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14. ABSTRACT:

The DNA damage-signaling pathway has been implicated in the development of prostate cancer since germline mutations in several genes (*BRCA1, BRCA2, and CHEK2*) whose products are involved in this pathway have been associated with increased risk for this cancer. We previously isolated a novel p73 up-regulated gene (*p73R1*) and identified p73R1 mutations in prostate cancer. In this report, we screened 856 unselected prostate cancer specimens and detected a frequency of 2.6% (22/856) truncation mutations in prostate cancers in contrast to 0.6% (2/327) in 327 population-based controls (Fisher's exact test, $P = 0.036$), with an odds ratio of 4.3 (95% confidence interval 1.2 – 21.2). In addition, we also demonstrated that mutant *p73R1* was unable to induce apoptosis and suppress cell growth in HeLa and Cos7 cells. The loss of function mutation in *p73R1* is due to the inability of the mutant to induce cytochrome c release from mitochondria. These results suggest that loss of function mutations in p73R1 predispose men to prostate cancer and further support the concept that the genetic defects in the DNA damage-response genes play an important role in the development of prostate cancer.

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p73R1, DNA damage-signaling, Prostate cancer risk, Apoptosis

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2006 Annual Report
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Clinical and Functional Analysis of *p73R1* Mutations in Prostate Cancer

Wanguo Liu, Mayo Clinic, Rochester MN 55905

Introduction:

Prostate cancer is a complex genetic disease and its etiology very likely involves many genetic loci with no major gene with high penetrance. To identify such prostate cancer susceptibility genes, we have been using a novel approach based on mutation screening of candidate genes involved in the DNA damage-signaling pathway. Genomic instability is a common feature of all human cancers. The DNA damage-signaling pathway plays a critical role in maintaining genomic stability in response to DNA damage. The integrity of this pathway is essential for the prevention of neoplastic transformation, since several proteins involved in this pathway (such as p53, BRCA1, and ATM) are frequently mutated in human cancer. P73 is a newly identified DNA damage-signaling pathway gene. In a search for p73-dependent DNA damage-responsive genes, we isolated a p73-upregulated gene (*p73R1*) that is identical to the *p53AIP1* gene (reference 1) activated by cisplatin-induced DNA damage. We have identified several deleterious germline mutations in this gene in approximately 3.2% (17/532) of primary or sporadic prostate cancer but only in 0.6% (2/331) of unaffected men (Fisher's exact test, $P = 0.016$). We, therefore, propose to study in detail the involvement of this gene in familial prostate cancer families which will be the first step towards providing evidence that *p73R1* is a prostate cancer susceptibility gene. The **objective** of this project is to identify the genetic role of p73R1 in prostate cancer development and to determine the functions and mechanisms of p73R1 in tumorigenesis. Our **hypothesis** is that *p73R1* is a candidate prostate cancer susceptibility gene. **Three Aims** for this proposal are: (1) To determine whether *p73R1* mutations co-segregate with prostate cancer phenotype in familial prostate cancer families; (2) to explore whether *p73R1* mutations are associated with any clinical and pathological characteristics in patients with prostate cancers; and (3) to determine the functional role of p73R1 in tumorigenesis. We have developed an accurate and sensitive Denaturing HPLC protocol to detect *p73R1* mutations. We will screen for *p73R1* mutations in 163 familial prostate cancer families and in 1,000 tumor samples collected at Mayo Clinic. Model-free genetic linkage analysis and statistical analysis will be performed to determine the co-segregation of *p73R1* mutations in prostate cancer families and any clinicopathological significance in patients with mutations and those without. *p73R1* mutants will be generated and expressed in mammalian cell systems to determine if the mutations fail to induce apoptosis and suppress cell growth.

Body: The tasks, which we proposed to fulfill in Year 2 & 3 and the accomplishments associated with each task, are summarized below:

<u>Specific Aim 2:</u> To determine the clinicopathological significance of *p73R1* mutations in patients with sporadic CaP: We plan to screen for *p73R1* mutations in an additional 1,000 unselected CaP tumor samples to determine possible associations with age, disease stage, PSA levels, aneuploidy, etc. Statistical analyses will be performed to assess the increased risk in patients with *p73R1* mutations compared to those without or to 332 unaffected men in the normal population.

Task 4. Determination of the clinicopathological significance of *p73R1* mutations (Months 11-15).

a) DNA from 1,000 freshly frozen tumor samples will be isolated using an approach that will enrich for tumor cells.
b) Mutation detection will be performed following the steps described in Task 1.

c) DNA from the matched normal tissues of the tumors containing the *p73R1* mutations will be analyzed to determine whether the mutation is somatic or germline.
d) Clinical and pathological information for 969 patients have already been gathered. The age of onset, PSA levels, aneuploidy, and the disease stage will be compared between patients with *p73R1* mutations and those without to determine the clinicopathological significance of the mutations.

To date, tissue slices from 416 paired CaP tumor/normal and additional 440 CaP tumor specimens have been prepared and DNAs from these tissue slices have been isolated. Due to the labor consuming and overwhelming demand for macro-dissection of tumor tissues in Mayo tissue processing facility, our preparation of DNA from 1,000 tumor tissues is behind the schedule. The remaining 144 CaP tumors are being processed and the analysis of the remaining group of CaP for *p73R1* mutations will be completed by March of this year.

The screen for *p73R1* mutations in the 856 CaP tumors has been completed to date. We have identified two truncation mutations (Ser32Stop and Arg21insG) in 22 specimens and three missense mutations in many other specimens (Table 1). By analysis of the matched normal tissues from the same patients, we found that all of these mutations are germline mutations.

The clinical information of these mutation carriers has already been extracted. Statistical analysis will be performed to assess the increased risk in patients carrying the p73R1 truncation mutations compared to those without mutations and to 327 unaffected men at the end of this project (see Task 7 in Aim 3).

Table 1. *P53AIP1* mutations identified in 856 prostate tumor specimens and controls

Mutations	*Amino Acid Change*	*Unselected CaP Tumor Specimens (n=856)*	*Unaffected Men (n=327)*	*Normal Controls (n=95)*
C95A	Ser32Stop	7	1	0
64insG	Arg21insG	15	1	0
C20T	Ala7Val	21	7	3
A304G	Arg102Glu	304	ND	28
C313T	Pro105Ser	289	ND	26

Specific Aim 3: To determine the functional role of p73R1 in CaP tumorigenesis: We will perform colony formation analysis, immunofluorescence microscopic analysis, and TUNEL assays to determine if mutant p73R1 facilitates tumorigenesis because of its inability to participate in apoptosis in mitochondria. In addition, we will perform LOH studies to determine whether p73R1 functions as a tumor suppressor by deletion of the wild-type allele in CaP harboring *p73R1* mutations.

Task 5. Determination of the functions of *p73R1* mutations in tumorigenesis (Months 16-23)
a) All of the mutations will be cloned into expression vector for colony formation assay.
b) TUNEL and flow cytometry analyses will be performed on all mutants generated for apoptosis analysis.

To date, we have identified five different *p73R1* mutations in prostate cancer. Expression constructs for wild-type (wt-) and the five mutants (mut-p73R1) have been generated. Colony formation analysis and TUNTL assays have been performed. As shown in Figure 1, left, only the two mutants carrying the truncating mutations lost the ability to suppress cell growth. The other mutants did not impair the suppressive function and thus are very likely the polymorphisms (data not shown). The results from TUNEL assays also support the results from colony formation analyses. Only the truncation mutation was unable to induce apoptosis in the TUNEL assay (Figure 1, right).

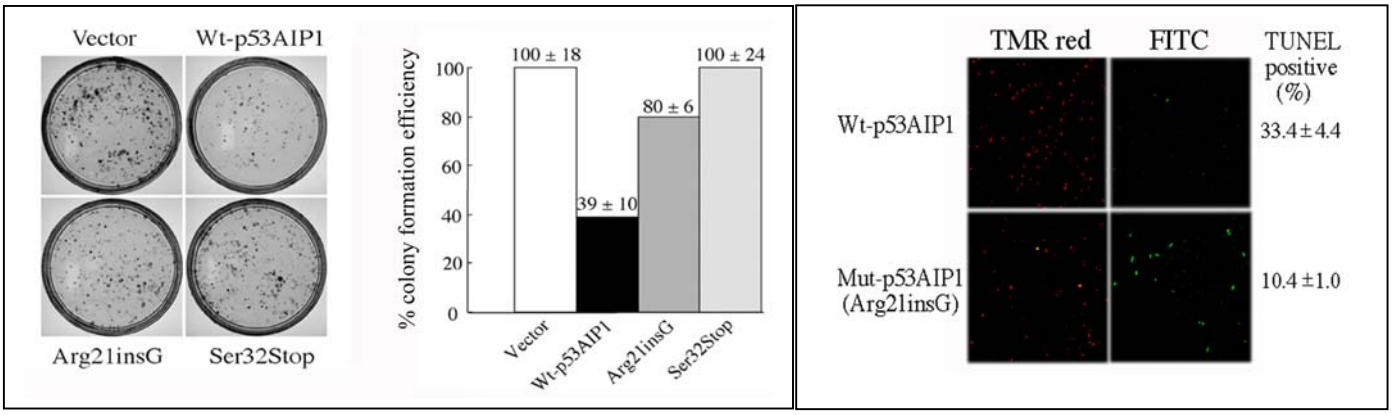

Figure 1. Ser32Stop and Arg21insG of p73R1 are loss-of-function mutations in programmed cell death. *Left,* Colony formation analyses by ectopic expression of wt- or mut-p73R1 in HeLa cells. The expression vector alone was used as the negative control. *Right,* TUNEL assay by extopic expression of wt- or mut-p73R1 in T98G cells. The cells expressing wt- p73R1 generated 3-4-fold more dead cells typified by positive TMR red staining than those expressing mutant protein.

