Original Article
Evaluation of Argonaute protein as a predictive marker for human clear cell renal cell carcinoma

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Abstract: Argonaute subfamily proteins are involved in human organ growth and development. Recent studies found its association with human breast cancer, however, its expression profile and its prognostic value in clear cell renal cancer (ccRCC) have not been investigated. Methods: Expression of the Argonaute proteins were assessed by immunohistochemistry (IHC) in tissue microarrays (TMA), containing paired tumor tissue and adjacent non-cancer tissue from 176 patients who had undergone surgery in hospital for histologically proven ccRCC. Prognostic value and correlation with other clinico-pathologic factors were evaluated in two classifications. Results: Data showed a significant higher expression of Argonaute 1 and Argonaute 2 present in neoplastic tissues compared with that in adjacent tissue; A significant correlation existed between the higher expression of Argonaute 1 protein with the T stage, lymph node metastasis and clinical TNM (cTNM); Survival analysis by Kaplan-Meier survival curve and log-rank test demonstrated that elevated Argonaute 1 and Argonaute 2 expression in cancer tissue predicted poorer overall survival (OS) compared with group in lower expression (36.3% VS 67.1%; 37.3% VS 53.9%; respectively). Notably, multivariate analyses by Cox's proportional hazard model revealed that expression of Argonaute 2 was an independent prognostic factor in renal cancer. Conclusions: In summary, our present study clarify that the aberrant expression of Argonaute in human RCC is possibly involved with tumorigenesis and development, and the Argonaute protein could act as a potential biomarker for prognosis assessment of renal cancer. Related mechanism is worthy of further investigation.

Keywords: Renal cancer, Argonaute protein, immunohistochemistry, tissue microarray

Introduction

Renal cell carcinoma (RCC) is the most lethal urologic tumor and the sixth leading cause of cancer deaths in Western countries. Each year, around 200,000 patients are diagnosed with this malignancy resulting in approximately 100,000 deaths, and its incidence is increasing steadily in recent years [1, 2]. RCC is represented by 80% by clear cell RCC (ccRCC), originating from the renal proximal tubule [3]. The curative effect of surgical resection varied differently in patients population: the 5-year survival rate in stage I, II, III and IV is about 93%, 65%, 53% and 27% respectively [4]. So it is clear that early diagnosis and medical intervention seems vital in decreasing mortality and promoting total quality of life, novel molecular markers about kidney cancer that can help individually evaluate risk of patient outcome and predict the prognosis are urgently required, as well as the prediction of therapy effect and advocating personalized treatment.

Argonaute protein (AGO protein) is one of the most indispensible components in the RNA induced silencing complex (RISC), which is responsible for the gene silencing phenomenon known as RNA interference (RNAi) [5]. These proteins bind different classes of small noncoding RNAs, including microRNAs (miRNAs), small interfering RNAs (siRNAs) and PIWI interacting RNAs (piRNAs), then small RNAs guide Argonaute proteins to their specific target through sequence complementarity, which typically leads to silencing of the target. Previous

Table 1. Clinical-pathological characteristics of renal cell carcinoma patients

Characteristics	N=176	%
Gender		
Male	126	71.6
Female	50	28.4
Age (yrs)		
<65	93	52.8
>65	83	47.2
Tumor Size (cm)		
<4	92	52.3
≥4	84	47.7
Histological grade		
I-II	56	31.8
III-IV	120	68.2
cTNM		
I-II	65	36.9
III-IV	111	63.1
T stage		
T_1-T_2	87	49.4
T_3-T_4	89	50.6
Lymph nodes metastasis		
Absence	122	69.3
Presence	54	30.7
Distant Metastasis		
Absence	163	92.6
Presence	13	7.4
Follow-ups		
Dead	61	34.7
Survival or lost	115	65.3

