Regulation of spindle and kinetochore-associated protein 1 by antitumor miR-10a-5p in renal cell carcinoma

Takayuki Arai,1,2 Atsushi Okato,1,2 Satoko Kojima,3 Tetsuya Idichi,4 Keiichi Koshizuka,5 Akira Kurozumi,1,2 Mayuko Kato,1,2 Kazuto Yamazaki,5 Yasuo Ishida,6 Yukio Naya,3 Tomohiko Ichikawa2 and Naohiko Seki1

Departments of 1Functional Genomics; 2Urology, Chiba University Graduate School of Medicine, Chiba; 3Department of Urology, Teikyo University Chiba Medical Center, Ichihara; 4Department of Digestive Surgery, Breast and Thyroid Surgery, Graduate School of Medical Sciences, Kagoshima University, Kagoshima; 5Department of Pathology, Teikyo University Chiba Medical Center, Ichihara, Japan

Key words
MicroRNA, miR-10a-5p, renal cell carcinoma, spindle and kinetochore-associated protein 1, tyrosine kinase inhibitor resistance

Correspondence
Naohiko Seki, Department of Functional Genomics, Chiba University Graduate School of Medicine, 1-8-1 Inohana, Chuo-ku, Chiba 260-8670, Japan. Tel: +81-43-226-2971; Fax: +81-43-227-3442; E-mail: naoseki@faculty.chiba-u.jp

Funding information
This study was supported by KAKENHI grants 15K10801, 16K20125, and 16H05462.

Received May 22, 2017; Revised July 20, 2017; Accepted July 23, 2017

Cancer Sci 108 (2017) 2088–2101
doi: 10.1111/cas.13331

Analysis of our original microRNA (miRNA) expression signature of patients with advanced renal cell carcinoma (RCC) showed that microRNA-10a-5p (miR-10a-5p) was significantly downregulated in RCC specimens. The aims of the present study were to investigate the antitumor roles of miR-10a-5p and the novel cancer networks regulated by this miRNA in RCC cells. Downregulation of miR-10a-5p was confirmed in RCC tissues and RCC tissues from patients treated with tyrosine kinase inhibitors (TKI). Ectopic expression of miR-10a-5p in RCC cell lines (786-O and A498 cells) inhibited cancer cell migration and invasion. Spindle and kinetochore-associated protein 1 (SKA1) was identified as an antitumor miR-10a-5p target by genome-based approaches, and direct regulation was validated by luciferase reporter assays. Knockdown of SKA1 inhibited cancer cell migration and invasion in RCC cells. Overexpression of SKA1 was observed in RCC tissues and TKI-treated RCC tissues. Moreover, analysis of The Cancer Genome Atlas database demonstrated that low expression of miR-10a-5p and high expression of SKA1 were significantly associated with overall survival in patients with RCC. These findings showed that downregulation of miR-10a-5p and overexpression of the SKA1 axis were highly involved in RCC pathogenesis and resistance to TKI treatment in RCC.

Renal cell carcinoma accounts for approximately 2–5% of adult malignancies worldwide, and approximately 350 000 new cases are diagnosed, with over 140 000 deaths, each year.1,2 Most cases of RCC (approximately 80%) are classified as ccRCC.3 In the majority of patients, ccRCC is associated with dysfunction of the VHL gene.4 Lack of VHL function causes activation of HIF and VEGF pathways in ccRCC cells.5,6,7 The mTOR pathway is also activated by dysregulation of HIF and VEGF pathways in patients with ccRCC.8,9

Based on this information regarding the molecular pathogenesis of ccRCC, molecular targeted therapies for patients with advanced and metastatic RCC have been developed during the past decade.10 The molecular-targeted agents sorafenib, sunitinib, pazopanib, axitinib, bevacizumab, and cabozantinib inhibit VEGF and VEGF receptor pathways, and temsirolimus and everolimus inhibit the mTOR pathway; treatment with these agents has resulted in significant benefits to patients with advanced RCC.10,11 However, the curative effects of these treatments are limited because cancer cells exhibit activation of several alternative signal cascades and acquire resistance to these treatments during therapeutic processes.11,12 Treatment strategies for drug-resistant cancer cells are limited, and the prognosis of these patients is extremely poor. However, the molecular mechanisms of resistance to molecular-targeted therapies in RCC cells are still unclear.

miRNAs act as pivotal players that regulate the expression control of protein-coding/protein-noncoding RNAs in a sequence-dependent manner.13,14 Notably, a single miRNA can directly control many mRNAs in human cells.15 Therefore, aberrantly expressed miRNAs can disrupt the tight control of RNA expression in cancer cells. Moreover, dysregulation of miRNAs is deeply involved in cancer cell progression, metastasis, and drug resistance.16–19

In RCC, miRNAs are closely related to the development of cancer, and previous studies have reported the relationships among many miRNAs and RCC. For example, the miR-200 family, containing miR-141, miR200a/b/c, and miR-429, forms two clusters, miR-200a/200b/429 and miR-141/200c, and expression of the miR-200 family is markedly downregulated in RCC tissues.20 Additionally, the miR-200 family has been reported to be involved in the EMT in several cancers, and miR-141 and miR-200c function as tumor suppressors in RCC by inhibiting the EMT through targeting of ZFHX1B, a transcriptional repressor for CDH1/E-cadherin.21 In this way, investigation of molecular networks based on miRNAs may help to elucidate the molecular mechanisms mediating the progression of RCC.
Fig. 1. Kaplan-Meier survival curves based on miR-10a-5p expression in patients with clear cell renal cell carcinoma (ccRCC), and schematic representation of the chromosomal location of human miR-10a. (a) Kaplan-Meier survival curve for overall survival rate based on miR-10a-5p expression in patients with ccRCC from The Cancer Genome Atlas (TCGA) database. (b) miR-10a is located on human chromosome 17q21.32. Mature microRNAs (miRNAs), miR-10a-5p (guide strand) and miR-10a-3p (passenger strand), are derived from pre-miR-10a.

