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## **Protein shedding in urothelial bladder cancer: prognostic implications of soluble urinary EGFR and EpCAM.**

Richard T. Bryan<sup>1#</sup>, Hannah L. Regan<sup>1#</sup>, Sarah J. Pirrie<sup>1</sup>, Adam J. Devall<sup>1</sup>, KK Cheng<sup>2</sup>, Maurice P. Zeegers<sup>1,3</sup>, Nicholas D. James<sup>4</sup>, Margaret A. Knowles<sup>5</sup>, Douglas G. Ward<sup>1</sup>

<sup>1</sup>School of Cancer Sciences, University of Birmingham, Birmingham, B15 2TT, UK.

<sup>2</sup>School of Population and Health Sciences, University of Birmingham, Birmingham, B15 2TT, UK.

<sup>3</sup>Department of Complex Genetics, NUTRIM School for Nutrition, Toxicology and Metabolism, Maastricht University Medical Centre+, Maastricht, The Netherlands.

<sup>4</sup>Clinical Trials Unit, University of Warwick, Coventry, CV4 7AL, UK.

<sup>5</sup>Section of Experimental Oncology, Leeds Institute of Cancer and Pathology, St James's' University Hospital, Beckett Street, Leeds, LS9 7TF, UK.

#Joint first authors.

Running title: Urinary EGFR and EpCAM in bladder cancer

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## **ABSTRACT**

**Background:** Better biomarkers must be found to develop clinically useful urine tests for bladder cancer. Proteomics can be used to identify the proteins released by cancer cell lines and generate candidate markers for developing such tests.

**Methods:** We used shotgun proteomics to identify proteins released into culture media by 8 bladder cancer cell lines. These data were compared with protein expression data from the Human Protein Atlas. EGFR was identified as a candidate biomarker and measured by ELISA in urine from 60 non-cancer control subjects and from 436 patients with bladder cancer and long-term clinical follow-up.

**Results:** Bladder cancer cell lines shed soluble EGFR ectodomain. Soluble EGFR is also detectable in urine and is highly elevated in some patients with high-grade bladder cancer. Urinary EGFR is an independent indicator of poor bladder cancer specific survival with a hazard ratio of 2.89 (95% CI 1.81-4.62,  $p < 0.001$ ). In multivariable models including both urinary EGFR and EpCAM both biomarkers are independent predictors of bladder cancer specific survival and have prognostic value over and above that provided by standard clinical observations.

**Conclusions:** Measuring urinary EGFR and EpCAM may represent a simple and useful approach for fast-tracking the investigation and treatment of those patients with the most aggressive bladder cancers.

## INTRODUCTION

Urothelial bladder cancer (UBC) is the seventh commonest cancer in Western societies (Burger *et al*, 2013). At presentation 75-85% will be non-muscle-invasive tumours (NMIBC, stages pTa/pT1/pTis), with the remainder being muscle-invasive (MIBC, stages pT2-4) (Kaufman *et al*, 2009). Progression to, or presentation with, MIBC represents the critical step in the disease course, necessitating radical therapies, including chemoradiotherapy or neoadjuvant chemotherapy followed by radical cystectomy (Witjes *et al*, 2014). Despite such approaches, outcomes have changed little for three decades (Kaplan *et al*, 2014). Reliably identifying patients with the most aggressive UBCs early (both NMIBC and MIBC) and expediting aggressive therapeutic strategies could go some way to improving outcomes (Bryan *et al*, 2014a), yet this remains a significant challenge despite sophisticated clinico-pathological algorithms (Babjuk *et al*, 2013; Witjes *et al*, 2014).

Since urine directly contacts UBCs it is an attractive biospecimen for developing non-invasive tests to detect and characterise bladder tumours. Challenges in developing such tests include UBC heterogeneity, such that different tumours may release different biomarkers (thus necessitating multimarker tests), and early-stage and low-grade tumours may only release very small amounts of such markers, potentially impairing test sensitivity. Additionally, markers must be highly tumour-specific so that haematuria and other non-malignant conditions do not generate false positives. In the search for better urinary biomarkers genomic, proteomic and metabolomic approaches have all yielded promising results (Huang *et al*, 2011; Kandimalla *et al*, 2013; Orenes-Piñero *et al*, 2007).

Protein ectodomain shedding is becoming increasingly recognised as an important cancer-related phenomenon, and may be a useful source of biomarkers (Hansen *et al*, 2013; Zatovicova *et al*, 2005). We have previously reported that the ectodomain of EpCAM is shed into the urine by bladder tumours (Bryan *et al*, 2014b). Here, we use proteomics to identify the proteins released by a panel of UBC cells *in vitro*, and compare them with tissue expression data. One of the shortlisted proteins,

EGFR, is released into the urine in a soluble form and is a promising biomarker for rapidly identifying UBC patients with the most aggressive disease. This work represents the first description of the prognostic value of soluble urinary EGFR in UBC, alone and in combination with soluble urinary EpCAM (Bryan *et al*, 2014b).

## **METHODS**

### **Materials and samples**

The cell lines used in this study were selected on the basis that they exhibit diverse mutation profiles and may capture some of the heterogeneity of UBC (Supplementary Information, Table S1). Urine samples were prospectively collected for biomarker research between 2006 and 2009 as part of the Bladder Cancer Prognosis Programme (BCPP, ethics approval 06/MRE04/65) (Zeegers *et al*, 2010). Patients were enrolled on the basis of initial cystoscopic findings suggestive of primary UBC. All UBC patients were newly-diagnosed, had not received treatment for UBC prior to urine collection, and were subsequently treated according to current standard practice. Inclusion and exclusion criteria are detailed elsewhere (Zeegers *et al*, 2010). Samples were placed on ice, centrifuged at 2000 x g for 10 minutes within 8 hours of collection, and supernatants stored at -80°C. Since patient recruitment occurred prior to histopathological confirmation of UBC, a proportion of patients were ultimately diagnosed with non-malignant conditions and these serve as non-cancer 'controls'. All patients were followed-up to July 2014 with causes of death notified to the BCPP study office.

