Soluble HER3 predicts survival in bladder cancer patients

ASHFAQUE A. MEMON[1,2], STEPHEN C. GILLIVER[2], MICHAEL BORRE[3], JAN SUNDQUIST[2], KRISTINA SUNDQUIST[2], EBBA NEXO[1] and BOE S. SORENSEN[1]

[1]Department of Clinical Biochemistry, Aarhus University Hospital, Aarhus DK-8200, Denmark; [2]Center for Primary Health Care Research, Lund University, 205 02 Malmö, Sweden; [3]Department of Urology, Aarhus University Hospital, Aarhus DK-8200, Denmark

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Abstract. The role of soluble human epidermal growth factor receptor (sHER3) in bladder cancer remains unclear. In the present study, an ELISA was developed for the quantification of sHER3 and its role was investigated in patients with bladder cancer (n=82) followed for 10 years. Furthermore, the effects of sHER3 on bladder cancer cell growth and migration were also investigated. The results demonstrated that plasma sHER3 levels were significantly higher in non-invasive tumours (Ta) compared with muscle-invasive tumours (T2-T4). Higher sHER3 levels were associated with a more improved survival rate. However multivariate Cox regression analysis, adjusted for clinical stage, grade, type and size of the tumour, demonstrated that sHER3 was not an independent biomarker of survival. Exogenous sHER3 significantly inhibited bladder cancer cell growth and migration. These results suggest that high sHER3 levels are associated with improved survival rates in patients with bladder cancer, and that sHER3 inhibits bladder cancer cell growth and migration.

Introduction

Bladder cancer is the fifth most common type of cancer in men and ninth in women (1). Various factors have been investigated for their involvement in the pathogenesis of bladder cancer. Such factors include the epidermal growth factor (EGF) family, which consists of four transmembrane tyrosine kinase receptors, human epidermal growth factor receptor (HER)1, HER2, HER3 and HER4, and a dozen ligands (2). While HER1, HER2 and HER4 activate intracellular signals following ligand binding, HER3 (also named ErbB3 and encoded by the *ErbB3* gene) lacks a kinase domain, and activates intracellular signals by forming heterodimers with other EGF receptors (3). HER3 also exists as soluble HER3 (sHER3), a truncated secreted form of either 45 kDa (p45-sHER3) or 85 kDa (p85-sHER3) (4). p85-sHER3 is a potent negative regulator of HER2, HER3 and HER4 activation (5).

We previously demonstrated that increased tumour expression of HER3 mRNA was associated with a more improved survival rate in bladder cancer (6,7). The aim of the present study was to explore the role of sHER3 as a potential non-invasive predictor of prognosis in patients with bladder cancer and to identify the possible mechanisms involved.

Materials and methods

Patients. Eighty-two patients with primary bladder cancer were included with a median age of 70 years (range, 53-88). A total of 66 patients were male and 16 were female. Biopsies were consecutively collected between November 1995 and June 2002 as part of the Molecular Oncology of the Bladder Bio-bank (Aarhus, Denmark). No patients were excluded from the present study. The tumours were staged according to the Union for International Cancer Control Tumour Node Metastasis staging system (7th edition) (8) and graded according to the World Health Organisation/International Society of Urological Pathology 2004 classification system (9). Patients were allocated to one of three groups according to tumour stage: Ta, superficial tumours; T1, superficial invasive tumours; and T2-T4, muscle-invasive tumours. At the time of inclusion, 18 patients had previously received treatment in the form of radical radiotherapy, chemotherapy or intravesical bacillus Calmette-Guérin therapy. The follow-up period was between the date of biopsy and the day of mortality or the end of the study. Patients were censored if they were alive at the time of analysis. The median follow-up was 54 months (range, 1-141 months). The Regional Committee of Scientific Ethics in Aarhus (Aarhus, Denmark) approved the study (approval no. 1994/2920) and written informed consent was provided by all participants in the study. All the procedures were performed in accordance with the Declaration of Helsinki. The same cohort was previously used in multiple studies (6,7,10).