Table 1. Characteristics of primary ccRCC clinical specimens

| No. | Age (years) | Sex | Pathology | Grade | pT | INF | v | ly | e.g or ig | fc | im | rc | rp | s |
|-----|-------------|-----|-----------|-------|----|-----|---|----|-----------|----|----|----|----|---|
| 1   | 71          | F   | Clear cell| G2    | T1a| a   | 0 | 0  | e.g      | 1  | 0  | 0  | 0  | 0 |
| 2   | 74          | M   | Clear cell| G1>G2 | T1a| a   | 0 | 0  | e.g      | 1  | 0  | 0  | 0  | 0 |
| 3   | 59          | M   | Clear cell| G3>G2 | T1b| a   | 0 | 0  | e.g      | 1  | 0  | 0  | 0  | 0 |
| 4   | 79          | M   | Clear cell| G2>G3 | T1a| a   | 0 | 0  | e.g      | 1  | 0  | 0  | 0  | 0 |
| 5   | 52          | M   | Clear cell| G2>G3 | T1b| a   | 0 | 0  | e.g      | 1  | 1  | 0  | 0  | 0 |
| 6   | 64          | M   | Clear cell| G2>G3 | T3a| b   | 1 | 0  | ig      | 0  | 1  | 1  | 0  | 0 |
| 7   | 67          | M   | Clear cell| G2>G3 | T3a| b   | 1 | 0  | ig      | 1  | 0  | 0  | 0  | 0 |
| 8   | 59          | M   | Clear cell| G3    | T3a| b   | 1 | 0  | ig      | 0  | 0  | 0  | 0  | 0 |
| 9   | 73          | M   | Clear cell| G1>G3 | T2a| a   | 0 | 1  | e.g      | 1  | 0  | 0  | 0  | 0 |
| 10  | 77          | M   | Clear cell| G1>G2 | T1b| a   | 0 | 0  | e.g      | 1  | 0  | 0  | 0  | 0 |
| 11  | 51          | F   | Clear cell| G2>G1 | T3a| b   | 1 | 0  | ig      | 0  | 0  | 0  | 0  | 0 |
| 12  | 84          | F   | Clear cell| G2    | T1a| a   | 0 | 0  | e.g      | 0  | 0  | 0  | 0  | 0 |
| 13  | 78          | M   | Clear cell| G2>G3 | T1b| b   | 0 | 0  | e.g      | 1  | 0  | 0  | 0  | 0 |
| 14  | 44          | M   | Clear cell| G2    | T1a| b   | 0 | 0  | e.g      | 1  | 0  | 0  | 0  | 0 |
| 15  | 57          | M   | Clear cell| G2    | T1b| a   | 0 | 0  | e.g      | 0  | 0  | 0  | 0  | 0 |

ccRCC, clear cell renal cell carcinoma; e.g, expansive growth; fc, capsular formation; ig, infiltrative growth; im, intrarenal metastasis; INF, infiltration; ly, lymph node; rc, renal capsule invasion; rp, pelvis invasion; s, sinus invasion; v, vein.
Table 2. Characteristics of ccRCC autopsy specimens after TKI treatment

| Treatment | Survival from diagnosis (months) | Pathological feature of autopsy | Histological type | Grade | Histological type of ccRCC | Pathological feature | Patient Specimen | Location | Age (years) | Stage | Stage at diagnosis | Histological type | Treatment |
|-----------|---------------------------------|-------------------------------|------------------|-------|--------------------------|---------------------|------------------|----------|--------------|-------|------------------|----------------|------------|
| A         | 9.1                             | Multiple lung metastasis      | Clear cell carcinoma | 4     | 4                         | Clear cell carcinoma | Sunitinib        | Kidney   | 69           | IV    | 2               | Clear cell carcinoma | Sunitinib  | Temsirolimus |
| B         | 8.5                             | Multiple bone metastasis      | Clear cell carcinoma | 3c    | 0                         | Clear cell carcinoma | Sunitinib        | Kidney   | 80           | III   | 3               | Clear cell carcinoma | Sunitinib  | Axitinib      |
| C         | 0.7                             | Bone metastasis              | Clear cell carcinoma | 0     | 0                         | Clear cell carcinoma | Sunitinib        | Mesenterium | 62           | I     | 1b              | Clear cell carcinoma | Sunitinib  | Pazopanib    |
| D         | 34                              | Pleural metastasis           | Clear cell carcinoma | 0     | 0                         | Clear cell carcinoma | Sunitinib        | Pleura    | 69           | IV    | X               | Clear cell carcinoma | Sunitinib  | Pazopanib    |

ccRCC, clear cell renal cell carcinoma; TKI, tyrosine kinase inhibitor.
SKA1 plasmid vectors were designed and provided by ORIGENE (cat. no. RC202370; Rockville, MD, USA). miRNAs and siRNAs were incubated with Opti-MEM (Invitrogen) and Lipofectamine RNAiMax transfection reagent (Invitrogen), as previously described.\(^{(22,25–28)}\) Plasmid vectors were incubated with Opti-MEM and Lipofectamine 3000 reagent (Invitrogen) by forward transfection following the manufacturer’s protocol.

Cell proliferation, migration, and invasion assays. 786-O and A498 cells were transfected with 10 nM miRNAs or siRNAs by reverse transfection. Cell proliferation was determined by XTT assays using a Cell Proliferation Kit II (Sigma-Aldrich, St Louis, MO, USA). Cell migration was evaluated with wound healing assays 48 h after transfection with 10 nM miR-10a-5p. \(^*P < 0.0001, \**P < 0.01, \***P < 0.001.\) Cell invasion activity was characterized by invasion assays 48 h after transfection with 10 nM miR-10a-5p. \(P < 0.0001.\)

Selection of putative target genes regulated by miR-10a-5p in ccRCC cells. To identify miR-10a-5p target genes, we used in
Selection strategy for identification of miR-10a-5p-regulated genes

Target genes of miR-10a-5p from TargetScan Database
(release 7.1)
3661 genes

Gene expression analysis
miR-10a-5p restoration in 786-O and A498 cells
(GSE 93290)
Downregulated genes by miR-10a-5p
(Log2 FC < -1.5)
31 genes (Table 3)

TCGA_OncoLnc
Kaplan-Meier analysis 25:25
Log-rank P-value < 0.05
Poor prognosis with a high expression
8 genes

Fig. 3. Identification of miR-10a-5p target genes. Flow chart of the strategy for identification of miR-10a-5p target genes.

