Epigenetic alterations of Krüppel-like factor 4 and its tumor suppressor function in renal cell carcinoma

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Krüppel-like factor 4 (KLF4) is a transcription factor that can have divergent functions in different malignancies. The expression and role of KLF4 in renal cell cancer remain unclear. The purpose of this study is to determine epigenetic alterations and possible roles of KLF4 in renal cell carcinoma. The KLF4 expression in primary renal cell cancer tissues and case-matched normal renal tissues was determined by protein and messenger RNA analyses. The epigenetic alterations were detected by methylation-specific PCR and Sequenom MassARRAY. Kaplan–Meier curves and the log-rank test were used for the survival analysis. The effects of KLF4 on cell growth and epithelial-to-mesenchymal transition (EMT) were determined in renal cancer cell lines after viral-based and RNA activation-mediated overexpression of KLF4. In vivo antitumor activity of KLF4 was evaluated by using stably KLF4-transfected renal cancer cells. KLF4 expression was dramatically decreased in various pathological types of renal cancer and associated with poor survival after nephrectomy. Hypermethylation of KLF4 promoter mainly contributed to its expression suppression. In vitro assays indicated that KLF4 overexpression inhibited renal cancer cell growth and survival. KLF4 overexpression also suppressed renal cancer cell migration and invasion by altering the EMT-related factors. In vivo assay showed that ectopic expression of KLF4 also inhibited tumorigenicity and metastasis of renal cancer. Our results suggest that KLF4 is a putative tumor suppressor gene epigenetically silenced in renal cell cancers by promoter CpG methylation and that it has prognostic value for renal cell progression.

Introduction

Renal cell carcinoma (RCC) is a common urologic tumor and accounts for about 3% of all human malignancies in adults. It is the third most common urological cancer after prostate and bladder cancer, and it has the highest mortality rate at over 40% and significant increase in its incidence during the last decades (1,2). Clear cell carcinoma is the most common subtype of RCC and accounts for approximately 75–80% of these tumors (3). Apart from surgery, it is both chemotherapy and radiotherapy resistant. The present absence of biomarkers for early detection and follow-up of the disease is responsible for late diagnosis and subsequent poor prognosis. Therefore, an increased investigation of genetic and epigenetic biological changes is necessary to improve understanding the pathogenesis and identify new biomarkers of RCC.

Abbreviations: DFS, disease-free survival; dsRNA, double-stranded RNA; EMT, epithelial-to-mesenchymal transition; GAPDH, glyceraldehyde 3-phosphate dehydrogenase; KLF4, Krüppel-like factor 4; mRNA, messenger RNA; OS, overall survival; RCC, renal cell carcinoma; siRNA, small interfering RNA; SEM, standard error of the mean.

Krüppel-like factor 4 (KLF4), also known as gut-enriched Krüppel-like factor, is a member of the KLF family of zinc-finger-containing transcription factors. Inactivation or silencing of KLF4 has been observed in a number of human cancers including gastric, colorectal, pancreas, esophageal, lung, prostate and hepatocellular cancer (4–10). Deletion of KLF4 in mouse models leads to abnormal differentiation, increased proliferation and formation of intestinal adenomas in the colon and gastric epithelia (11–13). Ectopic expression of KLF4 has been shown to inhibit cell proliferation, colony formation, cancer cell migration and invasion, promote cell cycle arrest and induce apoptosis in vitro and suppress carcinogenesis and metastasis in vivo (6,14). Moreover, accumulating clinical evidences also suggest that KLF4 functions as a tumor suppressor and has potential prognostic value of lymph node metastasis (4,9). These observations provided compact evidences that KLF4 has putative tumor suppressor function in a variety of malignancies. On the other hand, KLF4 expression is increased in primary breast ductal carcinoma and in oral and dermal squamous cell carcinomas (15–17). All these results suggested that KLF4 played a pivotal role in tumor development and progression.

Recently, we and others have reported that double-stranded RNA (dsRNA) can activate gene expression by targeting promoter sequence in a process termed RNA activation (RNAa). RNAa is a small RNA-guided gene regulation phenomenon in which promoter-targeted short dsRNA induces target gene expression at the transcriptional level (18–21). In our previous studies, RNAa has been used as a novel tool for interrogating gene function by serving as an alternative to traditional vector-based systems and an attractive strategy to activate tumor suppressor genes for the treatment of cancer.