Task 6. Determination of the mechanism of p73R1 in prostate cancer development (Months 24-32)

a) Indirect immunofluorescence analysis of mutant p73R1 will be performed to determine whether mutant p73R1 still localizes to mitochondria.
b) The ability of the mutant p73R1 to interact with bcl-2 by immunoprecipitation and Western blot analyses will be tested.
c) LOH in tumors with *p73R1* mutations will be analyzed using microsatellite markers D11S3463 (~6kb flanking the c-terminal of the *p73R1* gene) or the sequence variations identified in exon 4 using ABI 377 DNA sequencer and ABI Genotyper 2.5 software.

Wt-p73R1 has been shown to localize in mitochondria participating in apoptosis through disruption of the membrane potential (dissipation of mitochondrial $\Delta\Psi$m)(reference 1). We therefore hypothesized that the two truncation mutations unable to suppress cell growth and induce apoptosis might be due to the mutant unable to localize into mitochondria or unable to disrupt the mitochondrial membrane potential. We performed immunofluorescence analysis of the two mutant p73R1 expression constructs to determine their subcellular localization. Interestingly, both of the mutants and the Wt-p73R1 localize in mitochondria (Figure 2). However, both of the mutants were unable to disrupt mitochondrial membrane potential as indicated by good mito-tracker CMXRos labeling of mitochondria (Figure 2).

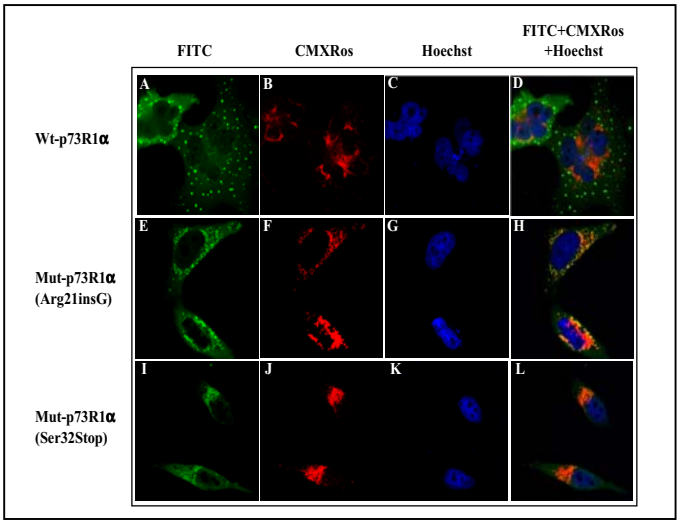

Figure 2. Mitochondrial localization of wt- and mut-p73R1 and membrane potential. *Upper panel*, HeLa cells transfected with wt-p73R1α and stained with 12CA5 (anti-HA) antibody (green), mito-tracker CMXRos (red) and Hoechst (blue). The fourth image is a merged picture to show disrupted mitochondrial membrane potential. *Middle panel*, representative HeLa cells overexpressing mut-p73R1α Arg21insG (green). The fourth merged image shows colocalization of the mutant protein (green) and the mito-tracker (red), indicating that the membrane potential is preserved. *Lower panel,* representative HeLa cells overexpressing mut-p73R1α Ser32Stop. Similar results were obtained when β forms of mut-p73R1 were expressed in HeLa cells or T98G cells (data not shown).

To further confirm that the two mutants unable to disrupt the membrane potential is the mechanism to lose the ability to induce apoptosis, we performed cytochrome c release analysis in the mutant transfectants. As shown in Figure 3, mitochondria can release cytochrome c in cells transfected with Wt-p73R1 while cytochrome c was retained in mitochondria in cells transfected with truncation mutations.

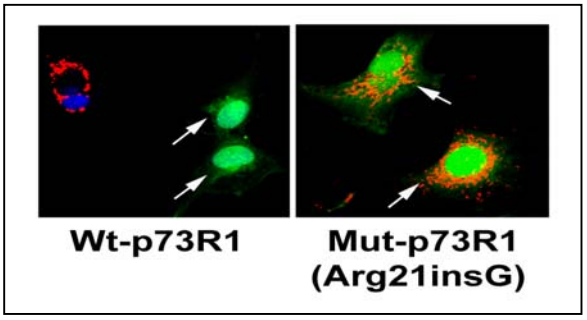

Figure 3. Mutant p73R1 unable to induce cytochrome *c* release from mitochondria in COS-7 cells. Fixation and staining of the cells were done 24 hrs after transient transfection of wt- and mut-p73R1 in pIRES vectors. The transfected cells were those with green fluorescence of EGFP. Cytochrome *c* staining is indicated by red fluorescence. *Left,* cytochrome *c* release in COS-7 cells transfected with wt-p73R1. Arrows indicate diffused cytochrome *c* staining. *Right,* cytochrome *c* staining in COS-7 cells transfected with mut-p73R1. Arrows indicate cytochrome *c* retaining in mitochondria.

Matsuda et al. (reference 2) demonstrated that the mechanism of p73R1 mediating apoptosis is because it interacts with Bcl-2 in mitochondria. We expect that the mutant p73R1 might not be able to bind to Bcl-2. However, we were not able to repeat the Matsuda's experiment even with the reagents that his laboratory provided. Instead, we found that overexpression of p73R1 repel the endogenous Bcl-2 (data not shown). We are now trying to figure out why p73R1 and Bcl-2 were unable to co-exist in cells.

So far, we are still ahead of the schedule of our proposal although the p73R1 mutation status of the remaining 144 CaP tumor specimens remain to be determined. We are sure that we will finish the LOH analysis proposed in Task 6c and the statistical analysis of the results that we proposed in Tasks 7 and 8 by the end of this year as we proposed.

Key Research Accomplishments:

1) We have screened for *p73R1* mutations in 856 CaP tumor specimens and identified 22 truncation mutations. The frequency of *p73R1* truncation mutations in this group of CaP is 2.6% in contrast to 0.6% (2/327) population-based controls (Fisher exact test, $P = 0.036$), with an odds ratio of 4.3(95% confidence interval 1.2-21.2).

2) We have demonstrated that the p73R1 truncation mutations lost ability to suppress cell growth by colony formation analyses and TUNEL assays.

3) We have determined that the p73R1 truncation mutations like wt-p73R1 localize in mitochondria but unlike wt-p73R1, they were unable to disrupt mitochondrial membrane potential and unable to induce cytochrome c release from mitochondria.

Reportable outcomes:

1) Germline *p73R1* truncation mutations are identified in ~3.0% of CaP tumor specimens and men carrying these mutations have a 4-5 fold increased risk to develop prostate cancer.

2) *P73R1* truncation mutations are loss of function mutations in regulation of apoptotic pathway in mitochondria.

Conclusions:

We have identified novel germline *p73R1* truncating mutations in ~3.0% of sporadic CaP samples collected between 1996-98 and about 2.6% in unselected CaP tumor samples collected between 2001-05

in Mayo. But the mutations only present in 0.6% of unaffected men, suggesting a 4-5 fold increased risk for mutation carriers to develop CaP cancer. In addition, we demonstrated that the *p73R1* truncating mutations are loss of function mutations. They were unable to disrupt mitochondria membrane potential, unable to induce apoptosis, unable to suppress cell growth, and unable to induce cytochrome c release in mitochondria. The inability of the *p73R1* truncation mutations to induce apoptosis probably decreases the capacity of prostatic cells to undergo apoptosis after DNA damage and hence these damaged cells with accumulating somatic mutations may eventually survive and develop into prostate cancer.

References:

1. Oda K, Arakawa H, Tanaka T, Matsuda K, Tanikawa C, Mori T, Nishimori H, Tamai K, Tokino T, Nakamura Y, Taya Y. p53AIP1, a potential mediator of p53-dependent apoptosis, and its regulation by Ser-46-phosphorylated p53. **Cell** 102(6):849-62, 2000.

2. Matsuda K, Yoshida K, Taya Y, Nakamura K, Nakamura Y, and Arakawa H. P53AIP1 regulates the mitochondrial apoptotic pathway. **Cancer Res.** 62:2883-89, 2002.

Appendices:

In this period, we submitted two manuscripts related to the subjects proposed in this study. They are included as appendices.

1. Wang X, Taniguchi K, Seelan RS, Wang L, McDonnell SK, Qian C, Pan K, Lu Y, Shridhar V, Couch FJ, Tindall DJ, Cooney KA, Isaacs WB, Jacobsen SJ, Schaid DJ, Thibodeau SN, and Liu W. Germline *p53AIP1* Mutations Disrupting DNA Damage-induced Apoptosis are Associated with Sporadic Prostate Cancer. **Cancer Res** (submitted).

2. Wu X, Dong X, Chen J, and Liu W. Characterization of Chk2 mutations in prostate cancer. **Hum Mut** (in press).

Germline *p53AIP1* Mutations Disrupting DNA Damage-Induced Apoptosis are Associated with Prostate Cancer Susceptibility

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Running title: *p53AIP1* mutations confer susceptibility to prostate cancer

Key Words: *p53AIP1*, germline mutations, apoptosis, prostate cancer risk.

