Downregulation of FOXO3a promotes tumor metastasis and is associated with metastasis-free survival of patients with clear cell renal cell carcinoma

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Translational relevance

The mechanisms leading to clear cell renal cell carcinoma (ccRCC) metastases are still unclear. This study identified FOXO3a as a key factor in ccRCC metastasis using transcriptional profiling of ccRCC samples. FOXO3a was found to be attenuated in primary metastatic ccRCC samples. The loss of FOXO3a was an independent prognostic factor for ccRCC metastasis. Overexpression of FOXO3a inhibited renal cancer cell lines metastasis in vitro and in vivo. Moreover, FOXO3a downregulation was found to up-regulate SNAIL1, thereby promoting renal cancer cell metastasis. Taken together, FOXO3a could be considered as an independent prognostic factor for ccRCC metastasis and could be a marker of occult metastases.
Abstract

Purpose: To explore the mechanisms underlying clear cell renal cell carcinoma (ccRCC) metastasis using transcriptional profiling and bioinformatics analysis of ccRCC samples, and to elucidate the role of FOXO3a in ccRCC metastasis.

Experimental Design: Gene expression profiling was performed using four primary metastatic and five primary non-metastatic ccRCC samples. The mRNA and protein levels of FOXO3a in ccRCC samples were investigated by real-time RT-PCR and immunohistochemistry, respectively. The association between metastasis-free survival of ccRCC patients and FOXO3a mRNA levels was analyzed. Biological functions of FOXO3a in renal cancer cell lines were investigated. The influence of FOXO3a on tumor metastasis was also studied in vivo orthotopic xenograft tumor model. Finally, the mechanism by which FOXO3a attenuation could increase invasion and migration of tumor cells was explored.

Results: Bioinformatics analysis of the profiling data identified FOXO3a as a key factor in ccRCC metastasis. FOXO3a expression was decreased in primary metastatic ccRCC samples. Patients with low FOXO3a mRNA levels had poor metastasis-free survival ($P=0.003$). Knocking-down FOXO3a induced tumor cell invasion and migration in the non-metastatic ccRCC cells. Induced FOXO3a overexpression in SN12-PM6 cells could inhibit tumor metastasis in vivo. Downregulation of FOXO3a increased SNAIL1 expression, thereby activating the epithelial-mesenchymal transition (EMT) of RCC cell lines.

Conclusions: The loss of FOXO3a induced EMT of tumor cells by upregulating
SNAIL1, which promoted tumor cells metastasis in *vitro* and in *vivo*. Thus, FOXO3a could be considered as an independent prognostic factor in ccRCC metastasis and could be a marker of occult metastases.
Introduction

Renal cell carcinoma (RCC) represents approximately 2-3% of all human malignancies, and its most common histological subtype is the clear cell RCC (ccRCC), accounting for 80-90% of all RCCs (1). Localised and metastatic ccRCC considerably differ in terms of prognosis and therapeutic approach. Indeed, the 5-year cancer-specific survival is less than 27.1% in metastatic ccRCC, but exceeds 70% in non-metastatic ccRCC (2). Currently, the only method for diagnosing metastatic ccRCC is based on imagery techniques, because of the lack of reliable molecular markers that could be used for the early detection of metastatic ccRCC.

Previous studies showed that ccRCC was associated with the loss of von Hippel-Lindau (VHL) gene, and the subsequent accumulation of hypoxia-inducible factors (HIFs) (3-4). Moreover, the target genes of HIFs were found to promote ccRCC metastasis (5-6). However, a clinical study by Young et al. (7) showed that the VHL status was not correlated with poor disease outcomes. Therefore, other mechanisms beyond the VHL-HIFs signal might promote ccRCC metastasis and could be used as a metastases marker.

Therefore, the aim of the present study was to explore the underlying mechanisms of ccRCC metastasis using primary metastatic (PM) and primary non-metastatic (PN) patients. Patients in the PN cohort were followed up for 14.1-41.5 months to identify metastasis development. Bioinformatics analysis of the differentially expressed genes (DEGs) was performed and we focused on the biological functions of FOXO3a. We hypothesized that FOXO3a downregulation...
might promote ccRCC metastasis. To demonstrate this hypothesis, the functions and mechanisms of FOXO3a in tumor cell migration and invasion were investigated in a series of in vitro studies. The inhibitory effect of FOXO3a on tumor metastasis was also examined in vivo using an orthotopic tumor nude mice model.

**Materials and Methods**

*Human tissue specimens*

Tissue specimens were obtained from ccRCC patients who underwent partial or radical nephrectomy at the Chinese People’s Liberation Army (PLA) General Hospital (Beijing, China) from January 2009 to May 2012. Specimens were immediately snap-frozen in liquid nitrogen after surgical removal. The pathological diagnosis of ccRCC was confirmed by a pathologist. Patients with negative chest and abdomen CT or MRI and without symptoms of metastasis were defined as PN ccRCC, whereas patients with the appropriate evidence of metastatic lesions were identified as PM ccRCC. A total of 114 patients with PN ccRCC and 21 patients with PM ccRCC were included in the study. We also included 17 normal tissue samples adjacent to tumor from the PM group, and 21 normal tissue samples adjacent to tumor from the PN group. PN ccRCC patients were followed up for a median period of 28.2 months (from 14.1 to 41.5 months); these patients were under observation for postoperative metastasis or recurrence. The T stages of the specimens were determined according to the 2009 TNM staging classification system (8). Histopathological classification of tumor grades was performed according to Fuhrman (9). Patients' demographic data
are provided in Supplementary Table S1. This study was approved by the ethics committee of the Chinese PLA General Hospital. Written informed consent was obtained from all included patients.