Results

Expression levels of miR-10a-5p in ccRCC specimens and cell lines. The public miRNA database (miRbase: release 21) showed that miR-10a-5p was located on chromosome 17q21.32. The mature sequence of miR-10a-5p was found to be 5'-UACCCUGUAUCGGAAUUUGUG-3' (Fig. 1b). We evaluated the expression of miR-10a-5p in clinical kidney specimens (noncancerous tissues, ccRCC tissues, and autopsy specimens of ccRCC) and cell lines. Expression levels of miR-10a-5p were significantly downregulated in primary cancer tissues and TKI-treated tissues compared with those in noncancerous tissues ($P = 0.0010$, $P = 0.0009$, respectively; Fig. 2a). In 786-O and A498 cells, expression levels of miR-10a-5p were relatively low compared with those of clinical specimens (Fig. 2a).

Effects of restoring miR-10a-5p on cell proliferation, migration, and invasion activities in ccRCC cell lines. To investigate the functional efficacy of miR-10a-5p, we carried out gain-of-function studies using miRNA transfection into 786-O and A498 cells. XT assays showed that cell proliferation was significantly inhibited in miR-10a-5p transfectants compared with that in mock or miR-control transfectants (Figs 2b; S1a). Migration assays showed that cell migration activity was significantly inhibited in miR-10a-5p transfectants in comparison with those in mock or miR-control transfectants (Figs 2c; S2a). Similarly, Matrigel invasion assays showed that cell invasion activity was significantly inhibited in miR-10a-5p transfectants in comparison with those in mock or miR-control transfectants (Figs 2d; S2b).

Identification of candidate genes regulated by miR-10a-5p in ccRCC cells. To further elucidate the molecular mechanisms and pathways regulated by antitumor miR-10a-5p in ccRCC cells, we carried out a combination of in silico analyses and oligo microarray analyses using miR-10a-5p transfectants. The strategy for selection of miR-10a-5p target genes is shown in Figure 3. First, we used TargetScanHuman 7.1 database and identified that 3661 genes had putative target sites for miR-10a-5p. We used the TargetScanHuman 7.1 database and narrow down putative miRNA target genes. An oligo microarray (Human Ge 60K; Agilent Technologies) was used for gene expression analysis. The microarray data were deposited into the GEO database (https://www.ncbi.nlm.nih.gov/geo/; accession number: GSE93290).

Western blotting. Cells were harvested 48 h after transfection, and lysates were prepared. Immunoblotting was carried out with rabbit anti-SKA1 antibodies (1:1000 dilution, SAB2701430; Sigma-Aldrich), anti-AKT antibody (1:1000, #4060; Cell Signaling Technology), anti-p-AKT (Y473) antibody (1:1000, #4060; Cell Signaling Technology), anti-ERK1/2 antibody (1:1000, #4370; Cell Signaling Technology), anti-FAK antibody (1:1000, #3285; Cell Signaling Technology) and anti-p-FAK antibody (1:1000, #3285; Cell Signaling Technology) and anti-p-AKT antibody (1:1000, #4060; Cell Signaling Technology), anti-SRC antibody (1:1000, #2123; Cell Signaling Technology) and anti-p-SRC antibody (1:1000, #6943; Cell Signaling Technology). Anti-glyceraldehyde 3-phosphate dehydrogenase (GAPDH) antibodies (1:10000, ab8245; Abcam, Cambridge, UK) were used as an internal loading control. Experimental procedures were carried out as described previously. Protein expression was quantified by using NIH-ImageJ.

Plasmid construction and dual-luciferase reporter assays. The partial wild-type sequence of the SKA1 3'-UTR or that with deletion of the miR-10a-5p target site was inserted between the XhoI-PmeI restriction sites in the 3'-UTR of the hRluc gene in the pSCEH-2 vector (C8021; Promega, Madison, WI, USA). The procedures were described previously.

Immunohistochemistry. Tissue specimens were incubated overnight at 4°C with anti-SKA1 antibodies (1:500 dilution, SAB2701430; Sigma-Aldrich). The slides were treated with biotinylated goat antibodies (Histofine SAB-PO kit; Nichirei, Tokyo, Japan). The procedures were described previously.

TCGA database analysis of ccRCC. To explore the clinical significance of miRNAs and target genes, we used the TCGA database. Gene expression and clinical data were retrieved from cBioportal (http://www.cbioportal.org/) and OncoLnc (data downloaded on April 30, 2017). We selected high and low target genes expression groups defined by the median value, and data were analyzed by Kaplan-Meier survival curves and log-rank statistics.

Statistical analysis. Relationships between two groups and the numerical values obtained by qRT-PCR were analyzed using Mann-Whitney U-tests and paired t-tests. Spearman’s rank test was used to evaluate the correlation between the expression levels of miR-10a-5p and SKA1. Relationships among more than three variables and numerical values were analyzed using Bonferroni-adjusted Mann-Whitney U-tests. Survival analysis was carried out using the Kaplan-Meier method and log-rank tests with JMP software (version 13; SAS Institute Inc., Cary, NC, USA); all other analyses were carried out using Expert StatView (version 5; SAS Institute Inc.).
Table 3. Candidate target genes regulated by miR-10a-5p in clear cell renal cell carcinoma (ccRCC)

