

Original article

$\alpha$ B-Crystallin in clear cell renal cell carcinoma: Tumor progression and prognostic significance<sup>☆</sup>

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**Abstract**

**Objectives:** AlphaB-crystallin ( $\alpha$ B-crystallin), a small heat shock protein, has been reported to be involved in the growth, antiapoptosis, migration, and chemoresistance of human malignancies.

**Materials and methods:**  $\alpha$ B-crystallin expression in normal renal and clear cell renal cell carcinoma (ccRCC) tissues was examined with two-dimensional (2D) gel electrophoresis assays. Immunohistochemistry was conducted to determine the presence of  $\alpha$ B-crystallin-positive tumor cells and staining intensity in 50 cases of ccRCC tissue samples. The association of  $\alpha$ B-crystallin protein expression, clinicopathologic parameters and prognosis of ccRCC patients was also analyzed with Student's *t*-test and Kaplan-Meier analysis. Moreover, Western blot assays were performed to detect the protein expression of  $\alpha$ B-crystallin in normal and tumor tissues and the alteration of cell cycle regulators in  $\alpha$ B-crystallin-overexpressing cells. MTT (3-[4,5-dimethylthiazol-2-yl]-2,5-diphenyltetrazolium bromide), BrdU, and transwell assays were performed to demonstrate the effects of  $\alpha$ B-crystallin overexpression on cell growth, DNA synthesis and cell migration of ccRCC cells, respectively.

**Results:** The results showed the up-regulation of  $\alpha$ B-crystallin expression in ccRCC tissues. Overall survival of ccRCC patients was significantly correlated with  $\alpha$ B-crystallin expression in tumor tissues. We found that  $\alpha$ B-crystallin overexpression increased the expression of cyclin A and the incorporation of BrdU, which may be related to the enhancement of cell growth. Transwell analyses demonstrated that presence of  $\alpha$ B-crystallin overexpression enhanced cell migration in ccRCC cells. Furthermore, rapamycin-resistance of tumor cells was induced when  $\alpha$ B-crystallin was overexpressed.

**Conclusions:** Our experimental findings highlight the importance of  $\alpha$ B-crystallin in the tumor growth, migration, and target therapy-resistance of ccRCC cells. © 2013 Elsevier Inc. All rights reserved.

**Keywords:** Clear cell renal cell carcinoma;  $\alpha$ B-crystallin; Prognostic factor; Proliferation; Migration; Drug resistance

**1. Introduction**

Renal cell carcinoma (RCC) is the most common type of kidney cancer in adults, and mainly arises from the proximal renal tubules [1,2]. Histopathologically, about 80% of RCC are clear-cell type (ccRCC), about 15% of RCC are papillary type, and the remaining 5% are other types [3]. Angiogenesis has been reported to play a cen-

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tral role in both primary tumor growth and distant metastasis in ccRCC. This could result from the mutation or hypermethylation of the von Hippel-Lindau (VHL) gene, with subsequent activation of hypoxia-inducible factor (HIF) accompanied by the downstream induction of vascular endothelial growth factor (VEGF) [4]. Therapies targeted at the VEGF and mammalian target of rapamycin (mTOR) pathways have become the standard of care in advanced RCC. However, acquired resistance typically occurs in patients after a period of 6–15 months of treatment [5]. Currently, there are only limited amounts of reliable diagnostic, prognostic, and therapeutic markers for ccRCC. The mechanism of drug-resistance of clear cell renal cell carcinoma (ccRCC) also requires further investigations.

AlphaB-crystallin ( $\alpha$ B-crystallin), a small heat shock protein (sHSP), is a major component of vertebrate lens, and is also expressed in nonlenticular tissues, such as renal tubular epithelium [6,7]. The structure of  $\alpha$ B-crystallin contains a conserved, approximately 90-aa region (termed the  $\alpha$ -crystallin core) with divergent amino- and carboxyterminal domains [8,9]. It has been observed that  $\alpha$ B-crystallin is overexpressed in gliomas [10], breast carcinomas [8,11], and ductal carcinoma in situ compared with the matched normal breast tissues [12]. However, down-regulation of  $\alpha$ B-crystallin has also been reported in highly malignant anaplastic thyroid carcinomas [13].

$\alpha$ B-crystallin is activated in response to stresses, such as heat shock, radiation, oxidative stress, and exposure to anticancer drugs. Previous studies have demonstrated that  $\alpha$ B-crystallin serves as an antiapoptotic molecule because it suppresses the activity of various apoptotic proteins, including caspase-3 [14,15]. Overexpression of wild-type  $\alpha$ B-crystallin promotes xenograft tumor growth and inhibits tumor necrosis factor-related apoptosis-inducing ligand (TRAIL)-induced apoptosis *in vivo* in nude mice, whereas a pseudophosphorylation  $\alpha$ B-crystallin mutant, whose anti-apoptotic function is impaired, abolishes xenograft tumor growth [15]. Another characteristic of  $\alpha$ B-crystallin is that this protein may result in chemoresistance in patients, which was suggested by a positive correlation between  $\alpha$ B-crystallin expression and resistance to neoadjuvant chemotherapy in breast cancer, found by Ivanov et al. [16]. Since  $\alpha$ B-crystallin could cause chemo-resistance, it would be of clinical interest to know whether  $\alpha$ B-crystallin also results in resistance to target therapy.

The aim of this study is to investigate the expression of  $\alpha$ B-crystallin in ccRCC tissues and determine its prognostic significance. We also evaluate the association between  $\alpha$ B-crystallin expression and cellular growth, migration, and rapamycin-resistance of ccRCC.

## 2. Materials and methods

### 2.1. Tissue sample

For immunohistochemistry, paraffin-embedded specimens from 50 patients with ccRCC were obtained from the Department of Pathology of our Institute with the approval of the Research Ethics Committee (200,812,064R). Only patients who underwent partial or radical nephrectomy in our institute during the period from 2002 to 2009 and agreed to participate in clinical studies were included. To prevent selection biases, no other criteria were applied to selection and inclusion of patients.

