

Growth differentiation factor-9 expression is inversely correlated with an aggressive behaviour in human bladder cancer cells

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Abstract. Growth differentiation factor-9 (GDF-9) is a family member of bone morphogenetic proteins (BMPs), which belong to the TGF- β superfamily. There has been a recent surge of interest in the role of growth differentiation factors and other BMPs in the development and spread of cancer. However, the role of GDF-9 in bladder cancer remains unknown. The present study investigated the expression of GDF-9 in normal and malignant human bladder tissue and its molecular interactions within bladder cancer cells. The expression of GDF-9 in human bladder tissues and bladder cancer cell lines was assessed at both the mRNA and protein levels using RT-PCR and immunohistochemistry, respectively. Full-length GDF-9 cDNA was amplified from normal mammary tissues. GDF-9 was overexpressed in bladder cancer cell lines using a mammalian expression construct. The effect of GDF-9 on cellular functions, was examined in bladder cancer cells overexpressing GDF-9 using a variety of *in vitro* assays. In normal bladder tissues, stronger staining of GDF-9 was seen in transitional cells, both in the cytoplasm and in the nucleus. In contrast, the staining of GDF-9 was notably weak or absent in cancer cells of tumour tissues. Similarly, the bladder cancer cell lines RT112 and EJ138, expressed very low levels of GDF-9. Moreover, overexpression of GDF-9 reduced the growth, adhesion and migration of bladder cell lines *in vitro*. However, the overexpression of GDF-9 had little bearing on the invasion of bladder cell lines *in vitro*. In conclusion, GDF-9 is expressed at lower levels in human bladder cancer cells compared with normal transitional cells of the bladder. GDF-9 levels are inversely correlated with the growth, adhesion and migration of bladder cancer cells *in vitro*. The results of the present study suggest that GDF-9 is a potential tumour suppressor in human bladder cancer.

Introduction

Tumour metastasis is the most significant contributor to the mortality of patients with bladder cancer. Although this metastatic process is poorly understood, several protein families have been implicated in the progression and development of bladder cancer. One of these families is known as the transforming growth factor β (TGF- β) superfamily (1-3). TGF- β has mainly been demonstrated to as a potent growth inhibitor in variety of cell types, including bladder cancer cells (4). Bone morphogenetic proteins (BMPs), meanwhile, a subfamily of the TGF- β superfamily, have been shown to be important in the bone formation process and several members have been implicated in the pathogenesis of different types of cancer (5-9).

BMPs are multi-functional growth factors that can regulate a variety of important cellular processes, including cell growth, apoptosis, differentiation, and invasion, in a range of cell types (10). Subsequently, BMPs were investigated in some types of cancer. In human liver cancer, small interfering RNA (siRNA)-targeting BMP-2 markedly inhibited the expression of BMP-2 in liver cancer cells, and decreased the abilities of migration and invasion of liver cancer cells (11). In ovarian cancer, BMP-2 efficiently increased the motility of epithelial ovarian cancer cell lines. In contrast, BMP-2 treatment decreased the ability of epithelial ovarian cancer cell lines to form spheroids indicating an inhibition of cell-cell adhesion. The expression of BMP-2 in tumour tissues from patients with ovarian cancer was inversely correlated with survival (12). In breast cancer, BMP-10 inhibited the aggressiveness of breast cancer cells and its reduced expression was correlated with poor prognosis (9). In prostate cancer, BMP expression has been shown to be variable, with BMP-2, -4, -7, -9 and -10 expression being reduced compared to normal prostate epithelial cells (13,14). In addition, BMP-6 and -2 have been shown to inhibit the growth of prostate cancer cells (15). We have recently shown that BMP-9 and -10 can inhibit the growth, adhesion, invasion, and migration of prostate cancer cells by inducing apoptosis via Smad1, 5 and 8 mediated up-regulation of the pro-apoptotic factor Par-4, and the Smad-independent pathway, respectively (14,16).