It remains unknown whether and, if so, how KLF4 contributes to the development and progression of renal cell cancer. In this study, we detected the KLF4 expression and promoter methylation in renal cell lines and cancer tissues. Viral-based and RNAa-mediated overexpression of KLF4 was utilized to investigate its function in vitro and in vivo. Our observations indicated that KLF4 is considerably decreased or lost in renal cancer mainly due to the aberrant promoter methylation, and KLF4 loss is associated with renal cancer progression. Loss or promoter hypermethylation of KLF4 in renal cancer tissues was significantly associated with a relatively lower overall survival (OS) and disease-free survival (DFS) in 5 years. These findings suggested that KLF4 functions as a tumor suppressor in renal cell cancer in vitro and in vivo. We also demonstrate that RNAa can be utilized as a research tool to upregulate endogenous gene expression and function in a manner similar to vector-mediated overexpression.

Materials and methods

Population and sample collection

Formalin-fixed, paraffin-embedded tissue specimens. In this study, we included three tissue microarrays (CHAOYING Biotechnology). Each tissues spot was accompanied with cases material including sex, age, histologic type, pathologic grade and clinic stage. We also included 12 successive cancer adjacent normal renal tissues, 16 successive primary renal clear cell cancer with lymph node metastasis and 19 successive primary renal clear cell cancer with venous tumor thrombus (one of them was also included in patients with lymph node metastasis). The specimen sections were confirmed by pathologist and obtained from the Department of Pathology of Tongji Hospital in Wuhan, China. Specimens from 149 consecutive patients with renal clear cell carcinoma were treated by radical nephrectomy and confirmed by pathological examination in Department of Urology, Tongji Hospital from March 2003 to December 2004 and follow-up were also included for the survival assay. The study protocol was approved by the ethics committee of Huazhong University of Science and Technology and Tongji Hospital and a written informed consent was obtained from all participants involved in this study.
Fresh-frozen samples. A total of 16 paired renal cancer and cancer adjacent normal renal tissues were obtained sequentially from patients undergoing radical nephrectomy from the period of 2010–2011. Normal renal tissues were acquired at least 5 cm away from the tumor site. Tissues specimens were snap frozen in liquid nitrogen before DNA and RNA extraction.

Cell culture and transfection
ACHN, 786-O, SN12-PM6 and HK-2 cells were maintained in Dulbecco’s modified Eagle’s medium supplemented with 10% fetal bovine serum and 2 mmol/l l-glutamine in a humidified atmosphere of 5% CO₂ maintained at 37°C. OS-RC-2 and CaKi-1 cells were cultured in RPMI-1640 supplemented with 10% fetal bovine serum and 2 mmol/l l-glutamine. dsRNA transfection was taken by X-tremeGENE small interfering RNA (siRNA) Transfection Reagent (Roche), according to the manufacturer’s protocol.

dsRNA design
dsRNA was designed as reported previously (18,22). All dsRNA sequences are listed in Supplementary Table S1, available at Carcinogenesis Online.

DNA methylation analysis by massarray
Genomic DNA was isolated using QIAamp DNA Mini Kit (Qiagen) and bisulfite modification of the genomic DNA was carried out using an Epitect Bisulfite Kit (Qiagen) according to the manufacturer’s instructions. Quantitative methylation analysis of the promoter of KLF4 was performed using the Sequenom MassARRAY platform (CapitalBio, Beijing, China) as described previously (23). PCR primers were designed with Methprimer (http://epidesigner.com). Primer sequences that amplified 466 bp of the KLF4 promoter are listed in Supplementary Table S1, available at Carcinogenesis Online. The spectra methylation ratios were generated by EpiTyper software version 1.0 (Sequenom, San Diego, CA).

In vivo tumorigenicity assay
Tumorigenicity in nude mice was determined as described previously (24,25). Two groups of eight mice each were injected subcutaneously with prepared cells at the same site. Tumor onset was measured with calipers at the site of injection every 3 days by two trained laboratory staffs at different times on the same day 3 weeks after injection when appreciable tumor formed subcutaneously. Tumor volume was calculated using the formula, \( V = \frac{a \times b^2}{2} \), where \( a \) represents the larger and \( b \) represents the smaller of the two perpendicular indexes. Animals were killed 56 days after injection and tumors were weighed. Nude mice were manipulated and cared according to NIH Animal Care and Use Committee guidelines in the Experimental Animal Center of the Tongji Medical College of Huazhong University of Science and Technology, China.