### **Proteomic analysis of secretomes**

Cells were grown to >80% confluence (apart from RT4 cells which grow as islands) in 4 x T75 flasks. Adherent cells were then washed 4 times with serum-free media and incubated overnight in serum-free media ± 200nM phorbol 12-myristate 13-acetate (PMA) (2 flasks for each condition equating to 100-200 µg protein). The low protein concentration in the conditioned media and the fact that >95% of the cells remained adherent indicate that any cell death during secretome generation was minimal. Cells and debris were removed by centrifuging the conditioned media at 2000 x g for 20 minutes prior and the entire supernatant processed using filter-aided tryptic digestion. Proteins were reduced with 20mM DTT in 8M Urea, 1% CHAPS, 100mM triethylammonium bicarbonate (1

hour at room temperature), alkylated with 50mM iodoacetamide and then concentrated in 30 kDa centrifugal filters. Following 4 washes with 100mM triethylammonium bicarbonate, 10 µg of sequencing grade trypsin were added to the filters (<50:1 protein:trypsin) and incubated at 37°C overnight. The peptides were centrifuged through the filters and their primary amines dimethylated by incubating with 25mM sodium cyanoborohydride and 0.2% formaldehyde (control secretome) or 0.2% deuterated formaldehyde (+PMA secretome) for 30 min at room temperature. Following quenching with 100mM ammonium bicarbonate the samples were separated into 24 fractions by mixed-mode HPLC (Phillips *et al*, 2010) and each fraction analysed by LC-MS/MS. For each LC-MS/MS run peptides were separated using a linear gradient of 1-37% acetonitrile in 0.1% formic acid over 1 hour at a flow rate of 350 nl/min (25 cm x 75 µm Acclaim PepMap C18 3µm 100Å column, Ultimate 3000 RS HPLC) and sprayed into a maXis impact (Bruker Daltonics) via a captive source with the instrument operating in data dependent mode (8 MS/MS per cycle). The data was searched against a database containing Swissprot human and bovine protein sequences and randomised versions thereof with MASCOT. Search parameters were: enzyme = trypsin, up to 2 missed cleavages, precursor ion tolerance = 20 ppm, fragment ion tolerance = 0.02 Da , mowse score >25, fixed modifications = cysteine carbamidomethyl, variable modifications = methionine oxidation and light/heavy dimethylation on N-termini and lysines. Multiple datasets were combined using Proteinscape 3 (Bruker Daltonics) and filtered at 1% protein false discovery. Relative quantitation (± PMA) was performed using WARP-LC software (Bruker Daltonics) with expression ratios normalised to the median ratio in each experiment. Cell compartment analysis was performed using <http://string-db.org/>.

### **EGFR ELISA and urine testing**

EGFR was measured with a sandwich ELISA utilising goat polyclonal antibodies against the extracellular domain of EGFR (R&D Systems, Cat no. DYC1854-5). All urine samples were measured using 50 µl of urine plus 50 µl of 1% BSA in PBST per well. Urinary EGFR is expressed as pg EGFR per mg creatinine (determined by the Jaffe method). Cell lysates were prepared in PBS containing 1% Igepal CA-630 and Complete Protease Inhibitor Cocktail (Roche) and the protein concentrations determined using the bicinchoninic acid assay calibrated with bovine serum albumin. Secretome and lysate EGFR concentrations were determined using appropriate volumes of sample (2-100 µl) for the concentration range of the assay. Secretome and lysate EGFR concentrations are expressed as ng EGFR per mg of total cell protein. For Western blotting proteins were separated with SDS-PAGE on 4-12% gels using MOPS running buffer (Life Technologies), blotted onto PVDF probed with 0.2 µg/ml biotinylated anti-EGFR antibody (R&D Systems, Cat no. BAF231) followed by streptavidin-HRP.

### **Human Proteome Atlas analysis**

Data was downloaded from <http://www.proteinatlas.org> (Uhlen *et al*, 2010). For a protein to be a candidate biomarker it should be absent or expressed at low levels in normal urothelium and highly expressed in UBC; however, due to the heterogeneity of UBC, clinically useful biomarkers may be over expressed in a sub-set of tumours rather than in all cases. We arbitrarily set our selection criteria for candidate biomarkers as high or medium staining in at least 4 cases of UBC (out of 11 or 12 cases depending on the protein) and low or undetectable staining in normal urothelium generating a list of 54 proteins.

### **Statistical Analyses**

Urinary EGFR levels are presented as medians and statistical significance was calculated using Mann–Whitney tests. UBC-specific survival was defined as the time from registration into the BCPP study to date of death from UBC. Patients were censored at the date last known to be alive or date of UBC-related death. Univariable Cox proportional hazards models were employed (alpha 0.1) to identify factors to be included in a Cox multivariable model; significance was set at 0.05. Univariable factors included age (years), tumour number (1 vs 2+), grade (1, 2 vs 3), size of largest tumour ( $p < 3$  cm vs  $> 3$  cm), CIS (present vs absent), stage (pTa or pT1 vs pT2+), sex (male vs female), urinary EGFR (normal vs elevated) and urinary EpCAM (normal vs elevated). Analysis was done in Stata 12.1 (StataCorp, College Station, TX, USA).

## RESULTS

### Proteomic Analysis of Secretomes

In total, 2078 unique proteins were identified in the conditioned media of one or more of the UBC cell lines with 1338, 1025, 794, 593, 443, 312 and 188 proteins identified in 2, 3, 4, 5, 6, 7 or 8 UBC cell lines respectively (Tables 1, S2 and S3). All secretomes were analysed  $\pm$  PMA to stimulate secretion and shedding. Relative quantitation was achieved using stable isotope labelling and is shown for all proteins quantitated  $\pm$  PMA in at least 4 UBC cell lines in Figure 1a and Table S4. The cellular compartmentalisation of the 39 proteins with a  $>2$ -fold median upregulation in the secretomes due to PMA is shown in Figure 1b. The upregulated proteins are almost exclusively secreted and cell surface proteins (34 out of 39 = 87%), whereas the corresponding percentage for all proteins found in at least 4 UBC cell lines was 69% and for all proteins found in at least 2 UBC cell lines it was 63%. We found reports of 11 of these proteins previously being investigated as urinary biomarkers for UBC with 8 being significantly elevated in patients with UBC (Table S4).

### Selection of EGFR as a Candidate Biomarker

Whilst PMA helps to identify proteins which are genuinely secreted or shed from UBC cells it does not necessarily indicate that they are UBC specific, especially as release of the same proteins was also stimulated in the immortalised 'normal' urothelial UROtsa cell line (Table S4). To address this issue we compared the secretome data with immunohistochemistry data in the Human Protein Atlas. This approach, requiring proteins to be overexpressed in UBC and released from UBC cell lines generated a shortlist of 5 candidates (epidermal growth factor receptor or EGFR, glucose-6-phosphate dehydrogenase, peroxiredoxin 6, LY6/PLAUR domain containing 3 and fibulin 1); EGFR was unique in that it was the only one of these 5 proteins that was identified in the cancer cell line secretomes and not in the UROtsa secretome. Tryptic peptides from EGFR were detected in 7 of the

8 UBC cell lines with a tendency for more peptides (suggestive of a higher EGFR protein concentration) in the cell lines derived from higher grade tumours (Table 1). Both 'light' and 'heavy' EGFR peptides were identified at similar levels in the secretomes indicating that EGFR is released into the conditioned media in the absence and presence of PMA. These findings were confirmed by ELISA: all of the cell lines express and release some EGFR although levels range from very low in UROtsa and several of the UBC lines through to extremely high in HB-CLS-2. PMA has a modest effect (on average < 2-fold increase) on secretome EGFR levels and the amount of EGFR released into the conditioned media varies from cell line to cell line between approximately 1 and 10% of the total cellular EGFR.