Growth differentiation factor-9 (GDF-9) is a member of the BMP family that has been identified as an oocyte growth factor with an important role in the regulation of folliculogenesis

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and ovulation (17). In breast cancer, highly aggressive breast cancer cells were shown to express very low levels of GDF-9 (18). On forced expression of GDF-9, breast cancer cells became less invasive (18). Furthermore, another group demonstrated up-regulated levels of GDF-9 in an aggressive oral carcinoma cell line (19). It has been reported that GDF-9 can promote the growth rate of both PC-3 and DU-145 prostate cancer cells by protecting the cells from caspase-3-mediated apoptosis, suggesting that GDF-9 may aid in the progression of prostate cancer by acting as a survival factor (20). Collectively, these studies suggest that GDF-9 plays contrasting roles in different malignancies. Although GDF-9 has been implicated in disease progression of certain tumours, its role in bladder cancer remains unknown. In the present study, we first examined the expression of GDF-9 in normal and malignant human bladder tissues, and the effect of GDF-9 on invasion, growth, adhesion and migration of bladder cancer cells.

Materials and methods

Materials, cell lines and tissue samples. All cell lines used in this study were obtained from the European Collection of Animal Cell Culture (ECACC; Porton Down, Salisbury, UK). Cells were routinely maintained in DMEM-F12 medium supplemented with 10% foetal bovine serum and antibiotics. Polyclonal goat anti-GDF-9 and monoclonal mouse anti-GAPDH were obtained from Santa Cruz Biotechnology (Santa Cruz, CA, USA). Other reagents or kits were obtained from Sigma-Aldrich (Poole, UK) unless otherwise stated. Twenty-one bladder samples were collected from patients with bladder cancer, immediately after surgery at the University Hospital of Wales, Cardiff University School of Medicine, including 12 bladder tumour tissues and 9 normal background bladder tissues. These tissues were collected immediately after radical cystectomy and transurethral resection of bladder tumour. All protocols were reviewed and approved by the Ethics Committee and all patients gave written informed consent.

Immunohistochemical staining procedure for bladder tissues. Frozen sections (8–10 μm thickness) were fixed using a mixture of acetone and methanol (50:50). The sections were then placed in Optimax wash buffer for 5–10 min to rehydrate and incubated for 60 min in a blocking solution that contained horse serum and probed with the primary antibody (anti-GDF-9a, diluted at 1:100). Following extensive washing, sections were incubated for 30 min with a biotinylated secondary antibody (multilink swine anti-goat/mouse/rabbit immunoglobulin, Dako Inc., Carpinteria, CA). Following washing, an avidin-biotin complex (Vector Laboratories) was applied to the sections followed by extensive washing. Subsequently, the diaminobenzidine chromogen (Vector Laboratories) was added to the sections, which were incubated in the dark for 5 min. Sections were then counterstained in Gill's haematoxylin and dehydrated in ascending grades of methanol before clearing in xylene and mounting under a coverslip.

Construction of GDF-9 expression vectors and transfection. These procedures were performed as previously described (20). Briefly, the first strand cDNA was synthesized from

RNA isolated from normal human mammary tissues using a DuraScript™ RT-PCR kit. PCR was then used to amplify the coding sequence of human GDF-9 using the Extensor Hi-Fidelity PCR master mix (ABgene Ltd., Epsom, UK). The sequences of the primers are shown in Table I. The verified GDF-9 insert was cloned into a mammalian expression plasmid vector (pEF/His TOPO® TA plasmid vector; Invitrogen, Inc., Paisley, UK). The recombinant plasmid vectors were transformed into chemically competent TOP10 *E. coli* (Invitrogen, Inc.), and the colonies were then analysed. Colonies carrying correct recombinant plasmids were amplified and plasmids were extracted. Purified GDF-9 transgenes and control plasmid vectors were then transfected into RT112 and EJ138 cells individually using an Easyjet Plus electroporator (Equibio Ltd., Kent, UK). After up to 3 weeks of selection with blasticidin the transfectants were verified for their expression of GDF-9 and successful clones were used in subsequent studies.

