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Original Article

Annexin A1 expression is correlated with malignant potential of renal cell carcinoma

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Abbreviations & Acronyms
Anxa1 = annexin A1
ccRCC = clear cell renal cell carcinoma
DFS = disease-free survival
ECM = extracellular matrix
FBS = fetal bovine serum
FIH-1 = factor inhibiting hypoxia-inducible factor 1
HIF-1α = hypoxia-inducible factor 1-alpha
MEM = minimum essential medium
MMP = matrix metalloproteinase
MT1 = membrane-type 1
MTS = 3-(4,5-dimethylthiazol-2-yl)-5-(3-carboxyme-thoxyphenyl)-2-(4-sulfophenyl)-2H-tetrazolium
qRT-PCR = quantitative real-time polymerase chain reaction
RCC = renal cell carcinoma

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Objectives: To evaluate the expression of annexin A1 protein in patients with renal cell carcinoma.
Methods: Annexin A1 expression was examined in renal cell carcinoma specimens from 27 patients, and their disease-free survival was analyzed using the log–rank test. Annexin A1 knockdown in the human renal cell carcinoma cell line Caki-1 was carried out, and its proliferation, invasion, motility and adhesion were compared with those of control cells.
Results: In 13 out of 27 patients, annexin A1 was highly expressed in the membrane of renal cell carcinoma tumor cells, whereas in the rest of the patients, annexin A1 expression was weak or negligible in the membrane of those cells. Patients with high annexin A1 expression had significantly poorer disease-free survival than those with weak or negligible annexin A1 expression ($P = 0.031$). In the renal cell carcinoma cell line, annexin A1 knockdown cells showed significantly decreased proliferation, invasion, motility and adhesion relative to control cells, and expressed lower relative levels of membrane-type 1 matrix metalloproteinase and hypoxia-inducible factor 1-alpha transcripts, showing a potential pathway regulated by annexin A1.
Conclusion: Annexin A1 is associated with renal cell carcinoma malignant potential and could serve as a marker of poor prognosis.

Key words: annexin, invasion, kidney, proliferation, renal cell carcinoma.

Introduction

RCC is the most common malignancy of the kidney.[1] In Japan, 13% of curative nephrectomy patients develop metastasis after surgery.[2] In contrast to non-metastatic RCC patients, the median survival of metastatic RCC patients is significantly worse.[3] Therefore new markers for RCC prognosis and therapeutic options targeting invasion and metastasis are required to improve RCC treatment.

Anxa1 was identified as the first member of the annexin superfamily of Ca^{2+}-dependent phospholipid-binding proteins, which are preferentially located on the cytosolic side of the plasma membrane.[4] Anxa1 protein has an apparent relative molecular mass of 40 kDa with inhibiting phospholipase A2 activity, which causes cell proliferation and migration.[5–7] The function of constitutively expressed Anxa1 in normal kidneys has not been fully understood.

In cancer, Anxa1 is overexpressed in human gastric and hepatocellular carcinoma, and its upregulation is related to malignancy.[8,9] Furthermore, Anxa1 antibody labeled blood vessels of the human prostate, liver, breast and lung tumors, but not matched normal tissues.[10,11] Based on these observations, we have developed molecular therapy targeting Anxa1 by using mouse cancer models.[12] In the present study, we evaluated Anxa1 expression in human RCC specimens and correlated its expression with patients' DFS. We then used the human RCC cell lines to assess whether Anxa1 expression is associated with RCC cell proliferation, migration or invasion.

Methods

Patients and tissue samples

The present study evaluated RCC specimens obtained from 27 patients who underwent radical nephrectomy from 1995 to 2009 at Asama General Hospital, Saku, Japan. None of the patients

had received any preoperative treatment or postoperative molecular-targeted therapy. Among the patients, 20 were men and seven were women, the median age was 69 years (range 46–82 years). Specimens were retrieved from the pathology files of the Department of Pathology of the same hospital. Pathological staging and grading were based on the American Joint Committee on Cancer Staging System, 7th edition.[13] Histological types were determined based on the 2016 World Health Organization classification.[14] Among the 27 cases, 22 were diagnosed as ccRCC, two as papillary RCC, two as chromophobe RCC and one as multilocular RCC. Nuclear grades were determined according to Fuhrman classification.[15] Patient survival was followed at Asama General Hospital, except for one individual, who was not re-admitted to the hospital after surgery. Tumor recurrence in the remaining 26 patients was diagnosed by clinicians on the basis of physical examination, imaging and scintigraphy. The mean observation period was 2983 days. The clinicopathological parameters for all 27 patients are summarized in Table 1. The present study was approved by the ethics committees of Asama General Hospital (no. 15–29) and Shinshu University School of Medicine (no. 3627), and was undertaken in accordance with the Declaration of Helsinki.

Immunohistochemistry

Anti-Anxa1 antibody (polyclonal, rabbit immunoglobulin G; Invitrogen, Carlsbad, CA, USA) diluted 1:100 was used as a primary antibody. Immunohistochemistry was carried out using the EnVision system (DakoCytomation, Carpinteria, CA, USA). Immunohistochemical evaluation was assessed by two authors (MY and KY). Specifically, when RCC tumor cell membranes were more strongly stained than non-neoplastic glomeruli, we judged the Anxa1 expression "positive." Specimens were judged "negative" when the Anxa1 expression level in tumor cell membranes was the same or weaker than non-neoplastic glomeruli. The same criteria were applied to the analysis of tumor vascular endothelial cells (tumor vasculature).

