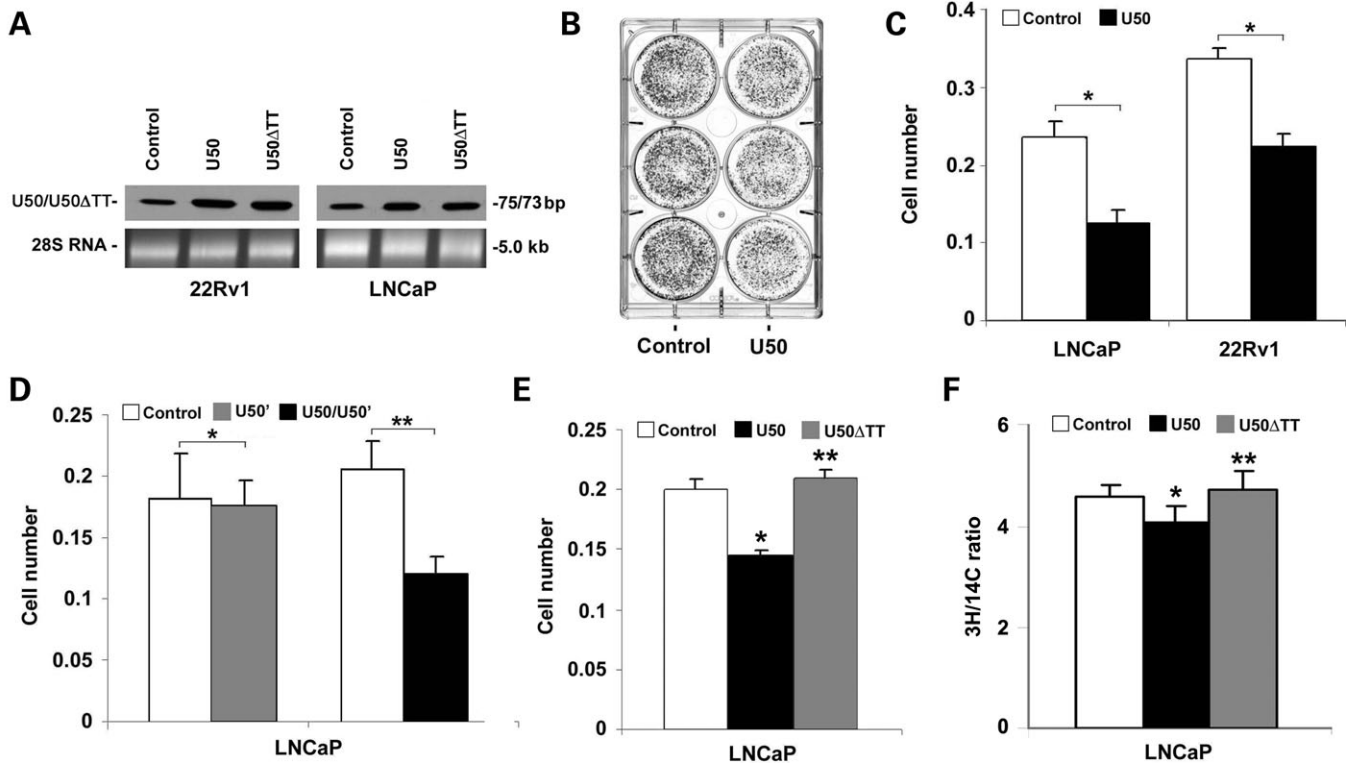


Figure 3. Ectopic expression of snoRNA *U50*, but neither *U50'* nor mutant *U50* (*U50ΔTT*) inhibited colony formation in prostate cancer cells. (A) Verification of *U50* expression by northern blot analysis upon the transfection of *U50* expression plasmid into 22Rv1 and LNCaP cells. (B) Image of a six-well plate showing *U50*-expression-reduced colony number in 22Rv1 cells at 12 days after transfection. (C) Cell numbers estimated by the SRB staining and the measurement of optical densities (y axis) after *U50* transfection into LNCaP and 22Rv1 cells. The readings were from day 12 post-transfection. $*P < 0.005$. (D) Expression of *U50'* did not alter colony forming efficiency, whereas a mixture of *U50* and *U50'* still did. $*P = 0.547$; $**P = 0.037$. (E) The TT deletion abolished the function of *U50* in suppressing colony formation in LNCaP cells. $*P < 0.005$ when compared with vector control; $**P = 0.44$ when compared with vector control but $P < 0.005$ when compared with wildtype *U50* control. (F) The TT deletion abolished the function of *U50* in suppressing the proliferation of LNCaP cells. $*P = 0.007$ when compared with vector control; $**P = 0.457$ when compared with vector control but $P = 0.007$ when compared with wildtype *U50*.

the matched normal cells showed only the wildtype allele, which indicates that the TT deletion occurred somatically in these cases (Fig. 4C). Two of the nine tumors also showed a homozygous TT deletion in their matched non-cancer cells, indicating that the mutation occurred in the germline of these men (Fig. 4D). Although it is rare for a somatic mutation to be identical to a sequence alteration present in germline DNA, somatic occurrence of the same mutation can be frequent in human malignancies (16,17). Somatic mutation of *U50* in three of 89 prostate cancers suggests that this mutation provides a survival advantage for cancer cells.

For the remaining four of the nine tumors with homozygous TT deletion, their matched normal cells showed a heterozygous genotype for the mutation (Fig. 4E), indicating that, during carcinogenesis, the wildtype allele was either mutated somatically, as in cases 52, 86 and 112, or lost through somatic deletion of 6q14.3. Loss of heterozygosity is common at 6q14–15 in prostate cancer (6), and, at random, both wildtype allele and the allele with the deletion should be lost at an equal frequency. The fact that the loss or somatic mutation only occurred in the wildtype allele but not in the mutant allele in the cases with a germline heterozygous genotype further suggests that loss of the wildtype *U50* allele provides a survival advantage for cancer cells.

