Engrailed-2 (EN2): A Tumor Specific Urinary Biomarker for the Early Diagnosis of Prostate Cancer

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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 Engrailed-2 (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-quantitative 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 center.

Conclusions: Urinary EN2 is a highly specific and sensitive candidate biomarker of prostate cancer. A larger multicenter study to further evaluate the diagnostic potential of EN2 is justified.
cancer compared to noncancer. 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 nonmalignant 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 and also potentially as a simple screening tool in the community.

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

(a) “low PSA group A”: men presenting to hematuria clinics where no urothelial malignancy was found (single episode of hematuria; radiology, cystoscopy, and cytology negative) but in whom digital rectal examination (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. 5–10 mL 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 noncancer 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 uropathologist. The 2003 UICC TNM classification was applied. Gleason scores, clinical stage and PSA were available in all cancer cases.

EN2 protein detection – Western blotting

An amount of 1.5 mL urine was centrifuged at 10,000 × g for 5 minutes to remove cells and cellular debris. Twenty microliters of the supernatant were then mixed directly with gel running buffer (Invitrogen). Proteins were resolved by 10% SDS–polyacrylamide gel electrophoresis and transferred to a polyvinylidene fluoride membrane (Invitrogen). Anti-EN2 antibody (ab45867; Abcam) was used at concentration of 0.5 μg/mL, and a goat-anti human IgG peroxidize-labeled antibody was used together with the ECL chemiluminescent system for detection.

EN2 protein detection – ELISA

Two monoclonal mouse anti-EN2 antibodies were raised using the synthetically produced C-terminal 100 amino acids (Biosynthesis Inc.) of EN2 as an antigen (Antibody Production Services Ltd.). One of these, APS1, was conjugated to alkaline phosphatase using the Lightning Link alkaline phosphatase conjugation kit (Innova Biosciences), whilst the other, APS2, was conjugated to biotin using the Lightning Link Biotin Conjugation kit (Innova Biosciences). APS2-biotin was captured onto a 96-well streptavidin plate (Nunc 436014) at a concentration of 4 μg/mL. After washing, 100 μL 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 4 μg/mL (1 hour at room temperature). After a final wash step a colorimetric agent – pNPP (Sigma) was added and the absorption of light at 405 nm was measured after 1 hour. The dilution series was used to generate a standard curve by which the concentration of EN2 in each sample was measured (Supplementary Fig. S2).

cDNA synthesis

RNA from prostate tumors 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°C for 5 minutes. One to five micrograms of RNA was incubated in a volume of 50 μL at 37°C for 1 hour with final concentrations of 10 mM DTT, 1 mM dNTP mix, as well as 100 ng/μL polyT primers, 200 units of reverse transcriptase (Invitrogen) and 40 units of RNaseOUT (Invitrogen). The cDNA synthesis reaction was terminated by placing tubes at 80°C for 5 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°C for 10 minutes, followed by 40 cycles of 30 seconds at 94°C, 1 minute at 60°C and 30 seconds at 72°C. The forward and reverse primers for En2 were 5′ GAACCGGACAAAGAGGACA 3′ and 5′ CGCTTG TCTCTGGAACCAAAT 3′, and for Beta actin they were 5′ ATGTA CCGTGGCATTTGGACG 3′ and 5′ GACIFGTCATACTCC TGCITGG 3′.

Immunohistochemistry

Expression of EN2 in PC and normal prostate was investigated using 3-μm-thick formalin fixed, paraffin embedded tissue array (PR2085a, US Biomax) 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 (DakoCyto- mation). Brain sections were used as a positive control. Antigen retrieval was performed using pH9.0 Tris/EDTA buffer (DakoCyto- mation) 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 nonmalignant 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 noncancer cells was studied by measuring the number of En2 transcripts in the human PC cell lines PC3, DU145, and LNCaP together with a nonmalignant 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, 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 tumor (0%, n = 11), or in normal prostate cores (0%, n = 9). We found no evidence of EN2 staining in normal prostate tissue, benign hypertrophy

Figure 2. A, core biopsy of a prostatic adenocarcinoma stained with anti-EN2 antibody. EN2 positive staining (brown) is present in tumor cells. Magnification, ×30. B, higher magnification of a tumor duct. EN2 is present in the cytoplasm of tumor 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, ×100. 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, ×120. D–F, section through a prostate tumor; tumor cells are stained using the prostate cancer specific antigen Alpha-methylacyl-coenzyme A racemase (AMACR; D). The same section was also stained with fluorescently labeled anti-EN2 (green; E). The merged image reveals an almost identical pattern of staining (F). Magnification (D–F), ×60.
nor in men with HGPIN (high grade prostatic intraepithelial neoplasia) in any tissue array section or biopsy from our patients. Analysis of larger tumor sections taken by biopsy revealed EN2 expression is most intense in the duct like structures of tumors (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 and c). This widespread distribution contrasts markedly with normal adult purkinje neurons where EN2 protein, as expected, is confined to the nucleus (ref. 27; Supplementary Fig. S1). We also compared the staining of EN2 to that of a known prostate cancer specific antigen, alpha-methylacyl-coenzyme A racemase (AMACR; ref. 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 noncancer individuals.

