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# Loss of Annexin II Heavy and Light Chains in Prostate Cancer and Its Precursors[1]

**Albert Chetcuti, Sienna H. Margan, Peter Russell, Stephen Mann, Douglas S. Millar, Susan J. Clark, John Rogers, David J. Handelsman, and Qihan Dong[2]**

*Department of Medicine, the University of Sydney, NSW 2006 [A. C., S. H. M., Q. D.]; Departments of Anatomical Pathology [P. R.] and Urology [J. R.] and Kanematsu Laboratories [D. S. M., S. J. C.], Royal Prince Alfred Hospital, NSW 2050; and ANZAC Research Institute and Departments of Andrology [D. J. H.] and Anatomical Pathology [S. M.], Concord Repatriation General Hospital, NSW 2139, Australia*

## Abstract

**Annexin II mRNA coding for a calcium binding protein was found to be absent in prostate cancer by subtractive hybridization and Northern analysis. In contrast to high expression in normal and benign hyperplastic glandular and basal epithelium, Annexin II heavy (p36) and light (p11) chains in 31/31 prostate cancer specimens were lost immunohistochemically. In glands involved by prostate intraepithelial neoplasia, 65% lost both chains in glandular epithelial cells, whereas basal cells were all positively stained. Southern analysis of cancer DNA showed no noticeable deletion in *p36* gene. LNCaP cells treated with 5-azacytidine re-expressed p36, suggesting methylation could be responsible for the silencing.**

## Introduction

Identification of genetic alterations is a necessity to gain insight into neoplasia and can provide new tools for diagnosis, treatment, and prevention. Prostate cancer is one of the most frequently occurring carcinomas in men and has become the second most common cause of cancer-related death. However, our understanding of its etiology and the multistep progression is very limited. Oncogenes and tumor suppressor genes known to be associated with other malignancies have a remarkably low frequency of mutation or deletion in prostate cancer. The most common change is the loss of Glutathione S-Transferase pi-1 expression attributable to hypermethylation in the gene promoter (1–3). We have now identified that Annexin II heavy (p36) and light (p11) chains are also commonly lost in prostate cancer and in some of its precursor, PIN.[3] The underlying cause is likely attributable to hypermethylation of the *p36* gene.

## Materials and Methods

**Prostate Specimens.** The entire prostate gland from radical prostatectomy patients was taken to pathologists immediately after surgical removal. Biopsies were made from the posterior prostate surface (4). Fresh normal prostate tissue was obtained from two sources. One was a biopsy specimen where no cancer cells were found. The other was a brain-dead organ donor. The peripheral zone of the prostate from the organ donors was dissected from the transitional/central zone using urethra and ejaculatory ducts as the landmarks. Specimens used for immunohistochemistry were fixed in 10% formaldehyde solution and paraffin embedded. Benign hyperplastic tissue was obtained from transurethral resection. Informed consent was obtained from the patients or families, and the study protocol was approved by the Central Sydney Area Health Service Ethics Review Committee.

**RNA/DNA Extraction.** Before nucleic acid extraction, fresh cancer specimens and normal prostate were microselected (5). The tissue was homogenized in TRI-reagent (Sigma Chemical Co., Sydney, NSW, Australia). Total RNA and DNA were isolated following the manufacturer's protocol with the following modification regarding DNA. After precipitation of DNA, the pellet was washed 3 times with 75% ethanol to remove residual phenol. The pellet was then digested overnight at 55°C in a lysis buffer [50 mM Tris, 100 mM EDTA, 0.5% SDS, and 0.3 mg/ml proteinase K (pH 8)]. DNA was isolated by standard phenol:chloroform:isoamyl alcohol technique.