Expression profiling

Total RNA from 4 PM and 5 PN ccRCC samples were prepared for oligonucleotide expression profiling. RNA samples were purified using the RNeasy Mini Kit (Qiagen, Venlo, Netherlands). RNA quality was assessed by denaturing gel electrophoresis. Subsequently, each RNA sample was processed using the GeneChip 3’ IVT Express Kit (901228, Affymetrix, Santa Clara, CA, USA). Products were hybridized to the GeneChip Human Genome U133 Plus 2.0 Array, washed, and stained using the GeneChip Hybridization, Wash, and Stain Kit (900720, Affymetrix). The array chips were then read using the GeneChip Scanner 3000 7G (Affymetrix). Data from these samples were extracted, normalized, and summarized using the Microarray Suite version 5.0 (MAS 5.0) algorithm implemented in the Affymetrix Expression Console software (10). We used a random variance model (RVM) to filter the DEGs because the RVM t-test performs much better than the standard t-test in identifying significantly different genes between groups (11). A p-value <0.05 was considered significantly different. Complete microarray data were deposited in the Gene Expression Omnibus website (accession code GSE47352).

Bioinformatics analysis
Gene Ontology (GO) analysis was performed on the Database for Annotation, Visualization and Integrated Discovery (DAVID) (12) to analyze the main function of the DEGs. We used the 1257 identified DEGs for the query, with the whole human genome as the background. The analysis was performed using the DAVID default parameters.

Pathway-analysis was performed based on the Kyoto Encyclopedia of Genes and Genomes (KEGG), Biocarta, and Reatome databases to identify the significantly enriched pathways of DEGs, as previously described (13). The Fisher’s exact test and χ² test were used to select the significant pathways.

Signal-net analysis was gene-gene interaction network constructed based on the DEGs dataset, as previously described (13). A repository of interactions derived from the KEGG database was based on the evidence of molecule interactions that were experimentally validated. The repository differed from the pathway maps on the KEGG database because this repository breaks down the protein interactions by pathway class and supplies the integrated protein interactions involved in multiple pathway categories. Therefore, we were able to search for DEGs using the repository. Two nodes were connected if their encoded gene products were directly connected or indirectly connected by a linker gene in the signal-net. The betweenness centrality of nodes in the signal-net was calculated as previously described (14). A higher betweenness centrality score of a gene indicated that it was connected to more other genes, implying it was more important in the signal-net.
Cell lines

The RCC cell lines 786-O, 769-P, A-498, Caki-2, Caki-1 and ACHN were purchased from the National Platform of Experimental Cell Resources for Sci-Tech (Beijing, China) in 2011. According to the American Type Culture Collection (ATCC, Manassas, VA, USA), the 786-O, 769-P, A-498 and Caki-2 cell lines were non-metastatic cells, while the Caki-1 and ACHN were metastatic cells. The SN12-PM6 cell line was kindly provided by Dr. XP Zhang of the Department of Urology, Union Hospital, Wuhan, China. Detailed information of cell lines and reagents is listed in the Supplementary Data.

Plasmid constructs and transfection

Transfections were performed using the jetPRIME reagent (Polyplus transfection, Illkirch, France), according to the manufacturer’s protocol. The expression vector pCR3.1-FOXO3a and the empty vector pCR3.1 were kindly provided by Christopher R. Herzog (Pennsylvania State University College of Medicine, Hershey, Pennsylvania) (15). The green fluorescence protein (GFP)-expression vectors pIRES2-EGFP (Clontech Laboratories Inc., Mountain View, CA, USA) and pGFP-V-RS (OriGene, Rockville, MD, USA) were used to establish the stably transfected cell lines. The coding domain sequence (CDS) of FOXO3a was amplified from the pCR3.1-FOXO3a by high-fidelity PCR amplification. The amplified products were cloned into pIRES2-EGFP. The short hairpin RNA (shRNA) kit targeting FOXO3a (TG320359) and the pGFP-V-RS Vector containing non-effective
29-mer scrambled shRNA (TR30013) were purchased from OriGene (Rockville, MD, USA).

The shRNA sequences inserted into pGFP-V-RS are shown in Supplementary Table S3. Transfections were conducted using the jetPRIME reagent as described above. At 24 hours after transfection, cells were subcultured in selective medium (2 μg/ml Puromycin for PEGF-V-RS or 800 μg/ml G418 for pIRES2-EGFP) for two weeks to select stable cell lines. All plasmids used were analyzed by direct sequence analysis to confirm the identities of the inserted sequences. The efficiency of transfection was evaluated by Western-blot assay and real-time RT-PCR.

The 1#sh-FOXO3a binding sites of pCR3.1-FOXO3a were synonymously mutated for the genetic rescue experiment. The 1# sh-FOXO3a sequence was: 5’-ATG GCA AGC ACA GAG TTG GAT GAA GTC CA-3’; and in the pCR3.1-FOXO3a-mut plasmid, the binding sites of 1# sh-FOXO3a were synonymously mutated to: 5’-ATG GCT AGT ACC GAA CTG GAC GAG GTG CA-3’. The pCR3.1-FOXO3a-mutated plasmid was transfected into 786-O cells transfected with the pGFP-V-RS-1#sh-FOXO3a plasmid.

