Short Communication

Identification of a Novel Gene with Increasing Rate of Suppression in High Grade Prostate Cancers

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Prostate cancer is the second leading cause of cancer-related deaths in the United States. However, the underlying molecular events for prostate cancer development are not clear. In this study, we applied the recently developed technology known as differential subtraction chain (DSC) to identify a novel gene whose expression is inactivated in high grade prostate cancer. This gene, designated as *SAPC*, is expressed in normal prostate acinar cells. Its expression is dramatically down-regulated in high grade prostate cancers (4/4) but is unaltered in low grade prostate cancers. It encodes a 7.7-kd protein. Its sequence shares some homology with the cysteine-rich domain of 2–5A-dependent RNase L, which is a critical component of the interferon-induced apoptosis cascade. The selective inactivation in the more aggressive prostate cancers holds promise for *SAPC* as a potential prognostic marker for high grade prostate cancer. (*Am J Pathol* 2001, 158:19–24)

Prostate cancer is one of the most frequently diagnosed malignancies in American men. Approximately 40,000 people die from this disease annually.¹ Despite the recent advances in our understanding of the environmental, hormonal, and nutritional etiologies of prostate cancer, much remains to be learned about the pathogenesis of prostate cancer.

The prostate gland is an organ with a unique predilection for abnormal growth along with advancing age. Although the incidence of prostate cancer is frequent, occurring in almost one-third of men over age 45,^{2,3} the proportion of cases reaching the stages with clinical symptoms varies widely among different areas of the world.^{4,5} There is strong evidence for both genetic and environmental factors in prostate cancer development.² However, it is not clear what molecular events are re-

sponsible for the progression of prostate cancer from a relatively indolent disease to one that could be life-threatening.

Recently we developed a new technology, termed differential subtraction chain (DSC), that detects the differences of genomic DNA or mRNA expression between two types of tissues.⁶ In comparison with differential display, DSC has the advantage of evaluating all species of mRNA in a single trial. With this technique, there is no need to handle radio-isotope, run sequencing gels, or excise manual gels for DNA fragment isolation. We applied this technology to prostate cancer and have identified expression sequences whose expressions were down-regulated in prostate cancer. One of these genes, designated *SAPC* (suppressed in aggressive prostate cancer), was found to have its expression limited to the epithelium of the normal prostate gland, but inactivated in prostate cancer with high Gleason score at advanced clinical stages.

Materials and Methods

Full Length cDNA Synthesis

Fresh surgical specimen of prostate cancer tissue was macrodissected to procure a tumor nodule and was confirmed to be free of normal prostate glandular tissue by examining multiple levels of frozen sections. Epithelial cells were separated from stromal cells by Percoll gradient centrifugation. Total RNA was extracted from epithelial cells with the Trizol method (Gibco-BRL, Rockville, MD). The extraction procedure was performed according to manufacturer's recommendation. Oligo-d(T) (TTTTTTTTTTTTTTTTTTTTTTTTT) and a switching primer *Bgl*IIaG (AGGCAACTGTGCTATCCGAGG-GAAGGG) were used in the first strand cDNA synthesis with 1 µg of total RNA and Superscript II (Gibco-BRL).

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For cDNA synthesis of normal prostate tissue, a pool of total RNA from prostate tissue of 8 normal organ donors was used. This was followed by polymerase chain reaction (PCR) of 94°C for 1 minute, then 94°C for 30 seconds, 56°C for 1 minute, and 72°C for 4 minutes for 26 to 30 cycles to amplify the full length cDNA fragments.

cDNA Amplicon Generation

One microgram of cDNA from tumor or normal prostate tissue was digested with 10 units of DpnII at 37°C for 6 hours. The digestion mixture was purified by QIAquick PCR purification kit (Qiagen, Valencia, CA), and ligated with adapter sequences *Bam*la/Ib (for normal prostate) (*Bam*la, ATGAAGTGCACCCTACGATTCGAG, *Bam*lb, pGATCCTCGAATCGTAGTGGGCACT) and *Bam*IIa/IIb (for tumor prostate) (*Bam*IIa, ATGAGACATGTTTCGTAGCCTAGG, *Bam*IIb, pGATCCCTAGG CTACG AAACATGTC) by T4 DNA ligase (New England Biolabs, Beverly, MA) at 25°C for 6 to 16 hours. The ligation mixture was purified and amplified by PCR (94°C for 1 minute, then 94°C for 30 seconds and 68°C for 3 minutes for 30 cycles).

