Reduced Expression of Growth and Differentiation Factor-9 (GDF9) Is Associated with Aggressive Behaviour of Human Clear-cell Renal Cell Carcinoma and Poor Patient Survival

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Abstract. *Background: Growth and differentiation factor-9 (GDF9) is a member of the bone morphogentic protein (BMP) family. GDF9 was recently shown to be a regulator of the development and spread of cancer cells, including kidney cancer cells. However, the clinical implication of GDF9 in human clear-cell renal cell carcinoma (CCRCC) remains unknown. In the present study, the expression of GDF9 in human CCRCC tissues, and correlation between GDF9 and pathological grade and stage of the tumours were examined in CRCC specimens. Materials and Methods: The expression of GDF9 was examined in paired human normal renal and CCRCC tumour tissues (n=86). The expression of GDF9 in human renal tissues was assessed at both the mRNA and protein levels using reverse transcription-polymerase chain reaction and western blot. Furthermore, the survival curve was constructed using Kaplan–Meier method. Results: Decreased GDF9 protein levels were seen in CCRCC tissues compared with normal tissues. Low protein levels were seen in tumours with high clinical stages and with high pathological nuclear grade of CCRCC. Likewise, levels of GDF9 transcript in normal renal specimens was significantly higher than that in CCRCC tissues. The transcript levels of GDF9 differed significantly amongst different clinical stages and different pathological nuclear grade of CCRCC: The higher the clinical stage or pathological nuclear grade of CCRCC, the lower the transcript level of GDF9. Cumulative survival curves indicated that GDF9 mRNA expression was negatively correlated with cumulative survival time. Patients with high level of GDF9 had significantly longer survival time than the patients with low level of GDF9 ($p<0.001$). Conclusion: GDF9 expression is markedly decreased in CCRCC, and is linked to pathological grade, clinical stage and long-term survival of the patients. This suggests that GDF9 is a potential tumour suppressor in CCRCC.*

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Renal cell carcinoma (RCC) is the third most common cancer in the urinary system and accounts for approximately 90% of all malignant renal tumors. Over 120,000 cases of RCC are diagnosed every year in Europe and the USA, and the incidence of RCC appears to be rising. RCC is a highly heterogeneous disease, with different histological subtypes and varying prognosis. Although the wide use of advanced imaging and surgical techniques have improved the clinical outcome of patients to some degree, the molecular mechanisms involved in RCC are yet not clear.

Growth and differentiation factor-9 (GDF9) is a protein factor belonging to the bone morphogenetic protein family (BMP) and the transforming growth factor (TGF)-β superfamily (1-3). GDF9 was initially identified as an oocyte growth factor with an important role in the regulation of folliculogenesis and ovulation (4), a number of studies have since shown that it is expressed in a range of other tissues, including testis, pituitary gland, adrenal gland and adrenocortical cancer in mouse (5), and in human brain, liver, kidney, prostate, bladder, skin cancer, breast cells and tissues (6-9). In recent years, GDF9 was also shown to have a close relationship with cancer progression. For example, Hanavadi *et al.* found an inhibitory effect of GDF9 expression on the progression of human breast cancer (10). Meanwhile, up-regulation of GDF9 protein level in an aggressive oral carcinoma cell line has been reported (11).

Recent research showed that GDF9 can promote the growth rate of both PC-3 and DU-145 prostate cancer cells by protecting cells from caspase-3-mediated apoptosis (12), and also promotes cell invasiveness of PC-3 cells (13). In our previous study, we found reduced or loss of GDF9 expression in human kidney cancer cells and that overexpression of GDF9 in kidney cancer cell lines suppresses the invasiveness, growth and migration of kidney cancer cells *in vitro*, suggesting that GDF9 may act as a potential tumour suppressor (14). In the present study, we investigated the expression of GDF9 in human renal clear-cell carcinoma (CCRCC) and herein report that expression of GDF9 in human CCRCC tissues was reduced in comparison to normal kidney tissues and that the reduction of GFD9 correlated with pathological grade, clinical stage and long-term survival of patients.

Materials and Methods

CCRCC specimens. A total of 86 (52 males and 34 females) pairs of CCRCC and normal renal tissue samples were snap-frozen in liquid nitrogen immediately after open radical nephrectomy. The average age of patients was 58.1±10.2 (range=28-76) years. The pathological features and staging were verified by a consultant pathologist. Each tumour underwent pathological staging based on the Union for International Cancer Control/American Joint Committee on Cancer (UICC and AJCC) 2002 classification of primary RCC and Fuhrman nuclear grading (15, 16). In the cohort, 35 (40.7%), 24 (27.9%) and 27 (31.4%) cases were staged as T1, T2 and T3, respectively. Fuhrman grades of the cohort were G1 in 29 cases (33.7%), G2 in 36 (41.9%) and G3 in 21 (24.4%). All protocols were reviewed and approved by the Research Ethics Committee of Peking University Cancer Hospital (2006021) and all patients gave their written informed consent.

