Hyaluronic acid family in bladder cancer: potential prognostic biomarkers and therapeutic targets

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Background: Molecular markers of clinical outcome may aid in designing targeted treatments for bladder cancer. However, only a few bladder cancer biomarkers have been examined as therapeutic targets.

Methods: Data from The Cancer Genome Atlas (TCGA) and bladder specimens were evaluated to determine the biomarker potential of the hyaluronic acid (HA) family of molecules – HA synthases, HA receptors and hyaluronidase. The therapeutic efficacy of 4-methylumbelliferone (4MU), a HA synthesis inhibitor, was evaluated in vitro and in xenograft models.

Results: In clinical specimens and TCGA data sets, HA synthases and hyaluronidase-1 levels significantly predicted metastasis and poor survival. 4-Methylumbelliferone inhibited proliferation and motility/invasion and induced apoptosis in bladder cancer cells. Oral administration of 4MU both prevented and inhibited tumour growth, without dose-related toxicity. Effects of 4MU were mediated through the inhibition of CD44/RHAMM and phosphatidylinositol 3-kinase/AKT axis, and of epithelial–mesenchymal transition determinants. These were attenuated by HA, suggesting that 4MU targets oncogenic HA signalling. In tumour specimens and the TCGA data set, HA family expression correlated positively with β-catenin, Twist and Snail expression, but negatively with E-cadherin expression.

Conclusions: This study demonstrates that the HA family can be exploited for developing a biomarker-driven, targeted treatment for bladder cancer, and 4MU, a non-toxic oral HA synthesis inhibitor, is one such candidate.

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The divergent molecular pathways of low- and high-grade tumour development, heterogeneity in tumour progression and frequent recurrence are a few of the unique features of bladder cancer (BCa) that contribute to the challenges and high cost associated with its clinical management (Leal et al., 2016). The relative ease of urine-based tests has led to the discovery of potential BCa biomarkers. However, the functional links of many of these biomarkers to BCa growth and progression remain unknown. Furthermore, very few of these biomarkers have been targeted for therapy (Schmitz-Drager et al., 2015; Leal et al., 2016; Nagata et al., 2016). Our previous studies have demonstrated that hyaluronic acid (HA) family members are potentially accurate biomarkers for BCa diagnosis, as well as for predicting disease recurrence and progression (Lokeshwar et al., 2000; Kramer et al., 2010, 2011).

Hyaluronic acid is a non-sulphated glycosaminoglycan. Hyaluronic acid and HA family members – HA syntheses (i.e., HAS1, HAS2, HAS3), HA receptors (i.e. CD44, RHAMM) and the glycosaminoglycan family members – HA synthases (i.e., HAS1, HAS2, HAS3) – promote tumour growth, metastasis and angiogenesis (Jordan et al., 2015; Karousou et al., 2000, 2002; Ekici et al., 2007, 2008; Ozturk et al., 2006). We have previously shown that urinary HA levels detect BCa with >85% accuracy (Lokeshwar et al., 1997, 2000, 2002). As a combined marker, elevated HAS2 and HYAL-1 mRNA levels detect BCa with ~82% accuracy (Kramer et al., 2011). In BCa tissues, HA is synthesised by tumour stroma and tumour cells (Hautmann et al., 2001; Lokeshwar et al., 2001). Owing to HYAL-1 activity, pericellular HA in tissues and patients’ urine consists of both high molecular mass and small HA fragments; some of these HA fragments are angiogenic (Lokeshwar et al., 1997, 2001). In preclinical models, while HA syntheses and/or HYAL-1 expression enhances tumour growth, angiogenesis and metastasis, their downregulation suppresses these tumour phenotypes (Lokeshwar et al., 2005a, b; Golshani et al., 2008; McAtee et al., 2014). Interestingly, HAS2 is a target of the glycogen debranching enzyme AGL, a tumour suppressor. HAS2 upregulation due to AGL downregulation promotes BCa growth (Guin et al., 2016). Pericellular HA–HA receptor interaction stimulates tumour cell functions by inducing oncogenic signalling, including the phosphatidylinositol 3-kinase (PI3-K)–AKT pathway and the epithelial–mesenchymal transition (EMT). Epithelial–mesenchymal transition markers such as Twist may have the potential in BCa diagnosis and predicting its progression or recurrence (Fantony et al., 2015; Singh et al., 2016). However, the correlation between HA family and EMT marker expression has not been examined.