In addition to the nine cases with homozygous TT deletion in tumor cells, five of the 89 (6%) cases that showed a heterozygous genotype in both normal and cancer cells, which brought the total number of cases with a heterozygous genotype to nine (10% of the 89 samples), further indicating that the TT deletion can be a germline event. To ensure that DNA samples for normal and cancer cells in the seven cases showing somatic mutation or deletion of the wildtype *U50* allele in cancer cells were from the same individual, we analyzed each pair using the AmpFLSTR® Identifiler® PCR Amplification Kit that has been optimized for human identification. Each pair of normal and cancer cells were indeed from the same individual (Fig. 4C and E, lower panel), excluding any possibilities of mismatching the samples for the TT deletion and indicating that homozygous TT deletion in *U50* is a cancer-related alteration. No association was found between *U50* homozygous deletion and clinicopathological characteristics of the clinical samples analyzed, including tumor grade, tumor stage and recurrence.

In total, 11 of 119 (9%) prostate cancers examined had the homozygous TT deletion (Table 1). Seven of the 11 cases involved a somatic alteration, two resulted from a germline mutation and the two in cell lines/xenografts had an unknown origin because no DNA from matched normal cells was available for analysis. Considering that the mutation

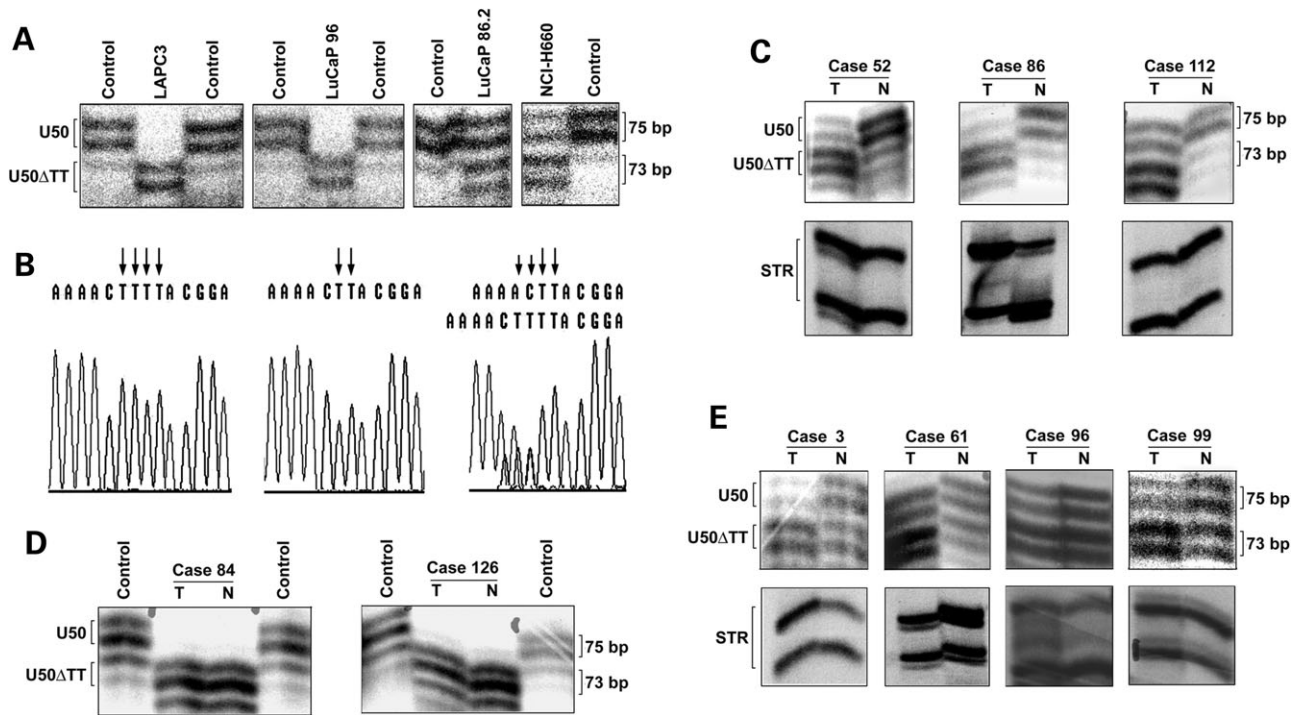


Figure 4. Detection of a *U50* mutation in prostate cancer. (A) At the genomic DNA level, xenografts LAPC3 and LuCaP 96 show a homozygous genotype for the TT deletion in *U50* (*U50*ΔTT), whereas cell line NCI-H660 and xenograft LuCaP 86.2 are heterozygous for the deletion. (B) DNA sequencing results showing wildtype, homozygous mutant and heterozygous mutant of *U50* in a normal sample, xenograft LAPC3 and xenograft LuCaP 86.2, respectively. Arrows point to the affected nucleotides. (C) Somatic mutations of *U50* in three primary prostate cancers. Lower panels show representative results from a STR (short tandem repeat) marker verifying the same origin of normal and cancer cells for each of the cases, as detected by the AmpFLSTR Identifier PCR Amplification Kit. (D) Homozygous genotype of the TT deletion detected in both cancer and normal cells from two prostate cancer patients. (E) Tumor-specific loss of the wildtype allele in four cases that had a heterozygous genotype for the TT deletion. Lower panels also show representative results from an STR marker verifying the same origin of normal and cancer cells for each case. In (A), (C), (D) and (E), sample names or case numbers are at the top, and all mutations were detected by sequencing gel electrophoresis of PCR products. T, tumor cells; N, matched non-cancer cells.

also occurred in germline, we evaluated the incidence of TT deletion in a normal population. We analyzed 104 control men who did not have prostate cancer at the time of blood collection. None of the 104 (0%) control men showed a homozygous TT deletion in *U50*. Because the prostates from the 104 control men did not have detectable cancer and deletion of 6q is primarily somatic in prostate cancer, we could thus compare the 89 cases and 104 controls to determine cancer-specific incidence of homozygous *U50* deletion in sporadic prostate cancer. The frequency of 7/89 or 8% for the homozygous *U50* deletion was significantly higher than the 0/104 incidence in the controls ($P = 0.02$, χ^2 test), suggesting that somatic *U50* mutation is a cancer-specific alteration.