Migration and invasion assays

Cellular migration and invasion assays were performed using a Boyden Chamber containing 24-well transwell plates (Corning Inc., Corning, NY, USA) with 8-μm pores on the membrane. All experiments were performed in duplicate and repeated three times.
For the migration assays, approximately $5 \times 10^4$ cells in 200 μl culture medium supplemented with 5% FBS were seeded into the upper chamber. The lower chamber was filled with the complete medium (with 10% FBS) as a chemoattractant. After 12 h of incubation at 37 °C in a 5% CO$_2$ atmosphere, the membranes containing the cells were fixed and stained with crystal violet. The lower surfaces of the membranes were photographed at 100× magnification. Five random fields were photographed for each chamber to determine the migration.

For the invasion assays, the membrane was coated with 50 μl 1:3 diluted matrigel (BD Biosciences). After the matrigel had solidified at 37 °C, approximately $1 \times 10^5$ cells in 200 μl culture medium supplemented with 1% FBS was seeded into the upper chamber, whereas the lower chamber was filled with the complete medium. Subsequently, the Boyden chamber was incubated at 37 °C with a 5% CO$_2$ atmosphere for 24 h. The subsequent staining and observation procedures were identical to those of the migration assays.

**In vivo orthotopic xenograft tumor model**

Four- to six-week old male BALB/c nude mice (Charles River Laboratories, Wilmington, MA, USA) with a mean body weight of 20 g were used to establish orthotopic xenograft tumor models. The mice were housed and fed under specific pathogen-free conditions. The animal models in this study were approved by the Animal Ethical Committee of the Chinese PLA General Hospital. The orthotopic xenograft tumor model was established as previously described (16). Specifically,
SN12-PM6 cells with the stably transfected IRES2-EGFP-FOXO3a or the respective empty vectors were harvested and washed twice with serum-free medium. Subsequently, the cells were suspended in PBS. Approximately $1 \times 10^6$ suspended cells were injected into the right kidney of the nude mouse. All mice were sacrificed at 7 weeks after the injection, and the kidneys and lungs were harvested. Immediately after the harvest of the xenografts and lung tissues, the GFP fluorescence of the samples was detected and analyzed in vitro using a molecular imaging system (NightOWL II LB 983, Berthold, Germany). The signal intensity of the GFP fluorescence from the lung tissues represented the amount of lung metastatic lesions. Subsequently, the xenografts and lung tissues were fixed in 10% neutral formalin and embedded in paraffin. Hematoxylin-eosin (HE) staining was performed on 4 μm sections by routine procedures to identify the tumor lesions.

**Statistical analysis**

Statistical analysis was performed using SPSS 12.0 (SPSS Inc., Chicago, IL, USA). Normally distributed data are expressed as mean ± standard deviation (SD) and comparisons were performed using Student’s t-tests. Abnormally distributed data are expressed as median with interquartile range and comparisons were performed using Mann-whitney U tests. The Kaplan-Meier and log-rank test were used for the metastasis-free survival analysis. The Cox regression analysis was used for univariate and/or multivariate survival analysis. Fisher’s exact test was used to evaluate the significance of differential metastasis rates in the nude mice orthotopic xenograft...
tumor models. The correlation of the FOXO3a and SNAIL1 mRNA levels was analyzed by linear regression analysis. $P$-values $<0.05$ were considered statistically significant.

Other materials and methods are listed in the Supplementary Data.

**Results**

**Bioinformatics analysis of the DEGs between PM and PN ccRCC tissues**

To explore the biological mechanism promoting ccRCC metastasis, we performed mRNA expression profiling of 4 PM and 5 PN ccRCC tissues. A total of 1257 DEGs were identified between the two groups. To further define the biological processes involving these DEGs, we performed gene ontology (GO) enrichment analysis using the DAVID database. Significantly enriched GO terms were clustered according to their functional annotations. Results from the database showed that among all 1257 DEGs, 863 genes were significantly enriched for 165 functional annotation clusters. The top 6 clusters had malignancy-related functions: regulation of gene expression, apoptosis, cell cycle, cell adhesion, cytoskeleton organization, and RNA transcription regulation (Supplementary Table S4). We further analyzed the pathways that were significantly enriched with DEGs, based on the KEGG database. These pathways and their interaction net are shown in Supplementary Figure S1. Similar to the GO analysis results, identified pathways included those related to cell adhesion, cell cycle, cytoskeleton regulation and apoptosis.

To obtain specific genes promoting ccRCC metastasis, we performed a
signal-net analysis to generate a network based on the experimentally validated interactions between molecules, with as many DEGs as possible (Supplementary Figure S2A and Supplementary Table S5). The central genes within the network were defined as key factors influencing other DEGs. Imbalance of the central genes was the underlying mechanisms promoting ccRCC metastasis. To obtain more reliable results, we combined several parameters, including the $P$-value, the fold change, and the betweenness centrality to screen the DEGs. The screening flow chart is shown in Supplementary Figure S2B. Therefore, LAMA4, JUN, ATR, FOXO3a, ETS1 and SORBS2 were considered as candidate central genes involved in ccRCC metastasis after the filtering process. The suggested functions of these DEGs were subsequently obtained by searching PubMed. Functional information of the six genes compelled us to focus on FOXO3a in a larger sample size.

