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Original Article

Study on the clinical significance of Argonaute2 expression in colonic carcinoma by tissue microarray

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Abstract: Background: To study the expression levels and clinical significance of Argonaute2 (EIF2C2) on colonic carcinomas and normal tissues. Methods: Colon tissue samples from 90 cases of colonic carcinomas and 90 normal subjects were accumulated and made into a tissue microarray containing 360 dots. Expression of Argonaute2 (EIF2C2) was detected by immunohistochemical staining of the tissue microarray. Results: There was significant difference in the expression levels of Argonaute2 (EIF2C2) between colonic carcinomas and normal tissues ($P<0.01$). However, the expression of Argonaute2 (EIF2C2) was not related to sex, age, position, differentiation, lymphatic metastasis and clinical stage of the tumor ($P>0.05$). Conclusion: Abnormal expression of Argonaute2 (EIF2C2) may be correlated with colon tumorigenesis.

Keywords: Tissue mircroarray, immunohistochemstry, colonic carcinoma, Argonaute2

Introduction

Colonic carcinoma is one of the most frequent cancers in the Western world. At the present time, with the changes in living conditions and life style, colonic carcinoma has become more frequent in China. The prognosis in advanced cases is poor and more than one-third of the patients die from progressive disease and the overall survival is about 40% after 5 years [1]. Given the high level in incidence rate and mortality rate of colonic carcinoma, it would be important to better understand the biological basis of tumor development and progression, to develop markers for assessing onset or prediction of therapy outcome, as well as to identify targets for the development of novel therapies. Colonic carcinoma may be considered the final step of a progressive imbalance between mucosal cell proliferation and apoptosis due to the activation of oncogenes and the inactivation of tumor suppressor genes [2-4]. The evaluation of the clinical utility of each of these genes would require multiple experiments with hundreds of tumor specimens. This would be both time-consuming as well as impractical for more than a handful of genes. Microarray technology provides a new and promising tool that allows the detection of multiple parameters simultaneously, and could be of importance in treating colonic carcinoma.

There are endogenous non-protein-coding RNA molecules in the human genome, which include transfer RNA, ribosomal RNA and various small non-coding RNAs. MicroRNAs (miRNAs) are small, non-coding, single-stranded RNAs ~22 nucleotides in length, which regulate gene expression by pairing with messenger RNA of target genes [5-8]. After being first identified in C.elegans, miRNAs were subsequently found in plants and animals, showing that they are highly conserved [5-8]. Regulation of miRNA is a complex process and is well orchestrated with many cellular components [5, 7, 8]. From genomic DNA, miRNAs are transcribed to primary miRNAs, which are subsequently processed to precursor miRNAs by Drosha RNAse III endonuclease in complex with the double-stranded RNA-binding domain protein DGCR8 [9, 10]. After export from nucleus to cytoplasm by exportin5, precursor miRNAs are further cleaved by Dicer in a complex with TARBP proteins to generate a short RNA duplex [9, 10]. Of the duplex, one strand becomes a mature miRNA, while the other strand is rapidly degrad-

ed. The mature miRNA directs a RNA-induced silencing complex (RISC) to 3'-untranslated region (UTR) of its complementary target genes and causes inhibition of translation and degradation of the messenger RNA [13-15]. The major components of the RISC are the Ago proteins. Argonaute2 is a member of a family of eight proteins in mammals, four of which are germ line specific [16]. Ago proteins contain a P-element induced wimpy testis (PIWI) domain that can adopt a ribomuclease H fole with potentially innate endonuclease activity [17-20]. However, Argonaute2 is the only Ago protein shown to mediate miRNA-dependent cleavage/degradation of target mRNAs in mammals. Recently, studies have also implicated functional roles for Argonaute2 independent of its endonuclease activity [21-25]. Argonaute2 has been observed to be diffuse within the cytoplasm and localized in both processing bodies (P bodies) and the nucleus [26].

Several lines of evidence indicate that miRNA is important in development, cell proliferation and cell death, deregulations of which contribute to the pathogenesis of cancers [27-30]. Some miRNAs act as either a tumor suppressor or a tumor promoter and alterations of miRNA have been identified in human cancers [27-30]. Transcriptional and epigenetic alterations, gene mutation and DNA copy number alteration of miRNAs have been reported in human cancers [27-30, 31]. In addition, altered expression and mutation of components in miRNA biogenesis have been reported [32-35]. However, data on the expression of Argonaute2 in colonic carcinoma is lacking. In the present study we analyzed the expression level of Argonaute2 in colonic carcinoma tissues by immunohistochemistry using a tissue microarray (TMA) approach.