### 2.2. Cell culture

Caki-2 cells were grown in the RPMI-1640 Medium (HiMedia Laboratories, Pvt., Mumbai, India) containing 10% fetal bovine serum (FBS). Cells were maintained as monolayers and incubated in a humidified 37°C incubator. After the cells had grown to confluence, they were disaggregated in a trypsin solution (GIBCO, Grand Island, NY), washed with media containing 10% FBS, centrifuged at  $125 \times g$  for 5 minutes, resuspended, and then subcultured according to standard protocols.

### 2.3. Two-DE and image analysis

Samples from 9 patients with ccRCC were prepared in 4% CHAPS, 9 M urea, 2% ampholytes (pH 3–10 nonlinearity), and 40 mM dithiothreitol (DTT), and the protein content was quantified by bicinchoninic acid (BCA) protein assay (Pierce). A total of 300- $\mu$ g protein was loaded onto immobilized pH gradient (IPG) gel strips (pH 3–10, 13 cm; GE Healthcare). The IPG strips were rehydrated overnight. For the first-dimensional separation, isoelectric focusing (IEF) was carried out using Ettan IPGphor II (GE Healthcare) at 300–8,000 V for 16 hours. After IEF, the IPG strips were equilibrated for 10 minutes each in 2 equilibration solutions (50 mM Tris-HCl, pH 8.8, 6 M urea, 2% SDS, 30% glycerol containing 100 mg DTT, or 250 mg iodoacetic acid). The 2-D electrophoresis was conducted at 130–250 V for 5–6 hours. The gels were stained with Sypro-Ruby overnight. The 2-DE gel images were scanned using a fluorescence image scanner Typhoon 9400 (GE Healthcare) and analyzed by the PDQuest software (Bio-Rad).

### 2.4. In-gel digestion and LC-MS/MS

Based on the 2-DE analysis of samples from ccRCC patients, we selected 14 differentially expressed proteins in RCC samples ( $P < 0.05$ ) for further identification with matrix-assisted laser desorption/ionization-ion mobility separation-mass spectrometry (MALDI-MS/MS) at the proteomic core facility of the Institute of Biological Chemistry, Academia Sinica. The protein spots were cut from the 2-DE

gels, and then destained 3 times with 25-mM ammonium bicarbonate buffer (pH 8.0) in 50% acetonitrile (ACN) for 1 hour. The gel pieces were dehydrated in 100% ACN for 5 minutes and then dried for 30 minutes in a vacuum centrifuge. Enzyme digestion was performed by adding 0.5  $\mu$ g trypsin in 25-mM ammonium bicarbonate per sample at 37°C for 16 hours. The peptide fragments were extracted twice with 50  $\mu$ l 50% ACN/0.1% trifluoroacetic acid (TFA). After removal of ACN and TFA by centrifugation in a vacuum centrifuge, samples were dissolved in 0.1% formic acid/50% ACN and analyzed by MALDI-MS/MS. Proteins were identified in the National Center for Biotechnology Information (NCBI) databases based on the peptide mass fingerprint mode or the MS/MS ion search mode with the MASCOT program.

### 2.5. Immunohistochemistry

Tumor and normal tissue samples were trimmed into 5-mm slices, fixed overnight in 4% paraformaldehyde in phosphate-buffered saline (PBS), embedded in paraffin, and cut into 4- $\mu$ m thick tissue sections.  $\alpha$ B-crystallin was immunolocalized by anti- $\alpha$ B-crystallin (1:100) and an appropriate secondary antibody. The signal of  $\alpha$ B-crystallin was developed with a catalyzed signal amplification system. Human skeletal muscle tissues were used as positive controls. The primary antibody, diluted to 1:50, was applied to conducting immunohistochemistry. The percentage of positively stained cells was semiquantitatively scored by 1 pathologist who was unaware of the outcome of the patients. Staining index is determined by percentage multiplied by intensity of staining (range, 0–200).

### 2.6. Cell transfection

Transfection was performed using Lipofectamine 2000 Transfection Reagent (Invitrogen, Carlsbad, CA) and following the manufacturer's protocol.  $\alpha$ B-crystallin overexpression plasmid was a gift from Dr. Shyh-Horng Chiou (Graduate Institute of Medicine, Kaohsiung Medical University, Taiwan). Briefly, Lipofectamine 2000/DNA mixture was added dropwise onto the Dulbecco's modified Eagle medium (DMEM) without FBS, mixed gently, and incubated in a humidified 37°C incubator for 4 hours. The transfected Caki-2 cells were incubated in fresh RPMI1640 with 10% FBS for additional 24 hours. In these experiments, cells transfected with empty vector (pcDNA) served as a control. The transfection efficiency was up to 80%.

### 2.7. Determination of cell growth curve

For a measurement of cell proliferation, the cells were seeded onto 24-well plates and grown in the RPMI1640 medium supplemented with 10% FBS. Media were changed daily until cell counting. The cell number was assessed with

the MTT (3-[4,5-dimethylthiazol-2-yl]2,5-diphenyltetrazolium bromide) assay [17]. Four samples were analyzed in each experiment.

### 2.8. Quantification of DNA synthesis

DNA synthesis was quantified by the colorimetric measurement of bromodeoxyuridine (BrdU) incorporation into DNA using the BrdU cell proliferation assay kit (Chemicon) according to the manufacturer's protocol. Cell cultures were set up in 96-well tissue culture plates at  $2 \times 10^6$  cells/well in 100  $\mu$ l medium, and incubated for 24 hours in 5% CO<sub>2</sub> at 37°C. At various times of incubation, the cell proliferation rate was measured based on the measurement of BrdU incorporation during DNA synthesis. Cells were labeled with 20  $\mu$ L BrdU reagent for 2 hours at 37°C. Thereafter, 200  $\mu$ l/well of fixing solution was added and incubated for 30 minutes at room temperature. Subsequently, immunostaining to detect incorporated BrdUrd was performed for 1 hour at 4°C in the dark with mouse anti-BrdUrd primary antibody, followed by peroxidase-conjugated goat anti-mouse secondary antibody. At the end of the assay, 100  $\mu$ l of tetramethylbenzidine (TMB) peroxidase substrate was filled into each well. After 30 minutes of incubation at room temperature, the reaction was stopped with 100  $\mu$ l acid stop solution, and the absorbance of the samples was assessed. Results were expressed as the mean absorbance of the samples in an enzyme-linked immunosorbent assay (ELISA) plate reader at 450 nm.

