Expression of Engrailed-2 (EN2) protein in bladder cancer and its potential utility as a urinary diagnostic biomarker

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Abstract

Despite significant advances in our understanding of the molecular pathology of bladder cancer, it remains a significant health problem with high morbidity and mortality associated with muscle-invasive bladder cancer (stages T2+), and high costs associated with the surveillance of non-muscle-invasive bladder cancer (NMIBC, stages Ta/T1/Tis). Moreover, current diagnostic biomarkers are suboptimal and of poor utility for low grade disease and surveillance. In this study we show that the *Engrailed-2* (*EN2*) transcription factor is expressed in, and secreted by, bladder cancer cell lines and patient tumor specimens, justifying an evaluation of urinary EN2 as a diagnostic biomarker in bladder cancer using archived samples from an established biospecimen collection. In patients with NMIBC, urinary EN2 was detected in most cases with an overall sensitivity of 82% and specificity of 75%. The sensitivity for stage Ta and T1 tumors was 71% and 76%, respectively, and 94% for stage T2+ tumors. This compares favorably with existing markers. The sensitivity for tumor grades 1, 2, and 3 was 69%, 78% and 87% respectively. Thus urinary EN2 has the potential to be a more sensitive and specific protein biomarker for NMIBC than currently available tests.

Keywords: Bladder Cancer, EN2, biomarker, diagnostic

Introduction

Urothelial carcinoma of the urinary bladder (UCB) is the fourth and ninth most common cancer amongst men and women, respectively, in Europe and North America, with an estimated prevalence of 500,000[1]. Long term surveillance is required in the majority of cases, involving regular cystoscopy. UCB results in significant mortality, with overall 5-year survival rates of 57% and 47% for men and women respectively when the disease presents as muscle-invasive[1]. As a result, UCB also represents a significant economic burden to healthcare systems as the most expensive malignancy to manage on a per patient basis from diagnosis to death[2,3] Around 75-80% of patients present with non-muscle-invasive disease (NMIBC), but recurrence and progression are significant issues, compelling current guidelines to recommend long-term surveillance[4]. There is therefore an urgent and unmet need to identify and validate accurate urinary biomarkers for the detection of disease recurrence in order to improve quality of life for NMIBC patients and reduce costs for healthcare providers, whilst maintaining or improving current outcomes.

Characteristics of effective biomarkers include cancer-specific expression and release from tumors. One gene that has recently been shown to have these properties is the transcription factor *Engrailed-2* (*EN2*), which is expressed in both breast cancer[5] and prostate cancer[6]. The former study showed that *EN2* has oncogenic potential, as its forced expression in a non-malignant mammary epithelial line increased cell proliferation, survival and invasion[5]. We have recently shown that *EN2* is also expressed in human prostate

cancer cell lines and patient tumors. Furthermore, full length EN2 protein can be released from prostatic cancer acini and ducts, and detected in the urine of prostate cancer patients, thus representing a potential diagnostic biomarker [6,7]. In this study we show that *EN2* is also expressed by bladder cancer tumors and is potentially a specific and sensitive biomarker for bladder cancer, even when the disease presents at early, non-invasive stages.

Materials and Methods

Cell Lines

Forty-three bladder tumor-derived cell lines, one line (HCV-29) derived from the non-malignant ureteric epithelium of a patient with bladder cancer, one telomerase immortalized NHU cell line (NHU-BTERT) and a pool of cultured primary normal human urothelial cells (NHU-Pool) isolated from 3 different donors were cultured for use in expression array experiments. Eight cell lines (LUCC1-8) were established in the Knowles laboratory. Cell lines were authenticated by short tandem repeat (STR) DNA typing using a Powerplex 16 kit (Promega). Profiles were compared to publically available data (ATCC, DSMZ) or where no reference profile was available, were confirmed as unique.

Growth conditions for the cell lines were as previously described[6,8]. The conditioned media used for EN2 detection was taken from cells grown to 90% confluence and then put in serum-free medium for 2 hours.

Paraffin-Embedded Tissues and Immunohistochemistry

Expression of EN2 in UCB and normal bladder tissue was investigated using 3µm thick formalin fixed, paraffin embedded tissue array sections (BL2081, US Biomax, Rockville, MD, USA). 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). Antigen retrieval was performed

using pH9.0 Tris/EDTA buffer (DakoCytomation) and heating in a microwave for 23 minutes.

Immunostaining was assessed by a uropathologist and scored (independently by a second observer) on a 0-3 scale, with 0 representing no apparent staining and 3 very strong staining. The levels of expression in the tissue array cores were scored as no staining (score = 0), tumor with low level staining, cytoplasmic only (score = 1), tumor with moderate staining, cytoplasmic only (score = 2), and tumor with high staining both cytoplasmic and nuclear (score = 3).

