

## **THE MORAN FOUNDATION**

### **PROGRESS REPORT FOR FINANCIAL SUPPORT 2004-2005**

**TITLE: Determination of the activity of CDC25C and its biologically active spliced variant as prognostic tools for prostate cancer.**

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#### **SUMMARY:**

Alterations in cell cycle progression, controlled by the sequentially activities of cyclin dependent kinases, have been implemented in a wide variety of malignant neoplasms, including prostate cancer. CDC25 phosphatases belong to the tyrosine phosphatase family and play a critical role in regulating cell cycle progression by dephosphorylating cyclin dependent kinases at inhibitory residues. CDC25C plays an important role in the G2/M transition by activating Cdc2/Cyclin B1 complexes. To determine whether CDC25C activity is altered in prostate cancer, we have examined the expression of CDC25C and its alternatively spliced variant in human prostate cancer. CDC25C protein is upregulated in prostate cancer in comparison to normal prostate tissue and is present almost exclusively in its active dephosphorylated form. Expression of a biologically active alternatively spliced CDC25C isoform is also increased in prostate cancer. By expression of dominant negative fibroblast growth factor receptors (FGFR), we have found that loss of fibroblast growth factor signaling decreases CDC25C activity in prostate cancer by multiple mechanisms, implying that the increased CDC25C activity in prostate cancer is linked to increased FGF signaling in this malignancy. A better

understanding of the mechanism by which FGF signaling regulates cell cycle progression will facilitate the development of cancer therapies that target FGF signaling pathway.

This project has been completed and the results are in press in *Clinical Cancer Research*. Please refer to the attached manuscript for details.

## Increased Expression and Activity of CDC25C Phosphatase and an Alternatively Spliced Variant in Prostate Cancer

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**Abstract** Alterations in the control of cell cycle progression have been implicated in a wide variety of malignant neoplasms, including prostate cancer. CDC25 phosphatases belong to the tyrosine phosphatase family and play a critical role in regulating cell cycle progression by dephosphorylating cyclin-dependent kinases at inhibitory residues. CDC25C plays an important role in the G<sub>2</sub>-M transition by activating Cdc2/Cyclin B1 complexes. To determine whether CDC25C activity is altered in prostate cancer, we have examined the expression of CDC25C and an alternatively spliced variant in human prostate cancer samples and cell lines. CDC25C protein is up-regulated in prostate cancer in comparison with normal prostate tissue and is present almost exclusively in its active dephosphorylated form. Expression of a biologically active alternatively spliced CDC25C isoform is also increased in prostate cancer and expression of alternatively spliced CDC25C is correlated to occurrence of biochemical (prostate-specific antigen) recurrence. We have also developed a quantitative reverse transcriptase-PCR analysis of Ki-67 expression as a method of measuring proliferative activity in prostate cancer from RNA samples. Based on this analysis of Ki67 expression, some but not all of this increase in CDC25C and its alternatively spliced variants is correlated with increased proliferation in prostate cancer. This data suggests that CDC25C might play an important role in prostate cancer progression and could be used to monitor and predict the aggressiveness of this disease.

Abnormal expression and/or activity of cell cycle regulatory proteins have been identified in a wide variety of malignant neoplasms, including prostate cancer. Cell cycle progression is controlled by the sequential activities of cyclin-dependent kinases, whose activities are tightly regulated by cyclins, cyclin-dependent kinase inhibitors, and a variety of other proteins. Several groups have shown increased expression of cyclin B1, which plays a critical role in the G<sub>2</sub>-M transition, in human prostate cancers (1, 2). Recent work by Maddison et al. (3) has shown increased levels of cyclin B1 in poorly differentiated and androgen-independent prostate cancers in the TRAMP mouse model of prostate cancer. During G<sub>2</sub>, the Cdc2/Cyclin B complex is kept inactive by phosphorylation of Cdc2 by Wee1. At the onset of mitosis, Cdc2/Cyclin B complexes are dephosphorylated by CDC25 phosphatase

leading to increased kinase activity (4–6). Our laboratory has shown previously that disruption of fibroblast growth factor signaling in prostate cancer cells leads to decrease in Cdc2 kinase activity and arrest in G<sub>2</sub> followed by cell death (7). These findings imply that the G<sub>2</sub>-M transition may be a critical checkpoint in prostate cancer.