Cell culture and Anxa1 knockdown

The human ccRCC cell lines (Caki-1, VMRC-RCW, KMRC-20 and KMRC-1) were obtained from the JCRB Cell Bank (Osaka, Japan) and cultured in MEM or RPMI1640, supplemented with 10% FBS (Hyclone, Logan, UT, USA) and 1% penicillin-streptomycin solution. Cells were cultured at 37°C in a humidified incubator containing 5% CO_2. Anxa1-silencing vectors were constructed using pSINsi-hU6 shRNA retroviral vectors, which harbor a neomycin resistance gene (Takara Bio, Shiga, Japan). Human *ANXA1* sequences that were targeted corresponded to nucleotides 857–875 (5′-GACGTAAACGTGTTCAATA-3′). The negative control plasmid contained a sequence with no significant homology to the human gene. For retroviral infection, retroviral packaging cells were transfected with recombinant retroviral vectors. Thereafter, the cells were cultured in fresh medium for 48 h, during which time the supernatant containing retrovirus was collected twice. Supernatants were concentrated and used to infect target cells. Caki-1 cells were infected with shRNA containing retrovirus using Amphotropic receptor booster (Takara Bio). Infected cells were then subcultured in fresh medium containing 1 mg/mL G418. G418-resistant cell pools were readily established within 14 days. The mRNA and protein expression levels were verified using quantitative PCR and western blotting, respectively.

qRT-PCR

Total RNA was extracted with an RNeasy mini kit (Qiagen, Hilden, Germany) and then reverse-transcribed with Prime Script RT Master Mix (Takara Bio). We carried out qRT-PCR using TaqMan Universal PCR Master Mix (Applied Biosystems, Foster City, CA, USA) and a TaqMan probe (Applied Biosystems) for *ANXA1* quantification, and using SYBR Premix Ex Taq II (Takara Bio) for other molecules. mRNA expression was analyzed using an Applied Biosystems 7300 Fast Real-Time PCR System, and the relative expression of transcripts to *GAPDH* mRNA was calculated, based on the ΔΔct method.

Cell proliferation assay

We carried out the cell proliferation assay using MTS solution (Promega, Madison, WI, USA). Control and shRNA Caki-1 cells were seeded in 96-well plates at 500 cells per well. After 24 h, MTS solution was added to each well, and

Table 1 Relationship of clinicopathological variables and Anxa1 expression on RCC cell membranes

	Anxa1 expression (%)			
	Positive (n = 13)	Negative (n = 14)	P-value	Statistical analysis
Age (years)				
≤70	6 (46.2)	7 (50.0)	0.573	Fisher's exact probability test
>70	7 (53.8)	7 (50.0)		
Sex				
Male	11 (84.6)	9 (64.3)	0.298	Fisher's exact probability test
Female	2 (15.4)	5 (35.7)		
T stage				
pT1	9 (69.2)	9 (64.3)	0.922	χ^2-test
pT2	2 (15.4)	3 (21.4)		
pT3	2 (15.4)	2 (14.3)		
Nuclear grade†				
G1	4 (30.8)	4 (28.6)	0.989	χ^2-test
G2	8 (61.5)	9 (64.3)		
G3	1 (7.7)	1 (7.1)		
Histological type‡				
Clear cell	13 (100)	9 (64.3)	0.127	χ^2-test
Papillary	0 (0.0)	2 (14.3)		
Chromophobe	0 (0.0)	2 (14.3)		
Multilocular	0 (0.0)	1 (7.1)		
Venous invasion				
Positive	5 (38.5)	3 (21.4)	0.293	Fisher's exact probability test
Negative	8 (61.5)	11 (78.6)		

†Graded by Fuhrman classification. ‡Clear cell, clear cell RCC; chromophobe, chromophobe RCC; papillary, papillary RCC; multilocular, multilocular RCC.

color density was measured at 490 nm using a micro-plate reader (Dainippon Pharmaceutical, Osaka, Japan). This assay was carried out daily for 5 days in sextet, and the means ± SD were calculated.

Matrigel invasion assay

Cells in serum-free MEM were seeded on the top of transwell inserts with an 8-μm pore size membrane coated with 25 μg of Matrigel (BD Biosciences, Franklin Lakes, NJ, USA), whereas the lower chamber was filled with MEM supplemented with 20% FBS as a chemoattractant. Cells were cultured for 24 h and the non-migrating cells in the inserts were scraped off with a cotton swab. Cells that had invaded the lower surface of the membranes were fixed and stained using crystal violet. Cells that had invaded the Matrigel-coated membrane were detected using light microscopy (×200 magnification), and the cell number was counted in five randomly chosen visual fields.

Scratch assay

For the scratch assay, 4×10^5 cells were seeded into 60 mm dishes and cultivated for 24 h to prepare the monolayer cells. These layers were wounded using a sterile 200-μL tip. After washing away the suspended cells, the remaining cells were cultured in MEM with 0.1% FBS. The migration progress was photographed in six regions, immediately (0 h) and 14 h after wounding, using an inverted microscope (EVOS; AR BROWN, Tokyo, Japan). The wounded areas were measured using ImageJ software (http://rsb.info.nih.gov/ij/), and the percentage of wound closure was calculated for each area.

In vitro cell adhesion assay

For *in vitro* cell adhesion assay, 96-well plates were pre-coated with either collagen type I (Nitta Gelatin, Osaka, Japan), collagen type IV (Nitta Gelatin), fibronectin (Sigma-Aldrich, St. Louis, MO, USA), laminin (Sigma-Aldrich) or FBS, and then blocked with MEM containing 0.5% BSA. Thereafter, 1×10^4 cells were seeded onto the coated plates and incubated for 30 min at 37°C. Unadhered cells were removed, and adherent cells were fixed with paraformaldehyde, stained with crystal violet and washed. Finally, crystal violet was solubilized with sodium dodecyl sulfate, and absorbance at 550 nm was determined using a microplate reader (Dainippon Pharmaceutical).