| Gene Symbol | Gene name                                      | Location  | No. conserved sites | No. poorly conserved sites | Log₂ ratio (A498) | Log₂ ratio (786-O) | Log₂ ratio (average) | TCGA-KIRC Oncolnc | P-value |
|-------------|------------------------------------------------|-----------|--------------------|---------------------------|-------------------|--------------------|---------------------|-----------------|---------|
| SKA1        | Spindle and kinetochore-associated protein 1    | 18q21.1   | 0                  | 1                         | -5.06             | -2.01              | -3.54               |                 | *2.72E–08|
| SLC7A1      | Solute carrier family 7 (cationic amino acid transporter, y+ system), member 1 | 13q12.3   | 0                  | 1                         | -3.38             | -2.23              | -2.81               |                 | 2.62E–01|
| SUMF1       | Sulfatase modifying factor 1                   | 3p26.1    | 0                  | 1                         | -2.35             | -2.65              | -2.50               |                 | 9.42E–01|
| P4HB        | Prolyl 4-hydroxylase, beta polypeptide          | 17q25.3   | 0                  | 1                         | -2.61             | -2.35              | -2.48               |                 | *2.75E–04|
| USP46       | Ubiquitin specific peptidase 46                | 4q12      | 1                  | 0                         | -1.96             | -2.9               | -2.43               |                 | 6.62E–02|
| ELOVL2      | ELOVL fatty acid elongase 2                    | 6p24.2    | 1                  | 0                         | -2.25             | -2.39              | -2.32               |                 | *6.39E–04|
| KCTD13      | Potassium channel tetramerization domain contain 13 | 16p11.2   | 0                  | 1                         | -2.69             | -1.72              | -2.21               |                 | *4.00E–06|
| LINCO0908   | Long intergenic non-protein coding RNA 908     | 18q23     | 0                  | 1                         | -2.62             | -1.79              | -2.21               |                 | 1.52E–01|
| SUN2        | Sad1 and UNC84 domain containing 2             | 22q13.1   | 0                  | 3                         | -2.10             | -2.31              | -2.21               |                 | 1.31E–01|
| FCF1        | FCF1 rRNA-processing protein                   | 1q42.3    | 0                  | 1                         | -2.51             | -1.84              | -2.18               |                 | 8.74E–04|
| ARG2        | Arginase 2                                     | 1q24.1    | 0                  | 1                         | -2.06             | -2.25              | -2.16               |                 | 6.91E–01|
| SLAMF7      | SLAM family member 7                          | 1q23.3    | 0                  | 1                         | -1.52             | -2.77              | -2.15               |                 | 1.23E–01|
| PRKAA2      | Protein kinase, AMP-activated, alpha 2 catalytic subunit | 1p32.2    | 1                  | 3                         | -1.66             | -2.58              | -2.12               |                 | 2.10E–09|
| FANCC       | Fanconi anemia, complementation group C        | 9q22.32   | 0                  | 3                         | -2.23             | -2.00              | -2.12               |                 | 4.29E–03|
| PDCL        | Phosducin-like                                  | 9q33.2    | 0                  | 1                         | -2.03             | -2.03              | -2.03               |                 | 1.19E–03|
| PSIP1       | PC4 and SFRS1 interacting protein 1            | 9p22.3    | 0                  | 1                         | -2.51             | -1.53              | -2.02               |                 | 4.96E–02|
| CD3D        | CD3d molecule, delta (CD3-TCR complex)         | 11q23.3   | 0                  | 2                         | -2.20             | -1.82              | -2.01               |                 | 9.89E–02|
| C1QL4       | Complement component 1, q subcomponent-like 4  | 12q13.12  | 0                  | 1                         | -1.97             | -2.04              | -2.01               |                 | 7.02E–03|
| SDC1        | syndecan 1                                     | 2p24.1    | 1                  | 0                         | -1.94             | -2.05              | -2.00               |                 | 6.76E–01|
| DNA14       | Dynene, axonemal, light chain 4               | 22q13.1   | 0                  | 1                         | -1.91             | -2.06              | -1.99               |                 | 7.34E–01|
| NUP62CL     | Nucleoporin 62kDa C-terminal like              | Xq22.3    | 0                  | 2                         | -1.7              | -2.03              | -1.87               |                 | 4.12E–02|
| MTUS1       | Microtubule associated tumor suppressor 1      | 8p22      | 0                  | 1                         | -1.93             | -1.76              | -1.85               |                 | 4.03E–03|
| RTN4R       | Reticulon 4 receptor                           | 22q11.21  | 0                  | 1                         | -2.07             | -1.6               | -1.84               |                 | *1.24E–05|
| PM20D2      | Peptidase M20 domain containing 2              | 6q15      | 0                  | 1                         | -2.04             | -1.56              | -1.80               |                 | 2.92E–02|
| PCD1        | Pterin-4-alpha-carbinolamine dehydratase/dimerization cofactor of hepatocyte nuclear factor 1 alpha | 10q22.1   | 0                  | 1                         | -1.55             | -2.03              | -1.79               |                 | 4.00E–01|
| APAF1       | Apoptotic peptidase activating factor 1        | 12q23.1   | 0                  | 3                         | -1.90             | -1.63              | -1.77               |                 | *1.18E–03|
| DOHH        | Deoxyhypusine hydroxylase/monooxygenase        | 19p13.3   | 0                  | 2                         | -1.78             | -1.56              | -1.67               |                 | *6.16E–03|
| AIM1        | Absent in melanoma 1                           | 6q21      | 0                  | 1                         | -1.71             | -1.55              | -1.63               |                 | 2.67E–01|
| ANXA7       | Annexin A7                                    | 10q22.2   | 1                  | 0                         | -1.50             | -1.65              | -1.58               |                 | 5.17E–02|
| HS3ST1      | Heparan sulfate (glucosamine) 3-O- sulfotransferase 1 | 4p15.33  | 0                  | 1                         | -1.54             | -1.56              | -1.55               |                 | 2.99E–03|
| IMPAD1      | Inositol monophosphatase domain containing 1    | 8q12.1    | 0                  | 1                         | -1.52             | -1.56              | -1.54               |                 | *5.59E–03|

* Kaplan–Meier survival analysis P < 0.05. Poor prognosis with high expression in ccRCC.