CDC25 phosphatases belong to the tyrosine phosphatase family and play a critical role in regulating cell cycle progression by dephosphorylating cyclin-dependent kinases at inhibitory residues. In human cells, CDC25 proteins are encoded by a multigene family, consisting of *CDC25A*, *CDC25B*, and *CDC25C* (8). In late G<sub>2</sub>, CDC25C dephosphorylates Cdc2 on both Thr<sup>14</sup> and Tyr<sup>15</sup>, leading to the activation of Cdc2/Cyclin B complexes (9–11) and progression through the G<sub>2</sub>-M checkpoint. Phosphorylation of Ser<sup>216</sup> of CDC25C throughout interphase and upon G<sub>2</sub> checkpoint activation has been found to negatively regulate the activity of CDC25C by cytoplasmic sequestration (12, 13) and a positive feedback loop has been proposed between Cdc2 and CDC25C (11, 13–15). Activated Chk kinases can phosphorylate CDC25C at Ser<sup>216</sup>, blocking the activation of Cdc2 and transition into the M phase (16). Another aspect of CDC25 regulation is alternative splicing that may produce at least five CDC25B variants (17), and splice variants are also reported for CDC25A and CDC25C (18, 19). The activity and regulation of CDC25C in prostate carcinoma has not been previously examined, despite its potentially important role in the G<sub>2</sub>-M transition in this common malignancy.

To determine whether CDC25C plays a role in prostate cancer, we have examined the expression of CDC25C and an alternatively spliced variant in human prostate cancer samples

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Received 12/10/04; revised 3/18/05; accepted 4/1/05.

**Grant support:** Department of Defense Prostate Cancer Research Program grant DAMD17-03-1-0006 (M. Ozen), Department of Veterans Affairs Merit Review funding (M. Ittmann), National Cancer Institute grant P50CA058204 (Baylor prostate cancer Specialized Programs of Research Excellence program), and Moran Foundation award.

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and cell lines at both the protein and RNA levels. CDC25C protein is up-regulated in comparison with normal prostate tissue and is present predominantly in its active dephosphorylated form. At the transcriptional level, CDC25C and an alternatively spliced variants were both overexpressed in prostate cancer. The expression of the spliced variants were correlated with biochemical recurrence.

## Materials and Methods

**Tissue acquisition and extraction.** Normal peripheral zone, hyperplastic transition zone (benign prostatic hyperplasia), and cancer tissues were collected from men undergoing radical prostatectomy for clinically localized prostate cancer by Baylor prostate cancer Specialized Programs of Research Excellence Tissue Core and snap frozen. Benign tissues were confirmed to be free of cancer and cancer tissues contained at least 70% carcinoma. RNAs were extracted from 17 normal peripheral zone tissues, seven benign prostatic hyperplasia tissues, and 58 prostate cancers using TRIzol Reagent (Invitrogen, Carlsbad, CA) as described in the manufacturer's protocol. We analyzed 20 cancers with no evidence of prostate-specific antigen (PSA) recurrence after 5 years of follow-up, 19 cancers with delayed PSA recurrence (mean time to recurrence of 34.1 months), and 19 cancers with early recurrence (i.e., <1 year; mean time to recurrence of 4.5 months). PSA recurrence was defined as serum PSA of >0.2 ng/mL. Protein extracts were prepared as described previously (20) from 10 cancers and eight normal peripheral zone tissues.

**Reverse transcriptase-PCR and agarose gel electrophoresis.** RNAs extracted from the prostate tissues were first reverse transcribed as previously described (21) and analyzed for the presence of two different cDNAs for CDC25C using the following primers flanking the deletions of the CDC25C sequence: forward, 5'-AGAGAGAAGCITATGTCTACG-GAACTCTTCATCC-3'; reverse, 5'-CCCAAATATITTCATTCCTGTC-3' as described previously by Bureik et al. (19). The  $\beta$ -actin primers were as described previously (22). Thirty-five cycles with the following program were done: denaturation at 94°C for 1 minute, annealing at 60°C for 1.5 minutes, elongation at 72°C for 1 minute followed by 5 minutes extension at 72°C. The reaction was done with the Takara kit (Takara Bio, Inc., Otsu, Japan) following the manufacturer's protocol. The PCR products were analyzed on a 1.5% agarose gels and stained with ethidium bromide.

**Cell lines.** DU145, PC3, and LNCaP human prostate cancer cell lines were cultured in RPMI 1640 supplemented with 1% antibiotic and antimycotic (Invitrogen Life Technologies, Carlsbad, CA) and 10% fetal bovine serum.