Since tumour-associated HA promotes tumour growth and progression, and BCa cells express more than one HA synthase, inhibition of HA synthesis by a small molecule may be a better treatment approach for controlling BCa than targeting individual HA synthases. 4-Methylumbelliferone (4MU; 7-hydroxy-4-methylcoumarin or hymecromone) is a HA synthesis inhibitor. 4-Methylumbelliferone is sold as a supplement in Europe and Asia to improve liver health. 4-Methylumbelliferone is not associated with any toxicity and has a maximum tolerated dose in mice of 2.8–7.3 g kg⁻¹ (National Institute for Occupational Safety; RETGs No. GN7000000). The two building blocks of HA synthesis are UDP-glucuronic acid and N-acetyl-D-glucosamine. Uridine 5’-diphospho-glucuronosyltransferase-glucuronic acid is a substrate of UDP-glucuronolyltransferases (Nakamura et al., 1997; Kakizaki et al., 2002, 2004). In mammalian cells exposed to 4MU, UDP-glucuronolyltransferase causes glucuronidation of 4MU, which depletes the intracellular UDP-glucuronic acid pool, inhibiting HA synthesis (Nakamura et al., 1997; Kakizaki et al., 2002, 2004). Since the Kₘ values of UDP-glucuronolyltransferases and HA-synthases range between 0.1 and 0.9 mM, the IC₅₀ of 4MU for inhibiting HA synthesis is ~0.4 mM (Nakamura et al., 1997; Kakizaki et al., 2002, 2004; Lokeshwar et al., 2010; Yates et al., 2015). In addition, 4MU downregulates the expression of HA syntheses and UDP-dehydrogenase, which are key enzymes in HA synthesis (Kuliti et al., 2009; Saiito et al., 2013). In tumour models, 4MU has shown antitumour activity (Lokeshwar et al., 2010; Arazi et al., 2011; Piccioni et al., 2012; Yates et al., 2015; Guin et al., 2016; Nagase et al., 2016; Ito et al., 2017). Our previous studies on preclinical models of prostate cancer demonstrated the chemopreventive and therapeutic efficacies of 4MU to prevent and inhibit prostate cancer development, growth, and its progression at doses comparable to those used in humans (Lokeshwar et al., 2010; Yates et al., 2015). In this study, we evaluated the expression of the HA family molecules in clinical specimens and in The Cancer Genome Atlas (TCGA) data set and targeted it for treatment using preclinical models of BCa. Moreover, we confirmed a correlation between HA family and the effectors of (i.e., EMT markers) the oncogenic HA signalling in clinical specimens.

### MATERIALS AND METHODS

**Cells and reagents.** BCa cell lines HT1376, J82, HT1197, T24 and UMUC-3 and immortalised normal urothelial cells SV-HUC1 were purchased from American Type Culture Collection (Manassas, VA, USA). 253J-Parent and 253J-Lung cells were provided by Dr Colin Dinney (MD Anderson Cancer Center, Houston, TX, USA). Immortalised normal bladder epithelial cell line UROtsa was provided by Dr Donald Sens (University of North Dakota, Grand Forks, ND, USA). Bladder cell lines were authenticated by Genetica DNA Laboratories Inc. (Cincinnati, OH, USA). Sodium salt of 4MU (MW: 198) was purchased from Sigma-Aldrich (Saint Louis, MO, USA); other reagents and antibodies as described before (Yates et al., 2015; Jordan et al., 2016). All experiments were conducted between passages 2 and 10.

**HA ELISA-like assay.** Serum-free conditioned medium (CM) of BCa cells was subjected to HA activity ELISA-like assay, as described before (Lokeshwar et al., 1997). Hyaluronic acid levels (ng ml⁻¹) were normalised to cell number. In some cases, CM was collected following 48 h treatment with 4MU (0.4 mM).

**Cell proliferation, apoptosis, motility and invasion assays.** Bladder cancer and normal urothelial cells (1.5 × 10⁴ cells per well) cultured in growth medium were exposed to 4MU (0–0.6 mM) either alone or in the presence of HA (50 μg ml⁻¹) or a PI3-K inhibitor LY294002 (10 and 10 μM) for 48 to 72 h. Following incubation, viable cells were counted (Trypan blue staining). Induction of apoptosis was examined in cells treated with 4MU for 48 h using the Cell Death ELISA Plus Kit (Roche Diagnostics, Indianapolis, IN, USA); results were expressed as apoptosis index (per 5000 cells) (Yates et al., 2015; Chanmee et al., 2016). Matrigel invasion and motility assays were carried out as described previously, except that 4MU and/or HA were added in both chambers of the Transwell. Incubation times for motility and invasion assays were 18 and 48 h, respectively (Lokeshwar et al., 2005b, 2006, 2010; Yates et al., 2015; Jordan et al., 2016). In scratch wound assay, 253J-Lung cells were cultured in 0.1% FBS-containing medium and treated with 4MU. The wound closure was photographed at different time intervals and analysed using the ImageJ Software (https://imagej.nih.gov/ij/). Per cent wound closure was calculated as: (A₀ − Aₘ)/A₀ × 100 (where A₀ is area at time 0 and Aₘ is area at time of measurement). Data are represented as mean ± s.d. (triplicate determination).

