

## Expression of the apoptotic calcium channel P2X<sub>7</sub> in the glandular epithelium is a marker for early prostate cancer and correlates with increasing PSA levels

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### Summary

In the current study, expression of the apoptotic calcium channel receptor P2X<sub>7</sub> and prostate-specific antigen (PSA) levels were studied in biopsy cores from 174 patients as well as 20 radical prostatectomy cases. In clinical biopsies, we have previously demonstrated that P2X<sub>1</sub> and P2X<sub>2</sub> calcium channel receptors are absent from normal prostate epithelium that does not progress to prostate cancer within 5 years. In cases that did progress to prostate cancer however, P2X<sub>1</sub> and P2X<sub>2</sub> labeling was observed in a stage-specific manner first in the nucleus, then the cytoplasm and finally on the apical epithelium, as prostate cancer developed. These markers were present up to 5 years before cancer was detectable by the usual morphological criteria (Gleason grading) as determined by H&E staining. In the current study, the apoptotic calcium channel receptor P2X<sub>7</sub> yielded similar results to that of P2X<sub>1</sub> and P2X<sub>2</sub>. Using radical prostatectomy tissue sections as well as biopsies, these changes in calcium channel metabolism were noted throughout the prostate, indicating a field effect. This finding suggests that the presence of a prostate tumor could be detected without the need for direct sampling of tumor tissue, leading to detection of false negative cases missed by H&E stain. The reliability of PSA levels as a prognostic indicator has been questioned in recent years. In the current study, PSA levels were correlated with the P2X<sub>7</sub> labeling results. All patients who exhibited no P2X<sub>7</sub> labeling had a prostatic serum antigen (PSA) level of <2. Patients who exhibited stage-specific P2X<sub>7</sub> expression, and who later developed obvious prostate cancer as diagnosed by H&E stain, all had a PSA > 2. This finding suggests that increasing PSA may be an accurate indicator of cancer development.

### Introduction

Prostate cancer is the second highest cause of cancer death in males after lung cancer in Western society. Although the incidence of prostate cancer is increasing in many countries, there is a lack of reliable prognostic indicators able to predict the behavior of individual tumors. Latent prostate cancer and the pre-invasive form of neoplasia known as prostatic intraepithelial neoplasia (PIN), has been found in 45% of autopsy specimens following death from trauma from men in their 50s to 82% of men aged in their 80s (Sakr *et al.* 1996). More effective early screening and improved accuracy of early diagnosis are high priorities, particularly among those men who present with elevated prostate specific antigen (PSA) levels and who appear negative for cancer at biopsy.

We have previously demonstrated that major changes in calcium regulatory mechanisms, as shown

by the expression of the P2X<sub>1</sub>, P2X<sub>2</sub> and P2X<sub>7</sub> calcium channel receptors, anticipate the diagnostic morphological changes associated with developing neoplasia over times that ranged from as little as 6 months to up to 5 years in individual prostate biopsies (Slater *et al.* 2003, 2004). These receptors were present in the glandular epithelium of cores that initially appeared normal by H&E stain but progressed to cancer at a later stage, as well as epithelium that was obvious cancer by Gleason grading. The first stage of this expression was in the nucleus only. The second stage was characterized by a punctate label throughout the cytoplasm. In the final stage, the label was concentrated on the apical epithelium of the glandular acini. The nuclear and cytoplasmic stages of P2X<sub>1</sub> and P2X<sub>2</sub> calcium channel expression were not accompanied by any abnormal morphological changes as seen by H&E stain. The third apical epithelial stage however, was always accompanied by an

obviously cancerous morphology as diagnosed by H&E stain (Slater *et al.* 2004).