**Quantitative real-time PCR and primer design.** Real-time reverse transcriptase-PCR (RT-PCR) was carried out in iCycler real-time thermal cycler (Bio-Rad, Hercules, CA) as described previously (22), incorporating the optimized PCR reaction conditions for each primer set. Oligonucleotide primers for CDC25C were carefully designed to cross exon/intron regions and to avoid self-complementarity or the formation of primer-dimers and hairpins. Two primer sets, one detecting only the full length CDC25C by binding in the region deleted in the alternatively spliced variant (forward, 5'-GCCACT-CAGCTTACCCTTC-3'; reverse, 5'-ATTTTCATTCCTGTCACCAAG-3') and the other detecting both spliced variant and the full-length CDC25C (forward, 5'-GACACCCAGAAGAGAATAATCATC-3'; reverse, 5'-CGACACCTCAGCAACTCAG-3') were used. Alternatively spliced transcript levels were calculated by subtraction of full-length CDC25C transcript levels from the total CDC25C levels. This approach allows quantitation of the major alternatively spliced isoforms detected in prostate cancer cells, specifically the C5 and C4 variants described by Wegener et al. (18). The primers for Ki67 were as follows: forward, 5'-ACGAGACGCCTGGTTACTA TC-3'; reverse, 5'-GCTCATCAATAACA-GACC CATTTAC-3'. The  $\beta$ -actin primers were as described previously (22). The threshold cycle ( $C_t$ ) values in log linear range representing

the detection threshold values was used for quantitation and expressed as copy numbers based on a standard curve generated using plasmid DNA.

**Western blot analysis.** The tissue samples were homogenized and lysed in lysis buffer (20) and cleared by centrifugation for 10 minutes in a microcentrifuge at 4°C. Protein concentration was determined using a Bio-Rad protein assay. The lysates were then boiled in sample buffer, centrifuged, and 30  $\mu$ g of supernatant protein subjected to SDS-PAGE electrophoresis using a 10% gel. The resolved proteins were electrotransferred to nitrocellulose membranes and then blocked with PBS with 0.5% Tween 20 (PBST) containing 5% fat-free milk. Western blot for CDC25C was done using 500 ng/mL of polyclonal anti-CDC25C antibody (C20, Santa Cruz Biotechnology, Santa Cruz, CA) and anti-phosphoCDC25C (Ser<sup>216</sup>) antibody (901, Cell Signaling Technology, Beverly, MA) at 4°C for 16 hours. The membranes were then washed with PBST and treated with appropriate secondary antibody. The antigen-antibody reaction was visualized using an enhanced chemiluminescence assay (Amersham, Arlington Heights, IL) and exposure to enhanced chemiluminescence film (Amersham). Control antibody was an anti- $\beta$ -actin monoclonal antibody (A5316, Sigma, St. Louis, MO) used at a 1:5,000 dilution. To determine the specificity of the bands observed in Western blots for CDC25, anti-CDC25C antibody was preincubated with 5-fold molar excess of the blocking peptide (sc-327 P, Santa Cruz Biotechnology) for 2 hours at room temperature before use in the Western blot protocol. For quantitative Western blotting studies, the intensities of the bands on the Western blots were quantified as densitometric units by using the GelExpert software package supplied with the Nucleovision gel imaging system (Nucleo Tech Corp., Hayward, CA).

## Results

**Activity of CDC25C protein in clinically localized human prostate cancers.** To evaluate the *in vivo* activity of CDC25C in prostate cancer tissues, we determined the levels of total and phosphorylated CDC25C protein in the lysates from normal prostate peripheral zone and prostate cancer tissue samples. Out of nine evaluable cancer samples, only two had readily detectable amounts of phospho-CDC25C (Fig. 1). In contrast, in the same blots normal prostate peripheral zone samples had detectable phospho-CDC25C protein in six of eight samples. Total CDC25C protein was detectable in six of nine cancer cases, whereas only two of eight normal tissue samples showed detectable total CDC25C when analyzed in the same blots (Fig. 1). Thus, in cancer tissues there is both markedly increased CDC25C protein and much less of its inactive phosphorylated form.