Gene expression profiling

Total RNA for gene expression analysis was extracted using TRIZOL (Invitrogen, Paisley, UK), DNase-treated and cleaned up using a Qiagen RNeasy Mini Kit. Whole-genome expression profiling was performed using GeneChip Human Genome U133 Plus 2.0 Arrays (Affymetrix, Santa Clara, CA, USA). 5 μg of total RNA from each cell line was labelled using the WT-Ovation Pico Target Prep v1.0 system (NuGen Technologies Inc., San Carlos, CA, USA) and hybridised according to the array manufacturer's instructions. The arrays were scanned and CEL files were imported into Partek Genomics Suite 6.5. Data were normalised and probe intensity measures were generated using the Robust Microarray Analysis (RMA)[9]. EN2 mRNA expression levels were represented as $\log_{10}$ (cell line intensity/NHU-Pool intensity).

cDNA Synthesis and RT-PCR

RNA was extracted from the bladder cancer derived cell lines using the Qiagen RNeasy kit (Qiagen, USA). Three paired biopsy samples of human UCB and normal adjacent bladder were evaluated (one from each of three patients). Ethical approval for the evaluation of these samples was in place (10/H1101/7). All were confirmed histologically as bladder cancer or normal by a specialist uropathologist. cDNA synthesis and RT-PCR were performed as previously described[6].

EN2 protein detection

EN2 detection by western blotting[6] was carried out as previously described. For the ELISA, a monoclonal mouse anti-EN2 antibody, APS1, was generated (Antibody Production Services Ltd, Haywards Heath, Sussex, UK) using the synthetically produced EN2 C-terminal 100 amino acids (Biosynthesis Inc, Lewisville, Texas, USA). An APS1-Alkaline phosphatase conjugate was used to detect EN2 captured onto a 96-well plate (Nunc 436014, Rochester, New York, USA). 100 µl of the urine supernatant samples or a dilution of the EN2 fragment in buffer was tested in duplicate. A standard curve was generated from dilution series to allow the concentration of EN2 in each sample to be measured.

Patients and Controls

Urine samples were collected prospectively for biomarker research between 2006 and 2009 as part of the Bladder Cancer Prognosis Programme (BCPP,

ethics approval 06/MRE04/65)[10]. Previous biomarker discovery work on urine samples from BCCP has been published[11].

Statistical analysis

The GraphPad prism package was used in statistical calculations. 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 the area under the curve 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). For the qRT-PCR data we used the mean of 3 independent experiments and tested for significance using Student's t-test with Welch's correction.

Results

EN2 expression in UCB

The previous findings that *EN2* is expressed by prostate[6] and breast[5] cancer cells led us to determine whether *EN2* was also expressed in UCB. Evaluation of gene expression microarray data from a panel of UCB cell lines, including several derived from low grade and low stage UCB revealed that a majority of cell lines express higher levels of *EN2* mRNA compared with normal urothelium controls (Fig 1a).

A small panel of UCB derived cell lines (RT4, UMUC3, LUCC8, J82, EJ and RT112) and a telomerase immortalized NHU cell line as normal control (NHU-BTERT) were subsequently cultured and the relative expression of *EN2* in each was determined by quantitative qRT-PCR (Fig 1b). This panel included cell lines representing high grade (J82, EJ) and low grade disease (RT112, LUCC8, RT4). After 24 hours of cell culture EN2 secretion was determined by ELISA. Moderate to high expression of EN2 was observed in RT4, UMUC3, EJ and RT112 lines. Low level expression was evident in the other lines including the NHU-BTERT line. However, only the 4 cell lines with the highest expression of *EN2* secreted EN2 into supernatant: RT4, UMUC3, EJ and RT112. EN2 expression at the protein level was determined using a fluorescently labeled anti-EN2 antibody and high levels of expression were seen (Fig 1c). To confirm these *in vitro* findings, we demonstrated that *EN2* RNA expression was restricted to tumor in three tumor/normal paired samples from patients with UCB ($p=0.0134$), Fig 1d.