The P2X purinergic receptors are fast, ligand-gated cation channels that open in response to the binding of extracellular ATP. The seven subtypes designated P2X<sub>1-7</sub> exhibit extensive homology (30–40%), particularly in structurally important elements such as the extracellular disulphide bridges. The purinergic (P2X) receptors contribute significantly to Ca<sup>2+</sup> influx to the cytoplasm and therefore apoptosis as well as regulating at least 40 intracellular calcium-binding proteins. These control protein secretion, cell motility and adhesion, invasiveness, cytoskeletal modification, cell junction assembly, tissue differentiation, and phosphorylation as well as nuclear matrix protein composition and expression, cell cycle regulation and DNA transcription (Dethlefsen *et al.* 1998). The ionotropic subtypes P2X<sub>1</sub> and P2X<sub>2</sub> are expressed in prostate epithelium when tumors are found immediately or develop later but not in normal tissue (Slater *et al.* 2003). These changes occurred simultaneously with the appearance of telomerase-associated protein (Matthews & Jones 2001, Slater *et al.* 2003). The appearance of low levels of telomerase activity in apparently normal tissue adjacent to tumors including lung, skin, gut, bladder, pancreas, kidney, cervix, vulva and prostate is suggestive of a field-effect of biochemical changes associated with a biochemical transformation that is undetectable with the use of common histological stains (Ueda *et al.* 1997, Lin *et al.* 1998, Matthews & Jones, 2001). It is unsurprising that several of the P2X receptor subtypes alter their expression in the acinar epithelial cells in the prostate in advance of the morphological changes associated with the development of prostate cancer, given that major biochemical changes occur in tissue that is undergoing developing neoplasia.

In the current study, 174 clinical biopsies and 20 radical prostatectomy cases were immunolabeled with anti-P2X<sub>7</sub> as well as P2X<sub>1</sub> and P2X<sub>2</sub> for comparison. The results were compared with the PSA levels for the corresponding patient at the time of biopsy/surgery.

#### *Antibody production*

Using identical methods as described earlier (Kukley *et al.* 2001), an antibody was raised in rabbit to the epitope G200 - C216 in human P2X<sub>7</sub>. Specificity of the antibody was checked in the usual ways (Kukley *et al.* 2001) with adsorption with conjugate peptide and free peptide eliminating binding. Affinity purification of the protein G-purified IgG fraction was carried out using the epitope bound to Affigel-10 (Bio-Rad). Western blots of HEK cells transfected with mutant E496A or K193A P2X<sub>7</sub> receptor show a single band corresponding to the full-length protein at 80 kDa. In addition,

labeling controls in which the primary antibody was omitted resulted in no labeling.

#### *Immunohistochemistry*

Tissue sections were cut from 1034 paraffin-embedded prostate cores, with an average of 6 cores (apex, mid, base from left and right peripheral zones) from each of 174 patients with a mean age of 67.7 ± 9.1 years. In addition, sections from 20 cases of radical prostatectomy were immunolabeled. Each section was immunolabeled as previously described (Slater *et al.* 2002). Each slide was de-waxed in two changes of fresh HistoClear for 15 min each and then rehydrated. All sections were immersed in 1% hydrogen peroxide for 5 min, washed and then incubated in anti-P2X<sub>7</sub> primary antibody at a concentration of 0.25 µg/ml IgG in PBS for 30 min. Thereafter, slides were washed three times in PBS for 5 min each, followed by a 30 min incubation with anti-rabbit secondary antibody (DAKO). All slides were then washed in PBS for 5 min and visualized using a 0.05% solution of diaminobenzidine (DAB) for 5 min, washed, dried and mounted in Entellan mounting medium (Merck). Approximately serial sections were stained with a standard H&E protocol. For practical, ethical and legal reasons, only formalin-fixed, paraffin-embedded and previously diagnosed biopsies were available for this study.

#### *Statistical analysis*

PSA concentrations were obtained for each patient at the time of biopsy and correlated with the following purinergic receptor translocation (PRT) categories: PRT0 (no label), PRT1 (nuclear label), PRT2 (cytoplasmic label) and PRT3 (apical label on prostate epithelial cells). The nonparametric Mann-Whitney test was used to compare the PSA concentrations from the normal tissue category PRT0 and from the combined P2X<sub>7</sub> positive categories PRT1-3 as no differences were detected between sub-categories. A *p* value < 0.05 was considered statistically significant.

