ORIGINAL ARTICLE

GATA-3 is down-regulated in patients with clear cell renal carcinoma☆

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KEYWORDS
Renal cancer; Kidney; GATA binding protein 3; Transcription factor

Abstract
Background: GATA-3 is a transcription factor involved in human growth and development. Recent studies found its association with breast cancer, however, its expression profile in renal cell carcinoma (RCC) has not been investigated.

Material and method: The study included 35 patients submitted to radical nephrectomy with confirmed pathological diagnosis of RCC. Normal control kidney tissues were obtained from 25 living kidney donors and tissues were biopsied before implantation. The majority of RCC samples were diagnosed as clear cell renal cell carcinoma (94.3%) except for 1 case of papillary RCC and 1 case of collecting duct carcinoma. GATA-3 expression was evaluated by quantitative PCR and Western blotting (WB) in RCC samples and normal kidneys respectively, immunohistochemical staining was performed as well. Meanwhile, the GATA-3 expression in two cancer cell lines (786-O, ACHN) and normal kidney epithelial cells (HK-2) was detected by PCR and WB. In addition, renal cancer cells and HK-2 cells were cultivated and detected by confocal microscopy for the exact intra-cellular localization of GATA-3.

Results: Data showed a significant down-regulation of GATA-3 expression present in neoplastic tissues compared with normal tissues; similarly, GATA-3 was significantly attenuated in all renal cancer cell lines compared with normal HK-2 cells. Confocal displayed a strong cytoplasmic immuno-fluorescence activity of GATA-3 with peri-nuclear zone in HK-2, whereas the intensity in cancer cells was markedly weaker than that of HK-2.

Conclusions: In summary, our present study clarifies that the aberrant expression profile of GATA-3 in human RCC is possibly involved with tumorigenesis, and the complicated mechanism is worthy of further investigation.

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Abbreviations: RCC, Renal cell carcinoma; ccRCC, clear cell renal cell carcinoma; PCR, polymerase chain reaction; 786-O, cultivated cells of human renal cell cancer; ACHN, human kidney adenocarcinoma cell line; HK-2, an immortalized proximal tubule epithelial cell line from normal adult human kidney; IHC, Immunohistochemistry; TBS, trisbuffered-saline; RT, room temperature; DAPI, 4,6-Diamidino-2-PH enylidole dihydrochloride; Caki-1, isolated cells from a skin metastasis of renal clear cell carcinoma; GDNF, glial cell line-derivedneurotrophic factor; Ret, the tyrosine kinase receptor ‘REarranged during Transfection’.


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Gata-3 es subregulado en pacientes con carcinoma renal de células claras

Resumen
Antecedentes: GATA-3 es un factor de transcripción implicado en el crecimiento y desarrollo humano. Estudios recientes han demostrado su asociación con el cáncer de mama, sin embargo, su perfil de expresión en el carcinoma de células renales (CCR) no se ha investigado.

Material y método: El estudio incluyó a 35 pacientes sometidos a nefrectomía radical con diagnóstico patológico confirmado de CCR. Se obtuvieron tejidos renales de control normales de 25 donantes vivos de riñón y los tejidos se biopsiaron antes de la implantación. La mayoría de las muestras de CCR fueron diagnosticadas como carcinoma renal de células claras (94,3%), excepto para un caso de CCR papilar y otro caso de carcinoma del sistema colector. La expresión de GATA-3 se evaluó por PCR cuantitativa y Western blot (WB) en muestras de CCR y riñones normales, respectivamente; también se realizó tinción inmunohistoquímica. Mientras tanto, la expresión de GATA-3 en 2 líneas celulares de cáncer (786-O, ACHN) y células epiteliales de riñón normales (HK-2) se deteció por PCR y WB. Además, las células renales cancerosas y las células HK-2 se cultivaron y se detectaron por microscopia confocal para la exacta localización intracelular de GATA-3.