### **Urinary EGFR as a diagnostic marker**

EGFR was measured in the urine of 436 UBC patients and 60 non-cancer controls. The data are summarised in Table 2. The median urinary EGFR concentration was 153pg/mg creatinine in the non-cancer controls and was not significantly increased in patients with pTa UBC. There were significantly higher levels of EGFR in the urine of patients with pT1 or pT2+ UBC (224 and 317pg/mg creatinine, respectively). The data for individual patients stratified according to stage and grade is shown in Figure 2. High levels of urinary EGFR were observed in a proportion, but not all, patients with high grade and high stage UBC. Defining the upper limit of the reference range as the mean + 2SD in the non-cancer control group gives a threshold of 630 pg EGFR/mg creatinine, and the percentage of positive cases is 7, 4, 13, and 27 in non-cancer controls, pTa, pT1 and pT2+ UBC, respectively.

### **Characterisation of 'soluble' EGFR**

Peptides from the extracellular domain, but not the intracellular domain, of EGFR were identified in secretomes from MGH-U3, RT112, 5637, RT-4 and SW-780 cell lines. VM-CUB-1 and HB-CLS-2

secretomes contained the highest levels of EGFR, and peptides from both the intracellular and extracellular domains were identified; however, both spectral counting and peptide intensity suggested a higher concentration of the extracellular domain than the intracellular domain i.e. the extracellular domain of EGFR is shed into the conditioned media but full-length membrane-bound EGFR is also present. To test this hypothesis we ultracentrifuged the secretomes at 136,000 x g for 90 minutes to pellet membranes and membrane-bound proteins and measured the EGFR remaining in the supernatants. On average, across the secretomes, 73% of the EGFR remained in the supernatant confirming that most of the EGFR is a soluble rather than membrane-bound form (Figure 3). Western blotting of the proteins released by HB-CLS-2 cells indicated that the predominant form of EGFR in the secretome has a molecular weight of approximately 100 kDa. A small amount of full-length EGFR (approximately 170 kDa) is also present but is removed by ultracentrifugation (Figure 3).

Ultracentrifugation was also used to test whether urinary EGFR was shed extracellular domain or membrane-bound EGFR: 12 urine samples with a range of elevated EGFR levels were ultracentrifuged and EGFR measured in the supernatants. On average, 93.5% (SD 7.8%) remained in the supernatant, indicating that the predominant form of EGFR in urine is the soluble extracellular domain.

### **Urinary EGFR as a prognostic marker**

Potential prognostic factors (age, number of tumours, grade, size of largest tumour, presence of CIS, stage, gender and urinary EGFR) were initially analysed in univariable models. EGFR was found to be highly significant (HR 6.9 90% CI 4.7, 10.1,  $p < 0.001$ ) and this can be seen in the Kaplan-Meier curves for UBC-specific survival in both NMIBC and MIBC (Figure 4). In the univariable analyses grade, stage, age, tumour size, CIS and urinary EGFR all reached statistical significance and were included in

a multivariable analysis. The multivariable analysis showed that elevated urinary EGFR has prognostic value (HR 2.9 95% CI 1.1, 4.6:  $P < 0.001$ ) over and above that provided by grade and stage for UBC-specific survival (Table 3a).

### **Combination of urinary EpCAM and EGFR for prognostication**

We have previously reported that the urinary concentration of the extracellular domain of EpCAM is associated with a shorter UBC-specific survival time (Bryan *et al*, 2014b). The urinary concentrations of EGFR and EpCAM have quite different distributions in our cohort of UBC patients: many patients have high levels of either EpCAM or EGFR (Figure 5) suggesting that they are not-redundant as biomarkers and might be used in combination to improve prognostication. Multivariable modelling including both biomarkers and all univariable factors was performed; grade and stage were both highly prognostic ( $p = 0.011$ ,  $p < 0.001$  respectively), as were EGFR (HR=6.69 95%CI 3.41, 13.12  $p < 0.001$ ) and EpCAM (HR=2.13 95%CI 1.19, 3.82  $p = 0.011$ ). In addition, the interaction between EGFR and EpCAM was statistically significant ( $p = 0.001$ ). It might be predicted that being 'double-positive' (high levels of both biomarkers) would carry an extremely poor prognosis, however, we found this not to be the case: compared to being 'double-negative' (low levels of both biomarkers), the risk of death from UBC doubles if a patient is EpCAM high and EGFR low, increases 6.7-fold with high EGFR and low EpCAM and for 'double-positive' the risk is increased 2.6-fold. Therefore, although the risk associated with being double-positive is greater than only being EpCAM positive it is less than being only EGFR positive ( $p = 0.001$ ) (Table 3b and Figure S2). Utilising the two biomarkers enables the identification of a double-negative group of patients who have the best prognosis and those patients who are EGFR high and EpCAM low who have the worst prognosis.

## DISCUSSION

Combining proteomic analysis of UBC cell line secretomes and immunohistochemistry data available in the Human Protein Atlas identified EGFR as a potential urinary biomarker for UBC. We then tested the diagnostic and prognostic utility of urinary EGFR in a cohort of 496 patients with long-term clinical follow-up. Urinary EGFR is elevated in 27% of MIBC cases and 17% of grade 3 UBCs but normal levels are observed in pTa and low-grade disease; thus, overall, urinary EGFR is not a good diagnostic marker for UBC. However, in patients with confirmed UBC, urinary EGFR is a strong independent prognostic indicator of UBC-specific survival. Urinary EGFR could have utility for rapidly identifying patients with the most aggressive disease, and expediting their subsequent investigation and management.

Shedding of EGFR from cancer cell lines (although not UBC cell lines) has been reported to be related to overexpression, to be stimulated by PMA (and hence may be under the control of protein kinase C), and to be blocked by metalloprotease inhibitors (Perez-Torres *et al*, 2008). Adamczyk reported that pancreatic cancer cell lines release both exosomal (full length, 170 kDa) and ectodomain (110 kDa) forms of EGFR (Adamczyk *et al*, 2011). Evidence suggests that EGFR ectodomain is shed as the result of proteolytic cleavage and that it is probably cleavage of an alternatively spliced 3kb transcript of EGFR that generates the soluble ectodomain (Wilken *et al*, 2013). Although this splice variant lacks both the transmembrane and intracellular domains it seems that it remains membrane associated until cleaved (Wilken *et al*, 2013). Our UBC cell line data is consistent with the body of evidence suggesting that both full-length and ectodomain EGFR are released from cancer cells by a regulated mechanism involving proteolytic cleavage.

Soluble EGFR has been detected in plasma and investigated in several cancers (Baron *et al*, 2003; Hudelist *et al*, 2006; Lemos-González *et al*, 2007; Müller *et al*, 2006; Zampino *et al*, 2008). However, plasma levels appear to be lower in cancer patients than healthy controls, are not related to EGFR

expression in tumours and there is little evidence of diagnostic, predictive or prognostic value. Although EGF has been measured in the urine of UBC patients (Chow *et al*, 1997), the only case of EGFR being measured in urine that we have encountered looks at a number of distal tumour sites and not UBC (Witters *et al*, 1995). Thus, we believe that the current work is the first exploration of soluble urinary EGFR as a biomarker for UBC, with a much clearer relationship between urinary EGFR and UBC than in studies of plasma EGFR in other solid malignancies.