## **Results**

In summary, tumor cells were found with standard H&E examination in 82/174 patients. All 82 were positive for P2X<sub>7</sub> in the epithelial cell cytoplasm. The appearance of this label was identical to that found in previous studies using P2X<sub>1</sub> and P2X<sub>2</sub> as demonstrated. The remaining 92/174 patients appeared normal by H&E. Of these, 33/92 appeared to be cancer cases based on a cytoplasmic P2X<sub>7</sub> staining pattern previously associated with cancer development (Slater *et al.* 2003). 38/92 exhibited a less-advanced staining

Table 1. P2X<sub>7</sub> labeling of 92 biopsies that appeared normal by H&E stain.

38 Biopsies	Nuclear P2X <sub>7</sub> label
33 Biopsies	Cytoplasmic P2X <sub>7</sub> label
21 Biopsies	No P2X <sub>7</sub> label (truly normal)

pattern in which only epithelial cell nuclei stained positively. The remaining 21/92 patients showed no staining in the 174 biopsies examined. These results are summarized in Table 1. The average PSA associated with P2X<sub>7</sub>-free tissue was <1 ng/ml while the presence of any positive label (nuclear or cytoplasmic) was associated with an average PSA of 13.7 ng/ml ( $p < 0.00001$ ).

Of the total 174 patients, 92 cases were originally diagnosed as benign because no cancer was detected in any of the available biopsy cores using H&E stain. Of the total of 82 cases diagnosed with cancer by H&E with Gleason scores in the range 4–10, 70 of the patients had a PSA > 4 ng/ml with 12 of these recording a PSA > 10 ng/ml. In no case previously diagnosed as benign were prominent nucleoli observed with H&E. However, in 66% of cases in which the Gleason score was 6 or more, prominent nucleoli were observed. Patient diagnoses and case notes were used as a guide to separate the cases of benign tissue (which were all collected from patients obviously suspected of having cancer) from both early and late stage cancer cases. Staining patterns in prostate tissue obtained from radical prostatectomy, TURP chips and biopsy core specimens were identical so the staining patterns were not just confined to a zone but were present in all sampled glandular structures. In benign tissue, as originally diagnosed by H&E staining as well as from observation of long-term patient outcome (ie no cancer detected within 5 years of the date of biopsy), P2X<sub>7</sub> receptor expression was totally absent.

The prostate biopsy cores examined in this study revealed a distribution of P2X<sub>7</sub> receptors that was essentially identical to the distribution found in previous work using P2X<sub>1</sub> and P2X<sub>2</sub> receptors (Slater *et al.* 2002). The current study had a substantially increased sample size. Previously diagnosed prostate cancer clinical specimens were used to identify three distinctly separate patterns of purinergic receptor expression that appeared with increasing grade of cancer. Benign tissue appeared entirely devoid of P2X<sub>7</sub> label, verifying the specificity of the antibody. In PRT1 (nuclear label only) cancerous morphology was never found in any biopsied region of the prostate by H&E stain whereas a PRT 2 staining pattern (punctate cytoplasmic) with no residual stage PRT1 (nuclear) staining indicated that a tumor would be found if sampling was sufficiently comprehensive. Since most tumors visible by H&E stain were confined to a small region of the prostate, the majority of sampled cores obtained from

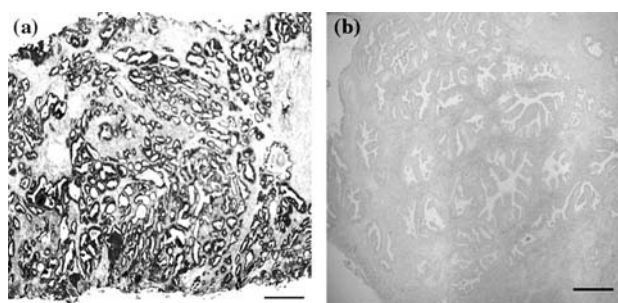


Figure 1. Typical section of radical prostatectomy prostate cancer tissue labeled with P2X<sub>7</sub> shows that the apical glandular epithelium is heavily labeled in all epithelial cells (Figure 2a). A serial section of the tissue obtained shows that when the antibody is added in the presence of 10 μm of the peptide epitope, staining is abolished (Figure 2b). Bars are 0.5 mm.

