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

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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...
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 23.1/3.8/23.12, 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 (HGNCl) 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, HTRIE, NTSE, 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, HTRIE 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 HTRIE, SNX14 or TBX18 was not detectable in normal prostates (data not shown). These results suggest that the genes of LOC389415, LOC441163, LOC441165, HTRIE, 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
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,
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 thymines 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,
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
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, $\chi^2$ 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 in U50. Because the prostate 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 ($P = 0.02, \chi^2$ test), suggesting that somatic U50 mutation is a cancer-specific alteration.

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...
Genotype All cases Clinically significant cases
prostate cancer (OR 2.90; 95% CI 1.17–7.21) was stronger.
cancer (OR 2.03; 95% CI 0.91–4.55) and clinically significant
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score
Clinically significant prostate cancer was defined by Gleason
cally significant prostate cancers from total prostate cancers.
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at 6q is often large and involves multiple loci (6). For
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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
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 clini-
cally 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. 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.

### 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 candi-
date 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 cul-
tured prostate cancer samples grown in culture or in mice,
we were able to localize the gene to 2.5 Mb at 6q14.3–15 (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 consider-
ation (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 pro-
liferation 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

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

<table>
<thead>
<tr>
<th>Samples (n)</th>
<th>Genotype(^a) distribution of U50 deletion</th>
<th>Genotype(^a) distribution of U50 deletion</th>
</tr>
</thead>
<tbody>
<tr>
<td>Cancer xenografts and cell lines (30)</td>
<td>2 (6.7)</td>
<td>2 (6.7)</td>
</tr>
<tr>
<td>Primary tumors from patients (89)</td>
<td>9 (10.1)</td>
<td>5 (5.6)</td>
</tr>
<tr>
<td>Normal tissues from patients (89)</td>
<td>2 (2.2)</td>
<td>9 (10.1)</td>
</tr>
<tr>
<td>Men without prostate cancer (104)</td>
<td>0 (0)</td>
<td>12 (11.5)</td>
</tr>
<tr>
<td>Men without prostate cancer (104)</td>
<td>92 (88.5)</td>
<td></td>
</tr>
</tbody>
</table>

\(^a\) –/–, +/+ 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

<table>
<thead>
<tr>
<th>Genotype</th>
<th>All cases/Controls</th>
<th>OR(^b) (95% CI)</th>
<th>Clinically significant cases(^b)</th>
<th>OR (95% CI)</th>
</tr>
</thead>
<tbody>
<tr>
<td>+/-</td>
<td>1131/131</td>
<td>1.00 (Reference)</td>
<td>426/1131</td>
<td>1.00 (Reference)</td>
</tr>
<tr>
<td>+/−</td>
<td>222/230</td>
<td>0.97 (0.79–1.19)</td>
<td>98/230</td>
<td>1.15 (0.88–1.49)</td>
</tr>
<tr>
<td>−/−</td>
<td>18/10</td>
<td>1.85 (0.85–4.03)</td>
<td>10/10</td>
<td>2.63 (1.08–6.38)</td>
</tr>
</tbody>
</table>

\(^b\) ORs on the basis of analysis adjusted for birth year, year of blood draw and race/ethnicity.

\(^b\) Clinically 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.
Homologous deletion of \textit{U50} occurs both somatically and in germline

\textit{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 \textit{6q14–15} tumor-suppressor gene. Seven of the 89 (8\%) cancers showed the same homologous deletion, whereas none of their matched normal cells did. Among the seven cancer-specific homologous deletions, three originated from mutation and four originated from either mutation or chromosomal loss (Fig. 4). Chromosomal loss is common at \textit{6q14.3–15} in prostate cancer (6), but the loss at \textit{U50} did not occur in any of the nine prostates that were heterozygous for the deletion allele, suggesting that homologosity of the deletion at \textit{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 homologous deletion of \textit{U50} plays a role in prostatic carcinogenesis.

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

Germinate homologous genotype of the \textit{U50} deletion is associated with clinically significant prostate cancer

The deletion also occurs in germline. We therefore evaluated whether the homologous deletion of \textit{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 \textit{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 homologous for the deletion had an increased risk of being diagnosed with clinically significant prostate cancer (Table2). 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 homologous 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 homologous deletion of \textit{U50} could be more relevant to true clinically significant prostate cancer and could potentially predict prostate cancer.

\textit{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 \textit{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 \textasciitilde{}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 \textit{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, \textit{U50} could be inactivated and contribute to the development of prostate cancer, because a homologous 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 \textit{U50} snoRNA is one of over 300 known human snoRNAs. It is encoded by intron 5 of the \textit{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 \textit{h5sn2} is highly expressed in normal brain, but its expression is drastically decreased in meningioma, suggesting a role for the loss of snoRNA \textit{h5sn2} in brain tumorigenesis (27). An snoRNA gene can be located at a chromosomal breakpoint involved in carcinogenesis. For example, the \textit{U50} snoRNA was originally discovered from the breakpoint of chromosomal translocation \textit{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 normal cell controls. DNA isolation was as described previously (13). Total DNA 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 germine 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 KAI1 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'–ACTGAGACAGGCCATTTGTTCG-3' and 5'–GGGTGGTAGGTGAGTGGGTATTGCG-3' for LOC441164; 5'-TTGGGCGGAATCCATGTGGTGTATG-3' and 5'–TCCAATCGGTGAGTGGGATGATTGCG-3' for NTSE; 5'-TACCTCCAGCAGGCCACGAGGACGTTGTATG-3' and 5'-AGCTGGACCTATATGGGAACTTG-3' for SYNCRIP. For the expression analysis of U50 by PCR, a primer with a linker sequence attached to a U50-specific sequence (5'-TCGAGCCGCGGCCGAGGTATCCTAGAAGCCAGATCCG-3', linker sequence is in boldface), along with a primer specific for GAPDH (5'-GTGTTCCAGGGTCTTACTC-3'), was used to direct cDNA synthesis using the SuperScript II reverse transcriptase (Invitrogen, Carlsbad, CA, USA). The following pairs of primers, 5'-TCGAGCCGCGGCCGAGGTATCCTAGAAGCCAGATCCG-3' (complementary to the linker sequence) and 5'-TATCTGTGATGATCTTATCCCGAAGATCCTG-3' for U50 and 5'-GGGTGGTAGGTGAGTGGGTATTGCG-3' for LOC441164, 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 the 18S ribosomal RNA copy number, 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.

Colonies formation and cell proliferation assays

The coding regions for LOC441164, NTSE 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 NTSE, we transfected FLAG-tagged construct into the MDA-MB-231 breast cancer cells and performed colony formation assay. The results with a tagged NTSE were similar to that from untagged NTSE in a previous study (32), which indicates that the FLAG tag did not affect NTSE 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 was 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, NTSE 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, NTSE 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 33P-dATP. PCR products were separated in a 35 × 45 cm2 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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Conflict of Interest statement. None declared.

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REFERENCES


