

Original Article

The PIWI protein acts as a predictive marker for human gastric cancer

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Abstract: Purpose: To investigate the expression of the human PIWI subfamily proteins in gastric cancer and their potential roles in the occurrence, development and prognosis of gastric cancer. Methods and patients: Expression of the PIWI proteins were assessed by immunohistochemistry (IHC) in tissue microarrays (TMA), containing paired tumor tissue and adjacent non-cancer tissue from 182 patients who had undergone surgery in hospital for histologically proven gastric cancer (GC). Prognostic value and correlation with other clinicopathologic factors were evaluated in two classifications. Results: The expression of PIWI1-4 was significantly higher in tumor tissue than that in adjacent tissue; A significant correlation was observed between the higher expression of PIWI 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 PIWI1 and PIWI2 expression in cancer tissue predicted poorer overall survival (OS) compared with group in lower expression (36.5% VS 67.6%; 37.4% VS 54.2%; respectively). Notably, multivariate analyses by Cox's proportional hazard model revealed that expression of PIWI1 was an independent prognostic factor in gastric cancer. Conclusions: The PIWI subfamily protein is an absolutely key molecular factor along with the tumor occurrence and development. And the PIWI protein could act as a potential biomarker for prognosis evaluation of gastric cancer.

Keywords: Argonaute protein, tissue microarray, Immunohistochemistry, gastric cancer

Introduction

A latest data published by the International Agency for Research on Cancer shows that gastric cancer was still a disease with a high death rate making it the second most common cause of cancer death worldwide after lung cancer [1]. In addition there was a domestic epidemiological report showing that in mainland China between 2004-2005, the mortality is 24.71/100,000, accounting for 18.19% of the cause of malignancy death, much higher than developed countries in the same period [2]. The curative effect of surgical operation varied greatly from patient to patient: the 5-year survival rate in stage IA, stage IB, stage II, stage IIIA, stage IIIB and stage IV is 93.2%, 65.1%, 52.3%, 41.4%, 16.5% and 10.6% respectively [3]. These observations collectively demonstrate that early diagnosis and corresponding intervention come to the only way to decrease mortality and promote the qualities of life. There is now a

general consensus that a lot of biomarker such as Alpha-fetoprotein (AFP) in liver cell carcinoma can predict carcinogenesis and prognosis accurately. Consistent with this notion, novel molecular markers about gastric cancer that can help classify the risk of patient outcome individually in early stage and identify tumor progression and predict the prognosis in the view of the biological basis are urgently needed, as well as the prediction of therapy outcome and the development of personalized treatment.

Argonaute protein (AGO protein) is one of the most indispensable components in the RNA-induced silencing complex (RISC), which is responsible for the gene silencing phenomenon known as RNA interference (RNAi) [4]. These proteins bind different classes of small non-coding RNAs, including microRNAs (miRNAs), small interfering RNAs (siRNAs) and PIWI-interacting RNAs (piRNAs), then small RNAs guide Argonaute proteins to their specific tar-

gets through sequence complementarity, which typically leads to silencing of the target. Previous studies have indicated that these proteins are also partially responsible for a series of biological processes such as cell proliferation, differentiation and apoptosis [5, 6]. All the Argonaute proteins have two main structures: PAZ domain and PIWI domain [7]. The PAZ domain contains a specific binding site that can anchor the characteristic two-nucleotide 3' overhang with small RNA [8, 9], while PIWI domain shows a high homology to RNase H. In fact, it is well accepted that Argonaute proteins have shown that some are endonucleases referred as "slicers" [10]. 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 [11, 12]. Moreover, some other studies have reported that this newly-found domain may be a key role in some protein-protein interactions [13]. This protein family was first identified in plants, classified by the presence of PAZ and PIWI domains [14]. In mammals there are eight Argonaute proteins which can be divided into the Ago subfamily and the PIWI subfamily [15, 16]. 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 [6]. In contrast, in most organisms such as the *Drosophila*, the zebrafish and the mouse by now, the expression of PIWI subfamily maintains a high consistency to the germ line, where they bind PIWI-interacting proteins known as piRNA. The human PIWI subfamily comprises PIWI L1/HIWI, PIWI L2/HILI, PIWI L3, and PIWI L4/HIWI2, involved in regulation of a widespread biological process such as epigenetics of cancer [17], RNA stability [18], transposon silencing [19-21], translation suppression and so on [22]. For the past few years, the Argonaute family has drawn more and more attention as a potential factor related to tumorigenesis in our study, which has established an objective theoretical basis for specific molecular diagnostics of colonic cancer [23]. Therefore this research is concentrated to investigate the expression of the human PIWI subfamily proteins by Immunohistochemistry (IHC) and identify their potential

roles in tumor occurrence, development and prognosis for patients with gastric cancer.