RNA isolation and reverse transcription followed by quantitative real-time polymerase chain reaction (PCR). RNA was isolated using Total RNA Isolation Reagent (Fisher Scientific, Epsom, UK). Reverse transcription was performed using the durascripttm RT-PCR kit, followed by quantitative real-time PCR (Q-PCR) using a ReadyMix PCR reaction mix (Bio-Rad, Hemel Hemstead, England, UK). Primers used for quantification were based on the Ampliflor technology and sequences for *GDF9* (5'-3') were: gcagaggtcaggaaactgt and actgaacctgaccgtacaatggagctcaca; and for *Glyceraldehyde-3-phosphate dehydrogenase* (*GAPDH*) were: aaggtcatccatgacaact and actgaacctgaccgtacagccatccacagtcttctg. Cycling conditions were 95˚C for 10 min, followed by 50 cycles of 95˚C for 10 s, 60˚C for 1 min, and 72˚C for 15 s. The results for *GDF9* were normalised against the level of *GAPDH*.

Western blot analysis of GDF9 expression. The protein concentration in tissue lysates were determined using the DC Protein Assay kit (Bio-Rad, Hemel Hemstead, England, UK) and an ELx800 spectrophotometer (BIO-TEK™) (Wolf Laboratories, York, England, UK). Equal amounts of protein were separated by sodium dodecyl sulfate-polyacrylamide gel electrophoresis and blotted onto nitrocellulose sheets. Proteins were then respectively probed with either an antibody against GDF9 or GAPDH (Santa Cruz Biotechnologies Inc., Santa Cruz, CA, USA) 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 an UVITech imager (UVITech, Inc., Cambridge, UK).

Statistical analysis. Quantitative analysis of western blot was performed using the GelDoc-2000 Imaging System (UVi Company, Cambridge, England, UK). For protein expression levels, the protein ratio (band density of protein/band density of GAPDH) was considered as 100% in the normal kidney tissue group, and that of the other group was expressed as a percentage of that of the normal kidney tissue group. Statistical analysis was conducted by one-way analysis of variance, followed by all pairwise multiple-comparisons procedures using the Bonferroni test. A survival curve was constructed using the Kaplan–Meier method. All data are presented as the mean±standard deviation, and significance was reached at $p<0.05$.

Results

Expression of GDF9 protein in CCRCC and normal kidney tissues. In order to estimate the protein levels of GDF9 in human kidney tissues, we conducted western blot analyses using proteins extracted from normal and malignant kidney tissues. As shown in Figure 1A and B, decreased GDF9 expression was seen in the CCRCC tissues compared with normal kidney tissues

The protein levels of GDF9 were significantly different in CCRCC with different clinical stage: the higher the clinical stage of CCRCC, the lower the protein level of GDF9 (T2 *vs*. T1, $p<0.001$ and T3 *vs*. T2, $p<0.001$) (Figure 1C).

Meanwhile, the protein levels of GDF9 significantly differed by pathological nuclear grade of CCRCC, namely lower GDF9 protein levels were seen in tumors with high pathological grade (G2 *vs*. G1, $p<0.001$ and G3 *vs*. G2, $p<0.001$) (Figure 1D).

Transcript of GDF9 in CCRCC and normal kidney tissues. The transcript level of GDF9 was examined in human renal tissues using Q-PCR. The number of copies of GDF9 transcript in normal kidney tissues was significantly higher than that of the CCRCC tissues ($p<0.0001$) (Figure 2).

The transcript level of GDF9 was inversely correlated with both clinical stage of CCRCC (T1 *vs*. T2, $p<0.001$ and T2 *vs*. T3, $p<0.001$) (Figure 3) and pathological nuclear grade of CCRCC (G1 *vs*. G2, $p<0.0001$ and G2 *vs*. G3, $p<0.0001$) (Figure 4).