The expression of EN2 was subsequently studied in primary UCB tissue, including a high density tissue array comprising 180 tumor cores and 16 normal bladder cores; EN2 protein was expressed by transitional cell carcinoma (TCC, Fig 2a), squamous cell carcinoma (SCC) (Fig. 2b) and adenocarcinoma of the bladder (Fig 2c). In TCC tumors, the pattern of EN2 expression was cytoplasmic and nuclear, similar to that observed in prostatic adenocarcinoma. In adenocarcinoma and SCC expression was also cytoplasmic and nuclear but levels more heterogeneous within the same tumor deposit with areas of focal positivity. Scoring the tumor core immunostaining (Fig 2d-g) revealed no staining for normal bladder tissue but 22-fold higher staining for all tumor types combined and 24-fold higher staining in TCC. SCC and adenocarcinoma had 17-fold and 6.4-fold higher staining than normal bladder, respectively (Fig 3). Higher grade tumors had progressively less EN2 expression than lower grade tumors - Grade 1 and Grade 2 tumors showed 2.3-fold and 1.9-fold more staining, respectively, than Grade 3 tumors (Fig 3). No differences in staining were apparent between tumors taken from male and female patients, nor was there any apparent relationship between staining and patient age (data not shown).

EN2 protein is present in the urine of bladder cancer patients

Given the expression of *EN2* in UCB cell lines and tissue sections, secretion of EN2 by cell lines and our previous findings in prostate cancer, we assessed urinary EN2 levels in an independent cohort of patients with and without UCB (BCPP[10]). The characteristics of the patients included in the study are shown

in Table 1. For those patients with UCB there was an expected distribution of histological grades and stages, and a mean age of 72 years (range 29 to 94 years, n=466); for the non-UCB (control) subjects the mean age was 71 years (range 34 to 88 years, n=52).

Full length EN2 protein could be detected in the urine of cancer patients by western blotting (Fig 4a); to quantify the EN2 concentration in all of the samples, the urine was also analyzed by ELISA. The mean urinary EN2 concentration in patients with UCB was 197ng/ml, whilst that for control subjects was 34ng/ml (Fig 4b). The majority of UCB patients were diagnosed with stage Ta or T1 tumors, and there was no significant difference between the mean urinary EN2 concentrations in these patients (171ng/ml and 184ng/ml, respectively). The concentration of EN2 in the urine of patients with T2+ tumors was significantly higher than in those with Ta and T1 tumors, at 261ng/ml. Higher grade tumors (Grades 2 and 3) were associated with higher mean urinary EN2 concentrations (192ng/ml for Grade 2 and 215ng/ml for Grade 3) than Grade 1 tumors (168ng/ml).

Urinary EN2 concentrations are predictive for UCB

The difference in urinary EN2 concentrations in patients with and without UCB indicated potential diagnostic value. A ROC analysis of these data gave an area under the curve of 0.844, which rises to 0.908 when comparing cancer patients to those individuals with entirely normal histology (Fig 4c). The ROC analysis indicated that the optimal urinary EN2 concentration threshold for cancer versus non-cancer was 55.5ng/ml in order to maximize the sensitivity

and specificity of the test. Using this cut-off gives a sensitivity for all UCB of 82% with a specificity of 75%. The sensitivity for stages Ta, T1 and T2+ are 71%, 76% and 94%, respectively (Table 1). Amongst the non-UCB patients, 19% had >55.5ng/ml EN2 in their urine (4% for patients with entirely normal histology).

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Discussion

In this study we have shown that EN2 is expressed by and secreted from UCB tumors. These results highlight a potential role for EN2 in UCB pathogenesis (as seen in breast and prostate cancer[5,6]), and they also highlight the potential utility of EN2 as a urinary biomarker for UCB. Specifically, EN2 appears to be a more sensitive marker for NMIBC than existing markers. As EN2 expression was also detected in non-transitional cell tumors (squamous cell and adenocarcinoma), it will be important to establish whether EN2 is also secreted into urine by patients harboring these forms of bladder cancer. A variety of molecular changes are associated with squamous cell and adenocarcinoma histology and, combined with evidence of EN2 expression/secretion in NMIBC and MIBC, it suggests a common regulatory defect. This is currently being investigated by our group. Squamous cell histology is common in countries where bladder cancer is associated with schistosomiasis[13] and therefore EN2 may be useful in this high risk population, in contrast with other currently used markers which have been shown to be more specific for TCC histology.

Current urinary biomarkers for UCB have important, significant limitations and none are reliable for post-treatment surveillance. In the evaluation of urinary EN2 as a diagnostic biomarker for UCB, EN2 was detected in the urine of most patients with NMIBC with an overall sensitivity of 82% and specificity of 75%. In comparison, urine cytology has a high diagnostic specificity (78-100%), but lacks robust sensitivity (12.2-84.6%), especially for low and

intermediate grade tumors[14]. The sensitivity of EN2 for stage Ta and T1 tumors was 71% and 76%, respectively, and 94% for stage T2+ tumors. This compares favorably with existing markers. The sensitivity for tumor grades 1, 2, and 3 was 69%, 78% and 87% respectively. This latter result apparently contradicts our IHC findings where EN2 staining was found to be relatively weaker in higher grade tumors. The most likely explanation for this would be that the structure of high grade tumors, and / or some aspect of their physiology allows for a more efficient release of EN2 protein into the urine.