Materials and methods

Patients and follow-up

A total of 182 patients who underwent surgery at hospitals for histologically proven gastric cancer (GC) that cooperated with National Engineering Center for Biochip at Shanghai during 2006-2008 are selected in this research. They were totally 129 men and 53 women, whose age range from 34 to 84 years (median: 65 years). Clinicopathological characteristics in our study are presented in **Table 1**. Paraffin-embedded tumor specimens and paired adjacent non-tumor specimens (≤ 1.5 cm away from the tu-

Table 1. Clinicopathological characteristics of gastric cancer cases

Characteristic	GC, N=182	%
Sex		
Male	129	70.9
Female	53	29.1
miss	0	0
Age (years)		
<65	96	52.7
≥ 65	85	46.7
miss	1	0.5
Tumor Size(cm)		
<5	94	51.6
≥ 5	84	46.2
miss	4	2.2
Histological grade		
I-II	48	26.4
III-IV	134	73.6
miss	0	0
cTNM		
I-II	67	36.8
III-IV	103	56.6
miss	12	6.6
T stage		
T1-T2	27	14.8
T2-T3	144	79.1
miss	11	6.0
Lymph nodes metastasis		
Absence	42	23.1
Presence	136	74.7
miss	4	2.2
Distant metastasis		
Absence	169	92.9
Presence	13	7.1
miss	0	0
Follow-ups		
Dead	105	57.7
Survival or lost	77	42.3
miss	0	0

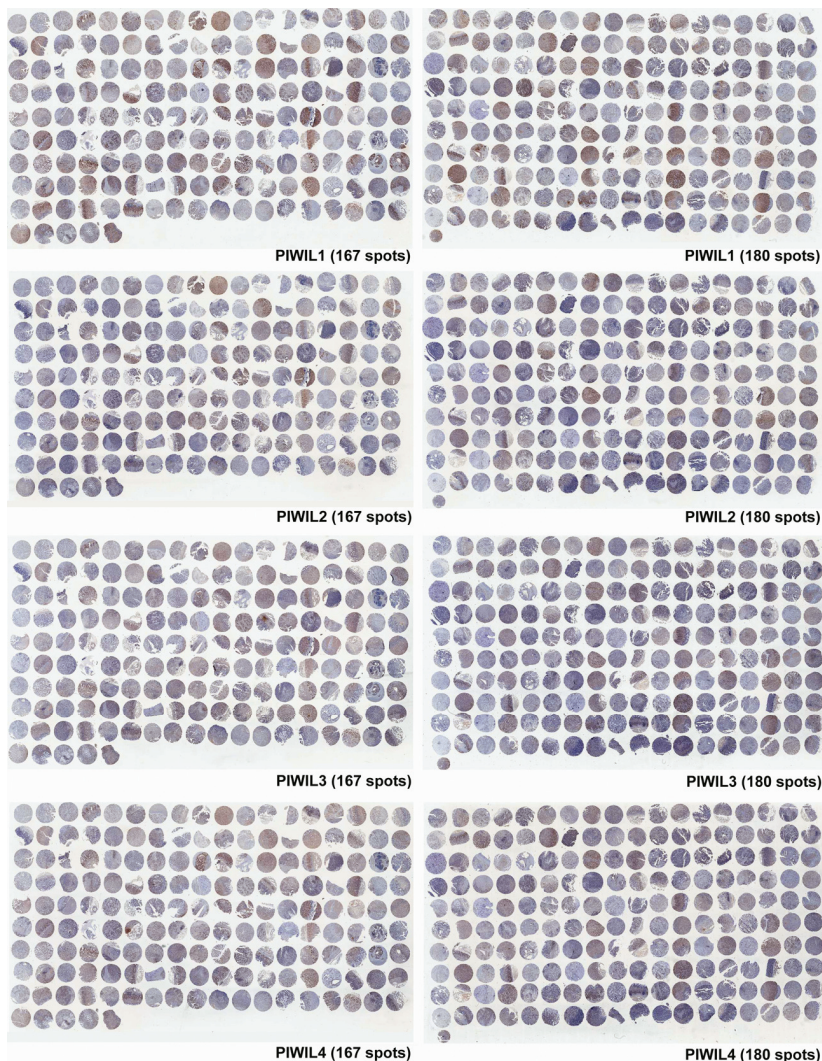


Figure 1. Tissue microarrays containing totally 182 samples in this study (IHC staining).

mor) were carefully collected before any treatment. All patients were followed up until August 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 brought into our study. Approval for the study was received in advance from the Ethics Committee of the host institution.

Tissue microarray construction

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 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- μ m 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 182 samples for each monoclonal antibody against PIWI proteins (Figure 1).

Preparation of antibodies against PIWI proteins

Antibodies against PIWI proteins in this research were prepared previously by immunizing rabbits with synthetic peptides derived from the sequences of Argonaute sub-family 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 [23].

Immunohistochemistry

The immunohistochemical staining procedure was cautiously conducted by using the two-step method. The sections must be deparaffinized and rehydrated first, and the slides were heated to boiling in EDTA buffer by autoclave as antigen retrieval then returned to room temperature. Antibodies against PIWI proteins in the study were centrifuged and diluted to a reasonable concentration as follows: PIWIL1(1:30), PIWIL2

(1:300), PIWIL3(1:250) and PIWIL4(1:250). In this research we selected the goat anti-rabbit immunoglobulin/HRP from DAKO as primary antibody and secondary antibody. The reaction product was visualized with diaminobenzidine as the chromogen and counterstained by hematoxylin. Targeting peptides will express in the cell cytoplasm for all of PIWIL1, PIWIL2, PIWIL3 and PIWIL4, then the immunohistochemical expression of each marker was assigned to the intensity and percentage of positive staining of the cytoplasm. The percentage of positive cells, as the extent of immunostaining, was quantified classified into five groups under microscope: 0% positive cells for 0; 1% to 25% positive cells for 1; 26% to 50% positive cells for 2; 51% to 75% positive cells for 3 and $\geq 76\%$ positive cells for 4. Intensity was scored as 0 as absence of staining, 1 for weak, 2 for moderate, and 3 for strong staining. The sum of the percentage of positive staining and the intensity was used to define expression levels. Samples were negative when the final scores were 0 to 3 and positive when 4 to 7.