cDNA DSC

Five hundred nanograms of normal prostate cDNA amplicons were mixed with 10 µg of prostate cancer cDNA amplicons, which had been digested with *Dpn*II to remove the attached adapter sequences, in a total volume of 8 µl. The mixture was then heated to 98°C for 3 minutes, and 2 µl of 5 mol/L NaCl were added to the reaction to give the final concentration of 1 mol/L while maintaining the temperature at 98°C. The mixture was then incubated at 98°C for an additional 2 minutes and hybridized at 67°C for 20 hours. The hybridization mixture was purified by sodium acetate/ethanol precipitation. The pellet was then washed with 70% ethanol. The dry pellet of the hybridization mixture was reconstituted in 50 µl of 1× Mung bean nuclease buffer and digested with 10 units of Mung bean nuclease at 30°C for 30 minutes. The digestion products were treated with 0.5 µl of 10% sodium dodecyl sulfate (SDS) and purified with ethanol precipitation. The DNA was resuspended in 9 µl of 3× EE (3 mmol/L EDTA, 3mmol/L EPPS, pH 8.0) buffer. One microliter was removed for PCR (quality control). The remainder of the sample was reheated to 98°C for rehybridization. This procedure was repeated twice.

DSC Product Cloning, Colony Screening, and Sequencing

Round 3 DSC products were amplified by PCR (94°C for 1 minute, then 35 cycles of 94°C for 30 seconds, 68°C for 3 minutes). The amplified products were visualized by agarose gel electrophoresis. An aliquot of the amplified products was ligated with TOPO TA cloning vector (Invitrogen, Carlsbad, CA) and transfected into *Escherichia coli*. Fifteen colonies were randomly picked and grown

overnight in LB broth with 100 µg/ml of ampicillin. Aliquots of the bacterial culture were spotted onto colony screen filter and hybridized with amplicons generated from normal or tumor prostate tissues. To identify which DSC fragment was not present in the tumor specimen, only those colonies without hybridization signal for tumor amplicons but positive for normal amplicons were selected. DNA was extracted from the selected colonies and sequenced using M13 forward and reverse primers.

Obtaining Full Length cDNA

Rapid amplification of cDNA end (RACE) was used. 5' and 3' ends of *SAPC*, a gene fragment identified in the previous procedure, were amplified with pairs of gene-specific primers and adapter/primers (gene-specific sense primer/AP1, gene-specific anti-sense primer/AP2) in PCR (94°C for 1 minute, then 35 cycles of 94°C for 30 seconds, 68°C for 4 minutes.) from Marathon cDNA library of normal prostate tissue (Clontech, Palo Alto, CA). The RACE products were visualized by agarose electrophoresis. An aliquot of the RACE products was ligated to TOPO TA cloning vector. Two hundred colonies were randomly picked and cultured in Luria-Bertani broth. The overnight cultures were spotted on genescreen filter and hybridized with ³²P-labeled *SAPC* DNA fragments. DNA was extracted from colonies that showed positive reactions and sequenced.

In Situ RNA Hybridization

5' end rhodamine-labeled 24-mer oligonucleotides corresponding to anti-sense or sense sequence (control) of *SAPC* were incubated with pronase digested, prehybridized, 3-µm-thin sections of prostate tissue from polyester-embedded tissue blocks at 37°C for 12 hours in hybridization solution. The slides were then washed twice with 2× standard saline citrate for 15 minutes, and then twice with 1× standard saline citrate for 15 minutes.⁷

In Vitro Transcription and Translation

A 1.5-kb PCR product of *SAPC* was ligated to pCR2 vector. One microgram of the purified DNA from the selected colonies was used in a TNT Couple Wheat Germ Extract system (Promega, Madison, WI) for *in vitro* transcription and translation assay. The procedure was performed according to the manufacturer's instruction.