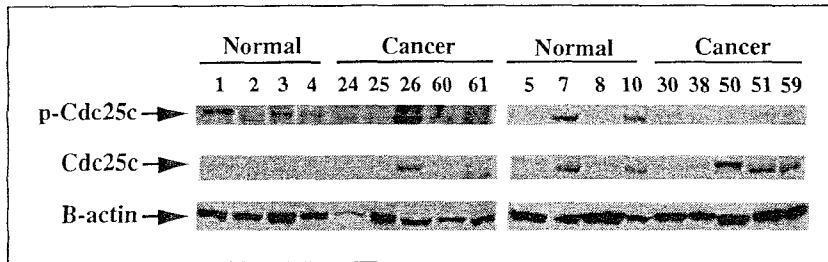
**Alternatively spliced CDC25C variant is detected in prostate cancer RNA and in prostate cancer cell lines.** In addition to the full-length CDC25C protein examined above, there are alternatively spliced of CDC25C transcripts that have been detected in a number of cancer cell lines (18, 19). The immunoglobulin heavy chain that is present in large amounts in some patient samples may interfere with direct measurement of the alternatively spliced proteins in clinical samples. We therefore analyzed RNAs from prostate cancer cell lines and a second set of clinically localized prostate cancers for the presence of the CDC25C alternatively spliced variants using RT-PCR and electrophoresis on agarose gels (Fig. 2A). The full-length wild-type (WT) and alternatively spliced CDC25C transcripts are present in both LNCaP and DU145 prostate cancer cell lines (Fig. 2A). The major alternatively spliced isoform corresponds to the C5 variant described by Wegener

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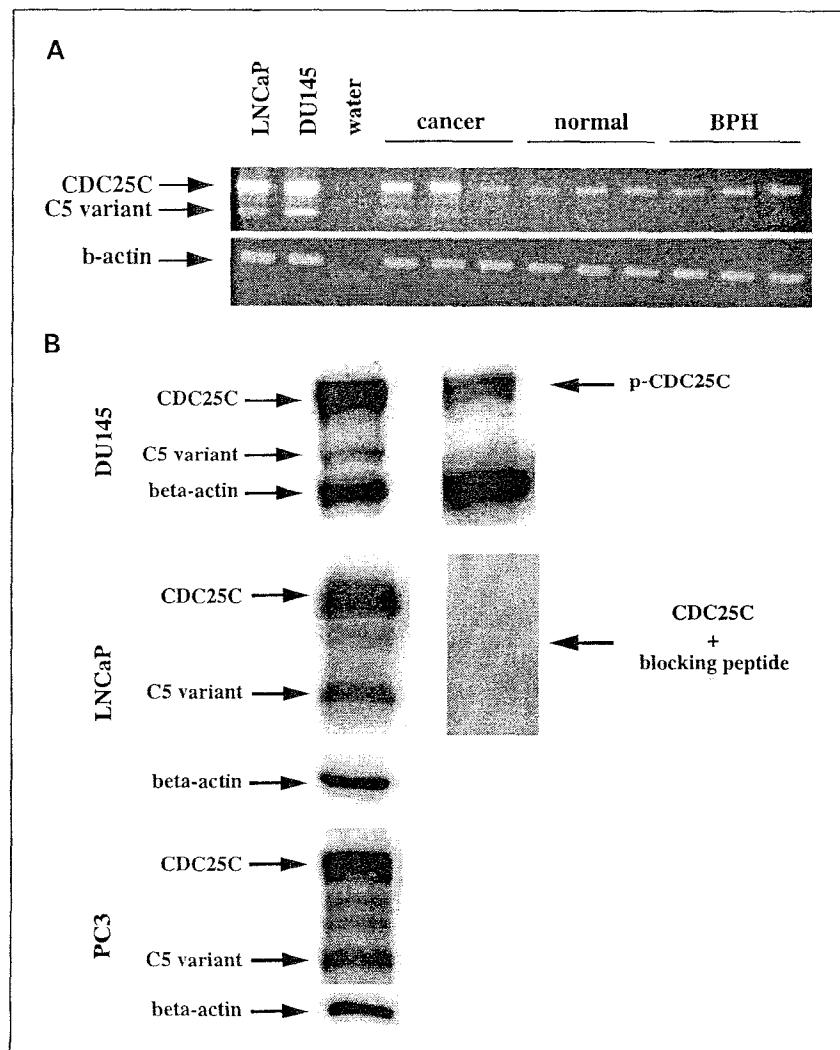
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**Fig. 1.** CDC25C protein levels in clinically localized human prostate cancers. Normal peripheral zone and cancer tissues were collected from men undergoing radical prostatectomy for clinically localized prostate cancer by Baylor prostate cancer Specialized Programs of Research Excellence Tissue Core and snap frozen. Protein lysates were prepared as described in Materials and Methods. Western blot for CDC25C was done using polyclonal anti-CDC25C antibody or a Ser<sup>216</sup> phosphorylation site specific antibody. An anti- $\beta$ -actin monoclonal antibody was used as loading control. The numbers representing samples are obtained from our clinical database. Sample 24 was not included in the analysis due to low  $\beta$ -actin signal, indicating inadequate protein in this lane.