On the other hand, 12 of the 104 control samples (12%) showed the presence of both a wildtype allele and the TT deletion allele, which is similar to the incidence of nine of 89 (10%) in patients with prostate cancer described earlier and thus raises the possibility that the TT deletion could be a benign polymorphism. To further evaluate whether the TT deletion is a cancer-related mutation or a benign polymorphism, we manipulated *U50* expression and performed functional assays. We attempted to knock down the expression of *U50* (75 bases) by RNA interference in the DU-145 and RWPE1 prostatic cell lines, which express higher levels of *U50*. Two small interfering RNAs (5'-CCUGAACUUCUGUUGAA AA-3' and 5'-ACUUUUACGGAUCUGGCUU-3') were

designed and tested. None of them altered *U50* expression or caused any changes in colony formation (data not shown). We also expressed both wildtype and mutant *U50*, along with the vector control, in LNCaP prostate cancer cells, and performed colony formation and cell proliferation assays. As shown in Figure 3E and F, the TT deletion abolished the function of *U50* in suppressing colony formation and cell proliferation, indicating that the mutation affects its function. The expression of *U50* and its mutant in transfected cells was verified by northern blot analysis (Fig. 3A).

Association study of the *U50* mutation in a cohort of cases and controls

To further determine the role of the *U50* deletion in prostate cancer and rule out the possibility of homozygous TT deletion as a benign polymorphism, we genotyped 1371 men with prostate cancer and 1371 matched control men for the 2 bp TT deletion and associated different genotypes with prostate cancer and clinically significant prostate cancer, using a well-established epidemiologic cohort reported previously (18,19). Both prostate cancer cases and controls in this analysis were predominantly white (~99% of both cases and controls) and elderly at the time of diagnosis (median age 70 years). Genotype distribution and results of regression models are presented in Table 2. In the analysis adjusted for the matching

Table 1. Summary of *U50* deletion in different tissue samples from prostate cancer patients and men without cancer

Samples (<i>n</i>)	Genotype ^a distribution of <i>U50</i> deletion		
	-/- (%)	+/- (%)	+/+ (%)
Cancer xenografts and cell lines (30)	2 (6.7)	2 (6.7)	26 (86.6)
Primary tumors from patients (89)	9 (10.1)	5 (5.6)	75 (84.3)
Normal tissues from patients (89)	2 (2.2)	9 (10.1)	78 (87.7)
Men without prostate cancer (104)	0 (0)	12 (11.5)	92 (88.5)

^a-/-, +/- and +/+ indicate homozygous, heterozygous and wildtype genotypes for the 2 bp deletion in *U50* genome.

Table 2. ORs for total and clinically significant prostate cancer incidences determined by homozygous (-/-) and heterozygous genotypes (+/-) of the 2 bp deletion in *U50* at 6q14.3 in a prospective analysis

Genotype	All cases Cases/Controls	OR ^a (95% CI)	Clinically significant cases ^b	
			Cases/Controls	OR (95% CI)
+/+	1131/1131	1.00 (Reference)	426/1131	1.00 (Reference)
+/-	222/230	0.97 (0.79–1.19)	98/230	1.15 (0.88–1.49)
-/-	18/10	1.85 (0.85–4.03)	10/10	2.63 (1.08–6.38)

^aORs on the basis of analysis adjusted for birth year, year of blood draw and race/ethnicity.

^bClinically significant prostate cancer cases were defined by Gleason score ≥ 7 or grade 3–4, stage C or D at diagnosis or men who had prostate cancer as their underlying cause of death. When the 24 control men who were diagnosed with prostate cancer during follow-up were excluded in statistical analysis, the association between homozygous deletion and risk of total prostate cancer (OR 2.03; 95% CI 0.91–4.55) and clinically significant prostate cancer (OR 2.90; 95% CI 1.17–7.21) was stronger.

factors, men homozygous for the 2 bp deletion had an increased risk of being diagnosed with prostate cancer that was not statistically significant [odds ratio (OR) 1.85, 95% confidence interval (CI) 0.85–4.03].

Considering that most prostate cancers are benign and do not pose a danger to life (1), which is a unique feature not seen in other common non-skin cancers, we separated clinically significant prostate cancers from total prostate cancers. Clinically significant prostate cancer was defined by Gleason score ≥ 7 or grade 3–4, stage C or D at diagnosis, or men who had prostate cancer as their underlying cause of death. The risk of clinically significant prostate cancer was significantly increased among men who were homozygous for the deletion (OR 2.63, 95% CI 1.08–6.38) (Table 2). Having a single copy of the deletion (heterozygous) was not significantly associated with risk of total or clinically significant prostate cancer. Results did not change meaningfully when we adjusted for prostate cancer risk factors in this study population.

Some of the control men, although cancer-free at the time of the diagnosis of their matched cases, were diagnosed with prostate cancer during subsequent follow-ups. When these men ($n = 24$) were excluded in statistical analysis, the association between homozygous deletion and risk of total prostate cancer (OR 2.03; 95% CI 0.91–4.55) and clinically significant prostate cancer (OR 2.90; 95% CI 1.17–7.21) was stronger.

DISCUSSION

In this study, we first attempted to identify a tumor-suppressor gene from chromosome 6q, which is frequently deleted in different types of human cancers including prostate cancer (5,6), and identified snoRNA *U50* as a reasonable candidate.

We also evaluated *U50* for its mutation in sporadic prostate cancer as well as the association of its germline deletion with prostate cancer susceptibility.