A number of other investigational urine markers have been described by Tilki *et al* in their recent review[15,16], but currently the presence of Nuclear Matrix Protein 22 (NMP22) protein in urine represents the most sensitive protein-based non-invasive test for early stage UCB . NMP22, a regulator of mitosis that escapes from bladder cancer cells undergoing apoptosis, can be detected in the urine and has been studied as a potential biomarker [15]. It has a sensitivity of 46.7% and 48.2% for stages Ta and T1, respectively (a superior sensitivity to urine cytology), and a specificity of 90.3% in the absence of any urinary tract disease (reduced to 77.6% in inflammatory conditions)[17]. The fact that its sensitivity for stages Ta and T1 does not exceed 50% may reflect the primary mechanism for NMP22 release, thought to be the breakdown of cell membranes during apoptosis. Since earlier stage tumors undergo less apoptosis than more advanced tumors[18], NMP22 release might also be expected to be less, limiting its diagnostic utility for NMIBC. Conversely, EN2 is actively secreted from viable cells[19,20] and its presence in urine is likely to be associated with live tumor cells. Like NMP22, a number of other diagnostic tools also have a relatively high sensitivity for more advanced

stage cancers (stages T2+), but none of them currently allow for detection of more than 50% of earlier stage cancers. Upon validation, the first clinical application for urinary EN2 would be as a diagnostic test for patients either presenting for the first time (at a haematuria clinic), in order to increase diagnostic sensitivity and specificity, or for those under surveillance with an aim to reducing the number of screening cystoscopies. For both of these scenarios, the majority of patients will have NMIBC. EN2 may also be used as one of a panel of urinary markers, to provide a combined risk assessment[21,22].

We have found that EN2 is expressed in NMIBC and MIBC, and also in metastatic bladder cancer, by analysis of tissue microarrays (manuscript in preparation). Our pilot study has shown that EN2 is expressed in, and secreted by, recurrent NMIBC and a prospective study of this specific group is on-going. The microarray data of cell lines confirmed EN2 expression in cells representing different stages and grades of disease. This suggests that EN2 expression / secretion may be an early event in bladder tumorigenesis and independent of the specific molecular dysregulation associated with either NMIBC or MIBC. The molecular basis of the observed *de novo* EN2 expression and physiology of EN2 secretion by bladder cancer cells is currently being investigated.

In previous studies, we demonstrated the potential utility of urinary EN2 for the diagnosis of prostate cancer[6,7] which raises the question of how to interpret elevated urinary EN2 levels. This may be addressed in a number of ways. Firstly, the mean urinary concentration of EN2 in prostate cancer was

329ng/ml compared to 197ng/ml for UCB in this study, although the optimal cut-off values were similar (42.5ng/ml for prostate cancer and 55.5ng/ml for UCB[6]). Future prospective trials will be designed to include a further comparison of urinary EN2 levels between prostate cancer patients and UCB patients. Secondly, the clinical presentation and diagnostic pathways are very different for UCB and prostate cancer. Bladder cancer usually presents with hematuria, whilst prostate cancer is diagnosed when investigating lower urinary tract symptoms, a strong family history of prostate cancer, a PSA test (due to symptoms or a scheduled general male health screen) or an abnormal digital rectal examination. Prostate cancer is only rarely associated with haematuria. Whilst the symptoms associated with both bladder and prostate cancer may also be attributable to non-cancer causes, their presence in conjunction with a positive EN2 test would strongly indicate the presence of bladder or prostate cancer and the need for further investigation. The adoption of EN2 as a new standard diagnostic biomarker will require multi-center prospective evaluation, either as a single marker or in conjunction with existing tests. Although it has potential in screening, surveillance and early detection, initial evaluation would most likely be undertaken in the haematuria clinic setting as a diagnostic test. This would establish its negative and positive predictive values which are directly related to sensitivity and specificity, and the prevalence of the disease in a defined population.

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Author contributions:

RM and HP conceived idea and wrote final manuscript with RTB. RM and FL carried out experiments. RTB, MPZ, KKC, NDJ, MAW, MAK, CDH, DGW, MAW provided samples and carried out experiments. SJ provided samples. All authors were involved in writing the paper and had final approval of the submitted and published versions.