studies have indicated that these proteins are also partially responsible for a series of biological processes such as cell proliferation, differentiation and apoptosis [6]. In fact, it is well accepted that Argonaute proteins have shown that some are endonucleases referred as "slicers" [7]. Recent structural analysis of the Ago protein has revealed a third functionally important domain which located between the former two domains, called the MID domain. This domain can bind the characteristic 5' phosphates of small RNAs, then anchors these small RNAs onto the Ago protein [8, 9]. Moreover, some other studies have reported that this newly-found domain may be a key role in some protein-protein interactions [10]. This protein family was first identified in plants, classified by the presence of PAZ and PIWI domains [11]. In mammals there are eight Argonaute

proteins which can be divided into the Ago subfamily and the PIWI subfamily [12, 13]. Ago subfamily is ubiquitously expressed in many organisms such as animals, plants, and fission yeast and can be divided into AGO1, AGO2, AGO3 and AGO4. Proteins of this subfamily could interact and bind with siRNA and miRNA to activate gene silencing pathway, which regulate gene expression in the transcription and post-transcription steps. For the past few years, the Argonaute family has drawn more and more attention as a potential factor related to tumorigenesis in colonic cancer study, as well as in prostate cancer [14, 15]. Therefore this research is concentrated to investigate the expression of the human Ago subfamily proteins by Immunohistochemistry (IHC) and identify their potential roles in tumor occurrence, development and prognosis for patients with kidney cancer.

Mateirals and methods

Patients

A total of 176 patients who underwent surgery at hospitals for histologically proven renal cell carcinoma (ccRCC) that cooperated with National Engineering Center for Biochip at Shanghai during 2006-2008 are selected in this research. They were totally 126 men and 50 women, whose age range from 32 to 79 years (median: 61 years). Clinico-pathological characteristics in our study are presented in **Table 1**. Paraffin-embedded tumor specimens and paired adjacent nontumor specimens (≤1.5 cm away from the tumor) were carefully collected immediately after nephrectomy operation. All patients were followed up until September 2011 with a median observation time of 35 months. Patients had to provide a written consent to participate and after receiving written information regarding its course and purpose, then they could be enrolled into our observation. Approval for the study was received in advance from the Ethics Committee of our institution (**Table 1**).