GDF9 gene expression and survival in CCRCC. After 48 months of follow-up, patients were analyzed for survival time based on the level of *GDF9* mRNA expression. The mean time of follow-up for the cohort (n=86) was 41.59±1.22 months (range=1-48 months). Cumulative survival curves were calculated using the Kaplan–Meier

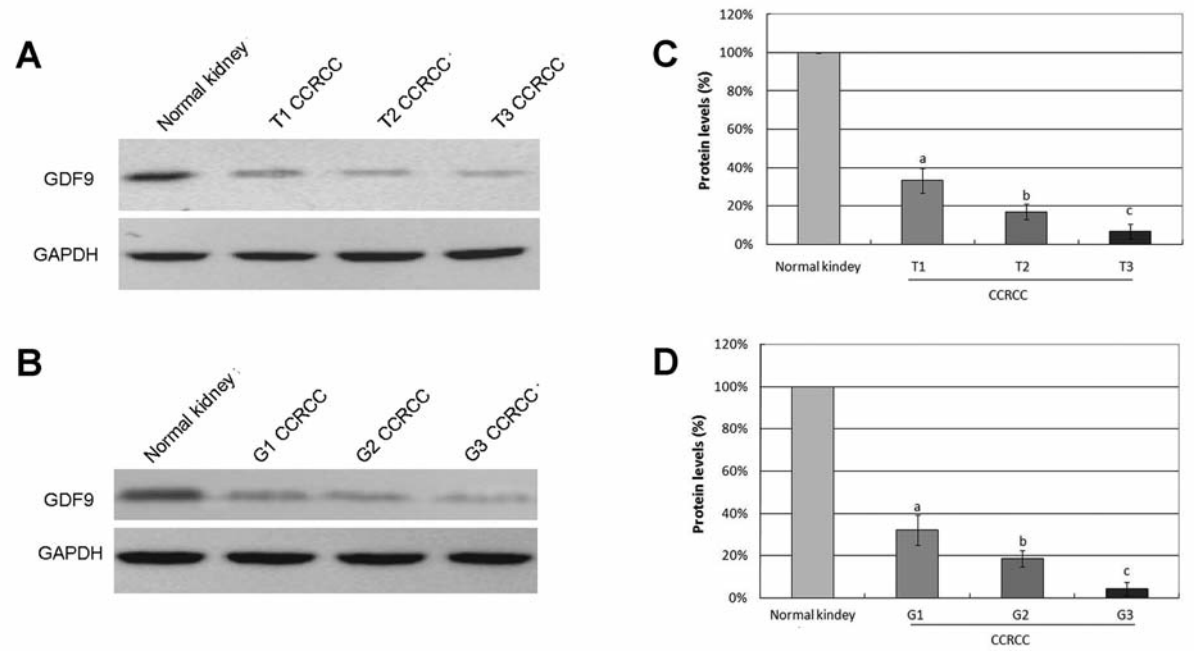

Figure 1. *Western blot results of Growth and Differentiation Factor-9 (GDF9) in clear cell renal cell carcinoma (CCRCC) and normal kidney tissues. A and B: Representative images of western blots demonstrate the changes of GDF9 with clinical stage and pathological nuclear grade of CCRCC and in normal kidney tissues. C and D: Quantitative analysis of western blot results.*

method. The mean survival time for those with high expression of *GDF9* mRNA was 46.53 months (95% confidence interval=45.14-47.92 months). The mean survival time for those with low expression of GDF9 was 35.65 months (95% confidence interval=31.45-39.84 months). The level of *GDF9* mRNA expression was negatively correlated with cumulative survival time. Patients with high level of GDF9 had significantly longer survival time than those with a lower level of GDF9 ($p<0.001$) (Figure 5).

