Engrailed-2 (EN2): a tumour specific urinary biomarker for the early diagnosis of prostate cancer

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Statement of Translational Relevance

Despite recent publication of numerous putative biomarkers of prostate cancer we are still completely dependent clinically on serum PSA. There is still an urgent need for new markers and particularly those identifying significant cancers. This study evaluates a transcription factor secreted by prostate cancer as a simple ELISA test without the requirement for DRE. The sensitivity, specificity and stability of EN2 at room temperature would make this test attractive for diagnosis of prostate cancer and also potentially as a simple screening tool in the community.

Abstract

Purpose: Prostate cancer (PC) is the second most common cause of cancer related death in men. A number of key limitations with prostate specific antigen (PSA), currently the standard detection test, has justified evaluation of new biomarkers. We have assessed the diagnostic potential of EN2 protein, a homeodomain-containing transcription factor expressed in PC cell lines and secreted into the urine by PC in men.

Experimental Design: EN2 expression in PC cell lines and prostate cancer tissue was determined by semi-quantative RT-PCR and immunohistochemistry. First pass urine (without prior digital rectal examination (DRE)) was collected from men presenting with urinary symptoms (referred to exclude/confirm the presence of prostate cancer) and from controls. EN2 protein was measured by ELISA in urine from men with PC (n=82) and controls (n=102).

Results: EN2 was expressed and secreted by PC cell lines and PC tissue but not by normal prostate tissue or stroma. The presence of EN2 in urine was highly predictive of PC, with a sensitivity of 66% and a specificity of 88.2%, without requirement for DRE. There was no correlation with PSA levels. These results were confirmed independently by a second academic centre.

Conclusions: Urinary EN2 is a highly specific and sensitive candidate biomarker of prostate cancer. A larger multicentre study to further evaluate the diagnostic potential of EN2 is justified.

Introduction

Prostate cancer (PC) is the second most common cause of cancer related death in men, with approximately 913,000 new cases world wide in 2008 (1). Localised, organ-confined PC can be cured in a large proportion of patients by surgery or radiotherapy. Advanced and metastatic PC continues to be associated with a poor prognosis (2). Serum prostate specific antigen (PSA) has been used as a cancer marker for initial diagnosis, monitoring of response to treatment, prediction of PC risk and of treatment outcome. As a prostate-specific and not prostate *cancer*-specific marker, it lacks both sensitivity and specificity to accurately detect the presence of PC, requires adjustment for age and prostate volume, is frequently raised in non-cancer conditions such as benign hypertrophy and prostatitis and so far has been controversial as a screening tool (3-5). A conventional cut off level of 4ng/ml has predictive value for detection of the prostate cancer (6,7) but only 15% of cancers were detected at prostate biopsy in the Prostate Cancer Prevention Trial (PCPT) (8). Higher detection rates of up to 44% have been reported at this PSA level in other studies, but these, unlike PCPT, did not evaluate healthy asymptomatic men (8-10). Studies of men at different ages also suggests differential incidence of prostate cancer. For the third through eighth decades of life, the incidence of prostate cancer in the cohort of 1051 subjects studied by Sakr *et al* was 7%, 23%, 39%, 44%, and 65%, respectively (11). Lowering PSA cut off levels results in higher sensitivity at the expense of much lower specificity and true negative rates of 70-80% (8, 12, 13). No refinement of PSA (e.g. free: total PSA ratio) or other biomarkers have

reduced this true negative rate (13).There is therefore an urgent need for new markers to overcome at least some of the limitations of serum PSA.

Biomarkers signifying the presence of any cancer may be defined on the basis of gene products uniquely expressed or overexpressed in tissue, serum or urine, in cancer compared to non-cancer. A number of genes are involved in early embryonic development and are subsequently re-expressed in cancer, for example the *HOX* genes, a family of homeodomain-containing transcription factors that determine the early identity of cells and tissues (14). We and others have shown that HOX gene dysregulation occurs in most common cancers, with evidence that targeting HOX/PBX binding has therapeutic value (15-19). We have studied *Engrailed-2* (*En2*), another member of this group which show a very high degree of functional conservation during development (20). *En2* is a transcriptional repressor, but is also has a role in translational regulation (20). In addition to its developmental role, *En2* has recently been shown to be a potential oncogene in breast cancer, as forcing its expression in the non-malignant mammary cells induces a malignant phenotype including increased cell proliferation and a loss of contact dependence (21).

In this study we show that *En2* is expressed in, and secreted by, prostate cancer but not normal prostatic tissue. The presence of EN2 protein in urine has been evaluated as a diagnostic biomarker for PC.

Materials and Methods

Patient and controls

This study was approved by the local research ethics committee (reference 09/H1109/84), and took place between June 2007 to June 2010. In total 194 urine samples were collected by the Surrey site. Men with lower urinary tract symptoms, individuals concerned they may have an asymptomatic PC (e.g. a positive family history) and men with an abnormal PSA test reading conducted by their family physician were referred into our specialist Uro-Oncology clinic. The purpose of the referral was to exclude or confirm the presence of prostate cancer. Men with known PC on treatment, with any other known concurrent or previous cancers within 10 years or urinary tract infection (as determined by the presence of leukocytes using a dipstick test) were excluded. Urine samples from patients and controls were collected prospectively; samples were blinded to laboratory staff at the time of EN2 measurement.

Two control groups of men >40 years were also assessed:

(a) ‘low PSA group A’; men presenting to haematuria clinics where no urothelial malignancy was found (single episode of haematuria: radiology, cystoscopy and cytology negative) but in whom DRE and PSA (routinely performed in this clinic) were normal, PSA below 2·5 ng/ml.