Experimental lung metastasis model
The antimetastatic activity of KLF4 was tested in the mouse ACHN lung metastasis model as described previously (26). ACHN cell was stably infected with lenti-KLF4 or lenti-NC containing green fluorescent protein label. Treated cells (2×10⁶) were suspended in 100 μl of phosphate-buffered saline and injected intravenously via the tail vein. Mice were killed and lungs were resected 30 days later after injection. The incidence and volume of metastases were estimated by imaging of mice for bioluminescence using the Living Image software (Xenogen, Baltimore, MD). The photon emission level was used to assess the relative tumor burden in the mice lungs. All animal studies were conducted under approved guidelines of the animal care and use committee of the Tongji Hospital.

Statistical analysis
Statistical significance was determined by using the SPSS 12.0. The Fisher’s exact test was utilized to assess the significance between different proportions. Values are expressed as mean ± standard error of the mean (SEM) unless otherwise indicated and \( t \)-test was used to examine the difference between treatment and negative control group. The Spearman coefficient of rank correlation was also used to measure correlation between methylation ratio and KLF4 expression. Kaplan–Meier curves and the log-rank test were used for the survival analysis. OS was measured from surgical resection day until death from any cancer and was only censored for the patients who were alive without evidence of recurrence at the last follow-up. DFS was measured from the surgical resection day until either recurrence or death without recurrence, and it was only censored for the patients who were alive without evidence of recurrence at the last follow-up. Difference of tumor weight in tumorigenicity assay was assayed by \( t \)-test. Significance was defined as \( P < 0.05 \).

Additional methods are listed in Supplementary Materials and Methods, available at Carcinogenesis Online.

Results
KLF4 is downregulated in various types of RCC and renal cancer cell lines
To determine the effect of KLF4 expression on renal cancer development and progression, we did immunohistochemistry staining of tissue microarrays, which contained 243 renal cancer and normal renal tissue specimens. 230 of them were confirmed qualified and contained enough cancer tissue for immunohistochemistry staining by pathologist, and tissue specimens from 12 cancer adjacent normal renal tissues, 16 renal clear cell cancer with lymph node metastasis, 19 renal clear cell cancer with vena cava tumor thrombus (one of them was also included in patients with lymph node metastasis) and 149 primary renal cancer specimens were obtained from Department of Urology, Tongji Hospital, which were involved in further survival assay. The immunohistochemistry staining results demonstrated that KLF4 is mainly expressed in the cytoplasm and nucleus in most of the cells in the cancer adjacent normal renal tissues specimens. In glomerulus cells, almost no KLF4 expression was observed. In sharp contrast, KLF4 expression was significantly decreased or lost in the cytoplasm of various types of RCC, such as renal clear cell carcinoma (\( P < 0.01 \)), papillary cell carcinoma (\( P < 0.01 \)) and squamous cell carcinoma (\( P < 0.01 \)). In renal granular cell carcinoma, KLF4 was also decreased markedly (\( P < 0.01 \)) although increased in some specimens (Figure 1A a–f and Supplementary Table S2, available at Carcinogenesis Online).

Further, quantitative PCR and western blot analysis were used to examine the expression of KLF4 in 16 paired renal cancer tissue and tumor adjacent renal tissue specimens and a panel of six renal cell lines including five cancerous (ACHN, OS-RC-2, CaKi-1, 786-O and SN12-PM6) and one non-tumorigenic (HK-2) cell lines. Quantitative PCR confirmed that KLF4 messenger RNA (mRNA) expression was noticeably downregulated by about 90% or even more in each renal cancer cell line compared with levels in HK-2 cells (Figure 1B a). KLF4 mRNA in renal cancer tissues was also decreased considerably compared with matched tumor adjacent tissues, and representative data were also shown in Figure 1B. Immunoblot analysis indicated that KLF4 protein levels were also decreased obviously and correlated to that of mRNA in nearly all renal cancer cell lines and renal cancer tissues (Figure 1B b).

Loss of KLF4 expression is associated with poor prognosis in patients with renal clear cell carcinoma
We systematically analyzed KLF4 expression in renal clear cell cancer as mentioned above. In the primary renal clear cell cancer tissue specimens, the level of KLF4 expression was divided into three categories: negative, weak and strong. No remarkable difference of KLF4 expression was found in the distribution according to sex, age and vena cava tumor thrombus formation. However, we did observe significant difference in the distribution of the patients according to pathologic grade (\( P = 0.041 \)), clinic stage (\( P = 0.023 \)) and lymph node metastasis (\( P = 0.020 \); Table I).