Results

To identify gene expression inactivation in prostate cancer, prostate cancer cell cDNA amplicons were used as drivers in a DSC reaction to subtract the counterparts from a pool of normal prostates. After 3 rounds of DSC as shown in Figure 1A, a distinct banding pattern was obtained. These DSC products were subcloned into TOPO TA cloning vector. Fifteen colonies were randomly picked and hybridized with amplicons from tumors and normal

Figure 1. Identification of *SAPC* through differential subtraction chain. **A:** Agarose electrophoresis of PCR amplified DSC products after rounds 0 (lane 1), 1 (lane 2), 2 (lane 3), and 3 (lane 4). **B:** PCR on cDNA amplicons generated from normal prostate tissues (lane 1) and prostate cancer (lane 2) with primers specific for *SAPC*. The primer sequences are AAAACTCTGGAT-TGCCGACTCTGC and CCATTAGGCAAGTCAAAGCATTTTC. **C:** RT-PCR on total RNA isolated from normal prostate and prostate cancer using primers specific for *SAPC* as for **B**. Primers specific for ubiquitin were used as control. The primer sequences are GACGCAAACATGCAGATCTTTGTG and AAT-GAAAGGGACACTTTATTGAGG. **D:** Northern blot analysis of *SAPC* expression on total RNA isolated from normal prostate tissues (lanes 1 and 3) and prostate cancer (lanes 2 and 4). Random primed ^{32}P labeled *SAPC* fragment was used as a probe.

prostate tissues. The DNA inserts from those colonies that gave positive signals in hybridization with amplicons from normal cells but negative from cancer cells were interpreted as specific for normal prostate; thus, expression was inactivated in prostate cancer. From those colonies that contain inserts specific for normal prostate tissues, the DNA fragments were extracted and sequenced. Most of the DSC cDNA fragments obtained

through this procedure represent previously unknown sequences. Three of the 15 colonies were determined to contain the same sequence. This sequence, designated *SAPC*, was used as a probe to hybridize with total RNA from normal and tumor samples that were used to produce cDNA amplicons. As shown in Figure 1D, a band corresponding to a 1.6-kb mRNA is identified in the normal prostate tissue in a Northern blot analyses. To further confirm the specificity of this DNA fragment, PCR was performed on amplicons generated from prostate cancer or normal prostate tissues. No PCR product was amplified from the tumor sample. In contrast, a 120-bp DNA fragment was identified in the normal prostate tissue (Figure 1B). Similar results were also obtained in RT-PCR (Figure 1C), confirming the inactivation of expression of this cDNA fragment in prostate cancer.

A survey study was performed to investigate whether inactivation of this cDNA fragment also occurs in other prostate cancer cases. A panel of 16 prostate cancer cases of various clinical stages and Gleason scores was tested by reverse transcriptase (RT) PCR to examine the expression status of *SAPC* (Table 1). As shown in Figure 2, 3 of 16 prostate cancer cases from frozen tissues had the inactivation of expression of this gene. Interestingly, the clinical stage, Gleason score and seminal vesical involvement of two of these prostate cancer cases were similar to the case where *SAPC* was originally identified, whereas the third case presented with a distant metastasis. All four cases where *SAPC* expression was inactivated shared the features of higher Gleason scores,^{8,9} extensive seminal vesicle involvement and multiple foci of capsular penetration. In three cases, there were metastases of prostate cancer. These features generally characterized more aggressive prostate cancer. In contrast, the 13 other cases of prostate cancer that were positive for *SAPC* expression were characterized with lower Glea-

Table 1. Expression of *SAPC* in Various Stages and Grades of Prostatic Adenocarcinoma

Case no.	Gleason score*	Capsular invasion	Seminal vesicle invasion	Metastasis status	Clinical stage	<i>SAPC</i> /UB ratio
1	4 + 5 = 9	Multifocal	Extensive	Lymph nodes	T3b	0.02
2	3 + 3 = 6	—	—	—	T1c	0.72
3	3 + 4 = 7	—	—	—	T1c	0.65
4	3 + 4 = 7	—	—	—	T2b	0.58
5	3 + 4 = 7	Focal	—	—	T3a	1.09
6	3 + 4 = 7	Multifocal	—	—	T3a	1.32
7	4 + 4 = 8	N/A	N/A	Liver	T4	0.02
8	3 + 3 = 6	Focal	—	—	T3a	0.56
9	3 + 3 = 6	—	—	—	T1c	0.49
10	3 + 2 = 5	—	—	—	T2a	0.61
11	3 + 3 = 6	—	—	—	T1c	0.69
12†	2 + 5 = 7	Focal	—	—	T3a	0.58
13	3 + 4 = 7	Multifocal	—	—	T3a	0.68
14	3 + 3 = 6	Focal	—	—	T3a	0.59
15	3 + 4 = 7	—	—	—	T3a	0.52
16	5 + 3 = 8	Focal	Extensive	SLL	T3b	0.05
17‡	4 + 4 = 8	Multifocal	Extensive	Lymph nodes	T3b	0.01

*Cells from dominant pattern account for over 70% of the microdissected samples or otherwise specified.

