

Clinical potential of *TCF21* methylation in the diagnosis of renal cell carcinoma

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Abstract. The aim of the present study was to investigate the clinical potential of transcription factor (*TCF*) 21 methylation in the diagnosis of renal cell carcinoma (RCC). *TCF21* methylation levels were quantified in renal tissues (55 cases of RCC tissue and 22 cases of normal tissue) and urine samples (33 cases of urine samples with RCC and 15 cases of normal urine samples) using pyrosequencing. Spearman's rank correlation coefficient was used to investigate the correlation between *TCF21* methylation levels and clinical parameters (gender, age, smoking history, Fuhrman grade and clinical stage). The receiver operating characteristic (ROC) curve was utilized to evaluate the accuracy of predictive diagnosis of RCC. *TCF21* methylation levels were significantly increased in RCC samples compared with normal renal tissues and urine samples. The Spearman's correlation analysis revealed that the *TCF21* methylation level was positively associated with age ($P=0.002$), smoking ($P=0.017$) and Fuhrman grade ($P=0.045$) in RCC tissues and was positively associated with tumor size ($P<0.001$), Fuhrman grade ($P=0.017$) and clinical stage ($P=0.017$) in urine samples. ROC curves revealed that the cut-off value, sensitivity and specificity were 23.61, 89.00 and 61.90%, respectively in tissue samples, and 26.84, 79 and 100%, respectively in urine samples. Furthermore, there were significant differences in the area under the curve between the tissue and urine samples ($P=0.004$). The results of the present study indicate that *TCF21* may be used as a biomarker

for diagnosing RCC, and *TCF21* methylation levels in urine samples may be a useful means of diagnosing RCC.

Introduction

Renal cell carcinoma (RCC) accounts for ~3% of malignant tumors in adults and >90% of neoplasms originating from the kidney (1,2). It has been reported that RCC is the ninth most common cancer worldwide, with approximately 337,860 new cases diagnosed in 2012 (3). The incidence of RCC differs geographically and the highest incidence occurs in developed countries (1). In addition, it possesses the highest mortality rate of the genitourinary cancers and up to 30% of patients exhibit metastases at the time of initial diagnosis (4). The increasing incidence and mortality predicts that RCC will continue to be a significant health burden in the future (5). Although a minority of RCC patients have a high risk of recurrence, RCC is largely curable by surgery if detected early (6). However, detection of cancerous cells at early stages is challenging due to the lack of early symptoms and incorrect distinction between benign and malignant masses through imaging or needle biopsies (7). Therefore, investigation of innovative noninvasive approaches allowing for early detection of RCC has been performed (8).

Previously, promoter hypermethylation involving DNA methylation of CpG islands has been considered to be a critical mechanism during cancer development (9-11). Aberrant DNA methylation in the regulatory region of cancer-associated genes has now been established as an alternative mechanism to heritably silence gene transcription (12,13). Transcription factor 21 (*TCF21*) is a validated target of aberrant promoter hypermethylation in cancer, and is crucial for the differentiation of epithelial cells adjacent to the mesenchyme (14). *TCF21* is a member of the basic helix-loop-helix transcription factor family and has a significant role in the regulation of cell differentiation and cell fate decisions during development of the lung, kidney and spleen (15). Furthermore, it has been considered to be a candidate tumor suppressor at 6q23-q24 that is epigenetically inactivated in several types of human cancer (16-18). A previous study reported that the methylation level of *TCF21* was markedly increased in patients with clear cell RCC (ccRCC) and was additionally an independent

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prognostic factor for poor survival (16). Costa *et al* (19) identified that *TCF21* was part of an innovative panel of biomarkers for simultaneous detection of bladder cancer, RCC and prostate cancer; however, to the best of our knowledge, correlations between *TCF21* methylation levels and clinical parameters have rarely been reported since then.

In the present study, the clinical potential of *TCF21* methylation in the diagnosis of RCC was investigated and the correlations between *TCF21* methylation levels and certain clinical parameters in renal tissues and urine samples were analyzed. The results of the present study indicate that detection of *TCF21* methylation may provide an effective novel method for diagnosing RCC.