In comparison of the KLF4 expression in normal renal tissue with its in primary tumors, metastatic lymph nodes and vena cava tumor thrombus, we found a significantly lower expression in the primary tumors, metastatic lymph nodes (\( P < 0.01 \)) and vena cava tumor thrombus (\( P < 0.01 \)) than in the normal renal tissues. Moreover, the expression of KLF4 was even lower in the metastatic lymph nodes than that in the primary tumors (\( P < 0.05 \)), whereas there was no significant difference between in vena cava tumor thrombus and in the primary tumors (Supplementary Table S3, available at Carcinogenesis Online).

Further, we analyzed the relationship between KLF4 expression and survival in renal clear cell cancer cases. In contrast, loss of KLF4 expression was associated with an inferior OS (\( P = 0.0134 \)) and DFS (\( P = 0.0122 \) duration (Figure 1C)). Overall, these findings strongly indicated that KLF4 decreased expression plays a critical role in renal clear cell cancer development and progression and is a valuable biomarker for this disease.
KLF4 promoter methylation contributed to expression loss and the renal clear cell cancer progression

The promoter region of KLF4 contains typical CpG islands (4,27). To explore the mechanism for the decrease of KLF4 expression in renal clear cell cancer, we determined KLF4 promoter methylation detection of genomic DNA extracted from surgically resected renal cancer specimens and matched cancer adjacent normal renal tissues, as well as the six renal cell lines using Sequenom MassARRAY. In total, 17 of 24 CpG sites (which were divided into 13 CpG units) were examined in the promoter region, except for the 2nd, 10th, 12th, 16th, 21st, 22th, 23th and 24th CpG sites that missed detection in the massarray (Figure 2A a). The 17 CpG sites were divided into 13 CpG units. The methylation ratio of a unit containing several CpG sites represents the average methylation of the CpG sites. The average methylation

**Fig. 1.** KLF4 expression is downregulated in renal cancer cell lines and tissues samples. (A) Representative microphotographs of KLF4 staining in cancer adjacent normal renal tissue (a), papillary cell carcinoma (b), granular cell carcinoma (c), squamous cell carcinoma (d), primary renal clear cell carcinoma (e) and renal clear cell cancer metastatic lymph node (f; original magnification: x400 for the inserts and x200 for all others). (B) (a) Relative expression of KLF4 mRNA expression levels were evaluated by real-time PCR in non-tumorigenic and renal cancer cell lines and paired case specimens; B (b) KLF4 and glyceraldehyde 3-phosphate dehydrogenase (GAPDH) protein levels were detected by immunoblot analysis. GAPDH served as an internal control. (C) Both OS and DFS rates in patients with KLF4-negative primary tumors were significantly worse than those in patients with KLF4-positive primary tumors. **P < 0.01.
ratio of each CpG unit in the three kinds of specimens was shown in Figure 2A b. Methylation ratio is the lowest in cancer adjacent renal tissue, median in renal cancer and the highest in renal cancer cell lines in all CpG units, but the 13th CpG unit in which renal cancer tissue shows a highest methylation ratio is related low in the absolute value.

In this study, hypermethylation was defined as the methylation ratio being >50%. The mean methylation ratio of the second unit (containing the third, fourth, fifth and sixth CpG sites) was >50% in renal cancer cell lines and renal cancer tissues. To identify the relationship between methylation status and KLF4 expression, we did correlation analysis between the KLF4 mRNA expression level and the methylation level of second CpG unit, which is the most highlighted in the KLF4 promoter region for the highest CpG site density and hypermethylation status. Picking the methylation ratio as x-axis and KLF4 expression level as y-axis, significance was achieved between methylation ratio and KLF4 mRNA expression level (Spearman correlation coefficient = −0.8985, P < 0.0001; Figure 2B). Further, we determined whether blockade of gene hypermethylation reactivates KLF4 expression in human renal cancer cell lines. Five renal cancer cell lines were incubated in medium or medium containing 5-aza-2-deoxycytidine, an inhibitor of DNA methyltransferase. As shown in Figure 2B, the DNA methyltransferase inhibitor significantly increased KLF4 expression in all the five renal cancer cell lines compared with that in mock group.

We also detected the association of KLF4 promoter methylation with renal clear cell cancer progression. As shown in Supplementary Table S4, available at Carcinogenesis Online, KLF4 promoter hypermethylation was significantly associated with pathological grade (P = 0.010), lymph node metastasis (P = 0.025), OS (P = 0.033) and DFS (P = 0.025) rate in 5 years after radical nephrectomy (Supplementary Table S4, available at Carcinogenesis Online). Kaplan–Meier curves and the log-rank test also showed significance of KLF4 promoter hypermethylation with OS (P = 0.0109) and DFS (P = 0.0392; Figure 2C and D). Therefore, promoter hypermethylation may contribute to the reduced KLF4 expression in a subset of renal cancer tissues and renal cancer cell line, and significantly associated with renal clear cell progression after radical nephrectomy.