Identification of snoRNA *U50* as a better candidate for the 6q tumor-suppressor gene

A series of assays were performed to identify the best candidate for the 6q tumor-suppressor gene. First, we applied the approach of deletion mapping, which was successfully used to map multiple tumor-suppressor genes including *PTEN* (20), to better define the location of this gene. Using 30 cultured prostate cancer samples grown in culture or in mice, we were able to localize the gene to 2.5 Mb at 6q14–15 (Fig. 1). Second, we examined the expression of all the genes located in the minimal region of deletion in normal prostates and excluded all but four genes for further consideration (Fig. 2). Third, we analyzed 30 prostate cancer samples for cancer-specific mutations (Fig. 4) and identified the snoRNA *U50* as the best candidate for the 6q tumor-suppressor gene because a homozygous 2 bp deletion was detected in multiple samples. Fourth, functional analysis showed that wildtype but not mutant *U50* inhibited cell proliferation or survival in the colony formation assay (Fig. 3). Among the 11 genes located in the minimal region of deletion, snoRNA *U50* became the best candidate because it had mutations, was down-regulated and reduced colony numbers in prostate cancer. However, there should be other genes at 6q that are affected by chromosomal deletion and play a role in prostatic carcinogenesis because the region of deletion at 6q is often large and involves multiple loci (6). For example, a recent study showed that another region of deletion at 6q involving the *MAP3K7* gene, which is at 6q16, about

4.8 Mb telomeric to *U50*, is significantly associated with high-grade prostate cancers (21).

Homozygous deletion of *U50* occurs both somatically and in germline

U50 was then examined in 89 localized prostate cancers for its role in sporadic prostate cancer and for better evaluation of its candidacy for the 6q14–15 tumor-suppressor gene. Seven of the 89 (8%) cancers showed the same homozygous deletion, whereas none of their matched normal cells did. Among the seven cancer-specific homozygous deletions, three originated from mutation and four originated from either mutation or chromosomal loss (Fig. 4). Chromosomal loss is common at 6q14.3–15 in prostate cancer (6), but the loss at *U50* did not occur in any of the nine prostates that were heterozygous for the deletion allele, suggesting that homozygosity of the deletion at *U50* is selected during carcinogenesis. In addition, it is rare for a somatic mutation to be identical to a germline alteration, and such an identity also suggests the relevance of a gene to cancer development, as reported for other cancer genes (16,17). Taken together, these results further suggest that the homozygous deletion of *U50* plays a role in prostatic carcinogenesis.

In addition to the seven localized prostate cancers with somatic alterations in *U50*, two of the 89 cancers had the homozygous deletion in both their normal and cancer cells, which brought the total number of cancers with the homozygous deletion to nine (10% of the 89 cases). Meanwhile, none of 104 verified cancer-free men had the homozygous deletion in their germline DNA. More frequent homozygous deletion of *U50* in cancers further supports the candidacy of *U50* for the 6q14–15 tumor-suppressor gene. Furthermore, these results suggest that homozygous deletion of *U50* is involved in ~10% of sporadic prostate cancers.

Germline homozygous genotype of the *U50* deletion is associated with clinically significant prostate cancer

The deletion also occurs in germline. We therefore evaluated whether the homozygous deletion of *U50* plays a role in prostate cancer susceptibility. Using a well-established epidemiologic cohort (18,19), we genotyped 1371 men with prostate cancer and 1371 matched control men for *U50* deletion and associated different genotypes with prostate cancer and clinically significant prostate cancer. Considering that most prostate cancers are benign and do not pose a danger to life (1), which is a unique feature not seen in other common non-skin cancers, it was necessary to separate clinically significant prostate cancers from total prostate cancers. Our results showed that men homozygous for the deletion had an increased risk of being diagnosed with clinically significant prostate cancer (Table 2). Because of the prospective nature of the cohort, some of the control men were diagnosed with prostate cancer during follow-up. When these men were excluded in statistical analysis, the association between homozygous deletion and risk of total prostate cancer and clinically significant prostate cancer was stronger. The prostate cancer patients in the cohort analyzed were primarily elderly at the time of diagnosis (median age 70 years), yet a genetic risk

factor is usually associated with younger age at diagnosis in prostate cancer patients. In addition, more of the control men could be diagnosed with prostate cancer at a later time. It is thus likely that homozygous deletion of *U50* could be more relevant to true clinically significant prostate cancer and could potentially predict prostate cancer.

U50 could be a typical recessive tumor-suppressor gene

In Knudson's (22) two-hit theory for the inactivation of a recessive tumor-suppressor gene, both alleles need to be mutated and/or deleted, which is referred to as 'two hits', in order to functionally inactivate a tumor-suppressor gene. The first hit is often a germline mutation, whereas the second hit is a somatic mutation or allelic loss. Our results in this study suggest that *U50* is a typical recessive tumor-suppressor gene that requires the loss of both wildtype alleles, or 'two hits', to be inactivated in cancer. The relatively common germline TT deletion in one of the two alleles, as seen in ~10% of the populations that had a heterozygous genotype for the TT deletion, could be the first hit. The first hit appears to be recessive and has no effect on *U50* function when the wildtype allele is present. When the second hit occurs through either somatic mutation or chromosomal deletion or germline mutation in some cases, as described in this study, *U50* could be inactivated and contribute to the development of prostate cancer, because a homozygous but not heterozygous genotype of the deletion was significantly associated with clinically significant prostate cancer.

Malfunction of snoRNA and oncogenesis

The majority of the human genomes encode for a large number of non-coding RNA (ncRNA) species excluding ribosomal RNAs and transfer RNAs (23). Although ncRNAs were considered functionless in the structure and function of cells for many years, emerging studies suggest that ncRNAs can have important biological functions (24). SnoRNAs represent a common class of ncRNAs abundantly expressed in mammalian cells. They constitute a major component of small nucleolar ribonucleoprotein complexes and guide site-specific modifications of nucleotides in target RNAs (25). The *U50* snoRNA is one of over 300 known human snoRNAs. It is encoded by intron 5 of the *U50HG* gene (14). The expression of snoRNA can be tissue-specific (26). For example, some snoRNAs are exclusively expressed in the brain, and the absence of their expression could be associated with a human disease (26). Another study also showed that different snoRNAs are differentially expressed in different tissues (27).