Statistical analysis

Patient age, sex and venous invasion status were compared using Fisher's exact probability test. Other categorical data

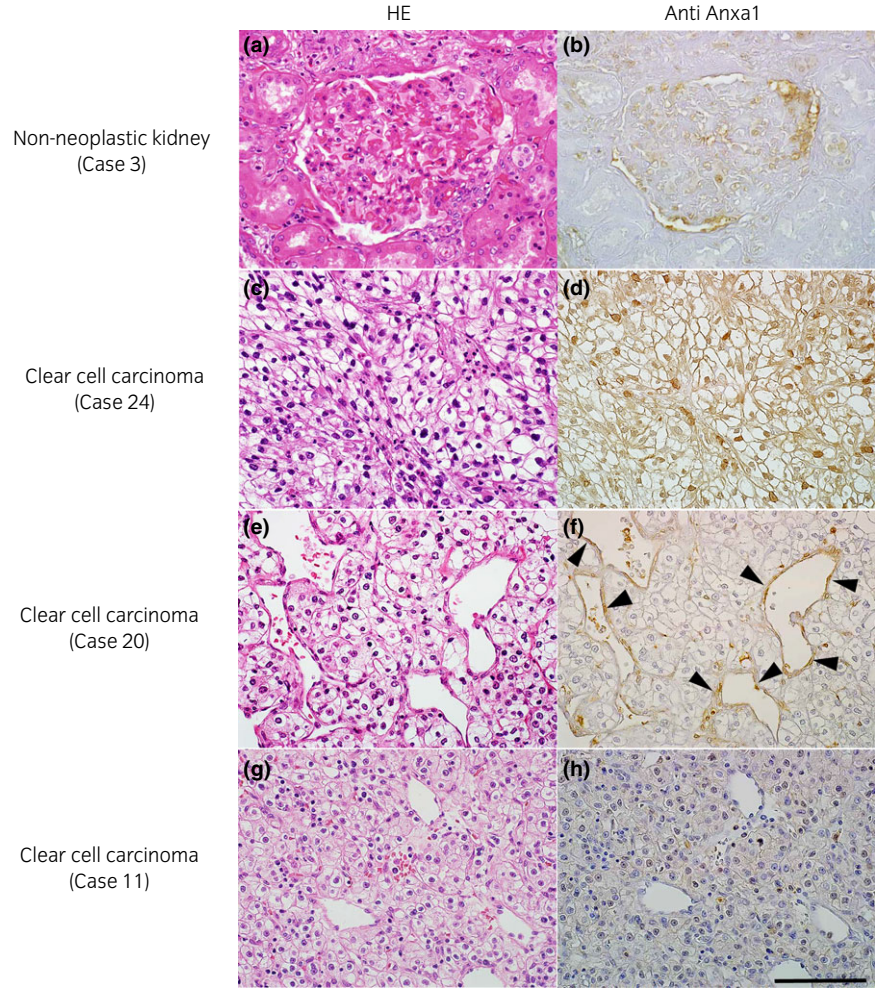

Fig. 1 Immunohistochemical analysis of Anxa1 expression in RCC. (a,c,e,g) Hematoxylin–eosin staining, and (b,d,f,h) immunohistochemistry for Anxa1. (b) Weak Anxa1 expression in endothelial cells of the glomerulus and distal tubules in control non-neoplastic kidney tissue adjacent to RCC. (c–h) RCC samples are shown. As seen in (d, f,h), Anxa1 expression levels in RCC vary from case to case. (c,d) Case 24, immunoreactivity is seen in the tumor cell membrane and/or nucleus. (e,f) Case 20, immunoreactivity is absent or weak in tumor cells, but apparent in endothelial cells of the tumor vasculature (black arrowheads). (g,h) Case 11, immunoreactivity is not detectable in tumor cells and the tumor vasculature. Scale bar, 100 μm.

in the clinicopathological examinations were compared using the χ^2-test. Survival curves were constructed using the Kaplan–Meier method, and the difference between the curves was evaluated by a log–rank test. Statistical analysis for *in vitro* experiments was carried out using the Student's *t*-test. All analyses were carried out using Microsoft Office Excel 2010 (Microsoft Corporation, Redmond, WA, USA). *P*-values < 0.05 were considered statistically significant.

Results

High Anxa1 expression in RCC specimens is correlated with poor prognosis

We first evaluated Anxa1 expression in RCC specimens using immunohistochemistry. We found that Anxa1 was strongly expressed on the tumor cell membrane of some specimens and in the tumor vasculature in others. In yet other cases, Anxa1 expression was not observed in either the tumor cell membrane or the tumor vasculature (Fig. 1; Table S1). Anxa1 expression levels in RCC specimens differed considerably from case to case, and the number of cases showing tumor cell positivity was not always correlated with those showing tumor vasculature positivity (Fig. 1; Table S1). In adjacent non-cancerous tissues, Anxa1 was weakly expressed in the endothelial cells of glomeruli (Fig. 1). Relevant to tumor cell membrane status, among the 27 RCC cases, 13 cases were judged as positive and 14 as negative (Table 1; Table S1). To determine whether Anxa1 expression correlates with clinicopathological aggressiveness, we evaluated the differences between the clinicopathological variables in Anxa1-positive and -negative cases (Table 1). However, we found no significant differences between these two groups in any clinicopathological factors, including age, sex, T stage, nuclear grade, histological type and venous invasion status (Table 1). We also evaluated the differences in DFS between the two groups of 26 cases from 27 cases, including 12 positive and 14 negative cases. All Anxa1-negative cases were free from RCC recurrence in the follow-up period. Log–rank test analysis confirmed that patients with negative Anxa1 had significantly better DFS than those with positive Anxa1 ($P = 0.031$; Fig. 2a). Focusing on the ccRCC cases ($n = 21$), a similar result was obtained (Fig. 2b).