Discussion

The incidence and mortality rates of RCC are increasing by 2-3% per decade globally (17, 18), and skeletal metastases occur in about one-third of patients with advanced or metastatic CCRCC (19). Factors linked to the metastatic spread of CCRCC are rather poorly-understood, although the BMP/TGFβ family proteins have been indicated in this process. TGFβ superfamily proteins are extracellular, secreted growth factors that function *via* autocrine or paracrine signaling, and include two chief sub-groups: the GDFs/BMPs and activins/TGFβs (20). Out of these proteins, TGFβ has been demonstrated to be a potent growth inhibitor in a variety of cell types, including renal cell carcinoma (CCRCC) cells (21). BMPs, a sub-family of the TGFβ superfamily initially characterized in bone, have been shown to regulate cell death, growth, and differentiation in different tissues (22). The BMP signalling transduction pathway is triggered when its ligands binds to type I and II serine/threonine kinase receptors, resulting in induction of either the SMAD-dependant or SMAD-independent pathways. BMP proteins have been shown to be important in bone formation and several members have been implicated in the pathogenesis of cancer (9, 23, 24). In human liver cancer, small interfering RNA targeting BMP2 markedly inhibited the expression of BMP2 in liver cancer cells, and reduced the migration and invasion of liver cancer cells (25). In ovarian cancer, BMP2 efficiently increased the motility of epithelial ovarian cancer cell lines. In contrast, BMP2 treatment reduced the ability of epithelial ovarian cancer cell lines to form spheroids, indicating an inhibition of cell–cell adhesion. The expression of BMP2 in tumor tissues from patients was also inversely correlated with survival (26). In breast and prostate cancer, BMP10 inhibits aggressiveness of breast cancer cells and is correlated with poor prognosis (7, 24). In prostate cancer, BMP expression has been shown to be variable, with BMP2, -4, -7, -9, and -10 expression being reduced compared to normal prostate epithelial cells (27, 28). In addition, BMP2 and -6 have been shown to inhibit the growth of prostate cancer cells (29). We recently showed that BMP9 and -10 can inhibit growth, adhesion, invasion, and migration of prostate cancer cells by inducing apoptosis *via* SMAD1-, -5-, and -8-mediated up-regulation of pro-apoptotic factor PAR4 (prostate apoptosis response 4), and the SMAD-independent pathway, respectively (28, 30).

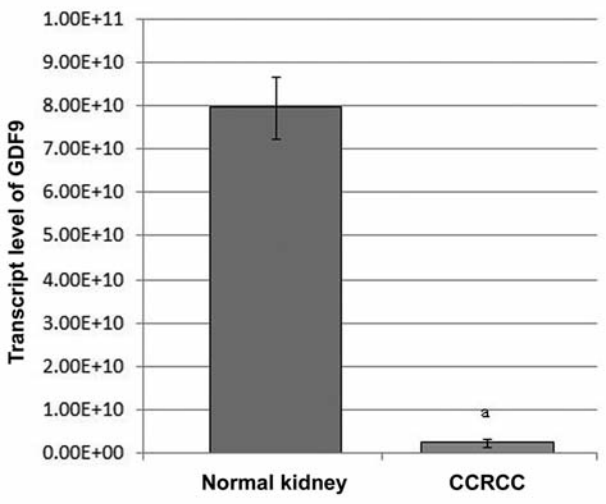

Figure 2. *Transcripts of Growth and Differentiation Factor-9 (GDF9) in clear cell renal cell carcinoma (CCRCC) and normal kidney tissues. The transcript level of GDF9 was examined in human renal tissues using Q-PCR. The number of copies of GDF9 transcript (normalised to GAPDH) in normal kidney tissues was significantly higher than that in CCRCC tissues. [a]p<0.001 compared with normal kidney tissues.*

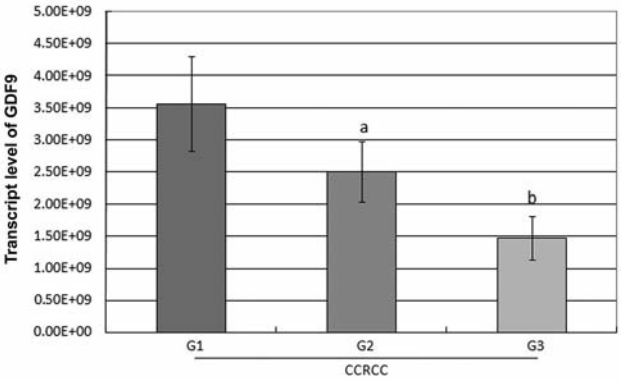

Figure 4. *Transcripts of Growth and Differentiation Factor-9 (GDF9) in clear cell renal cell carcinoma (CCRCC) of different pathological nuclear grades. The transcript level of GDF9 was examined in CCRCC tissues with different pathological nuclear grade using Q-PCR. The number of copies of GDF9 transcript in G3 CCRCC tissues was significantly lower than that in G2 CCRCC tissues, and that in G2 CCRCC tissues was significantly lower than that in G3 CCRCC tissues. [a]p<0.001 compared to G1 CCRCC tissues, [b]p<0.001 compared to G2 CCRCC tissues.*