With regard to a link between snoRNA and carcinogenesis, one earlier study showed that snoRNA *h5sn2* is highly expressed in normal brain, but its expression is drastically decreased in meningioma, suggesting a role for the loss of snoRNA *h5sn2* in brain tumorigenesis (27). An snoRNA gene can be located at a chromosomal breakpoint involved in carcinogenesis. For example, the *U50* snoRNA was originally discovered from the breakpoint of chromosomal translocation t(3,6)(q27;q15), which is involved in human B-cell lymphoma (14). A recent study demonstrated that adeno-associated viruses integrate their genome into mouse

genome, which causes liver cancer, and the integration sites identified in tumors were all located within a DNA interval encoding some snoRNAs (28). The expression of snoRNA has been associated with growth arrest of cells (29). For example, the host gene for *U50*, *U50HG*, possesses an oligopyrimidine tract that is characteristic of the 5'-terminal oligopyrimidine (5'TOP) class of genes, which have been shown to be coordinately regulated in response to cell growth (14). The *gas5* gene, which hosts multiple snoRNAs, is also a member of the 5'TOP gene family and has been reported as a growth arrest-specific gene, because the accumulation of *gas5*-generated snoRNAs was associated with an arrest of cell growth (30). These findings are consistent with our results in this study and suggest that snoRNA could be associated with growth arrest and likely tumor suppression. Our findings in this report also suggest that function-altering mutation in snoRNA could naturally occur in humans and thus play a role in human disease.

In summary, we narrowed a common region of deletion in 6q14–15, evaluated all expressed genes in the common region for cancer-specific mutations and found that snoRNA *U50* has a homozygously 2 bp deletion in ~10% of sporadic prostate cancers. Furthermore, homozygous genotype of the deletion was significantly associated with clinically significant prostate cancer in a prospectively analyzed cohort of prostate cancer cases and controls. Our findings suggest that snoRNA *U50* is a reasonable candidate for the 6q14–15 tumor-suppressor gene in human prostate cancer, its homozygous deletion is involved in ~10% of sporadic prostate cancers and that germline homozygosity of the deletion could predict clinically significant prostate cancer.

MATERIALS AND METHODS

Cell lines, xenografts, tissue specimens and blood DNA samples

Six prostate cancer cell lines (DU-145, NCI-H660, LNCaP, 22Rv1, MDAPCa2b and PC-3) and two immortalized and untransformed prostatic epithelial cell lines (PZ-HPV7 and RWPE1) were purchased from the American Type Culture Collection (Manassas, VA, USA). Cells were propagated following standard protocols from ATCC. Twenty-seven xenografts from 24 prostate cancers, described in detail in our previous study (13), were also used, including CWR21, CWR22, CWR91, LAPC3, LAPC4, LAPC9, PC82, LuCaP 23.1, LuCaP 23.8, LuCaP 23.12, LuCaP 35, LuCaP 35V, LuCaP 41, LuCaP 49, LuCaP 58, LuCaP 69, LuCaP 70, LuCaP 73, LuCaP 77, LuCaP 78, LuCaP 81, LuCaP 86.2, LuCaP 92.1, LuCaP 93, LuCaP 96, LuCaP 105 and LuCaP 115. For mutation analysis, genomic DNA for matched cancer and normal cells was isolated from 89 localized prostate cancers that were treated by prostatectomy and did not have lymph node involvement or distant metastasis at the time of surgery. Briefly, 10 consecutive sections were cut from each tissue block and mounted on slides. The first one was cut at 5 μ m and stained with hematoxylin to identify tumor and normal cells from each sample. Sections 2–10 were cut at 12 μ m and stained with hematoxylin. Regions rich in tumor cells were microdissected from these sections,

and the surrounding normal tissues were also isolated from the same slides as matched normal cell controls. DNA isolation was as described previously (13). Total RNA samples from normal human prostates and 13 other normal tissues (Clontech, Palo Alto, CA, USA) were used for expression analysis. In addition, total RNA was isolated from 15 fresh prostate cancers and used for expression analysis. Briefly, fresh prostate tissue was sectioned with a sterile scalpel blade to identify and collect a piece of cancer tissue into RNA-later solution (Ambion, Austin, TX, USA). A piece of normal tissue was also collected. After pathological verification of the tissue, total RNA was isolated following a standard protocol. Finally, we used genomic DNA from the blood cells of 104 unrelated individuals without any cancer to evaluate *U50* germline mutation. Genomic DNA for all the samples and RNA for all the cell lines and some of the xenografts were extracted following standard procedures. Use of human materials in this study was approved by the Institutional Review Board at Emory University.

Prospective study of *U50* mutation in prostate cancer

Men in the association analysis were participants in the Cancer Prevention Study II (CPS-II) Nutrition Cohort, a prospective study of cancer incidence including approximately 184 000 US men and women, established by the American Cancer Society (18). At enrollment into the Nutrition Cohort in 1992 or 1993, all participants completed a self-administered questionnaire that included questions on demographic, medical and life-style factors. Most participants were 50–74 years at the time of enrollment. Beginning in 1997, follow-up questionnaires were sent to cohort members every 2 years to update exposure information and to ascertain newly diagnosed cancers. Incident cancers reported on questionnaires were verified through medical records, linkage with state cancer registries or death certificates. The recruitment, characteristics, and follow-up of the CPS-II Nutrition Cohort are described in detail elsewhere (18).

From June 1998 through June 2001, participants in the CPS-II Nutrition Cohort were invited to provide a blood sample. After obtaining informed consent, blood samples were collected from 39 071 participants, including 17 411 men. Among men who had provided a blood sample, we identified 1452 cases that had been diagnosed with prostate cancer between 1992 and 2003 and had not been diagnosed with any other cancer (other than non-melanoma skin cancer). For each case, we selected one control from men who had provided a blood sample and were cancer-free at the time of the case diagnosis. Each control was individually matched to a case on birth date (± 6 months), date of blood collection (± 6 months) and race/ethnicity (white, African/American, Hispanic, Asian, other/unknown). A total of 81 prostate cancer cases and 81 of the controls initially selected were later excluded because of low DNA or contaminated sample. A total of 1371 cases and controls remained for analysis. Among the cases, we defined clinically significant prostate cancer (534 cases) as those with Gleason score ≥ 7 or grade 3–4, stage C or D at diagnosis or men who had prostate cancer as their underlying cause of death.