†Gleason 2 accounts for 95% of the sample.

‡The case where DSC was performed.

SLL, small lymphocytic lymphoma; *SAPC*/UB ratio, *SAPC*/ubiquitin expression ratio; N/A, prostatectomy is not applicable.

Figure 2. Expression of *SAPC* in prostate cancer. **A:** Agarose electrophoresis of RT-PCR products generated from 1 μ g total RNA obtained from microdissected prostate cancer cells (**middle panel**) and paired normal prostate tissues (**upper panel**) with primers specific for *SAPC*. Sixteen cases of prostate cancer were shown from case #1 (**lane 1**) through #16 (**lane 15**). **Lower panel** represents RT-PCR products of ubiquitin from the same tumor RNA samples of the middle panel. PCR was performed under the following condition: 94°C for 1 minute, then 26 cycles of 94°C for 30 seconds and 68°C for 3 minutes. **B:** Hematoxylin and eosin (H&E) stains and RNA *in situ* hybridization with *SAPC* on normal prostate tissue. H&E stain (**upper**) and RNA *in situ* hybridization of *SAPC* (**lower**) on normal prostate tissue. **C:** H&E stain (**upper**) and RNA *in situ* hybridization with *SAPC* (**lower**) on an intermediate grade prostate cancer with a Gleason score of 3 + 3 = 6. **D:** H&E stains and RNA *in situ* hybridization with *SAPC* on a high grade prostate cancer with a Gleason score of 4 + 4 = 8.

Figure 3. Expression of *SAPC* in 24 different types of human organs. RT-PCR products of *SAPC* were produced from total RNA of 24 organ tissues with primers specific for *SAPC*. The specific organs are indicated under each lane. PCR was performed for 35 cycles. Positive controls with primers specific for actin were performed (data not shown).

son score,⁵⁻⁷ no seminal vesicle involvement, negative lymph node metastasis, and few with capsular penetration.

To identify cells or structure within the prostate glands that are expressing *SAPC*, *in situ* hybridization with oligonucleotides corresponding to *SAPC* coding region as probes was performed on normal prostate tissue. As shown in Figure 2B, the expression of *SAPC* mRNA was restricted to prostate acinar cells, with the strongest expression occurring at the top layer of the epithelium. In contrast, the expression of *SAPC* was strongly suppressed in a high grade prostate cancer (Gleason score 4 + 4 = 8; Figure 2D), whereas the expression of *SAPC* was essentially unchanged in an intermediate grade prostate cancer (Figure 2C). To investigate whether the expression of *SAPC* is specific for prostate tissue, RT-PCR was performed on the tissues of 24 different human organs. As demonstrated in Figure 3, in addition to prostate, the expression of *SAPC* was also identified in placenta, kidney, lung, adrenal gland, spleen, and heart.

To obtain the full length *SAPC* cDNA from normal prostate tissue, RACE with gene-specific primers derived from *SAPC* sequences was performed. A 1.6-kb cDNA fragment was obtained. As shown in Figure 4A, this DNA fragment contains an open reading frame that encodes a 64-amino acid protein. A homology search of this protein indicates that it shares weak (60% positive) homology with the cysteine-rich domain of 2-5A-dependent RNase L (Figure 4B). To determine the orientation of *SAPC* cDNA and to rule out the potential artifacts generated during the library construction process, reverse transcriptions were performed using primers corresponding to the putative sense and anti-sense orientation of *SAPC*, respectively. Subsequently, PCRs were performed to detect the presence of reverse transcription products. As shown in Fig-