KLF4 inhibits growth and survival in vitro and in vivo

To further investigate the KLF4 function in renal cell cancer development, we overexpressed KLF4 gene by lentiviral vector carrying human KLF4 gene and downregulated KLF4 by siRNA. Quantitative analysis by CCK-8 assay indicated that ACHN and 786-O cell viability steadily decreased following lenti-KLF4 infection, whereas HK-2 cell viability steadily increased following siRNA-KLF4 infection (Figure 3A a). Colony formation assays also revealed that KLF4 overexpression reduced the number and size of colonies formed by ACHN and 786-0 cells, and KLF4 downregulation promoted colony formation by HK-2 (Figure 3A b).

Next, we checked whether KLF4 overexpression exerts an effect on the cell cycle distribution and apoptosis in RCC. After lentiviral-KLF4 infection for 96 h, overexpression of KLF4 caused a significant increase in G1/G0 populations with concurrent declines in S and G2/M populations as compared with control treatments (P < 0.05). Arrest in G1/M phase was associated with KLF4 overexpression in 786-O cells with proportional declines in S and G1/G0 populations (P < 0.05). KLF4 overexpression also led to an increase in cells with subdiploid (<2C) DNA content, a marker for DNA fragmentation/apoptosis (Figure 3B). In line with flow cytometry result, Terminal Deoxynucleotidyl Transferase-Mediated dUTP Nick End Labeling assay also showed that lenti-KLF4 infection treatment for 96 h significantly increased the portion of apoptotic cells in ACHN and 786-0 cells (Figure 3C). The KLF4 overexpression induced apoptosis in both cell types.

To determine whether the KLF4 overexpression modulated the expression of downstream cell cycle genes and mitotic checkpoint-related genes in renal cancer cells, mRNA levels of p21, p27, p57, CCND1, CCNB1, CENPE, MAD2L1 and BUB1B were evaluated in ACHN and 786-0 cells following lenti-KLF4 infection and in HK-2 cells following siRNA-KLF4 transfection. As shown in Supplementary Figure S2, available at Carcinogenesis Online, p21, p27 and p57 expression was upregulated, whereas CCND1, CCNB1, CENPE, MAD2L1 and BUB1B were downregulated in ACHN and 786-0 cells. The reverse tendency of mRNA expression changes was shown in HK-2 after KLF4 downregulation. Immunoblot analysis of protein was consistent with the mRNA changes except p57 and CCNB1. CCNB1 was only selectively downregulated in 786-0, which would induce G2/M cell cycle arrest. Besides that, p57 protein was increased in ACHN cells but markedly decreased in 786-0 cells. The protein levels of p57 did not correlate to p57 transcription for its mRNA expression increased in all these two cell lines, which suggests that p57 is differentially regulated by post-transcriptional mechanisms in 786-0 cells following KLF4 activation (Figure 3D).

Tumorigenicity assay was also carried out to confirm the tumor suppressive function of KLF4 in vivo. About 5 × 106 ACHN cells infected with lentiv-KLF4 or lentiv-NC were injected subcutaneously into the axilla of nude mice (n = 8 per group). Subcutaneous tumor formation assay