EGFR has been reported to be overexpressed in many epithelial cancers and was reported as a poor prognostic indicator in UBC as early as 1990 (Neal *et al*, 1990), representing a late event in the progression of the disease (Lipponen & Eskelinen, 1994). More recently, EGFR overexpression has been shown to be a characteristic of basal-like aggressive MIBC, and in mouse models these tumours respond to anti-EGFR therapy (Rebouissou *et al*, 2014). Several clinical trials using anti-EGFR therapies in combination with chemotherapy or radiotherapy in MIBC are currently underway. Although further work is required to determine whether urinary levels of EGFR are entirely determined by the level of expression in tumour cells, or whether regulation of shedding is also important, the fact that many bladder tumours release detectable levels of EGFR into the urine suggests that urinary EGFR should be considered as a possible predictive marker in clinical trials evaluating anti-EGFR therapy in UBC.

## **CONCLUSIONS**

We present the first description of the prognostic value of soluble urinary EGFR in UBC, both alone and in combination with soluble urinary EpCAM (Bryan *et al*, 2014b). These ELISA-based tests have significant potential clinical utility and we intend to evaluate this potential prospectively in UBC, as well as determining whether other urological malignancies also increase urine EGFR levels. Mechanisms of ectodomain shedding may add an extra layer of complexity to the molecular

pathology of UBC that may not be uncovered by genomic approaches and appear to unmask potentially important prognostic markers. Better understanding of these phenomena may also reveal new therapeutic targets, targets that are desperately needed for UBC (Bryan *et al*, 2014a).

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## Titles and Legends to Tables and Figures

Table 1. UBC cell line secretome analysis. The total number of unique peptides identified by LC-MS/MS in each secretome and the resulting protein identifications are shown alongside the number of EGFR peptides identified. Proteomic analysis was performed once per cell line. The concentration of EGFR (determined by ELISA on biological triplicates) in paired secretomes and cell lysates is shown in ng EGFR per mg of cellular protein (determined by BCA assay). The percentage of secreted EGFR (% Sec) is relative to cellular EGFR.

Table 2. Patient data and urinary EGFR summary. The total numbers of patients in each stage group and the numbers of males and females and number of patients with grade 1, 2 or 3 UBC are shown. Age and urinary EGFR for each stage group are shown as median (IQR). P-values and ROC areas are provided for each stage of UBC *versus* non-cancer controls. The number of positive cases in each group is calculated using a threshold of 630 pg EGFR/mg creatinine (mean + 2 SD of the urinary EGFR concentrations in the non-cancer group).

Table 3a. Cox multivariable analysis of prognostic indicators.

Table 3b. Cox multivariable analysis of prognostic indicators including EpCAM.

Figure 1. The effect of PMA on UBC cell line secretomes. Figure 1a shows the log<sub>2</sub> of median fold-change for proteins quantitated in 4 or more UBC cell line secretomes ± PMA (each datapoint is for a single protein and the proteins have been sorted from largest increase to largest decrease): 39 proteins show a >2-fold increase and 6 proteins a >2-fold decrease. The cell compartment for the 39 upregulated proteins is shown in Figure 1b.

Figure 2. Urinary EGFR in UBC patients. The left hand panel shows urinary EGFR data for patients stratified according to stage and the right hand panel according to grade.

Figure 3. The effect of ultracentrifugation on EGFR. The histogram shows the percentage of EGFR remaining in the supernatant following ultracentrifugation. Secretome data is shown as the mean  $\pm$  SD across all 9 cell lines ( $\pm$  PMA) and urine data as the mean  $\pm$  SD for urine from 12 patients with UBC and elevated EGFR. The Western blot for shows EGFR in HB-CLS-2 cell lysate and whole (W) and ultracentrifuged (U) HB-CLS-2 conditioned media.

Figure 4. The prognostic value of urinary EGFR. Kaplan-Meier curves are shown for UBC-specific survival with patients stratified according to low/high urinary EGFR (>630 pg/mg creatinine).

Figure 5. Relationship between urinary EGFR and EpCAM in UBC: the creatinine-normalised concentrations of EGFR and EpCAM in the urine of patients with MIBC.

- Adamczyk K, Tehrani M, Warnken U, Schmiegel W, Schnölzer M, Schwarte-Waldhoff I (2011) Characterization of soluble and exosomal forms of the EGFR released from pancreatic cancer cells. *Life Sci* **89**: 304-312
- Babjuk M, Burger M, Zigeuner R, Shariat S, van Rhijn B, Compérat E, Sylvester R, Kaasinen E, Böhle A, Palou Redorta J, Rouprêt M (2013) EAU guidelines on non-muscle-invasive urothelial carcinoma of the bladder: update 2013. *Eur Urol* **64**: 639-653
- Baron A, Cora E, Lafky J, Boardman C, Buenafe M, Rademaker A, Liu D, Fishman D, Podratz K, Maihle N (2003) Soluble epidermal growth factor receptor (sEGFR/sErbB1) as a potential risk, screening, and diagnostic serum biomarker of epithelial ovarian cancer. *Cancer Epidemiol Biomarkers Prev* **12**: 103-113
- Bryan R, Kirby R, O'Brien T, Mostafid H (2014a) So Much Cost, Such Little Progress. *European Urology* **66**: 253-262
- Bryan R, Shimwell N, Wei W, Devall A, Pirrie S, James N, Zeegers M, Cheng K, Martin A, Ward D (2014b) Urinary EpCAM in urothelial bladder cancer patients: characterisation and evaluation of biomarker potential. *Br J Cancer* **110**: 679-685
- Burger M, Catto J, Dalbagni G, Grossman H, Herr H, Karakiewicz P, Kassouf W, Kiemenev L, La Vecchia C, Shariat S, Lotan Y (2013) Epidemiology and risk factors of urothelial bladder cancer. *Eur Urol* **63**: 234-241
- Chow N, Liu H, Lee E, Chang C, Chan S, Cheng H, Tzai T, Lin J (1997) Significance of urinary epidermal growth factor and its receptor expression in human bladder cancer. *Anticancer Res* **17**: 1293-1296
- Hansen A, Freeman T, Arnold S, Starchenko A, Jones-Paris C, Gilger M, Washington M, Fan K, Shyr Y, Beauchamp R, Zijlstra A (2013) Elevated ALCAM shedding in colorectal cancer correlates with poor patient outcome. *Cancer Res* **15**: 2955-2964
- Huang Z, Lin L, Gao Y, Chen Y, Yan X, Xing J, Hang W (2011) Bladder cancer determination via two urinary metabolites: a biomarker pattern approach. *Mol Cell Proteomics* **10**: 1-10
- Hudelist G, Köstler W, Gschwantler-Kaulich D, Czerwenka K, Kubista E, Müller R, Helmy S, Manavi M, Zielinski C, Singer C (2006) Serum EGFR levels and efficacy of trastuzumab-based therapy in patients with metastatic breast cancer. *Eur J Cancer* **42**: 186-192
- Kandimalla R, Masius R, Beukers W, Bangma C, Orntoft T, Dyrskjot L, van Leeuwen N, Lingsma H, van Tilborg A, Zwarthoff E (2013) A 3-plex methylation assay combined with the FGFR3 mutation assay sensitively detects recurrent bladder cancer in voided urine. *Clin Cancer Res* **19**: 4760-4769
- Kaplan A, Litwin M, Chamie K (2014) The future of bladder cancer care in the USA. *Nat Rev Urol* **11**: 59-62
- Kaufman D, Shipley W, Feldman A (2009) Bladder cancer. *Lancet* **374**: 239-249
- Lemos-González Y, Rodríguez-Berrocal F, Cordero O, Gómez C, Páez de la Cadena M (2007) Alteration of the serum levels of the epidermal growth factor receptor and its ligands in patients with non-small cell lung cancer and head and neck carcinoma. *Br J Cancer* **96**: 1569-1578