Figure 4. Open reading frame of *SAPC* and its homology with 2-5A-dependent RNase L. **A:** Nucleotide sequence and the correspondent amino acid sequence of *SAPC*. **B:** Amino acid sequence homology of *SAPC* with cysteine-rich domain of 2-5A-dependent RNase L. **C:** Identifying the orientation of *SAPC* cDNA. One microgram of total RNA from normal prostate tissues was reverse transcribed with a primer (CCATTAGGCAAGTCAAGCATTTTC, lane 1) corresponding to the putative sense orientation of *SAPC* DNA fragment and a primer (AAAACCTCTGGATTGCCGACTCTGC, lane 2) corresponding to the putative anti-sense, respectively. The *SAPC* DNA was subsequently amplified with primers corresponding to the downstream *SAPC* DNA (GCATTTCTCTAGAACTGCGCTGAGGCGAG/GATTGCCGACTCTGCATCCTGGTTCC). **D:** *In vitro* transcription and *in vitro* translation of *SAPC* gene product. Vectors pCR2.1 containing full length *SAPC* cDNA fragment (lane 2) or containing no insert (lane 1) were *in vitro* transcribed and translated with ³⁵S-methionine. The translation products were separated with SDS-PAGE.

ure 4C, RT-PCR with the primer of the putative anti-sense direction (lane 2) generated a *SAPC* PCR product, whereas the sense primer (lane 1) did not. This confirms the orientation of *SAPC* mRNA, where the 64-amino acid open reading frame is located. To test whether this open reading frame is indeed functioning, *in vitro* transcription and translation with a plasmid containing the full length cDNA of *SAPC* were performed. A 7.7-kd protein was identified in a SDS-PAGE (Figure 4D), consistent with the predicted molecular weight of the *SAPC* gene product.

Discussion

Currently, histological classification of prostate cancer, specifically Gleason grading and pathological staging, is the mainstay of prognostic assessment once a cancer is verified by tissue sampling. However, to produce an accurate Gleason score and staging, extensive tissue sampling is required. This may limit the usefulness of histological classification when the amount of diagnostic tissue is small, such as when needle core biopsy samples are used. Molecular markers with prognostic significance may be of great value in assessing the invasive-

ness of a tumor. Suppression of *SAPC* expression in cancer cells correlates well (4/4) with the metastatic and aggressive behavior of prostate cancers. Examining the expression of *SAPC* in prostate cancer tissues may help to predict the stages of the disease. It will be of interest to see whether change in expression status of *SAPC* occurs in the early stages of aggressive prostate cancers.

Although the functional role of *SAPC* has not been established, there are some probabilities that *SAPC* may interact with 2–5A-dependent RNase L *in vivo*. First, *SAPC* is expressed mostly in terminally differentiated cells, as evidenced by the most intense staining in the top layer of epithelial cells of prostate glands. Its expression is dramatically decreased when cells are stimulated with serum growth factors (data not shown). Second, *SAPC* contains weak but distinctive homology with the cysteine-rich region of 2–5A-dependent RNase L, which is thought to be essential for dimerization and for protein-protein interaction.⁸ It has been shown that expression and activation of 2–5A-dependent RNase L is required for cells undergoing apoptosis and differentiation.^{9–11} Decreased expression of 2–5A-dependent RNase L is found in proliferating cells.¹² The regulation of cell cycle by 2–5A-dependent RNase L is thought to occur through regulating the overall level of RNA.¹³ It is tempting to hypothesize that *SAPC* regulates the cell cycle through its interaction with 2–5A-dependent RNase L. However, the direct evidence of physical interaction between *SAPC* and 2–5A-dependent RNase L, and the effect of *SAPC* on RNA level must await the ongoing complex formation study between *SAPC* and 2–5A-dependent RNase L and the *SAPC* functional study.

The significance of suppression of *SAPC* expression is not clear. However, in view of the fact that most of its expression inactivation occurs exclusively in high Gleason score prostate cancer, it is reasonable to postulate that the suppression of *SAPC* expression represents a distinctive genetic event in the prostate cancer development that underlies a more aggressive behavior. Expression of *SAPC* is restricted to several organs. *In situ* hybridization suggests that its expression is present in prostate acinar cells, in certain area of proximal tubules of the kidney and the lobules of the breast (data not shown). The predominant expression of *SAPC* in mature and differentiated epithelium is consistent with the hypothesis that expression of *SAPC* is associated with terminal cell differentiation and possibly programmed cell

death. A correlation between *SAPC* expression, differentiation, and/or apoptosis will help to address the hypothesis.

In conclusion, using DSC methodology, we have identified a novel gene whose expression is frequently inactivated in high grade prostate carcinoma and may represent an important component in cell differentiation and apoptosis. It is possible that suppression of *SAPC* expression in prostate cancer can be used as a prognostic factor to evaluate the potential behavior of a prostate cancer.

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