Statistical analysis

In this study, values are expressed as the mean $\pm$ standard error of the means. The differential expression of PIWI proteins between tumorous tissue and non-tumorous tissue was determined by Mann-Whitney U-test. Relationships between clinicopathological and molecular parameters were statistically analyzed using Spearman's rank correlation coefficient. Survival curves were estimated using the Kaplan-Meier method and compared by the log-rank test. Overall survival (OS) was defined as the time from the date of histological diagnosis to the date of last contact or death from any cause. A multivariate analysis was performed by using the Cox regression model to assess whether a factor was an independent predictor of prognosis of gastric cancer. A two-tailed P value of less than 0.05 was considered statistically significant. All statistical analyses were performed with SPSS v17.0 software (SPSS Inc, Chicago, IL).

Results

The expression of PIWIL1-4 was significantly higher in tumorous tissue than in adjacent tissue

By using of a large tissue microarray (182 cores) we investigated the protein expression of PIWIL1

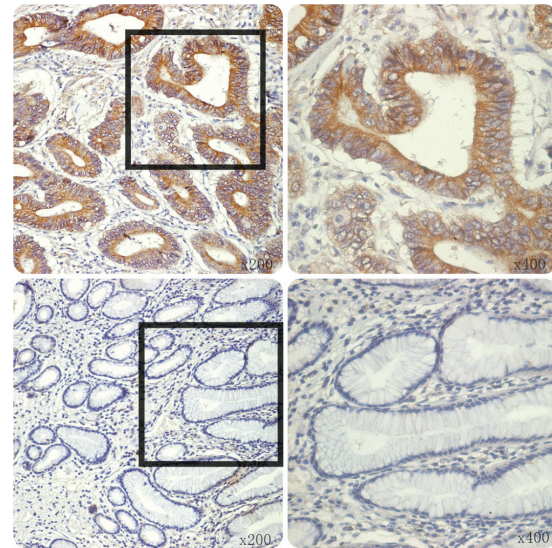


Figure 2. Immunohistochemical expression of PIWIL1 in gastric cancer and adjacent non-cancer tissue. Above: gastric cancer tissue; Below: adjacent non-cancer tissue; Magnifications: $\times 200$ (Left); $\times 400$ (Right).

- 4 in gastric cancer specimens and adjacent non-tumorous tissue. The tumorous or non-tumorous mucosa-specific staining was semi-quantitatively scored by the intensity and the percentage of positive staining. PIWI proteins expressions were detected mainly in cytoplasm of malignant cells. The positive expression of PIWIL1 ($P < 0.05$), PIWIL2 ($P < 0.001$), PIWIL3 ($P < 0.001$) and PIWIL4 ($P < 0.001$) in tumorous tissue was significantly higher than in adjacent non-tumorous tissue. Images of representative immunostaining are presented in **Figure 2-5**. 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 PIWI proteins with the T stage, lymph node metastasis and cTNM. IHC was employed to investigate the association between PIWI expression and clinicopathological features in the 182 gastric cancer specimen. The expression level of PIWIL2 in cytoplasm was significantly associated with T stage ($r=0.193$, $P=0.014$); The expression level of PIWIL4 in cytoplasm was significantly associated with T stage ($r=0.202$, $P=0.01$), lymph node metastasis ($r=0.261$, $P=0.001$) and cTNM ($r=0.37$, $P=0.000$). There were no statistical differences

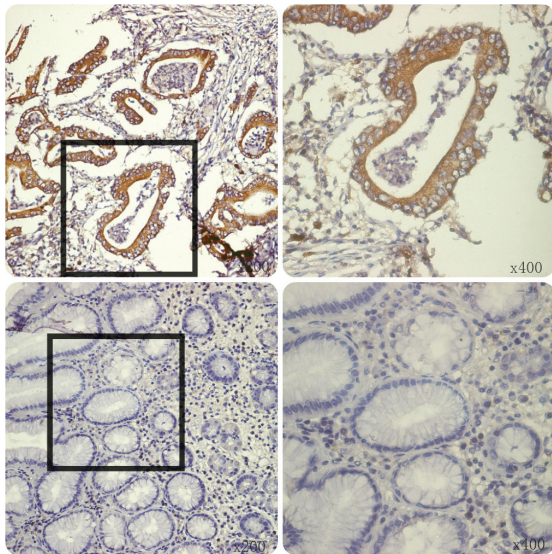


Figure 3. Immunohistochemical expression of PIWIL2 in gastric cancer and adjacent non-cancer tissue. Above: gastric cancer tissue; Below: adjacent non-cancer tissue; Magnifications: $\times 200$ (Left); $\times 400$ (Right).