**Transient transfections, immunoblotting, ELISA and proximal ligation assays.** 253J-Lung and HT1376 cells treated with 4MU (0
or 0.4 mM) for 48 h were subjected to immunoblotting for various proteins or to reverse transcription quantitative polymerase chain reaction (qPCR) assays for HA receptor and EMT marker transcripts, as described before (Yates et al., 2015; Jordan et al., 2016). In some cases, HA (50 μg·mL⁻¹) was added during 4MU incubation. 253J-Lung cells treated with 4MU (0 and 0.4 mM) were subjected to PI3-K activity assay using a colourimetric ELISA Kit (FACE PI3-K p85; Active Motif, Carlsbad, CA, USA) or to a PLA, using anti-PI3-K and CD44 antibodies as described before (Yates et al., 2015; Jordan et al., 2016). Alternatively, 253J-Lung cells were transiently transfected with a pcDNA3-Myr-HA-Akt1 or vector plasmid using Lipofectamine 2000 (Invitrogen, Carlsbad, CA, USA). Twenty-four hours following transfection, cells were treated with 4-MU (0 and 0.4 mM) for 48 h and analysed (Yates et al., 2015; Jordan et al., 2016). For NF-κB reported assay cells were transiently transfected with pNF-κB-luc and pGL4.74[hRluc/TK] plasmids. Eight hours following transfection, the cells were exposed to 4MU (0.4 mM). Following 16–18 h incubation, the firefly luciferase and Renilla luciferase activities were assayed. Results were expressed as a ratio of firefly luciferase to Renilla luciferase activities. (Jordan et al., 2016).

To quantify the levels of protein in immunoblot analysis, the blots were scanned and quantified using the ImageJ Software (https://imagej.nih.gov/ij/). For a specific protein, the intensity of the band in each sample was normalised to the corresponding (β-actin, α-tubulin and GAPDH bands; these three proteins were used as loading controls. The normalised intensities were calculated as described in Supplementary Tables 2 and 3.

Tumour xenografts. HT1376 cells (2 × 10⁶ cells per 0.1 ml) were implanted subcutaneously on the dorsal flank of 5–6-week-old athymic mice (n = 15). When tumours reached ≥ 100 mm³, mice were randomly divided into three groups and orally gavaged with either vehicle (2% sucrose) or 4MU (200 or 400 mg kg⁻¹) until day 35, when the mice in the vehicle group were killed. In 4MU groups, tumour growth was monitored until day 50. To test the effect of 4MU on tumour generation, in the 253J-Lung subcutaneous model, 4MU treatment commenced on the day of tumour cell injection. Immunohistochemical localisation of microvessels, activated caspase-3, E-cadherin, β-catenin and pβ-catenin(S552) was performed on HT1376 tumour specimens from vehicle and 4MU (400 mg kg⁻¹) treatment groups, as described before (Lokeshwar et al., 2005b, 2006; Yates et al., 2015). For immunohistochemistry, specimens were stained for microvessels (anti-CD34 1:20 dilution), activated caspase-3 (1:50 dilution), E-cadherin (anti-E-cadherin 1:1000), β-catenin (1:300) and anti-pβ-catenin(S552) (1:300), using the IHC procedure described before (Lokeshwar et al., 2005b, 2006, 2010; Yates et al., 2015; Jordan et al., 2016). Microvessel density (MVD) was determined by counting microvessels, respectively, using a Nikon H550L microscope (Nikon, Melville, NY, USA) with a video screen camera, under × 400 magnification.

Clinical specimens and TCGA database. Set 1: Normal bladder (NBL; n = 30) and bladder tumour (TBL; n = 40) specimens were collected at the University of Miami under the University of Miami’s institutional review protocol. These specimens were transferred to Augusta University under an approved protocol. Set 2: TCGA data set contains 407 unique BCa patients on whom HA family and EMT marker transcript levels, and overall survival status were available. Therefore, the TCGA data set marker levels were analysed only for the overall survival data; Supplementary Table 1 describes patient/specimen characteristics for both sets.