Detection of homozygous and hemizygous deletions

A total of 69 STS markers spanning the region of 6q14–q22 were used to detect homozygous and hemizygous deletions by regular and duplex PCR, as described in our previous study (13). A hemizygous deletion was considered to be present when the ratio of signal intensity for a 6q marker to that for the control marker in a tumor sample was less than half of the ratio in the normal human placenta DNA (Clontech) or matched normal cells. The control marker was from exon 5 of the *Kall1* gene, which is rarely altered at the genomic level in human prostate cancer (13).

Expression analysis

Total RNA was converted into cDNA using the Iscript cDNA synthesis kit (Bio-Rad Laboratories, CA, USA) according to the manufacturer's protocol. PCR amplification was then performed on the cDNA, with primers spanning different exons of different genes except for *U50*. Primer sequences are 5'-ACTGAAGACAGCGCCATTGTTCTG-3' and 5'-GGGTGGTAGGTGAGTGGGTATTGCG-3' for *LOC441164*; 5'-TGGGCGGAATCCATGTGGTGTATG-3' and 5'-TCCA CCATTGGCCAGGAAGTTTGG-3' for *NT5E*; 5'-TACCTCC ACGCCCTCGACC-3' and 5'-AGCTGGACCTATATGGGA TCTTCG-3' for *SYNCRIP*. For the expression analysis of *U50* by PCR, a primer with a linker sequence attached to a *U50*-specific sequence (5'-**TCGAGCGGCCGCCGGGC AGGTATCTCAGAAGCCAGATCCG-3'**, linker sequence is in boldface), along with a primer specific for *GAPDH* (5'-GTGGTCCAGGGGTCTTACTC-3'), was used to direct cDNA synthesis using the SuperScript II reverse transcriptase (Invitrogen, Carlsbad, CA, USA). The following pairs of primers, 5'-TCGAGCGGCCGCCGGGC-3' (complementary to the linker sequence) and 5'-TATCTGTGATGATCTT ATCCGAACCTGAAC-3' for *U50* and 5'-GTGGTCC AGGGGTCTTACTC-3' and 5'-TTCAACAGCGACACCC ACTC-3' for *GAPDH*, were used to detect gene expression. In addition to regular RT-PCR, we also performed real-time PCR with the ABI SYBR Green Kit and the ABI Prism 7000 Sequence Detection System (Applied Biosystems, Foster City, CA, USA) to detect gene expression in prostate cancer samples. Expression of a gene in each sample was indicated by the ratio of gene-specific reading to the reading of *GAPDH*, which was normalized by the normal control.

In the northern blot analysis for *U50* and *U50ΔTT* expression, 15 μg total RNA for each sample was separated by gel electrophoresis in a 6% denaturing polyacrylamide gel containing 7 M urea, transferred to Hybond-C nylon membrane (Amersham) and hybridized with ³²P-labeled probe in QuikHyb Hybridization solution (Stratagene, La Jolla, CA, USA) following standard protocols. The probes were generated by PCR amplification with primers used for *U50* and *U50ΔTT* expression constructs and radiolabeled by PCR amplification in the presence of ³²P-dCTP with the primer complementary to *U50* (5'-ATCTCAGAAGCCAGATCCG TAAAAG-3') or *U50ΔTT* (5'-ATCTCAGAAGCCAGATC CGTAAG-3'). The same amount of RNA for each sample was separated on a denaturing agarose gel for 28S RNA as a loading control.

Colony formation and cell proliferation assays

The coding regions for *LOC441164*, *NT5E* and *SYNCRIP* were cloned into the FLAG-pcDNA3 expression vector (Invitrogen). The inframe FLAG tag enabled the detection of protein expression by western blot analysis with anti-FLAG antibody (Sigma). On the basis of previous studies, a tag did not appear to affect the function of *SYNCRIP* in different analyses (31). Therefore, the inframe FLAG tag in our study should not affect *SYNCRIP* function either. For *NT5E*, we transfected FLAG-tagged construct into the MDA-MB-231 breast cancer cells and performed colony formation assay. The results with a tagged *NT5E* were similar to that from untagged *NT5E* in a previous study (32), which indicates that the FLAG tag did not affect *NT5E* function in our study. For *LOC441164*, it is not clear whether a FLAG tag affects its function or not. *U50*, its mutant with the TT deletion (*U50ΔTT*) and *U50'* sequences were cloned into the pSIREN-RetroQ vector (Clontech), which was designed to accurately express small RNA molecules.

For *U50*, the 22Rv1 and LNCaP prostate cancer cell lines, which express little *U50*, were seeded into six-well tissue culture plates. The next day, the Lipofectamine Plus reagent (Invitrogen) was used to transfect 1.6 μg of pSIREN-RetroQ-*U50* plasmid or the pSIREN-RetroQ vector control into cells. Forty-eight hours after transfection, puromycin was added into the media at a final concentration of 2 μg/ml, which completely killed parental 22Rv1 or LNCaP cells in 12 days. One set of cells were used to verify the expression of *U50* by real-time PCR and northern blot analysis. At days 8 and 12 after selection started, cells were fixed and stained with sulforhodamine B, and optical densities, which indicated cell numbers, were measured as described previously (13). *U50'* and *U50ΔTT* were analyzed in the same manner. The effect of *U50* or *U50ΔTT* on the proliferation of LNCaP cells was determined by measuring ³H-thymidine incorporation following a standard protocol. Briefly, LNCaP cells were seeded in 24-well plates with the medium containing ¹⁴C-thymidine. On the following day, cells were washed three times with PBS to remove free ¹⁴C-thymidine and then transfected with *U50*, *U50ΔTT* or pSIREN-RetroQ control plasmid as described earlier. Forty-eight hours after transfection, cells were incubated with fresh medium containing ³H-thymidine for 4 h and were fixed and measured for ³H and ¹⁴C radioactivity. The ratio of ³H radioactivity to that of ¹⁴C indicates the rate of DNA synthesis or cell proliferation. Statistical significance was determined using Student's *t*-test. A *P*-value of 0.05 or smaller was considered statistically significant.