(b) ‘low PSA group B’; men over 40 years with no symptoms or family history of PC and who had undergone routine health screening by family physicians (conducted once or twice yearly: physical examination, diabetes screening of serum and urine, serum cholesterol and serum PSA levels even in the

absence of lower urinary tract symptoms and family history of prostate cancer) and had a documented PSA below 2·5 ng/ml.

The majority of samples were donated the day after consultation. 5-10mls of first pass urine samples were donated pre-biopsy, prior to any hormone therapy and not immediately after DRE (range 4-24 hours post DRE). For the non-cancer control groups no DRE was performed prior to urine donation. Urine was stored in 1·5 ml aliquots at -80°C. Blood for PSA analysis was always drawn before urine collection. Histological examination of prostate biopsies was performed by a specialist uro-pathologist. The 2003 UICC TNM classification was applied. Gleason scores, clinical stage and PSA were available in all cancer cases.

EN2 protein detection – western blotting

1·5ml of urine were centrifuged at 10,000g for five minutes to remove cells and cellular debris. 20μl of the supernatant were then mixed directly with gel running buffer (Invitrogen, Paisley, UK). Proteins were resolved by 10% SDS–polyacrylamide gel electrophoresis and transferred to a polyvinylidene fluoride membrane (Invitrogen, Paisley, UK). Anti-EN2 antibody (ab45867; Abcam, Cambridge, Cambridgeshire, UK) was used at a concentration of 0·5μg/ml, and a goat-anti human IgG peroxidise-labelled antibody was used together with the ECL chemiluminescent system for detection.

EN2 protein detection – Enzyme-linked Immunosorbent Assay (ELISA)

Two monoclonal mouse anti-EN2 antibodies were raised using the synthetically produced C-terminal 100 amino acids (Biosynthesis Inc, Lewisville, Texas, USA) of EN2 as an antigen (Antibody Production Services

Ltd, Haywards Heath, Sussex, UK). One of these, APS1, was conjugated to alkaline phosphatase using the Lightning Link alkaline phosphatase conjugation kit (Innova Biosciences, Cambridge, Cambridgeshire, UK), whilst the other, APS2, was conjugated to biotin using the Lightning Link Biotin Conjugation kit (Innova Biosciences, Cambridge, Cambridgeshire, UK). APS2-biotin was captured onto a 96-well streptavidin-coated plate (Nunc 436014, Rochester, New York, USA) at a concentration of 4μg /ml. After washing, 100μl of urine or a dilution of the EN2 fragment in buffer was incubated in each well for 1 hour at room temperature. The plate was then washed 8 times in buffer (PBS with 0·1% Tween-20) and the secondary detection antibody – APS1-Alkaline phosphatase was added to each well at a concentration of 4μg/ml (1 hour at room temperature). After a final wash step a colormetric agent – pNPP (Sigma, St Louis, MO, USA) was added and the absorption of light at 405nm was measured after one hour. The dilution series was used to generate a standard curve by which the concentration of EN2 in each sample was measured (supplemental figure 2).

cDNA Synthesis

RNA from prostate tumours and normal adjacent tissue was obtained from urology clinic patients. Prostate biopsy tissue was taken contiguous to a routine sextant biopsy (from which histology was later confirmed), placed in OCT (optimal cutting compound), snap frozen and stored in liquid nitrogen. RNA was reverse transcribed as described previously (16). RNA was first denatured by heating at 65^{o}C for five minutes. 1-5μg of RNA was incubated in a volume of 50μl at 37^{o}C for one hour with final concentrations of 10mM DTT,

1mM dNTP mix, as well as 100ng/μl polyT primers, 200 units of reverse transcriptase (Invitrogen, Carlsbad, CA, USA) and 40 units of RNaseOUT (Invitrogen, Carlsbad, CA, USA). The cDNA synthesis reaction was terminated by placing tubes at 80^{o}C for five minutes.

Semi-quantitative RT-PCR

Semi-quantitative RT-PCR was performed using the Stratagene MX4000 Real Time PCR machine, measuring PCR product accumulation during the exponential phase of the reaction by SYBR green fluorescence. Reaction conditions were 1 cycle of 94^{o}C for 10 minutes, followed by 40 cycles of 30 seconds at 94^{o}C, 1 minute at 60^{o}C and 30 seconds at 72^{o}C. The forward and reverse primers for *En2* were 5' GAACCCGAACAAAGAGGACA 3' and 5' CGCTTGTTCTGGAACCAAAT 3', and for *Beta actin* they were 5' ATGTACCCTGGCATTGCCGAC 3' and 5' GACTCGTCATACTCCTGCTTG 3'.

Immunohistochemistry

Expression of EN2 in PC and normal prostate was investigated using 3μm thick formalin fixed, paraffin embedded tissue array (PR2085a, US Biomax, Rockville, MD, USA) and patient prostate biopsies. Immunohistochemical analysis was performed using a polyclonal rabbit anti-EN2 antibody (Abcam, Cambridge, Cambridgeshire, UK #28731) diluted 1:100 and the ABC detection method with peroxidase block (DakoCytomation). Brain sections were used as a positive control. Antigen retrieval was performed using pH9.0 Tris/EDTA buffer (DakoCytomation) and heating in a microwave for 23

minutes. The tissue arrays described in this study were stained using the same method and were obtained from Biomax US (PR2085a).

Cell culture

PC3 (22), DU145 (23), and LNCaP (24), prostate cancer cell lines, together with WPMY-1, a non-malignant fibroblast line derived from prostate stroma (25), were obtained from the ATCC (through LGC Standards UK), and maintained according to ATCC protocols supplied for each cell line. The conditioned media used for EN2 detection was taken from cells grown to 90% confluence without a media change after seeding 10% confluence.