Reverse transcription qPCR. Total RNA isolated from tissues was subjected to reverse transcription qPCR using primers specific for each HA family or EMT marker transcripts and normalised to β-actin levels; primer sequences have been published before (Lokeshwar et al., 2000; Chi et al., 2012; Yates et al., 2015; Jordan et al., 2016). The primers used for the detection of Snail and Twist were specific for Twist1 and Snail1. Normalised transcript levels were calculated as (1/2^ΔCq) × 100 where ΔCq = Cq (transcript) − Cq (β-actin). CD44 variant primers detect all variant isoforms.

Statistical analysis. Mean ± s.d. was computed for quantifiable parameters (e.g., cell number, apoptosis index, % motility, % invasion, tumour volume). Differences among vehicle/control and treatment groups were compared by one-way ANOVA followed by either unpaired t-test (e.g., control vs treatment) or Tukey’s multiple comparison test when comparing more than two groups; P-values were two-tailed. Statistical analysis for biomarker levels in both sets were performed as described before (Kramer et al., 2011). Differences between NBL and TBL specimens from patients who either developed (met +) or did not develop (met −) metastasis were compared using the Mann–Whitney U-test because the data showed a non-normal distribution: two-tailed P-values. In both sets, a logistic single parameter model (i.e., univariate analysis) was used to determine the association between a biomarker level and metastasis or survival. A Cox proportional hazards model (i.e., multivariate analysis) was used to determine which of the pre- and postoperative parameters or biomarkers predicted clinical outcome. Receiver operating characteristic curve was generated for each marker to determine the optimal cutoff limits based on the Youden’s index. These cutoff limits were then used to stratify high- and low-marker expression for generating Kaplan–Meier curves. Statistical analyses were performed using the GraphPad Prism (La Jolla, CA, USA) and JMP software (Cary, NC, USA).

RESULTS

HA family expression in bladder specimens. We have previously reported that among the seven HA family members (i.e., HAS1, HAS2, HAS3, CD44 standard form (CD44s), CD44 variant (CD44v), RHAMM and HYAL-1), HAS1, HAS2, HAS3 and HYAL-1 transcript and protein levels were significantly elevated in bladder tumour tissues (Kramer et al., 2011). Reanalysis of the expression data with updated clinical follow-up showed that HAS1 (3-fold), HAS2 (2.6-fold) and HYAL-1 (4.2-fold) transcript levels were significantly elevated in BCa tissues from patients who developed metastasis, when compared with those who did not. HAS3, CD44 and RHAMM transcript levels did not correlate with metastasis (data not shown). In univariate analysis, HAS1 and HYAL-1 levels significantly correlated with metastasis (Table 1). Kaplan–Meier plots showed that HAS1 and HYAL-1 levels significantly stratified BCa patients into low- and high-risk groups for developing metastasis (Figure 1A and B). Furthermore, only HYAL-1 transcript levels also correlated with disease-specific mortality (Figure 1C; χ²: 5.31; P = 0.0212). In multivariate analysis that included clinical, pathological parameters and biomarker levels, only HYAL-1 (χ²: 7.51; P = 0.006) was an independent predictor of metastasis.

We also analysed the expression of HA family molecules in a TCGA data set consisting of 407 BCa patients. In this data set, transcript levels of HAS2, HAS3 and HYAL-1 significantly correlated with overall survival (Table 1). Among HA receptors RHAMM (gene symbol HMMR) significantly correlated with overall survival (χ²: 4.57; P = 0.0325). HAS1, HAS2 and HYAL-1 transcript levels stratified patients into low- and high-risk groups for survival (Supplementary Figure 1A–C); HAS3 also stratified patients into low- and high-risk groups for survival (data not shown). These results show that HA family members are potential prognostic predictors of clinical outcome in BCa patients.
Table 1. Correlation between clinical outcome and HA or EMT markers in BCA specimens