ELISA. Whole-blood samples were collected into EDTA tubes and centrifuged at 2,000 x g for 15 min at 4˚C to collect the plasma. The samples were then aliquoted and stored at -80˚C for ELISA analysis as described below.

Correspondence to: Dr Ashfaque A. Memon, Centre for Primary Health Care Research, Lund University, CRC, Floor 11, Building 28, 35 Jan Waldenströms Street, 205 02 Malmö, Sweden
E-mail: ashfaque.memon@med.lu.se

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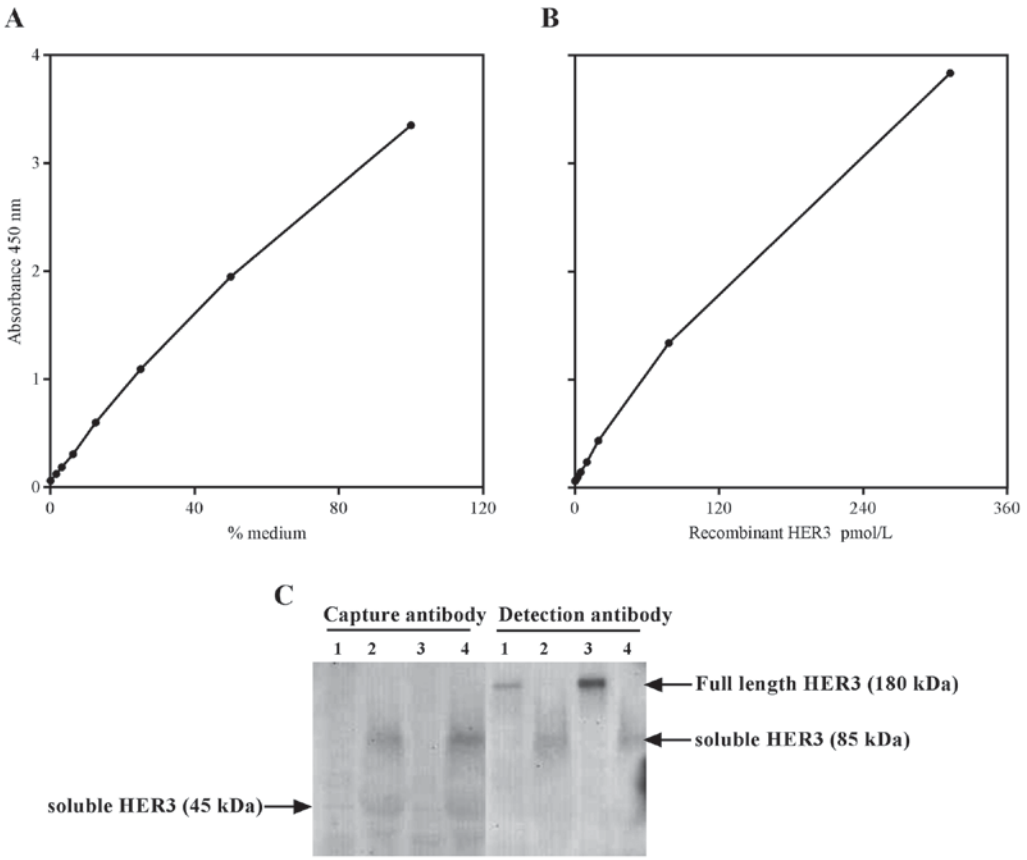

Figure 1. Calibration curve for capture and detection antibodies used in sHER3 ELISA. Calibration curve for sHER3 ELISA using (A) conditioned medium from WM266 melanoma cells or (B) recombinant HER3 as the calibrator. (C) Western blotting with capture (catalogue no. MAB3481) and detection (catalogue no. AF234) antibodies, showing full-length HER3 (180 kDa) in cell lysates (lanes 1 and 3) and sHER3 (~85 kDa) in conditioned media (lanes 2 and 4). A 45 kDa band was also detected with capture antibody, but not with detection antibody. Two cell lines, DU145 (lanes 1 and 2) and WM266 (lanes 3 and 4), were used. sHER3, soluble human epidermal growth factor receptor.