### 2.9. Protein extraction and Western blotting analysis

To determine the expression level of  $\alpha$ B-crystallin, the total proteins extracted from cells and tissues were analyzed with Western blotting as described previously [18]. In brief, protein extracts were separated on 12% SDS-polyacrylamide gels, transferred to polyvinylidene fluoride (PVDF) membranes, treated with 5% fat-free milk powder to block the nonspecific IgGs, and incubated for 1 hour with specific primary antibodies. The blot was then incubated with anti-mouse or anti-rabbit IgG linked to horseradish phosphatase (1:1,000) for 1 hour. Subsequently, proteins were visualized with the enhanced chemiluminescence (ECL) as a substrate in a digital imaging system. The quantitative data were obtained using a computing densitometer with Image-Pro Plus software.

### 2.10. Adhesion assay

Adhesion assays were performed as previously described [19]. Briefly, cells were plated onto a gelatin (10 mg/ml; Sigma, Steinheim, Germany)-coated 24-well plate, and then washed with PBS after plating for 1, 4, 6, or 24 hours. After washing, the adherent cells were allowed to grow in the cultured medium for another 18

hours before cell counting. The cell number was estimated by the MTT assay [17].

### 2.11. Migration assay

Migration assay was performed as described previously with minor modifications [19,20]. To assess the migration potential of tumor cells, transwells were assembled in a 24-well plate, and the lower chambers were filled with 600  $\mu$ l of RPMI1640 containing 10% FBS. Two hundred microliters of cells ( $10^5$  cells/ml) were inoculated onto the upper chamber of each transwell. The plate was then placed at 37°C in 5% CO<sub>2</sub>/95% air for 24 hours. After removing the nonmigrating cells with a cotton swab, cells that had migrated to the lower surface of the filters were fixed and stained with 0.1% crystal violet/20% (vol/vol) methanol. All assays were performed in triplicate. Three random fields were chosen in each insert, and the cells were counted and photographed under a light microscope ( $\times 200$ ).

### 2.12. Statistics

All statistical analyses were performed with the SPSS software ver. 17.0 (SPSS Inc., Chicago, IL). The overall survival rates were calculated using the Kaplan-Meier method and the differences between the curves were tested using the log-rank test. The Student's *t*-test was applied to analyze the statistical significance of the associations between  $\alpha$ B-crystallin expression and clinicopathologic parameters, and of cell growth, migration, and rapamycin-resistance between  $\alpha$ B-crystallin-overexpressing and control cells. Marker independence for prediction of survival was evaluated by univariate and multivariate analyses. Significance was accepted at  $P < 0.05$ .

## 3. Results

### 3.1. Protein profiling in ccRCC tissues

To identify differentially expressed proteins in ccRCC tissues, 9 pairs of tissues from the RCC lesions and adjacent normal tissues were subject to protein profiling using 2-DE of unfractionated whole protein extract. Of the 500 detectable protein spots, 14 spots were differentially expressed in RCC samples ( $P < 0.05$ ). Nine of these 14 spots, which showed a more than 2-fold increase or decrease in intensity, were further analyzed by MALDI-MS/MS. The results revealed that a total of 6 differentially expressed proteins, including  $\alpha$ B-crystallin (Fig. 1), were identified (Table 1). Although overexpression of  $\alpha$ B-crystallin in ccRCC has been reported in other studies [6,21,22], there were no further investigations of its role in ccRCC. Therefore, in this study, we focused on the clinical implications and functions of  $\alpha$ B-crystallin in ccRCC progression.

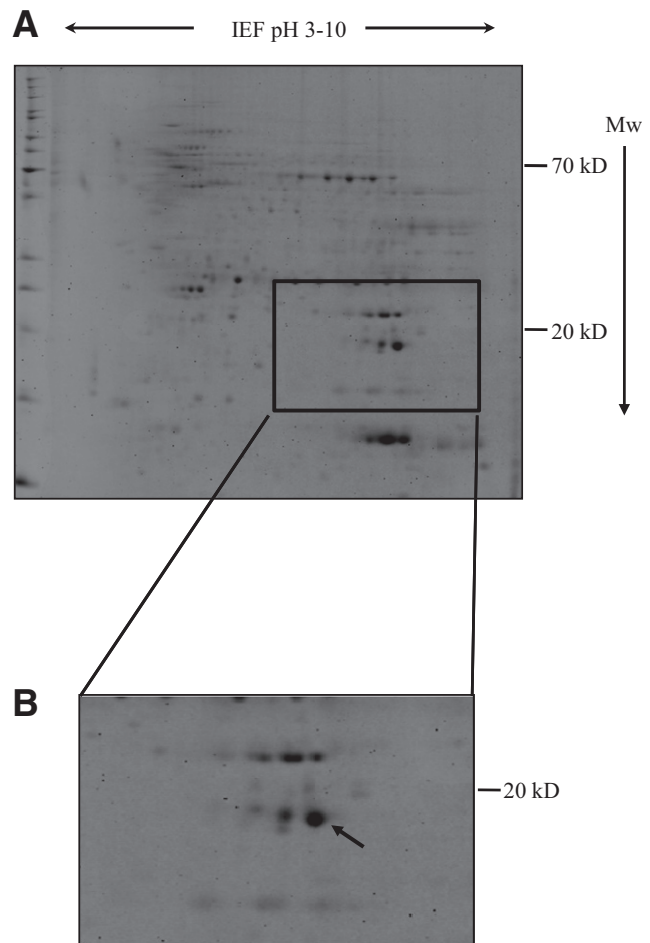


Fig. 1. Ruby fluorescence stained 2DE-gel (pH 4–10). Representative protein pattern of ccRCC tissues (A) with the blowup region (B) containing the protein spot of  $\alpha$ B-crystallin (arrow).

