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# Detection of preneoplasia in histologically normal prostate biopsies

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**P2X immunolabeling of prostate detected preneoplastic changes in apparently normal tissue. Labeling occurred in two well-defined stages before the diagnostic histological markers of cancer were visible. As cancer progressed, the location of P2X expression changed from confinement within individual nuclei in the acini (stage 1) to a cytoplasmic punctate label in the acinal epithelium, with an associated removal of nuclear stain (stage 2). Finally, in advanced cases, where clear morphological evidence of cancer was apparent, the P2X label condensed exclusively on the apical epithelium (stage 3). BPH/normal tissue was entirely devoid of P2X label. Biopsy samples (77) were tested in three categories. One group (35) were diagnosed as normal benign prostatic hyperplasia (BPH) on the basis of haematoxylin and eosin (H&E) stain, although underlying disease was suspected. Of these, 14 (40%) were clearly normal and appeared entirely devoid of label, 13 (37%) exhibited the first stage of P2X receptor labeling and the remaining eight (23%) exhibited second stage labeling. The accompanying H&E-stained sections of all these cases had a normal appearance. Low grade cancer biopsy samples with Gleason scores G4–7 (25) all revealed widespread second stage receptor labeling in areas of both normal and cancerous morphology, while 17 high grade cancer biopsy samples (Gleason G8–10) all showed third stage labeling along with some residual second stage labeling. The features of each P2X labeling stage occupied the entire histological area affected, offering more opportunity to diagnose the tissue than was supplied by the more-localised diagnostic features identified by H&E-stain. Besides detecting cases of preneoplasia in biopsies with a normal H&E appearance, this technique was also able to rule out the presence of neoplasia in purely hyperplasic prostates by the absence of any P2X labeling.** *Prostate Cancer and Prostatic Diseases* (2001) **4,** 92–96.

**Keywords:** prostate cancer; diagnosis; P2X receptor; calcium channels

## Introduction

The autonomic nervous system plays a major role in the homeostatic, growth and secretory functions of the prostate.[1] In vertebrates, the autonomic P2X receptors/channels of the purinergic nervous system constitute a newly-described class of fast response, ligand-gated, calcium-permeable and cation-selective ion channels activated by extracellular ATP from nerve terminals or a local tissue source.[2] The P2X receptors are widely-distributed in mammalian tissues.[3] P2X receptor binding on the cell membrane triggers an influx of cytosolic $Ca^{2+}$ into the cytoplasm, producing ionic concentrations that vary in different cellular compartments.[4] The intracellular $Ca^{2+}$ signal is transduced via more than 40 $Ca^{2+}$-binding proteins including the S100 proteins, tenascin, calmodulin, integrin and annexin.[5] These calcium-bound proteins activate and regulate the cell cycle, protein secretion, the composition of nuclear proteins, DNA transcription, apoptosis, adhesion protein binding, cell differentiation and phosphorylation.[6] Changes in cytosolic $Ca^{2+}$ therefore control a wide range of cellular responses.

Purines and pyrimidines can either stimulate or inhibit proliferation depending on the extracellular microenvironment, the physiological state of the target cells, cell cycle and the expression of P2X receptors.[7] Several lines of evidence suggest that P2X receptor expression, and the

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resulting variations in $Ca^{2+}$ homeostasis, may contribute to the progression of prostate cancer.[7–10] Extracellular $Ca^{2+}$ influx also activates invasion and malignancy-associated enzyme metalloproteinases.[11] As an extension of studies on the role of P2X receptors in the urogenital system[12–16] we examined their expression in clinical biopsies that had been previously diagnosed, using a unique suite of subtype-specific antibodies.

## Methods

Antibodies against unique epitopes in the extracellular domains of each P2X subtype were developed and their specificity determined using cDNA expressed in *Xenopus* oocytes and HEK293 cells. Each antibody exhibited specific labeling in rat, mouse and human tissue.[12–16] After optimisation and standardisation of P2X subtype antibody production and labeling characteristics, we evolved a standardised protocol. $P2X_1$ and $P2X_2$ had similar distributions. $P2X_3$ and $P2X_7$ labeled the same structures but obscured the clarity of the epithelial label because they exhibited denser staining of the prostatic nerve supply in the stroma. $P2X_4$, $P2X_5$ and $P2X_6$ did not label any components of normal or neoplastic tissue. Accordingly, we chose a mixture of affinity-purified $P2X_1$ and $P2X_2$ subtypes, each at a concentration of 0.5 μg/mL lgG in PBS. This $P2X_{1,2}$ mixture resulted in increased clarity of the features described, without an increase in stromal labeling.