these cancer patients were generally devoid of morphological changes. All these cancer cases however revealed distinct P2X<sub>7</sub> labeling features in all sampled cores, not just the cores in which obvious cancer was detected. In more developed cancer cases where the tumor was widespread and clear morphological evidence of cancer was apparent by H&E staining throughout all or most sampled cores, the P2X<sub>7</sub> receptors were found condensed exclusively on the apical epithelium (Figures 1 and 5). Labeling of P2X<sub>7</sub> receptors expressed in all prostate tissue that contained a tumor was always observed throughout the entire gland as demonstrated by radical prostatectomy tissue (Figure 1), showing the stage 2 (cytoplasmic) pattern of receptor expression even in those cores that appeared normal by H&E. This field-effect allows cancer anywhere in the prostate to be identified from a single core, regardless of whether the tumor is sampled directly. Only in advanced cases of cancer was the stage 3 pattern (apical deposition) of receptor expression observed.

Figure 1a shows a radical prostatectomy section from a patient with Gleason grade 7. It has been labeled with anti-P2X<sub>7</sub> label. All the glandular acini show apical labeling (PRT3) which is typical of advanced prostate cancer that is easily diagnosed by H&E stain. Note that all the glands are labeled, constituting a field effect. A biopsy core taken from any part of this prostate would yield the same result, preventing false negatives or positives. Figure 1b is a control and is a serial section of that shown in 1a. The P2X<sub>7</sub> antibody was added in the presence of 10 μm of the peptide epitope, causing staining to be abolished.

Figure 2 shows two approximately serial sections of a prostate biopsy core from a 71 year old man with prostatism. The clinical diagnosis was benign prostatic hyperplasia (BPH). No P2X<sub>7</sub> receptor expression was observed in the tissue stroma between acini. The complete absence of label for both P2X<sub>1,2</sub> receptors (Figure 2a) and P2X<sub>7</sub> receptors (Figure 2b) in epithelial

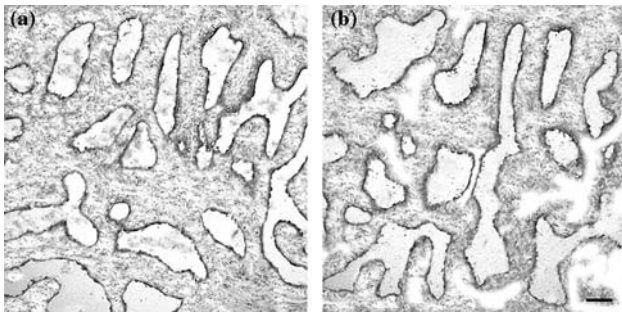


Figure 2. Approximately serial sections of a prostate biopsy core from a 71 year old man with prostatism. The clinical diagnosis using H&E was benign. The complete absence of epithelial expression of both P2X<sub>1,2</sub> (a) and P2X<sub>7</sub> (b) indicates that no early neoplasia or established cancer is present, confirming the diagnosis of benign tissue. These patients did not develop prostate cancer within 5 years. This lack of receptor expression in the epithelial cells has been designated purinergic translocation stage zero or PRT0. Bar = 200  $\mu$ m.

cells indicates that no early neoplasia or established cancer is present, confirming the diagnosis of benign tissue including BPH. We have previously designated this labeling pattern as purinergic receptor translocation stage zero or PRT0. This complete lack of staining for receptors was seen in only 21/92 cases originally diagnosed as benign by H&E (23%) and significantly in 0/82 cases diagnosed with cancer. These 21 patients had an average PSA of  $0.81 \pm 0.10$  ng/ml (mean  $\pm$  sem) with 12/21 in the range 0.2–0.8 ng/ml and 9/21 in the range 1.1–1.8 ng/ml. None of these patients were diagnosed with cancer within 5 years of these biopsies.