| Parameter | Metastasis (set 1) | Survival (set 2) |
|-----------|-------------------|-----------------|
|           | χ² | P-value | Odds ratio | 95% CI | χ² | P-value | Odds ratio | 95% CI |
| HAST      | 5.89 | 0.0159 | 1.63 | 1.2-2.6 | 3.00 | 0.083 | 
| HAS2      | 1.93 | 0.1649 | – | – | 4.68 | 0.0305 | 3.44 | 1.13-10.57 |
| HAS3      | 0.74 | 0.3891 | – | – | 7.56 | 0.006 | 3.97 | 1.5-10.75 |
| HYAL-1    | 7.58 | 0.0059 | 1.86 | 1.3-3.3 | 5.01 | 0.0252 | 4.4 | 1.2-16.1 |
| β-catenin | 2.65 | 0.1033 | – | – | 4.49 | 0.0340 | 3.57 | 1.1-11.82 |
| Twist     | 7.94 | 0.0055 | 4.64 | 1.9-17.1 | 11.49 | 0.0007 | 5.29 | 2.02-13.9 |
| Snail     | 3.48 | 0.0622 | – | – | 8.14 | 0.0043 | 6.2 | 1.77-21.7 |
| E-cadherin| 0.26 | 0.607 | – | – | 1.11 | 0.292 | – | – |

Abbreviations: BCA = bladder cancer, CI = confidence interval, EMT = epithelial-mesenchymal transition, HA = hyaluronic acid, TCGA = The Cancer Genome Atlas, Univariate analysis of HA family and EMT marker transcript levels to predict clinical outcome. Transcript levels of seven HA family and four EMT markers from clinical specimens in sets 1 (clinical specimens) and 2 (TCGA data set) were evaluated for the prediction of metastasis or survival by single parameter logistic regression analysis. Odds ratio and 95% CI are shown for significant parameters. As noted in the text in multivariate analysis that included clinical/pathological (age, gender), pathological (tumour grade, stage, concomitant carcinoma in situ, lymph node) and biomarker levels, only HYAL-1 and Twist were significantly correlated with metastasis.

*P<0.05.

Effect of 4MU on HA synthesis and cellular functions in BCA cells.

Measurement of HA levels by an HA ELISA-like assay showed that BCA cells secrete significant amounts of HA (100–1200 ng per 10^5 cells) in their conditioned media (Figure 1C). Incubation of 253J-Lung and HT1376 cells with 4MU (0.4 mM or 80 μg/ml –1 of sodium salt of 4MU) inhibited HA synthesis by >90% (P<0.0001; Figure 1C). This concentration of 4MU required for HA synthesis inhibition is consistent with those reported previously (Kakizaki et al, 2004; Lokeshwar et al, 2010). In cell proliferation assays, 4MU inhibited the growth of various BCA cells in a dose-dependent manner and at concentrations at which it inhibited HA synthesis; 56–60% growth inhibition was observed at 0.4 mM concentration (Figure 1D; P<0.0001 at each concentration when compared with untreated). 4-Methylumbelliferone did not significantly inhibit the growth of normal urothelial cells, UROtsa and SV-HUC1 (Supplementary Figure 2A). The growth inhibition caused by 4MU in BCA cells was due to a dose-dependent induction of apoptosis. At 0.4 mM concentration, 4MU caused a 3–4-fold induction of apoptosis (Figure 1E; P<0.0001). The addition of HA during the incubation of 253J-Lung and HT1376 cells with 4MU attenuated the growth inhibitory effects of 4MU; 58% vs 17% growth inhibition by 4MU in the absence or presence of HA (Figure 1F). The addition of HA prevented 4MU-induced apoptosis (data not shown).

Since HA regulates cell adhesion, migration and invasion (Jordan et al, 2015; Chanmee et al, 2016; Karousou et al, 2016) and 4MU inhibited HA synthesis by ~90% at 0.4 mM concentration (Figure 1C), we examined the effect of 4MU on migration, chemotactic motility and invasive activity at this concentration. In these and subsequent assays, 253J-Lung and HT1376 cells were chosen, because these cells synthesise high levels of HA, and are invasive, but express different isoforms of CD44; 253J-Lung express CD44s, whereas HT1376 cells express CD44s isoforms (CD44v3, CD44v6, and CD44E (contains variant exons 8–10; Figure 2D) (Lokeshwar et al, 2005b, 2006; Golshani et al, 2008; Jordan et al, 2016). Furthermore, while 253J-Lung cells have mutated/activated Pi3-K (E545G), but wild-type AKT, PTEN and p53, HT1376 cells express wild-type Pi3-K and AKT, but have mutated p53, and are PTEN null (Pagliaro et al, 2003; Pinto-Leite et al, 2014; Ross et al, 2016). As discussed below, the Pi3-K/AKT pathway is activated by HA–HA receptor interaction.