### Table I. Patients characteristics and KLF4 expression of renal clear cell carcinoma

<table>
<thead>
<tr>
<th>Characteristic</th>
<th>Total (n = 370)</th>
<th>KLF4 staining</th>
<th>P</th>
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</thead>
<tbody>
<tr>
<td></td>
<td></td>
<td>Negative (%)</td>
<td>Weak (%)</td>
</tr>
<tr>
<td>Sex</td>
<td></td>
<td>n = 321</td>
<td>n = 48</td>
</tr>
<tr>
<td>Men</td>
<td>268</td>
<td>235 (87.7%)</td>
<td>33 (12.3%)</td>
</tr>
<tr>
<td>Women</td>
<td>102</td>
<td>84 (82.4%)</td>
<td>17 (16.7%)</td>
</tr>
<tr>
<td>Age (years)</td>
<td></td>
<td>61.3 (15.1)</td>
<td>60.9 (14.9)</td>
</tr>
<tr>
<td>Mean (SD)</td>
<td></td>
<td>301</td>
<td>253 (84.1%)</td>
</tr>
<tr>
<td>Grade</td>
<td></td>
<td>69</td>
<td>66 (95.7%)</td>
</tr>
<tr>
<td>1–2</td>
<td>151 + 130</td>
<td>124 (82.1%)</td>
<td>26 (17.2%)</td>
</tr>
<tr>
<td>3–4</td>
<td>70 + 19</td>
<td>67 (95.7%)</td>
<td>3 (4.3%)</td>
</tr>
<tr>
<td>Lymph node metastasis</td>
<td></td>
<td>331</td>
<td>280 (84.6%)</td>
</tr>
<tr>
<td>T1</td>
<td></td>
<td>39</td>
<td>39 (100.0%)</td>
</tr>
<tr>
<td>T2</td>
<td></td>
<td>19</td>
<td>17 (89.5%)</td>
</tr>
<tr>
<td>N0</td>
<td></td>
<td>351</td>
<td>302 (86.9%)</td>
</tr>
<tr>
<td>N1</td>
<td></td>
<td>19</td>
<td>17 (89.5%)</td>
</tr>
<tr>
<td>Vena cava tumor thrombus</td>
<td></td>
<td>17</td>
<td>17 (89.5%)</td>
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Two-tailed Fisher’s exact test was done to determine the statistical significance of the relationship of KLF4 expression with various variables. SD, standard deviation.

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H.Li et al.

was used to examine the proliferative ability of KLF4 overexpressed ACHN cells in nude mice. Compared with cells infected with lenti-NC, in which detectable tumor growth emerged within 21 days, cells infected with lenti-KLF4 did not show detectable tumor growth until 33 days and led to dramatically decreased tumor size \((P < 0.001)\) and tumor weight \((P < 0.001)\) in 57 days (Figure 4A). In intravenous injection assay, bioluminescence imaging revealed that fluorescence signal in lenti-KLF4 group were significantly weaker than lenti-NC group, which mean that less metastasis is formed in lung after KLF4 overexpression (Figure 4C). Immunoblot assay confirmed that KLF4 overexpression also influenced cell cycle-related gene p21, CCND1 and epithelial-to-mesenchymal transition (EMT)-related gene E-cadherin and Snail expression \(\text{in vivo}\) and the effect was consistent with that \(\text{in vitro}\) (Figure 4C). These assays \(\text{in vivo}\) suggested that KLF4 had a potential to inhibit tumorigenicity and metastasis of renal cancer cells.

KLF4 overexpression inhibits renal cancer cell migration and invasion by modulating the expression of EMT-related proteins

To assess changes in cell migration, ACHN and 786-0 cells were infected with lenti-KLF4 or lenti-NC and allowed to migrate through a transwell membrane into complete media. Compared with the negative control, overexpression of KLF4 significantly inhibited cell migration of ACHN and 786-0 cells. To evaluate cell invasion, ACHN and 786-0 cells were infected with KLF4 and plated on the matrigel surface coated upon the membrane. As shown in figure, KLF4 overexpression also reduced ACHN and 786-0 cell invasion. The reverse effect was shown in SN12-PM6 after KLF4 downregulation with siRNA (Figure 5A a and b).

In this study, EMT-related genes mRNA expression was examined after KLF4 restoration or downregulation. As shown in Figure 5B, E-cadherin and TIMP2 mRNA expression were upregulated, whereas N-cadherin, β-catenin, Vimentin, Snail and Slug mRNA was downregulated in ACHN and 786-0 cells after KLF4 restoration, whereas the reverse tendency was shown in SN12-PM6 after siRNA-KLF4 transfection. E-cadherin and β-catenin were also detected with immunofluorescence in 786-0 cells. Results show that KLF4 overexpression increased E-cadherin expression but decreased β-catenin expression (Figure 5C). Besides that, E-cadherin, N-cadherin, Snail and Slug protein expression were also detected with immunoblot assay. As shown in Figure 5D, mesenchyme markers N-cadherin, Snail and...
Slug were downregulated, whereas epithelial marker E-cadherin was upregulated after KLF4 overexpression in ACHN and 786-0, and KLF4 downregulation contributed to the reverse effect in SN12-PM6. Taken together, these findings indicated that KLF4 inhibits renal cancer cell migration/invasion and is associated with its EMT suppression function.