RNA isolation and reverse transcription PCR. RNA was isolated using total-RNA isolation reagent (ABgene Ltd.). Reverse transcription was performed using the DuraScript™ RT-PCR kit, followed by PCR using a REDTaq™ ReadyMix PCR reaction mix (primer sequences shown in Table I). Cycling conditions were 94°C for 5 min, followed by 30 cycles of 94°C for 30 sec, 55°C for 30 sec, and 72°C for 40 sec. This was followed by a final 10 min extension period at 72°C. The products were visualized on 1.5% agarose gel stained with ethidium bromide.

Western blot analysis of GDF-9 expression. The protein concentration in cell lysates were determined using the DC Protein Assay kit (Bio-Rad) and an ELx800™ spectrophotometer (Bio-Tek). Equal amounts of protein were separated by sodium dodecyl sulfate-polyacrylamide gel electrophoresis (SDS-PAGE), and blotted onto nitrocellulose sheets. Proteins were then respectively probed with anti-GDF-9 antibody (1:400) or anti-GAPDH (as the loading control) and peroxidase-conjugated secondary antibody, with stringent washings between each step. Protein bands were visualized using the SuperSignal™ West Dura system (Pierce Biotechnology, Inc., Rockford, IL, USA), and photographed using a UVITech imager (UVITech, Inc., Cambridge, UK).

In vitro cell growth assay. Cell growth was assessed using a previously reported method (24,25). Briefly, the cells were plated into a 96-well plate (3,000 cells/well). Cell growth was assessed after a period of incubation (up to 5 days). Crystal violet was used to stain cells. Following washing, stained crystal was extracted with 10% acetic acid and absorbance was determined at a wavelength of 540 nm using a spectrophotometer (ELx800).

In vitro invasion assay. This assay was performed according to a standard procedure (21–23). Transwell inserts with 8 μm pore size were coated with 50 μg Matrigel (BD Matrigel™ Basement Membrane Matrix) and air dried. The Matrigel was rehydrated before use. Subsequently, 30,000 cells were added to each insert, and the same number of cells were loaded into another well as control. After 72 h, cells that had migrated through the matrix to the other side of the insert were fixed in 4% formalin and stained with 0.5% (w/v) crystal violet. The

Table I. Sequences of the primers used in this study.

Primer	Sense (5'-3')	Antisense (5'-3')
GDF-9 screening	ATGGCACGTCCCAACAAAT	ATTTGACAGCAGAGGAAAAA
GDF-9 expression	GCGCTTTTCAAAGTTCTATC	GGTCACATCAATCTGAATCC
GAPDH	ATGATATCGCCGCGCTCGTC	GCTCGGTGAGGATCTTCA

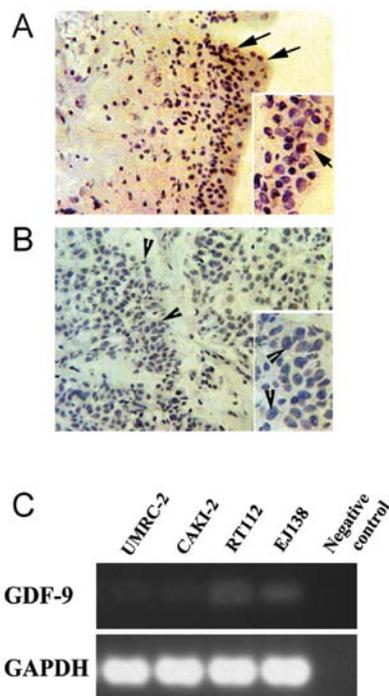


Figure 1. Expression of GDF-9 in bladder cancer. (A and B) Immunohistochemical staining of GDF-9 in (A) normal human bladder tissues and (B) invasive human bladder cancer tissue. The staining for GDF-9 was mainly seen in the cytoplasmic region of the transitional cells (indicated by arrows) and was absent in the cancer cells of tumour tissues (arrowheads). (C) The mRNA expression of GDF-9 was examined in bladder and kidney cancer cell lines using RT-PCR. In bladder cancer cell lines, RT112 and EJ138, expressed low levels of GDF-9; in kidney cancer cell lines, UMRC-2 and CAKI-2, the expression of GDF-9 was absent.

stained crystal was extracted with 10% acetic acid and the absorbance was determined at a wavelength of 540 nm using a spectrophotometer (ELx800).