Materials and methods

Patient and tumor sample collection. The present study was approved by the ethics committee of First Hospital of Quanzhou Affiliated Fujian Medical University (Quanzhou, China)(batch number, 20131016) and written informed consent was obtained from all participants. Between February 2011 and December 2013, 55 consecutive patients with RCC, including 30 men and 25 women, who had received treatment at the First Hospital of Quanzhou Affiliated Fujian Medical University were enrolled in the present study. Tumor samples were obtained from these patients subsequent to resection. Samples were instantly snap-frozen, stored at -80°C in liquid nitrogen and cut in a cryostat (Reichert Jung Cryocut 1800; Leica Microsystems, Inc., Buffalo Grove, IL, USA) for subsequent DNA extraction. The normal renal cell tissue samples from 22 RCC-free individuals were used as controls.

Urine sample collection and processing. First morning voided urine samples (1 sample per patient; 20-50 ml) were collected from 33 patients (28 males and 5 females) with RCC, who had been diagnosed and treated between February 2011 and December 2013 in the Department of Urology of First Hospital of Quanzhou Affiliated Fujian Medical University. Healthy individuals with no history of occupational exposure to carcinogens or personal/familial history of cancer were selected as the control group (n=15; 10 males and 5 females). Written informed consent was obtained from all subjects prior to enrolling into the study. The storage and processing procedures for urine samples were standardized. Briefly, samples were centrifuged at 1,776 x g for 10 min, and the pelleted urine sediment was rinsed with phosphate-buffered saline (Shanghai Qifa Biotechnology Co., Ltd., Shanghai, China) 2 times for 10 min each and stored at -80°C until required.

Isolation of nucleic acids and bisulfite treatment. DNA was extracted from the frozen tissue and urine samples using the AllPrep DNA Mini kit (Qiagen, Inc., Valencia, CA, USA) according to the manufacturer's protocol. The concentration and purity of DNA were assessed by determining the OD₂₆₀/OD₂₈₀ ratio using a Nanodrop 1000 spectrophotometer (Thermo Fisher Scientific, Inc., Waltham, MA, USA). DNA was treated by bisulfite using an EZ-96 DNA Methylation-Gold™ kit (Zymo Research, Irvine, CA, USA) according to the manufacturer's protocol, as well as the previously described protocol (20), and stored at -80°C until use.

Quantitative pyrosequencing methylation analysis. In order to detect the methylation level of the *TCF21* gene in urine and tissue samples, a total of 22 CpG loci of 3 fragments in the *TCF21* gene were selected for methylation. Polymerase chain reaction (PCR) and *TCF21* sequencing primers were designed with the PyroMark Assay Design 2.0 software (Qiagen, Inc.). The primers were as follows: Forward, TTAGTTAGGAGG GGAAGTAGGTTT; reverse, ACACCCAAAACAAA TAATCTTAAATCT; and sequencing, GGGGAAGTAGGT TTAG for *TCF21* 1; forward, AGTGTTTTAGGGGTTGTA GTTGTAGTTTA; reverse, CACACCCCACTCCCAAC; and sequencing, GTTGTAGTTGTAGTTTAGG *TCF21* 2; and forward, GGTGGAAGGTTTAGAAAGAGTTA; reverse, ACCACCTTCTCCCAACTA; and sequencing, GGAAGG TTTAGAAAGAGTTAA *TCF21* 3. PCR was performed with the HotStarTaq Master Mix kit (Qiagen, Inc.) according to the manufacturer's protocol, with 1 µl of bisulfate-converted DNA. PCR amplification was performed as follows: Denaturation at 95°C for 5 min, followed by 45 cycles at 95°C for 1 min, annealing at 36°C for 1 min and a final extension at 72°C for 5 min. Following PCR amplification, pyrosequencing was performed using a PyroMark Gold Q96 SQA Reagents kit (Qiagen, Inc.) and a PSQ96 HS DNA analyzing system (Qiagen, Inc.) according to the manufacturer's protocol. Pyro Q-CpG software version 1.0.9 (Qiagen, Inc.) was used to calculate the percentage of methylation w, as follows: Methylation (%) = methylated cytosine / total cytosine x 100.