Statistical analysis

The Graphpad prism package was used in statistical calculations. In order to test the significance of differences between mean EN2 concentrations in different patient groups we used an unpaired t-test with Welch's correction. Receiver operator characteristics (ROC) curves were generated for the EN2 and PSA values. The area under the curve was tested for significance using an unpaired t-test against the hypothesis that the real area under the curve was 0·5 (i.e. no diagnostic value).

Results

The complex regulatory functions and oncogenic potential (20) of *En2* led us firstly to study the expression of *En2* in PC cell lines. The differential expression of *En2* by malignant versus non-cancer cells was studied by measuring the number of *En2* transcripts in the human PC cell lines PC3, DU145, and LNCaP together with a non-malignant cell line derived from normal prostate fibroblasts, WPMY-1 (Fig 1a). Of these lines, PC3, DU145 and LNCaP all expressed *En2*. In addition, RNA was extracted from prostate biopsy cores and *En2* expression quantified by quantitative PCR. Of 12 cores that were histologically positive for cancer 10 expressed *En2*, whilst none of the 8 cores negative for cancer showed *En2* expression (Fig 1b). As EN2 protein can be secreted physiologically from some types of cell (20, 26) we looked for the protein in the conditioned medium surrounding PC3, DU145, LNCaP and WPMY-1 cells by Enzyme-linked Immunosorbent Assay (ELISA). PC3, LNCaP and DU145 cell lines release EN2 protein into the surrounding medium, but not WPMY-1 (Fig 1c), and immunofluorescent imaging of EN2 in PC3 cells revealed a high concentration of this protein close to the membrane consistent with its secretory potential (Fig 1d).

These findings were supported by immunohistochemical study of PC and normal prostatic tissue using an anti-EN2 antibody stain in a large representative 195 core tissue array and in patient biopsies. In the tissue array EN2 was highly expressed in prostate cancer (92%, n=184 prostate cancer cores), but not in normal tissue adjacent to the tumour (0%, n=11), or

in normal prostate cores (0%, n=9). We found no evidence of EN2 staining in normal prostate tissue, benign hypertrophy nor in men with HGPIN (high grade prostatic intra-epithelial neoplasia) in any tissue array section or biopsy from our patients. Analysis of larger tumour sections taken by biopsy revealed EN2 expression is most intense in the duct like structures of tumours (Fig 2a-c), and that EN2 protein is present in the cytoplasm, and in some cases in the basal membrane, but not in the nucleus (Fig 2c). Furthermore, blebs containing EN2 protein are apparent in prostatic acini and ducts (Fig 2b, c). This widespread distribution contrasts markedly with normal adult purkinje neurons where EN2 protein, as expected, is confined to the nucleus (27) (supplemental Fig 1). We also compared the staining of EN2 to that of a known prostate cancer specific antigen, alpha-methylacyl-coenzyme A racemase (AMACR) (28); merged images of each staining pattern show an almost complete overlay (Fig 2d-f).

Given the secretory properties of EN2 in embryonic development (29) and the observation of EN2-positive blebs within the lumen of malignant prostatic ducts, we looked for EN2 protein in the urine of men with biopsy-confirmed but untreated PC and controls. EN2 protein in untreated, unconcentrated urine from PC patients could be detected by western blotting with a band corresponding to full length EN2 protein (33kDa; Fig 3), but not after prostatectomy in the same patients and not in non-cancer individuals.

The ELISA assay for EN2 (supplemental Fig 2) was used to screen representative populations of patients with prostate cancer and relevant

controls. The stability of EN2 protein in urine was shown to be at least four days at room temperature (supplemental Fig 3), allowing postal collection of some samples. The demographics of patients and control groups are shown in table 1. The mean age of men in the study was similar (range 57-67 years). Median PSA (6·3-7·6 ng/ml) was similar in men suspected of PC versus the two low PSA control groups (0·9-1·1 ng/ml), as expected. Using a cut off of 42·5μg/L, EN2 protein was detected in 54 of the 82 (66%) men with PC confirmed by biopsy ('Biopsy (+)'). Notably, in 9 men in this EN2 positive group the PSA was < 2.5 ng/ml. In men with high PSA and no cancer on biopsy ('Biopsy (-)'), EN2 was detected in 6 of 58 (10·3%). In our control groups EN2 detection was also infrequent: in 'low PSA group A' EN2 was detected in 2 of 17 (11·7%); in low PSA group B EN2 was detected in 4 of 27 (14·8%). An exceptionally high level of EN2 was found in the urine of one individual in control 'low PSA group B' who had a PSA of 1.2ng/ml.

An ROC analysis for this data (Biopsy (+) v Biopsy (-)) revealed that the area under the curve was 0.8021 ($p<0·001$), indicating a high diagnostic potential for EN2 (Fig 4b). The mean concentration of EN2 protein was 10·4 fold higher in PC patients compared to that in all the men that were not known to have cancer (Fig 4a). We found no significant correlation between EN2 expression and combined Gleason score, although the majority of patients in our cohort had disease with a combined Gleason score of 6 or 7 (Fig 5a). There was also no significant correlation between serum PSA level and presence or absence of urinary EN2 (Fig 5b). EN2 was also found in the urine of 3 of 10 men (30%) with high grade prostatic intraepithelial neoplasia (HGPIN; Table

1; Fig 4a); two of these 3 men were found to have PC within 6 months upon re-biopsy.

In order to validate our findings with respect to urinary EN2 secretion, a similar study was completed independently at Cambridge University following the same collection protocol. EN2 detection by ELISA followed exactly the same protocol as for the Surrey University study. The results are summarised in Table 1 and are similar to those obtained for the Surrey patients. Of 13 control patients two were found to have EN2 in their urine (15%), whilst 47 of 81 patients with prostate cancer were positive for EN2 (58%).