The earliest appearance of P2X<sub>7</sub> receptors (purinergic receptor translocation stage 1 or PRT1) was manifest as dense, prominently labeled epithelial cell nuclei. These epithelial cells were surrounded by an unlabeled stroma in which no receptors were expressed. Dilution studies revealed this pattern formed from a dense

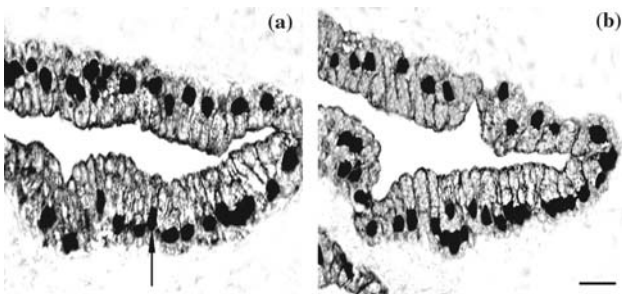


Figure 3. Approximately serial sections of a prostate biopsy core from a 56 year old man. The clinical diagnosis was benign hyperplasia by H&E. The stroma is devoid of labeled receptors. Expression of both the P2X<sub>1,2</sub> (a) and P2X<sub>7</sub> (b) receptors in the epithelium is in the form of prominent epithelial nuclei (PRT1), (a, b – arrows) indicating the presence of early neoplasia. As was the case shown in Figure 1, this labeling pattern was found throughout the prostate, constituting a field-effect. Bar = 20  $\mu$ m.

accumulation of P2X<sub>7</sub> receptors in the nucleus, giving rise to a punctate or granular appearance. These puncta were entirely absent in all genuinely benign tissue. P2X<sub>7</sub> receptor expression occurred in two well-defined stages before the diagnostic histological markers of cancer were visible by H&E staining. As cancer progressed, the location of P2X<sub>1,2</sub> (Figure 3a) and P2X<sub>7</sub> receptors (Figure 3b) changed from initial confinement within individual nuclei in the epithelial cells in the acini to a punctate cytoplasmic label accompanied by the removal of receptors expressed in the nuclei (Figures 3a, b – arrow) which we have termed PRT stage 2 or simply PRT 2.

Figure 3 shows approximately serial sections of a prostate biopsy core from a 56 year old man with a PSA of 8.4. The clinical diagnosis was again benign by H&E. No P2X<sub>1,2</sub> or P2X<sub>7</sub> receptors were found in the stroma, which consequently remained unlabeled with the antibody. Both the P2X<sub>1,2</sub> (Figure 3a) and P2X<sub>7</sub> (Figure 3b) antibodies bind to the separate receptor epitopes expressed in the nuclei (Figure 3a – arrow), previously described as PRT1 (Figure 3a – arrow).

The second stage of positive labeling (purinergic receptor translocation stage 2 or PRT2) was characterized by the appearance of P2X<sub>7</sub> receptors forming a punctate distribution throughout the cytoplasm of the epithelium. This apparent transport of receptors from nucleus to cytoplasm resulted in progressive de-expression of the (PRT1) nuclear label to an end-point where the nuclei became completely devoid of labeled receptors. These nuclei appeared outlined by the presence of the darkly staining punctate cytoplasmic label (see Figure 4). Some residual receptor label could occasionally be found on the nuclear membranes. PRT1 labeling was seen in 22% (38/174) of biopsied samples suspected of harboring some cancer but diagnosed as