et al. (18). Other variant transcripts, intermediate in size between the WT and C5 transcripts were present in lower amounts as well. The C5 CDC25C transcript was detectable by this methodology in 29 of 58 prostate cancer RNAs. In contrast, only 3 of 17 normal peripheral zone samples and none of the seven benign prostatic hyperplasia samples had detectable quantities of this variant. The difference between the cancer and benign samples was statistically significant ( $P = 0.002$ , Fisher

exact test). In addition, the presence of the variant was strongly associated with the occurrence of biochemical (PSA) recurrence. Overall, the variant was present in cancer samples from 23 of 38 patients with biochemical recurrence but only 6 of 20 without PSA recurrence. This difference was statistically significant ( $P = 0.027$ , Pearson's  $\chi^2$ ). Since PSA recurrence, particularly early PSA recurrence, is associated with aggressive disease and decreased patient survival, this observation implies



**Fig. 2.** Expression of full-length and alternatively spliced CDC25C mRNA and/or proteins in prostate tissues and prostate cancer cell lines. *A*, RNAs were extracted from normal peripheral zone tissues, benign prostatic hyperplasia (BPH) tissues, and prostate cancer tissue samples using TRIzol Reagent as described by the manufacturer's protocol and analyzed by RT-PCR as described in Materials and Methods. Bands corresponding to the full-length (512 bp) and variant mRNA (293 bp). Three samples each of cancer, normal, and benign prostatic hyperplasia groups. RT-PCR with  $\beta$ -actin primers was used as a control for cDNA quantity. *B*, protein lysates from prostate cancer cell lines are subjected to Western blot analysis using the appropriate antibodies as described in Materials and Methods. Preincubation with peptide immunogen for the CDC25C antibody was used to determine the specificity of the two CDC25C bands.

that expression of the variant mRNA is higher in aggressive prostate cancers. No statistically significant correlation of expression of the CDC25C variant with preoperative PSA or pathologic stage was detected. As illustrated in Fig. 2A, the cancer tissues also seemed to express increased amounts of WT mRNA in addition to expressing the variant mRNA, consistent with our observation of increased levels of WT CDC25C protein in the prostate cancer extracts.

We studied the presence of alternatively spliced CDC25C variant protein in prostate cancer cell lines to confirm its expression at the protein level (Fig. 2B). Both the WT and C5 variant are expressed in all three of the commonly used prostate cancer cell lines (DU145, LNCaP, and PC3). The specificity of the antibody for the WT and C5 variant was confirmed by preincubation of the anti-CDC25C antibody with excess peptide immunogen, which abolished both bands in a Western blot of LNCaP cell extract.

Other variant CDC25C proteins are also present in lower amounts, particularly in PC3 cells. To determine if the C5 variant is phosphorylated at the Ser<sup>216</sup> residue, which is associated with cytoplasmic sequestration and loss of biological activity, we analyzed Western blots of DU145 protein extracts with a Ser<sup>216</sup>-specific anti-phospho-CDC25C antibody. No phosphorylation at Ser<sup>216</sup> was detected in the C5 variant despite the readily detectable phosphorylation of the WT protein.

To assess the relationship between the level of WT and spliced variant proteins and mRNA levels, we carried out quantitative RT-PCR to detect WT or spliced variant mRNAs and did Western blots with serial dilutions of protein extracts from the same cells with anti-CDC25C antibody. We used both actively proliferating and confluent DU145 cells in these studies. As described in Materials and Methods, the quantitative RT-PCR assay used measures both the C4 and C5 mRNA variants (and potentially other variants) as alternatively spliced transcripts, although based on the gel electrophoresis in Fig. 2A, the C5 variant seems the dominant form. Western blots were scanned to quantitatively determine the band intensity of WT and alternatively spliced isoforms and the ratio of protein band intensity (as densitometric units) per  $\mu\text{g}$  protein to RNA copy number determined. For actively growing cells, this ratio was  $11.1 \times 10^{-3}$  for WT versus  $2.21 \times 10^{-3}$  for the spliced variant and for confluent cells this ratio was  $2.8 \times 10^{-3}$  for WT versus  $0.94 \times 10^{-3}$ . Thus, the WT transcript is associated with approximately three to five times more protein per transcript when compared with the alternative spliced transcript. Whether this is due to differences in translation efficiency or protein stability (or both) is not known.