We next evaluated Anxa1 expression in the tumor vasculature (Table S1). Further analysis of Anxa1-positive versus Anxa1-negative showed no significant difference in terms of any clinicopathological factors including age, sex, T stage, nuclear grade, histological type and venous invasion status (Table S2). Log–rank test analysis also confirmed no significant difference in DFS between positive and negative cases ($P = 0.990$).

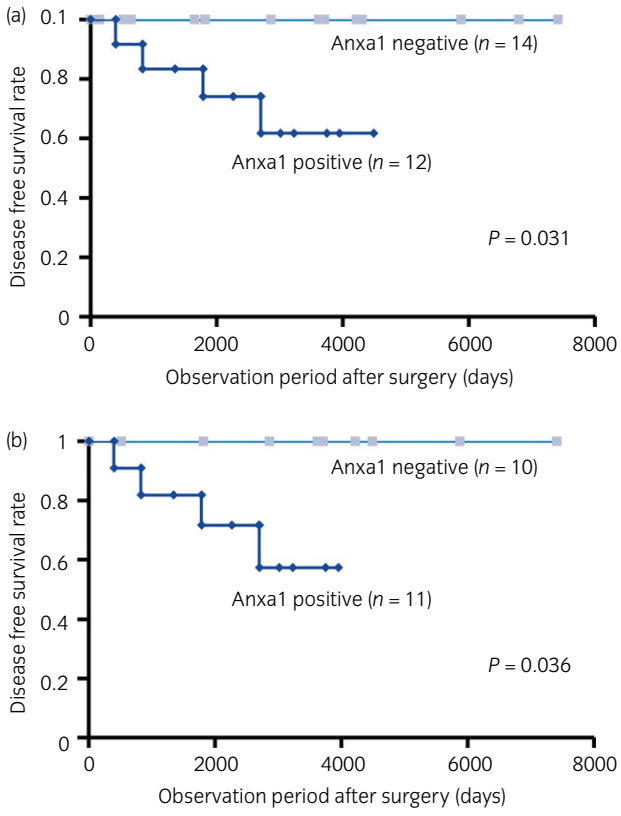

Fig. 2 (a) Kaplan–Meier curves of all studied RCC cases ($n = 26$). Anxa1-negative cases show significantly better prognosis than Anxa1-positive cases ($P = 0.031$). (b) Kaplan–Meier curves focusing on ccRCC cases ($n = 21$). Anxa1-negative cases show significantly better prognosis than do Anxa1-positive cases ($P = 0.036$).

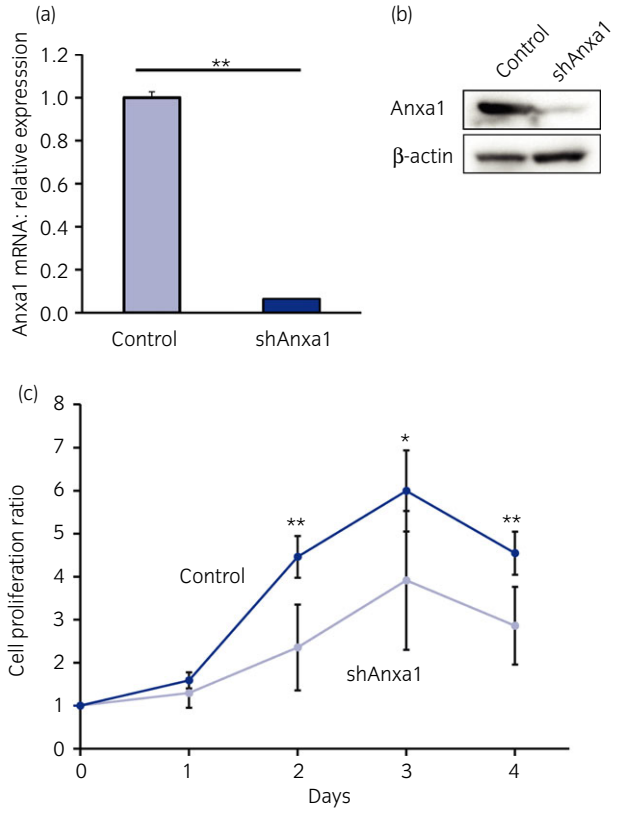

Fig. 3 (a) Relative expression of Anxa1 mRNA in Anxa1-knocked down Caki-1 cells. Expression levels of Anxa1 relative to GAPDH are shown. The value of control cells is set as 1. $^{**}P < 0.01$. (b) Western blotting analysis of Anxa1 knockdown Caki-1 cells. β-Actin serves as the loading control. (c) Proliferation of Anxa1 knockdown Caki-1 cells. A cell growth ratio is calculated as the A_{490} value each day divided by the value at day 0. Results are expressed as the mean ± SD. $^{*}P < 0.05$. $^{**}P < 0.01$.

Anxa1 knockdown decreases RCC cell proliferation

We next assessed Anxa1 function in Caki-1 cells, which are derived from human ccRCC. To do so, we knocked down the *ANXA1* gene with *ANXA1*-specific shRNA or control shRNA using a retroviral system. qRT-PCR analysis showed that *ANXA1* knockdown by *ANXA1*-specific shRNA efficiently decreased *ANXA1* mRNA expression compared with control shRNA (Fig. 3a), and western blotting analysis confirmed a significant decrease in Anxa1 protein levels in knockdown relative to control cells (Fig. 3b). Then, we assessed the effects of Anxa1 knockdown on Caki-1 cell proliferation *in vitro*. Proliferation in Anxa1 knockdown cells was significantly lower than that seen in control cells based on an MTS assay ($P < 0.05$; Fig. 3c).