Construction and section of tissue microarray

Briefly, a tissue arraying instrument (Beecher Instruments, Inc) was used to create cylinder-shaped holes in a square recipient paraffin block, then to remove tissue cores from the donor block such as clinical biopsies or tumor
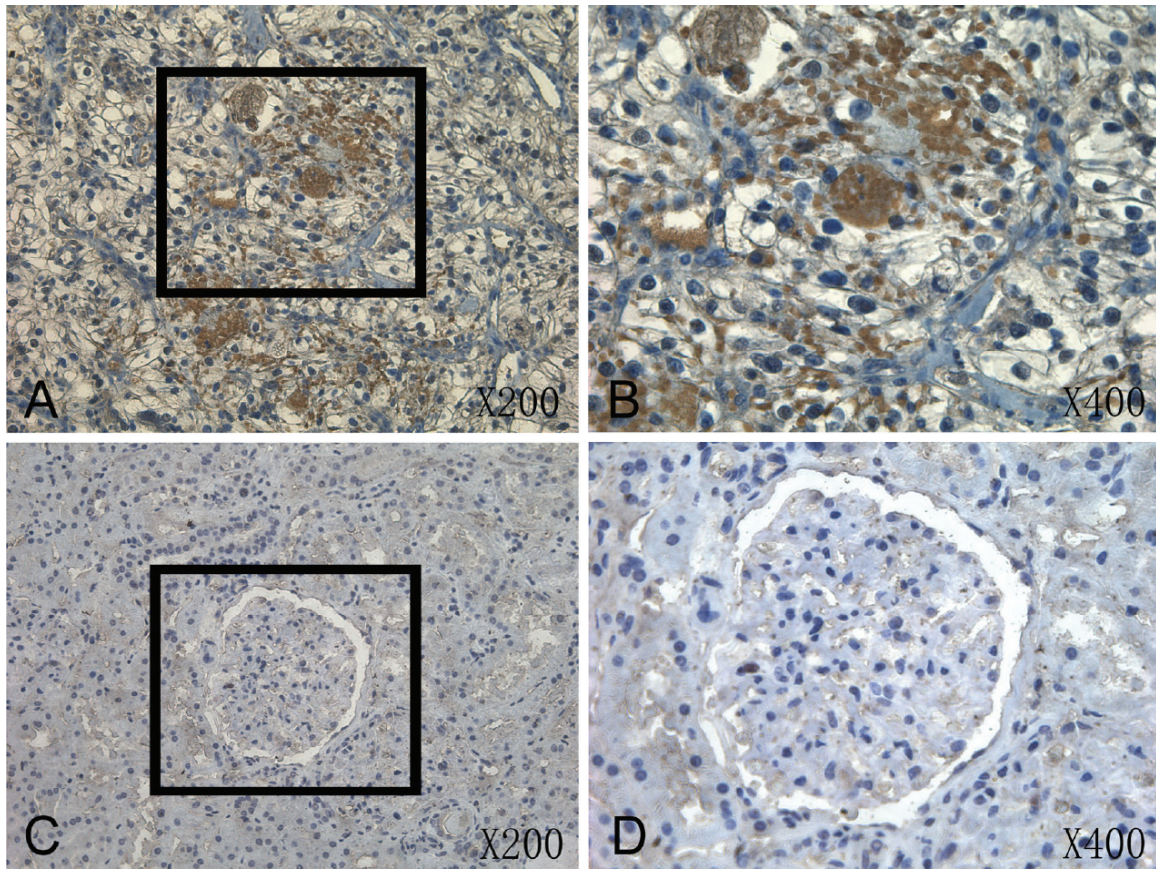
Figure 1. A: Immunohistochemistry expression of Argonaute 1 in tumor tissue (×200). B: Immunohistochemistry expression of Argonaute 1 in tumor tissue (×400). C: Immunohistochemistry expression of Argonaute 1 in adjacent non-tumor tissue (×200). D: Immunohistochemistry expression of Argonaute 1 in adjacent non-tumor tissue (×400).

samples by a hollow needle with an inner diameter of 1.5 mm, held in an X-Y axis precision guide. The cylindrical sample from a region in the donor block which is selected by an experienced pathologist was then inserted into a recipient paraffin block in a precisely spaced, array pattern. After the construction of arrayed block, a 5-µm section was cut with a microtome continuously with a high speed and picked a perfect piece to place on polylysine-coated slides. There are 2 tissue array blocks in this research containing totally 176 samples for each monoclonal antibody against Argonaute 1 and Argonaute 2 protein.

Preparation of antibodies against Argonaute proteins

Antibodies against Argonaute proteins in this research were prepared previously by immunizing rabbits with synthetic peptides derived from the sequences of Argonaute members. Then

the antibody solution was fractionated from the rabbit antisera and affinity-purified on peptide affinity columns. After verifying by ELISA and Western blot, the antibody solution was used in immunohistochemistry analyses.