Materials and methods

Preparation and identification of Argonaute2

We adopted the Ensemble database and the antibody screening software (Dragonfly, USA) to select the distinctive piece of Argonaute2 protein and then add aminothipropionic acid on C-terminal to connect peptide fragment and carrier. The peptide fragment was synthesized by C-Strong and the molecular weight by mass spectral analysis corresponded to the theoretical molecular weight. We coupled Argonaute2 peptide to keyhole limpet hemocyanin (KLH) to construct Argonaute2-KLH conjugate. The Argonaute2-KLH conjugate was injected into rabbits subcutaneously to produce polyclonal antibodies. Evolution of the antibody titer was controlled by measuring the binding of serial dilutions of the antisera to microtiter plates coated with peptide antigen. Eight weeks after the first injection, sera were collected and used to prepare purified antibodies. The IgG solution was fractionated from the rabbit antisera by precipitation with 40% saturated ammonium sulphate and then affinity-purified on peptide affinity columns. The flowthrough was collected and stored at -20°C for use in ELISA, Western blot, and immunohistochemistry analyses. The specificity and sensitivity of the antibodies were identified by ELISA and Western blotting after purification using affinity chromatorgraphy. The tissue chip array was used to study the distribution and expression levels of Argonaute2.

Patients and specimens

Demographic and clinical data were collected retrospectively. None of the patients received radiotherapy or chemotherapy before surgery. Formalin-fixed and paraffin-embedded tumors and normal tissues（≥5cm away from the tumor） specimens were obtained from the archives of the Department of Surgical Gastroenterology, the Tongji Hospital of Tongji University and National Engineering Center for Biochip at Shanghai. All specimens were viewed by one pathologist (Jing Fang). The specimens that were interpretable for IHC included: (1) Ninety colon cancer specimens including different grades, such as moderate to high (n=69) and low differentiated (n=21); (2) ninety normal colon tissues resected from areas of the colon ≥ 5 cms from the corresponding cancer tissues.

Patients were only included in the study if they had provided written consent to participate in the study after receiving oral and written information regarding its course and purpose. Approval for the study was received from the Ethics Committee of the host institution.

Construction and sectioning of tissue microarray

The colon cancer microarray was constructed as previously described [36]. Briefly, fresh sec-

tions were cut from the donor block and stained with hematoxylin-eosin (HE) and these slides were used to guide the samplings from morphologically representative regions of the tissues. A tissue array instrument was used to create holes in a recipient paraffin block and to acquire tissue cores from the donor block by a thin-walled needle with an inner diameter of 1.0 mm or 1.5 mm, held in an X-Y precision guide. The cylindrical samples were retrieved from the selected regions in the donors and extruded directly onto the recipient blocks with defined array coordinates. After the construction of the array block, multiple 4 mm thick sections were cut with a microtome using an adhesive-coated tape sectioning system.

Tissue loss was a significant factor for tissue array based analysis with previously reported rates of tissue damage ranging from 15% to 33% [37-39]. In our analysis the rates of lost cases attributable to tissue damage were less than 5% for the different markers and damaged tissues were excluded from clinicopathological analyses of the respective markers.

IHC on formalin-fixed tissue microarray sections

IHC staining for the Argonaute2 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 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 Argonaute2 was used. Each section was then incubated with Envision rabbit peroxidase (GeneTech) for 30min. Finally, the sections were treated with 0.02% 3,3'-diaminoberzidine and 0.005% H_2O_2 in 0.05mmol/L Tris-Hcl buffer and counterstained by hematoxylin.

Scoring of Argonaute2 expression

The evaluation of the immunohistochemical staining was performed independently by two authors without knowledge of the clinicpothological information. The immunoreactive scores besides Argonaute2 were determined by the sum of extension and intensity as reported previously [40]. 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 Argonaute2 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.

Results

Clinicopathological data

Complete histological and clinical data were collected from patient' records, which showed that the median age of the study population was 60 years (range, 28-93 years). There were 33 patients with median age less than 60 years. There was a male predominance in the cancer patients (male:female ratio=48:42). Forty-five patients had positive lymph node metastasis, whereas 45 were negative. The clinical stages of these patients were strictly identified as A (n=6), B (n=38), C (n=40) and D (n=6) according to Dukes stage. After the second diagnostic assessment there were 21 low and 69 moderate to highly differentiated tissues in the cancer tissue blocks according to the histological grades.