Expression constructs for *LOC441164*, *NT5E* and *SYNCRIP* were also transfected into LNCaP or 22Rv1 cells. Gene expression was confirmed by western blot analysis with anti-FLAG antibody, and the colony formation assay was conducted as described for *U50* earlier. Two previously established growth-suppressor genes, *FOXO1A* (33) and *ATBF1* (13), were used as the positive controls.

Mutation analysis

We first amplified the open-reading frames for the three protein-encoding genes, *LOC441164*, *NT5E* and *SYNCRIP*,

from cDNA and snoRNAs *U50* and *U50'* sequence from genomic DNA by PCR from 15 prostate cancer cell lines and xenografts and directly sequenced the PCR products (Macrogen, Seoul, Republic of Korea). With the detection of the 2 bp deletion in *U50*, we then performed PCR in combination with SSCP in all the samples, as described previously (13). For a shifted band in a sample, which indicated a sequence alteration, another round of PCR–SSCP was performed to confirm the shift. Once a band shift was confirmed in a sample, genomic DNA of that sample was amplified and the PCR products were purified using the Qiaquick PCR Purification Kit (Qiagen, Germany) and sequenced to reveal the sequence alteration. For all samples including clinical samples and blood DNA samples, we also performed PCR combined with denaturing polyacrylamide gel electrophoresis to detect the TT deletion.

Genotyping of the prospective cohort

DNA was extracted from buffy coat following standard protocols. For genotyping, each DNA sample was amplified by PCR using the same PCR primers for mutation detection in the presence of ^{33}P -dATP. PCR products were separated in a $35 \times 45 \text{ cm}^2$ denaturing polyacrylamide sequencing gel, which was then dried and exposed to X-ray film to detect *U50* alleles (the wildtype allele is 2-base longer than the mutant allele). Genotyping was performed at Emory University, and all investigators except those from the American Cancer Society were blinded to case–control status. In addition, 4% blind duplicates were randomly interspersed with the case–control samples for quality control. Concordance for these quality control samples was 100%. The genotyping success rate was 100% for both case and control. The genotype distribution among controls was in Hardy–Weinberg equilibrium ($P = 0.64$).

Statistical analysis in the prospective analysis of the cohort

This part of the study was conducted at the American Cancer Society. We used both conditional and unconditional logistic regression models in the analysis of the association between the deletion and prostate cancer and observed consistent results with both approaches. To make use of information from all genotyped cases and controls, we calculated ORs using an unconditional logistic regression model that was adjusted for each of the matching variables rather than using a matched pair analysis. All models were adjusted for birth year (in single-year categories), blood collection date (in single-year categories) and race/ethnicity (white, African-American, Hispanic, Asian, other/unknown). Other covariates that were considered for the analysis were family history in a father and/or brother, education, smoking, diabetes, NSAID use, total calcium intake and PSA screening.

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REFERENCES

- Scardino, P.T., Weaver, R. and Hudson, M.A. (1992) Early detection of prostate cancer. *Hum. Pathol.*, **23**, 211–222.
- Sakr, W.A., Grignon, D.J., Crissman, J.D., Heilbrun, L.K., Cassin, B.J., Pontes, J.J. and Haas, G.P. (1994) High grade prostatic intraepithelial neoplasia (HGPN) and prostatic adenocarcinoma between the ages of 20–69: an autopsy study of 249 cases. *In Vivo*, **8**, 439–443.
- Postma, R. and Schroder, F.H. (2005) Screening for prostate cancer. *Eur. J. Cancer*, **41**, 825–833.
- McGregor, M., Hanley, J.A., Boivin, J.F. and McLean, R.G. (1998) Screening for prostate cancer: estimating the magnitude of overdiagnosis. *Can. Med. Assoc. J.*, **159**, 1368–1372.
- Knuutila, S., Aalto, Y., Autio, K., Bjorkqvist, A.M., El-Rifai, W., Hemmer, S., Huhta, T., Kettunen, E., Kiuru-Kuhlefelt, S., Larramendy, M.L. *et al.* (1999) DNA copy number losses in human neoplasms. *Am. J. Pathol.*, **155**, 683–694.
- Dong, J.T. (2001) Chromosomal deletions and tumor suppressor genes in prostate cancer. *Cancer Metastasis Rev.*, **20**, 173–193.
- Trent, J.M., Stanbridge, E.J., McBride, H.L., Meese, E.U., Casey, G., Araujo, D.E., Witkowski, C.M. and Nagle, R.B. (1990) Tumorigenicity in human melanoma cell lines controlled by introduction of human chromosome 6. *Science*, **247**, 568–571.
- Welch, D.R., Chen, P., Miele, M.E., McGary, C.T., Bower, J.M., Stanbridge, E.J. and Weissman, B.E. (1994) Microcell-mediated transfer of chromosome 6 into metastatic human C8161 melanoma cells suppresses metastasis but does not inhibit tumorigenicity. *Oncogene*, **9**, 255–262.
- Sandhu, A.K., Kaur, G.P., Reddy, D.E., Rane, N.S. and Athwal, R.S. (1996) A gene on 6q 14–21 restores senescence to immortal ovarian tumor cells. *Oncogene*, **12**, 247–252.
- Theile, M., Seitz, S., Arnold, W., Jandrig, B., Frege, R., Schlag, P.M., Haensch, W., Guski, H., Winzer, K.J., Barrett, J.C. *et al.* (1996) A defined chromosome 6q fragment (at D6S310) harbors a putative tumor suppressor gene for breast cancer. *Oncogene*, **13**, 677–685.
- Morelli, C., Cardona, F., Boyle, J.M., Negrini, M. and Barbanti-Brodano, G. (1997) Mapping of 22 new ESTs around a tumor suppressor gene and a senescence gene at 6q16–>q21. *Cytogenet. Cell Genet.*, **79**, 97–100.
- Miele, M.E., Jewett, M.D., Goldberg, S.F., Hyatt, D.L., Morelli, C., Gualandi, F., Rimessi, P., Hicks, D.J., Weissman, B.E., Barbanti-Brodano, G. *et al.* (2000) A human melanoma metastasis-suppressor locus maps to 6q16.3–q23. *Int. J. Cancer*, **86**, 524–528.
- Sun, X., Frierson, H.F., Chen, C., Li, C., Ran, Q., Otto, K.B., Cantarel, B.L., Vessella, R.L., Gao, A.C., Petros, J. *et al.* (2005) Frequent somatic mutations of the transcription factor ATBF1 in human prostate cancer. *Nat. Genet.*, **37**, 407–412.
- Tanaka, R., Satoh, H., Moriyama, M., Satoh, K., Morishita, Y., Yoshida, S., Watanabe, T., Nakamura, Y. and Mori, S. (2000) Intronic *U50* small-nucleolar-RNA (snoRNA) host gene of no protein-coding potential is mapped at the chromosome breakpoint t(3;6)(q27;q15) of human B-cell lymphoma. *Genes Cells*, **5**, 277–287.
- Sun, X., Chen, C., Vessella, R.L. and Dong, J.T. (2006) Microsatellite instability and mismatch repair target gene mutations in cell lines and xenografts of prostate cancer. *Prostate*, **66**, 660–666.
- Levine, R.L., Wadleigh, M., Cools, J., Ebert, B.L., Wernig, G., Huntly, B.J., Boggon, T.J., Wlodarska, I., Clark, J.J., Moore, S. *et al.* (2005) Activating mutation in the tyrosine kinase JAK2 in polycythemia vera, essential thrombocythemia, and myeloid metaplasia with myelofibrosis. *Cancer Cell*, **7**, 387–397.
- James, C., Ugo, V., Le Couedic, J.P., Staerk, J., Delhommeau, F., Lacout, C., Garcon, L., Raslova, H., Berger, R., Bennaceur-Griscelli, A. *et al.* (2005) A unique clonal JAK2 mutation leading to constitutive signalling causes polycythaemia vera. *Nature*, **434**, 1144–1148.