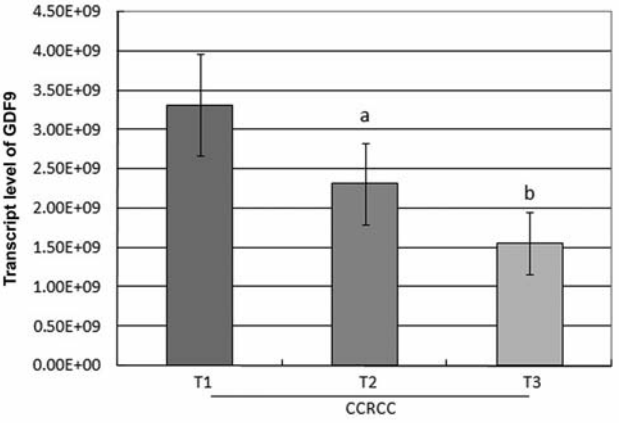

Figure 3. *Transcripts of Growth and Differentiation Factor-9 (GDF9) in clear cell renal cell carcinoma (CCRCC) of different clinical stages. The transcript level of GDF9 was examined in different clinical stages of CCRCC tissues using Q-PCR. The number of copies of GDF9 transcripts in T3 CCRCC tissues was significantly lower than that in T2 CCRCC tissues, and that in T2 CCRCC tissues was significantly lower than that in T3 CCRCC tissues. [a]p<0.001 compared with T1 CCRCC tissues, [b]p<0.001 compared with T2 CCRCC tissues.*

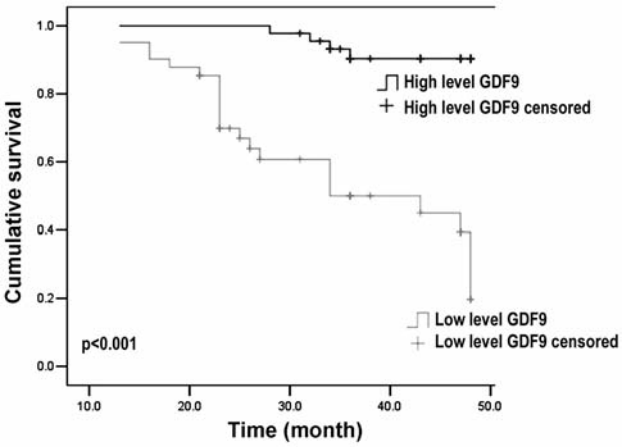

Figure 5. *Kaplan–Meier analysis of overall survival of patients with clear cell renal cell carcinoma (CCRCC) depending on the expression level of Growth and Differentiation Factor-9 (GDF9) mRNA. A high level of GDF9 was associated with significantly longer survival compared to those who had a lower level (p<0.001).*

As a member of the BMP family, GDF9 is known as a follicular growth factor, crucial for primary follicle growth (31). It is believed to regulate cell proliferation or differentiation in addition to its role in stimulating granulosa cell proliferation (32). Despite the importance of BMPs in cancer, the role of GDF9 in tumor progression remains unclear and controversial. GDF9 was not expressed in highly aggressive breast cancer cells, but breast cancer cells became less invasive after forced expression of GDF9 (10).

In CCRCC cells, GDF9 was seen in the cytoplasmic area of normal renal tubular epithelial cells, but staining was lower in or absent from CCRCC tissue cells, especially in specimens with higher nuclear grade, compared to normal renal cells, tubular epithelial cells. Moreover, the number of

GDF9 transcripts in CCRCC tissues were significantly lower than that in normal kidney tissues. Our results also show that the higher the pathological nuclear grade, the lower the copy number of *GDF9* transcripts. This inverse correlation is contrary to that observed in prostate cancer cells (9, 13), which indicates that in different human tumor types, GDF9 may have different expression patterns. It is interesting to note that both normal renal tubular epithelial cells and CCRCC cells had little GDF9 in the nucleus. The nuclear existence of GDF9 is particularly interesting as it has been suggested that the cytoplasmic/nuclear distribution pattern of GDF9 protein may be a key feature in cancer and important in the contrasting role of GDF9 in different cancer types. Thus, changes in the overall level of staining of GDF9 in kidney cancer cells and in intracellular distribution appear to be a feature in human kidney tumour tissues.

To the best of our knowledge, the current study is the first report to examine the RNA and protein expression of GDF9 in human CCRCC tissues and to test the correlations between GDF9 and pathological grade and clinical stage of CCRCC. In conclusion, our study shows that reduced expression of GDF9 in CCRCC, and GDF9 expression is linked to higher pathological nuclear grade and clinical stage, and poorer long-term survival in patients with CCRCC. This indicates that GDF9 plays a key role in the control of the aggressiveness of CCRCC tissues, which is further supported by our *in vitro* results, in which GDF9 exhibited an inhibitory effect on the growth of kidney cancer cells (14). These results suggest that GDF9 is a potential tumour suppressor in CCRCC.

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