Immunohistochemistry on formalin-fixed sections

IHC staining for the Argonaute 1/Argonaute 2 on sections of the formalin-fixed samples on the tissue microarray was carried out by using the Envision ready-to-use methods. Slides were deparaffinized in xylene and rehydrated through graded concentrations of ethanol to distilled water and endogenous peroxidase activity was blocked by incubation with 30 mL/L H_2O_2 in methanol for 10 min at room temperature. Then sections were submitted to antigen retrieval in a pressure cooker containing 0.01 mmol/L natrium citricium buffer for 10 min. Slides were subsequently incubated in 100
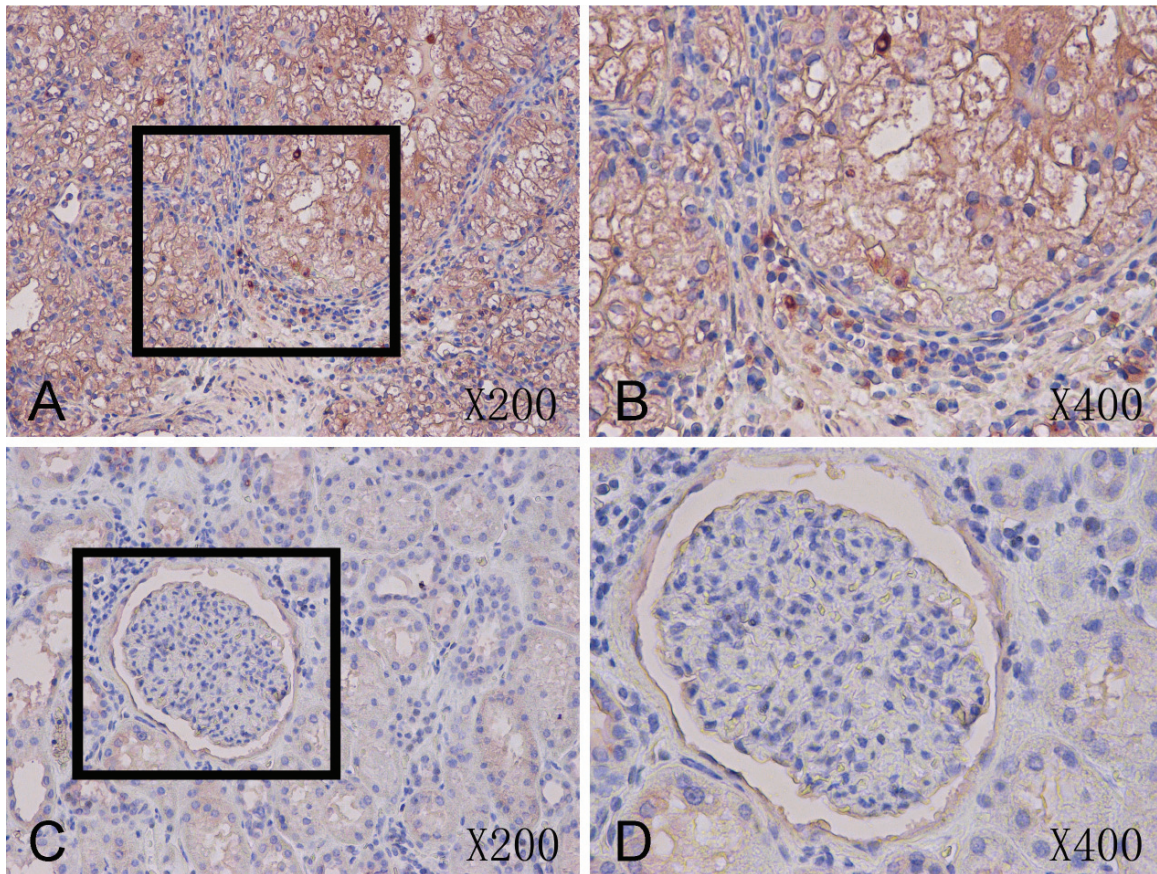
Figure 2. A: Immunohistochemistry expression of Argonaute 2 in tumor tissue (×200). B: Immunohistochemistry expression of Argonaute 2 in tumor tissue (×400). C: Immunohistochemistry expression of Argonaute 2 in adjacent non-tumor tissue (×200). D: Immunohistochemistry expression of Argonaute 2 in adjacent non-tumor tissue (×400).

mL/L normal goat serum for 20 min at room temperature. Sections were permeabilized in PBS-Triton and incubated overnight with primary antibody at 4°C. The antibodies were used in PBS-Trison with variable dilution. Rabbit anti-human polyclonal antibody to Argonaute 1/Argonaute 2 was used. Each section was then incubated with Envision rabbit peroxidase (GeneTech) for 30 min. Finally, the sections were treated with 0.02% 3,3'-diaminoberzidine and 0.005% H_2O_2 in 0.05 mmol/L Tris-HCl buffer and counterstained by hematoxylin.