In a scratch-wound assay with 253J-Lung cells, there was a significant inhibition of wound closure by 4MU in a dose-dependent manner. The wound closure was nearly complete (75.3 ± 4.2%) in the untreated 253J-Lung cells within 24 h (h) when compared with 4MU-treated cells (e.g., 33.3 ± 3.1% for 0.4 mM concentration; P<0.0001; Figure 1A). Similarly, 4MU inhibited the chemotactic motility of 253J-Lung and HT1376 cells by 52% and 61%, respectively (P<0.0001 in both cell types; Figure 2B). In addition, 4MU effectively abrogated invasive activity of 253J-Lung and HT1376 cells by 88.4% and 60.6%, respectively (Figure 2C). In both cell lines, these inhibitory effects of 4MU were attenuated by HA addition (Figure 2B and C). These results show that the inhibitory effects of 4MU on BCA cell functions are primarily due to the inhibition of HA synthesis.

Effect of 4MU on apoptosis effectors. In 253J-Lung and HT1376 cells, 4MU induced activation of proapoptotic effectors (caspase-3, -8 and -9), Fas and FADD induction, and PARP cleavage in a dose-dependent manner, but decreased antiapoptotic protein bcl-2 (Figure 2D). The levels of these pro- and antiapoptosis effectors were increased 2.5- to >10-fold in 4MU-treated cells (P<0.0001; Supplementary Table 2). Hyaluronic acid addition attenuated these effects of 4MU on various apoptosis effectors (Figure 2A and Supplementary Table 3).

Targeting of HA family biomarkers.

Cell surface interaction of HA with its receptors CD44 and RHAMM induces intracellular signalling that includes activation of Pi3-K, AKT and EMT. In both cell lines, 4MU downregulated (1.5–10-fold) transcript levels of HA receptors CD44 (CD44s/CD44v) and RHAMM and EMT markers – β-catenin, Snail and Twist by 1.5- to 10-fold, but upregulated E-cadherin levels (4.5–7-fold; P<0.0001; Supplementary Figure 2B). At the protein level, in both cell lines, 4MU downregulated CD44 and RHAMM and phosphorylation of AKT(S472) in a dose-dependent manner. Consequently, 4MU also downregulated phosphorylated glycosyn synthase-3 kinase (pGSK-3β), pβ-catenin(S552), Snail and Twist levels but upregulated β-catenin activation is regulated by AKT and GSK-3β/β (Xu et al, 2015a, b). Phosphorylation at S552 induces nuclear localisation of β-catenin, but phosphorylation at T41/S45 by activated GSK-3β/β causes its degradation; GSK-3β/β itself is phosphorylated by activated AKT and consequently degraded (Takahashi-Yanaga, 2013). Activated β-catenin upregulates Snail and Twist via the β-catenin TCF/LEF complex, this in turn negatively regulates E-cadherin expression in the presence of HA, the effect of 4MU on HA receptor and AKT activation, and downstream EMT effectors were attenuated (Figure 3A and Supplementary Table 3). This suggests that the effect of 4MU on the observed signalling events is due to the inhibition of HA synthesis.

Effect of 4MU on Pi3-K/AKT axis. To confirm the effect of 4MU on HA-receptor-mediated AKT activation and downstream signalling in 253J-Lung cells, we overexpressed myristoylated AKT (mAkt), which is constitutively active. Myristoylated AKT overexpression attenuated 4MU-induced alterations in apoptosis effectors, inhibition of cell growth and induction of apoptosis (Figure 3B and C and Supplementary Table 3). Furthermore, constitutively active AKT in mAkt transfectants abrogated the effects of 4MU on EMT determinants – downregulation of pβ-catenin (S552), Snail, Twist levels and upregulation of pβ-catenin.
treatment was started once the tumours reached 100 mm³, that is, day 22. As shown in Figure 4D, 4MU significantly inhibited tumour growth at 200 and 400 mg kg⁻¹ doses, even after 4MU treatment was stopped on day 35. The differences in tumour volume (mm³) in the vehicle group (day 35; 770.8 ± 218.9) and 4MU treatment groups (day 50; 200: 216.6 ± 44.1; 400: 128.6 ± 61.5) were significant (P < 0.0001). As we have reported previously, 4MU did not affect animal weight (Figure 4E). Immunohistochemistry on HT1376 tumour tissues confirmed the downregulation of β-catenin, pβ-catenin (S552) and the upregulation of cleaved caspase-3 and E-cadherin in the 4MU treatment group (400 mg kg⁻¹), when compared with the vehicle group (Figure 4F). Furthermore, MVD per high power field was significantly reduced in the 4MU treatment group (0.6 ± 0.72) when compared with the vehicle group (9 ± 2.3) (P < 0.001; Figure 4G). We also evaluated the efficacy of 4MU in preventing bladder tumour initiation and growth. In the 253J-Lung model, oral treatment with 4MU starting on the day of tumour cell injection also prevented tumour growth. 4-Methylumbelliferoene-treated animals did not form palpable tumours; tumour volumes (mm³) on day 33 for vehicle: 748 ± 348.4; 4MU: 24.2 ± 15.7.