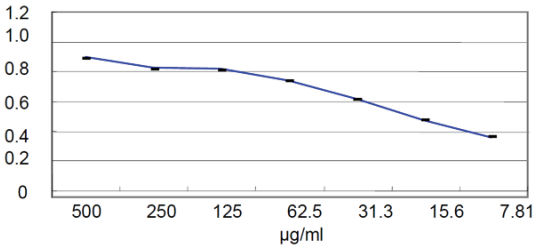

Figure 1. Detection of affinity chromatograph-purified Argonaute2 antibodies by ELISA. The peptide showed strong immune response after 4 immunizations in rabbits, and antisera exhibited high tier when tested by ELISA using immobilized peptide on 96-well microtiter plates. Antibodies from individual rabbits were purified separately by peptide affinity chromatography.

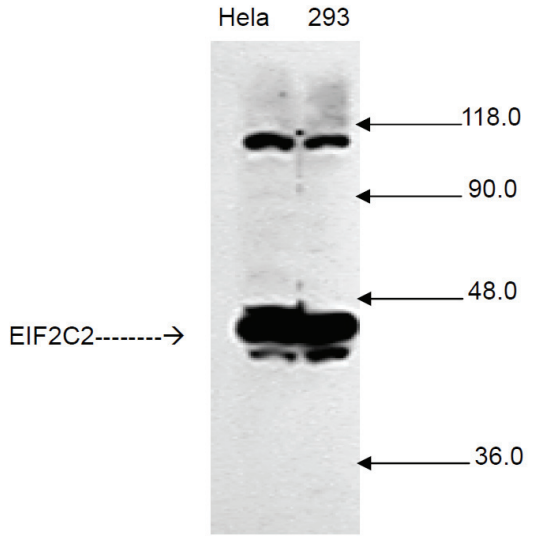

Figure 2. Western blot analysis of argonaute2 (EIF2C2) antibody using Hela, 293 cells. Total protein extracted from either Hela or 293 cells was used for immunoblotting with purified antibodies. Western blot analysis showed that these purified antibodies recognized the bands at expected molecule mass corresponding to the Argonaute2 protein.

Production and validation of antibodies against Argonaute2 protein

An in-house peptide selection database called Antibody Designer was used to select optimal peptide immunogens for production of antibodies against human Argonaute2 protein. A synthetic peptide derived from the sequence of Argonaute2 was conjugated to KLH for immunization. The peptide showed strong immune response after 4 immunizations in rabbits, and antisera exhibited high tier when tested by ELISA using immobilized peptide on 96-well microtiter plates. Antibodies from individual rabbits were purified separately by peptide affinity chromatography (**Figure 1**). Typically 2-20 milligrams of purified antibodies were obtained for each peptide. SDS-PAGE analysis showed that the molecular mass of purified antibodies was 50 kDa, which corresponded to the molecular mass of rabbit IgG. Total protein extracted from either Hela or 293 cells was used for immunoblotting with purified antibodies. Western blot analysis showed that these purified antibodies recognized the bands at expected molecule mass corresponding to the Argonaute2 protein (**Figure 2**). In contrast, no band was detected with preimmune rabbit serum. Furthermore, immunohistochemical analysis showed predominantly cytoplasmic staining in most tumorous tissues but very weak or no staining in normal human tissues.

Argonaute2 expression in colonic carcinoma

Expression of Argonaute2 was analyzed in normal and cancer tissues of the colon by immunohistochemical approach using TMA. The data on the immunostainings are summarized in **Table 1**. In colonic carcinoma different levels of Argonaute2 immunopositivity was observed in 89 of 90 tissues (**Figure 3A** and**3B**). In normal colonic tissues, weak immunopositivity was observed for Argonaute2 in 84 of 90 tissues (**Figure 3C** and **3D**). Statistically, Argonaute2 expression in colonic carcinoma tissues was significantly higher than those in normal tissues ($P<0.01$).

The spearman's analysis of clinicopathologic factors related to Argonaute2

We then analyzed the relationship of immunostaining with various pathologic parameters (age, sex, tumor location, degree of differentiation, metastasis to lymph node, Dukes' grade and stage). However, there was no significant association with any of the above factors ($P>0.05$) (**Table 2**).