Resultados: Los datos mostraron una importante subregulación de la expresión de GATA-3 presente en los tejidos neoplásicos en comparación con los tejidos normales; de manera similar, GATA-3 se atenuó significativamente en todas las líneas celulares de cáncer renal en comparación con las células HK-2 normales. La confocal mostró una fuerte actividad citoplasmática inmunofluorescente de GATA-3 con la zona perinuclear en HK-2, mientras que la intensidad en las células cancerosas fue notablemente más débil que la de HK-2.

Conclusiones: En resumen, nuestro estudio aclara que el perfil de expresión aberrante de GATA-3 en el CCR humano está posiblemente involucrado en la tumorogénesis, y el complicado mecanismo es digno de mayor investigación.

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Introduction

Renal cell carcinoma (RCC) is the most lethal urologic tumor and the sixth leading cause of cancer deaths in Western countries. Each year, around 200,000 patients are diagnosed with this malignancy resulting in approximately 100,000 deaths, and its incidence is increasing steadily in recent years.\(^1\)\(^2\) RCC is represented by 80% by clear cell RCC (ccRCC), originating from the renal proximal tubule.\(^3\)

The GATA family of transcription factors is involved in several aspects of developmental biology and cell differentiation. The GATA family name is derived from their ability to bind to the consensus DNA sequence (A/T) GATA (A/G). The DNA-binding domain consists of 1 or 2 zinc-finger domains with a highly conserved basic domain. Six GATA transcription factors have been identified in vertebrates. GATA-1 and -2 play key roles in erythroid and myeloid lineages. GATA-4 and -5 participate in cardiac development and myocyte hypertrophy. GATA-4, -5, and -6 are also involved in smooth muscle differentiation and endoderm formation. GATA-3 plays an important role in the development of T cells,\(^4\) the sympathetic nervous system\(^5\) and in the embryogenesis of kidney, it served as a key regulator of nephric morphogenesis and guidance in the pro/mesonephric kidney.\(^6\) There is evidence that GATA-3 plays a fundamental role in the process of tumor development,\(^1\)\(^2\)\(^3\) however, there is no evidence about GATA-3 profiling in the renal cell carcinoma.

The objective of the present study was to analyze GATA-3 expression in the RCC disease compared with the normal kidney tissues, and also to investigate the expression profile of GATA-3 in different renal cancer cell lines.

Methods

Patients

Two kidney samples were collected for analysis from 35 RCC patients admitted to Tongji University Affiliated Tenth hospital; respectively, tumor and control samples that were frozen in liquid nitrogen for RNA extraction and GATA-3 immuno-histochemical staining. A control cohort of 25 normal kidney tissues were obtained from living donor nephrectomy, we performed protocol kidney biopsies during kidney perfusion and trimming process before transplantation. All the urine samples from the control cohorts demonstrated no abnormality and their kidney pathology profile were free of renal malignancies.

All study protocols were approved by the Hospital Ethics Committee and we have obtained the permission of Institutional Review Board for working with the human tissues 15 years ago under the supervision of Tongji University medical school. All patients were fully informed and signed information-consent-form before operation and tissue-harvesting practice.

RNA extraction and quantitative PCR

Tissue samples were manually ground into a fine powder in liquid nitrogen with mortar and pestle. Total RNA was extracted from tissue samples using the single-step guanidinium isothiocyanate method. The integrity of all mRNA preparations was confirmed by gel electrophoresis. At sub-confluence, pancreatic cancer cell lines were rinsed twice
in ice-cold PBS and solubilized in MagNA Pure lysis buffer. mRNA was prepared by automated isolation using a MagNA Pure LC instrument and an isolation kit I (Roche Applied Science; Mannheim, Germany). cDNA was prepared using 1st Strand cDNA Synthesis Kit for RT-PCR (AMV) according to the manufacturer's instructions (Roche Applied Science). Reverse transcriptase products were amplified by real-time polymerase chain reaction (PCR) with a LightCycler Fast-Start DNA SYBR Green kit, as described previously (Roche Applied Science). The number of specific transcripts (GATA-3) was normalized to housekeeping genes (18s) before graph plotting (all primers obtained from Search-LC; Heidelberg, Germany).