The evaluation of the immunohistochemical staining was performed independently by two authors without knowledge of the clinicpathological information. The immunoreactive scores besides Argonaute 1/Argonaute 2 were determined by the sum of extension and intensity as literature reported previously [16]. The intensity of the staining was scored using the following scale: 0, no staining of the tumor cells; +, mild staining; ++, moderate staining and +++, marked staining. The area of staining was evaluated and recorded as a percentage: 0, less than 5%; +, 5%-25%; ++, 26%-50%; 3+, 51%-75% and 4+, more than 75%. The combined scores were recorded and graded as follows: -, 0; +, 1-2; ++, 3-5; +++, 6-7.

Statistical analysis

Computerized statistical analyses were performed using the Statistical Package for the Social Sciences (SPSS), version 12.0. Clinical and histopathologic information and the results from the immunohistochemical studies of the tissue microarray were entered into a database. The variances of Argonaute 1/Argonaute 2 expression among different tissues was analyzed using Mann-Whitney U-test and the clinicopathological data was analyzed with Spearman's correlation test. In all statistical analyses, a two-tailed *p* value ≤0.05 was considered statistically significant.

Table 2. Argonaute 1/Argonaute 2 expression in tumor tissue and adjacent non-tumor tissue

Markers	tumor tissue	adjacent non-tumor tissue	p	Z
Argonaute 1	4.67±2.013	4.21±1.753	0.042	-2.15
Argonaute 2	4.58±2.077	3.10±1.195	0.001	-5.869

Results

The expression of Argonaute 1 and Argonaute 2 were significantly higher in tumorous tissue than in adjacent tissue

By using of a large tissue microarray (176 cores) we investigated the protein expression of Argonaute 1 and Argonaute 2 in renal cancer specimens and adjacent non-tumor tissue. The tumorous or nontumor staining was semiquantitatively scored by the intensity and the percentage of positive staining. Argonaute proteins expressions were detected mainly in cytoplasm of malignant cells (**Figures 1** and **2**). The positive expression of Argonaute 1 ($p<0.05$), Argonaute 2 ($p<0.01$) in tumor tissue was significantly higher than in adjacent non-tumor tissue. Images of representative immunostaining are presented in **Figures 1** and **2**. The results are shown in **Table 2**.

Relationship between the expression of Argonaute proteins and clinicopathological parameters

A significant correlation was observed between the higher expression of Argonaute proteins with the T stage, lymph node metastasis and cTNM. IHC was employed to investigate the association between Argonaute expression and clinicopathological features in the 176 renal cancer specimen. The expression level of Argonaute 1 in cytoplasm was significantly associated with T stage (r=0.189, p=0.014); The expression level of Argonaute 2 in cytoplasm was significantly associated with T stage (r=0.192, p=0.012), lymph node metastasis (r=0.257, p=0.015) and cTNM (r=0.379, p=0.032). There were no statistical differences among each protein expression and age, sex, tumor size and distant metastasis. Detailed data is shown in following **Table 3**.

Argonaute expression and survival analysis: univariate survival analysis

Follow-up information was available for 176 patients until September 2011, within the observation period, there were 61 renal cancer related deaths with a median follow-up time of 20 months (0~54 months). And the remaining 105 patients were still alive or lost to follow-up with a median follow-up time of 53 months ranging (42~61 months). Survival analysis by Kaplan-Meier survival curve and log-rank test demonstrated that patients with lower expression of Argonaute 1 and Argonaute 2 in tumor tissue had a better overall survival than patients with tumor with higher expression (p=0.040 and p=0.038, respectively), the 5-years survival rate of patients with higher expression was significantly lower than that of patients with lower expression (36.3% VS 67.1%; 37.3% VS 53.9%; respectively, **Table 4**). The survival curve was demonstrated in **Figure 3**.