**FOXO3a was downregulated in primary metastatic ccRCC tissues**

To further confirm the microarray results of FOXO3a expression, 21 PM and 42 PN ccRCC tissue samples from patients were matched 1:2 according to tumor size, patient age, and gender. Reduced mRNA levels of FOXO3a were found in the PM ccRCC samples, compared with the PN ccRCC samples (Figure 1A, $P<0.001$). Furthermore, immunohistochemistry demonstrated that the FOXO3a protein was mainly localized in the cell nucleus of PM, PN and normal renal tissues. Significantly decreased levels of this protein in the nucleus were detected in 10 PM ccRCC tissue samples, compared with 10 PN ccRCC tissues ($P=0.004$) and normal renal tissues.
Reduction of FOXO3a protein levels was also found in a panel of metastatic RCC cell lines (Caki-1, ACHN and SN12-PM6), compared with non-metastatic cell lines (786-O, 769-P, A-498 and Caki-2 cells) (Figure 1C). FOXO3a protein localizing to the nucleus was significantly reduced in the metastatic cell lines, according to immunofluorescence staining (Figure 1D).

**Decreased FOXO3a levels were an independent prognosis factor for ccRCC metastasis.**

To study the association between clinicopathological variables and the prognosis of PN ccRCC, we followed up 114 PN ccRCC patients for 14.1-41.5 months (median of 28.2 months). We first analyzed the relationship between FOXO3a levels and prognosis in PN ccRCC patients through univariate Cox regression analysis. Since an arbitrary threshold of low or high FOXO3a level may strongly influence the statistical outcomes, we ranked the entire PN ccRCC dataset based on the FOXO3a expression levels and subsequently selected nine thresholds from the 10th percentile to the 90th percentile. Univariate Cox regression analysis based on these thresholds was performed. The corresponding *P*-values of these thresholds are shown in Figure 1E. Except for the extremely high threshold level (P80 and P90 in this dataset), the low-FOXO3a groups (P10 to P70) always had a poor prognosis (*P*<0.05). Therefore, we selected the median of the dataset as the threshold in subsequent studies. Furthermore, the effect of FOXO3a expression on prognosis was still significant in
the multivariate Cox regression analysis (high-expression of FOXO3a: HR=0.366, 95%CI: 0.161-0.829, \( P = 0.016 \)), even when TNM stage and Fuhrman grade were included, indicating the independent prognostic significance of FOXO3a protein levels (Table 1). The Kaplan-Meier analysis demonstrated that the patients with low FOXO3a levels (below the median) had a poorer metastasis-free survival than those belonging to the high FOXO3a levels (log-rank test, \( P = 0.003 \); Figure 1F).

FOXO3a inhibited the migration and invasion of RCC cell lines

The above mentioned data prompted further study of FOXO3a biological functions. We speculated that repression of FOXO3a was an underlying mechanism that promoted ccRCC metastasis. Based on the observed FOXO3a protein levels in the RCC cell lines (Figures 1C and 1D), the non-metastatic 786-O cells with high FOXO3a expression and the metastatic SN12-PM6 cells with low FOXO3a expression were selected for the following experiments. pGFP-V-RS-1#shFOXO3a and pGFP-V-RS-2#shFOXO3a plasmids were effectively and stably transfected into 786-O cells, as shown by green fluorescent protein (GFP) expression (Figure 2A), and significantly inhibited FOXO3a protein expression in the 786-O cells, compared with the 786-O cells stably transfected with pGFP-V-RS-sh-sramble or empty vector (pGFP-V-RS).(Figure 2B). Knockdown of FOXO3a significantly promoted the migration and invasion of 786-O cells, compared with 786-O cells stably transfected with pGFP-V-RS (migration: 1#shFOXO3a: 437.7±119.9; 2#shFOXO3a: 401.7±159.0; empty vector: 241.5±95.0; invasion: 1#shFOXO3a: 249.1±89.8; 2#shFOXO3a:
253.7±115.9; empty vector: 79.8±36.2) (all P<0.05) (Figure 2C). Moreover, a genetic rescue assay was performed, and the pCR3.1-FOXO3a-mutated plasmid significantly inhibited the silencing efficiency of pGFP-V-RS-1#shFOXO3a in 786-O cells (Figure 2B). The migration and invasion abilities of 786-O cells co-transfected with the pCR3.1-FOXO3a-mutated and pGFP-V-RS-1#shFOXO3a plasmids were not significantly different compared with cells transfected with pGFP-V-RS (migration: 189.0±81.6; invasion: 97.4±45.0; both P>0.05) (Figure 2C).

Likewise, the pIRES2-EGFP-FOXO3a plasmid was stably transfected into SN12-PM6 cells (SN12-PM6 FOXO3a+), as shown by GFP detection (Figure 2D), and significantly increased FOXO3a protein expression in the SN12-PM6 cells, compared with SN12-PM6 cells stably transfected with empty vector (pIRES2-EGFP) (Figure 2E). Overexpression of FOXO3a significantly inhibited the migration and invasion of SN12-PM6 cells (Figure 2F).

A wound-healing assay further demonstrated that knockdown of FOXO3a increased the migration capacity of the 786-O cells (Supplementary Figure S3A), but overexpression of FOXO3a reduced the migration capacity of the SN12-PM6 cells (Supplementary Figure S3B). Therefore, FOXO3a inhibited the migration and invasion of RCC cell lines in vitro.