In vitro cell matrix adhesion assay. Cell matrix adhesion was assessed according to a previously described method (21-23). A total of 30,000 cells were added to each well of a 96-well plate, previously coated with Matrigel (5 μ g/well). After 40 min of incubation, non-adherent cells were washed off using balanced saline solution (BSS) buffer. The remaining adherent cells were then fixed and stained with crystal violet. The number of adherent cells in random fields were observed and counted under a microscope.

Electric cell-substrate impedance sensing (ECIS) based attachment and migration assay. The ECIS Z-Theta instrument and 96W1E arrays (Applied Biophysics, Inc., Troy, NY, USA) were used in the study. We used a recently reported method (26).

Briefly, the same number of test cells (60,000/well) was added to each well of the ECIS arrays. Impedance and resistance of the cell layer was immediately recorded for a period of up to 20 h. When confluence was reached, the monolayer in each well was electrically wounded at 1,400 μ A and 6,000 Hz for 30 sec to create a 250 μ m wound/well. Impedance and resistance of the wounded cells as they migrated in the wound was then recorded for a period of up to 20 h. Data were analysed using the ECIS software, supplied by the manufacturer.

Statistical analysis. All statistical analysis was performed using the SPSS 16.0 software (SPSS Inc., Chicago, IL, USA). Non-normally distributed data were assessed using the Mann-Whitney test, while the two sample t-test was used for normally distributed data. Differences were considered to be statistically significant at $P < 0.05$.

Results

Expression of GDF-9 in human bladder tissues and cell lines. In order to estimate the protein levels of GDF-9 in human bladder tissues, we conducted immunohistochemical analysis using archival normal and malignant bladder tissues. Normal bladder transitional cells stained positively for GDF-9 (Fig. 1A, arrows). The staining was largely confined to the cytoplasmic region of the cells. The staining of GDF-9 was absent in cancer cells of tumour tissues (Fig. 1B, arrow heads). The mRNA expression of GDF-9 was also examined in two bladder cancer cell lines and two kidney cancer cell lines using RT-PCR. The two bladder cancer cell lines, RT112 and EJ138, expressed low levels of GDF-9; interestingly, in the kidney cancer cell lines, UMRC-2 and CAKI-2, expression of GDF-9 was absent (Fig. 1C).

Overexpression of GDF-9 in bladder cancer cells. To investigate the impact of GDF-9 on functions of bladder cancer cells, the constructed GDF-9 expression vectors were utilised to overexpress GDF-9 in bladder cancer cells. After the selection using blasticidin, the expression of GDF-9 in the transfected cells was verified using both RT-PCR and Western blotting (Fig. 2). Increased GDF-9 expression of both mRNA (Fig. 2A) and protein (Fig. 2B) was seen in RT112^{GDF-9exp}, in comparison with the controls, RT112^{WT} and RT112^{pEF}. Similarly, overexpression of GDF-9 was confirmed in EJ138^{GDF-9exp} cells, in comparison with EJ138^{WT} and EJ138^{pEF} control cells.

GDF-9 is associated with the growth rate but not with the invasion of bladder cancer cells. RT112 and EJ138 cells forced to overexpress GDF-9 displayed a slower growth rate, compared with the controls (Fig. 3) (A-RT112 and B-EJ138 cells). Using

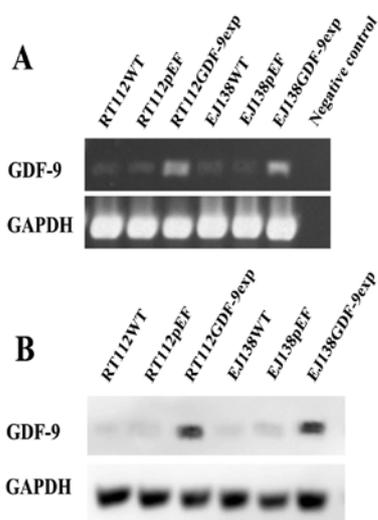


Figure 2. Overexpression of GDF-9 in bladder cancer cells. (A) RT-PCR shows increased GDF-9 transcripts in the GDF-9-transfected RT112 and EJ138 cells compared to the controls. GAPDH was examined as a housekeeping gene. NC is negative control. (B) Changes of GDF-9 protein by GDF-9 overexpression in the corresponding bladder cancer cells were verified using Western blot analysis. GAPDH was used as the housekeeping control.