Anxa1 knockdown antagonizes Caki-1 cell invasiveness and motility

We next evaluated the effects of Anxa1 knockdown on invasivity using a Matrigel invasion assay. Analysis showed that Anxa1 ablation decreased Caki-1 cell invasiveness (Fig. 4a,b). Furthermore, when we evaluated the cell motility using a scratch assay, the motility significantly decreased in Anxa1-knockdown cells within 14 h of making the scratch relative to control cells ($P < 0.01$; Fig. 4c,d). We further assessed the adhesiveness of Caki-1 cells to the ECM. We found that Caki-1 cell adhesion to the ECM was weakened by Anxa1 knockdown (Fig. 4e). Collectively, these results suggested that Anxa1 deficiency in Caki-1 cells attenuates invasiveness, cell motility and adhesion to the ECM. We further evaluated the proliferation, invasiveness and motility of VMRC-RCW cells, in which the *ANXA1* mRNA expression level was lower than Caki-1 (Fig. S1). These activities of VMRC-RCW were lower than Caki-1, consistent with the results of Anxa1 knockdown cells (Fig. S1).

MT1-MMP expression decreases in Anxa1-knocked down Caki-1 cells

We finally investigated the molecular basis for phenotypes described above using *in vitro* analysis. Based on our findings that Anxa1 knockdown decreases invasiveness and motility, we focused on the expression of MMPs. Interestingly, expression of the MT1-MMP transcript levels in Caki-

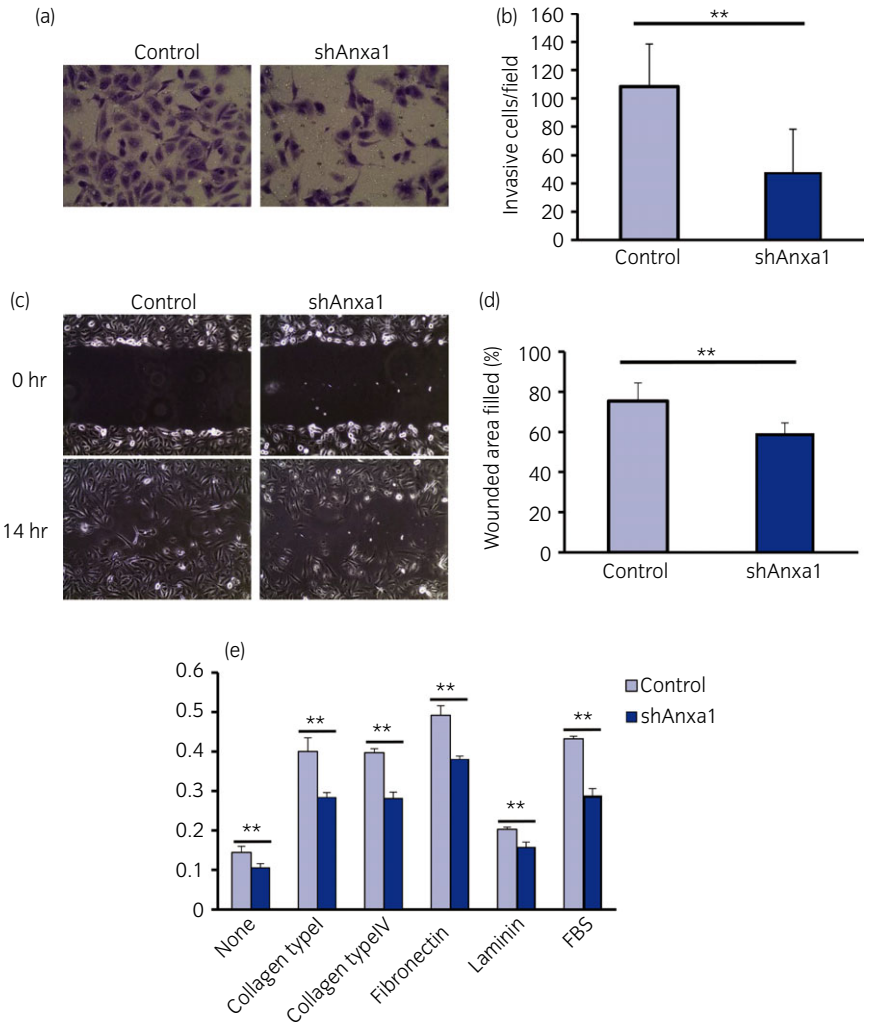

Fig. 4 (a,b) Matrigel invasion assay. (a) Representative images of control and Anxa1 knockdown invading Matrigel. (b) Invading cells were counted in five randomly chosen fields in triplicate wells. Results are expressed as the mean ± SD ($n = 15$). $^{**}P < 0.01$. (c,d) Scratch assay. (c) Representative photographs were taken immediately (0 h) and 14 h after wounding. (d) Filling of wounded areas was evaluated using ImageJ software, and the percentage of wound closure was calculated for each area. Results are expressed as the mean ± SD ($n = 6$). $^{**}P < 0.01$. (e) ECM adhesion assay. After pre-coating 96-well plates with the indicated ECM factors, adhesion assays were carried out as described in the Methods. Results are expressed as the mean ± SD ($n = 4$). $^{**}P < 0.01$.