RNAa-based overexpression of KLF4 in renal cancer cells
RNAa-based overexpression of KLF4 was identified in prostate cancer cells as we described previously. To explore whether endogenous KLF4 can be also activated by RNAs in renal cancer cells. We also tested the same four candidate dsRNAs (dsKLF4-525, dsKLF4-496, dsKLF4-261 and dsKLF4-168) as previous study suggested. Each dsRNA was transfected into ACHN cells and KLF4 expression was evaluated by real-time PCR 4 days later. Compared with controls, dsKLF4-496 and dsKLF4-525 induced KLF4 expression by approximately 5.6- and 1.3-fold, respectively, whereas dsKLF4-168 and dsKLF4-261 did not significantly alter KLF4 levels (Supplementary Figure S3A a, available at Carcinogenesis Online). Four days seem to be optimal interval after transfection for efficient activation (Supplementary Figure S3A b, available at Carcinogenesis Online). To determine whether KLF4 is susceptible to RNAa in other renal cell lines, we transfected 786-0 and OS-RC-2 cells with dsKLF-496. Four days after transfection, dsKLF4-496 induced KLF4 mRNA expression by approximately 9.5- and 6.2-fold in 786-0 and OS-RC-2 cells, respectively (Supplementary Figure S3A c, available at Carcinogenesis Online). Consistent with mRNA induction, immunoblot analysis revealed that KLF4 protein levels were also elevated by dsKLF4-496 in each of the sensitive cell lines (Supplementary Figure S3A d, available at Carcinogenesis Online).

We detected the proliferation and colony formation after RNAa-mediated overexpression of KLF4 and the similar tumor suppressive

Fig. 3. Overexpression of KLF4 by lentiviral vector inhibits renal cancer cell growth and survival. (A) (a) CCK-8 Kit was utilized to quantify cell viability at each time point. Data are plotted as the mean ± SEM of two independent experiments; A (b) representative photographs of cell culture plates following staining for colony formation of ACHN, 786-0 and HK-2 cells. (B) Flow cytometry data from ACHN and 786-0 cells were analyzed to determine cell cycle distribution. (C) Terminal deoxynucleotidyl transferase-mediated dUTP nick end labeling assay was also performed on ACHN cell after treating with phosphate-buffered saline, lenti-NC or lenti-KLF4. (D) Cell cycle-related protein was determined by immunoblot analysis. GAPDH was used as control. *P < 0.05; **P < 0.01.
Supplementary Figure S3B and C, available at Carcinogenesis Online. RNAa of KLF4 gene caused cell colony formation inhibition, migration and invasion suppression and modulation of several downstream target genes in a pattern similar to lentiviral vector-mediated overexpression of KLF4: (i) increased levels of p21, p27 and p57 and (ii) declines in CCND1, CCNB1, BUB1B, CENPE and MAD2L1 (Supplementary Figure S3D). RNAa-mediated overexpression of KLF4 recapitulated the results obtained by lentiviral-based KLF4 induction. These data validated RNAas as a useful technique to study and confirmed KLF4 functions as a putative tumor suppressor in renal cancers cells.

Discussion

In this study, we were the first to have investigated the expression and potential tumor suppressive role of KLF4 in human renal cancer development and progression. First, we discovered the distinct KLF4 expression patterns in cancer adjacent normal renal tissues and renal cancer tissues. Specifically, we observed strong or weak KLF4 expression mainly in the cytoplasm of the renal proximal and distal convoluted tubule epithelial cells but almost no expression in glomerulus cells, suggesting that KLF4 is selectively expressed in epithelium but not in endothelium. In contrast, we observed a substantially decreased or lost KLF4 expression in various pathologic types of renal cancer tissues and cancer cell lines and were significantly associated with progression after radical nephrectomy. Second, mechanism study showed that KLF4 promoter hypermethylation was found in all five renal cancer cell lines and a subset of renal cancer tissues, which played an important role in KLF4 downregulation and also showed significant prognostic value in renal cancer progression. Third, restoration of KLF4 expression significantly induced renal cancer cell apoptosis, inhibited cancer cell growth, migration and invasion in vitro. KLF4 downregulation promotes renal cancer cell growth, migration and invasion. Finally, KLF4 overexpression also inhibited tumorigenicity and metastasis in animal models. Therefore, we offered the first clinical and casual evidence and potential mechanisms of KLF4 alterations in renal cell cancer and demonstrated that KLF4 plays a tumor suppressive role in renal cancer development and progression, and KLF4 pathway may be a valuable prognostic biomarker for renal clear cell cancer progression and relatively shorter survival period, or may be a potential target for renal cancer treatment.