Discussion

In this study we have shown that the transcription factor *En2* is expressed by PC-derived cell lines and in primary prostate tumours, but not in normal prostate tissue. Our data indicates that EN2 protein is also *secreted* by both cell lines and primary tumours, and is found in the majority of PC patients in first pass urine collected without preceding DRE, but not in non-cancer controls. We found that EN2 is associated with a sensitivity of 66% and specificity of almost 90% using the 42·5 ng/ml cut off. The maximum specificity that can be achieved using this assay is 100%, using a cut off at 1927 ng/ml, but the resulting sensitivity is only 2·5%. The maximum sensitivity (73%) is achieved using a cut off at 1·5 ng/ml and the resulting specificity is 80%. The cut off value of 42·5 ng/ml was selected to give both high sensitivity and specificity.

PSA is a valuable tool for the early detection and monitoring of PC, but PSA values vary with prostate volume, age and raised levels are seen in non-cancer disorders such as prostatitis. The specificity and sensitivity of PSA has been difficult to fully determine as men with low PSA levels rarely undergo biopsy. The Prostate Cancer Prevention Trial (PCPT) biopsied 5112 men in the placebo arm irrelevant of PSA level. A PSA >4ng/ml had a sensitivity of 24% and specificity of 93% (30). The same study confirmed clinical experience that some PC are associated with very low levels of PSA production. PC was present in 6·6% of men where PSA <0·5ng/ml and 27% men had PSA of 3·1-4 ng/ml (8). Ultimately the choice of PSA ‘cut off’

balancing sensitivity against specificity results in false positive tests and large numbers of unnecessary biopsies (31, 32). A recent study has indicated that PC mortality may be reduced almost by half over 14 years by early detection by PSA in a screening program. Although this was achieved at the expense of 'over diagnosis' the potential value of biomarkers in early disease was demonstrated (5). There have been no new serum based prostate cancer detection tests sufficiently specific or sensitive to reach the clinic in the last decade. Recently published urine-based tests share the limitations of PSA to varying extents and mostly require urine collection post-DRE. Most notable amongst these is PCA3, a non-coding RNA present in urine (33) and Annexin A3 (34). PCA3 has a higher specificity than PSA (76% to 89%) and the detection rate of PC is reported to be 34%, but it still gives significant numbers of false positive results (33). Furthermore it requires urine post-DRE and is expensive and complex to use. It is used to help decide whether to proceed to repeat prostate biopsy in cases where the PSA is persistently high. Annexin A3 is a protein present at higher concentrations in patients that have PC, and has a sensitivity and specificity of 31.2% and 90%, respectively (34). It only has utility in conjunction with urinary PSA measurements, which themselves are subject to all the potential flaws of serum PSA discussed earlier. Other recently described urinary prostate cancer biomarker approaches that have been based on matrix modelling of multiple markers (35) are complex, not immediately applicable to routine clinical practice and may be more appropriate for stratifying patients in clinical trials. In contrast, EN2 can be detected in 100μl of unprocessed urine, collected without the requirement for DRE and employs a simple enzymatic detection method. DRE expels cells

into urine and is the basis of tests such as PCA3 where mRNA from these expelled cells is extracted. EN2 is secreted by the prostate cancer cells, the cellular component of the collected urine is discarded and only the supernatant evaluated after spinning down. We have seen negligible changes in EN2 concentrations pre-DRE and post-DRE urine to date (n=12). This is being further evaluated in our current follow up study in larger numbers of individuals. The clinical potential of EN2 is further supported by our findings of its stability in urine at room temperature for at least 4 days without preservative allowing routine transport of samples to diagnostic labs.

The high predictive value of urinary EN2 raises the possibility that it could be used alongside PSA in the primary diagnosis of PC in patients presenting with lower urinary tract symptoms or a family history of PC, and reduce unnecessary prostate biopsies. Further potential utility of EN2 as a prostate cancer biomarker was demonstrated by its presence in the urine of men with low PSA (<2.5 μg/L) but histologically confirmed prostate cancer on biopsy (n=9). Non-PSA secreting PC represent a small proportion of all cases but are problematic (36). This study found high levels of EN2 in the urine of one individual from our control group 1 (he was asymptomatic:no urinary symptoms, weight loss or pain suspicious of metastatic involvement, and had a PSA of 1·2 during a general health screening by his family physician). It is of course possible that he and individuals designated ‘false positive’ in this study may be harbouring non-PSA secreting PC or have PC present in transitional zone or the anterior horn of the peripheral zone. This patient was discussed with the ethics committee and referred to a urologist for further assessment.

The ELISA based assay we have developed will potentially allow a number of different detection platforms including a lateral flow application with a ‘dip stick’ test which could be performed quickly and cheaply in primary care or as a component of a large scale screening program (if justified by further studies). EN2 may therefore be positioned as an adjunct to PSA for diagnosis in symptomatic or ‘at risk’ individuals (e.g. positive family history, HGPIN), in men with ‘bordeline’ PSA levels or in general population screening in view of its simplicity and no requirement for DRE. The greatest uptake would most likely be by family physicians for these reasons. The expression of EN2 in the tissue and urine of individuals with other urothelial cancers (renal, bladder and ureteric) is currently being determined to address its ultimate specificity for prostate cancer. In addition, a larger multicentre study is planned to determine whether EN2 could be used as a monitoring tool (PSA was originally approved for this purpose), the effect of surgery, hormonal and radiotherapy on EN2 secretion into urine, and also whether levels of urinary EN2 correlate with tumour stage and Gleason grade. However, as with every PC biomarker in development, the ultimate question will be centred around the utility of EN2 (alone or in combination with other markers) in identifying ‘significant’ cancers that require early intervention.