Lipponen P, Eskelinen M (1994) Expression of epidermal growth factor receptor in bladder cancer as related to established prognostic factors, oncoprotein (c-erbB-2, p53) expression and long-term prognosis. *Br J Cancer* **69**: 1120-1125

Müller V, Witzel I, Pantel K, Krenkel S, Lück H, Neumann R, Keller T, Dittmer J, Jänicke F, Thomssen C (2006) Prognostic and predictive impact of soluble epidermal growth factor receptor (sEGFR) protein in the serum of patients treated with chemotherapy for metastatic breast cancer. *Anticancer Res* **26**: 1479-1487

Neal D, Sharples L, Smith K, Fennelly J, Hall R, Harris A (1990) The epidermal growth factor receptor and the prognosis of bladder cancer. *Cancer* **65**: 1619-1625

Orenes-Piñero E, Cortón M, González-Peramato P, Algaba F, Casal I, Serrano A, Sánchez-Carbayo M (2007) Searching urinary tumor markers for bladder cancer using a two-dimensional differential gel electrophoresis (2D-DIGE) approach. *J Proteome Res* **11**: 4440-4448

Perez-Torres M, Valle B, Maihle N, Negron-Vega L, Nieves-Alicea R, Cora E (2008) Shedding of epidermal growth factor receptor is a regulated process that occurs with overexpression in malignant cells. *Exp Cell Res* **314**: 2907-2918

Phillips H, Williamson J, van Elburg K, Snijders A, Wright P, Dickman M (2010) Shotgun proteome analysis utilising mixed mode (reversed phase-anion exchange chromatography) in conjunction with reversed phase liquid chromatography mass spectrometry analysis. *Proteomics* **10**: 2950-2960

Rebouissou S, Bernard-Pierrot I, de Reyniès A, Lepage M, Krucker C, Chapeaublanc E, Hérault A, Kamoun A, Caillault A, Letouzé E, Elarouci N, Neuzillet Y, Molinié V, Vordos D, Laplanche A, Maillé P, Soyeux P, Ofualuka K, Reyat F, Biton A, Sibony M, Paoletti X, Southgate J, Benhamou S, Lebret T, Allory Y, Radvanyi F (2014) EGFR as a potential therapeutic target for a subset of muscle-invasive bladder cancers presenting a basal-like phenotype. *Sci Transl Med* **6**: 244ra91

Uhlen M, Oksvold P, Fagerberg L, Lundberg E, Jonasson K, Forsberg M, Zwahlen M, Kampf C, Wester K, Hober S, Wernerus H, Björling L, Ponten F (2010) Towards a knowledge-based Human Protein Atlas. *Nat Biotechnol* **28**: 1248-1250

Wilken J, Perez-Torres M, Nieves-Alicea R, Cora E, Christensen T, Baron A, Maihle N (2013) Shedding of soluble epidermal growth factor receptor (sEGFR) is mediated by a metalloprotease/fibronectin/integrin axis and inhibited by cetuximab. *Biochemistry* **52**: 4531-4540

Witjes J, Compérat E, Cowan N, De Santis M, Gakis G, Lebret T, Ribal M, Van der Heijden A, Sherif A (2014) EAU guidelines on muscle-invasive and metastatic bladder cancer: summary of the 2013 guidelines. *Eur Urol* **65**: 778-792

Witters L, Curley E, Kumar R, Chinchilli V, Harvey J, Crebbin V, Harvey H, Lipton A (1995) Epidermal growth factor receptor ectodomain in the urine of patients with squamous cell carcinoma. *Clin Cancer Res* **1**: 551-557

Zampino M, Magni E, Santoro L, Zorzino L, Dell'Orto P, Sonzogni A, Fazio N, Monfardini L, Chiappa A, Biffi R, de Braud F (2008) Epidermal growth factor receptor serum (sEGFR) level may predict

response in patients with EGFR-positive advanced colorectal cancer treated with gefitinib? *Cancer Chemother Pharmacol* **63**: 139-148

Zatovicova M, Sedlakova O, Svastova E, Ohradanova A, Ciampor F, Arribas J, Pastorek J, Pastorekova S (2005) Ectodomain shedding of the hypoxia-induced carbonic anhydrase IX is a metalloprotease-dependent process regulated by TACE/ADAM17. *British Journal of Cancer* **93**: 1267-1276

Zeegers M, Bryan R, Langford C, Billingham L, Murray P, Deshmukh N, Hussain S, James N, Wallace D, Cheng K (2010) The West Midlands Bladder Cancer Prognosis Programme: rationale and design. *BJU Int* **105**: 784-788

Figure 1.