### 3.2. Immunostaining of $\alpha$ B-crystallin protein expression in ccRCC tissue samples

To determine the expression and distribution of  $\alpha$ B-crystallin in ccRCC tissues, immunohistochemistry was performed in 50 tumor tissues from ccRCC patients. Comprehensive clinicopathologic features of these patients are depicted in Table 2. Sixteen percent of these tumor tissues did not express  $\alpha$ B-crystallin. The median percentage of positive tumor cells was 20% (range: 0%–85%). The mean staining index for  $\alpha$ B-crystallin was 33.8 (range: 0–170) (Fig. 2A), and the staining intensity was varied (Fig. 2B–D). We also found that the tumor cells expressing  $\alpha$ B-crystallin were clustered instead of randomly distributed. These findings illustrated the diverse expression of  $\alpha$ B-crystallin in ccRCC tissues.

### 3.3. Correlations between $\alpha$ B-crystallin expression and clinicopathologic parameters

We analyzed the association between  $\alpha$ B-crystallin expression and the clinicopathologic features, such as patho-

Table 1  
Differentially expressed proteins in ccRCC

Protein name	Swiss-Prot accession no.	Score	Fold change*	P value
Vimentin	Q9NTM3	35	+2.83	0.005
Endoplasmin	P14625	50	+3.79	0.011
AP-4 complex subunit $\sigma$ -1	Q9Y587	32	−4.34	0.062
$\alpha$ -Enolase	Q8WU71	67	+2.04	0.008
Pyruvate kinase isozymes M1/M2	P14618	96	+4.13	0.003
$\alpha$ B-crystallin	P02511	76	+3.10	0.012

\* Fold change is given as the ratio of the spot intensity in normal kidney cortex over tumor tissue (negative variation or decrease) or tumor tissue over normal tissue (positive variation or increase).

logic stage, metastasis, and nuclear grade, of 50 ccRCC samples ( $P = 0.175, 0.322, \text{ and } 0.638$ , respectively). There was no specific correlation between the expression of  $\alpha$ B-crystallin and these clinicopathologic parameters.

### 3.4. Prognostic significance of $\alpha$ B-crystallin expression in ccRCC

Kaplan-Meier analysis was performed to explore the correlation between  $\alpha$ B-crystallin expression and patients' survival. The median follow-up period after nephrectomy was 44.5 (range 1–79) months. The result showed that the overall survival rate was significantly lower in the  $\alpha$ B-

crystallin-high ccRCC than in the  $\alpha$ B-crystallin-low ccRCC cases ( $P < 0.0001$  by log-rank test; Fig. 2E). Then, we investigated the independent factors related to prognosis with univariate and multivariate regression analyses (Table 3). Univariate Cox regression analysis revealed that high pathologic stage, distant metastasis, and higher  $\alpha$ B-crystallin expression were significantly correlated with poor overall survival ( $P = 0.045, 0.010, \text{ and } 0.001$ , respectively). Based on the multivariate Cox regression analysis, high  $\alpha$ B-crystallin protein expression and distant metastasis were independent prognostic factors for overall survival ( $P = 0.001 \text{ and } 0.006$ , respectively).

### 3.5. $\alpha$ B-crystallin overexpression promoted growth of ccRCC cells

To determine the role of  $\alpha$ B-crystallin in ccRCC growth, we transiently transfected Caki-2 cells, which expressed a low endogenous level of  $\alpha$ B-crystallin, with an  $\alpha$ B-crystallin overexpression plasmid. The protein level was compared with those in empty vector-transfected cells. Western blots indicated that the transfected cells showed overexpression of  $\alpha$ B-crystallin protein (Fig. 3A). To examine the effect of  $\alpha$ B-crystallin overexpression on cell growth, the MTT assay was conducted to estimate the cell number. After 48 hours, the number of  $\alpha$ B-crystallin-overexpressing cells was significantly higher than that of control cells (Fig. 3B).

### 3.6. $\alpha$ B-crystallin overexpression increased DNA synthesis in ccRCC cells

To better understand the actions of  $\alpha$ B-crystallin on a specific phase of the cell cycle, the cells were switched to the medium with 0.04% FBS for 24 hours to render them quiescent and to synchronize their cell cycle activities. After this procedure, they were first returned to the medium containing 10% FBS, and then treated with BrdU at various times for the observation of DNA synthesis. The result showed that the BrdU incorporation into  $\alpha$ B-crystallin-overexpressing cells was enhanced during the S phase of the cell cycle (Fig. 3C), implying that  $\alpha$ B-crystallin overexpression promotes DNA synthesis.

Table 2  
Patient characteristics

	Number of patients	%
Total	50	100
Sex		
Males	33	66
Females	17	34
Age (years)		
Mean age	58.8	
Range	39–82	
$\geq 65$ Years	18	36
$< 65$ Years	32	64
Pathologic stage		
pT <sub>1</sub> /pT <sub>2</sub>	42	84
pT <sub>3</sub> /pT <sub>4</sub>	8	16
Lymph node involvement		
N <sub>0</sub>	50	100
N <sub>1</sub> /N <sub>2</sub>	0	0
Distant metastasis		
Yes	7	14
No	43	86
Nuclear grade		
G <sub>1</sub> /G <sub>2</sub>	32	64
G <sub>3</sub> /G <sub>4</sub>	18	36
Survival		
Died	11	22
Alive	39	78
Staining index of $\alpha$ B-crystallin		
Low expression ( $\leq 40$ )	37	74
High expression ( $> 40$ )	13	26

According to the mean staining index of  $\alpha$ B-crystallin, 40 was recommended to use as cut-off by the pathologist.