A sandwich ELISA was developed employing monoclonal anti-human HER3 antibody (dilution, 1:1,000; catalogue no. MAB3481; R&D Systems Europe, Ltd., Abingdon, UK) as the capture antibody and the polyclonal goat anti-human HER3 antibody (dilution, 1:200; catalogue no. AF234; R&D Systems Europe, Ltd.) as the detection antibody. A total of 0.1 μg capture antibody was diluted in 100 μl of 15 mmol/l sodium carbonate and 35 mmol/l sodium bicarbonate (pH 9.6), and added to each well of Nunc MaxiSorp F96 immunoplates (catalogue no. 442404; Thermo Fisher Scientific, Inc., Waltham, MA, USA). Following incubation for 20 h at 4˚C, the supernatant was removed from the wells and the samples were blocked with 200 μl of 1 mol/l ethanolamine for 20 h at 4˚C. The plates were stored at -20˚C until use in subsequent experiments.

The detection antibody was biotinylated by dissolving 80 μg polyclonal goat anti-human HER3 antibody in 800 μl of dilution buffer (10 mM phosphate, 145 mM sodium chloride, pH 7.4) and dialysing it against 1 litre of sodium hydrogen carbonate (0.1 M, pH 8.3) for 24 h at 4˚C with one change of dialysis buffer after 3 h. The dialysed sample was incubated with 8 μl of 4.4 mM biotinaminocaproate N-hydroxysuccinimidyl ester in dimethyl sulfoxide both (Sigma-Aldrich; Merck KGaA, Darmstadt, Germany) in the dark with gentle agitation for 4 h at room temperature. Following the addition of 8 μl of 100 mM lysine-HCl (Fluka Chemie GmbH, Sigma-Aldrich; Merck KGaA), the samples were incubated for 15 min at room temperature. Finally, 8 μl of λ globulin [5% rabbit λ globulin (Calbiochem; Merck KGaA) and 10% bovine IgG (Sigma-Aldrich; Merck KGaA)] dissolved in dilution buffer was added, and the sample was dialysed for 3 days against 1 litre of dilution buffer with three changes of buffer, and with 0.1% sodium azide in the last volume of buffer. The biotinylated antibody was stored at -20˚C until further use.

WM266 melanoma cells were obtained from American Type Culture Collection (ATCC, Manassas, VA, USA) and were cultured in Dulbecco's Modified Eagle's Medium (DMEM; Gibco; Thermo Fisher Scientific, Inc.) at 37˚C with 5% CO_2. Conditioned media from these cells was used as a calibrator, and assigned a value of 100 arbitrary units to the highest calibrator. When comparing the signal to that of recombinant HER3 (catalogue no. 348-RB; R&D Systems Europe, Ltd.) 100 arbitrary units corresponded to ~300 pmol/l (Fig. 1A). The calibration curve covered 0.1-100 arbitrary units (Fig. 1B).

Each assay was performed as follows. First, the plates were washed three times with washing buffer [10 mmol/l sodium phosphate buffer containing 145 mmol/l NaCl and 1 g/l Tween 20 (pH 7.4); VWR International, Radnor, PA, USA; catalogue no. AMPQ15265)]. Then, 100 μl of calibrator or sample diluted (1:7) in assay buffer [0.1 M phosphate, 0.15 bovine serum albumin (catalogue no. A7030; Sigma-Aldrich; Merck KGaA),