The ELISA assay for EN2 (Supplementary Fig. S2) 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 4 days at room temperature (Supplementary Fig. S3), 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 to 67 years). Median PSA (6.3 to 7.6 ng/mL) was similar in men suspected of PC versus the 2 low PSA control groups (0.9 to 1.1 ng/mL), as expected. Using a cut off of 42.5 μg/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 1 individual in control "low PSA group B" who had a PSA of 1.2 ng/mL.

![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.](Image)

**Table 1. Summary of patient demographics and urine screening results using an ELISA to quantify EN2 protein**

<table>
<thead>
<tr>
<th>Group</th>
<th>n</th>
<th>Mean age (range)</th>
<th>Mean PSA (range)</th>
<th>Median PSA</th>
<th>%EN2 positive</th>
</tr>
</thead>
<tbody>
<tr>
<td><strong>Surrey University</strong></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Biopsy (+)</td>
<td>82</td>
<td>67 (44–83)</td>
<td>15 (1.9–175)</td>
<td>7.3</td>
<td>66</td>
</tr>
<tr>
<td>Biopsy (−)</td>
<td>58</td>
<td>66 (52–82)</td>
<td>9 (0–12)</td>
<td>7.6</td>
<td>10.3</td>
</tr>
<tr>
<td>Low PSA A</td>
<td>17</td>
<td>63 (42–84)</td>
<td>1.2 (0.4–2.5)</td>
<td>1.1</td>
<td>11.7</td>
</tr>
<tr>
<td>Low PSA B</td>
<td>27</td>
<td>57 (45–86)</td>
<td>1.1 (0.2–2.5)</td>
<td>0.9</td>
<td>14.8</td>
</tr>
<tr>
<td>All non-Ca</td>
<td>102</td>
<td>63 (42–86)</td>
<td>5.8 (0–12)</td>
<td>4.6</td>
<td>11.8</td>
</tr>
<tr>
<td>HGPIN</td>
<td>10</td>
<td>63 (50–78)</td>
<td>8.27 (2.5–16)</td>
<td>7.7</td>
<td>30</td>
</tr>
<tr>
<td><strong>Cambridge University</strong></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Biopsy (+)</td>
<td>81</td>
<td>67 (40–85)</td>
<td>151.8 (1.9–6510)</td>
<td>7.3</td>
<td>58</td>
</tr>
<tr>
<td>Biopsy (−)</td>
<td>13</td>
<td>60 (45–78)</td>
<td>6.4 (2.2–11.8)</td>
<td>6.3</td>
<td>15</td>
</tr>
</tbody>
</table>

*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 hematuria 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 2 centers.
An ROC analysis for this data [Biopsy (+) vs. 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 and Fig. 4a); 2 of these 3 men were found to have PC within 6 months upon rebiopsy.

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 summarized in Table 1 and aresimilar to those obtained for the Surrey patients. Of 13 control patients 2 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 tumors but not in normal prostate tissue. Our data indicates that En2 protein is also secreted by both cell lines and primary tumors, and is found in the majority of PC patients in first pass urine collected without preceding DRE, but not in noncancer 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 noncancer 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 PCPT biopsied 5,112 men in the placebo arm irrelevant of PSA level. A PSA >4 ng/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 <0.5 ng/mL and 27% had PSA of 3.1–4 ng/mL (31). Ultimately the choice of PSA “cut off” balancing sensitivity against specificity results in false positive tests and large numbers of unnecessary biopsies (32, 33). 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 noncoding RNA present in urine (34) and Annexin A3 (35). 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 (34). 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 (35). 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 modeling of multiple markers (36) 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 100 μL 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 (37). This study found high levels of En2 in the urine of 1 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 harboring 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 multicenter 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 tumor stage and Gleason grade. However, as with every PC biomarker in development, the ultimate question will be centered around the utility of EN2 (alone or in combination with other markers) in identifying "significant" cancers that require early intervention.

Disclosure of Potential Conflicts of Interest

No potential conflicts of interest were disclosed.

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