Statistical analysis. Data are expressed as the mean ± standard deviation. Statistical analyses were performed using SPSS for Windows, version 20 (IBM SPSS, Armonk, NY, USA). Pairwise comparison utilized the Mann-Whitney U test. Spearman's rank correlation was employed in order to examine the association between multiple clinical parameters. A receiver operating characteristic (ROC) curve was constructed in order to evaluate the accuracy of the predictive model. P<0.05 was considered to indicate a statistically significant difference.

Results

Methylation level of the *TCF21* gene. The data analysis of the first fragment of *TCF21* is presented in Fig. 1. A total of 22 CpG sites of 3 fragments in the *TCF21* gene promoter in each sample were detected. The results revealed that there were significant differences in the methylation level of the first fragment in the cancer and normal tissues at 9 sites (sites 8-11, 14-16, 18-19). In addition, *TCF21* methylation levels were significantly increased in cancerous renal tissue and urine samples compared with normal renal tissue and urine samples.

Clinicopathological analysis of *TCF21* methylation level in RCC tissue. An analysis of *TCF21* methylation levels in RCC tissues in relation to various clinical parameters is presented in Fig. 2. The results of the analysis demonstrated that *TCF21* methylation levels were increased in male patients, patients aged ≥50 years, smokers, patients with tumor size ≥7 cm and clinical stage III or IV (21) compared with female patients, patients aged <50 years, nonsmokers, patients with tumor size <7 cm and Fuhrman grade I or II (22). The Spearman's correlation coefficient between *TCF21* methylation levels and clinical

Table I. Correlation between transcription factor 21 methylation levels and clinical parameters in renal cell carcinoma tissue.

Clinical parameter	P-value	r
Gender	0.227	0.166
Age	0.002	0.403
Tumor size	0.423	0.11
Smoking	0.017	0.321
Fuhrman grade	0.045	0.271
Clinical stage	0.057	0.258

Table II. Correlation between transcription factor 21 methylation levels and clinical parameters in urine samples with renal cell carcinoma.

Clinical parameter	P-value	r
Gender	0.43	0.142
Age	0.713	0.067
Tumor size	0.000	0.622
Smoking	0.204	0.227
Fuhrman grade	0.010	0.441
Clinical stage	0.017	0.411

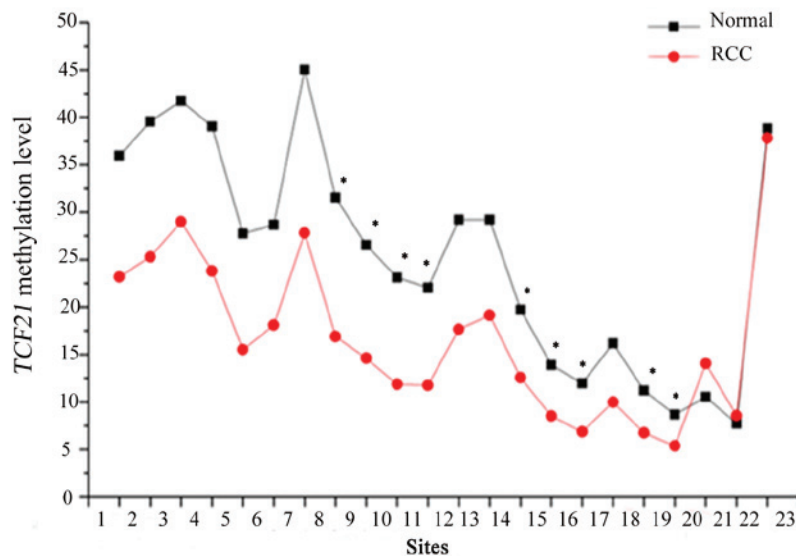


Figure 1. Data analysis of the first fragment of *TCF21*. The results revealed that there were significant differences between the cancer and normal tissues at 9 methylation sites (sites 8-11, 14-16 and 18-19) of the first fragment. *P<0.05 normal vs. control. *TCF21*, transcription factor 21; RCC, renal cell carcinoma.