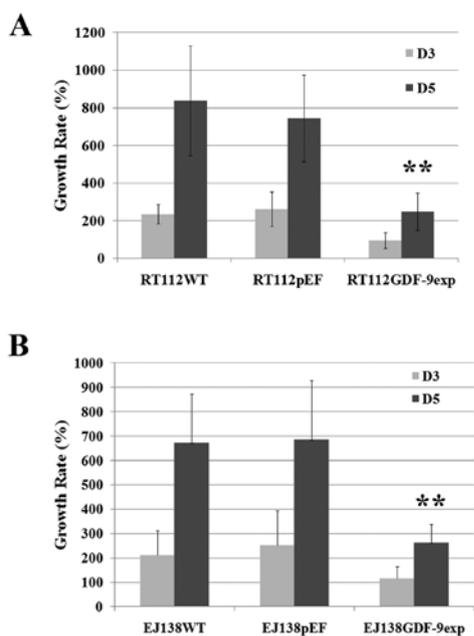


Figure 3. Effects of GDF-9 on *in vitro* growth of bladder cancer cells. (A) The cell growth rate (third and fifth day, both being normalized by first day) of RT112^{GDF-9exp} cells significantly decreased in comparison to the two controls, RT112^{WT} and RT112^{pEF}. (B) The cell growth rate of EJ138^{GDF-9exp} cells significantly decreased in comparison to the two controls, EJ138^{WT} and EJ138^{pEF}; ***P*<0.01. Shown are representative data from three independent experiments.

the same cells, the invasiveness was further examined in the genetically modified cells. However, the overexpression of GDF-9 had no significant bearing on the invasion of bladder cell lines, RT112 and EJ138 *in vitro* (Fig. 4).

Effect of GDF-9 overexpression on cell matrix adhesion in bladder cancer cells. We first examined the effect of GDF-9

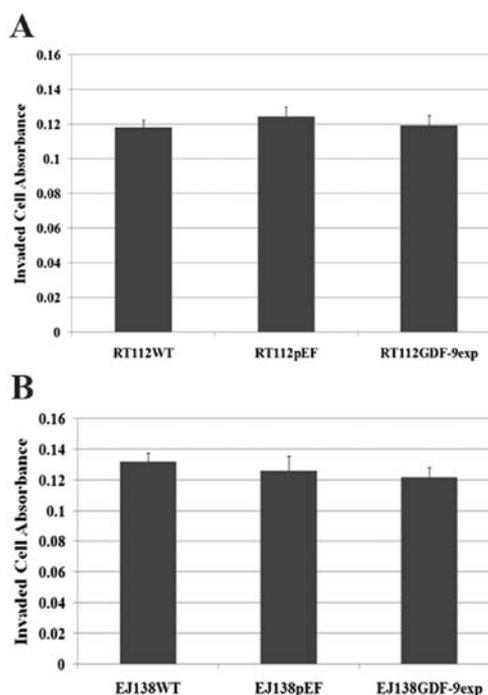


Figure 4. Effects of GDF-9 overexpression on the invasion of bladder cancer cells. (A) RT112 cells; (B) EJ138 cells. There was no significant difference among the three groups. Shown are representative data from three independent experiments.

on the cell matrix adhesion of bladder cancer cell lines. Overexpression of GDF-9 significantly inhibited the cell matrix adhesion of the RT112 cells (*P*<0.01 vs. both controls) (Fig. 5A). Similarly, compared with EJ138^{WT} and EJ138^{pEF}, the number of adherent cells for EJ138^{GDF-9exp} was significantly reduced (*P*<0.01 vs. both controls) (Fig. 5B).