**Suppressive Subtractive Hybridization.** Poly(A)$^+$ RNA was isolated from total RNA of a cancer specimen and a normal prostate of an organ donor using Dynabeads oligo(dT)$_{25}$ (DYNAL, Oslo, Norway). Using PCR-Select cDNA subtraction kit (Clontech, Palo Alto, CA), subtracted libraries were subcloned into pGEM T-easy vector (Promega, Madison, WI). Individual recombinant colonies were randomly picked and inoculated into 96-well plates containing L-broth and ampicillin. Cloudy medium was used as template for PCR amplification of the insert sequence. PCR products were dot blotted onto membranes. Duplicate membranes were hybridized to subtracted radiolabeled cancer and normal cDNA library, respectively. The sequence of differentially expressed clones was determined by single direction sequencing.

**RT-PCR.** Total RNA (2 $\mu$g each) was reverse transcribed into cDNA. For p36, PCR was performed using primers derived from the nucleotides 971–991 and 1213–1229 (GenBank accession no. D00017). For p11, PCR was performed using primers derived from the nucleotides 138–158 and 376–394 (GenBank accession no. M38591). HPRT was amplified as a loading control (5′-TTACTTTTCTAACACACGGTGGTA-3′ and 5′-TTGCTGACCTGCTGGATTACATCA-3′). Thermocycling included an initial denaturation at 94°C for 2 min; 24 cycles (p36 and p11) and 30 cycles (HPRT) of denaturation (94°C for 30 s); annealing (30 s) at 55°C (p36), 51°C (p11), and 52°C (HPRT); and elongation (72°C for 45 s). A water control consisted of all reagents except template was included in each experiment.

**Northern and Southern Analyses.** Total RNA (15 $\mu$g) was denatured for 15 min at 65°C and electrophoresed using a 1% formaldehyde agarose gel. The RNA was transferred onto Hybond N nylon membrane. A PCR product (nucleotide 971-1229) of p36 cDNA was used as a probe. The membrane was hybridized in ExpressHyb solution (Clontech) containing $1 \times 10^6$/ml cpm of denatured probe overnight at 68°C. Genomic DNA (10 $\mu$g each) was digested using *Hin*dIII, *Pst*I, and *Bgl*II. After overnight digestion at 37°C, DNA was electrophoresed in a 0.8% agarose gel and transferred onto nylon membrane after depurination, denaturation, and neutralization. Radiolabeled probes were prepared from two overlapping PCR products covering the entire open reading frame of *p36* gene. Hybridization condition was same as Northern.

**Immunohistochemistry.** Tissue sections (5 $\mu$m) were incubated for 1 h at 37°C after microwave antigen retrieval with a mouse monoclonal anti-p36 antibody (Zymed Laboratories) diluted 1/400 in 1% preimmune goat serum. Biotinylated goat antimouse IgG, diluted 1/200 in 1% preimmune goat serum, was used as the secondary antibody. The signal was amplified using the avidin-biotin-peroxidase complex system (Vector Laboratories, Burlingame, CA) and visualized using the liquid 3,3′-diaminobenzidine substrate-chromogen system (Dako, Carpinteria, CA). Isotype and method controls were performed for each sample by substituting the primary antibody with preimmune mouse IgG (Dako) and 1% preimmune goat serum, respectively. For p11, a mouse monoclonal anti-p11 antibody (Transduction Laboratories) diluted 1/1000 was used. For 34$\beta$E12 and PSA, the antibodies were diluted 1/100 (Dako) and 1/600 (Dako), respectively. Dako Envision + peroxidase (mouse

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[2] To whom requests for reprints should be addressed, at Department of Medicine, D06, University of Sydney, NSW 2006, Australia. Phone: 612-95155186; Fax: 612-95161273; E-mail: qhd@med.usyd.edu.au.

[3] The abbreviations used are: PIN, prostate intraepithelial neoplasia; RT-PCR, reverse transcription-PCR; HPRT, hypoxanthine phosphoribosyltransferase; DAB, 3,3′-diaminobenzidine; PSA, prostate-specific antigen; BPH, benign prostatic hyperplasia.

K4001 for 34$\beta$E12 and rabbit K4003 for PSA) was used as the signal detection system.