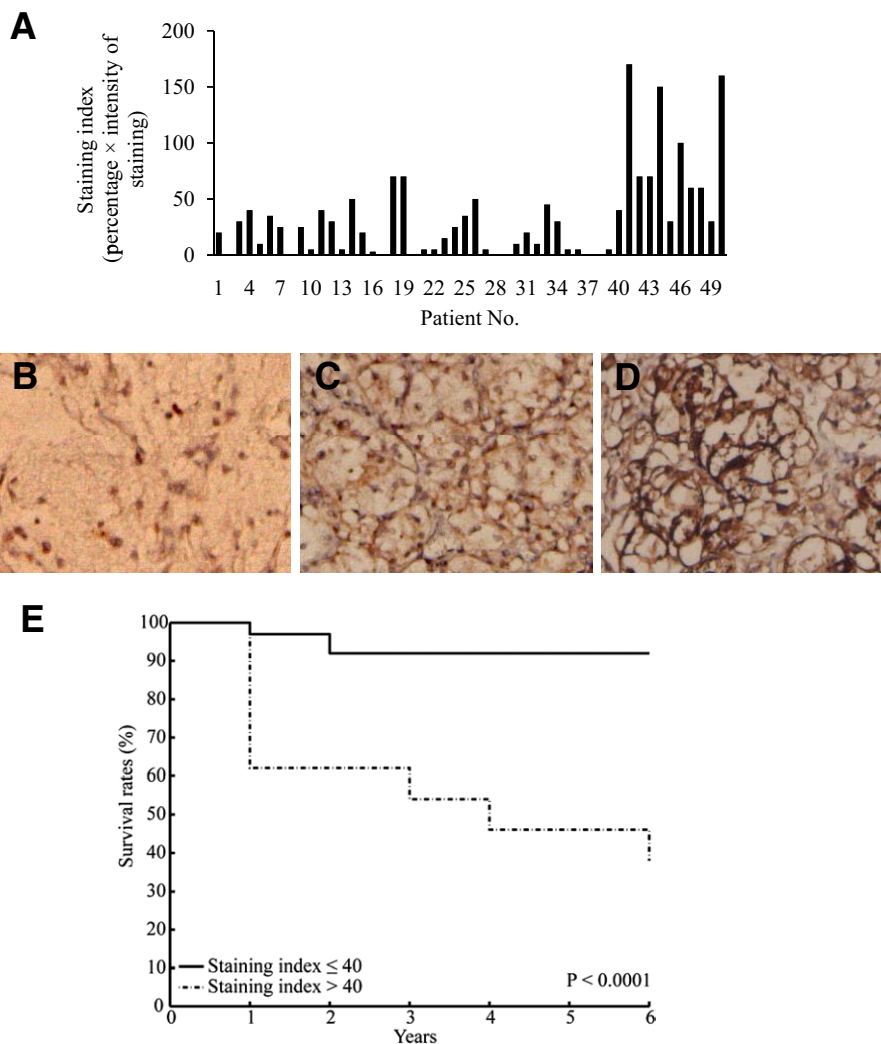


Fig. 2. Expression of  $\alpha$ B-crystallin in tumor tissues and the prognostic significance in ccRCC. (A) the percentage of  $\alpha$ B-crystallin-positive cells in 50 ccRCC tumor tissues.  $\alpha$ B-crystallin expression was analyzed by immunohistochemistry in ccRCC tissues, and each bar represents the percentage of  $\alpha$ B-crystallin in one ccRCC tumor sample that was semiquantitatively scored by one pathologist. (B)–(D) Immunostaining of  $\alpha$ B-crystallin expression in ccRCC tissues.  $\alpha$ B-crystallin was mostly localized in the cytoplasm of ccRCC tumor cells. (B) Negative expression (–,  $\times 200$ ); (C) weak expression (+1,  $\times 200$ ); (D) moderate expression (+2,  $\times 200$ ); (E) the overall survival rate was significantly lower in the  $\alpha$ B-crystallin-high ccRCC cases. Statistical analysis was done by using the log-rank test. The corresponding *P* values are indicated. (Color version of figure is available online.)

3.7. Alterations in cell cycle activity

To investigate the molecular mechanisms underlying the  $\alpha$ B-crystallin overexpression-induced DNA synthe-

sis, the cells with  $\alpha$ B-crystallin overexpression or empty vector transfection were switched to the medium containing 0.04% FBS for 24 hours to render them quiescent at the G0/G1 phase. They were then returned to the culture

Table 3  
Univariate and multivariate analysis of prognostic variables by Cox regression analysis

Variables	Univariate analysis		Multivariate analysis	
	HR (95% CI)	<i>P</i> value	HR (95% CI)	<i>P</i> value
Pathologic stage	3.57 (1.03–12.41)	0.045	1.36 (0.19–9.58)	0.758
Nuclear grade	2.53 (0.77–8.31)	0.127	4.26 (0.68–26.71)	0.121
Distant metastasis	5.41 (1.50–19.55)	0.010	7.36 (1.78–30.47)	0.006
Staining index	9.59 (2.54–36.24)	0.001	15.76 (2.94–84.59)	0.001