Based on gene expression data (GEO database accession number: GSE93290), 31 genes were markedly downregulated after transfection of 786-O and A498 cells with miR-10a-5p (fold-change Log₂ < -2.0; Table 3). Finally, we checked the expression status of these 31 genes and the clinical significance of ccRCC using the OncoLnc database (http://www.oncolnc.org/). Kaplan-Meier survival curves showed that high expression of eight genes was associated with poor prognosis in ccRCC (Table 3; Figs 4a, 5). Among these genes, we focused on SKA1, which was most downregulated by transfection of miR-10a-5p, and showed the most dramatic difference in OncoLnc prognostic analysis (P = 2.72E–08, Fig. 4a).

Expression of SKA1 in ccRCC clinical specimens. A total of 15 pairs of primary ccRCC specimens, adjacent noncancerous specimens, and TKI-treatment failure autopsy specimens from 15 sites were used for the expression analysis of SKA1 by qRT-PCR. Expression of SKA1 was significantly upregulated in primary cancer tissues compared with that in normal tissues (P = 0.0011; Fig. 4b) and was significantly higher in autopsy specimens than in primary cancer tissues (P = 0.0011; Fig. 4b). Additionally, Spearman’s rank test indicated a negative correlation between the expression levels of miR-10a-5p and SKA1 (Fig. 4c).

Furthermore, to analyze SKA1 protein expression, immunohistochemistry was carried out with a ccRCC tissue.
microarray (cat. no. KD806; US Biomax, Rockville, MD, USA) and autopsy specimens after TKI treatment (Patient D, Table 2). Patient characteristics for samples used in the tissue microarray are as described in http://www.biomax.us/tissue-arrays/Kidney/KD806. SKA1 was strongly expressed in primary ccRCC tissues and autopsy specimens after TKI treatment (Patient D, Table 2).

Fig. 4. Kaplan-Meier survival curves based on SKA1 expression in patients with clear cell renal cell carcinoma (ccRCC), and expression levels of SKA1 in ccRCC clinical specimens. (a) Kaplan-Meier survival curve for overall survival rate based on SKA1 expression in patients with ccRCC. (b) Expression levels of SKA1 in ccRCC clinical specimens and cell lines. GUSB was used as an internal control. TKI, tyrosine kinase inhibitor. (c) Negative correlation between miR-10a-5p and SKA1.

Fig. 5. Kaplan-Meier survival curves for overall survival rates based on expression of seven genes, excluding SKA1, in patients with clear cell renal cell carcinoma (ccRCC).
TKI treatment compared with that in normal kidney (Fig. 6).

**SKA1 was directly regulated by miR-10a-5p transfection in ccRCC cells.** We carried out qRT-PCR and western blotting to validate whether restoration of miR-10a-5p in 786-O and A498 cells reduced the expression of SKA1. Expression of SKA1 mRNA was significantly suppressed by miR-10a-5p transfection compared with that in mock- or miR-control-transfected cells (Fig. 7a). Similarly, SKA1 protein expression was repressed in the miR-10a-5p transfectants (Fig. 7b).

Next, we carried out luciferase reporter assays to determine whether SKA1 mRNA had a functional target site. The TargetScan database predicted that miR-10a-5p bound at position 28–35 in the 3′-UTR of SKA1. We used vectors encoding a partial wild-type sequence of the 3′-UTR of SKA1 mRNA, including the predicted miR-10a-5p target site, or a vector lacking the miR-10a-5p target site. Luminescence intensity was significantly reduced by cotransfection with miR-10a-5p and the vector carrying the wild-type 3′-UTR of SKA1. However, luminescence intensity was not suppressed when the target site of miR-10a-5p was deleted from the vectors (Fig. 7c).

**Effects of silencing SKA1 in ccRCC cell lines.** To examine the functional significance of SKA1, we carried out loss-of-function studies using si-SKA1 transfectants. First, we evaluated the knockdown efficiency of si-SKA1 transfection in 786-O and A498 cells. In this study, we used two types of si-SKA1 (si-SKA1-1 and si-SKA1-2). qRT-PCR and western blotting analyses showed that transfection with both siRNAs effectively downregulated SKA1 mRNA and SKA1 protein expression in 786-O and A498 cells (Fig. 8a,b). Furthermore, functional assays indicated that si-SKA1 transfection markedly inhibited cell proliferation, migration, and invasion in comparison with mock- or si-control-transfected cells (Figs 8c; S1b, S3a, S4a).

**Effects of cotransfection of SKA1/miR-10a-5p in 786-O cells.** To validate whether the molecular pathway of SKA1/miR-10a-5p was critical for the progression of ccRCC, we carried out SKA1 rescue experiments by cotransfection with SKA1 and miR-10a-5p in 786-O cells. SKA1 protein expression by Western blotting analysis is shown in Figure 9a. Functional assays showed that the migration and invasion abilities of ccRCC cells were recovered by SKA1 and miR-10a-5p transfection compared with cells with restored miR-10a-5p only (Figs 9b–

---

TKI treatment compared with that in normal kidney (Fig. 6).

**SKA1 was directly regulated by miR-10a-5p transfection in ccRCC cells.** We carried out qRT-PCR and western blotting to validate whether restoration of miR-10a-5p in 786-O and A498 cells reduced the expression of SKA1. Expression of SKA1 mRNA was significantly suppressed by miR-10a-5p transfection compared with that in mock- or miR-control-transfected cells (Fig. 7a). Similarly, SKA1 protein expression was repressed in the miR-10a-5p transfectants (Fig. 7b).

Next, we carried out luciferase reporter assays to determine whether SKA1 mRNA had a functional target site. The TargetScan database predicted that miR-10a-5p bound at position 28–35 in the 3′-UTR of SKA1. We used vectors encoding a partial wild-type sequence of the 3′-UTR of SKA1 mRNA, including the predicted miR-10a-5p target site, or a vector lacking the miR-10a-5p target site. Luminescence intensity was significantly reduced by cotransfection with miR-10a-5p and the vector carrying the wild-type 3′-UTR of SKA1. However, luminescence intensity was not suppressed when the target site of miR-10a-5p was deleted from the vectors (Fig. 7c).