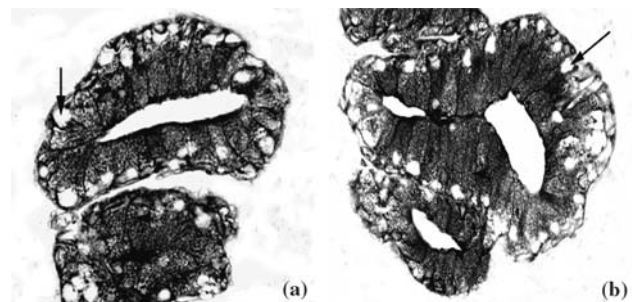
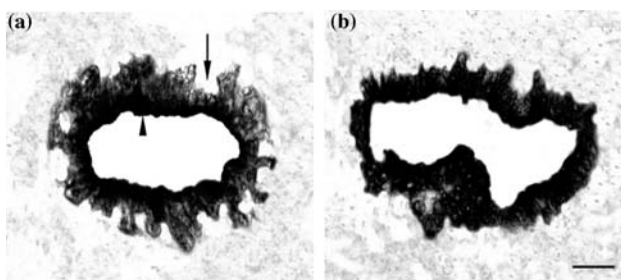


Figure 4. Approximately serial sections of a prostate biopsy core from a 57 year old. The overall clinical diagnosis was Gleason score 6 but the area depicted is from a core well-removed from the site of the tumor that was reported as benign by H&E stain. The stroma is devoid of expressed receptors. The expression of both the P2X<sub>1,2</sub> (a) and P2X<sub>7</sub> (b) receptors in the epithelium is in the form of translocating cytoplasmic receptors, which have the appearance of granules (puncta). The nuclei were devoid of label (arrows). This pattern is termed PRT2. It identifies the presence of established cancer elsewhere in the prostate due to the tissue-wide field-effect.

benign on the basis of H&E staining patterns. Once again the staining pattern was observed in each of the sampled biopsy cores collected from each of these patients. All 38 examples of PRT1 were detected in the cohort of 92 patients previously diagnosed as normal or benign. These 38 patients all exhibited a higher PSA ( $13.4 \pm 1.1$  ng/ml, mean  $\pm$  sem) at the time of biopsy than any of the 21 cases diagnosed by us as PRT0/normal or benign. Only 6/38 patients had a PSA  $< 4$  ng/ml (range 3.6–3.9 ng/ml) while 9/38 had PSA levels in the range 4–10 ng/ml and the remainder (23/38) had a PSA  $> 10$  ng/ml.

Figure 4 shows approximately serial sections of a prostate biopsy core collected from a 57 year old man with a PSA of 12.8. The overall clinical diagnosis, based on an analysis of all cores taken, was Gleason score 6 but the area depicted was from a core that was diagnosed as benign by H&E stain. No receptors were detected in the stroma. Both the P2X<sub>1,2</sub> (Figure 4a) and P2X<sub>7</sub> (Figure 4b) epithelial cell labels clearly show translocating receptor puncta in the cytoplasm forming a granular appearance. The nuclei in the epithelial cells were devoid of receptors and so appeared unstained (arrows). A similar pattern was observed in all the cores collected from this patient regardless of the presence of cancer detectable by H&E stain.

Figure 5 shows approximately serial sections of a prostate biopsy core from an 86 year old man with a PSA of 16.8. The clinical diagnosis was Gleason score 7. In this case, prostate cancer was obvious throughout the bulk of the prostate by both H&E stain and purinergic receptor labeling. This labeling pattern represents the end stage of receptor translocation and is designated PRT3. It describes intense labeling of P2X<sub>1,2</sub> (Figure 5a) and P2X<sub>7</sub> (Figure 5b) receptors on



**Figure 5.** Approximately serial sections of a prostate biopsy core from an 86 year old. The clinical diagnosis by H&E stain was Gleason score 7. In this case, prostate cancer was obvious throughout the prostate by both H&E stain and purinergic receptor labeling as also shown in Figure 1. This receptor labeling pattern represents the end stage of receptor translocation designated PRT3. It shows intense P2X<sub>1,2</sub> (a) and P2X<sub>7</sub> (b) receptor labeling on the apical epithelium (5a – arrowheads) while the nuclei and cytoplasm are now largely devoid of label (a – arrow). This pattern is associated with the distinct morphological changes that are usually diagnostic of advanced cancer. Other tissue in the same prostate that appeared normal by H&E exhibited PRT2 labeling. Bar = 20  $\mu$ m.