pH 8.0] was added to each well. After incubation for 2 h at room temperature, the plates were washed three times with washing buffer. The detection antibody was diluted to a concentration of 485 μg/l in assay buffer and 100 μl of diluted antibody was added to each well. The plates were then incubated for 2 h at room temperature. The plates were washed three times with washing buffer, and 100 μl of horseradish peroxidase-avidin (Dako; Agilent Technologies, Inc., Santa Clara, CA, USA; catalogue no. P0364) diluted 1:2,000 in 10 mmol/l sodium phosphate (pH 7.4), 400 mmol/l NaCl and 0.2 g/l lysozyme (catalogue no. L6876; Sigma-Aldrich; Merck KGaA) was added to each well. The plates were incubated for 30 min at room temperature and were washed three times with washing buffer. Next, 100 μl of TMB ONE ready-to-use substrate (catalogue no. 4380A; Kem-En-Tec Diagnostics, Taastrup, Denmark) was added. The colour reaction was stopped after 18 min by adding 100 μl of 1 mol/l phosphoric acid at room temperature. The colour developed was measured photometrically at a wavelength of 450 nm and corrected for absorbance at 620 nm. The calibration curve was computed by plotting the absorbance of the calibrators and constructing a cubic spline curve using GraphPrism for Windows version 7 (GraphPad Software Inc., La Jolla, CA, USA). The imprecision of the assay was 14% (n=82; mean, 4.5 arbitrary units) as determined from running the patient samples twice ~1 month apart. The antibodies were tested using western blotting with cell lysate and conditioned media from DU145 (ATCC) prostate cancer cells which were cultured in DMEM (Gibco; Thermo Fisher Scientific, Inc.) at 37˚C with 5% CO_2, and WM266 melanoma cells.

Cell culture and reagents. HCV-29 bladder cancer cells were obtained from the American Type Culture Collection (Manassas, VA, MD). Cells were seeded in T25 culture flasks (Nalge Nunc International, Penfield, NY, USA) and maintained at 37˚C in a humidified atmosphere of 5% CO_2 in Dulbecco's modified Eagle's medium (DMEM) supplemented with 10% foetal calf serum. Cells were checked routinely for Mycoplasma infection. At 80-90% confluence, cells were treated with 10-40 nM sHER3 (R&D Systems Europe, Ltd.). Cells untreated with sHER3 were used a control. Control and treated cells were washed with cold PBS [137 mM NaCl, 2.7 mM KCl, 10 mM Na_2HPO_4, 2 mM KH_2PO_4 (pH 7.4)] and harvested after 24 h with scraping buffer (PBS containing 4 mM iodoacetic acid, 1 mM orthovanadate, 1 μg/ml aprotinin, 1 μg/ml chymostatin, 1 μg/ml leupeptin and 1 μg/ml pepstatin). Cell pellets were stored at -80˚C for western blotting analysis.

Western blotting. Cell pellets were incubated on ice for 30 min in RIPA buffer (150 mM NaCl, 1% NP-40, 0.5% deoxycholate, 0.1% sodium dodecylsulfate, 50 mM Tris-HCl, pH 7.2, supplemented with 4 mM iodoacetate, 1 mM orthovanadate, 1 mg/ml eachpepstatin, chymostatin, leupeptin, and aprotinin), homogenised by gentle vortexing and cleared by centrifugation at 18,500 x g at 4˚C for 10 min (11). Protein concentration was determined using BCA reagent (Pierce; Thermo Fisher Scientific, Inc.). Equal amounts of protein (25 μg) were resolved by 8-12% SDS-PAGE. Resolved proteins were transferred onto polyvinylidene difluoride membranes, which were blocked overnight at 4˚C with 5% (w/v) non-fat dry milk in TBS-T solution [25 mM Tris (pH 7.5), 150 mM NaCl, 0.05 % (w/v) Tween 20]. After washing, the membranes were incubated with specific primary and secondary antibodies according to the manufacturer's protocol. Primary antibodies against the following proteins were used: Phosphorylated-HER3; total-HER3; and β-actin (Sigma-Aldrich; Merck KGaA). Immunoreactive bands were detected using ECL reagent (GE Healthcare, Chicago, IL, USA). Secondary horseradish peroxidase-conjugated goat anti-rabbit antibody (dilution, 1:2,000; catalogue no. P0448; Dako; Agilent Technologies, Inc., Santa Clara, CA, USA) for 40 min at room temperature followed by washing and enhanced chemiluminescence (ECL) for 2 min. Immunoreactive bands were detected by a Biospectrum® AC Imaging System (UVP, Inc., Upland, CA, USA). This experiment was repeated at least twice.