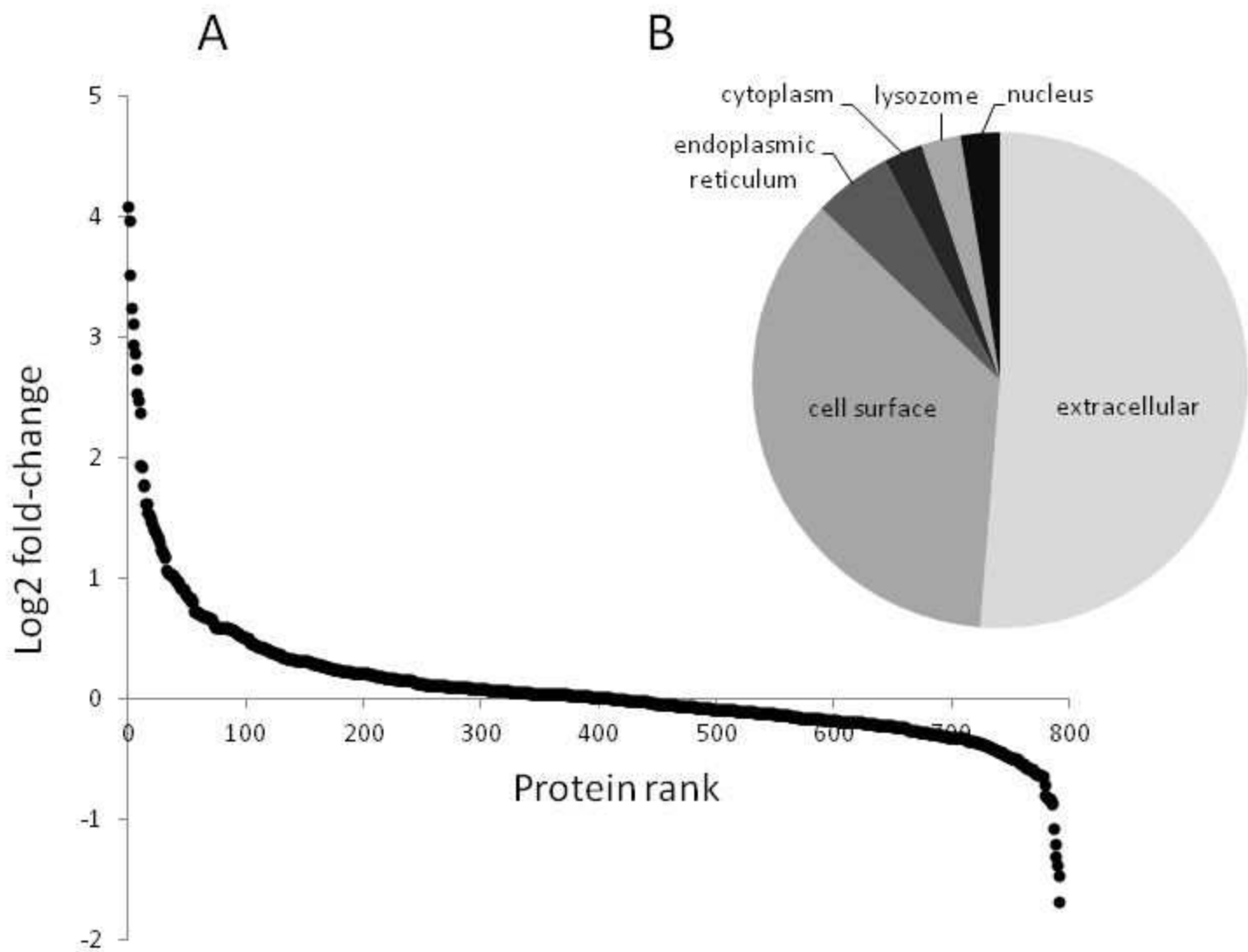


Figure 2.

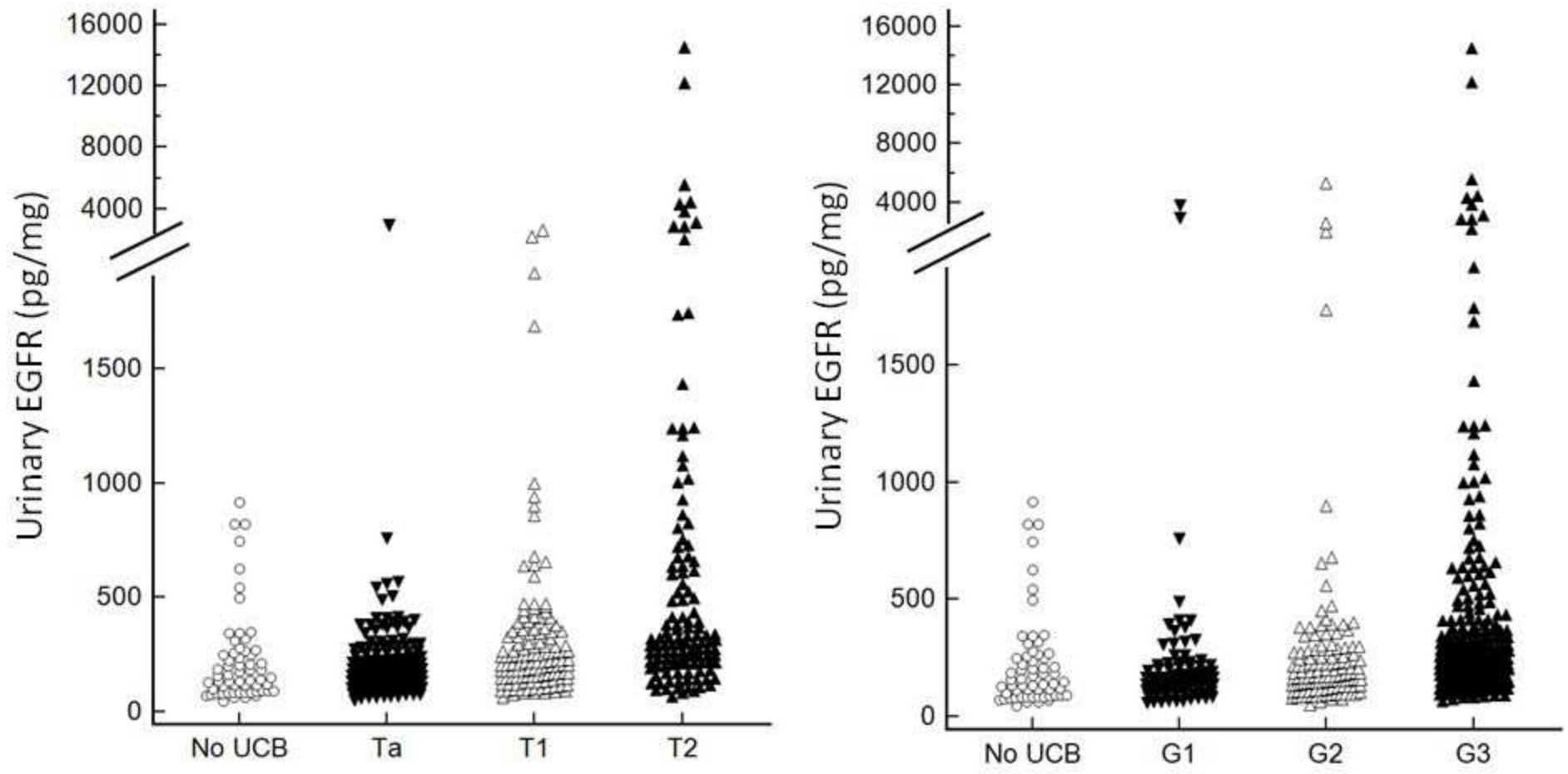


Figure 3.