For the typical CpG islands in promoter region and evidence of hypermethylation in gastric cancer, colon cancer and medulloblastoma tissues, KLF4 promoter methylation was also detected in renal cancer tissues and cell lines. Correlation analysis also showed a significant association between KLF4 promoter CpG unit methylation ratio and corresponding KLF4 mRNA level. Demethylation treatment induced robust re-expression in cancer cell lines at different degrees. This suggested that regional hypermethylation plays an important role in the alteration of KLF4 gene expression in carcinoma development and progression. Besides the hypermethylation, other epigenetic and genetic changes, such as hemizygous deletion, point mutation and upstream regulator genes such as APC and p53, may also contribute to the downexpression of KLF4 in renal cancer (4,5,27).

We also found that KLF4 decrease and promoter hypermethylation were significantly correlated with an advanced cancer biology, which was indicated by high pathologic grade, lymph node metastasis as well as poor OS and DFS rates. Therefore, we provide the first evidence that KLF4 can be used as a novel biomarker for outcome in patients with renal clear cell cancer after radical nephrectomy. This implies the possibilities that at the time of initial diagnosis of renal clear cell cancer, KLF4 expression or promoter methylation status can be used not only to design optimal, individualized treatment but also to distinguish patients who would benefit from close monitoring after surgery from those who would not.

KLF4 has been recognized as a tumor suppressor in several types of cancer and, more recently, has been shown to suppress the proliferation, migration and invasion of pancreatic (6), esophageal (7), prostate (9) and hepatocellular cancer cells (10), suggesting its potential role as a metastasis suppressor. However, there is no evidence of the potential contribution of altered KLF4 expression to renal cancer development and progression. In this study, clinical and experimental evidence strongly suggests that KLF4 functions as a tumor suppressor in human renal cancer and that its alteration of expression plays an important role in renal cancer development and progression.
Although little is known about the mechanisms by which KLF4 may influence cancer development and progression, there are a few lines of evidence indicating that altered KLF4 expression affects cell cycle, induces apoptosis and inhibits cell migration and invasion. In this study, we found that restoration or an increase of KLF4 expression significantly suppresses cell proliferation and colony formation by regulating several downstream cell cycle-related genes and mitotic checkpoints. KLF4 overexpression also significantly induces apoptosis of renal cancer cells, and which seemed to be consistent with those of previous studies on bladder cancer, colon cancer and leukemia cells. Tumorigenicity inhibition in nude mice confirmed the tumor suppressive role of KLF4 in renal cancer.

KLF4 has been shown to inhibit migration and invasion in several cancer models. Increased KLF4 expression also inhibited renal cancer cell migration and invasion. Recently, EMT became a focus point in cancer development and metastasis, and KLF4 is a transcriptional activator of E-cadherin and suppressor of EMT in breast cancer and activates an epithelial gene expression program during generation of induced pluripotent stem cells from mouse fibroblasts, in part, through repression of Snail. Besides, in the breast cancer study, few report has demonstrated the relationship between KLF4 and EMT in other malignancies. In this study, we did further investigation of metastasis inhibition mechanisms in relation to EMT factors. We detected EMT-related genes expression profiling demonstrated that KLF4 overexpression significantly induces epithelial marker E-cadherin expression and suppress mesenchymal marker N-cadherin, Snail and Slug.

RNAa-mediated overexpression described in our previous studies is a novel approach to upregulate genes in human cells. Compared with traditional vector-based approach, which are frequently created from complementary DNA libraries or amplicons and require ectopic expression from an exogenous construct, RNAa has the unique ability to enhance endogenous transcription of a targeted gene, which contain introns or untranslated region elements that can have pivotal effects on endogenous gene function. In support, downstream gene showed a greater sensitivity for RNAa than appeared to facilitate a greater measurable effect on downstream gene expression than viral transduction, whereas KLF4 overexpression efficiency in RNAa was lower than viral transduction result from the inherent differences between them.

In conclusion, both mRNA and protein expression profiling demonstrated that KLF4 expression was frequently decreased or lost in renal cancer tissues and cancer cell lines. By using viral-based and
RNAa-mediated overexpression and siRNA of KLF4, we revealed that KLF4 inhibits renal cancer cell growth/survival by modulating the expression of key downstream genes. KLF4 also suppressed renal cell migration/invasion through the suppression of EMT. KLF4 suppressed renal tumorigenicity and metastasis in vivo. This study reveals that KLF4 is downregulated and functions as a putative tumor suppressor in renal cancer genesis and progression, and demonstrates the applicability of RNAa to study gene function. KLF4 pathway is a potential therapeutic target for human renal cancer treatment.

Supplementary material
Supplementary Materials and Methods, Tables S1-S4 and Figures S1-S6 can be found at http://carcin.oxfordjournals.org/

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References

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