**GATA-3 immunohistochemical staining**

Immunohistochemical staining for GATA-3 on paraffin sections was performed by an alkaline phosphatase antialkaline phosphatase (APAAP) procedure using polyclonal rabbit anti-human GATA-3 antibodies as primary reagent (dilution 1:20; Santa Cruz Biotechnology). Paraffin sections of 1 mm thickness were cut and dewaxed, followed by microwave pretreatment in 0.01 M citrate-buffer, pH 8.0, for 15 min at 600 W. Primary antibodies were diluted in Trisbuffered-saline (TBS) with an additional 2.5 mg/ml of normal human immunoglobulins (g-venin; Behring, Marburg, Germany) and 0.2% BSA (Sigma) and then incubated overnight at room temperature (RT). Parallel incubations with normal rabbit immunoglobulins (DAKO; Hamburg, Germany) instead of the GATA-3 antibodies served as negative controls. After washing, polyclonal mouse anti-rabbit antibodies (1:50; DAKO) were incubated for 30 min in TBS/BSA, followed by polyclonal rabbit anti-mouse-bridging antibodies (1:25; DAKO) and the APAAP-complex (1:50; DAKO), which were added in two different incubation cycles of, respectively, 30 and 15 min. Naphthol-AS-biphosphate (Sigma) and new fuchsin (Merck; Darmstadt, Germany) were used as the substrate for alkaline phosphatase. Finally, the sections were counterstained with hematoxylin (Merck) and mounted.

**Western blot analysis**

Proteins from normal control kidneys and patients-oriented renal cancer tissues were homogenized in RIPA lysis buffer (Tris–HCl 50 mM, pH 6.8, NaCl 150 mM, 0.1% SDS, 1% NP-40, 0.5% Na-deoxycholate) and supplemented with 500 mM PMSF and protease inhibitor (Sigma; Deisenhofen, Germany). The lysate was collected and centrifuged at 4 °C for 5 min at 14,000 rpm to remove the insoluble material. The protein concentration of the supernatant was measured by spectrophotometry using the BCA protein assay (Pierce Biotechnology; Rockford, IL). Equal amounts of protein extracts were fractionated on a 10% Bis–Tris gel (Invitrogen; Karlsruhe, Germany). After electrophoresis, proteins were transferred to polyvinylidene difluoride membranes. Ponceau S (Sigma) staining of the membrane was used to ensure equal loading. For immunoblotting, unspecific binding was blocked with 5% non-fat milk, and the membranes were then incubated with the primary rabbit polyclonal anti-GATA-3 antibody (Santa Cruz Biotechnology; Santa Cruz, CA) in a dilution of 1:200 in 5% non-fat milk TBS-Tween at 4 °C overnight. Subsequently, the membranes were washed with TBS-Tween buffer (5% nonfat milk in 20 mM Tris–HCl, 150 mM NaCl, 0.1% Tween 20) and incubated with horseradish peroxidase-conjugated goat anti-rabbit IgG (Bio-Rad Laboratories; Hercules, CA) at RT for 1 h. After washing with TBS-Tween buffer, the signal detection was performed with an enhanced chemiluminescence reaction (ECL Western blotting detection; Amersham Life Science, Amersham, UK).