Sections cut from blocks from cases that had been previously diagnosed were supplied by private clinicians and hospital pathology departments, as 5–10 μm paraffin-embedded sections of prostate biopsy tissue on glass slides. The mean age of the patients was $72 \pm 9$ y. Of these cases, 35 had previously been diagnosed as normal and/or containing BPH. All these cases contained some degree of epithelial hyperplasia. Twenty-five cases were in the Gleason score range 4–7, while 17 were Gleason 8–10. Those that had Gleason scores in the range 4–10 all had PSAs over 8 and/or rising. In no case previously diagnosed as normal/BPH, were prominent nucleoli observed. However, in 66% of cases with a Gleason score of 6 or more, prominent nucleoli were observed.

The supplied cases were de-waxed in two changes of fresh Histoclear for 10 minutes each and rehydrated. Sections were incubated for 30 min in 3% hydrogen peroxide in 1% bovine serum albumin in phosphate buffered saline (PBS), and washed in PBS $3 \times 5$ min. Sections were then incubated with an equal mixture of $P2X_{1,2}$ subtypes at 0.5 μg/mL lgG in PBS, for 30 min. Thereafter, slides were washed three times in PBS for 10 min each, followed by a 30 min incubation with a 1:100 dilution of HRP-conjugated goat anti-rabbit secondary antibody (Dako, Carpentera, CA). All slides were then washed three times in PBS for
10 minutes each, visualised using a 0.05% solution of diamino benzidine (DAB) for 10 min, washed, dried and mounted in Entellin mounting medium (Merck, Darmstadt, Germany). Approximately serial sections were stained with a standard H&E protocol and labeled with anti-P2X antibodies as described, respectively. For practical, ethical and legal reasons, only formalin-fixed, paraffin-embedded and previously diagnosed biopsy samples were used. Conjugate epitopes completely blocked labeling in pre-absorption controls.

## Results

Previously-diagnosed prostate cancer clinical specimens were used to identify three distinctly separate patterns of purinergic receptor (P2X) expression with increasing grade of cancer. Of the P2X subtypes ($P2X_{1-7}$), a mixture of $P2X_{1,2}$ antibodies resulted in optimal labeling patterns. In normal (non-neoplastic) tissue containing varying degrees of benign prostatic hyperplasia (BPH), no $P2X_{1,2}$ label (or any other subtype) was present. In samples clinically diagnosed as high grade cancer (Gleason grade G8–10), a distinctive labeling pattern was routinely observed in different regions. In early stage cancer (Gleason grade G4–7) stage 1 and 2 P2X labeling patterns predominated, although stage 3 labeling was observed in advanced cancer, particularly G7 tissue. These three distinct patterns were designated as purinergic receptor translocation stages 1–3 (PRT 1–3). PRT 1 involved intense labeling of prostatic epithelial nuclei while in PRT 2, punctal cytoplasmic P2X receptor translocation predominated, with the (previously labeled) nuclei now unlabeled. All label within the nuclei had migrated outside the nuclei. PRT 3 involved intense P2X receptor labeling on the apical epithelium in those cells possessing clear morphological changes. In the most advanced cases of cancer only PRT 3 was observed. However, in regions of tissue from cases of advanced cancer where the cellular morphology was merely hyperplasic or appeared entirely normal, PRT 2 and very occasionally PRT 1 were observed.

Patient diagnoses and case notes were used as a guide to separate the cases of normal/BPH (taken from patients obviously suspected of harboring cancer) from early stage and from late stage cancer. Staining patterns in TURP chips and biopsy core specimens were identical. In normal and/or BPH tissue, as originally diagnosed by H&E staining as well as long-term patient outcome, P2X labeling was totally absent.

Figure 1a is an H&E-stained section of a core biopsy sample taken from a 71-y-old man diagnosed with BPH while Figure 1b shows an anti-P2X immunoperoxidase-labeled (approximately) serial section showing the complete lack of P2X receptor expression characteristic of non-neoplastic BPH tissue. This lack of staining was seen in 40% (14 of 35) of cases diagnosed as normal/BPH by H&E. Of the 14 normal, 12 had normal PSA levels, while in two cases the PSA levels were slightly increased. We propose that these cases represent genuinely normal tissue, with no evidence of any preneoplastic change.