**5-Azacytidine Treatment.** LNCaP cells ($0.5 \times 10^6$) were cultured in T-medium (6) with 10% heat-inactivated fetal calf serum (FCS) for 2 days. A final concentration of 5 $\mu$M of 5-azacytadine was added to the culture medium from a freshly prepared 5 M stock solution in DMSO. After 5 days the cells were harvested and RNA isolated using TRI-reagent. The RNA was DNase treated, and RT-PCR was performed as described above using p36 primers.

## Results

**Identification of Loss of Annexin II (p36) mRNA in Prostate Cancer.** Using suppressive subtractive hybridization between two microselection-derived prostate mRNAs, one from a previously untreated 64-year-old prostate cancer patient and the other from a 25-year-old brain-dead, cancer-free organ donor, we identified a cDNA sequence that was absent in prostate cancer. Sequencing revealed a full match with Annexin II heavy chain (p36) from nucleotide position 657 to 1357 (GenBank accession no. D00017). RT-PCR and Northern analysis using total RNA from additional six normal prostate and seven cancer specimens confirmed that expression of p36 was significantly reduced in prostate cancer (Fig. 1, *A* and *B*).

**Annexin II (p36) Protein in Normal Prostate, BPH, Prostate Cancer, and PIN.** We analyzed p36 immunohistochemically in normal and diseased prostate (Fig. 2). In peripheral ($n = 10$) and transitional/central zones ($n = 6$) of normal prostates from organ donors, the apical and lateral (*i.e.*, circumferential) plasma membrane of glandular and ductal epithelial cells showed high expression of p36. Similarly, the basal cells also expressed p36 protein. In BPH tissue derived from transurethral resection of prostate ($n = 12$), p36 was expressed in the same cell type as normal tissue with comparable intensity. In contrast, a loss of p36 expression in cancer cells was observed in 31/31 randomly selected individual cancer blocks with a Gleason score range of 3–9 ($5.7 \pm 1.4$, mean $\pm$ SD), whereas adjacent normal and hyperplastic glands in the same tissue block had positive expression. The proportion of cancer cells in each block that had no p36 expression was nearly 100% in 28 cases and close to 50–75% in the other 3 cases. The Gleason score of the 3 cases was 5, 6, and 5, respectively. One of them was the cancer case 5 in the Northern analysis (Fig. 1*B*). Cytokeratin 34$\beta$E12, a marker of prostate basal cells (7), was used to verify that the p36-positive cells were indeed cancer cells, because they lacked a participating basal cell component.

We additionally examined the p36 expression in high-grade PIN. Twenty-one of the 31 cancer blocks had foci of high-grade PIN at the interface between cancer and non-neoplastic glands. Each block had 6 or 7 glands involved by high-grade PIN, making the total 130 glands examined. There was a clear difference in p36 expression in this morphologically indistinguishable, high-grade PIN. Of them, 65% showed negative and 35% showed positive, apical staining whereas all of the basal cells were positively stained. Persistent expression of 34$\beta$E12 in p36-negative PIN ruled out the possibility of cancer (Fig. 2). PSA-positive staining confirmed that p36-negative PIN was prostate gland origin (data not shown).