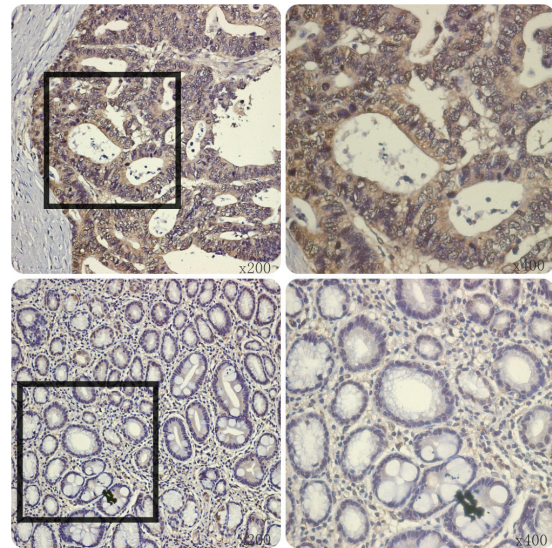


Figure 5. Immunohistochemical expression of PIWIL4 in gastric cancer and adjacent non-cancer tissue. Above: gastric cancer tissue; Below: adjacent non-cancer tissue; Magnifications: $\times 200$ (Left); $\times 400$ (Right).

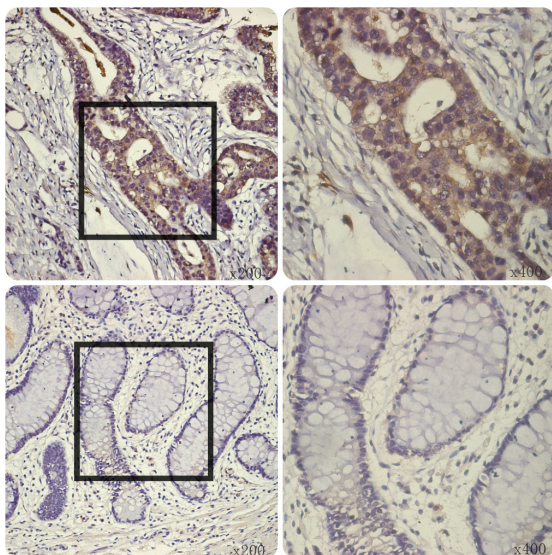


Figure 4. Immunohistochemical expression of PIWIL3 in gastric cancer and adjacent non-cancer tissue. Above: gastric cancer tissue; Below: adjacent non-cancer tissue; Magnifications: $\times 200$ (Left); $\times 400$ (Right).

among each protein expression and age, sex, tumor size and distant metastasis. Detailed data is shown in following **Table 3**.

PIWI expression and survival analysis: univariate survival analysis

Follow-up information was available for 182 patients until August 2011, within the observation period, there were 105 gastric cancer-related deaths with a median follow-up time of 20 months ranging 0 to 55 months. And the remaining 77 patients were still alive or lost to follow-up with a median follow-up time of 52 months ranging 42 to 61 months. Survival analysis by Kaplan-Meier survival curve and log-rank test demonstrated that patients with lower expression of PIWIL1 and PIWIL2 in tumorous tissue had a better overall survival than patients with tumor with higher expression ($p = 0.04$, $p = 0.037$, respectively), the 5-years survival rate of patients with higher expression was significantly lower than that of patients with lower expression (36.5% VS 67.6%; 37.4% VS 54.2%; respectively, **Table 5**), but we did not see any correlation between patients' outcome and the expression level of PIWIL3 or PIWIL4 ($p = 0.365$; $P = 0.555$, respectively) (Seen in **Figure 6**).

Multivariate cox regression analysis

To avoid the influence caused by univariate analysis, the expression of PIWIL1-4 as well as

Table 2. PIWIL1-4 expression in tumorous tissue and adjacent non-tumorous tissue

Markers	Tumorous tissue	Adjacent non-tumorous tissue	P*	Z
PIWI1	4.676±2.082	4.47±1.87	0.044	-2.014
PIWI2	4.15±2.031	3.145±2.233	0.000	-5.686
PIWI3	4.006±2.169	3.029±1.777	0.000	-4.471
PIWI4	4.121±1.977	3.462±1.349	0.001	-3.406

Table 3. Relationship between the expression of PIWI proteins and clinicopathological parameters

marker	correlation coefficient (r)							
	sex	age	tumor size	cTNM	T stage	LN metastasis	Distant metastasis	Histological grade
PIWIL1	0.021	0.066	0.088	0.082	0.070	0.041	0.039	-0.036
PIWIL2	0.113	0.027	0.032	0.125	0.193 ^a	0.029	0.040	-0.037
PIWIL3	0.017	0.080	0.082	0.044	0.071	0.021	0.044	-0.164
PIWIL4	-0.005	0.040	0.130	0.370 ^b	0.202 ^a	0.261 ^b	0.137	-0.086

^aP<0.05, ^bP<0.01, LN: lymph node

Table 4. Multivariate Cox's proportional hazards regression analysis of prognostic factors for gastric cancer

Variables in the Equation							95.0% CI for Exp(B)	
	B	SE	Wald	df	Sig.	Exp(B)	Lower	Upper
PIWIL1	.980	.451	4.715	1	.030	2.663	1.100	6.447
PIWIL2	-.190	.309	.377	1	.539	.827	.451	1.516
Tumor Size	.068	.029	5.486	1	.019	1.070	1.011	1.133
Histological grade	.500	.205	5.961	1	.015	1.648	1.104	2.461
Age	.024	.012	4.482	1	.034	1.025	1.002	1.048
cTNM	.560	.188	8.827	1	.003	1.751	1.210	2.533

other parameters was examined in multivariate Cox analysis. The PIWIL1 was indeed found to be a significant independent prognostic factor for poor OS in our study (B=0.98; P=0.03; Exp (B)=2.663; **Table 5**), which indicated that the PIWI protein could act as a potential biomarker for prognosis evaluation of gastric cancer. Of other parameters, tumor size, Histological grade, age and cTNM were also found to be independent prognostic factors for patient survival (**Table 4**).