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Abstract

Germline mutations in several genes (*BRCA1, BRCA2,* and *CHEK2*) whose products are involved in the DNA damage-signaling pathway have been implicated in prostate cancer risk. To identify additional genes in this pathway that might confer susceptibility to this cancer, we analyzed a recently identified DNA damage-response gene, *P53AIP1* (a gene encoding for p53-regulated Apoptosis-Inducing Protein 1), for mutations in prostate cancer. Five novel germline variants were identified. The two truncating mutations (Ser32Stop and Arg21insG) were found in 3% (4/132) of unselected prostate tumor samples. Genotyping of the two mutations in an additional 393 men with sporadic prostate cancer showed a frequency of 3.1% (12/393) in contrast to 0.6% (2/327) in 327 unaffected men (Fisher's exact test, $P = 0.018$), with an odds ratio of 5.1 (95% confidence interval 1.1 – 23.0). In addition, 2 of 6 tumors carrying the truncating mutations were associated with loss of heterozygosity of the wild-type alleles, suggesting that p53AIP1 may act as a tumor suppressor. We also demonstrated that mutant p53AIP1 was unable to induce apoptosis and suppress cell growth in HeLa and COS-7 cells. These results suggest that loss of function mutations in p53AIP1 predispose men to sporadic prostate cancer and further support the concept that the genetic defects in the DNA damage-response genes play an important role in the development of prostate cancer.

Introduction

Many lines of evidence have shown that genetics play an important role in the development of prostate cancer (1). Studies in the last several years have suggested that a number of rare, highly penetrant loci may contribute to Mendelian inheritance of prostate cancer and that other genetic alterations contributing to the majority of non-Mendelian inheritance of prostate cancer are likely to be multiple low-penetrance alleles (2). These alleles could bear function-associated polymorphisms in the regulatory genes such as the androgen receptor gene or mutations in genes associated with certain signaling pathways that are involved in prostate tumorigenesis (3, 4). The DNA damage-signaling pathway is essential for the prevention of genomic instability, a common feature of all human cancers including prostate cancer. Germline mutations in several key components of this pathway (*BRCA1*, *BRCA2*, and *NBS1*) have been shown to associate with prostate cancer risk (5-7). We and others recently reported that mutations in *CHEK2* have an increased risk for male carrier to develop prostate cancer (8, 9). These studies suggest that integrity of the DNA damage-signaling pathway is crucial for prevention of neoplastic transformation in the prostate and that the genes participating in the DNA damage-signaling pathway could be targets for mutations in prostate cancer tumorigenesis.

P53AIP1 is a downstream target of p53 and is induced by DNA damage (10). This gene has three transcripts (α, β, and γ). The α and β forms of *p53AIP1* are localized to the mitochondria and induce apoptosis through dissipation of mitochondria membrane potential. The expression of *p53AIP1* and p53AIP1-induced apoptosis are closely correlated with phosphorylation of p53 at Ser-46, indicating that p53AIP1 plays an important role in mediating p53-dependent apoptosis. Moreover, p53AIP1 interacts with bcl-2, an inhibitor of apoptosis, in mitochondria (11). This

event induces the release of cytochrome *c* from mitochondria and very likely regulates p53AIP1-mediated apoptosis through regulation of the mitochondria membrane potential. These findings suggest that p53AIP1 is crucial for regulation of p53-dependent DNA damage-signaling pathways and disruption of the function of p53AIP1 in p53-mediated apoptosis could play an important role in cancer development. Since the frequency of *p53* mutations in prostate cancer is low but much higher in other cancers, *p53AIP1* could be an ideal mutation target for prostate cancer susceptibility.

Here we report the identification of germline *p53AIP1* mutations in prostate cancer and show that the mutation carriers have increased risk of developing prostate cancer. We also provide evidence to reveal the functional aspects of the *p53AIP1* mutations in the development of prostate cancer.

Materials and Methods

Tumors and patients. The 132 prostate tumor specimens used in this study were unselected and were collected at the Mayo Clinic (Rochester, MN) between 1997-98. All of the specimens were obtained by surgery, quickly frozen in liquid nitrogen and transferred to –80°C freezer until further analysis. DNA was extracted from cryocut slices of frozen tissues with Easy-DNA kits (Invitrogen, Carlsbad, CA).

The group of patients with sporadic prostate cancer (n = 393) has been previously described in our other gene association studies, with a reduction of 7 cases because of availability at the time the study was conducted (8, 12). These patients were selected from respondents to a family history survey that reported no family history of prostate cancer (13, 14). The diagnosis of prostate cancer was confirmed by pathology reports. Prostate specific antigen (PSA) values at diagnosis were available for 317 men, with a median value of 7.1 and with 76% having values of 4 or greater. The median age of diagnosis for this group was 65.0 (range 46.0 – 79.0).

The unaffected control group (n = 327, all male) has also been described in our previous publications, though four controls have been dropped from the original group of 331 due to the occurrence of prostate cancer (8, 12). These control subjects were recruited from a sampling frame of the local population provided by the Rochester Epidemiology Project (15): 475 men were randomly selected for a clinical urologic examination (16). This clinical examination included digital rectal examination (DRE) and transrectal ultrasound (TRUS) of the prostate, abdominal ultrasound for post-void residual urine volume, measurement of serum levels of prostate-specific antigen (PSA) and creatinine, focused urologic physical examination, and cryopreservation of serum for subsequent sex hormone assays. Any patient with an abnormal

DRE, elevated serum PSA level, or suspicious lesion on TRUS was evaluated for prostatic malignancy. If the DRE and TRUS were unremarkable but the serum PSA level was elevated (>4.0 ng/ml), a sextant biopsy (three cores from each side) of the prostate was performed. An abnormal DRE or TRUS result, regardless of the serum PSA level, prompted a biopsy of the area in question. In addition, a sextant biopsy of the remaining prostate was performed. Those men who were found to be without prostate cancer on the basis of this extensive workup at baseline or at any of the follow-up examinations through 1994 were used for the control population; 327 of these individuals participated in this particular study. The median age of these men was 59.6 (range 49.0 – 89.0). All of the participants in this study gave full informed consent and were approved by the Mayo Institutional Review Board.

Mutation detection. The genomic sequences of the primers designed for the amplification of three *p53AIP1* exons are *p53AIP1*e2F/R (5'-AAATGAGGAGAAGCCAAGTT-3' and 5'-CGGCACCACGGTGAGA-3'), *p53AIP1*e3F/R (5'-AACCATCCAAGAGACGG-3' and 5'-ATCACTTAATTCTATCACGG-3'), and *p53AIP1*e4F/R (5'-AAGGACTCCATACGTTTTGC-3' and 5'-GCTGGAGCCATTTCTCGAC-3"). PCR products were obtained using AmpliTaq Gold (Applied Bioscience, Foster City, CA) and 35 cycles from 25 ng of genomic DNA per reaction. Denaturing high-performance liquid chromatography (DHPLC) analysis was performed on automated DHPLC instruments (Transgenomic or Varian) and PCR products containing heteroduplexes were subsequently sequenced as previously described (17).

Plasmid construction and transfection. Wild-type (wt) and mutant (mut) *p53AIP1* cDNA were PCR amplified with primers containing HA or FLAG sequence tags and NheI and XbaI

restriction sites. The corresponding PCR products were digested and inserted into PCI (Promega, Madison, WI) or IRES-EGFP (Clontech, Mountain View, CA) plasmids. HeLa, COS-7, Saos-2 and T98G cells were grown in Dulbecco's modified Eagle's medium/10% fetal calf serums supplemented with antibiotics. Transient transfections were performed when cells reached 60-80% confluence either in culture dishes or on slides with Superfect (Qiagen, Valencia, CA) or FuGENE 6 (Roche, Indianapolis, IN).

Immunoprecipitation, Western blotting, and immunostaining. These procedures were followed as we previously described (18). Briefly, to immunoprecipitate the ectopically expressed protein, whole cell lysates were obtained using Beach lysis buffer. The beads conjugated with anti-HA mouse monoclonal antibody [clone 12CA5 (Roche, Indianapolis, IN)] were mixed with cell lysates to pull down the recombinant protein. The precipitated protein was resolved on 15% SDS-PAGE gels and transferred onto PVDF membrane (Bio-Rad, Hercules, CA). The blot was then stained with anti-HA antibody and visualized by ECL (Amersham Biosciences, Piscataway, NJ). For the immunostaining of the epitope tagged wt- and mut-p53AIP1 protein, the transiently transfected HeLa cells were fixed with 3% paraformaldehyde in 1xPBS and permeablized with 0.2% Triton X-100 at room temperature. The cells were then blocked in 3% milk in 1xPBS and stained with mouse anti-HA, human AMA serum at 1:1000 dilution (personal gift, Dr. Mark McNiven) in 3% milk. Alexa 488 conjugated goat anti-mouse and Alexa 670 conjugated goat anti-human antibodies (Invitrogen, Carlsbad, CA) were used for the secondary staining. The indirect immunofluorescence was visualized and recorded on a Carl Zeiss Confocal Laser Scanning Microscope LSM510. To label the mitochondria with mito-

tracker CMXRos (Invitrogen), cells were first incubated with 100 nM fluorescent probe in the culture medium for 30 minutes in the incubator just before fixation.

Colony formation analysis and TUNEL assay. Wt- and mut-p53AIP1 cDNA were cloned into IRES-EGFP (Clontech) plasmids and transfected into COS-7 cells. EGFP-positive cells were sorted 48 h after transfection and seeded in 3.5 cm dishes with equal numbers. Colonies of cells were fixed in ethanol and stained with crystal violet around 10 days. The plates were photographed and the number of colonies counted. Tunnel staining was performed on T98G cells transfected with wt- and mut-p53AIP1 expression constructs in PCI vectors. After immunostaining of p53AIP1 by 12CA5 antibody, the nuclear DNA double-strand breaks were labeled with TMR-red labeled nucleotides catalyzed by terminal deoxynucleotidyl transferase (TdT) (*In situ* Cell Death Detection Kit, TMR red, Roche) and analyzed by fluorescence microscopy.