The ECIS system was used to further investigate the effect of enhanced expression of GDF-9 on RT112 and EJ138 cell adhesion. The attachment capacity was markedly reduced in RT112^{GDF-9exp} cells compared with RT112^{WT} and RT112^{pEF} cells (Fig. 5C and D). Similarly, the attachment capacity was markedly reduced in EJ138^{GDF-9exp} cells compared with EJ138^{WT} and EJ138^{pEF} cells (Fig. 5E and F).

Effect of GDF-9 on migration of bladder cancer cells. The effect of GDF-9 on RT112 and EJ138 cellular motility was assessed using the ECIS system. The migration capacity was markedly reduced in RT112^{GDF-9exp} cells compared with RT112^{WT} and RT112^{pEF} cells (Fig. 6A and B). Similarly, the migration capacity was markedly reduced in EJ138^{GDF-9exp} cells compared with EJ138^{WT} and EJ138^{pEF} cells (Fig. 6C and D).

Discussion

Bladder cancer ranks ninth in worldwide cancer incidence. It is the seventh most common malignancy in men and the seventeenth in women. It is the fourth most common cancer in men and the second most common malignancy affecting the genitourinary system (27). Approximately 356,000 new bladder cancer cases (274,000 males and 83,000 females)

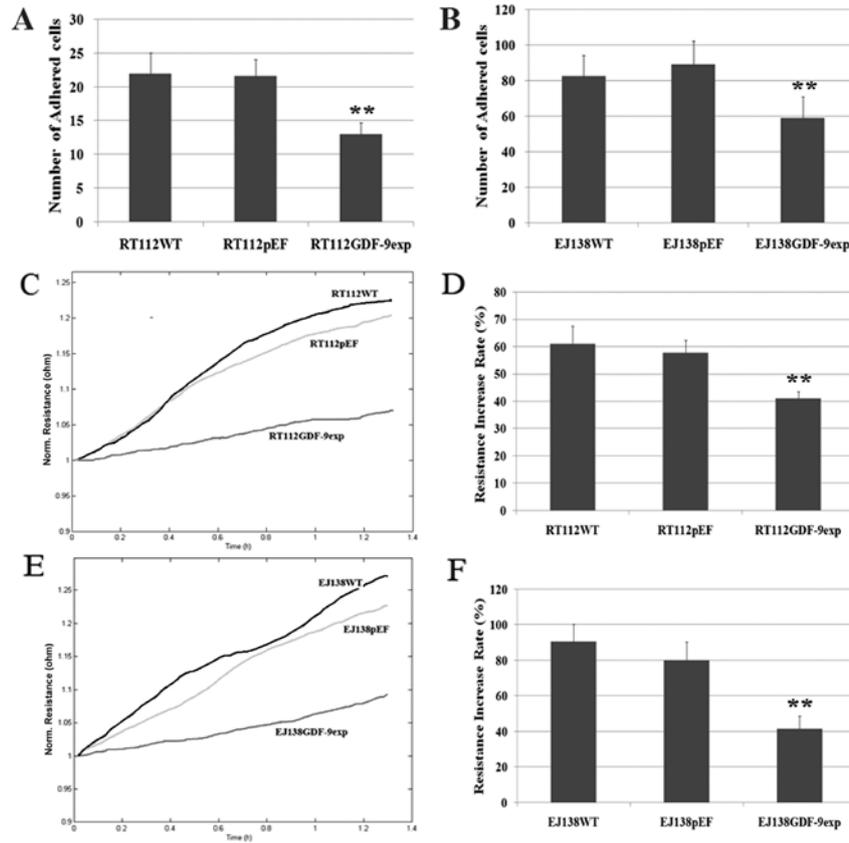


Figure 5. Effects of GDF-9 on adhesion of bladder cancer cells *in vitro*. (A) Overexpression of GDF-9 reduced the number of adherent cells in RT112^{GDF-9exp} cells. **P<0.01 vs. RT112^{WT} and RT112^{pEF} cells. (B) Overexpression of GDF-9 reduced the number of adherent cells in EJ138^{GDF-9exp} cells. **P<0.01 vs. EJ138^{WT} and EJ138^{pEF} cells. (C and D) RT112^{GDF-9exp} cells which overexpressed GDF-9 showed a markedly reduced attachment using an ECIS model. (E and F) EJ138^{GDF-9exp} cells which overexpressed GDF-9 showed a markedly reduced attachment. ECIS RbA modelling of cell attachment indicated a significant reduction of attachment in GDF-9 transfected cells. **P<0.01 vs. wild-type and empty plasmid control cells. All experiments were repeated 3 times.