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

[24], and all of these biological process needs the participation of the Argonaute proteins which has being an area of intense investigation. As a subfamily of Argonaute proteins, PIWI proteins were originally named after the *Drosophila* PIWI gene and have been found to be associated with a new class of small RNAs called the PIWI-interacting (pi)RNAs, which has been implicated in germ cell development, stem cell self-renewal, and retrotransposon silencing [25, 26, 19]. With More in-depth research, increasingly research findings demonstrate that the PIWI subfamily proteins combined with piRNAs have a more close relation with tumorigenesis and progression of human cancer. HIWI is a homologue of PIWI gene firstly found in human testis. HIWI gene maps to the long arm of chromosome 12, band 12q24.33, and this genomic region may show a compact genetic linkage to the occurrence and development of human tes-

Table 5. The 5-year survival rate of the PIWIL1-4 expression and other clinicopathologic features

5 year survival rate		survival rate	standard error	P value
PIWIL 1	Negative	0.676	0.090	0.040
	Positive	0.365	0.041	
PIWIL 2	Negative	0.542	0.073	0.037
	Positive	0.374	0.046	
PIWIL 3	Negative	0.367	0.071	0.365
	Positive	0.447	0.046	
PIWIL 4	Negative	0.429	0.071	0.555
	Positive	0.425	0.047	
Sex	Male	0.429	0.045	0.651
	Female	0.388	0.069	
Age	<65	0.523	0.056	0.005
	≥65	0.328	0.050	
Tumor Size	<5cm	0.589	0.061	0.000
	≥5cm	0.301	0.046	
Histological grade	I-II	0.576	0.074	0.022
	III-IV	0.361	0.043	
cTNM	TNM1	0.769	0.117	0.010
	TNM2	0.464	0.074	
	TNM3	0.326	0.050	
	TNM4	0.167	0.108	

testicular germ cell tumors of adolescents and adults (TGCTs). There are about 3.6 kb mRNA expressed abundantly encoded by HIWI in the adult testis, and it encodes an elementary 861-amino-acid protein that shares a high homology throughout its entire length with the other PIWI proteins. In normal human testes, HIWI is specifically expressed in human germline cells, and its expression is almost only observed in the spermatocytes and round spermatids during spermatogenesis, while a high level expression was also found in testicular seminomas-tumors which originate from embryonic germ cells with retention of germ cell phenotype. Consequently, as the expression of HIWI gene is specifically observed in both normal and malignant spermatogenic cells, an evident inference is acquired that the deregulation of HIWI expression could have a vital involvement in the occurrence and development of tumor [27].

To directly address the potential roles for PIWI proteins in the occurrence and development of tumor in this study, an elaborate experiment was conducted and a rigorous analysis was performed of human PIWI proteins on a cohort of 182 gastric cancer specimens. Our results reveal that the positive rate of PIWIL1-4 protein expression in gastric cancer tissue was remarkably higher than that in non-cancer tissue ($P<0.05$), which further proving the conclusion

gained by Liu [28] et al in 2006. In that study by contrasting the expression of HIWI in gastric cancer cell line and gastric cancer tissues respectively, they had shown that the percentage of HIWI positive cells was dramatically increased from 10% in normal gastric tissues to 76% in gastric cancers, which confirm the notion among oncologists in vitro study that PIWI proteins might be a significant factor in a series of oncogenic event. Moreover, Lee [29] et al found that PIWIL2 is expressed specificity only in testis and a wide variety of tumors. Therefore, PIWIL2 appears to fall in the category of cancer-testis antigens (CTAs). Even more important is that they have explored the relationship between PIWIL2 and some significant cellular signal transduction pathways such as Stat3/ Bcl-XL and Stat3/cyclin D1. Their research showed that PIWIL2 could activate and enhance the Stat3/Bcl-XL and Stat3/cyclin D1 signaling pathways to inhibit apoptosis and stimulate proliferation which results in the tumorigenesis. Meanwhile when they inhibited the expression of PIWIL2 protein, the constitutive signaling pathways was also blocked leading to the repression of tumor cell growth in vitro and in vivo. So this finding provides a novel therapeutic intervention for human cancer in the future.