Cytochrome *c* release by immunofluorescence staining. For cytochrome *c* immunostaining, 1,000 cells were seeded in each well of 12-well slides a day before experiment. Cells were transfected with wt- and mut-p53AIP1 expression constructs in pIRES vectors using Fugene 6 reagent; 48 hours later, cells were washed with PBS, fixed with 4% paraformaldehyde, and permeabilized in 0.1% Triton X-100 in PBS for 10 minutes at room temperature. Cells were then blocked with 3% non-fat milk in PBST for 1 hour, cultured with anti-cytochrome *c* antibody (BD, Franklin Lakes, NJ) at 1:100 dilution for 1 hour at room temperature, washed with PBS, cultured with secondary antibody Alexa 568 at 1:2000 dilution in PBST for 1 hour at room temperature. Cells were then washed with PBS and stained with Hoechst. Slides were mounted

with VECTASHIELD mounting medium (Vector Laboratories, Burlingame, CA) and observed using a confocal microscope.

Statistical analysis. The association of *p53AIP1* truncating mutations (Ser32Stop and Arg21insG) with prostate cancer was evaluated using Armitage's test for trend in the number of variant alleles (19).

Results

To determine the genetic roles of *p53AIP1* in prostate cancer susceptibility, we screened this gene for mutations in 132 primary prostate tumor specimens. The entire coding and exon/intron junction sequences of this gene were PCR amplified and screened for mutations by DHPLC followed by direct sequence analysis. Three unique non-synonymous sequence variations (C20T, Ala7Val; A304G, Arg102Glu; and C313T, Pro105Ser), one nonsense mutation (C95A, Ser32Stop), and one frameshift mutation due to a one base-pair insertion (64insG, Arg21insG) were identified (Figure 1). The two *p53AIP1* truncating mutations (Ser32Stop and Arg21insG) were present in 3% (4/132) of the tumor samples (Table 1). The three non-synonymous variants were present in 4.5-41% of prostate tumor specimens, respectively. All five variants identified in *p53AIP1* were present in both tumor specimens and matched normal tissues, indicating that they were germline in nature. To further evaluate the significance of the two *p53AIP1* truncating mutations in the predisposition to prostate cancer, we assessed their frequencies in an additional 393 men with sporadic prostate cancer and in 327 population-based controls. The two mutations were detected in 12 (3.1%) sporadic prostate cancer cases and in two (0.6%) controls (Table 1), suggesting that men who carry a germline *p53AIP1* truncating mutation have a 5-fold increased

risk of developing prostate cancer (odds ratio, 5.1; 95% confidence interval, 1.1 – 23.0; P = 0.018).

We also examined the *p53AIP1* mutations in other types of tumors by genotyping the two truncating mutations and the C20T (Ala7Val) missense alteration in 403 tumor specimens including 127 breast cancer, 110 ovarian cancer, 72 gastric cancer, and 94 neuroblastomas (Table 1). Although the C20T missense alteration identified in prostate cancer was also present in other cancers with a similar frequency, we did not detect the two truncating mutations in any of these non-prostate tumors, suggesting that the *p53AIP1* truncating mutations are probably specific for prostate cancer. However, more extensive studies will be required to analyze additional tumor types and more samples for each type in order to determine whether the *p53AIP1* truncating mutations are unique to prostate cancer.

Oda *et al.* has demonstrated that wt-p53AIP1 is localized in mitochondria and led to apoptosis through disruption of the membrane potential (dissipation of mitochondrial $\Delta\Psi m$) (10). We then asked whether *p53AIP1* mutations that we detected in prostate cancer alter its apoptotic function. We generated epitope-tagged expression constructs containing both α and β forms of wt- and mut-p53AIP1 (designated as FLAG-p53AIP1α-wt-HA, p53AIP1β-wt-HA, p53AIP1α-Arg21insG-HA, p53AIP1β-Arg21insG-HA, and p53AIP1α-Ser32Stop-HA) and transiently transfected into HeLa cells. Both α and β forms of wt- and Arg21insG mutant proteins were expressed (Figure 2A), except for the Ser32Stop mutant which was probably too small (only 31 aa) to be stable for the detection. Subsequent immunofluorescence staining of these recombinant proteins and mitochondria revealed that both α forms of wt-p53AIP1 and

Arg21insG mutants were localized to the mitochondria (Figure 2B). But, unlike wt-p53AIP1, the Arg21insG mutant was unable to disrupt the mitochondrial membrane potential as indicated by good mito-tracker CMXRos labeling of mitochondria. Similar results were obtained in T98G cells (data not shown). These results indicate that the mutant p53AIP1 without C-terminus is able to translocate to mitochondria but unable to disrupt mitochondria membrane potential.

Since disruption of mitochondrial membrane potential and subsequent cytochrome *c* release has been suggested as the mechanism of p53AIP1-induced apoptosis (11), we reasoned that mut-p53AIP1 might no longer be able to trigger programmed cell death. We therefore performed TUNEL assays on T98G cells transfected with the α form of wt- or mut-p53AIP1. As shown in Figure 3A, the cells expressing wt protein generated 3-4-fold more dead cells typified by positive TMR red staining than those expressing mutant protein. We subsequently transfected COS-7 cells with wt-p53AIP1 and Arg21insG mutant expression constructs in pIRES vectors that can express EGFP simultaneously and immunostained the cells with anti-cytochrome *c* antibody. As shown in Figure 3B, the cytochrome *c* staining in mitochondria is retained in mut-p53AIP1 expressing cells (EGFP-positive cells) while released in wt-p53AIP1 transfectants (EGFP-positive cells). These results demonstrate that mut-p53AIP1 (Arg21insG) is unable to induce cytochrome *c* release in mitochondria and therefore unable to trigger programmed cell death.

To further evaluate the role of mutant p53AIP1 in cell growth, we performed colony formation assay. Expression constructs of wt-p53AIP1, Arg21insG and Ser32Stop mut-p53AIP1 in pIRES vectors were transfected into COS-7 cells and the EGFP-positive cells were enriched by flow cytometry and continuously cultured for 10 days. As expected, wt-p53AIP1 strongly

suppressed cell growth shown by significantly less and smaller colonies in comparison with cells transfected with control plasmid. In contrast, the number and size of colonies transfected with mut-p53AIP1 constructs (Arg21insG or Ser32Stop) are more comparable with control (Figure 3C and 3D). These data demonstrated that *p53AIP1* truncating mutations lost the abilities to suppress cell growth.

Discussion

In this study, we provide evidence for the first time that *p53AIP1* is mutated in prostate cancer and the men carrying the *p53AIP1* truncating mutations have increased risk of developing prostate cancer. Although we estimate the risk to be high, with an odds ratio of 5.1, the rarity of the mutations translates to a wide confidence interval for this risk, 1.1 – 23.0. Larger studies are required to refine this risk estimate. Other than the two truncating mutations, we also identified three missense alterations in this gene. The Ala7Val alteration is present in both cases and controls with a similar frequency. Expression of the construct with this alteration localizes to mitochondria and dissipates mitochondrial membrane potential (data not shown). Thus, this is very likely a non-synonymous polymorphism. Two other missense alterations at the very C-terminus of the protein showed very high frequencies in prostate tumor samples. However, the roles of these two alterations in prostate cancer susceptibility remain to be elucidated.

The functional analysis of *p53AIP1* mutations provides some insights into the possible mechanism underpinning the association of these mutations with increased risk of prostate cancers. Our results demonstrate that Ser32Stop and Arg21insG are loss-of-function mutations in terms of inducing apoptosis. These deleterious mutations may decrease the capacity of prostatic

cells to undergo apoptosis after DNA damage and hence these damaged cells with accumulating somatic mutations may eventually survive and develop into tumor clones. On the other hand, we analyzed six available tumors carrying the *p53AIP1* mutations and loss of *wt-p53AIP1* alleles was observed in two of them (data not shown), indicating that *p53AIP1* is probably a tumor suppressor gene. Collectively, these results from our combination of genetic and functional studies suggest that *p53AIP1* is a prostate cancer susceptibility gene and mutations in this gene severely impair its function to induce apoptosis, probably causing tumorigenesis in patients with prostate cancer.

The results from this study and the studies of *BRCA1, BRCA2, CHEK2,* and *NBS1* in prostate cancer suggest that germline mutations in the DNA damage-response pathway genes contribute to genetic susceptibility of prostate cancer (5-9). Although the majority of the mutations for these genes were found in sporadic prostate cancer, some of the mutations were observed in both familial and sporadic prostate cancers (20, 21). To explore the possibility that the two truncating mutations of *p53AIP1* might also play a role in hereditary prostate cancer, we analyzed a total of 981 affected men from 426 families with prostate cancer. This included 160 families collected at Mayo Clinic, 142 at Johns Hopkins, and 124 at University of Michigan (22). The association *of P53AIP1* truncating mutations with familial prostate cancer was evaluated using a test for trend in the number of variant alleles, analogous to Armitage's test for trend in proportions, yet with the appropriate variance to account for correlated family data (23). Mutation frequencies among familial cases was higher than that in controls, with frequencies of 1.2%, 0.8%, and 2.7% detected among the familial prostate cancer cases from Mayo Clinic, Johns Hopkins, and University of Michigan, respectively (Table 2). None of these frequencies was significantly different than the frequency observed in controls (0.6%). However, all familial cases show a

slightly increased risk (odds ratio > 1) compared to controls. The frequency of the mutation carriers was not statistically different among the three groups of familial cases ($p = 0.19$), nor when contrasted with the sporadic cases ($p = 0.10$), although the power to detect differences is weak because of the rarity of the mutation. A test of differences among the odds ratios contrasting familial cases versus controls was not statistically significant ($p = 0.5$), and the Mantel-Haenszel stratified odds ratio comparing all familial cases to controls was 2.5 (95% CI 0.98 – 6.62). Other genes may play a more prominent role for prostate cancer susceptibility in the familial cases. Clearly, additional studies are needed to explain this difference. Finally, we must point out that the risk contribution of the rare mutations in each of the 5 DNA damage-response genes is relatively low. However, the cumulative risk contribution of these mutations in the group of genes in the same pathway could be high in the population if they are independent. Thus, identification of this type of mutation in more DNA damage-response genes in prostate cancer will improve our understanding of the etiology of this disease and also potentially identify men at increased risk of developing prostate cancer in whom prevention strategies might be targeted.