**Confocal microscopy**

786-O and ACHN cancer cells were grown for 24 h on Teflon-covered slides (Menzel-Gläser; Braunschweig, Germany) at a confluency of 5000 cells/well. The immortalized proximal tubule epithelial cell line from normal adult human kidney HK-2 cell was used as positive control for GATA-3 nuclear localization. After rinsing in PBS, cells were fixed in 4% formalin in PBS for 1 h at 4 °C, followed by ice-cold methanol for 5 min, and acetone for 2 min. Thereafter, slides were permeabilized by 0.05% Saponin for 20 min at RT. After blocking the slides in normal goat serum (KPL; Gaithersburg, MD) for 1 h at RT, the primary polyclonal rabbit anti-GATA-3 antibody (Santa Cruz Biotechnology) was added in a dilution of 1:50. Incubation was performed overnight at 4 °C. After thorough rinsing in PBS containing 0.1% BSA-C (Aurion; Wageningen, Netherlands) for 3 × 5 min, slides were incubated with goat anti-rabbit secondary antibody, conjugated with fluorescein dye Cy3 (Biomedica Corporation; Foster City, CA). Thereafter, for nuclear visualization, slides were incubated with DAPI (Sigma–Aldrich, MO, US) at a dilution of 1:1000 for 10 min. Slides were mounted and visualization was performed by scanning the slides with confocal microscopy (Leica GmbH; Wetzlar, Germany).

**Statistical analysis**

Because the hypothesis of "normal distribution of data" was rejected by the Shapiro–Wilks test, non-parametric statistical tests were used. The Mann–Whitney test was
used for comparison of mRNA expression levels between groups, and the Spearman’s rho coefficient test for evaluation of correlation between variables. \( p < 0.05 \) was defined as significant. All statistical analyses were done with SPSS 12.0 (SPSS Inc., Chicago, IL, USA).

### Results

#### Clinical and histological features

Table 1 shows that RCC tissues used in the present study represented initial stages of the disease (T1–T3), 91.4% of the samples were classified as clinical stages I and II, whereas 33 samples (94.3%) represented histological classification as renal clear cell tumors, 1 sample (2.9%) represented papillary renal tumors and 1 sample (2.9%) represented renal collecting duct carcinoma. Furthermore, the samples also represented low metastasis frequency (8.6%), low Fuhrman grade and just two cases of Gerota’s fascia invasion (5.7%) that correlated with more aggressive RCC tumors.

<table>
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<th>Case</th>
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<th>Clinical stage</th>
<th>Pathological stage</th>
<th>Histology</th>
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<th>Metastasis</th>
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</table>

#### Expression of GATA-3 mRNA in RCC tissue and cancer cells

As seen in Fig. 1, there is a significant less expression of GATA-3 mRNA in RCC tissues (0.24 ± 0.121) compared to normal-control kidney samples (0.99 ± 0.02; \( p < 0.05 \), Student’s t-test). Meanwhile, the expression of GATA-3 in 786-O cells and ACHN cells were both significantly lower than HK-2 cells (0.42 ± 0.09 vs. 0.99 ± 0.02 and 0.49 ± 0.10 vs. 1.01 ± 0.02; respectively, \( p < 0.05 \), Student’s t-test).

#### IHC for GATA-3

We immunohistochemically investigated the expression of GATA-3 in 35 RCC patients as well as 25 normal control kidneys. GATA-3 was positively stained predominantly in the peri-tubular area in normal kidney, the GATA-3-labeled cells were homogenously distributed in the brush-border area and interstitial space, however, the distribution pattern in the...
GATA-3 is down-regulated in patients with clear cell renal carcinoma

Figure 2  GATA-3 expression in normal kidney tissues (A, C, E) and in RCC samples (B, D, F). A, C, E: Strong staining of GATA-3 in healthy kidney; B, D, F: weak staining of GATA-3 in clear cell carcinoma. Original magnification: 200× (A - D); 100× (E and F).

RCC tissue was different, both the intensity and the density of GATA-3 expression was significantly weaker than that of normal kidney, as demonstrated in Fig. 2.