**Effects of silencing SKA1 in ccRCC cell lines.** To examine the functional significance of SKA1, we carried out loss-of-function studies using si-SKA1 transfectants. First, we evaluated the knockdown efficiency of si-SKA1 transfection in 786-O and A498 cells. In this study, we used two types of si-SKA1 (si-SKA1-1 and si-SKA1-2). qRT-PCR and western blotting analyses showed that transfection with both siRNAs effectively downregulated SKA1 mRNA and SKA1 protein expression in 786-O and A498 cells (Fig. 8a,b). Furthermore, functional assays indicated that si-SKA1 transfection markedly inhibited cell proliferation, migration, and invasion in comparison with mock- or si-control-transfected cells (Figs 8c; S1b, S3a, S4a).

**Effects of cotransfection of SKA1/miR-10a-5p in 786-O cells.** To validate whether the molecular pathway of SKA1/miR-10a-5p was critical for the progression of ccRCC, we carried out SKA1 rescue experiments by cotransfection with SKA1 and miR-10a-5p in 786-O cells. SKA1 protein expression by Western blotting analysis is shown in Figure 9a. Functional assays showed that the migration and invasion abilities of ccRCC cells were recovered by SKA1 and miR-10a-5p transfection compared with cells with restored miR-10a-5p only (Figs 9b–
These results supported that SKA1 affected the aggressiveness of ccRCC cells.

Clinical significance of SKA1 in ccRCC. To explore the clinical significance of SKA1 in ccRCC, we analyzed Kaplan-Meier curves of DFS rates according to the expression level of SKA1, and the relationships among SKA1 expression and cancer stage, tumor stage, and histological grade in ccRCC were evaluated using the TCGA-KIRC database. Kaplan-Meier curves for DFS rates showed that the DFS of the high SKA1 expression group was significantly shorter than that of the low expression group in ccRCC ($P < 0.0001$, Fig. 10a). Additionally, expression levels of SKA1 were significantly increased in cases of advanced disease stage, advanced T stage, and advanced histological grade (Fig. 10b–d). These analyses suggested that SKA1 affected disease progression and malignancy in ccRCC. Similarly, TCGA data analysis results of clinical significance for the other seven genes are shown in Figures S6–S9.

Effects of SKA1 downstream signaling by si-SKA1 knockdown or miR-10a-5p restoration in RCC cells. We investigated the

d; S1c, S5a,b). These results supported that SKA1 affected the aggressiveness of ccRCC cells.

Fig. 7. Direct regulation of SKA1 by miR-10a-5p in clear cell renal cell carcinoma (ccRCC) cells. (a) SKA1 mRNA expression was evaluated using qRT-PCR in 786-O and A498 cells 48 h after transfection with miR-10a-5p. GAPDH was used as an internal control. $*$ $P < 0.0001$. (b) SKA1 protein expression was evaluated by western blotting in 786-O and A498 cells 72 h after transfection with miR-10a-5p. GAPDH was used as a loading control. (c) miR-10a-5p binding site in the 3′-UTR of SKA1 mRNA. Dual luciferase reporter assays in 786-O using vectors encoding the putative miR-10a-5p target site of SKA1 3′-UTR (positions 28-35). Data were normalized by expression ratios of Renilla/firefly luciferase activities. $*$ $P < 0.0001.
downstream signals of miR-10a-5p/SKA1 axis in 786-O cells using mature miR-10a-5p or si-SKA1 transfectants. To explore the downstream survival pathways of miR-10a-5p/SKA1 axis, phosphorylation of ERK1/2 (Thr 202/Tyr 204), AKT (Ser 473), FAK (Tyr 397) and SRC (Tyr 416) was examined. Knockdown of SKA1 or restoration of miR-10a-5p markedly reduced the phosphorylation of ERK1/2, AKT, FAK and SRC (Fig. 11).

**Discussion**

Based on the underlying molecular oncogenic mechanisms of RCC, several molecular targeted agents have been developed to improve the prognosis of patients with advanced RCC. However, although almost all patients with RCC respond to initial treatment with molecular targeted therapies, cancer cells ultimately become resistant to these treatments. Several molecular mechanisms of drug resistance have been reported in RCC; however, all of these mechanisms are not sufficient to explain the observed changes in cancer cells. Understanding these molecular mechanisms using current genomic approaches is the first step to overcoming drug resistance in RCC cells.

To investigate the molecular mechanisms of drug resistance in RCC cells, we constructed a miRNA expression signature using autopsy specimens from patients with ccRCC who exhibited TKI-treatment failure. Our present data demonstrated that miR-10a-5p was associated with poor prognosis in patients with RCC. miR-10a belongs to the miR-10 family together with miR-10b, and miR-10a-5p is a guide strand of miR-10a. Previous studies have indicated that miR-10a is deregulated in several types of cancers. In different types of cancer, miR-10a has dual functions as either a cancer-promoting or cancer-suppressing miRNA. In RCC, a recent study showed that miR-10a-5p is a predictor of progression and survival for RCC; however, the functional significance and relevant
molecular mechanisms of miR-10a-5p in cancer progression are still unknown.

One of the main challenges in miRNA studies is identification of miRNA-target genes and RNA networks mediated by antitumor miRNAs in cancer cells. A total of 31 genes were identified as putative targets of miR-10a-5p regulation in ccRCC cells in this study. Among them, eight genes (SKA1, P4HB, ELOVL2, KCTD13, RTN4R, APAF1, ANXA7, and IMPAD1) were associated with poor prognosis in patients with RCC by TCGA analyses. We focused on the SKA1 gene because overexpression of this gene showed the most significant association with poor prognosis in RCC. Another seven genes may also be involved in the pathology of RCC. Analyses of these genes are important for elucidating the molecular mechanisms of RCC oncogenesis, metastasis and drug resistance.