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Figure legends

Figure 1. *EN2* expression in bladder cancer derived cell lines. (a) Relative EN2 expression measured by micro-array across a range of normal bladder and bladder cancer derived cell lines. (b) Upper panel – qRT-PCR analysis of *EN2* transcripts in the bladder cancer derived cell lines EJ, UMUC3, RT4, LUCC8, J82, and RT112, together with the transformed, normal bladder cell line B TERT. Expression is shown as a ratio with the house keeping gene *Beta-actin* (x1000). Error bars show the SEM (n=3). Lower panel - EN2 secretion by the same cell lines as measured by an ELISA. (c) Fluorescent micrograph of EJ cells stained with a FITC-labeled anti-EN2 antibody. EN2 staining is shown in green. Cell nuclei are stained blue (DAPI). Scale bar: 5µm. (d) qRT-PCR analysis of *EN2* expression in bladder tumors and normal adjacent tissue (NAT). Expression is shown as a ratio with the house keeping gene *Beta-actin*. Error bars show the SEM (n=3).

Figure 2. EN2 staining of bladder cancer sections with an anti-EN2 antibody (brown). Expression by immunohistochemistry in transitional (a) x10 magnification, squamous (b) x20 magnification, and adenocarcinoma histology (c) x 20 magnification. Expression pattern in transitional cell carcinoma was uniform cytoplasmic staining with some nuclear positivity. The expression in squamous cell and adenocarcinoma variants was much more heterogeneous within tumors with focal positivity in cytoplasm. Scoring of tissue expression of EN2 in tissue array: (d) no staining (score= 0), (e) tumor with low level staining, cytoplasmic only (score = 1), (f) tumor with moderate

staining, cytoplasmic only (score =2), (g) tumor with high staining both cytoplasmic and nuclear (score =3). Scale bar: 10μm.

Figure 3. Scoring of EN2 staining in bladder tumors. EN2 staining was scored from 0 to 4, whereby '0' is no staining and '4' is very strong staining tumor. The proportion of each tumor type or tumor grade having a given score (s) is shown. 'Normal' – normal bladder tissue (n=16), 'All ca' – all cancer types (n=180), 'TCC' – transitional cell carcinoma (n=149), 'SCC' – squamous cell carcinoma (n=16), 'Adenoca' – adenocarcinoma (n=15), tumor'G1' - Grade 1 (n=57), 'G2' - Grade 2 (n=65), and 'G3' - Grade 3 (n=31). It was not possible to grade all tissue samples on the array due to quality of staining.

Figure 4. EN2 in urine. (a) Western blot of urine from bladder cancer patients ('C') or a non-cancer patient ('N'). (b) Mean urinary EN2 concentrations for bladder cancer patients and non-cancer patients in Birmingham. 'All Ca' – all cancer stages / grades combined. 'Ta' –stage Ta, 'T1' –stage T1, 'T2' – stage T2, 'G1' – Grade G1, 'G2' – Grade G2, 'G3' – Grade 3, 'All non-Ca' – all non-cancer patients combined. Error bars show the SEM. (c) ROC analysis of all cancer urine samples v either all non-cancer urines (black) or only urine samples from patients with entirely normal bladder tissue (and thus no cystitis, grey).

Table 1. Summary of data from Birmingham patients. 'All Ca' – all cancer stages / grades combined. 'Ta' –stage Ta, 'T1' –stage T1, 'T2' – stage T2, 'G1' – Grade G1, 'G2' – Grade G2, 'G3' – Grade 3, 'Non Ca' – all non-cancer

patients combined. ‘ROC’ – area under the ROC curve generated from comparing the group to the non-cancer patients. ‘%EN2+’ - proportion of samples positive for EN2 (cut off = 55.75ng/ml). ‘%spec’ - % specificity.

Table 1 Summary of data from the BCPP patients.

Group	**n**	**Mean age (range)**	**Mean [EN2] ng/ml**	**ROC (95% CI)**	**% EN2+**	**% spec**
All Ca	466	72 (29-94)	197	0.844 (0.797-0.890)	82	75
Ta	251	71 (29-94)	171	0.811 (0.756-0.867)	71	75
T1	113	73 (47-93)	184	0.828 (0.765 to 0.890)	76	75
T2	102	75 (43-93)	261	0.927 (0.884 to 0.970)	94	75
G1	111	70 (36-92)	168	0.793 (0.725 to 0.861)	69	75
G2	140	70 (29-94)	192	0.826 (0.766 to 0.886)	78	75
G3	215	75 (43-93)	215	0.881 (0.834 to	87	75

				0.927)		
All Non Ca	55	71 (34-88)	35	-	19	-
Cystitis	27	73 (45-87)	53	-	33	-
Normal	28	69 (34-88)	15	-	4	-