The earliest stage of P2X labeling (PRT 1) was characterised by dense, prominently-labeled epithelial nuclei (PEN) surrounded by an unlabeled stroma. Dilution studies revealed this pattern was composed of a dense accumulation of P2X receptor/channel puncta in the nucleus, that were entirely absent in genuinely normal/BPH tissue. PRT 1 labeling was seen in 60% (21 of 35) of biopsied samples suspected of harboring some cancer but

diagnosed as normal/BPH on the basis of H&E staining. A total of 16 of these 21 cases had normal PSA levels while the other five showed a slight increase. Figure 2a is a micrograph of an H&E-stained core biopsy from a 56-y-old patient with a diagnosis of BPH with 'slowly increasing' PSA but with no cancer identified. The acini appear to be entirely normal. Figure 2b is a micrograph of the same group of acini, in an (approximately) serial section, immunolabeled with anti-P2X antibodies showing the characteristic prominent epithelial nuclei (PEN-arrow) of PRT 1 despite its benign H&E appearance.

The second stage (PRT 2) was characterized by the appearance of P2X receptor puncta throughout the cytoplasm. This transport of receptors resulted in progressive de-expression of the (PRT 1) nuclear label to an end-point where the nuclei were completely devoid of label. These nuclei appeared outlined by the presence of cytoplasmic label. Some residual label occasionally could be found on the nuclear membrane itself. Figure 3a shows a H&E-stained core biopsy from a 57-y-old man with an increasing PSA. The clinical diagnosis was (mild) BPH with an adjacent area of cancer diagnosed as Gleason score 6. The area depicted in Figure 3a is from the area diagnosed as BPH and devoid of cancer. Figure 3b is an anti-P2X immunoperoxidase-labeled (approximately) serial section showing the same acinal cluster as Figure 3a. PRT 2 is readily apparent. In some regions of tissue diagnosed as advanced cancer, PRT 2 was sometimes observed although stage PRT 3 labeling was far more common. Figure 4a shows a core biopsy from a 69-y-old patient with increasing PSA. The clinical diagnosis was Gleason score 9. All acini were abnormal by H&E stain. Figure 4b is a micrograph of an anti-P2X immunoperoxidase-labeled (approximately) serial section showing the same acinal cluster as Figure 4a. PRT 2 is readily apparent (arrow), appearing as a distinctive, punctate cytoplasmic-translocating receptor label.

The final stage (PRT 3) consisted of an apical deposition (AD) of intensely labeled receptors on the apical epithelium. The nuclei remained devoid of label but the translocating puncta in the cytoplasm disappeared as the receptors deposited on the apical epithelium. The AD label was characteristically dense, homogeneous rather than punctate and relatively thick—a feature probably intensified by the contribution of apical labeling in the section below the plane of focus. In cases where the histological appearance by H&E stain was obviously cancer, PRT 3 was predominent. Figure 5a is a core

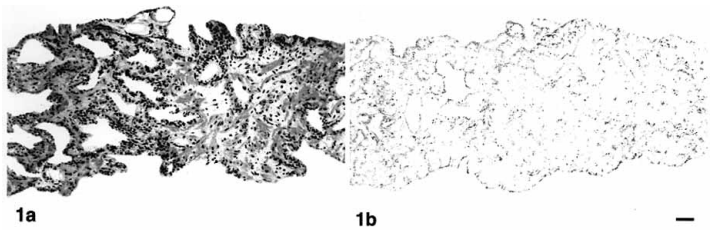


**Figure 1** *Panel (a) shows a H&E stained section of biopsy core sample from a 71-y-old man with increasing PSA. The clinical diagnosis was BPH. Figure 1(b) is an immunolabeled (approximately) serial section demonstrating non-expression of P2X receptors in BPH/normal tissue. Bar = 50 microns.*

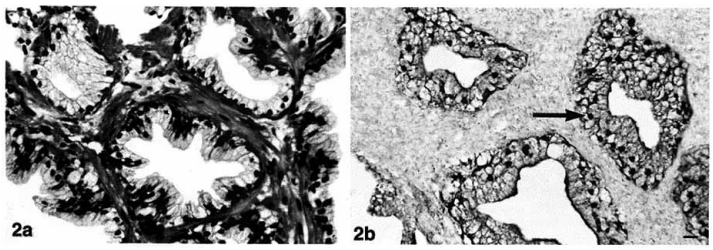


**Figure 2** *Panel (a) shows a H&E stained core biopsy specimen from a 56-y-old with 'slightly increasing' PSA. The clinical diagnosis was BPH. Figure 2(b) is an immunolabeled (approximately) serial section demonstrating PRT 1 (prominent epithelial nuclei or PEN—arrow), suggesting the onset of preneoplasia. Bar = 20 microns.*