Multivariate cox regression analysis

To avoid the influence caused by univariate analysis, the expression of Argonaute 1 and Argonaute 2 as well as other parameters was examined in multivariate Cox analysis. The Argonaute 2 was found to be a significant independent prognostic factor for poor overall survival in our study (B=0.97; p=0.03; Exp (B)=2.671; **Table 4**), which indicated that the Argonaute protein could act as a potential biomarker for prognosis evaluation of renal cancer. Of other parameters, tumor size, histological grade, age and cTNM were also found to be independent prognostic factors for patient survival (**Table 5**).

Discussion

Small non-coding RNA has drawn more and more attention due to its rapidly rising role in the gene transcriptional and posttranscriptional regulation. There is about approximately 30% gene expression in human body regulated by the mechanism of gene silencing such as RNAi [17], and all of these biological process needs the participation of the Argonaute proteins which has being an area of intense investigation. However, the presumed role of Argonaute proteins in the etiology of cancer remains unclear. The present study demonstrated that Argonaute proteins were up-regulated in the renal cell cancer, and explored available evidence of close correlation of Argonaute expres-

Table 3. Relationship between the expression of Argonaute 1/Argonaute 2 proteins and clinicopathological parameters

Markers	Correlation coefficient (r)							
	Gender	Age	Tumor size	cTNM	T stage	LN metastasis	Distant metastasis	Histological grade
Argonaute 1	0.111	0.027	0.032	0.126	0.189[a]	0.028	0.040	-0.036
Argonaute 2	0.021	0.049	0.071	0.379[a]	0.192[a]	0.257[a]	0.039	-0.037

[a]p<0.05; LN: lymph node.

Table 4. The 5-year survival rate of the Argonaute 1/Argonaute 2 expression and other clinicopathologic features

5-year survival rate

		Survival rate	Standard error	p value
Argonaute 1	Negative	0.671	0.088	0.040
	Positive	0.363	0.040	
Argonaute 2	Negative	0.539	0.073	0.038
	Positive	0.373	0.045	
Gender	Male	0.435	0.046	0.655
	Female	0.388	0.067	
Age	<65	0.522	0.056	0.008
	≥65	0.321	0.052	
Tumor size	<4 cm	0.579	0.060	0.000
	≥4 cm	0.302	0.046	
Histological grade	I-II	0.575	0.075	0.023
	≥III	0.357	0.044	
cTNM	TNM1	0.764	0.119	0.011
	TNM2	0.461	0.076	

their contribution to the miRNA pathway during mammalian development were under investigation in the recent year [20].

Genome-wide shotgun proteomics and absolute quantification of Argonauts demonstrates that Ago 2 is the most abundant Argonaute and so associates with the largest pool of miRNAs in both mouse epidermal cells and human melanoma cells. With more in-deph research, data demonstrated that miRNA activity can be quantitatively manipulated by controlling individual Argonauts [21].

Recent newly up surging findings demonstrated that the Argonauts proteins have a more close relation with tumorigenesis and progression of different kinds of human cancer [22, 23]. The interaction of Argonaute proteins with small RNAs or other parts of RISC involved in carcinogenesis has not been completely elucidated. Gene specific translational control induced by some miRNA species has been reported to have an effect on cancer development [24]. As a component of RISC, Argonaute 2 binds to miRNAs or piRNAs (Piwi-interacting RNA), and aberrant regulation of these small RNAs by Argonaute 2 might induce the malignant phenotype of these cells. SND1, also reported to be a component of RISC, is over-expressed in human colonic carcinoma tissues, even in early-stage lesions [25]. Identification of target mRNA species and interacting partners of Argonaute 2 might lead us to the further insights into the complex roles of Argonaute 2 in renal cancer carcinogenesis.

sion and the total patients' survival during a five-year follow-up survey.