1 cells were significantly lower in Anxa1 knockdown compared with control cells (Fig. 5a), but not MMP-2 and MMP-9 (Fig. S2), prompting us to monitor mRNA encoding HIF-1α, a transcription factor positively regulating MT1-MMP expression.[16] HIF-1α transcript levels decreased in Anxa1 knockdown relative to control Caki-1 cells (Fig. 5b). HIF-1α protein expression levels also decreased in Anxa1 knockdown relative to control Caki-1 cells, and enhanced HIF-1α expression by $CoCl_2$ further supported the above result (Fig. 5c). Collectively, these results suggest that Anxa1 controls RCC cell motility and invasion by regulating the expression of HIF-1α and MT1-MMP.

Discussion

The present study shows that Anxa1 expressed on the membrane of RCC cells from patients' specimens was significantly correlated with DFS. Furthermore, Anxa1 knockdown in the RCC cell line, Caki-1, attenuated cell proliferation, motility and invasion *in vitro*. Both of these lines of evidence suggest that Anxa1 expression in RCC is positively correlated with patients' malignant outcome.

Previous studies on non-neoplastic kidney tissues showed that Anxa1 was immunolocalized in the Bowman's capsule, the loop of Henle, collecting tubules and ducts.[17] Furthermore, Anxa1 expression in RCC was reportedly correlated with malignancy.[18] These results support the present findings of high expression of Anxa1 in RCC and weak expression of Anxa1 in non-cancerous glomeruli. Although Anxa1 expression was higher in RCC with poor prognosis, statistically significant differences in other clinicopathological indications, such as tumor stage, venous invasion and nuclear grade, were not observed between Anxa1-positive and -negative groups. We judged Anxa1 staining as positive when it was more intense in the membrane of tumor cells than in vessels of non-neoplastic glomeruli in the same section. In a previous study by others, the staining intensity was scored as A0 (no reaction), A1 (strong staining at the cell membranes and no or weak reaction in the cytoplasm) and A2 (strong staining at the cell membrane and cytoplasm).[18] Differences in these and the present results are likely due to the difference in scoring protocol for immunohistochemical staining of Anxa1. In fact, we examined Anxa1 expression in an additional three cases of rhabdoid-cell type RCC that show aggressive invasiveness and hence have poor prognosis, and found that Anxa1 was diffusely and highly expressed on the tumor cell membrane in all the three cases (data not shown). These studies show that Anxa1 is overexpressed in RCC specimens, and its upregulation is associated with malignancy and poor prognosis in patients. A larger number of patients will be required for further investigations.

For functional analysis, we used a human RCC cell line, Caki-1, which expresses Anxa1 constitutively. We found that constitutive Anxa1 knockdown using shRNA system decreased their proliferation, motility and invasion *in vitro*, strongly supporting the idea that Anxa1 is positively correlated with RCC malignancy. Another group also used Caki-1 cells for studying the potential effects of Anxa1 expression on renal cell migration.[18] They found that transient transfection of Anxa1 cDNA into Caki-1 cells did not alter haptotactic cell migration towards an ECM protein fibronectin.[18] They also reported that cell migration towards vitronectin and collagen type I increased slightly in Anxa1-overexpressing Caki-1 cells, but the change was not significant.[18] The

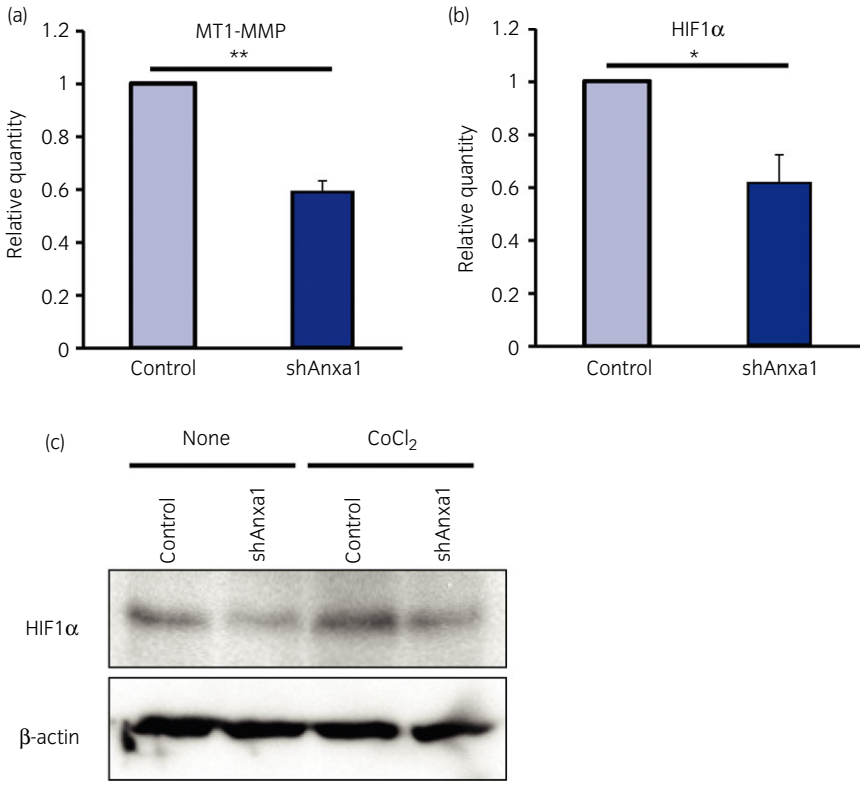

Fig. 5 The levels of (a) MT1-MMP and (b) HIF-1α mRNA transcripts relative to GAPDH in Anxa1 knockdown (shAnxa1) versus control Caki-1 cells. The value of control cells in each panel was set as 1. *$P < 0.05$, **$P < 0.01$. (c) Western blotting analysis of HIF-1α protein expression in shAnxa1 versus control Caki-1 cells with or without 100 μmol/L $CoCl_2$. β-Actin serves as the loading control.

difference between the present findings and theirs was probably due to the constitutive expression of Anxa1 in original Caki-1 cells. We used a constitutive knockdown system by a retroviral vector, whereas they used transient transfection of cDNA into originally Anxa1 expressed Caki-1 cells.