the apical epithelium (Figure 5a – arrowhead) while the nuclei and cytoplasm are largely devoid of labeled receptors (Figure 5a – arrow). The apical P2X<sub>7</sub> label was characteristically dense and homogeneous rather than punctate. In cases where the histological appearance by H&E stain was obviously cancer, PRT3 labeling was predominant (Figures 1 and 5). A total of 115/174 patients were found to exhibit PRT2 or PRT3 stage labeling. This represents an additional 33/174 patients who have cancer but were false negatives based on sampling of the prostate having missed the tumors. Of these 115 patients 17 presented with a low-range PSA (0.5–3.9 ng/ml) at the time of biopsy, 44 presented with mid-range PSA (4–10 ng/ml) and the remaining 47% or 54/115 presented with a higher-range PSA ( $> 10$  ng/ml). The overall average PSA readings for the 115 patients found to have either detected cancer by H&E as well as PRT labeling (82 cases) plus the additional 33 cases detected with otherwise hidden tumor by PRT alone was  $13.8 \pm 1.2$  ng/ml. This was not significantly different from the value found for patients exhibiting only PRT1 labeling. The combined average for all patients for whom any positive PRT stage labeling was detected was  $13.7 \pm 1.1$  ng/ml. The Mann–Whitney two-tail *t*-test analysis of the two populations PRT0 and the combined group PRT1–3 shows a level of significance at the level  $p < 0.00001$ . Table 2 summarizes the PSA *versus* PRT label results for all patients.

## Discussion

All biopsies were taken from patients who were strongly suspected of having prostate cancer by their clinicians. As expected from any such patient cohort, a significant proportion of positive cases are routinely missed due to the obvious difficulty of sampling a small tumor within the whole prostate, even with the assistance of ultrasound. Many small tumors can escape being sampled.

H&E stain analysis of each of the average of 6 cores collected for each patient uncovered 82/174 patients with demonstrable cancer by the usual morphological criteria (H&E stain). P2X<sub>7</sub> labeling revealed an additional 33 (or 19%) that were previously undetected by H&E stain. These biopsies had a cytoplasmic P2X<sub>7</sub> label, indicating established neoplastic change. A further 38/174 patients (22%) were found to have presented with early neoplastic changes as evidenced by PRT1 (nuclear P2X<sub>7</sub>) labelin. Only 21/174 patients (12%) showed no P2X<sub>7</sub> labeling at all and therefore free of developing or established neoplasia. These results strongly indicate the original clinicians' suspicions that prostate cancer was present in those of the patient cohort who had high or increasing prostate-specific antigen (PSA) concentrations or other clinical

Table 2. The PSA concentrations in the individual categories PRT1 (early neoplasia), PRT2 and PRT3 (advanced cancer) were statistically equal and were thus combined.

PRT0 staged tissue ( $n = 21$ )			PRT1-3 staged tissue ( $n = 153$ )		
PSA range (ng/ml)	$n$	%	PSA range (ng/ml)	$n$	%
PSA < 1	12	57.1	PSA < 1	1	0.6
PSA 1–2	9	42.9	PSA < 3	2	1.3
			PSA < 4	16	10.5
			PSA 4–10	47	30.7
			PSA > 10	90	58.8
Average			Average		
PSA			PSA		
$0.81 \pm 0.10$			$13.7 \pm 1.1$		

The Mann–Whitney analysis of the two populations categorized as PRT0 and PRT1-3 showed that PSA concentrations were significantly different ( $p < 0.00001$ ).

symptoms indicating the likelihood of prostate cancer. The expression pattern of P2X<sub>7</sub> receptors in the current study was identical to that found for the P2X<sub>1,2</sub> receptors, as previously described (Slater *et al.* 2002).