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FIGURE LEGENDS

Figure 1. *En2* expression in prostate cancer. (a) RT-QPCR analysis of *En2* expression in the PC-derived cell lines PC3, LNCaP, and DU145, and the non malignant, stromal derived fibroblast line WPMY-1. Expression is shown relative to the *Beta-actin* gene (x10000). (b) RT-QPCR analysis of *En2* in biopsies that were found to be histologically positive ('PC') or negative ('Non PC') for prostate cancer. *** denotes $p<0.001$. Expression is shown relative to the *GAPDH* gene (x10000). (c) EN2 protein is present in the conditioned medium from prostate cancer cell lines. EN2 protein in the media surrounding PC3, DU145, LNCaP and WPMY-1 cells was quantified using Enzyme-linked Immunosorbent Assay (ELISA). (d) Immunofluorescent microscopy showing the distribution of EN2 protein in PC3 cells. Arrowheads indicate high concentrations of EN2 adjacent to the membrane. Left image – EN2 staining only (green). Right image – EN2 staining (green), merged with DAPI staining (showing the position of the nucleus, blue) and a light micrograph. Scale bar: 5μm.

Figure 2 (a) Core biopsy of a prostatic adenocarcinoma stained with anti-EN2 antibody. EN2 positive staining (brown) is present in tumour cells. Magnification: x30. (b) Higher magnification of a tumour duct. EN2 is present in the cytoplasm of tumour cells, with strongest staining at the luminal border (1). EN2 positive blebs are visible attached to the luminal border (2) or free within the lumen (3). The nuclei are stained blue. Magnification: x100. (c) A malignant prostatic acinus showing strong EN2 staining in the cytoplasm and

within a secretory bleb (S), but not in the nuclei (N). Magnification: x120. (d-f) Section through a prostate tumour; tumour cells are stained using the prostate cancer specific antigen Alpha-methylacyl-coenzyme A racemase (AMACR) (d). The same section was also stained with fluorescently labelled anti-EN2 (green) (e). The merged image reveals an almost identical pattern of staining (f). Magnification (d-f): x60.

Figure 3. EN2 protein detection in urine by western blot. Urine was collected from individuals being examined for urinary symptoms consistent with prostate cancer, and controls. The presence of PC was confirmed by biopsy. The predicted MW of EN2 is 33 kDa and a band of this size was observed exclusively in the urine of some patients with prostate cancer. The representive blot shows a positive result for a patient with PC pre-prostatectomy but not post-prostatectomy, nor in men with BPH, HGPIN and men under 30 where PC would not reasonably be expected to be present.

Figure 4 (a) Quantitation of EN2 by ELISA. The concentration of EN2 was determined by ELISA for urine samples from each group. The mean value for [EN2] determined by ELISA is shown for each group and error bars represent the standard error of the mean. Patient group abbreviations are described in Table 1. An unpaired t-test with Welch's correction was used to calculate the significance of the mean EN2 concentration for PC and non-cancer diagnosis groups (*** denotes that $p<0.001$). (b) An ROC analysis of urine EN2 concentrations in men with biopsy proven prostate cancer (Biopsy (+)) v the men that were a biopsy did not find PC (Biopsy (-)) in the Surrey cohort.

Figure 5 (a) A comparison of mean EN2 concentrations in urine from PC patients subdivided into those with a Gleason score of G6 to G10. The error bars represent the standard error of the mean. The difference in the mean value for each group is not statistically significant ($p>0{\cdot}05$). (b) A vertical scatter plot representing the serum PSA concentrations of patients found to have PC. These are split into two groups, patients whose urine was positive for EN2 (EN2+), and negative for EN2 (EN2-). The horizontal bar represents the mean value for each group; the P value associated with these means is shown. 'Combined' – data for all patients in this group.

Table 1 Summary of patient demographics and urine screening results using an ELISA to quantify EN2 protein. 'Biopsy (+)', patients found to have PC upon biopsy. 'Biopsy (-)', men with a raised PSA but found to be negative for prostate cancer upon biopsy. 'Low PSA A', men with a low PSA (<2·5ng/ml) selected from patients attending a haematuria clinic that were not found to have disease; 'Low PSA B', men with a low PSA (<2·5ng/ml) that were assessed as part of a community screening program. 'Surrey' indicates Surrey patient cohort; 'Cambridge' indicates Cambridge patient cohort. The sample collection and EN2 assay were performed independently by these two centres.

Supplementary figure 1 EN2 in purkinje cells. A section through a human cerebellum showing the cell bodies of a number of purkinje cells (the large cells). There are small foci of immunoreactivity (brown speckled staining)

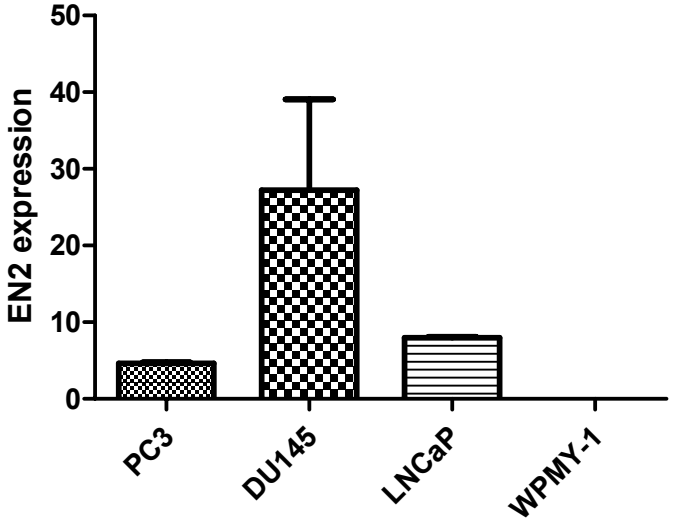
within cell nuclei of purkinje cells and not other neuronal cells or supporting tissue. Magnficiation: x200.