Although molecular mechanisms underlying Anxa1-dependent malignancy of RCC have been unknown, Anxa2 reportedly regulates RCC cell motility.[19] Anxa2 and Anxa1 have approximately >50% homology in their amino acid sequences, and both have Ca^{2+}-dependent actin filament-binding activity within cholesterol-rich membrane domains.[20] Anxa2 regulates the organization of membrane-associated actin by promoting association of lipid microdomains or in conjunction with other actin-binding proteins, such as α-actin, ezrin and actin.[21] Marjo *et al.* reported that Anxa1 silencing by shRNA reduced cell spreading and inhibited the formation of stress fibers at 24 h after cell seeding, whereas at 48 h, the stress fibers were short and the cells were irregularly shaped.[22] Therefore, Anxa1 might regulate RCC cell motility by regulating actin cytoskeletal remodeling. However, further studies are required to elucidate the molecular mechanisms of Anxa1 in RCC.

Others have shown that MT1-MMP is a key regulator of mesenchymal cell activity.[16] The same study also showed that the cytoplasmic tail of MT1-MMP binds FIH-1 and promotes a stable FIH-1–Mint3 interaction. Mint3-bound FIH cannot hydroxylate HIFα. Thus, MT1-MMP activates HIF during normoxia. HIF promotes the expression of many genes, including MT1-MMP and MT1-MMP expression, and HIF activation might form a positive feedback loop in both mesenchymal and cancer cells.[16] These results support our data that levels of HIF-1α and MT1-MMP mRNAs significantly decrease in Anxa1-knocked down versus control Caki-1 cells (Fig. 5).

In conclusion, the present study indicates overall that patients with RCC showing strong Anxa1 expression in the tumor cell membrane exhibited shorter DFS and worse prognosis than RCC patients whose tumor cells express weak or no Anxa1 expression. In this regard, exploitation of Anxa1 as a drug target might improve the prognosis for RCC patients.

Conflict of interest

None declared.

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Supporting information

Additional Supporting Information may be found in the online version of this article at the publisher's web-site:

Figure S1. A comparison between Caki-1 and the VMRC-RCW phenotype.

Figure S2. Relative expression of MMP-2 and MMP-9 mRNA in Anxa1-knocked down Caki-1 cells.

Table S1. Clinicopathological data and Anxa1 expression data in 27 RCC cases.

Table S2. Relationship between clinicopathological variables and Anxa1 expression status on RCC tumor vasculature.

Table S3. Recurrence site and treatment for recurrence in cancer recurrence-positive cases.