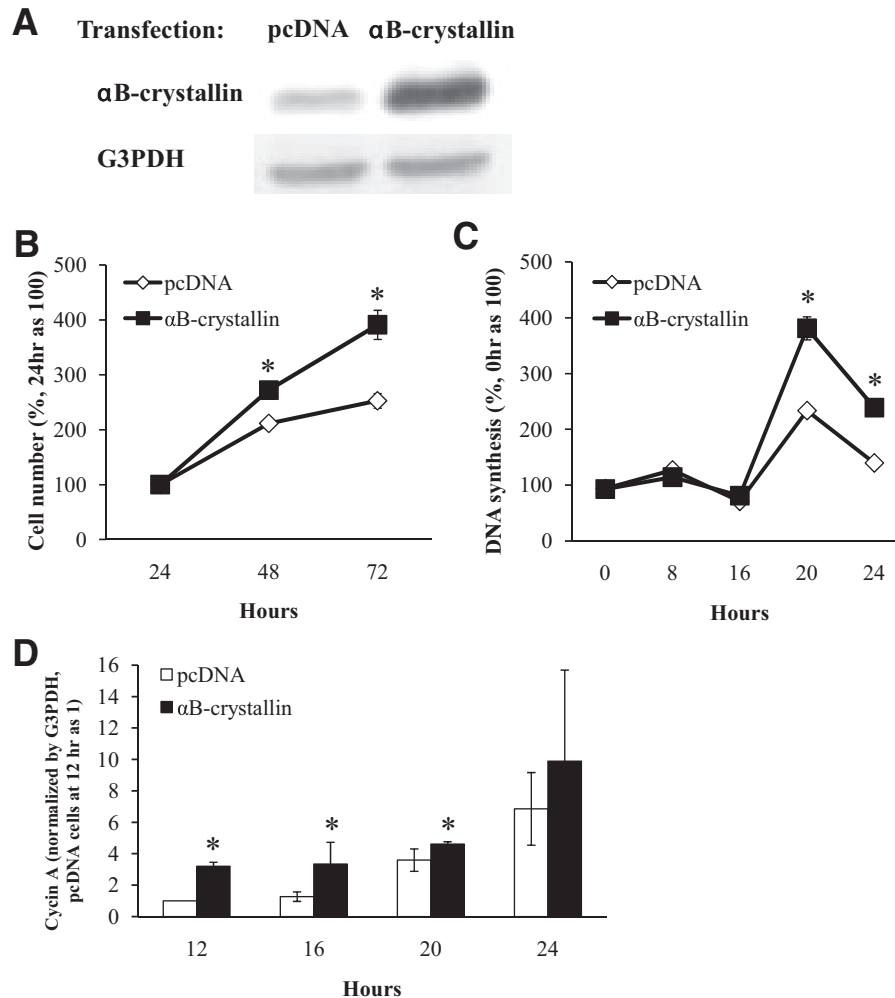


Fig. 3. Effects of  $\alpha$ B-crystallin overexpression on cell growth of ccRCC cells. (A) Western blot detection of  $\alpha$ B-crystallin expression in  $\alpha$ B-crystallin-overexpressing and control cells. (B)  $\alpha$ B-crystallin overexpression promoted the growth of ccRCC cells. The cell number was assessed by MTT assay. (C) DNA synthesis was increased when cells overexpressed  $\alpha$ B-crystallin. (D) Effect of  $\alpha$ B-crystallin overexpression on the protein level of cyclin A. Western blot analysis was carried out to examine the change of cyclin A expression in the  $\alpha$ B-crystallin-overexpressing cells. Proteins were extracted from the cultured cells at 12–24 hours after 10% FBS was added in the medium to allow for the re-entry of cell cycle, and probed with proper dilutions of specific antibodies.  $\alpha$ B-crystallin overexpression increased the protein level of cyclin A. Results from a representative experiment are shown. Membrane was probed with an anti-G3PDH antibody to verify equivalent loading. Three samples were analyzed in each group, and values represent the means  $\pm$  SEM. The quantified results were adjusted with G3PDH and expressed as relative fold change to control cells (pcDNA) at 12 hr. \* $P < 0.05$  vs. control group.

medium supplemented with 10% FBS; 12–24 hours later, these cells were harvested for protein extraction and Western blot analysis to examine the effects of  $\alpha$ B-crystallin overexpression on the expression of cell cycle regulatory proteins, such as cyclins, cyclin-dependent kinases (CDKs), and CDK inhibitors (CKIs). Fig. 3D illustrates that the protein levels of cyclin A, but not cyclin B, D1, D3, and E, CDK2 and CDK4 (data not shown) were increased in the  $\alpha$ B-crystallin-overexpressing cells. We also examined the protein levels of p21, p27, and p53, 3 known CKIs, in the transfected cells. The protein level of these CKIs showed no significant differences between the  $\alpha$ B-crystallin-overexpressing and control cells (data not shown). The above results suggested that  $\alpha$ B-crystallin might increase DNA synthesis by up-regulating cyclin A expression.

### 3.8. $\alpha$ B-crystallin overexpression enhanced migration of ccRCC cells

To examine whether  $\alpha$ B-crystallin also regulates the migration of RCC cells, transwell migration assay was performed in  $\alpha$ B-crystallin-overexpressing cells. As shown in Fig. 4A, the migration of RCC cells was enhanced when  $\alpha$ B-crystallin was overexpressed. Since the attachment and adhesion are critical steps for the process of migration and would affect the cell invasion, we further examined whether  $\alpha$ B-crystallin overexpression could affect the cell adhesion on the extracellular matrix. Adhesion of  $\alpha$ B-crystallin-overexpressing cells was found to be significantly higher than that of control cells at 1 to 6 hours after plating, but there was no difference at 24 hours (Fig. 4B), suggesting that  $\alpha$ B-crystallin overexpression enhanced cell migration but not adhesion.