18. Calle, E.E., Rodriguez, C., Jacobs, E.J., Almon, M.L., Chao, A., McCullough, M.L., Feigelson, H.S. and Thun, M.J. (2002) The American Cancer Society Cancer Prevention Study II Nutrition Cohort: rationale, study design, and baseline characteristics. *Cancer*, **94**, 2490–2501.
19. Patel, A.V., Calle, E.E., Pavluck, A.L., Feigelson, H.S., Thun, M.J. and Rodriguez, C. (2005) A prospective study of XRCC1 (X-ray cross-complementing group 1) polymorphisms and breast cancer risk. *Breast Cancer Res.*, **7**, R1168–R1173.
20. Li, J., Yen, C., Liaw, D., Podsypanina, K., Bose, S., Wang, S.I., Puc, J., Miliareis, C., Rodgers, L., McCombie, R. *et al.* (1997) PTEN, a putative protein tyrosine phosphatase gene mutated in human brain, breast, and prostate cancer. *Science*, **275**, 1943–1947.
21. Liu, W., Chang, B.L., Cramer, S., Koty, P.P., Li, T., Sun, J., Turner, A.R., Von Kap-Herr, C., Bobby, P., Rao, J. *et al.* (2007) Deletion of a small consensus region at 6q15, including the MAP3K7 gene, is significantly associated with high-grade prostate cancers. *Clin. Cancer Res.*, **13**, 5028–5033.
22. Knudson, A.G., Jr (1977) Genetics and etiology of human cancer. *Adv. Hum. Genet.*, **8**, 1–66.
23. Mattick, J.S. and Makunin, I.V. (2005) Small regulatory RNAs in mammals. *Hum. Mol. Genet.*, **14**, R121–R132.
24. Kishore, S. and Stamm, S. (2006) The snoRNA HBII-52 regulates alternative splicing of the serotonin receptor 2C. *Science*, **311**, 230–232.
25. Kiss, T. (2002) Small nucleolar RNAs: an abundant group of noncoding RNAs with diverse cellular functions. *Cell*, **109**, 145–148.
26. Cavaille, J., Buiting, K., Kiefmann, M., Lalande, M., Brannan, C.I., Horsthemke, B., Bachellerie, J.P., Brosius, J. and Huttenhofer, A. (2000) Identification of brain-specific and imprinted small nucleolar RNA genes exhibiting an unusual genomic organization. *Proc. Natl Acad. Sci. USA*, **97**, 14311–14316.
27. Chang, L.S., Lin, S.Y., Lieu, A.S. and Wu, T.L. (2002) Differential expression of human 5S snoRNA genes. *Biochem. Biophys. Res. Commun.*, **299**, 196–200.
28. Donsante, A., Miller, D.G., Li, Y., Vogler, C., Brunt, E.M., Russell, D.W. and Sands, M.S. (2007) AAV vector integration sites in mouse hepatocellular carcinoma. *Science*, **317**, 477.
29. Schneider, C., King, R.M. and Philipson, L. (1988) Genes specifically expressed at growth arrest of mammalian cells. *Cell*, **54**, 787–793.
30. Smith, C.M. and Steitz, J.A. (1998) Classification of gas5 as a multi-small-nucleolar-RNA (snoRNA) host gene and a member of the 5'-terminal oligopyrimidine gene family reveals common features of snoRNA host genes. *Mol. Cell Biol.*, **18**, 6897–6909.
31. Cho, S., Park, S.M., Kim, T.D., Kim, J.H., Kim, K.T. and Jang, S.K. (2007) BiP internal ribosomal entry site activity is controlled by heat-induced interaction of NSAP1. *Mol. Cell Biol.*, **27**, 368–383.
32. Zhi, X., Chen, S., Zhou, P., Shao, Z., Wang, L., Ou, Z. and Yin, L. (2007) RNA interference of ecto-5'-nucleotidase (CD73) inhibits human breast cancer cell growth and invasion. *Clin. Exp. Metastasis*, **24**, 439–448.
33. Dong, X.Y., Chen, C., Sun, X., Guo, P., Vessella, R.L., Wang, R.X., Chung, L.W., Zhou, W. and Dong, J.T. (2006) FOXO1A is a candidate for the 13q14 tumor suppressor gene inhibiting androgen receptor signaling in prostate cancer. *Cancer Res.*, **66**, 6998–7006.