Acknowledgements

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Table 1. Frequencies of *p53AIP1* mutations in patients with prostate cancer, non-prostate cancers, and unaffected control subjects

DNA samples	*Cases*	*Ala7Val*	*Ser32Stop*	*Arg21insG*	*Arg102Glu*	*Pro105Ser*
Clinic CaP tissues	132	6	2	2	54 (40.9%)	53 (40.2%)
Sporadic CaP blood	393	9	3	9	nd	nd
Unaffected men blood	327	7	1	1	nd	nd
Neuroblastoma tissues	94	3	0	0	nd	nd
Ovarian cancer tissues	110	4	0	0	nd	nd
Breast cancer tissues	127	5	0	0	nd	nd
Gastric cancer tissues	72	1	0	0	nd	nd

Table 2. *P53AIP1* germline mutations in men with prostate cancer and in unaffected men

	N	*Genotype n (%)*		*P-value‡*	*OR (95% CI)*
		VW†	*WW*		
Controls	327	2 (0.6%)	325 (99.4%)		
Familial CaP Cases					
Mayo Clinic	434	5 (1.2%)	429 (98.8%)	0.499	1.89 (0.32, 11.38)
JHU	246	2 (0.8%)	244 (99.2%)	0.797	1.33 (0.15, 11.61)
Michigan	301	8 (2.7%)	293 (97.3%)	0.071	4.44 (0.94, 21.0)
Sporadic CaP Cases	393	12 (3.1%)	381 (96.9%)	0.018	5.11 (1.14, 23.04)

† VW genotype refers to carriers of either the Ser32Stop mutation or the Arg21insG mutation .
‡ P-values are from Armitage's test for trend on allele counts accounting for related subjects. For the unrelated sporadic cases, this is exactly Armitage's test for trend.

Figure legends:

Figure 1. Identification of two novel *p53AIP1* mutations (*B, C*) and three non-synonymous variants (*A, D, E*) in prostate tumor specimens by DHPLC analysis and direct sequencing. *Top,* electropherograms displaying the wild-type sequence. *Bottom,* electropherograms displaying the sequence variants. The arrows mark the location of the mutations.

Figure 2. Ser32Stop and Arg21insG of p53AIP1 are loss-of-function mutations in programmed cell death. *A,* Ectopic expression of wt- and mut-p53AIP1 in HeLa cells. The expression vector alone was used as the negative control. *B,* Mitochondrial localization of mut-p53AIP1 Arg21insG and membrane potential. *Upper panel*, HeLa cells transfected with wt-p53AIP1α and stained with 12CA5 (anti-HA) antibody (green), mito-tracker CMXRos (red) and Hoechst (blue). The fourth image is a merged picture to show disrupted mitochondrial membrane potential. *Lower panel*, representative HeLa cells overexpressing mut-p53AIP1α Arg21insG (green). The fourth merged image shows colocalization of the mutant protein (green) and the mito-tracker (red), indicating that the membrane potential is preserved. Similar results were obtained when β form of mut-p53AIP1 Arg21insG was expressed in HeLa cells (data not shown).

Figure 3. Analysis of wt- and mut-p53AIP1 in apoptosis and cell growth. *A,* TUNEL assays of wt- and mut-p53AIP1α Arg21insG in T98G cells. Mut-p53AIP1 was tagged with a C-terminal HA epitope and wt-p53AIP1 with both an N-terminal FLAG and a C-terminal HA epitope. The cells were immunostained with anti-HA (12CA5) antibody and then labeled with TMR red fluorescence. Expression-positive and tunnel-positive cells are indicated by FITC green

fluorescence and TMR red fluorescence, respectively. Images were taken on a Carl-Zeiss Axiovert II fluorescence microscope. *B,* p53AIP1 mutant loses the ability to induce cytochrome *c* release from mitochondria in COS-7 cells. Fixation and staining of the cells were done 24 hrs after transient transfection of wt- and mut-p53AIP1 in pIRES vectors as described in Materials and Methods. The transfected cells were those with green fluorescence of EGFP. Cytochrome *c* staining is indicated by red fluorescence. Top image: cytochrome *c* release in COS-7 cells transfected with wt-p53AIP1 expression construct. Arrows indicate diffused cytochrome *c* staining. Lower image: cytochrome *c* staining in COS-7 cells transfected with mut-p53AIP1construct. Arrows indicate cytochrome *c* retaining in mitochondria. *C and D,* Effects of wt- and mut-p53AIP1 on cell growth as shown by the culture dishes after crystal violet staining. COS-7 cells were transfected with the corresponding *p53AIP1* plasmids and sorted by flow cytometry. Control cells were transfected with pIRES-EGFP vector alone to serve as a negative indicator of cell growth suppression. Expression of wt-p53AIP1 in COS-7 cells was used as a positive control. The colonies for each expression construct were counted and expressed as % colony formation efficiency and the results shown are the average mean of three experiments.

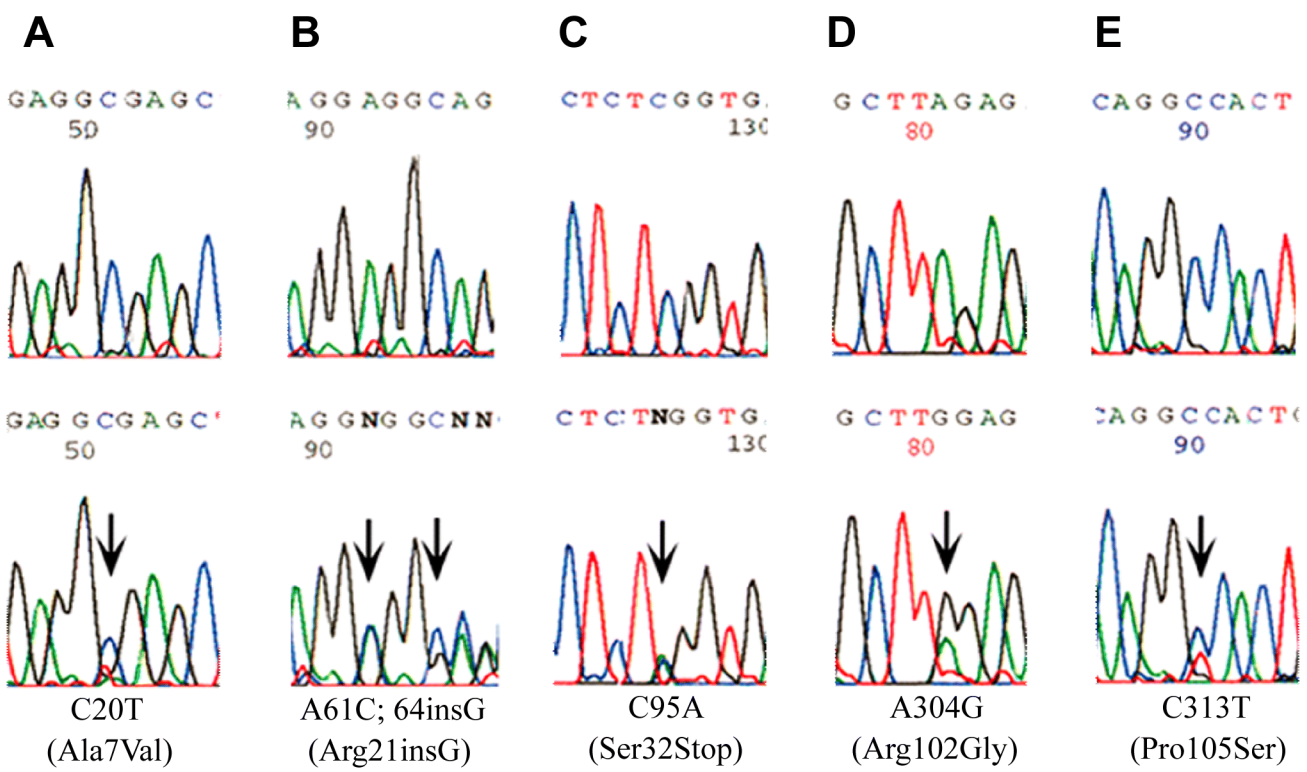
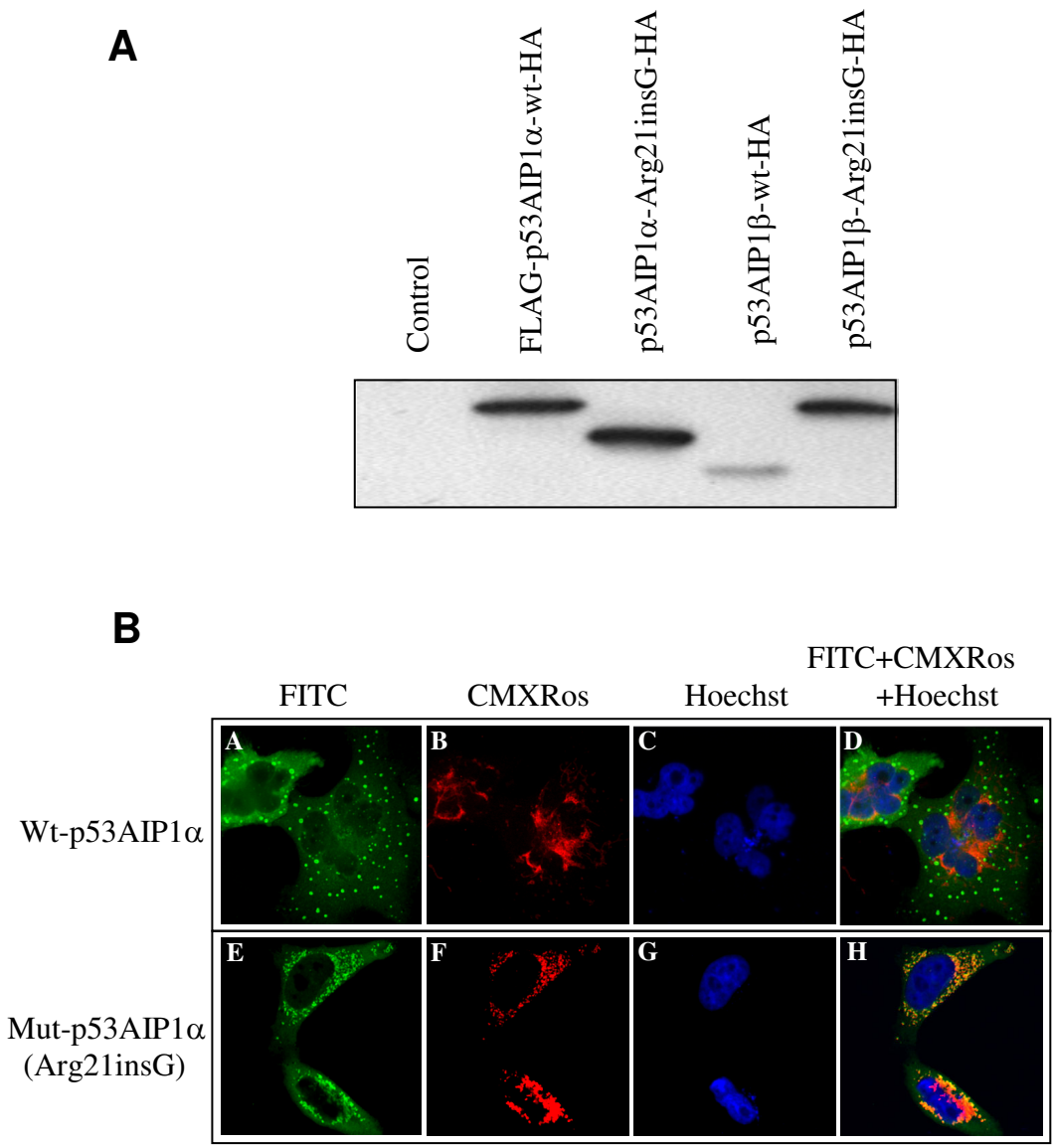