Besides, a significant correlation was observed between the higher expression of PIWI proteins

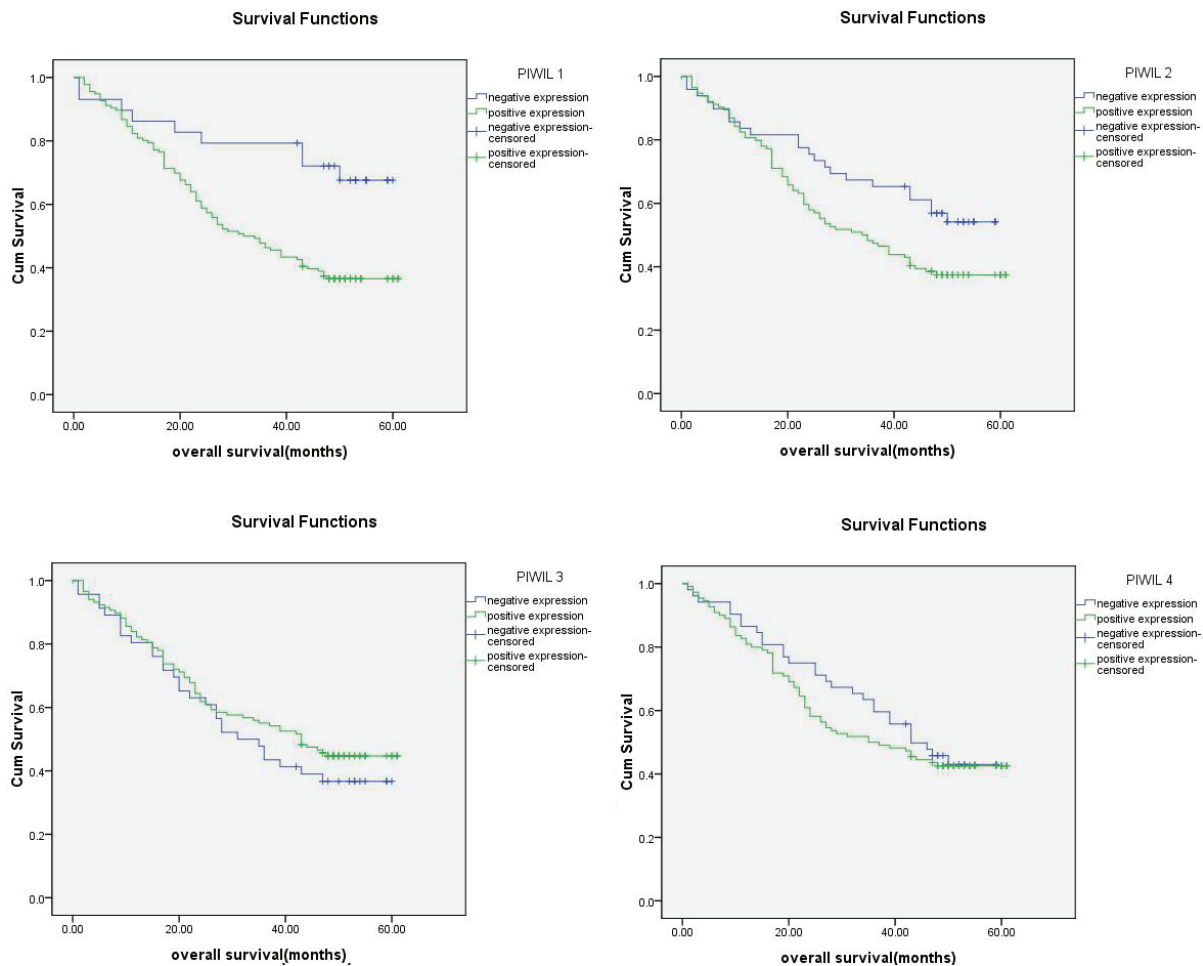


Figure 6. The survival analysis of PIWI1-4. Patients with higher PIWI1 and PIWI2 expression in tumorous tissue were closely correlated with poorer overall survival than patients with tumor with lower expression ($p = 0.004$, $p = 0.037$, respectively), while no correlation between patients' outcome and the expression level of PIWI3 or PIWI4 ($p = 0.365$; $P = 0.555$, respectively).

with the T stage, lymph node metastasis and cTNM in this research. So a possible involvement of PIWI subfamily in the development and progression of gastric cancer is proposed naturally. In the present study, we found the expression level of PIWI2 in cytoplasm was significantly associated with T stage ($r=0.193$, $P=0.014$); The expression level of PIWI4 in cytoplasm was significantly associated with T stage ($r=0.202$, $P=0.01$), lymph node metastasis ($r=0.261$, $P=0.001$) and cTNM ($r=0.37$, $P=0.000$). It is suggested that PIWI2 and PIWI4 are associated with tumor progression to advanced stage and may promote tumor invasion. Such as noted above, the PIWI subfamily may interfere the activation of cellular signal transduction pathway, cell division cycle and

tumor angiogenesis to influence biological behavior of tumor like proliferation and metastasis, and this had been well demonstrated in recent relevant researches. Liu [30] et al have demonstrated that PIWI2 is ubiquitously and uniquely expressed in various stages of breast cancers and its expression patterns are associated with ER and Ki67 as well as cancer development, suggesting that PIWI2 plays an important role in breast cancer development. Li S [31] et al were the first observation that HIWI was universally upregulated in many types of cancers and associated with tumor vessels, and the evidence suggested that the overexpression of HIWI might be a key element in stem cell division and caused malignancy development. moreover, in previous study by Liu X [28] cov-

ered that the growth of cancer cells could be inhibited and cell cycle arrest would be induced in G2/M phase by using RNAi or antisense technology to silence PIWI's gene in human gastric cancer and seminomas, and this work will further consolidate the thesis that overexpression of PIWI members had a compact relationship with the proliferation and apoptosis of cancer stem cell.