Supplementary figure 2 Enzyme-linked Immunosorbent Assay (ELISA) for EN2 protein in urine. (a) Standard curve for equating light absorbtion at 405nm (‘OD’) to the concentration of EN2. (b) Intra assay variation. A single urine sample was measured 22 times in the same assay. A summary of the primary output (i.e. OD at 405nm) is shown.

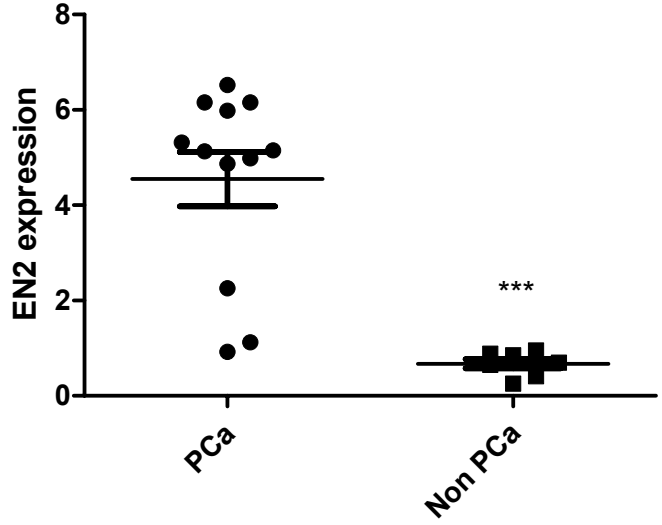
Supplementary figure 3 EN2 protein is stable for at least 4 days at room temerature. Urine samples that contained full length EN2 protein (as determined by western blotting) were stored at room tempertaure for two weeks and tested again for full length EN2 protein after 4, 7 and 13 days. ‘+’ indicates the presence of full length EN2 protein (i.e. a 33kDa band detected by western blotting) and ‘-‘ indicates its absence.

Figure 1

a



b



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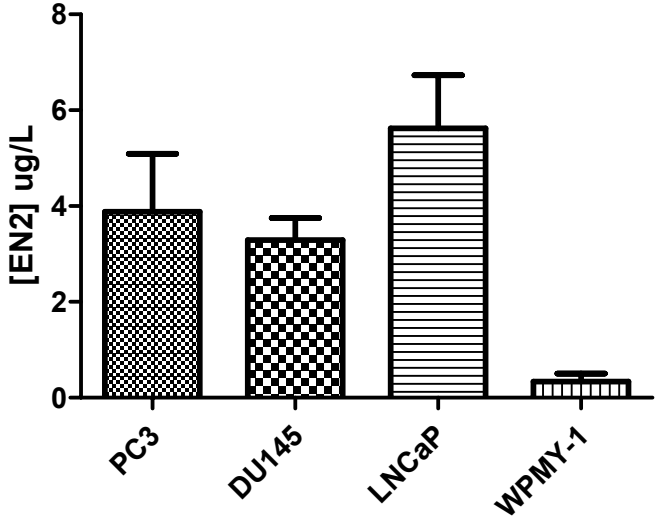

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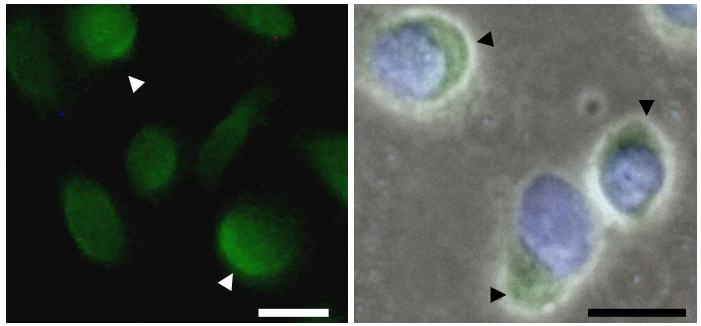