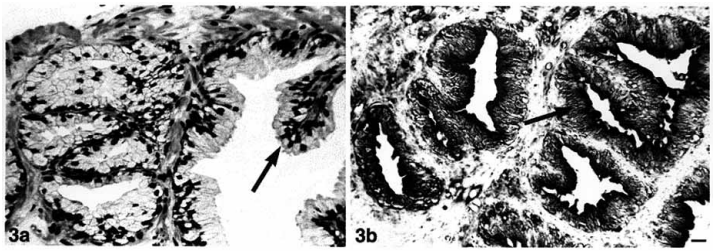


**Figure 3** *Panel (a) shows a H&E stained core biopsy specimen from a 57-y-old with 'increasing' PSA. The clinical diagnosis was BPH in the area depicted with an adjacent area of Gleason score 6. Figure 3(b) is an immunolabeled (approximately) serial section demonstrating PRT 2 indicating the likely presence of cancer within the tissue or an advanced case of preneoplasia suggesting the imminent onset of cancer. Bar = 20 microns.*

biopsy taken from an 86-y-old patient with increasing PSA. The clinical diagnosis was Gleason score 7. All acini in the biopsy were abnormal by H&E stain and contained numerous prominent nucleoli (arrow). Figure 5b shows an anti-P2X immunoperoxidase-labeled (approximately) serial section of the same acinal cluster as Figure 5a. PRT 3 predominates, as characterised by the intense deposition of receptors on the apical epithelium (arrow).

## Discussion

This study describes three stages in progressive prostate cancer consisting of P2X receptor/channel translocation from the nucleus to the plasma membrane via the cytoplasm. All biopsy specimens were taken from patients who presented with symptoms of prostate disease. The presence of PRT 1 or PRT 2 labeling features in tissue diagnosed as normal/BPH suggests that the neoplastic process begins at a metabolic level, some time prior to the appearance of H&E-stained diagnostic features. It is unlikely that these labeled receptors are acting as functional calcium channels in the nucleus at this early stage of expression and prior to being transported to epithelial cell surfaces.[17]

The observation of PRT 1 and PRT 2 in tissue diagnosed as normal by H&E staining is suggestive of preneoplasia given the presence of PRT 2–3 in cases of obvious cancer in which morphological changes are observed. Of the 35 cases previously diagnosed as normal/BPH, 40% (14 of 35) exhibited no P2X expression at all. This observation suggests that these tissues did not harbor any preneoplasia. A further 37% (13 of 35) contained PRT 1, while 23% (8 of 35) contained PRT 2. A finding of PRT 2 features in tissue diagnosed as normal was suggestive of comparatively aggressive or at least more advanced preneoplasia. No normal/BPH cases contained features of PRT 3, as this stage was always accompained by obvious carcinogenic changes to the cellular morphology. In each of the 25 cases previously categorised as Gleason score G4–7, PRT 2 was observed with some PRT 1 but not PRT 3. In the 17 cases previously diagnosed in the range G8–10, PRT 3 was always seen, with occasional or no features of PRT 2. In biopsy tissue that was well removed from a site of diagnosed cancer and that was normal by H&E staining, PRT 2 labeling and even some areas of PRT 1 labeling was noted. The PRT staining pattern was exclusive to the entire histological area of a particular stage. Often only a single core showed any cancer with all other cores appearing normal or BPH by H&E but all these cores contained clear signs of PRT 1 and/or PRT 2. We propose that all the cores that showed these features of PRT 1 and PRT 2 indicated the presence of underlying transformational change.