A
B
C
D
E
50
90
80
C20T
(Ala7Val)
A61C; 64insG
(Arg21insG)
C95A
(Ser32Stop)
A304G
(Arg102Gly)
C313T
(Pro105Ser)

Figure 1


A
Control
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p53AIP1α-Arg21insG-HA
p53AIP1β-wt-HA
p53AIP1β-Arg21insG-HA
B
FITC
CMXRos
Hoechst
FITC+CMXRos
+Hoechst
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(Arg21insG)
A
B
C
D
E
F
G
H

Figure 2

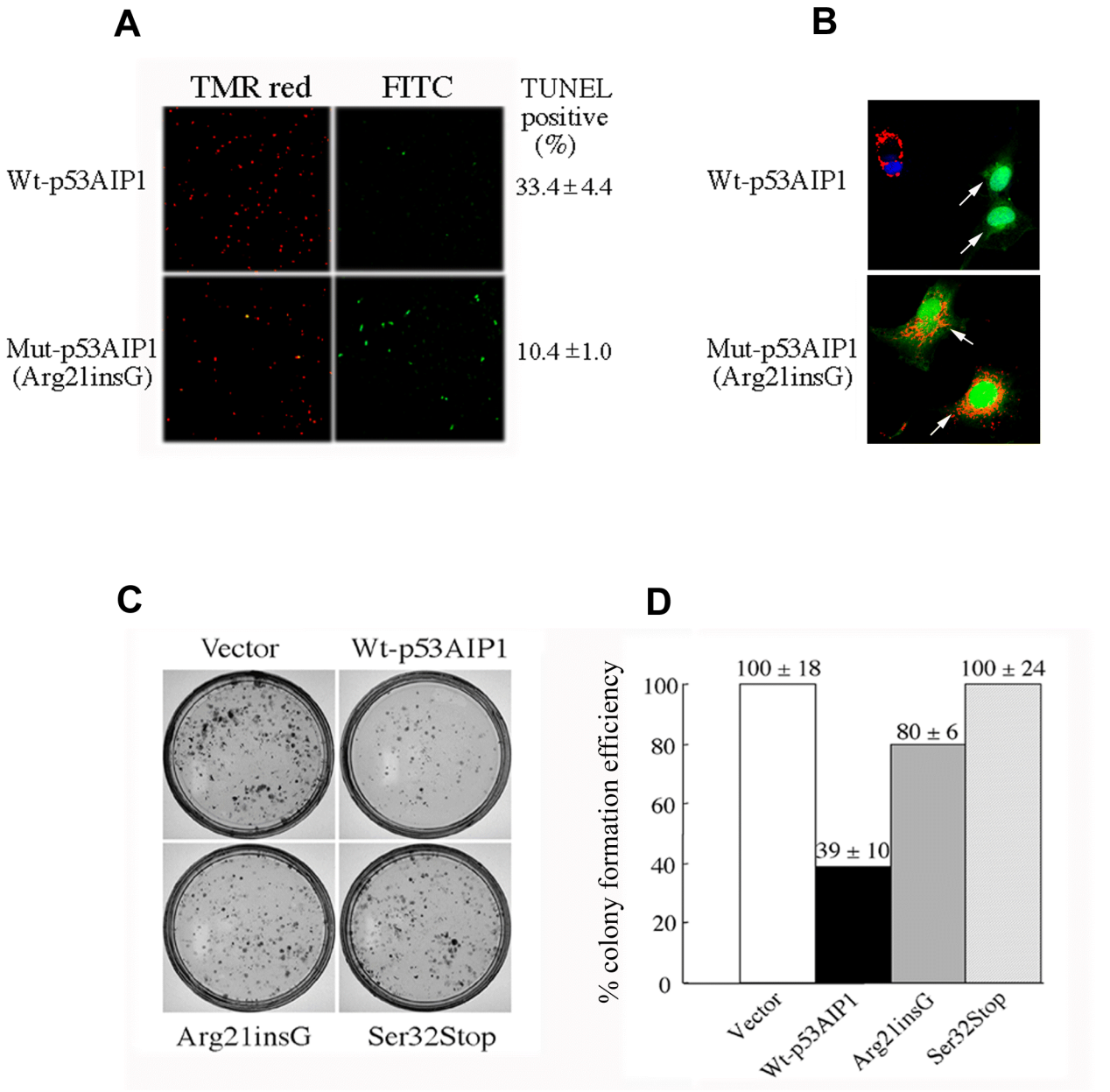
A
TMR red
FITC
TUNEL positive (%)
Wt-p53AIP1
33.4 ± 4.4
Mut-p53AIP1 (Arg21insG)
10.4 ± 1.0
B
Wt-p53AIP1
Mut-p53AIP1 (Arg21insG)
C
Vector
Wt-p53AIP1
Arg21insG
Ser32Stop
D
% colony formation efficiency
100 ± 18
39 ± 10
80 ± 6
100 ± 24
0
20
40
60
80
100
Vector
Wt-p53AIP1
Arg21insG
Ser32Stop

Figure 3

MUTATION IN BRIEF

Mutually Exclusive Mutations of *CHEK2* and *TP53* Implicated in Primary Prostate Tumors and Cancer Cell Lines

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Short Title: Mutually exclusive mutations of *CHEK2* and *TP53* in prostate cancer

Communicated by <Please don't enter>

Genetic defects in *CHEK2* and *TP53* have been implicated in prostate cancer development. However, the interaction of these two genes in prostate cancer tumorigenesis has not been investigated. We previously described 11 *CHEK2* mutations in a group of 84 primary prostate tumors. In this report, we screened the same group of tumors for *TP53* mutations and revealed 9 somatic and 2 germline mutations. One germline *TP53* mutation (A408T; Gln136His) and 2 somatic mutations (T1022G; Phe341Cys and 22bp duplication mutation) are novel and unique to prostate cancer. More interestingly, *CHEK2* and *TP53* mutations were observed to be mutually exclusive in these tumors. Analysis of 5 commonly used prostate cancer cell lines revealed that 4 cell lines harboring *TP53* mutations carry no *CHEK2* mutation while the only cell line (LNCaP) carrying wild-type *TP53* harbors a *CHEK2* mutation. The novel *CHEK2* mutation identified in LNCaP cells changes Thr387 to Asn which has been shown to impair CHEK2 autophosphorylation and activation. These results suggest that the *CHEK2* and *TP53* mutations can uniquely substitute each other in at least 25% (21/84) of prostate cancers and that DNA damage-signaling pathway plays an important role in prostate cancer tumorigenesis.