Figure 2

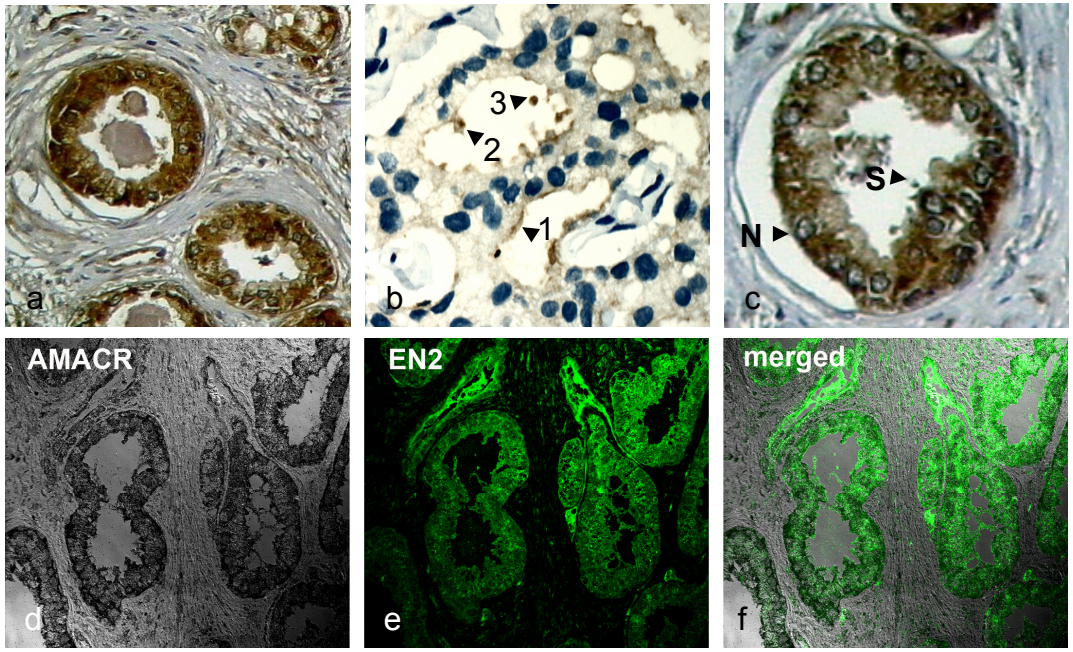


Figure 3

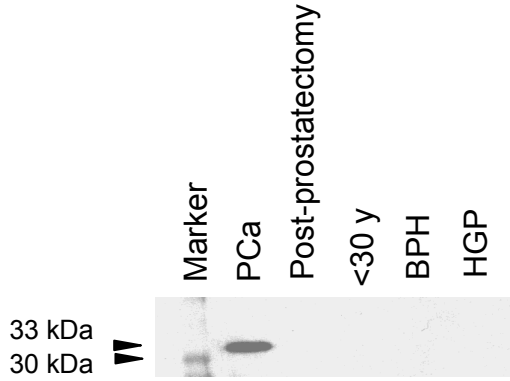
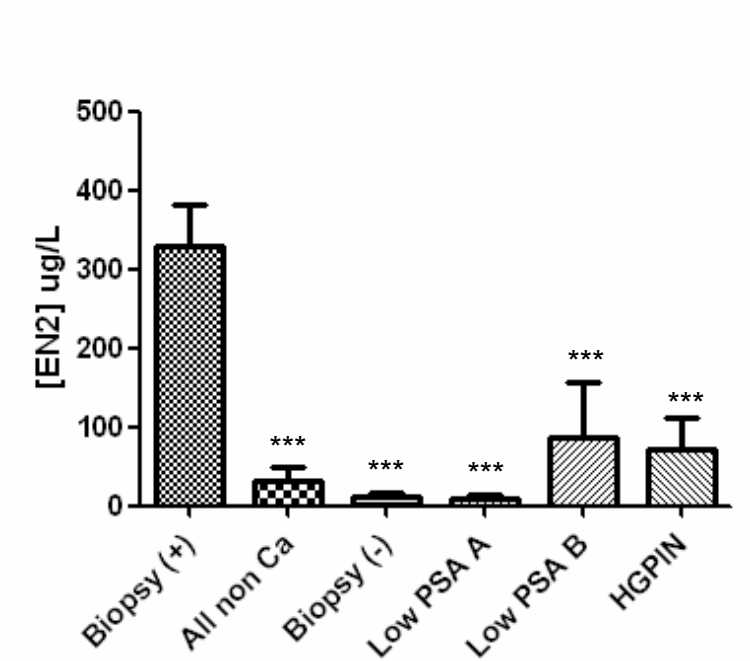


Figure 4

a


500
400
300
200
100
0
[EN2] ug/L
***
***
***
***
***
Biopsy (+)
All non Ca
Biopsy (-)
Low PSA A
Low PSA B
HGPIN

b

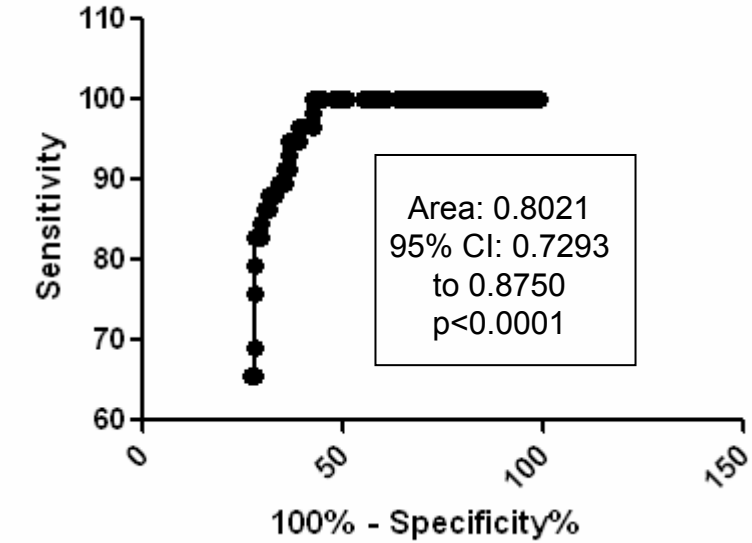
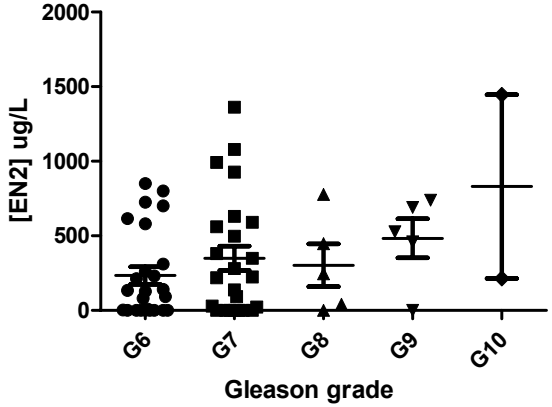
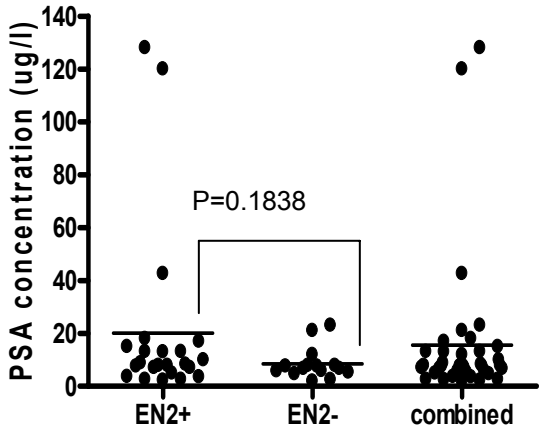