Discussion

Colonic carcinogenesis is characterized by distinct morphological, genetic and cellular events. Development and progression of colon cancer to metastasis and lethal state are believed to be driven by multiple genetic alterations, the

Table 1. Expression of Argonaute2 (EIF2C2) in tumor tissues and normal tissues

Tissue type	No.	Argonaute2(EIF2C2)				Z	P
		-	+	++	+++		
Tumor tissue	90	1	4	12	73	-8.498	0.000
Normal tissue	90	6	27	43	14		

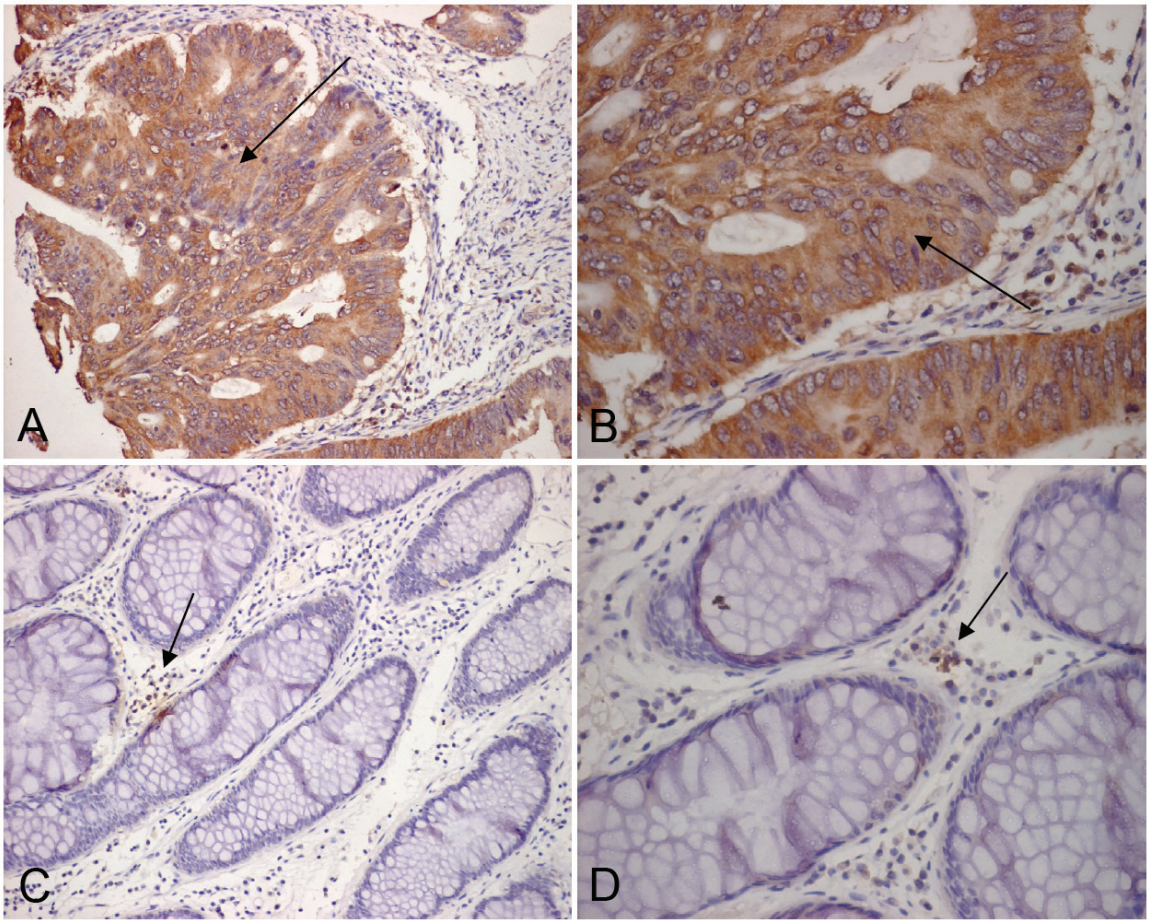

Figure 3. A: Immunohistochemistry expression of argonaute2 in tumor tissue (×200). B: Immunohistochemistry expression of argonaute2 in tumor tissue (×400). C: Immunohistochemistry expression of argonaute2 in normal tissue (×200). D: Immunohistochemistry expression of argonaute2 in normal tissue (×400).

nature of which has remained poorly understood.