**FOXO3a suppressed the metastatic behavior of RCC cells in vivo**

To further study the inhibitory effect of FOXO3a on metastases in vivo, we established a nude mouse orthotopic xenograft tumor model to observe its lung
metastasis ratio. The SN12-PM6 FOXO3a+ cell line and the corresponding control cell line with the empty vector (SN12-PM6 stable transfected with pIRES2-EGFP) were implanted into the right kidney of the respective nude mice. As shown in Figure 4D, FOXO3a expression in the kidney tissue of the FOXO3a+ group was higher than in the empty vector control group. Metastatic lesions were found in the peripheral lung field (Figure 3A f) and in the lung hilum (Figure 3A g) 7 weeks after implantation. As shown in Table 2, 100% (17/17) of the xenografts formed primary tumors. Tumor weight was similar between groups \( (P=0.49) \). In the empty vector control group, metastasis occurred in the lungs of 100% (8/8) of the mice. In the FOXO3a+ group, lung metastasis rate was only 55.6% (5/9), which was lower than that in the empty vector group. However, this difference was not significant \( (P = 0.082) \).

Lowly-aggressive xenografts developed less metastatic lesions at the same period. Therefore, the inhibitory effect of FOXO3a on metastasis could be assessed based on the metastasis rate and the amount of the metastatic lesions. The SN12-PM6 FOXO3a+ and empty vector control cell lines expressed GFP, which could be detected and quantified by a molecular imaging system. Thus, we measured all lung metastatic lesions using this system. As shown in Figures 3B, 3C and 3D, the mean GFP fluorescence intensity in the lung in the FOXO3a+ group was significantly decreased, compared with that in the empty vector group \( (P=0.026) \). Therefore, the amount of metastatic lesions was decreased in the FOXO3a+ group. Based on the above mentioned results, we concluded that FOXO3a could suppress tumor metastasis.
Attenuation of FOXO3a activated EMT through the upregulation of SNAIL1 expression in vitro and in vivo

Given that FOXO3a suppresses tumor cell metastasis in vitro and in vivo, we further explored the mechanism by which FOXO3a suppressed metastasis in ccRCC cell lines. GO enrichment and pathway analysis of the microarray data revealed a probable EMT in the metastatic ccRCC group. In Figure 2A, the shape of 786-O cells transfected with the sh-scramble and empty vectors were rounded. After FOXO3a silencing, the cell processes increased, and cells showed a change from rounded epithelial-like shape to a spindle fibroblast-like shape. In the genetic rescue experiment, 786-O cells silenced by 1#sh-FOXO3a co-transfected with the pCR3.1-FOXO3a-mutated plasmids displayed a reversal in cell shape, from spindle to rounded. In Figure 2D, SN12-PM6 cells transfected with the empty vector had a spindle fibroblast-like shape. After FOXO3a overexpression, they acquired a rounded epithelial-like shape. We also detected a series of EMT-related markers by immunofluorescence staining, including the mesenchymal markers, N-cadherin (17-18), and the epithelial marker E-cadherin (19). FOXO3a gene silencing in 786-O cells increased the expression of N-cadherin, but decreased E-cadherin expression; overexpression of FOXO3a in SN12-PM6 cells reversed the protein levels of N-cadherin and E-cadherin (Figure 4A). Thus, FOXO3a might suppress EMT of ccRCC cells and thereby inhibited metastasis.

We searched for a panel of key regulators of EMT, including TWIST1, HIF1a,
SNAIL1, and YBX1 (20-23). These genes were all transcription factors that regulated EMT. We found that knockdown of FOXO3a evidently raised the SNAIL1 levels, an EMT activator, in 786-O cells, whereas the overexpression of FOXO3a significantly suppressed SNAIL1 expression in SN12-PM6 cells (Figure 4B). To further confirm the relationship between FOXO3a and SNAIL1 in kidney tissues, we detected SNAIL1 expression in PM and PN tissues by immunohistochemistry. Significantly increased levels of SNAIL1 protein were detected in PM ccRCC tissue samples, compared with PN ccRCC tissues ($P=0.019$) and normal renal tissues ($P<0.001$) (Figure 4C). Moreover, the correlation between the FOXO3a and SNAIL1 mRNA levels of ccRCC tissue samples was further analyzed by linear regression analysis. As shown in Figure 4D, the SNAIL1 mRNA level was negatively correlated with the FOXO3a mRNA expression ($n = 133 \; r = -0.281, \; P = 0.031$). Subsequently, FOXO3a and SNAIL1 expression were detected by immunohistochemistry in orthotopic xenograft tumor tissues. FOXO3a was lowly expressed in empty vector (EV) tumors, while it was highly expressed in FOXO3a-overexpressing tumor tissues, as expected. SNAIL1 was expressed in the EV tumors, while SNAIL1 expression was lower in the FOXO3a-overexpressing tumors than that in the EV tumors (Figure 4E), which was consistent with the results from the ccRCC patients.

FOXO3a and SNAIL1 biological functions were further studied. Two siRNA effectively and specifically inhibited SNAIL1 or FOXO3a protein expression in 786-O cells at 48 h after transfection (Figure 4F). Transwell assays showed that knocking-down SNAIL1 abolished cell migration and invasion, which were increased
by knocking-down FOXO3a in the 786-O cells (Figure 4G). MMP2, N-cadherin, ZEB1, and SNAIL1 levels were evidently increased, whereas E-cadherin levels were decreased by knocking-down FOXO3a in the 786-O cells. Transfection of si-SNAIL1 reversed the protein levels of MMP2, N-cadherin, ZEB1, and E-cadherin in 786-O cells. (Figure 4H).