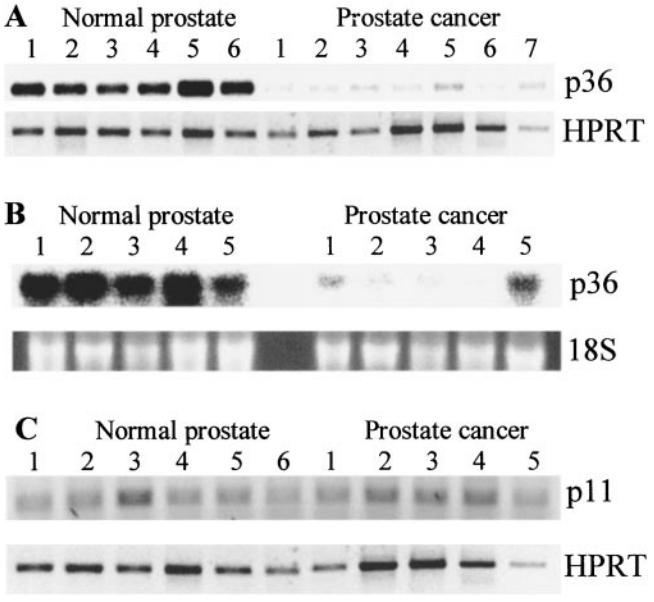


Fig. 1. RT-PCR and Northern analyses of p36 and p11. *A*, RT-PCR of p36 in individual normal prostate (*1–4* and *6* from cancer-free biopsy specimens; and *5* from organ donor) and prostate cancer-derived total RNA. HPRT was used to indicate comparable RNA loading. *B*, Northern analysis of p36 in individual normal prostate (*1–3* from cancer-free biopsy specimens; *4* and *5* from organ donors) and prostate cancer-derived total RNA. Ethidium bromide-stained 18S rRNA indicates comparable RNA loading. *C*, RT-PCR of p11 in individual normal prostate (same as p36) and prostate cancer-derived total RNA. Three PCR cycles (21, 24, and 27) were used, and there was no statistically significant difference in the intensity of PCR products between normal prostate and prostate cancer. Depicted are products with 24 PCR cycles.

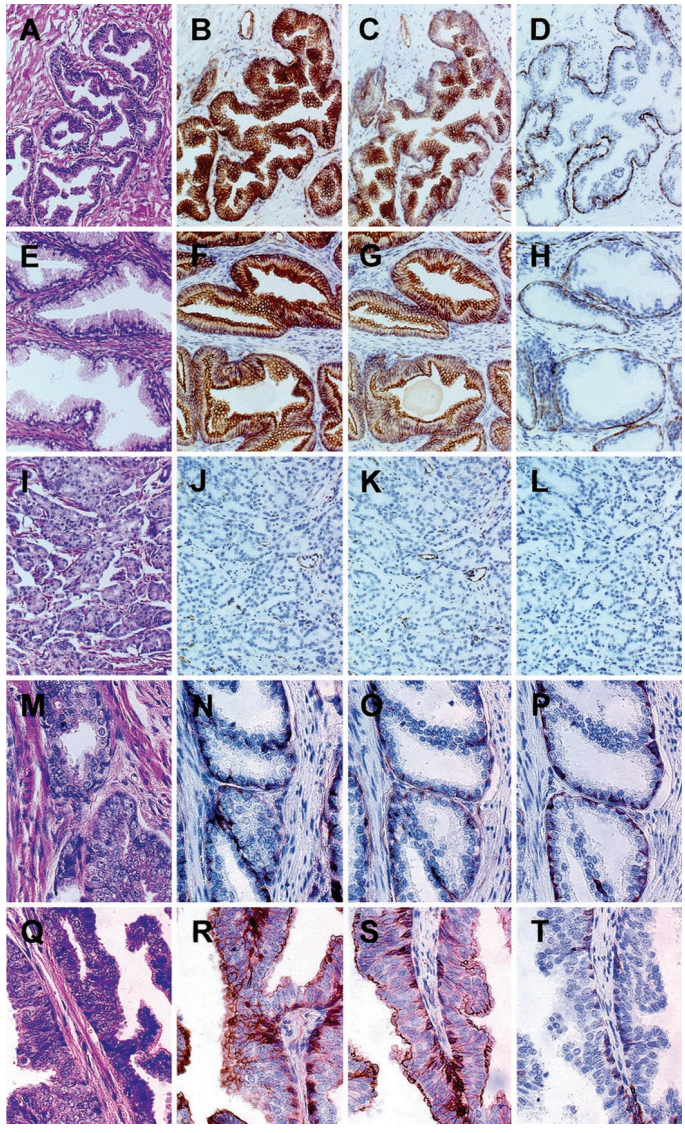


Fig. 2. p36 and p11 protein expression in prostate tissues. Depicted in each row are consecutive sections stained with H&E (*first column*), p36 antibody (*second column*), p11 antibody (*third column*), and 34$\beta$E12 Ab (*fourth column*). *A–D*, glands from peripheral zone of an organ donor (×20); *E–H*, BPH from transurethral resection (×20), *I–L*, cancer from radical prostatectomy (×20); *M–P*, p36/p11 negative PIN from radical prostatectomy (×40); *Q–T*, p36/p11 positive PIN (×40).