The German immunoreactive score was used to assess the GATA-3 expression. In detail, by multiplying the percentage of immunoreactive cells (0%, 0; 1–10%, 1; 11–50%, 2; 51–80%, 3; 81–100%, 4) by staining intensity (negative, 0; weak, 1; moderate, 2; strong, 3), we get the immunoreactive score for each section. The immunohistochemical scores (ranging from 0 to 12) were considered negative (0, 0–1), weakly positive (1+, 2–4), moderately positive (2+, 6–8), and strongly positive (3+, 9–12) for GATA-3 expression. And

| Table 2  IHC staining for GATA-3 in normal kidney and renal cell carcinoma. |
|---------------------------------|-----------------|-----------------|-----------------|
|                                 | Normal kidney (n = 25) | Renal cell carcinoma (n = 35) | *p* value      |
| Immunohistochemical score       | 9.0 ± 2.5         | 6.3 ± 3.1        | <0.05          |
| Positivity grading              | 2.8 ± 1.1         | 0.8 ± 0.5        | <0.05          |
Figure 3  A. Down-regulation of GATA-3 protein levels in human renal cell cancer samples. Equal amounts of total protein were fractionated on a 10% Bis-Tris Gel, blotted onto polyvinylidene difluoride membranes, and incubated with rabbit anti-human GATA-3 polyclonal antibody. Antigen-antibody interactions were visualized by ECL Western blotting detection reagents. (B) Abundant GATA-3 protein was detected in HK-2 cells, however, it was significantly down-regulated in three renal cancer cell lines: ACHN, Caki-1 and 786-O. Protein was extracted from three cancer cell lines and normal kidney HK-2 cells. Equal amounts of total protein were fractionated on a 10% Bis-Tris Gel, blotted onto polyvinylidene difluoride membranes, and incubated with rabbit anti-human GATA-3 polyclonal antibody. Antigen-antibody interactions were visualized by ECL Western blotting detection reagents. N: normal kidney; RCC: renal cell cancer.

the comparison of GATA-3 expression of RCC and normal kidney was listed in Table 2.

Western blot analysis

As shown in Fig. 3A, analysis of the GATA-3 protein content of same tissues revealed similar results: in normal kidney tissue samples, GATA-3 was detectable, however, in RCC tissues, it was below detection level, and this is independent of tumor stage (data not shown).

Equal amounts of total protein were processed and the expression of GATA-3 was significantly deficient in the three classical renal cancer cell lines (786-O, ACHN and caki-1) compared with normal HK-2 kidney cell (Fig. 3B).

Cytoplasmic localization of GATA-3 in renal cancer cells

To further determine the exact localization of GATA-3 in renal cancer cells, 786-O, ACHN and HK-2 cell lines were detected by confocal microscopy using double staining with the nuclear stain DAPI. All cell lines demonstrated a cytoplasmic immuno-fluorescence activity of GATA-3 with marked peri-nuclear zone, whereas the intensity of GATA-3 in 786-O and ACHN was slightly weaker than in HK-2 cells. In all cell lines, no GATA-3 immunoreactivity could be detected in the nucleus (Fig. 4 Fig. 4).

Discussion

GATA-3, a crucial transcription factor in T-cell development and differentiation, has been recently shown variable expression pattern in different human cancer studies; however, the presumed role of GATA-3 in the etiology of cancer remains unclear. The present study demonstrated that GATA-3 was down-regulated in the renal cell cancer, and explored available evidence of intra-cellular distribution of GATA-3 in two classical RCC cancer cells, for the first time, predicted the expression profile of GATA-3 in human renal cell cancer.