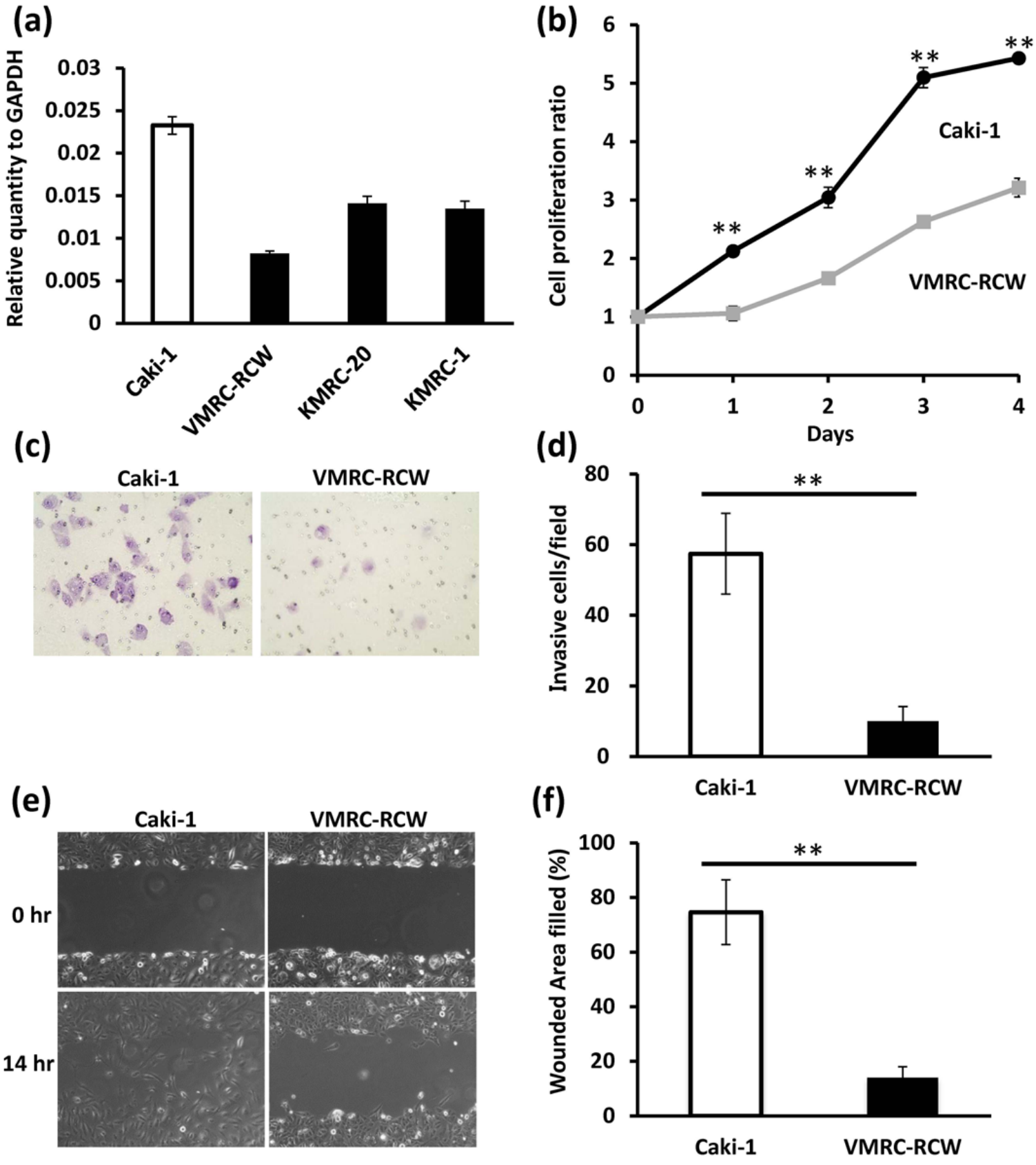

Fig. S1. A comparison between Caki-1 and VMRC-RCW phenotype. (a) Relative expression of Anxa1 mRNA in RCC cell lines. Expression levels of Anxa1 relative to GAPDH are shown. (b) Proliferation of Caki-1 and VMRC-RCW cell lines. A cell growth ratio is calculated as the A_{490} value each day divided by value at day 0. Results are expressed as means +/- SD. **, $P < 0.01$ (c and d) Matrigel invasion assay. (c) Representative images of Caki-1 and VMRC-RCW cells invading matrigel. (d) Invading cells were counted in 5 randomly chosen fields in triplicate wells. Results are expressed as means +/- SD (n = 15). **, $P < 0.01$ (e and f) Scratch assay. (e) Representative photographs were taken immediately (0 h) and 14 h after wounding. (f) Filling of wounded areas was evaluated using ImageJ software, and the percent of wound closure was calculated for each area. Results are expressed as means +/- SD (n = 6). **, $P < 0.01$

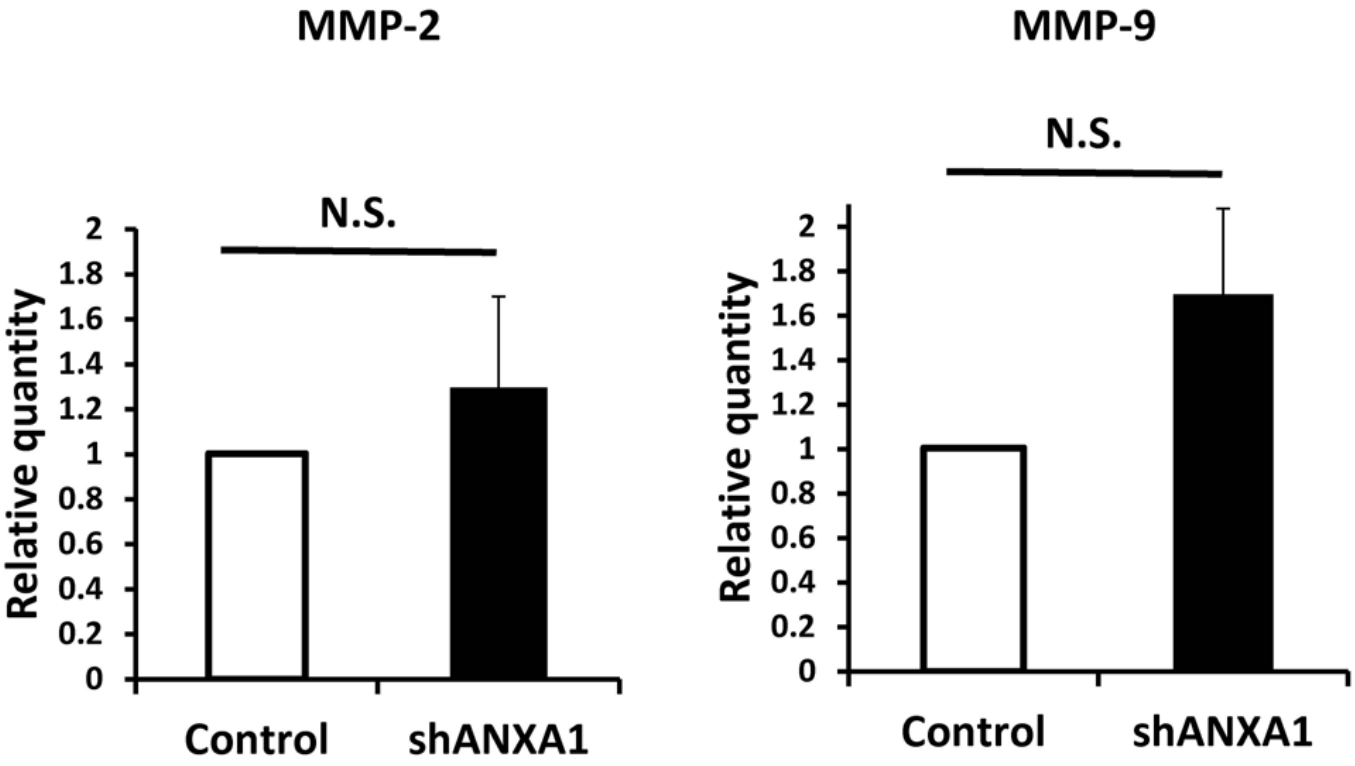

Fig. S2: Relative expression of MMP-2 and MMP-9 mRNA in Anxa1-knocked down Caki-1 cells. N.S., no significantly difference.