Fig. 3. CpG map of *p36* gene. *Vertical lines above bar* indicate the relative position of the CpG sites. *Nucleotide position below the bar* is based on the sequence from GenBank (Accession no. AC019146). The orientation of genomic DNA is reverse complimentary to p36 cDNA.

**Annexin II (p11) Protein in Normal Prostate, BPH, Prostate Cancer, and PIN.** Because Annexin II light chain (p11) is always in complex with p36, the absence of p36 expression in prostate cancer and in 65% of PIN prompted us to examine p11 expression immunohistochemically (Fig. 2). In normal and BPH, p11 was expressed in the same cell type as p36. Prostate cancer cells had also lost p11 expression. The small fraction of p36-positive cancer cells in three cases also expressed p11. However, we noticed in a few cancer cells that weak p11 expression was present, whereas p36 was lost. p36-negative or -positive PIN remained negative or positive for p11. Interestingly, despite the absence of p11 protein in prostate cancer, p11 mRNA levels remained unchanged when compared with normal prostate (Fig. 1*C*).

**Association of Hypermethylation with Silencing of Annexin II (p36) Gene Expression.** To address the question of whether the decrease in p36 mRNA level is a result of a homozygous deletion in p36 coding regions in cancer DNA, Southern analysis with three different endonucleases was conducted using p36 open reading frame sequence as the probe on genomic DNA from paired cancer tissue and blood leukocyte of the same patients ($n = 8$). We found no difference between cancer and blood DNA (data not shown). To establish if the *p36* gene has a CpG island spanning the promoter region and could therefore be susceptible to hypermethylation, we used the full-length p36 cDNA sequence to search for p36 genomic DNA sequences from a high throughput genomic DNA database and obtained a sequence from clone AC019146. Analysis of the upstream region of the gene has shown a CpG island spanning the promoter, first exon, and intron of the gene (Fig. 3). To determine whether methylation is associated with Annexin II silencing in prostate cancer, we treated prostate cancer LNCaP cells, which do not express p36, with 5-azacytidine (Fig. 4). p36 is expressed in normal prostate, blood, and DU145 prostate cancer cells but is silent in prostate cancer and LNCaP cells. Annexin II expression was reactivated in LNCaP cells after 5-azacytidine treatment suggesting that methylation is associated with Annexin II silencing in prostate cancer.

## Discussion

Annexin II can be found *in vivo* as p36, p36p11, and $p36_2p11_2$, and is involved in endocytosis, exocytosis, and membrane trafficking (8). p36 belongs to a family of calcium- and lipid-binding proteins and is a substrate for receptor and nonreceptor protein kinases (9, 10). Recently, membrane p36 has been shown to mediate steroid rapid action (11). p11 is a small calcium binding protein and shares sequence homologies with the S-100 family (12). Overexpression of p36 increases p11 protein level with no change in its mRNA levels (13), suggesting that p36 has a post-translational stabilizing effect on p11 protein. Thus, it is possible that the lack of p36 in prostate cancer gives rise to substantial degradation of p11 so that the rate of translation from p11 mRNA fails to compensate. The observation that negative p36 expression coexists with a weak expression of p11 in a small number of cancer cells may represent the early stage of p11 degradation. As the loss of p36 and subsequently p11 in prostate cancer obligates all forms and functions of Annexin II, it is difficult to know which function of Annexin II is related to prostate carcinogenesis. However, the importance of calcium, the key element for Annexin II to function, is well appreciated in programmed cell death in the prostate (14, 15). The distinctive expression pattern of Annexin II among BPH, PIN, and cancer indicates that the loss of Annexin II is specific for prostate cancer. The loss of expression in 31/31 individual prostate cancer blocks regardless of the Gleason score and 65% of high-grade PIN suggests that Annexin II is involved in an early stage or common pathway of prostate carcinogenesis. Recently, Annexin I has been found to be absent in prostate cancer and PIN by Western blot and immunohistochemistry (16), and Annexin VII can suppress proliferation of prostate cancer cells and is significantly reduced in metastatic and recurrent prostate cancer (17). Thus, Annexin could represent a previously unrecognized mechanism involved in prostate cancer. The clinical implication of residual Annexin II-positive cancer cells in three cases will be closely followed up.