To confirm our qualitative observations in the clinical with more rigorous quantitative data, we determined the expression levels of CDC25C WT and the spliced variants by real-time RT-PCR assay. Quantitative analysis of expression of WT CDC25C mRNA revealed a 4-fold increase in WT mRNA in cancer tissues relative to normal tissues ( $0.3 \pm 0.08$  CDC25C transcripts/ $10^4$   $\beta$ -actin transcripts in normal versus  $1.28 \pm 0.2$  CDC25C transcripts/ $10^4$   $\beta$ -actin transcripts in cancer tissues; mean  $\pm$  SE). This difference was statistically significant ( $P < 0.001$ , Mann-Whitney rank sum test). In addition, as can be seen in

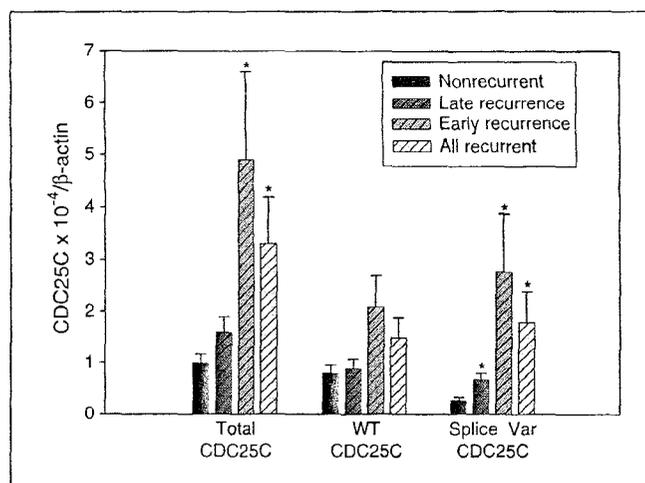


Fig. 3. Correlation of PSA recurrence with expression of CDC25C mRNAs relative to  $\beta$ -actin as determined by real-time RT-PCR. Total and WT mRNA transcript levels were determined by quantitative RT-PCR as described in Materials and Methods. Alternatively spliced transcript levels were calculated by subtraction of WT CDC25C transcript levels from the total CDC25C levels. Columns, means; bars,  $\pm$  SE. \*, statistically significant differences.

respectively, Mann-Whitney) when compared with nonrecurrent cancers. This increase is particularly marked in the prostate cancers with early recurrence. It should be noted that although the amount of spliced variant mRNAs in the recurrent cancers is about equal to the amount of WT mRNA, the amount of spliced variant protein(s) is probably 3- to 5-fold lower, based on the quantitative studies in DU145 cells described above.

**Determination of Ki-67 RNA levels by real-time quantitative reverse transcriptase-PCR as measurement for proliferative activity and normalization of CDC25C levels.** Because CDC25 is involved in control of exit from the G<sub>2</sub> phase of the cell cycle, it is likely that the differences between normal and cancer tissues and nonrecurrent and recurrent cancer tissues may be associated with differences in proliferative activity. To address this question, we designed a real-time RT-PCR assay to determine RNA levels of the proliferation marker, Ki-67. The monoclonal antibody recognizing Ki-67 is routinely used in oncology to assess the proliferative index of tumor cells. Ki-67 transcript levels were  $\sim 2$ -fold higher in the cancer tissues when compared with normal peripheral zone tissues ( $P = 0.001$ , Mann-Whitney). As seen in Fig. 4, relative expression of Ki-67 to  $\beta$ -actin was increased in Gleason score 7 to 9 cases (versus Gleason scores 5-6) and in cases with extracapsular extension and seminal vesicle invasion (versus organ-confined cancers), although not in cases with lymph node metastasis (Fig. 4). Ki-67 mRNA was also increased in prostate cancers that recurred (versus nonrecurrent cancers), particularly the cancers with early PSA recurrence (Fig. 4). The difference between the Ki-67 transcript levels in nonrecurrent versus those with early recurrence was statistically significant ( $P = 0.032$ , Mann-Whitney). Overall, as expected, increased Ki-67 transcript levels were associated with pathologic and clinical variables indicative of aggressive disease.