EMT marker expression and its correlation to HA family marker levels in BCa specimens. Measurement of EMT marker expression in bladder specimens, in which we had evaluated HA family marker expression, showed that β-catenin, Twist and Snail

Antitumour effects of 4MU. To determine whether 4MU inhibited tumour growth in the HT1376 xenograft model,
transcript levels were significantly upregulated but E-cadherin levels were significantly downregulated in BCa tissues, when compared with normal bladder tissues (Figure 5A and B). However, only Twist transcript levels were significantly higher (10-fold) in BCa tissues from patients who developed metastasis, when compared with those who did not (Figure 5B). In univariate analysis, Twist levels significantly correlated with metastasis and Kaplan–Meier plot showed that Twist levels significantly stratified BCa patients into low- and high-risk groups for developing metastasis (Table 1 and Figure 5C). In the TCGA data set, upregulation of β-catenin, Snail and Twist transcript levels significantly correlated with overall survival (Table 1). Furthermore Snail and Twist transcript levels stratified patients into low- and high-risk groups for survival (Figure 5D and E).

As HA signalling induces the expression of β-catenin, Snail and Twist and downregulates E-cadherin expression, we examined whether the expression of HA family markers correlated with that of EMT markers. As shown in Supplementary Table 4, although there were differences in the correlation of individual HA family markers with EMT markers, in both sets of specimens, upregulation of HA family significantly correlated with the upregulation of β-catenin, Snail and Twist levels (P < 0.01). Furthermore, there was a significant inverse correlation between HA family upregulation and E-cadherin downregulation in BCa specimens (P < 0.01–0.001 for correlations). The studies in clinical specimens support our mechanistic studies that oncogenic HA signalling supports an aggressive phenotype by upregulating EMT determinants.

### DISCUSSION

The present study demonstrates that a biomarker-based targeted treatment strategy that involves HA family biomarkers and their targeting by a non-toxic dietary supplement, 4MU, can potentially be useful in the treatment of BCa. In this study, multiple approaches encompassing functional, mechanistic and xenograft studies as well as biomarker analyses show that HA family molecules associate with and are drivers of BCa growth and progression. Our study confirms that the antitumour activity of 4MU is largely due to its inhibitory effects on HA synthesis. In the presence of HA, the phenotypic readouts such as inhibition of growth, motility and invasion by 4MU are attenuated. Furthermore, 4MU also prevented HA from exerting its effects on PI3-K/AKT signalling and EMT markers; these observations establish the targeted therapeutic efficacy of 4MU. These results are consistent with our previous report, in which we demonstrated that 4MU has significant chemopreventive and therapeutic efficacy in prostate
and/or HA (50 μg ml⁻¹); β-actin, GAPDH, Tubulin: loading controls. (B) Immunoblot analysis of vector and mAKT transfectants; β-actin, GAPDH, Tubulin: loading controls. (C) 253J-Lung vector and mAKT transient transfectants were treated with 4MU (0, 0.4 mM). Following 48 h incubation, viable cells were counted (left panel) and also subjected to apoptosis assay (right panel). (D) Transient transfectants treated with 4MU were assayed for invasion. Data in C and D: Mean ± s.d. (triplicate); P < 0.001 in untreated and 4MU treated samples for vector transfectants; P > 0.05 for mAKT transfectants.

Figure 3. Effect of HA and myr-AKT expression on 4MU-induced effects. (A) Immunoblot analysis of 253J-Lung cells treated with 4MU (0, 0.4 mM) and/or HA (50 μg ml⁻¹); β-actin, GAPDH, Tubulin: loading controls. (B) Immunoblot analysis of vector and mAKT transfectants; β-actin, GAPDH, Tubulin: loading controls. (C) 253J-Lung vector and mAKT transient transfectants were treated with 4MU (0, 0.4 mM). Following 48 h incubation, viable cells were counted (left panel) and also subjected to apoptosis assay (right panel). (D) Transient transfectants treated with 4MU were assayed for invasion. Data in C and D: Mean ± s.d. (triplicate); P < 0.001 in untreated and 4MU treated samples for vector transfectants; P > 0.05 for mAKT transfectants.