Finally, A total of 182 patients histologically proven gastric cancer (GC) with follow-up information were conducted a systematically analysis to confirm the relationship of the PIWI subfamily and outcome of patient with GC for the first time. Our finding has demonstrated that patients with lower expression of PIWIL1 and PIWIL2 in tumorous tissue had a better overall survival than patients with tumor with higher expression ($p = 0.004$, $p = 0.037$, respectively), providing an evidence that elevated expression of PIWIL1 or PIWIL2 in gastric cancer might facilitate an increased malignant and worse prognostic phenotype of this tumor. It is noteworthy that when the expression of PIWI proteins as well as other parameters was examined in multivariate Cox analysis, PIWIL1 is found as an independent prognostic factor ($P=0.03$) for patient survival in this research. The aberrant expression of PIWI protein linked to a poor prognosis of patients was also documented in other cancers. Taubert H [32] et al devoted themselves to demonstrate the connection between cancer genes and their prognosis, they applied the quantitative real-time-PCR to investigate the coexpression of three stem cell-associated genes, HIWI, hTERT and surviving in primary soft-tissue sarcomas (STS). They found that no matter what tumor stages of the patients with the poorest prognosis, an impressively increased risk of tumor-related death was observed accompanying with the coexpression of these three stem cell-associated genes in their research, which was also to imply that the risk caused by tumor is based upon expression of some relevant stem cell-associated genes rather than on tumor stage. In another research, Grochola LF [33] et al investigated the Hiwi mRNA transcripts and protein expression in pancreatic ductal adenocarcinoma (PDAC) tissues by quantitative real-time PCR and immunohistochemistry (IHC) respectively. There were 40 out of 56 tissues measured an elevated level of Hiwi mRNA transcripts and at the same time a positive expression of Hiwi gene was also detected in tumors of

21 out of 78 patients. Importantly, abnormal expression of Hiwi mRNA had resulted in a significantly increased risk for tumor-related death (relative risk (RR)=2.78; $P=0.034$). Briefly, this finding implied that aberrant expression of Hiwi can increase the risk of tumor-related death in male PDAC patients.

Conclusions

Thus far, the role of PIWI proteins with small RNAs or other part of RISC involved in carcinogenesis has been partly demonstrated in this study. We postulate that at least some members of the human PIWI family may be involved in the development and progression of gastric cancer and can serve as an independent biomarker for prognosis. We also place special emphasis on the overexpression of PIWI may identifies patients at high risk and be a novel therapeutic molecular target for gastric cancer. However molecular details of the process are still poorly understood and limitations of the study sample, which has so far only scratched the surface of this very fertile field, further study and more acknowledge about this protein is still urgently needed to make it real.

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References

- [1] Ferlay J, Shin HR, Bray F, Forman D, Mathers C, Parkin DM. Estimates of worldwide burden of cancer in 2008: GLOBOCAN 2008. *Int J Cancer* 2010; 127: 2893-2917.
- [2] Zou XN, Duan JJ, Huangfu XM, Chen WQ, Zhao P. Analysis of stomach cancer mortality in the national retrospective sampling survey of death causes in China, 2004 – 2005. *Zhonghua Yu Fang Yi Xue Za Zhi* 2010; 44: 390-397.
- [3] Li XB, Zhang HW, Zhao QC, Dong GL, Wang WZ. Risk factors for oncologic outcome after surgical treatment in patients with gastric cancer: a