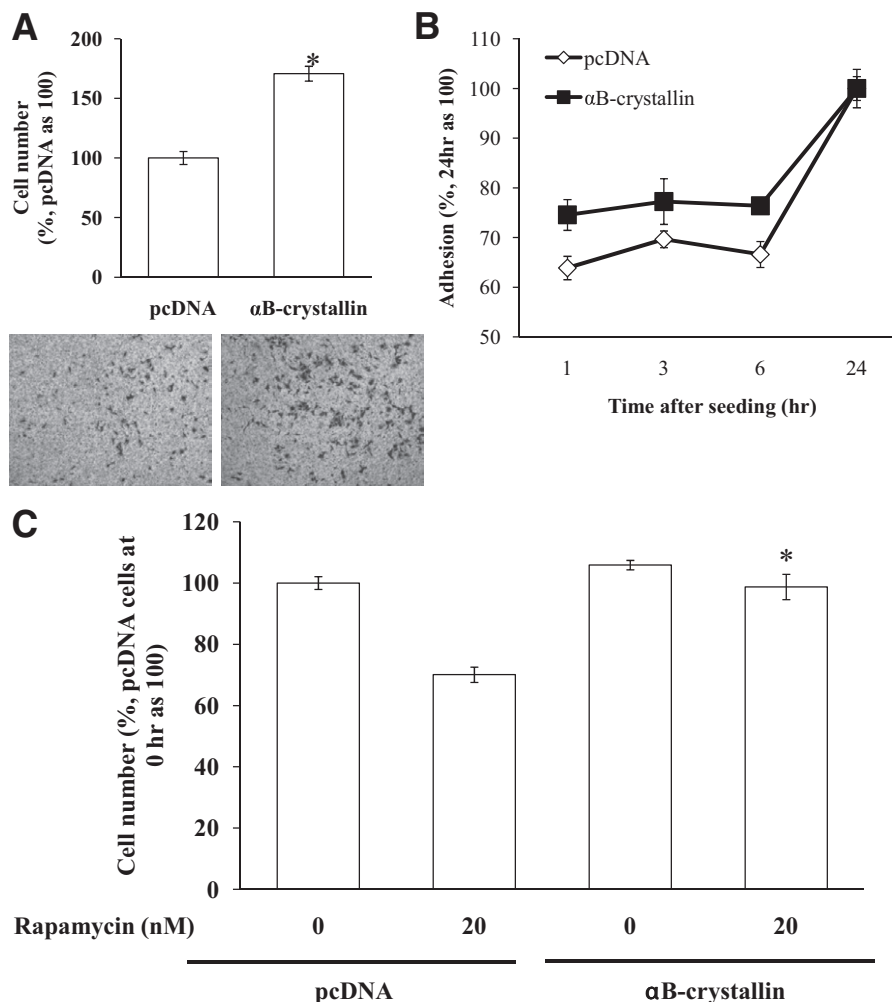


Fig. 4. Effects of  $\alpha$ B-crystallin overexpression on ccRCC cell migration, adhesion and rapamycin-resistance. (A) Upper panel,  $\alpha$ B-crystallin overexpression significantly enhanced the migration of ccRCC cells. Lower panel, representative photographs show the difference between  $\alpha$ B-crystallin-overexpressing and control cells. (B) Effects of  $\alpha$ B-crystallin overexpression on cell adhesion to extracellular matrix. Wells were coated with gelatin (10 mg/ml). Caki-2 cells were transfected with pcDNA or  $\alpha$ B-crystallin-overexpressing plasmid, and then seeded on the precoated plates and allowed to attach for 1, 4, 6, or 24 hours. Numbers of adherent cells were assessed by the MTT assay. (C)  $\alpha$ B-crystallin overexpression in ccRCC cells led to rapamycin-resistance. Caki-2 cells were transfected with  $\alpha$ B-crystallin-overexpressing or pcDNA plasmid, and then plated on the 24-well plates for 24 hours. After that, cells were treated with or without rapamycin (20 nM) for 2 days. The cell number was assessed by the MTT assay. Three samples were analyzed in each group, and values represent the means  $\pm$  SEM. \* $P < 0.05$  vs. control group.

### 3.9. $\alpha$ B-crystallin overexpression could induce rapamycin-resistance of ccRCC cells

It has been reported that some ccRCC patients are inherently resistant to mTOR-targeted approaches, and most patients acquire resistance over time [5]. Therefore, the biological basis for resistance to these target therapies and the clinical approach in this setting is of utmost interest. In the present study, the MTT assay was applied to evaluate the effect of  $\alpha$ B-crystallin overexpression on rapamycin-induced inhibition cell growth. The result showed that rapamycin caused a 30% inhibition of cell proliferation in the control group, whereas  $\alpha$ B-crystallin overexpression abolished the inhibitory effects of rapamycin on RCC cell growth (Fig. 4C), demonstrating the role of  $\alpha$ B-crystallin in rapamycin-resistance.

## 4. Discussion

Some characteristics of RCC, e.g., the lack of early-warning signs (which results in a high proportion of patients with metastases), diverse clinical manifestations, and resistance to radiotherapy and chemotherapy are of clinical significance [1]. Environmental and genetic factors play important roles in renal tumorigenesis [1,23]. A broadened and deepened understanding of molecular pathways underlying the RCC progress has made the search for prognostic markers possible [24]. Identification and validation of novel molecular markers will enable us to explore additional prognostic factors to identify RCC patients at high risk of tumor progression, and develop more effective therapeutic strategies.

Many antiapoptotic factors, such as Bcl-2, have been detected to be overexpressed in various solid tumors [25,26]. Recently,



$\alpha$ B-crystallin was reported to function as an antiapoptotic factor, as it can prevent TRAIL-induced and p53-dependent apoptosis by suppressing caspase-3 and RAS activation, respectively [15,27].  $\alpha$ B-crystallin overexpression has been documented in many human solid tumors and is usually associated with poor prognosis of tumor patients [28–30]. Chin et al. showed that  $\alpha$ B-crystallin overexpression is associated with high tumor stage, lymph node involvement, and local recurrences in patients with head and neck cancer [31]. Tang et al. demonstrated that the expression of  $\alpha$ B-crystallin is highly related to the transferability and invasive capacity of hepatocellular carcinoma cells, which may result in different survival rates in hepatocellular carcinoma patients [32]. Additionally, Moyano et al. reported that  $\alpha$ B-crystallin is commonly expressed in basal-like tumors, and can be used to predict poor survival in breast cancer patients [30]. In our study, we found that the expression of  $\alpha$ B-crystallin is an independent prognostic factor for ccRCC patients, suggesting that this protein could also be important for ccRCC tumorigenesis.