To address the question of whether the >50% decrease in p36 mRNA level is a result of a homozygous deletion in the p36 coding region in cancer DNA, we conducted Southern analysis on genomic DNA from paired cancer tissue and blood leukocyte. Because our prostate cancer cases are sporadic, a somatic homozygous deletion in the p36 coding region in the cancer DNA should be revealed by comparing it with the paired blood DNA. Any point mutations or small deletions within the coding region of the *p36* gene, which Southern analysis may not be able to detect, should not cause such a substantial decrease in p36 mRNA levels. We found no difference thus far between cancer and blood DNA, suggesting that the decrease in p36 mRNA level is likely attributable to alterations affecting the expression or processing of mRNA. Because DNA hypermethylation is commonly associated with silencing of tumor-related genes (1–3), a preliminary study was conducted to examine the possible association of methylation with Annexin II silencing in prostate cancer. The finding that the *p36* gene does have a CpG island in the promoter region and in the first exon and, more importantly, that p36 expression could be reactivated by treatment of prostate cancer LNCaP cells with a demethylation agent indicates that hypermethylation could be responsible for silencing of the *p36* gene *in vivo*. However, because 5-azacytidine can also affect Sp1 transcriptional activity (18), additional study by bisulphite sequencing of normal and cancerous prostate-derived DNA is needed to verify and identify methylation sites that distinguish cancer cells from normal cells. Although p36-positive cancer cells of case 5 was demonstrated by both Northern analysis and immunohistochemistry, it remains to be seen whether additional mechanisms besides mRNA transcription are involved in the down-regulation of the p36 protein, considering the fact that 28 of 31 cases had no p36 protein, whereas one of five cases showed unchanged p36 mRNA.

This study also provides evidence that high-grade PIN is not biochemically homogenous. It is known that not all PIN will progress to cancer (19); however, the lack of a progression-associated "marker" has prevented pathologists from stratifying the morphologically un-

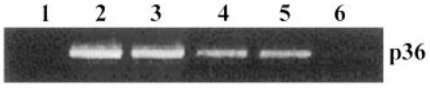


Fig. 4. 5-Azacytine reactivation of *p36* gene expression in LNCaP cells. RT-PCR was used to determine p36 expression using RNA isolated from (*1*) prostate cancer, (*2*) normal prostate, (*3*) blood, (*4*) DU145 cells, and (*5*) LNCaP cells treated with and (*6*) without 5 azacytidine.

distinguishable PIN. Although additional studies are required to correlate the p36-negative and -positive PIN with the incidence of cancer and prognosis, the fact that the vast majority of cancer cells show negative expression for p36 suggests that the p36-negative PIN is the true precursor of cancer. We speculate that p36-positive PIN cannot progress to cancer without loss of p36 expression first. However, because we have encountered three cases containing a small fraction of p36-positive cancer cells, it remains a possibility that p36-positive PIN can develop directly to p36-positive cancer.

A differential diagnosis among well-differentiated cancer, PIN, and non-neoplastic lesion is often required for needle-core biopsy, in particular when the number of glands is insufficient and/or the quality of a specimen is unsatisfactory (20). Currently, 34$\beta$E12 is the "gold standard" by highlighting the absence of basal cells in infiltrating cancer. However, it is problematic to additionally differentiate PIN from non-neoplastic glands because basal cells are present in both. The unique expression pattern of Annexin II-negative PIN, absent in glandular epithelial but present in basal cell, is in contrast to benign hyperplasia in which Annexin II is expressed in both cells and to the vast majority of cancer cells in which Annexin II expression is abolished and basal cells are no longer attached. Thus, identification of Annexin II provides a potential diagnostic marker for needle-core biopsy specimens.

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