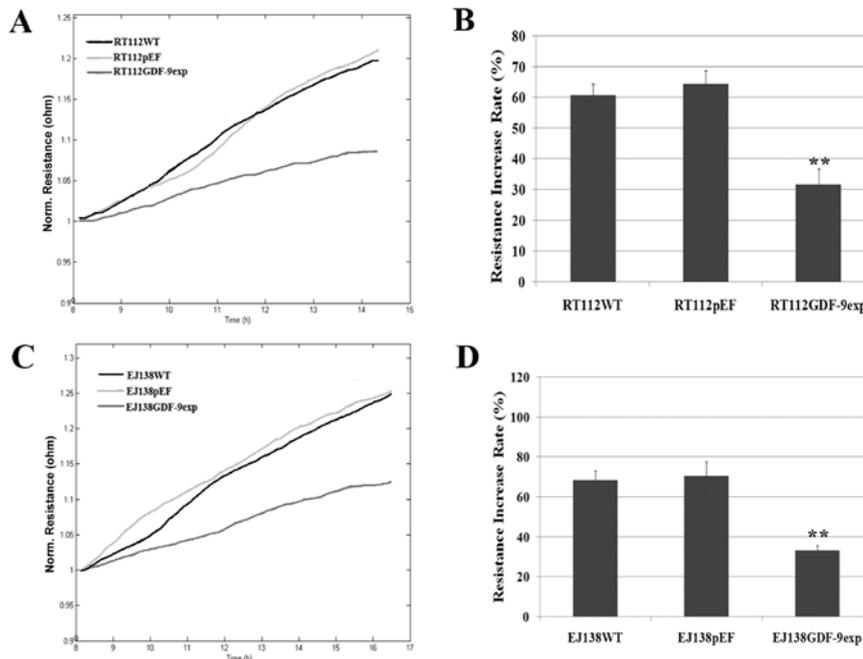


Figure 6. Effect of GDF-9 on migration of bladder cancer cells. Effect of GDF-9 expression on cell migration as analysed by ECIS (wounding assays). The impedance changes during the migration process are shown. (A) The UMRC -2^{GDF-9exp} cells which overexpressed GDF-9 showed a markedly reduced migration. (B) The EJ138^{GDF-9exp} cells which overexpressed GDF-9 showed a markedly reduced migration. (C) ECIS RbA modelling of cell attachment indicated a significant reduction of attachment in GDF-9 transfected RT112^{GDF-9exp} cells. **P<0.01 vs. RT112^{WT} and RT112^{pEF} cells. (D) ECIS RbA modelling of cell attachment indicated a significant reduction of attachment in GDF-9 transfected EJ138^{GDF-9exp} cells. **P<0.01 vs. EJ138^{WT} and EJ138^{pEF} cells. All experiments were repeated 3 times.

were reported worldwide in 2002. At the same time, bladder cancer is the most common malignancy of the urinary tract, accounting for 90-95% of urothelial carcinomas (28), which are also called transitional cell carcinomas (TCCs) (29). TCC is a heterogeneous disease with considerable variations of its natural history. For example, the five-year survival rate is 97-98% for patients with a monofocal, well-differentiated and small papillary tumour, whereas this could be 0% if patients have invasive bladder cancer extending throughout the bladder wall and with gross nodal metastases. It is also characterized as a carcinoma with multifocality and a high recurrence rate (30). At initial diagnosis, 75% patients present with non-muscle-invasive bladder cancer (NMIBC), the remaining 25% present with muscle-invasive bladder cancer (MIBC). The main problems of NMIBC are recurrence and progression, while MIBC is frequently associated with metastatic diseases and is the major cause of mortality. In NMIBC, disease recurrence will occur in ~50-80% of patients after management with transurethral resection (TUR) and intravesical therapy (31). Despite improvements in both early detection and treatment, management of this disease remains a challenge.