cancer models (Yates et al, 2015). It is noteworthy that HA and HYAL-1 expression is not only elevated in prostate cancer but also correlates with biochemical recurrence (Posey et al, 2003; Ekici et al, 2004; Gomez et al, 2009). The association of the HA family molecules with the aggressiveness of cancer, as well as the mechanism of action and efficacy of 4MU in two different cancer systems, suggest that targeting of HA signalling by a nontoxic orally bioavailable supplement such as 4MU, is likely to be effective in other cancer systems in which the expression of the HA family molecules is elevated. As the expression of HA family is elevated in a variety of cancers (Lokeshwar et al, 2002; Kramer et al, 2011; Chi et al, 2012; Peng et al, 2016), 4MU should potentially have a broader applicability as a targeted antitumour agent. To this effect, in this study 4MU showed significant antitumour efficacy in the therapeutic (treatment starting when tumours were palpable) and chemopreventive (253J-Lung model) settings. 4-Methylumbelliferone has also shown antitumour efficacy in a few other cancer models, although an in-depth analysis of its mechanism of action has been examined only in one other study from our group involving preclinical models of prostate cancer (Lokeshwar et al, 2010; Arai et al, 2011; Piccioni et al, 2012; Yates et al, 2015; Nagase et al, 2016; Ikuta et al, 2017). As a dietary supplement and in small clinical studies, the 4MU dose is about 1.5 g per day (Camarri and Marchettini, 1988; Abate et al, 2001). This dose is equivalent to the doses used in this study. Pharmacokinetics of 4MU shows rapid clearance by biotransformation into glucuronide (major) and sulphated derivatives; the glucuronidated derivative is inactive and excreted in urine (Kuipers et al, 2016). Based on the favourable toxicity profile and efficacy in HA family expressing tumours, 4MU could be potentially tested in clinical trials.

The EMT phenotype is a critical hallmark of cancer metastasis. Our study demonstrates a link between HA signalling and EMT signature (i.e., β-catenin upregulation/activation and E-cadherin downregulation) via the PI3-K/AKT axis. Furthermore, inhibition...
of a complex formation between CD44 and PI3-K by 4MU shows that by inhibiting HA synthesis, 4MU abrogates the first step in HA-signalling-mediated induction of EMT signature. A salient finding of our study is the analysis of the expression of HA family and EMT effectors in the same cohort of specimens and a TCGA database of BCa patients. These analyses confirm the prognostic potential of HA synthases and HYAL-1 in predicting metastasis and survival (DSM or overall) in BCa patients. As HYAL-1 degrades HA into angiogenic fragments, it is the tumour-associated HA-HAase system that is responsible for BCa growth and progression (Lokeshwar et al, 1997, 2001, 2005b; Jordan et al, 2016). As both the tissue and TCGA data analyses show that HA synthases are differentially expressed in BCa tissues, targeting of individual HA synthases is unlikely to be effective in blocking the tumour-associated HA-HAase system, and therefore HA inhibitors such as 4MU would make a better treatment option to control BCa.
Our study also identified Twist as a prognostic marker for predicting metastasis and overall survival in BCa patients. The prognostic potential of Twist has been reported previously (Fantony et al., 2015; Singh et al., 2016). In this study, we observed some differences in our clinical specimen data set and the TCGA data. For example, in our clinical specimens, HAS1, HYAL-1 and Twist transcript levels showed the potential to predict metastasis and/or DSM. In the TCGA database HAS1, HAS2, HAS3, HYAL-1, RHAMM and EMT markers, except E-cadherin, correlated with survival in univariate analysis and/or stratified patients into low- and high-risk groups for overall survival. The reasons for this discrepancy may be the parameters used for correlation – metastasis and DSM in our data set and overall survival in the TCGA data set. Nevertheless, in both data sets, the expression of individual HA family markers significantly correlated with different EMT markers. These observations on clinical specimens provide credence to the mechanistic studies that HA signalling regulates EMT signature, and therefore aggressive phenotype in BCa.

In summary, our study demonstrates that HA family biomarkers promote BCa growth and progression very likely by upregulating the EMT phenotype. Certain HA family molecules (i.e., HAS1, HYAL-1) and EMT markers (i.e., Twist) can potentially be useful in predicting outcome in BCa patients and perhaps for selecting patients who might benefit from treatments that block HA signalling, such as 4MU. Therefore, the HA family is a potential candidate for developing biomarker-driven targeted non-toxic oral treatment for BCa.

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**CONFLICT OF INTEREST**

The authors declare no conflict of interest.

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