**Discussion**

The aim of the present study was to explore the underlying mechanisms of ccRCC metastasis. Bioinformatics analysis, including GO enrichment and pathway analysis, indicated that EMT probably occurred in the PM ccRCC groups. EMT has been defined as a possible mechanism of metastasis (24). EMT could transform the epithelial tumor cells and confer the mesenchymal characteristics that would facilitate the dissemination of tumor cells, leading to metastases (25). Moreover, some studies reported that EMT influences ccRCC progression (26). Therefore, we assumed that ccRCC metastasis might be promoted by EMT in tumor cells.

GO enrichment and pathway analysis could only describe systematic information of biological processes, and the identification of specific genes with crucial functions from the list of redundant DEGs was a major challenge. Jia et al (27) successfully identified the crucial genes by combining the results of signal-net analysis with PubMed data. In our study, 6 DEGs were initially selected by the signal-net analysis. Of these 6 DEGs, LAMA4 is involved in blood vessel development and is a marker of vascularity in certain types of cancers (28). ATR has a role in p53 activation and renal cell apoptosis (29). JUN and ETS1 are reported
oncogenes promoting tumor cell metastasis (30). However, according to microarray results, both DEGs were downregulated in PM ccRCC. SORBS2 acts as an adapter protein to assemble signaling complexes for regulating actin-dependent processes, such as cell adhesion and migration (31). FOXO3a is a transcriptional factor belonging to the forkhead family. Among these DEGs, FOXO3a was considered the most attractive for further study.

In the immunohistochemistry assays, we found that the intracellular location of FOXO3a in the ccRCC tissues was different from that in the adjacent normal kidney tissues. In the adjacent normal tissues, FOXO3a was found strongly positive in the nucleus and slightly positive in the cytoplasm, whereas in the ccRCC tissues, FOXO3a was found mainly localized in the nucleus and was attenuated significantly. Moreover, the FOXO3a protein was further reduced in the PM ccRCC tissue cells compared with that in the PN ccRCC tissue cells (p = 0.004). The reduced expression and altered subcellular localization of FOXO3a in ccRCC tissues may be mediated by multiple levels of regulation. First, the FOXO3a expression was suppressed at the transcription level in metastatic ccRCC tissues, as we observed a significant reduced FOXO3a mRNA level in the PM group. Second, the FOXO3a protein level was reduced but the FOXO3a mRNA level was not obviously changed in PN group compared with the adjacent normal tissues, suggesting a posttranscriptional regulation might exist. Indeed, FOXO3a was reported as a target of miR-155 (32) and miR-182 (33), which could regulate FOXO3a expression through translation repression. Third, FOXO3a was found strongly positive in the nucleus and slightly positive in the
cytoplasm in the adjacent normal tissues, whereas it was found mainly localized in the nucleus in the ccRCC tissues. Previous studies showed that the intracellular localization of FOXO3a is closely related to its activity (34). Phosphorylation of FOXO3a by AKT, IKK, or ERK results in FOXO3a translocation from the nucleus to the cytoplasm, and subsequent degradation, whereas phosphorylation by c-jun-NH2-kinase results in retaining FOXO3a in the nucleus and thereby increasing FOXO3a activity (35). We believe that the detailed regulation mechanism of FOXO3a expression were complex and attractive, and further studies are required to shed light on this point.

The FOXO3a protein is significantly downregulated in renal cancer, compared with adjacent normal kidney tissues. Activation of FOXO3a could function as a tumor suppressor promoting cell cycle arrest and apoptosis in RCC cell lines (36). In the present study, we found that FOXO3a mRNA levels were dramatically suppressed in PM ccRCC compared with PN ccRCC. Immunohistochemistry confirmed that FOXO3a protein levels were decreased in PM ccRCC tissues, which correlated with the observed mRNA levels. Therefore, we present the first report suggesting that low FOXO3a mRNA levels are an independent prognostic factor for ccRCC metastasis, based on multivariate Cox regression analysis. This phenomenon implies that FOXO3a expression could serve as a possible marker for distinguishing ccRCC patients with a high risk of metastasis. However, more studies with larger sample sizes are required to draw robust conclusions about this point.

To date, reports on the role of FOXO3a in metastasis development are
controversial. Some studies reported that FOXO3a activation could reverse the invasive phenotype of breast cancer cells (37) and that FOXO3a silencing could increase the motility of urothelial cancer cells (38). However, FOXO3a was described as a MMP-9 and MMP-13 inducer promoting the invasion of Hela and MDA-MB-435 cell lines (39). In this study, we identified that FOXO3a silencing promoted renal cancer cells migration and invasion, while restoration of FOXO3a expression significantly suppressed the migration and invasion of renal cancer cells in vitro. The anti-metastasis function of FOXO3a was confirmed using a nude mice orthotopic tumor model. Therefore, we propose that the downregulation of FOXO3a might promote ccRCC metastasis. To the best of our knowledge, this is the first evidence that the loss of FOXO3a could promote metastasis in RCC cells.

Based on the results of the GO and KEGG analyses, alteration of the identified biological functions indicated a mesenchymal trait and suggested that a probable EMT occurred in the PM ccRCC group (25, 40). SNAIL1 is a powerful EMT inducer (41-43) associated with renal cancer invasion and prognosis (43-44). We found that FOXO3a silencing in 786-O cells increased SNAIL1 mRNA levels, whereas the restoration of FOXO3a in SN12-PM6 repressed SNAIL1 expression. Our analysis of gene expression in our clinical samples reinforced the inverse correlation of FOXO3a and SNAIL1 in ccRCC. Moreover, the increased cellular migration and invasion caused by FOXO3a silencing were reduced after SNAIL1 was knocked-down. The expression of EMT markers was remarkably altered by FOXO3a and/or SNAIL1 silencing in the 786-O cell line. Therefore, we concluded that FOXO3a
down-regulation induced EMT of tumor cells by upregulating SNAIL1 expression in ccRCC. FOXO3a might directly regulate SNAIL1 expression as a transcriptional suppressor or indirectly by other signals (45), and the exact involved mechanisms require further study.