GATA-3 is a highly conserved protein that plays critical roles in the development and cellular differentiation. Grote et al. proposed that GATA-3 is a key regulator of nephric duct morphogenesis and guidance in the pro/mesonephric kidney. In detail, the nephric (Wolffian) duct, which is a central constituent of both structures, elongates caudally along a stereotypical path to reach the hindlimb level where it induces metanephros (adult kidney) formation, while the remaining duct gives rise to the male genital tract (epididymis, vas deferens). During mesonephric development, GATA-3 is expressed in the Wolffian duct and in its derivatives but not in the mesonephric blastema. At the metanephric stage, GATA-3 is expressed in the excretory system and in mesangial cells but not in other structures derived from the metanephric mesenchyme. In a cDNA microarray screen for genes specifically expressed in the pro/mesonephros and
regulated by Pax proteins, GATA-3, a transcription factor gene was identified to be associated with hypoparathyroidism, deafness and renal anomaly (HDR) syndrome.\textsuperscript{12} Inactivation of Gata3 by insertion of an Ires-GFP reporter gene resulted in a massive increase in nephric duct cellularity, which was accompanied by enhanced cell proliferation and aberrant elongation of the nephric duct. Developmental biologists gave us convincible clues that at molecular level, the nephric duct of GATA-3 knockout embryos is characterized by the loss of Ret expression and signaling, which may contribute to the guidance defect of the nephric duct.\textsuperscript{13} As reported earlier, the transcripts of the GDNF receptor gene Ret, is an essential component of the GDNF signaling pathway involved in ureteric bud formation and nephric duct guidance.\textsuperscript{14,15} These data corroborate with our results showing that in normal kidney epithelial HK-2 cells, GATA-3 seems like "house-keeping" gene and it is strongly expressed in the cytoplasm. However, in the cases of RCC, the expression of GATA-3 was either significantly down-regulated or even absent, which suggested that high expression of GATA-3 in tubular epithelial cells means "normal", and a loss of GATA-3 expression might contribute to tumorigenesis.

Figure 4 Localization of GATA-3 in different cell lines. Confocal microscopy exposed GATA-3 immunoreactivity in the cytoplasm of two renal cancer cell lines (B) 786-O, (E) ACHN and in normal adult kidney cells (H) HK-2. (A) DAPI in 786-O, (D) DAPI in ACHN, (G) DAPI in HK-2; (C) Merge in 786-O, (F) Merge in ACHN, (I) Merge in HK-2. (A–I) Original magnification 160×. (B, E, H) All cell lines demonstrated a cytoplasmic immuno-fluorescence activity of GATA-3 with marked peri-nuclear zone, whereas the intensity of GATA-3 in 786-O and ACHN was slightly weaker than in HK-2 cells.
The latest bioinformatic analysis shows us the novel biological pathway signature and cellular differentiation program in RCC. Tun et al. performed gene expression profiling of early-stage RCC and patient-matched normal renal tissue using Affymetrix HG-U133a and HG-U133b GeneChips, and found that RCC is characterized by a lack of epithelial differentiation, mesenchymal/adipogenic transdifferentiation, and pluripotent mesenchymal stem cell-like differentiation capacity in vitro. Literature reported that GATA-3 serves as an inhibitor in adipocyte differentiation, and whether GATA-3 down-regulation is related to RCC development via interfering adipocyte transition, it is worthy of further investigation.\textsuperscript{17,18}

A limitation of our present study should be mentioned. Because of the fact that there are just one sample of the papillary renal tumor and one sample of the renal collecting duct carcinoma, no significant difference was observed between GATA-3 profile and histological classification for kidney cancer. Ultimately, emerging data illustrated the role of GATA-3 in the differentiation of the mammmary luminal cells in human breast cancer and growing body of evidence implicated the GATA family of transcription factors as key regulators of cell fate specification and maintenance.\textsuperscript{19,20} GATA-3 promotes the differentiation of luminal cells, while repressing other cell types such as adipocytes. A better understanding of how GATA-3 regulates luminal cell differentiation will be important in cancer therapy and shed further light on its role as a prognostic factor. GATA-3 defines a distinct class of cancer genes that are differentiation factors rather than conventional tumor suppressor genes, which affect the malignant phenotype by enforcing differentiation.\textsuperscript{21-23} In conclusion, our study evaluated the aberrant expression profile of GATA-3 in human RCC and this disturbance could give kidney cancer cells a distinct growth advantage and might be associated with dedifferentiation abnormality, which is beneficial to explore novel therapeutic strategies, especially for the challenging early indetectable and drug-resistant human RCC.

Conflict of interest
The authors declared no potential conflicts of interests with respect to the authorship and/or publication of this article.

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