It is well known that one mRNA was regulated by a number of miRNAs. TargetScan database searching showed that SKA1 was a putative target for miR-10a-5p, miR-24-3p and miR-23b-3p which were significantly downregulated in our miRNA signature of TKI treatment of RCC specimens. Our previous studies demonstrated that miR-24-3p and miR-23b-3p acted as antitumor miRNAs targeting several oncogenic genes. Also, miR-10b-5p functioned as an antitumor miRNA in several types of cancers including RCC. We made the following hypothesis from these findings, SKA1 is regulated by several antitumor miRNAs which contribute to RCC pathogenesis and drug resistance. From the analyses of these miRNAs, it can be expected to lead to elucidation of the mechanism of drug resistance of RCC.

Several studies have indicated that SKA1 is involved in the growth and proliferation of various types of cancer, including oral adenosquamous and hepatocellular carcinoma, bladder cancer, gastric cancer, prostate cancer, thyroid cancer, non-small cell lung cancer, and glioblastoma. In the human genome, SKA1, SKA2, and SKA3 form the SKA complex. During mitosis, the SKA complex is localized between the outer kinetochore interface and the spindle microtubules. This complex is indispensable for stabilizing adhesion of spindle microtubules to kinetochores and maintaining the metaphase plate; therefore, this complex is critical for appropriate chromosome segregation during mitosis. Interestingly, the expression of SKA1 contributes to cisplatin resistance in lung cancer cells by protecting the cells from cisplatin-induced apoptosis. Knockdown of SKA1 decreases the activation of extracellular signal-regulated kinase (ERK1/2) and AKT-mediated signaling pathways in lung cancer cells. Another study also demonstrated that knockdown of SKA1 alleviated the activation of ERK1/2 and AKT in bladder cancer cells. In adenoid cystic carcinoma, knockdown of SKA1 inhibited cell proliferation, invasion, and migration, and cell cycle arrest by regulating cell cycle-promoting genes and the matrix metalloproteinase-9 gene. These findings suggested that the expression of SKA1 may be induced by cancer-promoting genes and could contribute to cancer cell aggressiveness and drug resistance. Many reports have demonstrated that acquired resistance of RCC cells to molecular targeted therapies induces cancer-promoting genes and activates several alternative pathways. A previous study showed that sunitinib treatment significantly suppressed phosphorylation of ERK1/2 and AKT in TKI-sensitive RCC cells, whereas inhibition of phosphorylation was not observed in TKI-resistant RCC cells. In this study, phosphorylation of ERK1/2 and AKT was suppressed by knockdown of SKA1 and restoration of miR-10a-5p. This suggests that downregulation of miR-10a-5p and overexpression of the SKA1 axis may be involved in resistance to VEGF- and mTOR-targeted treatments in RCC.

Fig. 9. Effects of cotransfection of SKA1/miR-10a-5p in 786-O cells. (a) SKA1 protein expression was evaluated by western blotting analysis of 786-O cells 72 h after reverse transfection with miR-10a-5p and 48 h after forward transfection with the SKA1 vector. GAPDH was used as a loading control. (b) Cell proliferation was determined using XTT assays 72 h after reverse transfection with miR-10a-5p and 48 h after forward transfection with the SKA1 vector. **P < 0.01. (c) Cell migration activity was assessed by wound-healing assays 48 h after reverse transfection with miR-10a-5p and 24 h after forward transfection with the SKA1 vector. *P < 0.0001. (d) Cell invasion activity was characterized by invasion assays 48 h after reverse transfection with miR-10a-5p and 48 h after forward transfection with SKA1 vector. *P < 0.0001.

© 2017 The Authors. Cancer Science published by John Wiley & Sons Australia, Ltd on behalf of Japanese Cancer Association.
In conclusion, downregulation of miR-10a-5p was detected in the miRNA signature of TKI-failure RCC and acted as an antitumor miRNA in RCC cells. To the best of our knowledge, this is the first study showing that antitumor miR-10a-5p directly regulated SKA1 in RCC cells. Overexpression of SKA1 was observed in primary and TKI-failure RCC specimens. Moreover, downregulation of miR-10a-5p and overexpression of SKA1 were associated with poor prognosis in patients with RCC. Elucidation of miR-10a-5p/SKA1-mediated molecular networks may improve our understanding of the pathogenesis of primary RCC and molecular targeted treatment failure in RCC and facilitate the development of new treatment strategies.

**Disclosure Statement**

Authors declare no conflicts of interest.

**Abbreviations**

- ccRCC: clear cell renal cell carcinoma
- DFS: disease-free survival
- EMT: epithelial-to-mesenchymal transition
- HIF: hypoxia-inducible factor
- miRNA: microRNA
- RCC: renal cell carcinoma
- SKA1: spindle and kinetochore-associated protein 1
- TKI: tyrosine kinase inhibitor
- VEGF: vascular endothelial growth factor
- VHL: von Hippel-Lindau

**Fig. 10.** Kaplan-Meier survival curve based on SKA1 expression in patients with clear cell renal cell carcinoma (ccRCC), and expression levels of SKA1 according to TNM stage, T stage, and histological grade. (a) Kaplan-Meier survival curves for disease-free survival rate based on SKA1 expression in patients with ccRCC. (b-d) Expression levels of SKA1 were significantly increased in cases of advanced TNM stage, advanced T stage, and advanced histological grade. *P < 0.01, **P < 0.001, ***P < 0.0001.

**Fig. 11.** Effects of the gene encoding SKA1 protein on downstream signaling. Knockdown of SKA1 and restoration of miR-10a-5p in 786-O cells reduced the phosphorylation of ERK1/2, AKT, FAK and SRC. GAPDH was used as a loading control.
References

1. Capitanio U, Montorsi F. Renal cancer. *Lancet* 2016; 387: 894–906.
2. Ferlay J, Soerjomataram I, Dikshit R et al. Cancer incidence and mortality worldwide: sources, methods and major patterns in GLOBOCAN 2012. *Int J Cancer* 2013; 132: 891–908.
3. Rini BL, Campbell SC, Escudier B. Renal cell carcinoma. *Lancet* 2009; 373: 1119–32.
4. Gnarra JR, Tory K, Weng Y et al. The microRNA-23b-27b-24-1 cluster is a disease progression marker and tumor suppressor in prostate cancer. *Oncotarget* 2014; 5: 7748–59.