In conclusion, FOXO3a expression was decreased in PM ccRCC samples, and low FOXO3a levels were an independent prognostic factor for ccRCC metastasis. The loss of FOXO3a induced EMT of tumor cells by upregulating SNAIL1, which promoted tumor cells metastasis in vitro and in vivo. Thus, FOXO3a could be a predictor or a marker of occult metastases.

Acknowledgments

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Table 1. Metastasis-free survival of 114 PN ccRCC patients in Cox regression analysis

<table>
<thead>
<tr>
<th></th>
<th>Univariate analysis</th>
<th>Mutivariate analysis</th>
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<tbody>
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<td>HR</td>
<td>95% CI</td>
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<tr>
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<td></td>
</tr>
<tr>
<td>Male</td>
<td>1</td>
<td></td>
</tr>
<tr>
<td>Female</td>
<td>0.947</td>
<td>0.405-2.217</td>
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<tr>
<td>Age (years)</td>
<td></td>
<td></td>
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<tr>
<td>&lt;60</td>
<td>1</td>
<td></td>
</tr>
<tr>
<td>≥60</td>
<td>1.273</td>
<td>0.579-2.800</td>
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<tr>
<td>FOXO3a levels</td>
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</tr>
<tr>
<td>Low</td>
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</tr>
<tr>
<td>High</td>
<td>0.314</td>
<td>0.139-0.711</td>
</tr>
<tr>
<td>BMI</td>
<td></td>
<td></td>
</tr>
<tr>
<td>&lt;25</td>
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</tr>
<tr>
<td>≥25</td>
<td>0.910</td>
<td>0.437-1.894</td>
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<td>TNM Stage</td>
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<tr>
<td>&lt;3</td>
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</tr>
<tr>
<td>≥3</td>
<td>9.095</td>
<td>4.343-19.047</td>
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<td>Fuhrman Grade</td>
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<td>≤2</td>
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<tr>
<td>&gt;2</td>
<td>2.867</td>
<td>1.266-6.494</td>
</tr>
</tbody>
</table>

TNM stage grouping was assigned according to the 2009 TNM staging classification system. BMI: body mass index; HR: hazard ratio; CI: confidence intervals.
Table 2. Kidney tumor incidence, tumor weight and lung metastasis in the FOXO3a+ and EV control groups

<table>
<thead>
<tr>
<th>Group</th>
<th>Number of mice</th>
<th>Tumor incidence (%)</th>
<th>Tumor weight (mg) Mean ± SD</th>
<th>Metastasis rate (%)</th>
</tr>
</thead>
<tbody>
<tr>
<td>FOXO3a+</td>
<td>9</td>
<td>9/9 (100)</td>
<td>0.2853±0.1025</td>
<td>5/9 (55.6)</td>
</tr>
<tr>
<td>EV</td>
<td>8</td>
<td>8/8 (100)</td>
<td>0.3171±0.0797</td>
<td>8/8 (100)</td>
</tr>
</tbody>
</table>

Tumor incidence rate was calculated after mice were sacrificed at week 7. Tumor weight was evaluated by subtracting the weight of the normal kidney (left kidney) from the kidney implanted with the tumor. The Student’s t-test was used to compare tumor weight. The Fisher’s exact test was used for the metastasis rate. EV: empty pIRES2-EGFP vector.
Figure legends

Figure 1. FOXO3a was downregulated in PM ccRCC tissues and was an independent prognosis factor for ccRCC metastases

(A) Real-time RT-PCR analysis of FOXO3a mRNA levels in PM (n = 21) and PN (n = 42, matched to PM at 1:2) tissues. FOXO3a mRNA levels of the adjacent normal tissues of PM and PN groups were also evaluated. APM: primary metastasis adjacent normal tissue (n=17); APN: primary non-metastasis adjacent normal tissue (n=21). Data are presented as mean ± standard deviation (SD). (B) Representative images of FOXO3a immunohistochemistry in PM and PN tissues, and adjacent normal renal tissues (AT). NC: negative control (without primary antibody against FOXO3a). Scale bar: 20μm. Quantitative analysis of FOXO3a protein levels in the nucleus in 10 sets of paired PM and PN tissues, and normal renal tissues (n=10). Data are expressed as median and interquartile range. (C) Western-blot detection of FOXO3a expression in a panel of RCC cell lines. GAPDH was used as an internal control. (D) Localization of FOXO3a in both non-metastatic and metastatic ccRCC cells were determined by immunofluorescence staining (magnification:×600). Blue: DAPI; Red: CF™555. (E) Prognostic significance of FOXO3a, calculated using univariate Cox regression analysis. Different thresholds were used to divide the PN ccRCC patient into high or low FOXO3a group. The horizontal axis represents the percentile scale used as threshold; the vertical axis represents the corresponding P-values from the threshold. (F) Kaplan-Meier analysis demonstrated that metastasis-free survival rate of patients with low-FOXO3a group was lower than in the high-FOXO3a group (log-rank test,
Figure 2. FOXO3a suppressed RCC cell migration and invasion in the transwell assays.