There have been many examples of alterations of miRNAs and miRNA-related gene expressions in human cancers [27-29, 31-35]. Many miRNA genes are deleted or amplified in human cancers [31]. During miRNA biogenesis, failure at the Drosha processing step caused a widespread down-regulation in uterine cancers [32]. Dicer and other miRNA biogenesis genes are down-regulated in lung adenocarcinomas [33], while Dicer is up-regulated in lung squamous cell carcinomas and prostate carcinoma [34, 35]. These reports suggest that deregulations of miRNAs themselves and those of miRNA biogenesis machinery might be influencing factors in tumor development. Such frequent alterations led us to analyze expression levels of the RISC protein Argonaute2 in colonic carcinoma. Although several human Argonaute proteins have been identified, relatively little is known

Table 2. Relationship between the expression of Argonaute2 and clinicopathological parameters

Clinicpathological parameters	No.	Argonaute2				Rank sum test	P
		-	+	++	+++		
Age (year)							
>60	57	1	3	7	46	0.023	0.831
≤60	33	0	1	5	27		
Sex							
male	48	1	3	4	40	-0.042	0.693
female	42	0	1	8	33		
Location							
rectum	40	1	2	7	30	0.141	0.185
colon	50	0	2	5	43		
Differentiate							
high+moderate	69	1	3	8	57	-0.062	0.559
Low	21	0	1	4	16		
Lymph node transfer							
positive	45	1	2	5	37	-0.020	0.851
negative	45	0	2	7	36		
Staging (Dukes)							
CD	46	1	2	5	38	-0.031	0.773
AB	44	0	2	7	35		

about their functions in human disease. Argonaute subfamily members are components essential for siRNA-mediated gene silencing in mammalian cells and involved in the effecter step of mammalian RNAi. In the present study, we sought to determine whether there was any difference in Argonaute2 expression between normal and colon carcinoma tissues, which had not been studied previously. We found that Argonaute2 was weakly expressed in normal colonic tissues and differently expressed in colonic carcinoma tissues, suggesting that the increase in Argonaute2 expression may occur at an early stage of some colonic carcinomas.

An earlier study demonstrated that expression level of Argonaute2 is elevated in estrogen receptor (ER) α-negative ($ERα^-$) vs. ERα-positive ($Erα^+$) breast cancer cell lines and in $ERα^-$ breast tumors. In MCF-7 cells the low level of Argonaute2 was found to be dependent upon active Erα/estrogen signaling. Interestingly, the increased expression of Argonaute2 in $ERα^-$ cells was severely blunted by inhibition of the epidermal growth factor (EGF) receptor/MAPK signaling pathway. These data indicate that Argonaute2 is regulated at both the transcriptional and posttranslational level and also implicate Argonaute2 and enhanced miRNA activity in the tumorigenic progression of breast cancer cell lines [41].

Nam et al. analyzed the expression of Argonaute2 in 107 prostate carcinomas (PCA) and 58 esophageal squamous cell carcinoma (ESCC) tissues by immunohistochemistry using a tissue microarray (TMA) method [42]. In the prostate, Argonaute2 was not expressed in normal glandular cells, while it was expressed in 50.0% of prostate intraepithelial neoplasia (PIN) and 57% of the PCA. In the esophagus Argonaute2 was expressed in normal squamous cells, while Argonaute2 was expressed in 58.6% of the ESCC, respectively. However, the expression of Argonaute2 was not associated with pathologic characteristics of the cancers, including age, sex, Gleason score (PCA) and stage. The increased expressions of Argonaute2 in both PCA and ESCC compared with corresponding normal tissues suggested that overexpression of the protein may be related to miRNA functions and might play a role in tumorigenesis of PCA and ESCC.

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 [8]. As a component of RISC, Argonaute2 binds to miRNAs or piRNAs (Piwi-interactingRNA), and aberrant regulation of

these small RNAs by Argonaute2 might induce the malignant phenotype of these cells. SND1, also reported to be a component of RISC, is overexpressed in human colonic carcinoma tissues, even in early-stage lesions [43-45]. Identification of target mRNA species and interacting partners of Argonaute2 might provide us with further insights into more precise roles of Argonaute2 in colonic carcinogenesis [44].

Li et al. constructed a tissue microarray containing 75 specimens from colon cancer and 75 specimens from adjacent non-cancerous tissue and assayed the Argonaute family by immunohistochemistry on consecutive formalin-fixed tissue microarray sections [46]. The expression of EIF2C1-4 and PIWIL1-4 was significantly higher in tumors tissues than in adjacent tissues. Notably, a significant correlation was observed between the positive expression levels of EIF2C2, EIF2C3, EIF2C4, PIWIL4 and the presence of distant metastasis, suggesting that they may promote tumor invasion.

In the present study, we did not find any association between the clinicopathologic parameters of the patients and Argonaute2 expression, suggesting that the alterations in the levels of Argonaute2 expression might not directly result in discernible changes in the behavior of the cancers. To determine whether such over-expression is common feature of cancers, expression status of Argonaute2 should be further analyzed in other cancers.

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