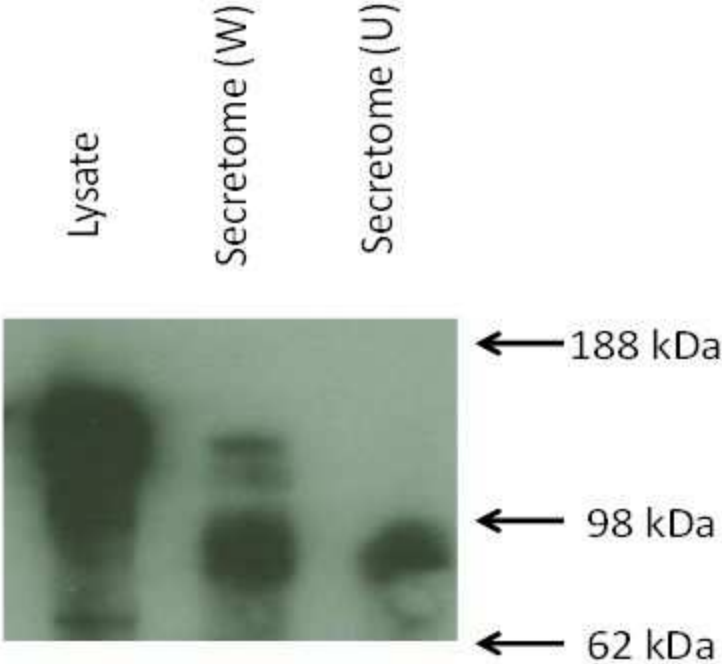
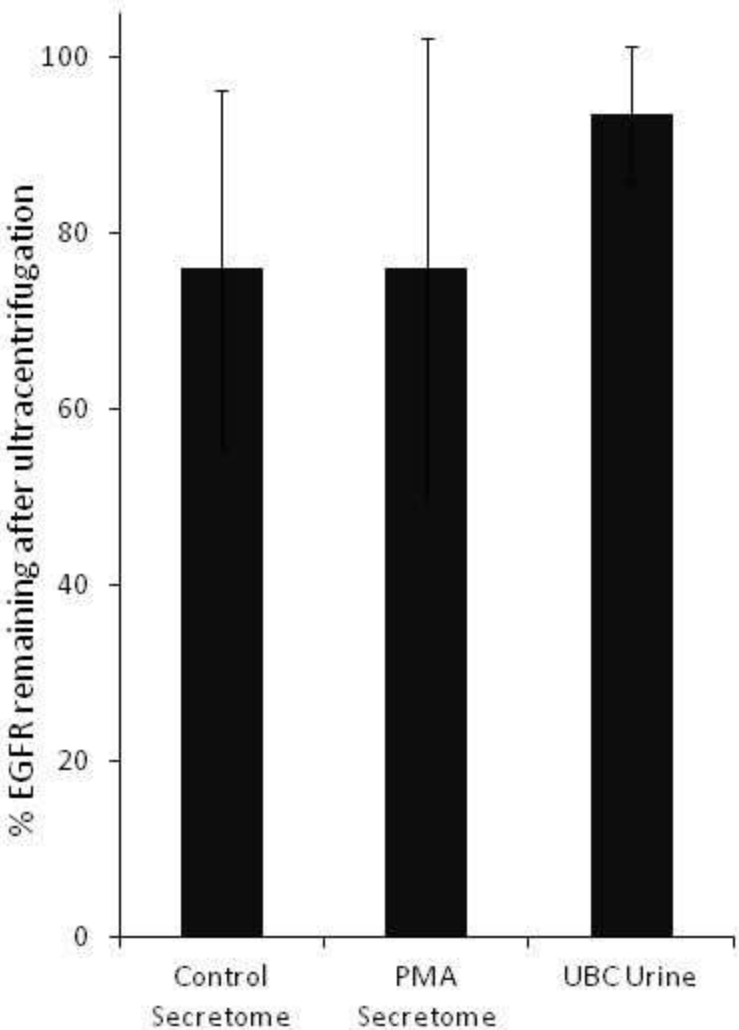
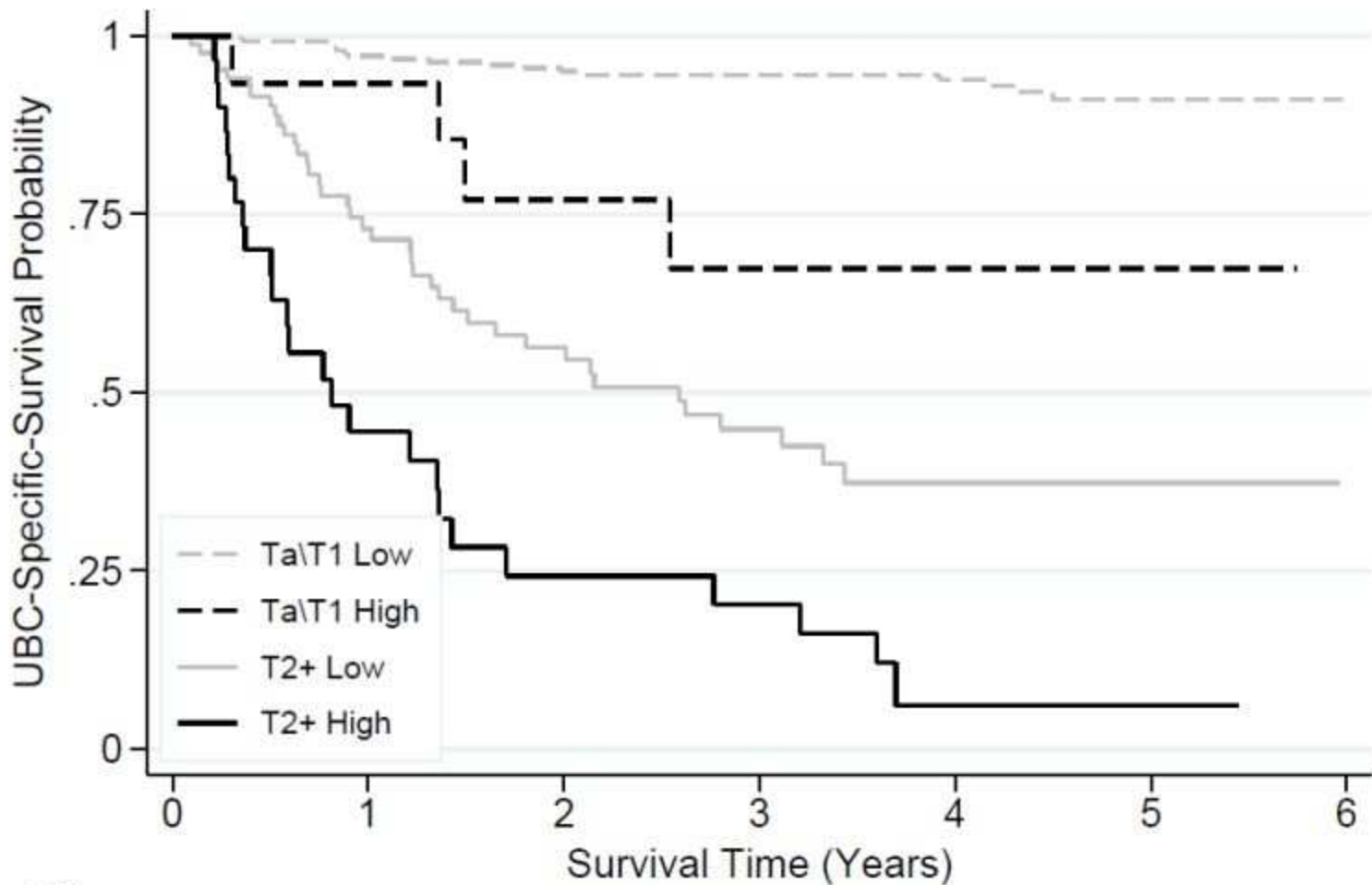


Figure 4.



Patients at risk

Ta\T1 Low	267	237	207	176	122	58	0
Ta\T1 High	15	13	8	7	5	3	0
T2+ Low	86	47	31	20	12	12	0
T2+ High	33	12	6	5	1	1	0

Figure 5.