The presence of P2X<sub>7</sub> nuclear (PRT1) or cytoplasmic (PRT2) labeling features in prostate tissue that appears normal or benign by standard H&E may be used to detect some of the early metabolic cellular changes that precede and accompany an existing underlying neoplastic process. As these processes advance they eventually manifest in morphological changes that become visible with H&E stain. The labeled receptors found in the nucleus (PRT1) and cytoplasm (PRT2) can only become functional after they have been transported to the apical epithelium (Dutton *et al.* 2000) and consequently the appearance of these early stages of receptor labeling is not yet associated with obvious changes to calcium metabolism.

The presence of nuclear and cytoplasmic P2X<sub>7</sub> (PRT1,2) staining features in otherwise apparently normal tissue is suggestive of early neoplasia given that P2X<sub>7</sub> receptor labeling in the acinar apical epithelium was detected in every case of obvious cancer as diagnosed by H&E stain. The reason for a nuclear label pattern is unclear. Some epitopes may form within the nucleus or on the nuclear membrane itself. Only some of the cases labeled in the categories PRT1-2 had any sign of PIN or other morphological abnormality. Of the 92 cases previously diagnosed as normal or benign by H&E, 23% (21 cases) with a PSA from 0.2–1.8 ng/ml exhibited no P2X<sub>7</sub> receptor expression at all in any of the sampled cores. We propose that these cases represent genuinely normal or benign prostate tissue, with no evidence of early neoplasia. Indeed, none of these patients were found to have cancer within 5 years following biopsy. The P2X<sub>7</sub> labeling pattern was of the same stage in all cores throughout the prostate of each case. A finding of PRT2 in prostate tissue that was apparently normal by H&E was also suggestive of the an unsampled tumor somewhere else in the prostate. H&E staining could only detect cancer by direct

sampling of the tumor. In contrast, labeling of P2X<sub>7</sub> receptors was correlated with the presence of unsampled cancer within the prostate gland. We propose that all the cores that showed these features of PRT1 and PRT2 indicate the presence of underlying early neoplasia and/or moderate grade cancer. PRT3 always accompanied high grade cancer that was easily diagnosed by H&E stain.

A significant finding in the current study is the fact that PSA concentrations appear to be far more accurate than had been previously thought. Generally about 10% of assumed benign or BPH cases exhibit a PSA > 4 ng/ml while about 15% of diagnosed cancer cases exhibit a PSA < 4 ng/ml. This anomaly currently makes the overall accuracy of PSA as a screen little better than 70%. The highest PSA recorded for the 21 normal (no P2X<sub>7</sub> label or PRT0) patients was 1.8 ng/ml. All cases with P2X<sub>7</sub> labeling had an average PSA of  $13.7 \pm 1.1$ . The accuracy of PSA screening in conjunction with P2X<sub>7</sub> labeling results in an accuracy of approximately 95% once the false negatives arising from unsampled tumors are eliminated. P2X<sub>7</sub> positive epithelial prostate tissue is also correlated strongly with a corresponding elevation in PSA measured in the serum of the patient at the time of the biopsy.

A major benefit of the P2X<sub>7</sub> label PRT staging technique is the field-effect that reveals the presence of unsampled cancer in the prostatic epithelium, even in areas that appear normal or benign by H&E stain. Regardless of whether the observed labeling pattern anticipates the development of cancer or is a consequence of the influence of cancer on the adjacent epithelium (Xue *et al.* 1997), it is clearly apparent that the presence of cancer in the prostate can be readily detected by P2X<sub>7</sub> labeling and that truly negative cases can be readily distinguished. This method may also be employed to provide data on the rate of progression of individual tumors within patients and to monitor the progress of treatments since only minimal tissue need be collected at biopsy due to the presence of the organ

wide field-effect. Consistent increasing PSA concentrations above 2 ng/ml should be considered highly likely to be associated with developing prostate cancer, although the rate of development of the disease cannot be determined by PSA alone. Elevated PSA may not be accompanied by an obvious tumor for several years, in which case only PRT1 stage (nuclear) label should be present.

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