Proliferation and migration assays. The proliferation of HCV-29 cells was assessed using xCELLigence technology as described previously (9). Briefly, a total of $5x10^3$ HCV-29 cells were seeded in quadruplets overnight in DMEM in a 96-well microelectronic cell sensor system plate (Roche Diagnostics, Basel, Switzerland). They were then treated with the recombinant sHER3 peptide (0 or 20 nM). After treatment, the plate was inserted into an xCELLigence RTCA proliferation instrument (Roche Diagnostics) connected to a computer. This system measures cell growth by analysing changes in electrical impedance in microelectrodes under the 96-well plate when cells attach to the bottom of the plate (12). Cell growth was analysed in real time for 96 h. Values are expressed as a cell index, normalised for differences in cell density immediately prior to treatment. The experiment was performed in triplets (n=3) and repeated at least three times.

HCV-29 cell migration was also assessed using xCELLigence technology. Cells were treated with recombinant sHER3 peptide (0, 20 or 40 nM) and their migration relative to untreated control cells was measured at 24 h. The experiment was run in quadruplet (n=4) and repeated at least twice.

Statistical analysis. Kruskal-Wallis test following by Bonferonni post hoc test was performed to compare overall differences in sHER3 levels in T-stages and migration assay results. The multiple comparisons' criterion was employed to identify differences between groups. $P<0.05$ were considered to indicate a significant difference (two-tailed). Kaplan-Meier survival curves were used to estimate the survival rate of the patients. The log-rank test was used to compare the survivals. The significance of various variables for survival was analysed by the Cox proportional hazards model in the multivariate analysis. Statistical analyses were performed using SPSS software version 23.0 (IBM Corp., Armonk, NY, USA). Graphs and figures were made using GraphPad Prism software version 7.0 (GraphPad Software, Inc., La Jolla, CA, USA).

Results

ELISA assay. An ELISA was developed for HER3 employing commercial antibodies (Fig. 1). To determine which isoform was detected by this novel ELISA, western blotting was performed using capture and detection antibodies in cell lysate, and conditioned media samples obtained from WM266

Table I. Baseline data and distribution of sHER3 according to T stage of bladder cancer.

T stage	Ta	T1	T2-4
Patients, n (%)	23 (28)	18 (22)	41 (50)
Sex, n (%)			
Male	15 (65)	16 (89)	35 (85)
Female	8 (35)	2 (11)	6 (15)
Age (years)			
Median	69	74	68
Range	53-88	60-88	54-83
sHER3, n (%)			
≤Median	8 (35)	6 (33)	27 (66)
>Median	15 (65)	12 (67)	14 (34)

sHER3, soluble human epidermal growth factor receptor; Ta, non-invasive tumours; T2-T4, muscle-invasive tumours.