(A) Representative fluorescence (GFP) micrographs of 786-O cells stably transfected with pGFP-V-RS-1#shFOXO3a, pGFP-V-RS-2#shFOXO3a, pGFP-V-RS-sh-sramble, empty vector (pGFP-V-RS) (NC) and pCR3.1-FOXO3a mutation against 1#shFOXO3a (magnification: ×100). (B) Western blot assays to identify the silencing efficiency of the plasmids presented in A. GAPDH was used as an internal control. (C) Representative images of transwell assays (magnification: ×100). Quantification of the numbers of migrating or invading cells are presented as mean ± SD from three independent experiments. (D) Representative fluorescence (GFP) micrographs of SN12-PM6 cells stably transfected with pIRES2-EGFP-FOXO3a and empty vector (pIRES2-EGFP) (magnification: ×100). (E) Western blot assays to identify the overexpression efficiency of the plasmids presented in D. GAPDH was used as an internal control. (F) Representative images of the transwell assays (magnification: ×100). Quantification of the numbers of migrating or invading cells is presented as mean ± SD from three independent experiments. ***P<0.001.

Figure 3. FOXO3a suppressed tumor metastasis in nude mouse models with orthotopic xenograft tumors.

(A) Macroscopic appearances of (a) a xenograft tumor (arrow) in the kidney and (b)
lung tissue with metastatic lesions. Microscopic appearances of (c) normal kidney and (d) normal lung tissues. (e) Histopathology of the xenograft tumor in the mouse kidney. N: adjacent normal tissue; T: tumor tissue. Metastatic lesions (arrows) were found in (f) the peripheral lung field and (g) the lung hilum 7 weeks after implantation. All sections were stained with hematoxylin and eosin (H&E). Scale bar: 100 μm. Representative images of the lung metastatic lesions (B) and renal xenograft tumor (C) using molecular imaging system. (D) Green fluorescence of lung tissues was decreased in the SN12-PM6 FOXO3a+ group (5 lungs with metastatic lesions) compared with the EV group (8 lungs with metastatic lesions). No significant difference was observed in green fluorescence signal of kidneys between the EV (n = 8) and FOXO3a+ (n = 9) groups. Values are shown as mean ± SD. EV: empty vector (pIRES2-EGFP). *P < 0.05.

Figure 4. Loss of FOXO3a expression induced the EMT of 786-O cells by up-regulating SNAIL1 expression

(A) A series of EMT-related markers, including the mesenchymal markers, N-cadherin, and the epithelial marker E-cadherin were detected by immunofluorescence staining (magnification: ×600). Red: CF™555; blue: DAPI. (B) Real-time RT-PCR results showing that SNAIL1 mRNA levels were increased in FOXO3a knocked-down 786-O cells, but were decreased in the FOXO3a-overexpressing SN12-PM6 cells. Data are shown as mean ± SD. (C) Representative images of SNAIL1 immunohistochemistry in PM and PN tissues, and adjacent normal renal tissues (AT).
NC: negative control (without primary antibody against SNAIL1). Scale bar: 20μm. Quantitative analysis of SNAIL1 protein levels in 10 sets of paired PM and PN tissues, and normal renal tissues (n=10). Data are expressed as median and interquartile range.

(D) Negative correlation of SNAIL1 and FOXO3a mRNA levels in ccRCC tissues samples (n =133, r=−0.281, P=0.031; linear regression analysis). (E) FOXO3a and SNAIL1 protein expressions were determined by immunohistochemistry in the kidney tissues of the mice implanted with the SN12-PM6 FOXO3a+ cells. N: adjacent normal tissue; T: tumor tissue. Scale bar: 50μm. (F) Western blot analysis indicating the silencing efficiency of si-FOXO3a and si-SNAIL1 in 786-O cells at 48 h after transfection. NC: 786-O cells without any treatment; PC: si-GAPDH as positive control. (G) 786-O cells were transfected with si-FOXO3a alone, both si-FOXO3a and si-SNAIL1, or si-SNAIL1 alone. 786-O cells transfected with scramble siRNA and the normal 786-O cells were used as negative control groups. Representative images of the transwell assays using 786-O cells (magnification: ×100). Quantification of the numbers of migrating or invading cells was presented as mean ± SD from three independent experiments. (H) 48 h after transfection, a series of EMT-related markers, including the mesenchymal markers, N-cadherin, ZEB1, and MMP2, and the epithelial marker E-cadherin were detected by western blot. GAPDH was used as an internal control. **P<0.01, ***P<0.001.
Figure 1

(A) Log2 Relative mRNA Expression

(B) FOXO3a

(C) Non-metastatic vs Metastatic

(D) FOXO3a, DAPI, Merged

(E) P Value

(F) Metastasis Free Survival

Log-Rank test: p = 0.003
Figure 2

A

1# sh  2# sh

d-sh-scramble  pGFP-V-RS  FOXO3a-mut

B

FOXO3a  GAPDH

1# sh  2# sh  sh-scramble  pGFP-V-RS  FOXO3a-mut

C

Invasion  Migration

1# sh  2# sh  d-sh-scramble  pGFP-V-RS  FOXO3a-mut

D

FOXO3a+  plRES2-EGFP

E

FOXO3a  GAPDH

PBS  plRES2-EGFP  FOXO3a+

F

Invasion  Migration

FOXO3a+  plRES2-EGFP  PBS

Figure 2
Figure 4
Downregulation of FOXO3a promotes tumor metastasis and is associated with metastasis-free survival of patients with clear cell renal cell carcinoma

Dong Ni, Xin Ma, Hong Zhao Li, et al.

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