Argonaute proteins are the essential components of the miRNA-induced silencing complex (miRISC) that directly recruit miRNAs and function as the interface between miRNAs and their mRNA targets. Mammals have four Argonauts (Ago 1-4) that are involved in the miRNA pathway and among them, Ago 2 is unique, with the slicer activity that mediates the cleavage of perfectly matched targets for miRNAs and siRNAs [18, 19]. To date, exhaustive bioinformatic and experimental analyses have failed to identify a large number of perfectly matched miRNA:mRNA regulatory sequences. It remains unclear why Ago 2 is universally important for miRNA functions in diverse organs and tissues of mammals. In addition the functional significance of Ago 1, Ago 3, and Ago 4 for miRNA activity is poorly understood, probably due to the lack of developmental phenotypes in knockout (KO) mouse models. The function of individual Argonauts and the quantitative nature of

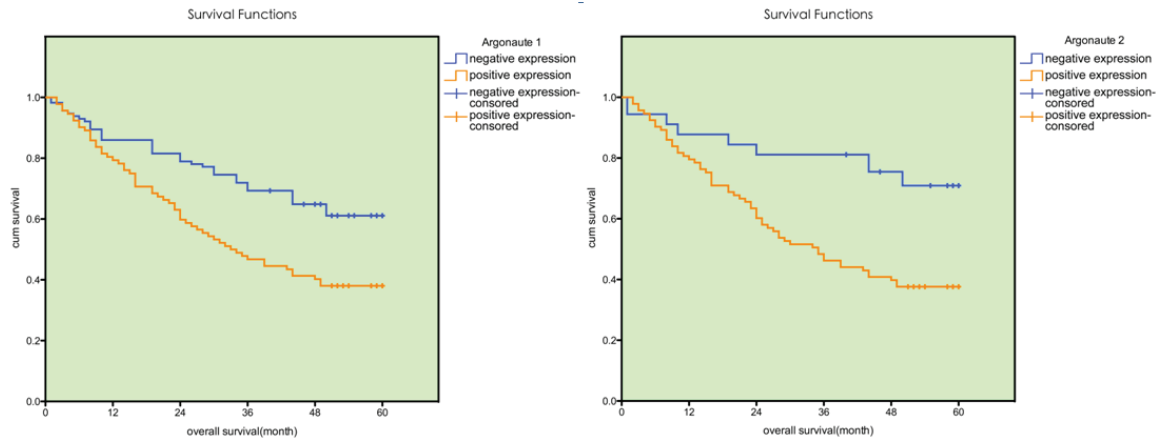To directly address the potential roles for Argonaute proteins in the occurrence and

Figure 3. The survival analysis of Argonaute 1 and Argonaute 2. Patients with higher Argonaute 1 and Argonaute 2 expression in tumor tissue were closely correlated with poorer overall survival than patients with tumor with lower expression (*p*=0.040, *p*=0.038, respectively).

Table 5. Multivariate Cox's proportional hazards regression analysis of prognostic factors for renal cancer

Variables								
							95.0% CI for Exp (B)	
	B	SE	Wald	df	Sig.	Exp (B)	Lower	Upper
Argonaute 1	-0.191	0.302	0.377	1	0.538	0.826	0.447	1.520
Argonaute 2	0.970	0.449	4.712	1	0.030	2.671	1.101	6.449
Tumor size	0.067	0.028	5.433	1	0.018	1.070	1.010	1.133
Histological grade	0.500	0.202	5.956	1	0.015	1.649	1.104	2.458
Age	0.024	0.012	4.479	1	0.035	1.026	1.003	1.048
cTNM	0.538	0.187	8.816	1	0.003	1.750	1.211	2.527

development of kidney cancer, an elaborate experiment was conducted and a rigorous analysis was performed of human Argonaute proteins on a cohort of 176 renal cancer specimens. Our results revealed that the positive rate of Argonaute 1 and Argonaute 2 expression in gastric cancer tissue was remarkably higher than that in non-tumor tissue (p<0.05), which further proving the conclusion gained by Wang [26] et al. In Wang's study, by contrasting the expression of Argonaute 2 in colonic cancer tissues and normal tissue, they had shown that the abnormal expression of Argonaute 2 might be correlated with colon oncogenic event.