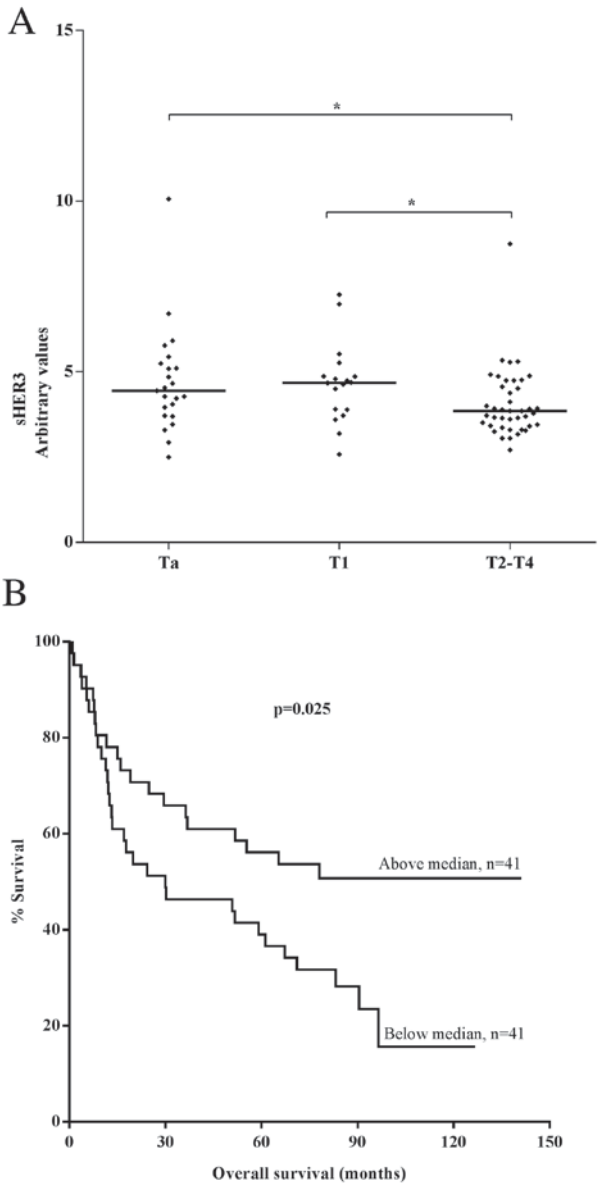

Figure 2. Association between sHER3 level, bladder cancer stage and survival rate. (A) Plasma sHER3 level according to clinical stage of bladder cancer. Analysis of significance for overall group differences was performed using the Kruskal-Wallis test (P=0.03). The multiple comparisons' criterion was employed to identify differences between groups Ta vs. T2-T4 and T1 vs. T2-T4. *P<0.05. (B) Kaplan-Meier analysis of overall survival in patients (n=82) with high (>median) and low (≤median) plasma sHER3 levels. P=0.025 (log-rank test); hazard ratio, 0.53; 95% confidence interval, 0.3-0.93. sHER3, soluble human epidermal growth factor receptor; Ta, non-invasive tumours; T2-T4, muscle-invasive tumours.

and DU145 cell lines. Results from the conditioned medium experiments (Fig. 1C; lane 3 and 4) demonstrated that both cell lines expressed the 85 kDa protein. However, a faint band at 45 kDa was also detected in conditioned media, but not by the capture antibody. Full length HER3 was detected by the detection antibody only in cell lysate (Fig. 1C; Lane 1 and 3). Therefore, based on these results, it was concluded that this ELISA detects the 85 kDa isoform of the HER3 receptor. The WM266 cell line has been used previously to investigate full length HER3 via western blotting with a different antibody compared with that used in this study and in agreement with the results of the present study they also demonstrated that HER3 is expressed in these cells (13).

sHER3 levels in bladder cancer patients. ELISA was used to measure sHER3 in 82 patients with bladder cancer. Patient characteristics and sHER3 levels according to tumour stages of bladder cancer are presented in Table I. Plasma sHER3 levels were significantly higher in patients with superficial tumours compared with in those with superficial invasive and muscle-invasive tumours (P<0.05 for Ta vs. T2-T4; Fig. 2A). No significant differences in sHER3 levels were identified between patients with Ta and T1 cancer (P=0.80).

Plasma sHER3 levels were dichotomised as 'high' (above median, >4.0 arbitrary units) and 'low' (median or below, ≤4.0 arbitrary units) for all subjects. Patients with high sHER3 levels demonstrated significantly longer overall survival times compared with those with low sHER3 levels in the Kaplan-Meier analysis (P=0.025; log-rank test; Fig. 2B). However, multivariate logistic regression analysis of the data revealed that sHER3 levels were not an independent factor for survival (data not shown). The clinical stage and the size of the tumour were identified as independent predictors of survival (data not shown).

Role of sHER3 in bladder cancer cells. To investigate possible mechanisms of increased survival in patients with higher sHER3 levels, HCV-29 cells were treated with a recombinant sHER3 peptide. Peptide treatment resulted in a dose-dependent reduction in the phosphorylation of full-length HER3 (Fig. 3A). The time point 24 h was selected based on our previous experiments on EGFRs, where it was evident that compared with chemical inhibitors, including drugs targeting the EGF receptors, other types of inhibitors, including calcium (14) and as in this study (HER3 peptide) may take longer to affect the inhibition of HER3 phosphorylation. Furthermore, western blotting clearly demonstrated that HER3 phosphorylation was partially inhibited at lower concentrations (10 nM) and it was only with higher concentrations (20 nM) that a significant inhibition was