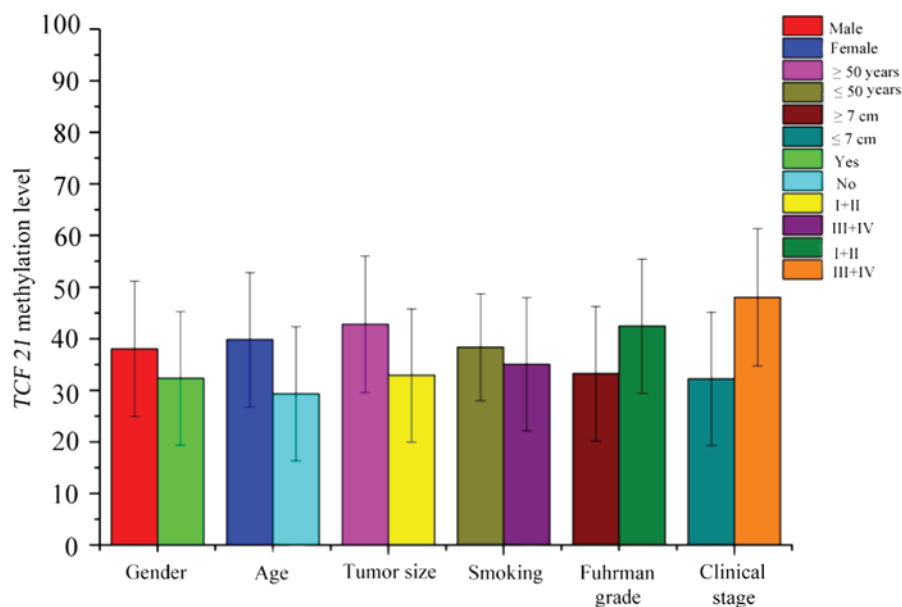


Figure 2. A comparison between *TCF21* methylation levels in RCC tissue and various clinical parameters. The results of the analysis demonstrated that *TCF21* methylation levels were increased in male patients, patients aged ≥50 years, smokers, patients with tumor size ≥7 cm and clinical stage III or IV. *TCF21*, transcription factor 21; RCC, renal cell carcinoma.

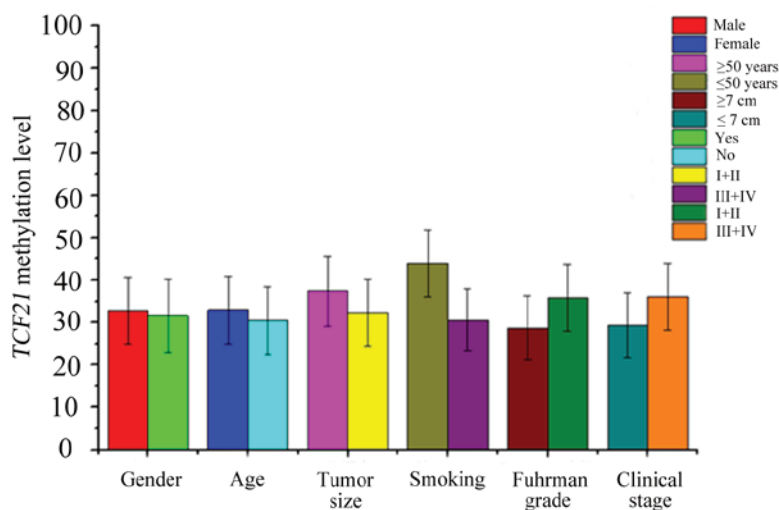


Figure 3. A comparison between *TCF21* methylation levels in urine samples with RCC and various clinical parameters. *TCF21* methylation levels were increased in male patients, patients aged ≥ 50 years, smokers, patients with tumor size ≥ 7 cm and clinical stage III or IV. *TCF21*, transcription factor 21; RCC, renal cell carcinoma.