Besides, a significant correlation was observed between the higher expression of Argonaute proteins with the T stage, lymph node metastasis and cTNM in this research. Argonaute subfamily might be involved in the development and progression of renal cancer as we sup-

posed before. In the present study, we found the expression level of Argonaute 1 in cytoplasm was significantly associated with T stage (r=0.189, p=0.014); the expression level of Argonaute 2 in cytoplasm was significantly associated with T stage (r=0.192, p=0.012), lymph node metastasis (r=0.257, p=0.015) and cTNM (r=0.379, p=0.032). It is suggested that Argonaute 1 and Argonaute 2 are associated with tumor progression to advanced stage and may promote tumor invasion. We further imagined that the Argonaute subfamily proteins may interfere with the activation of cellular signal transduction pathway, cell division cycle and tumor angiogenesis to influence biological behavior of tumor, and this had just been unraveled in the latest relevant researches. Chen et al. demonstrated the regulatory roles of Argonaute 1 in MDA-MB-231 injected SCID mice through promoting angiogenesis. In this study, the author combined bioinformatics in

conjunction with lab experimental validation and revealed that let-7 and miR-103/107 are robustly induced in vascular endothelial cells and consequently target Argonaute 1. Subsequently, a translational desuppression pathway up-regulates VEGF and enhances angiogenesis in vitro and in vivo. Specimens from human breast cancer provided evidence that this pathway may be implicated in tumor angiogenesis and progression. These findings suggest a novel role of hypoxia-responsive microRNAs (HRMs) in hypoxia-induced gene expression and the consequent angiogenesis via targeting Argonaute 1 [27].

Ultimately, A total of 176 patients histologically proven renal cancer with follow-up information were conducted a systematically analysis to confirm the relationship of the Argonaute proteins and outcome of patient initially. Our finding demonstrated that patients with lower expression of Argonaute 1 and Argonaute 2 in tumor tissue had a better overall survival than patients with higher expression (p=0.040, p=0.038, respectively), providing an evidence that elevated expression of Argonaute 1 or Argonaute 2 in renal cancer might facilitate an increased malignant and worse prognostic phenotype. It is noteworthy that by multivariate Cox analysis combining expression of Argonaute proteins with other parameters, Argonaute 2 was found as an independent prognostic factor (p=0.03) for patient survival. The aberrant expression of Argonaute protein linked to a poor prognosis of patients has never been investigated in renal cancer before.

Conclusions

In summary, the role of Argonaute proteins with small RNAs or other part of RISC involved in carcinogenesis has been partly demonstrated in the study. We postulate that at least some members of the human Argonaute family may be involved in the development and progression of renal cancer and can serves as an independent biomarker for prognosis. We also place special emphasis on the over-expression of Argonaute may identifies patients at high risk and be a novel therapeutic molecular target for renal cancer. However, molecular mechanisms are poorly understood and for the limitations of our study samples, which has so far only scratched the surface of the unknown arena, further investigations are urgently required.

Conflict of interest

The authors declared no potential conflicts of interests with respect to the authorship and/or publication of this article.

Abbreviations and acronyms

RCC, Renal cell carcinoma; ccRCC, clear cell renal cell carcinoma; ELISA, enzyme-linked immuno sorbent assay; TMA, tissue microarray; OS, overall survival; RISC, RNA induced silencing complex; IHC, Immunohistochemistry; siRNAs, small interfering RNAs; PBS, Phosphate buffer solution; HRMs, hypoxia-responsive microRNAs; VEGF, vascular endothelial growth factor; SCID, severe combined immune deficiency; MDA-MB-231, a type of frequently-used breast cancer cell line.

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