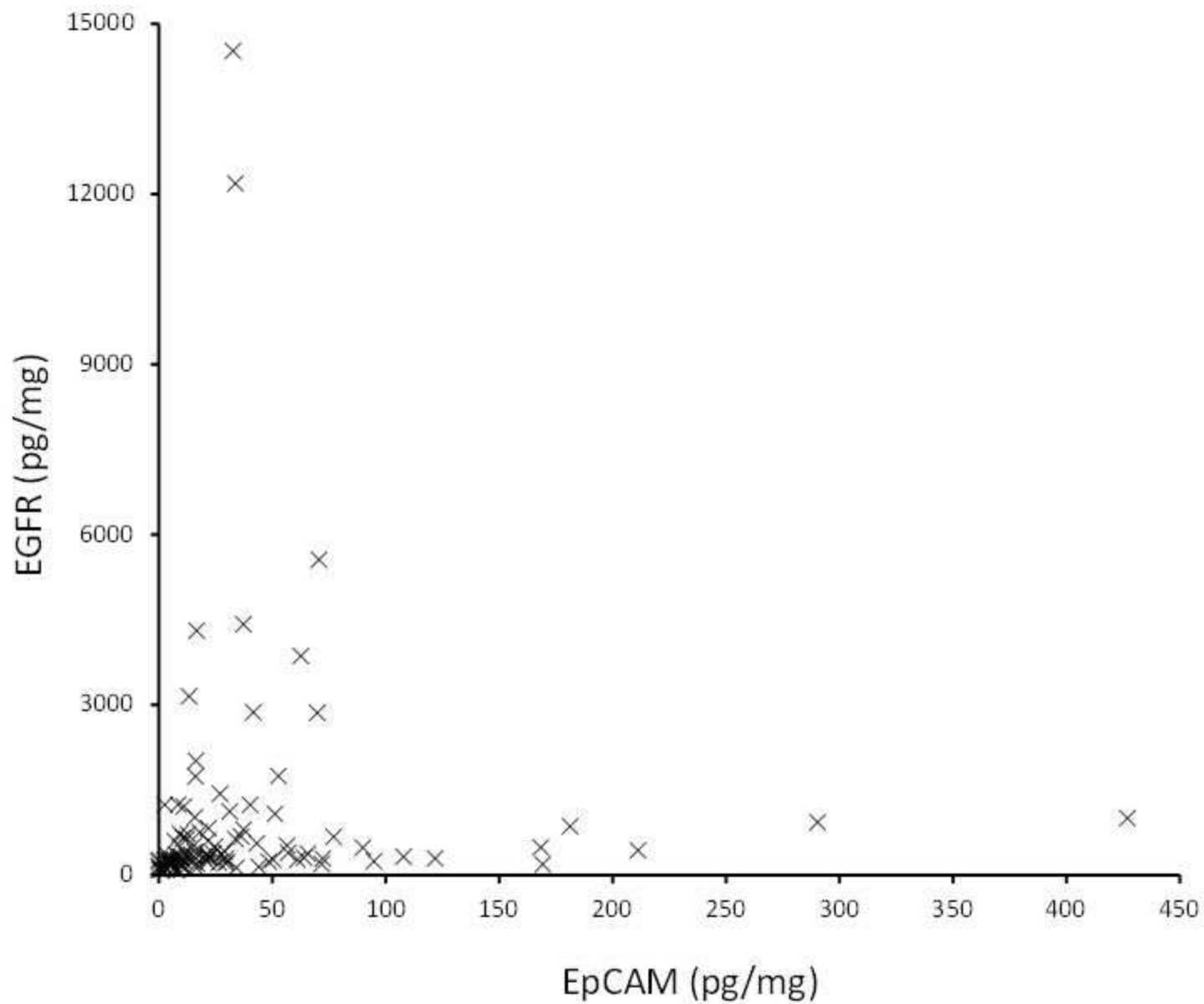


Table 1.

Cell line	Grade	No. human peptides	No. human proteins	No. EGFR peptides	Secretome [EGFR] ng/mg	Cellular [EGFR] ng/mg	% Sec
UROtsa	NA	5499	763	0	0.40 (0.03), 0.72 (0.08)	43.7 (1.3), 61.4 (3.7)	1.0
MGH-U3	1	10954	1041	5	4.29 (0.92), 4.46 (1.19)	90.8 (4.7), 94.7 (8.9)	4.7
R-T4	1	8145	1160	1	1.30 (0.36), 2.62 (0.29)	34.0 (18.0), 22.1 (11.8)	9.6
SW-780	1	4181	627	1	2.81 (0.32), 4.11 (0.62)	49.3 (4.50), 62.2 (16.7)	5.9
RT112	2	5900	787	4	0.66 (0.07), 1.18 (0.17)	82.4 (3.3), 62.0 (5.6)	1.3
VM-CUB-1	2	5915	765	16	10.8 (2.39), 9.40 (1.18)	227 (3.79), 151 (41.7)	5.5
5637	2	4188	628	3	0.41 (0.03), 0.68 (0.04)	102 (3.81), 72.8 (0.87)	0.7
T-24	3	7087	916	0	1.19 (0.32), 2.37 (0.57)	27.1 (1.36), 29.0 (3.18)	6.3
HB-CLS-2	3	7135	847	53	252 (92.8), 298 (22.3)	2720 (161), 2646 (927)	10.3

Table 2.

Stage	n	Male/Female	Grade (G1/G2/G3)	Age	EGFR (pg/mg creatinine)	p-value	ROC	No. positive cases
Non-cancer	60	45/15	na	75 (66-79)	153 (91-261)	na	na	4 (7%)
pTa	184	140/44	79/84/21	74 (65-81)	167 (124-236)	0.395	0.536	3 (2%)
pT1	130	112/18	2/37/91	75 (67-80)	224 (151-353)	0.0003	0.650	13 (10%)
pT2+	122	97/25	0/6/116	78 (68-83)	317 (215-658)	0.0001	0.766	33 (27%)

Table 3a.

Variable	$\beta$	Se( $\beta$ )	Haz Ratio	P-value	HR 95% CI
Grade 3 (reference grade 1 or 2)	0.982	0.395	2.670	0.013	1.231, 5.792
Stage pT2+ (reference pTa or pT1)	1.997	0.306	7.369	<0.001	4.043, 13.429
High EGFR (reference low EGFR)	1.062	0.239	2.891	<0.001	1.809, 4.620

Table 3b.

Variable	$\beta$	Se( $\beta$ )	Haz Ratio	P-value	HR 95% CI
Grade 3 (reference grade 1 or 2)	1.005	0.394	2.732	0.011	1.262, 5.912
Stage pT2+ (reference pTa or pT1)	1.892	0.301	6.632	<0.001	3.680, 11.953
High EGFR (reference low EGFR)	1.900	0.344	6.691	<0.001	3.411, 13.124
High EpCAM (reference low EpCAM)	0.756	0.298	2.131	0.011	1.189, 3.818
EGFR and EpCAM Interaction (High EGFR and EpCAM)	-1.689	0.487	0.185	0.001	0.071, 0.480