Albges L, Choueiri T, Escudier B et al. A systematic review of sequencing and combinations of systemic therapy in metastatic renal cancer. *Eur Urol* 2015; 67: 100–10.

2. Calvo E, Schmidinger M, Heng DY, Gruenwald V, Escudier B. Improvement in survival end points of patients with metastatic renal cell carcinoma genotyped for VEGF and VEGFR2 mutations and combinations of systemic therapy. *Oncotarget* 2015; 6: 3455–66.

3. Lund AH. miR-10 in development and cancer. *Cell Death Differ* 2010; 17: 209–14.

4. Khan S, Wall D, Curran C, Newell J, Kerin MJ, Dwyer RM. MicroRNA-10a is reduced in breast cancer and regulated in part through retinoic acid. *BMC Cancer* 2015; 15: 345.

5. Okuchida K, Mizumoto K, Lin C et al. MicroRNA-10a is overexpressed in human pancreatic cancer and involved in its invasiveness partially via suppression of the HOXA1 gene. *Ann Surg Oncol* 2012; 19: 2394–402.

6. Jia H, Zhang Z, Zou D et al. MicroRNA-10a is down-regulated by DNA methylation and functions as a tumor suppressor in gastric cancer cells. *PLoS One* 2014; 9: e88057.

7. Kowalik CG, Palmer DA, Sullivan TB et al. Profiling microRNA from nephrectomy and biopsy specimens: predictors of progression and survival in clear cell renal cell carcinoma. *BJU Int* 2017 (in press).

8. Gao J, Aksoy BA, Dogrusoz U et al. Integrative analysis of complex cancer genomics and clinical profiles using the cBioPortal. *Science Signaling* 2013; 6(269): pe2574.

9. Hou R, Wang D, Lu J. MicroRNA-10b inhibits proliferation, migration and invasion in cervical cancer cells via direct targeting of insulin-like growth factor-1 receptor. *Oncol Lett* 2017; 13: 5009–15.

10. Zhan B, Li KY, Chen HY et al. Spindle and kinetochore-associated complex subunit 1 regulates the proliferation of oral adenosquamous carcinoma CAL-27 cells in vitro. *Cancer Cell Int* 2013; 13: 83.

11. Qin X, Yuan B, Xu X, Huang H, Liu Y. Effects of short interfering RNA-mediated gene silencing of SKA1 on proliferation of hepatocellular carcinoma cells. *Sci Rep* 2013; 3: 1324–32.

12. Tian F, Xing X, Xu F et al. Downregulation of SKA1 gene expression inhibits cell growth in human bladder cancer. *Cancer Biother Radiopharm* 2015; 30: 271–7.

13. Sun W, Yao L, Jiang B, Guo L, Wang Q. Spindle and kinetochore-associated protein 1 is overexpressed in gastric cancer and modulates cell growth. *Mol Cell Biochem* 2014; 391: 167–74.

14. Li J, Xuan JW, Khattamifar V et al. SKA1 over-expression promotes centriole over-duplication, centrosome amplification and prostate tumorigenesis. *J Pathol* 2014; 234: 178–89.

15. Dong C, Wang XL, Ma BL. Expression of spindle and kinetochore-associated protein 1 is associated with clinical outcome and poor prognosis in papillary thyroid carcinoma. *DisMarkers* 2015; 2015: 615641.

16. Shen L, Yang M, Lin Q, Zhang Z, Miao C, Zhu B. SKA1 regulates the metastasis and cisplatin resistance of non-small cell lung cancer. *Oncol Rep* 2016; 35: 2561–8.

17. Shi X, Chen X, Peng H et al. Lentivirus-mediated silencing of spindle and kinetochore-associated protein 1 inhibits the proliferation and invasion of neural glioblastoma cells. *Mol Med Rep* 2015; 11: 3533–8.

18. Gaitanos TN, Santamaria A, Jeyaprakash AA, Wang B, Conti E, Nigg EA. The microRNA-23b/27b/24-1 cluster and combinations of systemic therapy in metastatic renal cell carcinoma. *BJU Int* 2017; 108: 900–17.
Supporting Information

Additional Supporting Information may be found online in the supporting information tab for this article:

**Fig. S1.** Proliferation curves over time according to the results of XTT assays. (a) Transfection with miR-10a-5p in 786-O and A498 cells. (b) Transfection of si-SKA1 into 786-O and A498 cells. (c) SKA1 rescue experiments. *P < 0.001, **P < 0.0001.

**Fig. S2.** Phase micrographs of wound healing and invasion assays by transfection with miR-10a-5p in RCC cell lines. (a) Phase micrographs of 786-O and A498 cells 14 h after monolayer wound healing. (b) Phase micrographs of invading 786-O and A498 cells.

**Fig. S3.** Phase micrographs of wound healing and invasion assays by transfection with si-SKA1 in 786-O cells. (a) Phase micrographs of 786-O cells 14 h after monolayer wound healing. (b) Phase micrographs of invading 786-O cells.

**Fig. S4.** Phase micrographs of wound healing and invasion assays by transfection with si-SKA1 in A498 cells. (a) Phase micrographs of A498 cells 14 h after monolayer wound healing. (b) Phase micrographs of invading A498 cells.

**Fig. S5.** Phase micrographs of wound healing and invasion assays by cotransfection with SKA1/miR-10a-5p in 786-O cells. (a) Phase micrographs of 786-O cells 14 h after monolayer wound healing. (b) Phase micrographs of invading 786-O cells.

**Fig. S6.** Kaplan-Meier survival curves for disease-free survival rates based on expression of seven genes, excluding SKA1, in patients with ccRCC.

**Figs S7–9.** Expression levels of seven genes, excluding SKA1, according to TNM stage, T stage, and histological grade in patients with ccRCC from TCGA database. *P < 0.01, **P < 0.001, ***P < 0.0001.