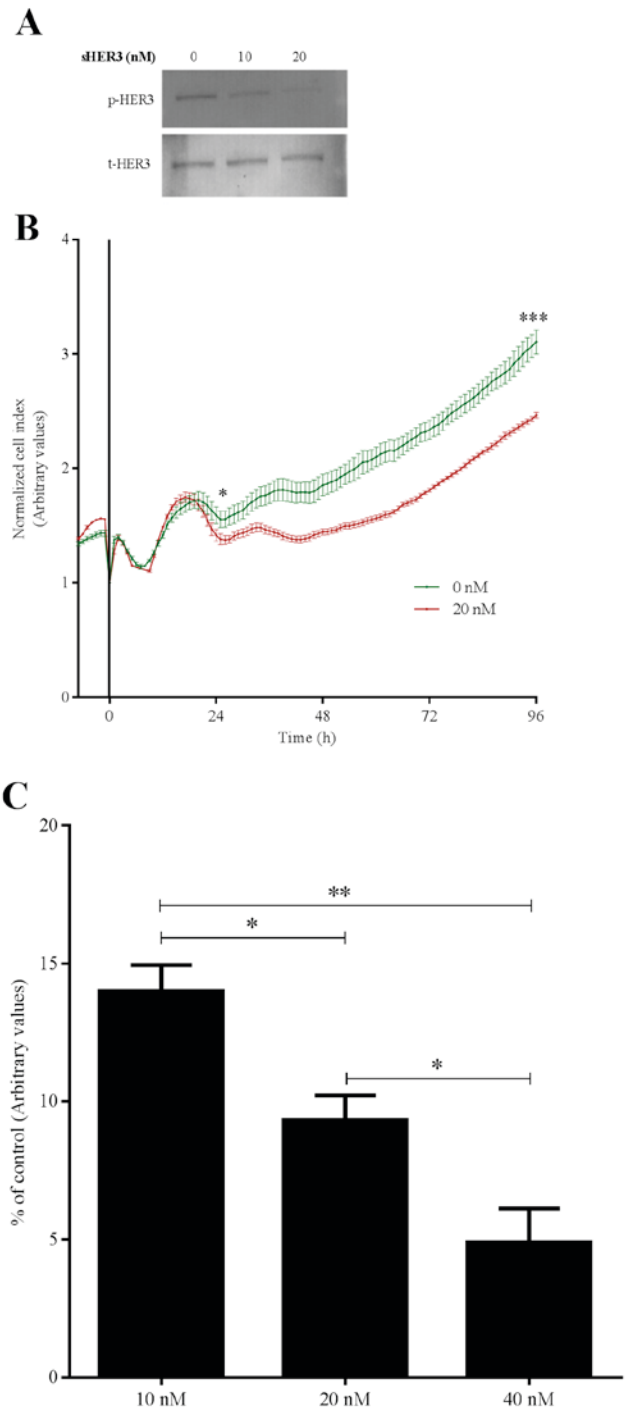

Figure 3. Effects of sHER3 on HER3 signalling, proliferation and migration of HCV-29 bladder cancer cells. (A) Western blotting analysis of p-HER3 (180 kDa) and t-HER3 (180 kDa) levels in HCV-29 cells treated with recombinant sHER3 peptide at the indicated concentrations: 0 (untreated control) 10 and 20 nM for 24 h. (B) xCELLigence data presenting the growth effect of recombinant sHER3 peptide on HCV-29 cells. Differences in cell density were normalised for at 0 h (the time of treatment, vertical black line). Significant differences (P<0.05) in cell proliferation were identified thorough-out, at and after 24 h treatment between cells treated with 20 nM sHER3 peptide compared with untreated cells. ***Denotes difference in control and treated cells at 96 h (P=0.0005), *denotes P<0.05. Data represent the mean ± standard error of the mean following three replicates. (C) xCELLigence data presenting the migration of HCV-29 cells at 24 h after HER3 treatment at the indicated concentrations, data represent the mean ± standard error of the mean following four replicates. Analysis of significance for overall group differences was performed by the Kruskal-Wallis test (P=0.0016). The multiple comparisons' criterion was employed to identify differences between groups and is indicated as *P<0.05 and **P<0.005. sHER3, soluble human epidermal growth factor receptor; p, phosphorylated; t, total

observed even after 24 h. Therefore, it was suggested that at an earlier point, no inhibition with 10 nM HER3 peptide may have been observed. Furthermore, the real-time proliferation assay revealed differences in cell growth from after 24 h treatment of sHER3 peptide. This inhibition of HER3 was accompanied by decreased growth (Fig. 3B) and migration (Fig. 3C) of HCV-29 cells.