GDF-9 is a member of the BMP family known as a well-established follicular growth factor that has been shown to be vital during early follicular development (32). BMPs signal via two types of specific receptors (BMPRs); type I and II serine/threonine kinase receptors (33). The BMP signal transduction pathway is triggered when a BMP ligand binds to its receptors resulting in induction of either the Smad-dependent or Smad-independent pathway. The former is activated by BMPs binding to a pre-formed heteromeric receptor complex (PFC) (34). This induces the type-II receptor to activate the type-I receptor, which in turn phosphorylates the receptor-mediated Smads (R-Smads) 1, 5 and 8 or Smads 2 and 3. These activated R-Smads then co-localize with Smad 4 and undergo nuclear translocation to induce BMP responsive gene expression (35). The Smad-independent pathway meanwhile, is activated by BMPs that first bind to the type-II receptor, followed by recruitment of the type-I receptor (34).

Despite the importance of BMPs in cancers however, the role of GDF-9 in cancer remains elusive. The role of GDF-9, in tumour progression remains unclear and somewhat controversial. Its tumour suppressive role has been demonstrated in human breast cancer (18), but the opposite has been seen in oral cancer and prostate cancer (19,20). To the best of our knowledge, the current study is the first report to examine the staining pattern of GDF-9 in human bladder tissues and to assess its impact on the invasion, growth, adhesion and migration of bladder cancer cells by genetically manipulating the expression of GDF-9.

Similarly to human breast cancer (18), GDF-9 was expressed at a lower level or was absent in bladder tumour cells of bladder tissues. Furthermore, the bladder cancer cell lines, RT112 and EJ138, expressed very low levels of GDF-9. Moreover, overexpression of GDF-9 reduced the growth, adhesion and migration of bladder cell lines *in vitro*. This is in contrast to that observed with prostate cancer cells (8,36). This would indicate that in some human tumours, GDF-9 exists at lower levels, although the opposite may also occur. It is interesting to note that GDF-9 is absent in both normal bladder transitional cells and bladder cancer cells. The nuclear

existence of GDF-9 is particularly interesting as it has been suggested that the cytoplasmic/nuclear distribution pattern of the GDF-9 protein may be a key feature in cancer and an important reason in determining the contrasting role of GDF-9 in different cancer types. The current study further indicates the possibility of a nuclear connection in the function of GDF-9. Thus, changes in the overall level of staining of GDF-9 in bladder cancer cells and in intracellular distribution appear to be a feature in human bladder tumour tissues.

In the present study, we employed methods to genetically alter the expression of GDF-9 in bladder cancer cells, namely the overexpression approach. In clear contrast, forced overexpression of GDF-9 in two bladder cancer cell lines, RT112 and EJ138, resulted in a reduction of adhesion and migration. This indicates that GDF-9 plays a key role in the control of the aggressiveness of bladder cancer cells. The current study has also demonstrated that overexpression of GDF-9 resulted in a decreased cell growth *in vitro*. In breast cancer, primary breast cancer samples from patients with good predicted prognosis had significantly higher levels of GDF-9 compared to samples from patients with poor prognosis including those who exhibited metastasis, local recurrence, or had died from breast cancer (18). These studies together with evidence presented herein, strongly suggest that GDF-9 is a potential tumour suppressor in bladder and breast tumours. This suggestion is further supported by our *in vitro* results, in which GDF-9 exhibited inhibitory effect on growth of bladder cancer cells.

In conclusion, the current study demonstrated the reduced expression of GDF-9 in bladder cancer. GDF-9 overexpression can suppress the aggressiveness of bladder cancer cells through inhibiting cell growth, adhesion and migration. This study thus suggests that GDF-9 may be putative tumour suppressor in bladder cancer.

Acknowledgements

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