We then examined the correlation between CDC25C and Ki-67 transcript levels using the Pearson Product Moment test. There was a statistically significant correlation between the Ki-67 transcript levels and total, WT and variant CDC25C transcript levels ( $P < 0.001$ ,  $P < 0.001$ ,  $P < 0.018$ ,

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respectively). The correlation coefficients ranged from 0.5 to 0.6, implying that a substantial fraction, but not all, of the variance in CDC25C levels is associated with differences in proliferation. As an alternative way to examine this association, we normalized expression of CDC25C expression levels using Ki-67 transcript levels determined on the same cDNAs rather than  $\beta$ -actin levels (Fig. 5). Using this normalization, expression levels of the CDC25C splice variants was still significantly increased in patients with subsequent biochemical recurrence, including patients with either early ( $P = 0.041$ ) and late recurrence ( $P = 0.044$ ). The level of the Ki-67 normalized alternatively spliced CDC25c mRNA was also significantly higher in cancers with higher Gleason score (Gleason 7-9 versus 5-6;  $P = 0.044$ , Mann-Whitney) and was higher in cases with extracapsular extension, seminal vesicle invasion, and lymph node metastasis, although these differences were not statistically significant.

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## Discussion

CDC25A and CDC25B have been shown to collaborate with either mutation in the RAS oncogene or loss of retinoblastoma protein in transformation and, in this initial report, CDC25B protein was increased in 32% of human breast cancers (23). Subsequently, increased expression of CDC25A has been shown in head and neck (24), non-small cell lung (25), gastric (26), and colon cancers (27), whereas CDC25B is increased in non-Hodgkin's lymphomas (28), as well as head and neck (24), non-small cell lung (25), gastric (26), colon (27), pancreatic (28), and prostate cancers (29). In contrast, increased expression of CDC25C has only been reported in a fraction of colon (27) and endometrial cancers (30). We have shown that the majority of prostate cancers have both increased total WT CDC25C protein and less phosphorylated CDC25C when compared with normal prostatic tissue. The level of WT CDC25C mRNA was increased 4-fold in cancer tissues

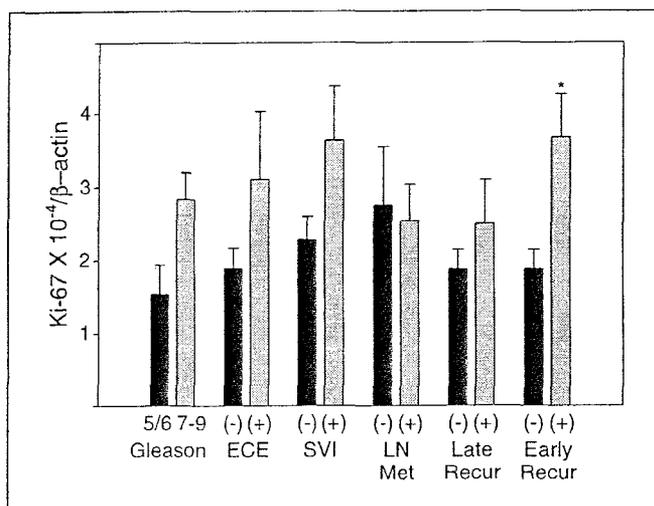


Fig. 4. Correlation of expression of Ki-67 mRNA relative to  $\beta$ -actin with clinical and pathologic variables in clinically localized prostate cancer. Clinical samples are grouped by Gleason grade, presence of extracapsular extension (ECE), seminal vesicle invasion (SVI), and lymph node metastasis (LN Met). Cases with PSA recurrence were compared with those without recurrence (-). \*, statistically significant differences.