Discussion

We previously revealed a dual role of HER in bladder cancer (6), but the role of sHER3 in patients with bladder cancer remains unclear. In the present study, an ELISA assay was developed to determine the levels of circulating sHER3 and investigate its role in bladder cancer. The results demonstrated that sHER3 is associated with a longer survival time of patients with bladder cancer, and that it inhibits the growth and migration of bladder cancer cells *in vitro*.

Transcripts of the extracellular domain of sHER3 of various lengths have been previously reported (4). Although it is not clear whether all of these transcripts are translated into proteins, p45-HER3 and p85-HER3 are well-characterised naturally occurring secreted products of the *ErbB3* gene (5). A cell culture study demonstrated that p45-HER3 and p85-HER3 inhibits ligand-dependent stimulation of HER3 tyrosine phosphorylation, and that p85-HER3 is a more potent inhibitor of HER3 phosphorylation compared with p45-HER3 (5). The ELISA protocol used in the present study was hypothesised to detect the various forms of sHER3 likely to be present in the samples; however, western blotting analysis using conditioned media, and capture and detection antibodies only detected the 85 kDa form of HER3. However, additional controls, including the use of recombinant protein for competition experiments and verification of the protein identity by mass spectrometry, are required to validate these results.

It was revealed that significantly higher levels of sHER3 were present in early-stage (Ta-T1) bladder cancers compared with in late-stage (T2-T4) bladder cancer. Furthermore, higher levels of sHER3 were associated with a longer overall survival time, suggesting a protective role for sHER3 in bladder cancer. To the best of our knowledge, no previous studies have analysed the role of sHER3 in patients with bladder cancer. In agreement with the findings in the current study, a study analysing the role of 45 kDa sHER3 in prostate cancer reported that higher levels of sHER3 were associated with longer progression-free survival times (15). However, another study suggests that sHER3 increased the invasiveness of PC-3 prostate cancer cells (16). Previously, using same cohort, we demonstrated that a higher expression of the full length HER3 was significantly associated with a more improved survival rate of patients with bladder cancer (6,7). Taken together, these results indicate that the full length and soluble forms of the HER3 receptor are associated with a more improved survival rate of patients with bladder cancer. However, further studies on a larger cohort and on other isoforms of the soluble HER3 are required to confirm these results.

To understand the role of sHER3 in bladder cancer, the effects of recombinant sHER3 were also investigated on bladder cancer cell growth and migration. Incubation of HCV-29 bladder cancer cells with recombinant sHER3

inhibited tyrosine phosphorylation of HER3. Cell growth and migration analyses revealed a significant decrease in the growth, and migration of HCV-29 cells treated with recombinant sHER3 compared with untreated cells. A previous study on sHER3 reported that it binds to heregulin (HRG), a ligand for HER3 and HER4, with an affinity equivalent to the affinity of HRG for full-length HER3 (5). This binding can effectively limit binding of HRG to its receptors on the cell surface (5). Furthermore, exposure of cells to high levels of sHER3 inhibited HRG-mediated activation of other HERs (HER2 and HER4) and thereby inhibited HRG-stimulated cell growth (5). Together these results suggest that sHER3 may be a negative regulator of HER3-mediated signalling.

In conclusion, the results of the current study suggest that higher levels of sHER3 are associated with a longer survival time of patients with bladder cancer, and that sHER3 decreases the growth and migration abilities of bladder cancer cells. These results identify sHER3 as a candidate biomarker in patients with bladder cancer and provide a foundation for further studies to explore the possible use of sHER3 in bladder cancer treatment.

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