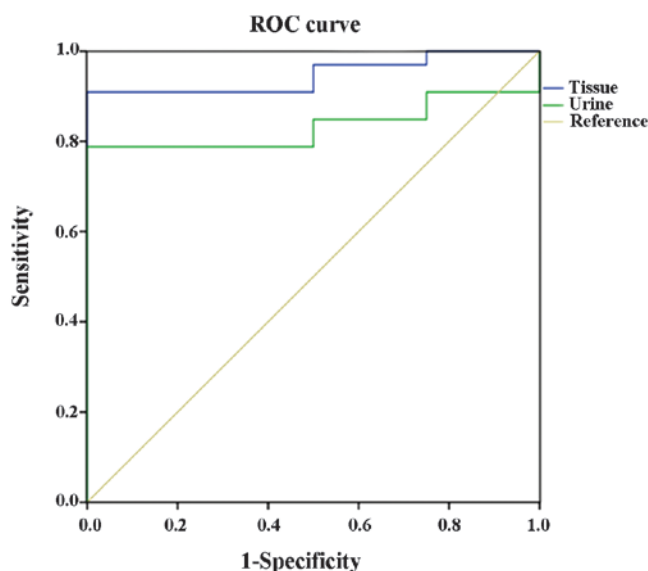


Figure 4. The ROC curve of *TCF21* methylation level in RCC tissue and urine samples. The diagnostic validity of the *TCF21* methylation level in RCC tissues was increased compared with RCC urine samples. ROC, receiver operating characteristic; *TCF21*, transcription factor 21; RCC, renal cell carcinoma.

parameters is summarized in Table I. The results demonstrated that *TCF21* methylation level was positively associated with age ($r=0.403$; $P=0.002$), smoking history ($r=0.321$; $P=0.017$) and Fuhrman grade ($r=0.271$; $P=0.045$). However, there were no significant differences for gender ($r=0.166$; $P=0.227$), tumor size ($r=0.110$; $P=0.423$) and clinical stage ($r=0.258$; $P=0.057$).

Clinicopathological analysis of *TCF21* methylation level in urine samples with RCC. A comparison between *TCF21* methylation levels in urine samples with RCC and various clinical parameters is presented in Fig. 3. Similarly, *TCF21* methylation levels of male patients, patients aged ≥ 50 years, smokers, patients with tumor size ≥ 7 cm and clinical stage III or IV were increased compared with female patients, patients

aged < 50 years, nonsmokers, patients with tumor size < 7 cm and Fuhrman grade I or II. Spearman's correlation analysis revealed that the *TCF21* methylation level was positively associated with tumor size ($r=0.622$; $P<0.001$), Fuhrman grade ($r=0.411$; $P=0.017$) and clinical stage ($r=0.411$; $P=0.017$). However, there were no significant differences for gender ($r=0.142$; $P=0.430$), age ($r=0.067$; $P=0.713$) and smoking history ($r=0.441$; $P=0.010$; Table II).

ROC curve of *TCF21* methylation level in RCC tissue and urine samples. The ROC curve of *TCF21* methylation level in RCC tissue and urine samples is presented in Fig. 4. In the ROC curve analysis of *TCF21* methylation level in RCC tissue, the cut-off value was 23.61% and the sensitivity and specificity were 89.00 and 61.90%, respectively for predicting RCC. The ROC curve analysis of *TCF21* methylation level in urine samples with RCC demonstrated a cut-off value of 26.84% and the sensitivity and specificity were 79.00 and 100.00%, respectively for predicting RCC. Furthermore, there were significant differences in the area under the curve between the tissue and urine samples ($P=0.004$), which may indicate that the diagnostic validity of the *TCF21* methylation level in RCC tissue was increased compared with urine samples with RCC.