110
100
90
80
70
60
Sensitivity
Area: 0.8021
95% CI: 0.7293
to 0.8750
p<0.0001
0
50
100
150
100% - Specificity%

Figure 5

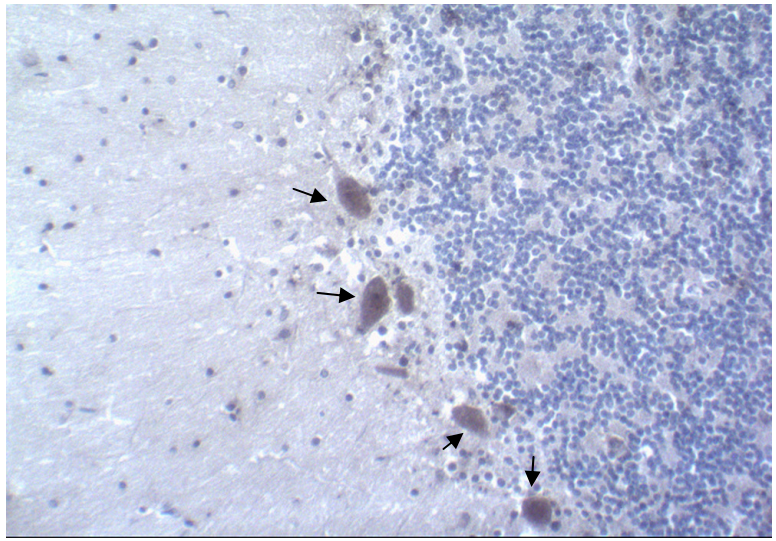
a


2000
1500
1000
500
0
[EN2] ug/L
G6
G7
G8
G9
G10
Gleason grade

b

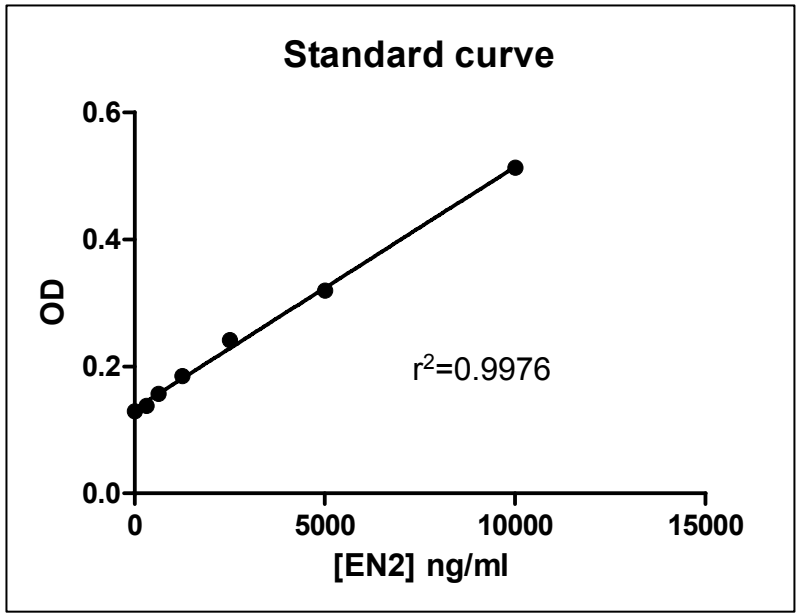

140
120
100
80
60
40
20
0
PSA concentration (ug/l)
P=0.1838
EN2+
EN2-
combined

Supplemental figure 1



Supplemental figure 2

a



b

Mean:	0.12235
SD:	0.009314107
Max:	0.162
Min:	0.1148
Range:	0.0472

Supplemental figure 3

Urine	Day 0	Day 4	Day 7	Day 13
1	+	+	+	+
2	+	+	-	-
3	+	+	+	+
4	+	+	+	+
5	+	+	+	+
6	+	+	-	-
7	+	+	+	-
8	+	+	-	-

Table 1

Surrey University

Group	n	Mean age (range)	Mean PSA (range)	Median PSA	%EN2 +ve
Biopsy (+)	82	67 (44-83)	15 (1.9-175)	7.3	66
Biopsy (-)	58	66 (52-82)	9 (0-12)	7.6	10.3
Low PSA A	17	63 (42-84)	1.2 (0.4-2.6)	1.1	11.7
Low PSA B	27	57 (45-86)	1.1 (0.2-3)	0.9	14.8
All non Ca	102	63 (42-86)	5.8 (0.2-30)	4.6	11.8
HGPIN	10	63 (50-78)	8.27 (2.5-16)	7.7	30

Cambridge University

Group	N	Mean age (range)	Mean PSA (range)	Median PSA	%EN2 +ve
Biopsy (+)	81	67 (40-85)	151.8 (1.9-6510)	7.3	58
Biopsy (-)	13	60 (45-78)	6.4 (2.2-11.8)	6.3	15