KEY WORDS: *CHEK2*; *TP5;* germline mutations; somatic mutations; prostate cancer

INTRODUCTION

Prostate cancer is the most common malignancy and the second leading cause of cancer-related death in the United States. Although the identification of genetic components contributing to the development of this cancer remains challenge, a growing body of evidence from mutation analysis and association studies of candidate genes have suggested that the genetic defects in the DNA damage-signaling pathway could be risk factors for prostate cancer development (Rosen, et al., 2001). Men carrying germline mutations in *BRCA1* (MIM# 113705) and

Received <date>; accepted <date>.

BRCA2 (MIM# 600185) have increased risk for developing prostate cancer (Gayther, et al., 2000). Non-synonymous variants in DNA damage-signaling pathway genes such as *ATM* (MIM# 607585), *XRCC1* (MIM# 194360), and *NBS1* (MIM# 602667) have also been shown to confer susceptibility to prostate cancer (Angele, et al., 2004). These studies suggest that the integrity of the DNA damage-signaling pathway is essential for the prevention of neoplastic transformation in prostate.

Cell cycle-checkpoint kinase 2 gene (*CHEK2*; also known as *CHK2*, MIM# 604373) and *TP53* (MIM# 191170) are two key regulators in the DNA damage-signaling pathway. CHEK2 is activated in response to various DNA-damage agents in an ATM-dependent fashion (Matsuoka, et al., 1998). Activated CHEK2, along with other DNA-damage-activated protein kinases, stabilizes TP53 or enhances degradation of Cdc25A (MIN# 116974) in the cell-cycle checkpoint control, through coordination of DNA repair, cell-cycle progression, and apoptosis (Falck, et al., 2001). Recently, heterozygous germline mutations in the *CHEK2* gene have been identified in patients with Li Fraumeni Syndrome (LFS; MIM# 151623), a highly penetrant familial cancer phenotype, usually associated with inherited mutations in *TP53* (Bell, et al., 1999). Subsequently, germline and somatic *CHEK*2 mutations were reported in many different types of human cancers including lung, breast, colon, bladder, and ovary cancers (Bartek and Lukas, 2003; Matsuoka, et al., 2001). We reported *CHEK2* mutations in prostate cancer and showed that germline *CHEK2* mutation confers susceptibility to prostate cancer (Dong, et al., 2003). We also demonstrated that the *CHEK2* mutations identified in prostate cancers impair kinase activity of CHEK2, a very likely cause of tumorigenesis in prostate (Wu, et al., in press). TP53 is the downstream target of CHEK2 in the DNA damage-signaling pathway. Somatic *TP53* mutations have been reported in 4-30% of prostate cancers and have been shown to associate with prostate cancer progression (Navone, et al., 1993). However, the two genes have never been screened for mutations in the same set of prostate tumors and the relationship of the two genes in prostate cancer tumorigenesis remains unknown.

In this study, we analyzed *CHEK2* and *TP53* mutations in the same group of 84 unselected primary prostate tumor specimens and 5 commonly used prostate cancer cell lines to provide a comprehensive assessment of the mutations in the two genes and to test the hypothesis that *CHEK2* and *TP53* mutation are mutually exclusive in prostate cancer and the integrity of the DNA damage-signaling pathway is crucial for the prevention of neoplastic transformation in prostate.

MATERIALS AND METHODS

Primary prostate tumor specimens and cell lines

Eighty-four unselected primary prostate tumors were collected at Mayo Clinic between 1997 and 1998 as previously described (Dong, et al., 2003). Fresh tissue specimens were collected at the time of operation and frozen at –80℃. Five prostate cancer cell lines (22Rv1b, DU145, PC3, NCI-H660, and LNCaP) were obtained from American Type Culture Collection. Cells were cultured in medium according to the standard protocol.

DNA isolation and mutation analysis

High molecular weight genomic DNA from these frozen tissues or cell lines were isolated using Easy-DNA Kit (Invitrogen, Carlsbad, CA) following the manufacturer's instruction. PCR was performed using 13 pairs of intronic primers covering the 14 coding exons of the *CHEK2* gene as previously described (Dong, et al., 2003) and 9 pairs of intronic primers covering 10 exons (exons 2-11) of the *TP53* gene (available upon request). PCR amplification was performed in a volume of 12.5 μl containing 5 ng of genomic DNA, each primer at 0.2 mM, dNTP at 0.2 mM, 2.0 mM $MgCl_2$, 0.5 U of *Taq* polymerase (Ampli*Taq* Gold, Applied Biosystems, Foster City, CA) with PCR reaction buffer provided by the manufacturer. Denaturing high-performance liquid chromatography (DHPLC) analyses followed by direct sequencing of the PCR products were performed as described previously (Liu, et al., 1997).

RNA preparation and RT-PCR

Total mRNA from the frozen tissue or cell lines was extracted with Trizol reagent (Invitrogen, Carlsbad, CA) according to the manufacturer's instruction. Total mRNA was transcribed to cDNA with the SuperScriptIII First-Strand Synthesis System (Invitrogen, Carlsbad, CA) according to the instruction of the manufacturer. RT-PCR for amplifying *TP53* exons 2-5 was performed using the primers as follows: Forward 5'-

TGGAGGAGCCGCAGTCAGAT-3' in exon 2 and Reverse 5'-GGGCGGGGGTGTGGAATCAA-3' in exon 5. *CHEK2* mutation in LNCaP was also confirmed by RT-PCR analysis using the following primers: Forward 5'-AATTGATTGGAAGGGGGAGAGCTGT-3' in exons 7-8 and Reverse 5'-AGAGCTGTGGATTCATTTTCCTCAGA-3' in exon 13.

TA cloning and sequencing

Genomic PCR products of *TP53* exon 4 and RT-PCR products covering *TP53* exons 2-5 were subcloned into the pGEM®-T Easy vector (Promega, Madison, WI) according to the manufacturer's instruction. Several white colonies were selected for PCR amplification and direct sequencing.

RESULTS AND DISCUSSION

Novel somatic and germline *TP53* mutations in prostate cancer

Mutations in *CHEK2* somatic *TP53* have been reported in prostate cancer (Dong, et al., 2003; Navone, et al., 1993). To better understand the relationship of these two genes in prostate cancer tumorigenesis, we screened for *TP53* mutations in a group of 84 primary prostate tumors in which *CHEK2* had been assessed for mutations (Dong, et al., 2003). Eleven *TP53* mutations in 84 (13%) prostate tumors were identified including 9 missense mutations, 1 pre-mature termination mutation (C637T; Arg213Stop), and 1 22bp duplication mutation in exon 4 (Table 1, Fig. 1A, 1B). Interestingly, although the frequency of *TP53* mutations identified in this group of prostate tumors is similar to those previously reported, 2 of 11 (18%) of our mutations were found in exon 4 and 10, out of exons 5-8, the most commonly mutated region in human cancers (Hsu, et al., 1991), suggesting that it is necessary to search the entire coding region of *TP53* for mutations when the role of TP53 in prostate cancer is investigated. We also established the somatic or germline nature for each of the *TP53* mutations by sequencing DNA derived from the matched normal tissue of the same patient. We found that 9 were somatic mutations and 2 were considered to be germline mutations since the mutations were present in both tumor and matched normal tissues.

To date, more than 21,500 *TP53* mutations have been reported in human cancer and documented in the *TP53* mutation database (IARC TP53 Mutation Database at http://www-p53.iarc.fr). Searching this database, we found that one of the germline *TP53* mutation (A704G; Asn235Ser) identified in one of the prostate tumor is a common germline *TP53* mutation described in patients with breast cancer (Cornelis, et al., 1997). However, the other germline *TP53* mutations (A408T; Gln136His) and two somatic *TP53* mutations (T1022G; Phe341Cys and 22 bp duplication mutation) have not been reported before in any other cancers, suggesting that these mutations are novel and probably unique to prostate cancer.

CHEK2 and *TP53* mutations are mutually exclusive in prostate tumors and cell lines

We previously reported 11 *CHEK2* mutations in the same group of prostate tumor specimens (Dong, et al., 2003). Interestingly, examination of the distribution of *CHEK2* and *TP53* mutations in the 84 tumors revealed that the *CHEK2* and *TP53* mutations were mutually exclusive. Only one case showed concomitant *CHEK2* and *TP53* mutation. This is the only case with a higher Gleason score (5+5) in this group and a higher tumor stage (T3b). Our data thus suggest that at least 25% (21/84) of primary prostate tumors harbors mutations in either *CHEK2* or *TP53* and the mutations in these two genes can substitute each other in prostate cancer.

Further evidence for mutually exclusive *CHEK2* and *TP53* mutations in prostate cancer is provided by nonoverlapping nature of *CHEK2* and *TP53* mutations in prostate cancer cell lines. We screened the two genes for mutations in 5 commonly used prostate cancer cell lines by direct sequencing all 14 coding exons of *CHEK2* and exons 2-11 of *TP53*. Not surprisingly, we found that *CHEK2* were not mutated in 4 prostate cancer cell lines that carry *TP53* mutations while mutated in LNCaP cells (Fig. 1C), the only cell line that carries a wild-type *TP53* (Table 1). Mutually exclusive mutations of *CHEK2* and *TP53* have been reported in patients with Li-Fraumeni syndrome (LFS) and also in patients with breast cancer (Staalesen, et al., 2004). Moreover, the particular *CHEK2* 1100delC mutation in breast cancer patients is mutually exclusive with mutations in the other two DNA damage-response genes, *BRCA1* or *BRCA2* (Meijers-Heijboer, et al., 2002). Our results from the analysis of *CHEK2* and *TP53* mutations in both primary prostate tumors and cell lines provide further evidence that loss of function in *CHEK2* might be functionally equivalent to *TP53* mutation in prostate and mutation in one of the key DNA damage-signaling pathway genes might be sufficient to promote tumorigenesis in prostate cancer.