Discussion

In the present study, the clinical potential of the observation of *TCF21* methylation for the diagnosis of RCC was investigated in tissue and urine samples. The results of the present study demonstrated that *TCF21* methylation levels were significantly increased in RCC tissue and urine samples compared with normal tissue and urine samples. Furthermore, *TCF21* methylation was additionally positively associated with age, smoking history and Fuhrman grade in RCC tissues and tumor size, Fuhrman grade and clinical stage in urine samples. In addition, the sensitivity was significantly increased in RCC tissue samples compared with urine samples. It was concluded that *TCF21* may be a useful biomarker for diagnosing RCC,

and *TCF21* methylation levels in urine samples may be a novel method of diagnosing RCC.

It is a challenging task to screen for the early stages of RCC. RCC tends to be clinically asymptomatic during its earliest stages; therefore, approximately 20-30% of cases are diagnosed at a locally advanced stage or with metastasis (23). Although noninvasive imaging (including computed tomography and ultrasonography) has been widely used in the detection of RCC, diagnostic challenges are arising due to problems with accurate discrimination of benign from malignant masses and accurate categorization (24). Thus, the development of more efficient and accurate detection of RCC at early stages is imperative. The role of epigenetic alterations and gene promoter hypermethylation in the early diagnosis of cancer is receiving increasing attention (7,25-28). An emerging class of cancer biomarkers, including prostaglandin-endoperoxide synthase 2, Ras association domain family 1 isoform A, retinoic acid receptor β and Kelch-like family member 35, has been previously reported to be methylated in RCC, with a sensitivity of 31-77% and specificity of 75-91% (29-32). In addition, Costa *et al* (19) demonstrated that protocadherin-17 and *TCF21* aberrant promoter methylation may have a critical role in urological cancers. Furthermore, Ye *et al* (16) indicated that *TCF21* may be a predictive biomarker of poor prognosis in patients with ccRCC. However, correlations between *TCF21* methylation levels and clinical parameters were not reported in this previous study (16).

TCF21 is a newly recognized target of aberrant promoter hypermethylation in malignancies, which encodes a basic helix-loop-helix transcription factor (33,34). It is involved in mesenchymal-epithelial cell transition and controls cell fate determination and tissue differentiation in the embryo (35). The present study investigated the clinical potential of *TCF21* methylation in the diagnosis of RCC, and additionally evaluated the correlations between *TCF21* methylation levels and certain clinical parameters in renal tissues and urine samples. The results of the present study revealed that *TCF21* methylation levels were significantly increased in RCC samples compared with those of normal renal tissues or urine samples, which was in line with the findings of previous studies (16,19). The Spearman's correlation coefficient revealed that the *TCF21* methylation level was positively associated with age, smoking history and Fuhrman grade in RCC tissues and associated with tumor size, Fuhrman grade and clinical stage in urine samples. The above results indicated that the *TCF21* methylation level was associated with the degree of malignancy and differentiation of RCC; the higher the level of *TCF21* methylation, the higher the degree of RCC malignancy. In addition, age and smoking history were associated with RCC; however, the mechanisms underlying these associations remain to be elucidated. The present study hypothesized that aging and smoking may act on the aberrant methylation of *TCF21*.

ROC curves revealed the cut-off value, sensitivity and specificity for predicting RCC were 23.61, 89.00 and 61.90%, respectively in tissue samples and were 26.84, 79.00 and 100.00%, respectively in urine samples. Significant differences were identified in the area under the curve between the tissue and urine samples. These results are similar to a previous finding which demonstrated a significant decrease in sensitivity in urine sample (36). One of the primary reasons for these observed results may be due to the varying amount

of neoplastic cells in tissues and urine samples. Therefore, the results might be improved by increase the volume of tested urine samples, because it seems to increase the content of DNA from RCC tumors. Despite these marked differences in the area under the curve, *TCF21* methylation levels in urine samples may be considered to be a novel and useful method of diagnosing RCC.

In conclusion, the results of the present study demonstrated that *TCF21* methylation levels were significantly increased in RCC tissue and urine samples compared with normal tissue and urine samples. *TCF21* may be utilized as a biomarker for the diagnosis of RCC and the detection of methylation levels in RCC urine samples may be a novel and efficient method of diagnosing RCC, due to the noninvasive nature of this method

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