$\alpha$ B-crystallin has been demonstrated to regulate the cell growth and migration. In normal lens epithelial cells, the expression of  $\alpha$ B-crystallin along the axis of the dividing cells during cytokinesis and the presence of abnormal mitotic profiles in  $\alpha$ B-crystallin<sup>-/-</sup> cells indicate the involvement of  $\alpha$ B-crystallin in mitosis [33]. That  $\alpha$ B-crystallin strongly interacts with FGF-2, NGF- $\beta$ , VEGF, and insulin in chaperone sequences suggests that  $\alpha$ B-crystallin affects cell differentiation and proliferation via its chaperone activity [34].  $\alpha$ B-crystallin overexpression also induces EGF- and anchorage-independent growth, increases cell migration and invasion, and constitutively activates the mitogen-activated protein kinase MAPK kinase/ERK (MEK/ERK) pathway in breast cancer cells [30]. Furthermore, Maddala et al. found that  $\alpha$ B-crystallin participates in actin dynamics during cell migration, as it is colocalized with actin,  $\beta$ -catenin, WAVE-1 (a promoter of actin nucleation), Abi-2 (a component of WAVE-1 protein complex), and Arp3 (a protein of the actin nucleation complex) [35]. Our study revealed that  $\alpha$ B-crystallin overexpression elevated cyclin A expression in ccRCC cells, and this elevated expression of cyclin A might have contributed to a 1.5-fold increase in DNA synthesis and, consequently, promoted cell growth. We also found that  $\alpha$ B-crystallin overexpression significantly enhanced cell migration without affecting cell adhesion.

Heat shock proteins, such as HSP27, HSP60, and HSP90, have been reported to be overexpressed and to participate in carcinogenesis and tumor progression in various tumors [36–39]. Moreover, the expression of HSPs is related to increased resistance to apoptosis induced by cytotoxic agents, and has therefore been implicated in drug resistance [40]. In prostate cancer, overexpression of HSP27 is highly correlated with castrate-resistance [41]. Zoubeidi et al. showed that HSP27 overexpression increases IGF-I-induced phosphorylation of Erk, p90Rsk, and Akt, and such a finding elucidates the interactions between HSP27 phos-

phorylation and the IGF-I receptor signaling pathway and highlights the role of HSP27 in castrate-resistant prostate cancer [38]. HSP60 overexpression has also been found in early prostate carcinogenesis [42], and has been demonstrated to be strongly associated with prognostic clinical parameters, such as Gleason score, initial serum PSA levels, lymph node metastasis, and with the onset of hormone-refractory disease and reduced cancer-specific survival [37]. HSP90 is overexpressed in many tumors [43], and plays a key role in folding and stabilization of oncoproteins, such as Wilms tumor 1 protein (WT1) [44,45]. According to Bansal's study, HSP90 may contribute to tumor growth and chemoresistance of myeloid leukemia through regulating the expression of WT1 [45]. Given the findings above, controlling the function of heat shock proteins with inhibitors represents a potentially attractive approach to cancer therapy. Some of the HSP inhibitors have also been reported to overcome radioresistance and target drug-resistance in non-small-cell lung cancer and mantle cell lymphoma *in vivo* [46,47].

Because of the chemo- and radio-resistant nature of ccRCC, target therapy has become the main treatment. However, resistance to target therapy was observed in a fraction of ccRCC patients [5]. It has been reported that  $\alpha$ B-crystallin might account for the poor prognosis of patients with resistance to the apoptosis-inducing agents because of its antiapoptotic functions [48], and that overexpressed  $\alpha$ B-crystallin could be a cause of chemo-resistance in other tumor cells (e.g., breast cancer) [16]. Up-regulation of  $\alpha$ B-crystallin in a cell line of radioresistant non-small-cell lung cancer was also found [49]. All these findings imply that  $\alpha$ B-crystallin is a powerful negative predictor of chemotherapy and radiotherapy responses. Recently, because of a more detailed understanding of the molecular basis of hypoxia and angiogenesis in renal carcinogenesis, target therapies against multiple HIF-related pathways, such as the phosphatidylinositol 3-kinase-AKT-mTOR, RAS/RAF/MAP, and VEGF signaling routes, were developed. Although these targeting agents improve progression-free survival, resistance to target therapies remains an issue in the treatment of ccRCC [23]. In the present study, we found that  $\alpha$ B-crystallin overexpression also contributed to mTOR inhibitor-resistance of ccRCC cells. One of the possible explanations is that  $\alpha$ B-crystallin activates the AKT kinase pathway to promote survival, and thus counteracts the mTOR inhibitor-induced apoptosis. However, the exact underlying mechanisms and the role of  $\alpha$ B-crystallin in mTOR inhibitor-resistance deserve further investigations.

Our results were based on a small number of patients who were referred to a single center, which is the main limitation of our study and might lead to an underestimation of the association between  $\alpha$ B-crystallin expression and the clinicopathologic features, such as pathologic stage and metastasis. Therefore, a prospective, large-scale study at multiple centers is needed in the future to confirm the current findings.

## 5. Conclusions

In this study, we demonstrated the expression of  $\alpha$ B-crystallin in ccRCC tissues, and investigated the effects of  $\alpha$ B-crystallin on resistance to mTOR inhibitor, growth, and migration of ccRCC cells. Our results showed a significant correlation between  $\alpha$ B-crystallin expression and overall survival of RCC patients.  $\alpha$ B-crystallin overexpression enhanced cell growth through enhancement of DNA synthesis, migration, and rapamycin-resistance, suggesting the involvement of  $\alpha$ B-crystallin in ccRCC progression. To the best of our knowledge, this is the first demonstration that  $\alpha$ B-crystallin could serve as an oncoprotein that predicts poor clinical outcomes in ccRCC. The observation that  $\alpha$ B-crystallin diminished the effect of rapamycin in cell growth implies that such a protein could be a promising prognostic marker and therapeutic target for mTOR inhibitor-resistant ccRCC.

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