The novel *TP53* or *CHEK2* mutations identified in prostate cancer are deleterious

We identified one novel *CHEK2* mutation and three novel *TP53* mutations in prostate tumor specimens and cancer cell lines. The *CHEK2* mutation identified in LNCaP cells is a C1160A transversion mutation which changes Thr to Asn at amino acid 387 (Thr387Asn). Several studies have shown that the Thr387 is critical for autophosphorylation of CHEK2 in response to DNA damage (Julie K. Schwarz, 2003). Mutation at this residue impairs the autophosphorylation and reduces the kinase activity of CHEK2 in response to DNA damage.

The majority of somatic *TP53* mutations identified in our prostate tumor specimens are previously described in either prostate cancer or other cancers. However, the 22 bp duplication mutation in exon 4 of *TP53* has never been reported in any cancer. This mutation duplicates 22 bp in the splice junction site including 9 bp in intron 3 and 13 bp in exon 4 (Table 1, Fig. 1A, 1B). This mutation is predicted to result in either aberrant alternative splicing or 22 bp insertion in exon 4. Either event will lead to a frameshift of TP53 open reading frame and create a premature stop termination at codon 42 resulting in the deletion of DNA binding domain and the tetramerization domain. This mutation was confirmed at both DNA and RNA levels by direct sequencing of several clones generated by TA cloning of the genomic PCR products and the RT-PCR products covering exons 2-5 (Fig. 1A, 1B). In addition, the mutant RNA was found to be more stable than wild-type *TP53* in the tumor (data not shown) and it probably plays a dominant negative effect for *TP53* in this tumor.

The two germline *TP53* mutations (A408T; Gln136His and A704G; Asn235Ser) identified in our prostate tumor specimens are within the DNA binding domain. To assess the functional importance of these two mutations, we compared the two amino acid residues within 20 TP53 protein sequences from rat down to fish and found that these two residues are conserved during evolution (Choisy-Rossi, et al., 1999). Replacement of these conserved amino acids in the DNA binding domain of TP53 may interfere with the binding ability of TP53 to its target genes (Cho, et al., 1994). These data suggest that the novel *TP53* and *CHEK2* mutations identified in prostate cancer are deleterious and are probably the cause of neoplastic transformation in these tumors.

Germline *TP53* mutations in prostate cancer have never been reported except in a few family members of the LFS families. We identified two germline mutations in two prostate tumor specimens from patients without LFS phenotype. Although we do not have blood DNA from the two patients for the confirmation of the nature of these two mutations, they were consistently detected in several tissue spots in their matched normal tissues obtained by LCM (data not shown). It will certainly be necessary to screen for germline *TP53* mutations in blood samples from patients with familial or sporadic prostate cancer to determine whether germline mutations in *TP53* play any roles in prostate cancer susceptibility.

DNA damage-response pathway has been suggested as a necessary molecular barrier for prevention of neoplastic transformation in human tissues (Bartkova, et al., 2005). The disruption of this pathway leading to tumorigenesis has been implicated in many human cancers shown by high frequency (>50%) of somatic *TP53* mutations. However, the mutation frequency of *TP53* in prostate cancer is rather low. We detected only 13% in our unselected prostate tumor specimens. Even though the combined mutation frequency of both *CHEK2* and *TP53* mutations reaches to 25% in our sample set, it is still much lower than that of *TP53* mutation alone reported in many other cancers. It is thus very likely that other DNA damage-response genes might be the mutation targets in prostate cancer. A systematic search for such mutations in other DNA damage-response genes may improve our understanding of the etiology of this disease and provide tools for potential identification of men at increased risk of developing the disease or for prediction of patient outcome in which prevention strategies or better disease management strategies might be targeted.

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Table 1. *CHEK2* and *TP53* mutations identified in primary prostate tumor specimens and prostate cancer cell lines

	***CHEK2* mutation**	***TP53* mutation**
Tumor samples		
PC2		*A704G; Asn235Ser
PC4	*C541T; Arg181Cys	
PC13	*A967C; Thr323Pro	
PC16	*1100delC	
PC17		22bp insertion in exon 4
PC21	*1100delC	
PC22	A349G; Arg117Gly	
PC23		G550A; Asp184Asn
PC25		*A408T; Gln136His
PC36		T1022G; Phe341Cys
PC38		C535T; His179Tyr
PC41		A578G; His193Arg
PC42	G961A; Glu321Lys	C637T; Arg213Stop
PC47	*G715A; Glu239Lys	
PC51		C799T; Arg267Trp
PC67		T645A; Ser215Arg
PC68	*T470C; Ile157Thr	
PC70		G845A; Arg282Gln
PC71	*G190A; Glu64Lys	
PC77	*C1427A; Thr476Lys	
PC84	*1100delC	
Cell lines		
LNCaP	C1160A; Thr387Asn	
PC-3		414delC; frameshift
DU145		C668T; Pro223Leu
22Rv1b		A992G; Gln331Arg
NCI-H660		Deletion exon 9-11

* Germline mutations

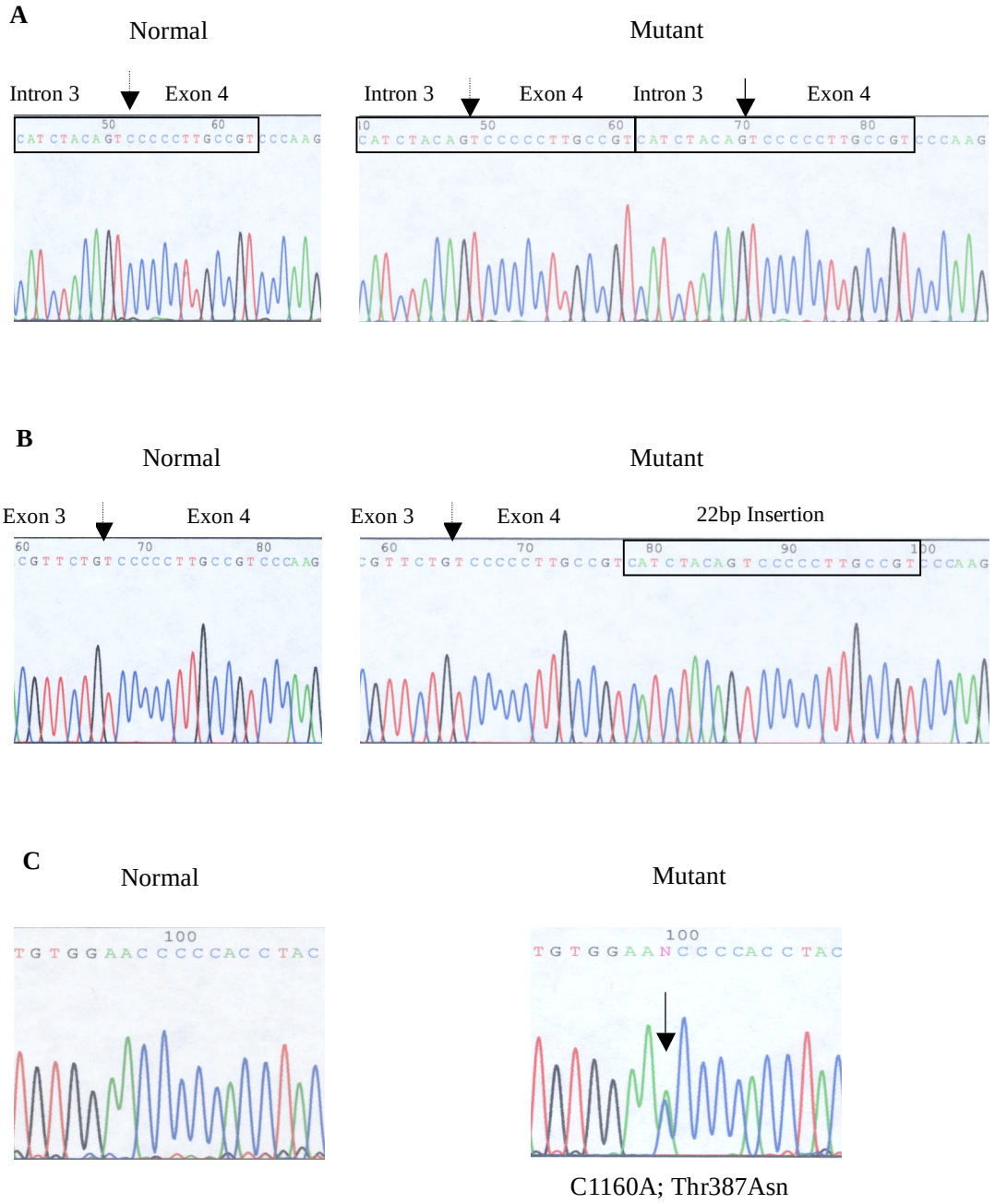

Figure 1. Novel *CHEK2* and *TP53* mutations identified in prostate tumors and cancer cell lines. **A, B,** Sequence analysis shows the 22bp duplication mutation in one prostate tumor: 22 bp duplication mutation (in panel) is shown in genomic sequence covering 9 bp in intron 3 and 13 bp in exon 4 (A) or 22 bp insertion in cDNA sequence (B). Arrows indicate the splice sites. **C,** *CHEK2* mutation (C1160A; Thr387Asn) found in LNCaP cell line. Arrow indicates the mutation site.