- multivariable analysis of 1031 patients. *Zhonghua Wei Chang Wai Ke Za Zhi* 2010; 13: 115-118.
- [4] Faehnle CR, Joshua-Tor L. Argonautes confront new small RNAs. *Curr Opin Chem Biol* 2007; 11: 569-577.
- [5] Pillai RS, Artus CG, Filipowicz W. Tethering of human Ago proteins to mRNA mimics the miRNA-mediated repression of protein synthesis. *RNA* 2004; 10: 1518-1525.
- [6] Carmell MA, Xuan Z, Zhang MQ, Hannon GJ. The Argonaute family: tentacles that reach into RNAi, Developmental control, stem cell maintenance and tumorigenesis. *Genes Dev* 2002; 16: 2733-2742.
- [7] Cerutti L, Mian N and Bateman A. Domains in gene silencing and cell differentiation proteins: the novel PAZ domain and redefinition of the PIWI domain. *Trends Biochem Sci* 2000; 25: 481-482.
- [8] Lingel A, Simon B, Izaurralde E, Sattler M. Structure and nucleic-acid binding of the Drosophila Argonaute 2 PAZ domain. *Nature* 2003; 426: 465-469.
- [9] Lingel A, Simon B, Izaurralde E, Sattler M. Nucleic acid 3'-end recognition by the Argonaute2 PAZ domain. *Nat Struct Mol Biol* 2004; 11: 576-577.
- [10] Parker JS, Roe SM, Barford D. Crystal structure of a PIWI protein suggests mechanisms for siRNA recognition and slicer activity. *EMBO J* 2004; 23: 4727-4737.
- [11] Ma JB, Yuan YR, Meister G, Pei Y, Tuschl T, Patel DJ. Structural basis for 5'-endspecific recognition of guide RNA by the A. fulgidus PIWI protein. *Nature* 2005; 434: 666-670.
- [12] Parker JS, Roe SM, Barford D. Structural insights into mRNA recognition from a PIWI domain-siRNA guide complex. *Nature* 2005; 434: 663-666.
- [13] Till S, Lejeune E, Thermann R, Bortfeld M, Hothorn M, Enderle D, Heinrich C, Hentze MW, Ladurner AG. A conserved motif in Argonaute-interacting proteins mediates functional interactions through the Argonaute PIWI domain. *Nat Struct Mol Biol* 2007; 14: 897-903.
- [14] Bohmert K, Camus I, Bellini C, Bouchez D, Caboche M, Benning C:AGO1 defines a novel locus of Arabidopsis controlling leaf development. *EMBO J* 1998; 17: 170-180.
- [15] Sasaki T, Shiohama A, Minoshima S, Shimizu N. Identification of eight members of the Argonaute family in the human genome. *Genomics* 2003; 82: 323-330.
- [16] Meister G, Tuschl T. Mechanisms of gene silencing by doublestranded RNA. *Nature* 2004; 431: 343-349.
- [17] Pal-Bhadra M, Leibovitch BA, Gandhi SG, Rao M, Bhadra U, Birchler JA. Heterochromatic silencing and HP1 localization in Drosophila are dependent on the RNAi machinery. *Science* 2004; 303: 669-672.
- [18] Grivna ST, Pyhtila B, Lin H. MIWI associates with translational machinery and PIWI-interacting RNAs (piRNAs) in regulating spermatogenesis. *Proc Natl Acad Sci USA* 2006; 103: 13415-13420.
- [19] Brennecke J, Aravin AA, Stark A, Dus M, Kellis M, Sachidanandam R, Hannon GJ. Discrete small RNA-generating loci as master regulator- of transposon activity in Drosophila. *Cell* 2007; 128: 1089-1103.
- [20] Kalmykova AI, Klenov MS, Gvozdev VA. Argonaute protein PIWI controls mobilization of retrotransposons in the Drosophila male germline. *Nucleic Acids Res* 2005; 33: 2052-2059.
- [21] Xu M, You Y, Hunsicker P, Hori T, Small C, Griswold MD, Hecht NB. Mice Deficient for a Small Cluster of PIWI-Interacting RNAs Implicate PIWI-Interacting RNAs in Transposon Control. *Biol Reprod* 2008; 79: 51-57.
- [22] Lau NC, Seto AG, Kim J, Kuramochi-Miyagawa S, Nakano T, Bartel DP, Kingston RE. Characterization of the piRNA complex from rat testes. *Science* 2006; 313: 363-367.
- [23] Lan Li, Chaohui Yu, Hengjun Gao, Youming Li. Argonaute proteins: potential biomarkers for human colon cancer. *BMC Cancer* 2010; 10: 38.
- [24] Lewis BP, Burge CB, Bartel DP. Conserved seed pairing, often flanked by adenosines, indicates that thousands of human genes are microRNA targets. *Cell* 2005; 120: 15-20.
- [25] Lin H, Spradling AC. A novel group of pumilio mutations affects the asymmetric division of germline stem cells in the Drosophila ovary. *Development* 1997; 124: 2463-2476.
- [26] Cox DN, Chao A, Baker J, Chang L, Qiao D and Lin H. A novel class of evolutionarily conserved genes defined by PIWI are essential for stem cell self-renewal. *Genes Dev* 1998; 12: 3715-3727.
- [27] Qiao D, Zeeman AM, Deng W, Looijenga LH, Lin H. Molecular characterization of hiwi, a human member of the PIWI gene family whose overexpression is correlated to seminomas. *Oncogene* 2002; 21: 3988-3999.
- [28] Liu X, Sun Y, Guo J, Ma H, Li J, Dong B, Jin G, Zhang J, Wu J, Meng L, Shou C. Expression of hiwi gene in human gastric cancer was associated with proliferation of cancer cells. *Int J Cancer* 2006; 118: 1922-1929.
- [29] Lee JH, Schütte D, Wulf G, Füzesi L, Radzun HJ, Schwyer S, Engel W, Nayernia K. Stem-cell protein PIWI2 is widely expressed in tumors and inhibits apoptosis through activation of Stat3/Bcl-XL pathway. *Hum Mol Genet* 2006; 15: 201-211.
- [30] Liu JJ, Shen R, Chen L, Ye Y, He G, Hua K, Jarjoura D, Nakano T, Ramesh GK, Shapiro CL, Barsky SH, Gao JX. PIWI2 is expressed in various stages of breast cancers and has the potential to be used as a novel biomarker. *Int J Clin Exp Pathol* 2010; 3: 328-337.

- [31] Li S, Meng L, Zhu C, Wu L, Bai X, Wei J, Lu Y, Zhou J, Ma D. The universal overexpression of a cancer testis antigen hiwi is associated with cancer angiogenesis. *Oncol Rep* 2010; 23: 1063-10688.
- [32] Taubert H, Würfl P, Greither T, Kappler M, Bache M, Bartel F, Kehlen A, Lautenschläger C, Harris LC, Kaushal D, Füssel S, Meye A, Böhnke A, Schmidt H, Holzhausen HJ, Hauptmann S. Stem cell-associated genes are extremely poor prognostic factors for soft-tissue sarcoma patients. *Oncogene* 2007; 26: 7170-7174.
- [33] Grochola LF, Greither T, Taubert H, Möller P, Knippschild U, Udelnow A, Henne-Bruns D, Würfl P. The stem cell-associated Hiwi gene in human adenocarcinoma of the pancreas: expression and risk of tumor-related death. *Br J Cancer* 2008; 99: 1083-1088.