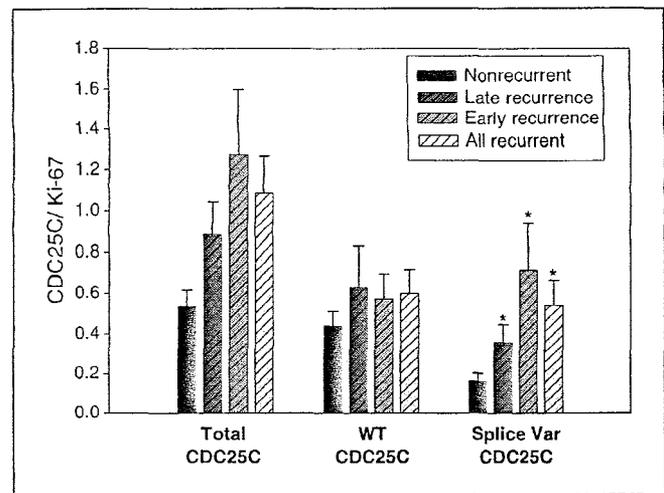


Fig. 5. Relative expression of CDC25C to Ki67 in prostate cancer. Total and WT mRNA transcript levels were determined by quantitative RT-PCR as described in Materials and Methods. Alternatively spliced transcript levels were calculated by subtraction of WT CDC25C transcript levels from the total CDC25C levels. Samples are grouped by their recurrence status and interval. \*, statistically significant differences in each group.

consistent with these increased protein levels. Thus, there are significantly higher levels of WT CDC25C protein in prostate cancer and much less of its inactive phosphorylated form, consistent with a marked increase in CDC25C phosphatase activity in prostate cancer.

In addition to the full-length CDC25C mRNA, we detected a major alternatively spliced CDC25C in both human prostate cancers *in vivo* and in prostate cancer cell lines. This alternatively spliced transcript has a deletion of exons 3, 5, and 6 of the CDC25C gene and encodes a smaller protein containing the COOH-terminal catalytic domain and 17 unique amino acids. This alternatively spliced variant can complement a CDC25C mutant strain of *Schizosaccharomyces pombe* (19). It is interesting to note that this variant leads to an increased uncoupling of the onset of mitosis and the completion of DNA synthesis in this mutant strain of *S. pombe*, implying poor regulation of the activity of this variant protein. This alternatively spliced RNA was detected as visible band in one half of the prostate cancers analyzed but in only 16% of the benign tissues. The presence of this variant was significantly correlated with biochemical (PSA) recurrence following radical prostatectomy. Quantitative RT-PCR studies confirmed a significant increase in variant CDC25C mRNAs in prostate cancer and its correlation with PSA recurrence, particularly early PSA recurrence that is associated with aggressive disease and worse patient outcome (31). It should be noted that the quantitative RT-PCR assay detects other splice variants, particularly the C4 variant, which may also contribute to the observed correlation, although the C5 variant was the most highly expressed form. Although the spliced variant mRNA are only a portion of total CDC25C mRNA *in vivo*, it may have significant biological effects that lead to more aggressive diseases, perhaps through poor regulation of its activity. Thus, prostate cancer is characterized by multiple alterations in CDC25C that can increase its activity *in vivo*.

Based on comparison of CDC25C and Ki-67 transcript levels, there was a significant correlation of WT and variant CDC25C

transcript levels with Ki-67 transcript levels. Increased expression of CDC25C would be expected to promote progression through the G<sub>2</sub>-M checkpoint and, particularly if associated with loss of other cell cycle checkpoint controls, would increase cellular proliferation. Further mechanistic studies to determine the basis of the increased CDC25C transcript levels in cancer may reveal whether this is a primary event associated with malignant transformation or a secondary event related other alterations in prostate cancer cells such as increased growth factor signaling.

The finding that the level of alternatively spliced CDC25C transcripts is correlated with recurrence, even after correction for proliferation by normalization to Ki67 levels indicates that expression of variant CDC25C has independent correlation with biochemical recurrence. Bureik et al. (19) have shown that expression of the major alternatively spliced CDC25C variant in *S. pombe* resulted in uncoupling of mitosis from completion of the S phase. Of note is the finding that the C5 variant does not seem phosphorylated at the inhibitory Ser<sup>216</sup> residue. This site

is phosphorylated by Chk kinase, which plays an important role in protecting the genomic integrity of cells following DNA damage (32). Thus, expression of the C5 variant of CDC25C could potentially result in genomic instability, which could facilitate emergence of aggressive disease. Further studies are needed to test this possibility.

In summary, we have described, for the first time, the increased activity of CDC25C phosphatase and overexpression of an alternatively spliced CDC25C mRNA in prostate cancer. Increased expression of the both total CDC25C mRNA and its spliced variant are correlated with biochemical recurrence, particularly early recurrence. Further studies need to be done to determine the role of CDC25C and its spliced variants in prostate cancer pathogenesis.

## Acknowledgments

We thank the Michael E. DeBakey Veterans Affairs Medical Center for the use of their facilities.

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