An explanation for our findings may be found in the unique metabolism of prostate cells. In cells from any other body tissue, citrate provides fuel for oxidation in the Krebs cycle and the production of ATP. The unique prostate cell Krebs cycle works in a fundamentally different manner with citrate being an end-product of oxidation rather than the fuel for oxidation.[18–20] Glucose and pyruvate are then used as substitutes for citrate. This results in an extremely inefficient energy metabolism and a 10–30 times greater citrate concentration in the prostatic cytosol than is found in all other cells of the body. Citrate and zinc, essential for the proper functioning of the sperm, are ultimately secreted into the seminal plasma where the $Zn^{2+}$ binds to citric acid and glycoproteins. Prostatic citrate production is regulated by testosterone and prolactin. The mechanisms of this process have not been elucidated, but are generally believed to involve

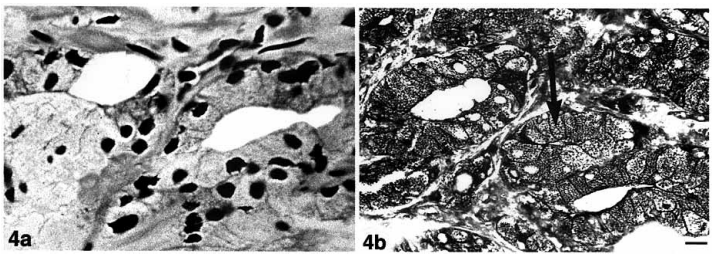


**Figure 4** *Panel (a) shows a high power detail of a core biopsy specimen from a 69-y-old with increasing PSA. Clinical diagnosis is Gleason score 9 with all acini appearing abnormal by H&E. Figure 4(b) is an immunolabeled (approximately) serial section that clearly shows the translocating receptors (arrow) in PRT 2. Most areas had already progressed to PRT 3. Bar = 20 microns.*

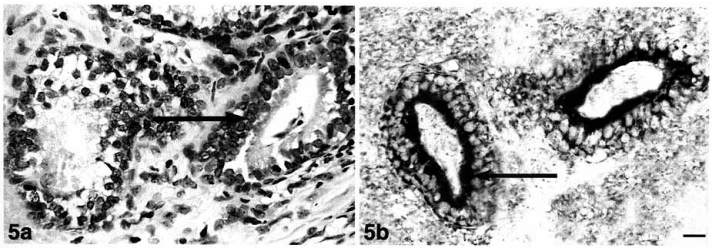


**Figure 5** *Panel (a) shows high power detail of a core biopsy sample from a 86-y-old with increasing PSA. Clinical diagnosis is Gleason score 7. All acini were abnormal by H&E stain and contained numerous prominent nucleoli (arrow). Figure 5(b) is an immunolabeled (approximately) serial section showing the same acinal clusters. PRT 3 predominates, as shown by intense concentration of receptors on apical epithelium (arrow). Bar = 20 microns.*

tyrosine kinase-mediated pathways. Both zinc and citrate are significantly increased in BPH tissue (due to the increased numbers of secretory cells) and markedly decreased in prostate cancer. Normal prostate epithelial cell citrate levels are typically 4000–6000 nmol/g. In the proliferating cells of BPH tissue they are extremely high (8000–12 000 nmol/g) but very low in cancer cells (1000–2000 nmol/g).[21] This results from the reversion of cancer cells to the normal somatic Krebs cycle. The neoplastic transformation of prostate cells therefore involves radical metabolic change. It is probable that the varying P2X $Ca^{2+}$ channel expression observed in the current study is the direct result of this altering metabolism. The phenomenon of calcium-associated receptor translocation in cancer is not unique. A similar translocation sequence to that described in this study has been reported for the malignancy-associated, calcium-binding S100 proteins in smooth muscle cells following intracellular $Ca^{2+}$ influx.[22] An antibody to this protein family, anti-human S100B, has been used as a test for malignant melanoma for some time. Moreover, keratin expression studies have shown that some BPH tissue possesses an intermediate phenotype which may have premalignant potential suggesting that BPH and cancer may be etiologically related.[23] Our results clearly support this suggestion.

We propose that the use of purinergic receptor translocation (PRT) staging constitutes an effective tool with which to readily differentiate patients with non-cancerous BPH from those with early cancer, as yet undetectable by H&E staining, in men who present with elevated PSA levels and/or enlarged prostates. These features are clearly stage-specific, appear in each lobe of the prostate and extend a large distance from sites of cancer as diagnosed by H&E stain. This feature reduces the need to take many needle biopsy samples in the hope of sampling a diagnostic group of cells, currently required for H&E diagnosis. Data obtained by P2X immunolabeling appears to be unrelated to that of H&E staining, as it flags the metabolic changes that accompany neoplasia before the appearance of morphological change.

Regardless of whether the observed pattern anticipates the development of cancer, or is a consequence of a field effect in which the cancer process influences the adjacent epithelium,[23] the result remains that the presence of cancer can be detected indirectly and truly negative cases can be readily distinguished. This method may also provide data on the rate of progression of individual tumours.

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