Small RNA sequencing revealed aberrant piRNA expression profiles in colorectal cancer

JIE YIN, WEI QI, CHEN-GUANG JI, DONG-XUAN ZHANG, XIAO-LI XIE, QIAN DING, XIAO-YU JIANG, JING HAN and HUI-QING JIANG

Department of Gastroenterology, The Second Hospital of Hebei Medical University, Hebei Key Laboratory of Gastroenterology, Hebei Institute of Gastroenterology, Shijiazhuang, Hebei 050000, P.R. China

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Abstract. Piwi-interacting RNAs (piRNAs), a novel class of non-coding RNAs, are enriched in germ cells and implicated in spermatogenesis. Emerging evidence demonstrated deregulated expression of piRNAs in numerous tumor types. However, changes in piRNA expression profiles in colorectal cancer (CRC) have not yet been investigated. In the present study, small RNA sequencing was used to evaluate the differences in piRNA expression profiles between CRC and adjacent non-tumor tissues, as well as to screen for differentially expressed piRNAs. The present results demonstrated that the percentage of unique piRNA reads had no notable difference between the paired CRC and adjacent non-tumor samples (0.12% vs. 0.13%); however, the counts of total piRNA reads in CRC samples were increased, compared with those in adjacent non-tumor samples (0.15% vs. 0.07%). Differential expression analysis identified 33 upregulated piRNAs and 2 downregulated piRNAs in CRC samples, among which piR-18849, piR-19521 and piR-17724 were the top three upregulated piRNAs. Reverse transcription-quantitative polymerase chain reaction further confirmed that the expression levels of piR-18849, piR-19521 and piR-17724 were increased in 80 CRC tissues, compared with paired adjacent non-tumor tissues. Furthermore, the high expression of piR-18849 and piR-19521 was associated with a poor degree of differentiation. The increased expression of piR-18849 was also associated with high lymph node metastasis. However, no associations were determined between piR-17724 expression and clinicopathological characteristics of patients. In summary, the present study is the first to provide an overview of the changes in piRNA expression patterns in CRC, shedding new light on the regulatory roles of piRNAs in colorectal carcinogenesis. piR-18849 and piR-19521 may be prognostic biomarkers for patients with CRC.

Correspondence to: Professor Hui-Qing Jiang, The Second Hospital of Hebei Medical University, Hebei Key Laboratory of Gastroenterology, Hebei Institute of Gastroenterology, 215 Heping West Road, Shijiazhuang, Hebei 050000, P.R. China
E-mail: jianghq@aliyun.com

Abbreviations: piRNA, Piwi-interacting RNA; CRC, colorectal cancer; ncRNA, non-coding RNA; miRNA, microRNA; lncRNA, long non coding RNA; RT-qPCR, reverse transcription-quantitative polymerase chain reaction

Key words: piRNA, colorectal cancer, non-coding RNA, deep sequencing, expression profile

Introduction

Colorectal cancer (CRC), one of the most common cancer types worldwide, remains a serious threat to human health with high incidence and mortality globally (1). According to the 2018 statistics by the American Cancer Society, CRC ranks third in the morbidity and mortality among all malignancy types in the United States (2). CRC is difficult to detect at an early stage due to a lack of typical symptoms and signs; therefore, only 39% of patients with CRC have no metastasis at the time of diagnosis, with a 5-year survival rate of up to 90%; however, the majority of patients are diagnosed when the disease has distant metastasis, and the 5-year survival rate of these patients drops to 14%, according to data released by the National Cancer Institute (2006-2012) (3). Metastasis and recurrence are considered the primary causes of mortality in patients with CRC (4). Therefore, it is urgent and necessary to elucidate the molecular mechanism underlying the onset and progression (metastasis and recurrence), which will provide novel therapeutic strategies for CRC.

The onset and progression of CRC are multi-step processes, starting with hyper-proliferation of epithelial cells, then forming carcinoma *in situ* and eventually progressing to invasive and metastatic carcinoma (5). Gene mutations, including adenomatous polyposis coli, c-MYC and V-Ki-ras2 Kirsten rat sarcoma viral oncogene homolog, and epigenetic changes, such as aberrant DNA methylation, serve key roles in these processes, and these alterations result in abnormalities in signaling pathways, including Wnt, mitogen-activated protein kinase/phosphatidylinositol 3-kinase, transforming growth factor-β and tumor protein P53, which will further influence a number of important biological functions of the cells (6-8). Notably, the nature of gene mutations and epigenetic changes is that both alter the expression of oncogenes or tumor suppressor genes (9,10). Gene expression is regulated at multiple levels,

including transcription and translation levels (11). Previously, non-coding RNAs (ncRNAs), such as microRNAs (miRNAs) and long ncRNAs (lncRNAs), have been identified as crucial regulators of gene expression in numerous human diseases, particularly tumors (12-15).

ncRNAs are generated from non-coding regions that were previously considered to be junk DNA, without the potential of translation into proteins (16). Previously, a novel class of ncRNAs has been identified, termed Piwi-interacting RNAs (piRNAs), which are characterized by a 3'-terminal 2'-O-methylation (17,18). piRNAs are named due to their characteristics of exclusive association with the Piwi subfamily, but not the Ago subfamily, and they maintain genome integrity by epigenetically silencing transposons (19,20). Although piRNAs were initially considered to be only expressed in germ cells, a growing number of studies identified piRNAs in various human tissues and cells, including brain tissues and cardiac progenitor cells (21,22), and they are also abnormally expressed in tumor cells (23,24), indicating that piRNAs may be involved in the onset and progression of tumors.

To date, it has been demonstrated that piRNAs are associated with gastric cancer, breast cancer, lung cancer, multiple myeloma and bladder cancer and piRNAs serve various roles in these tumor types, including tumor promotion and suppression in different tumor types (25-29). Additionally, the mechanisms underlying piRNAs in tumors are also diverse and include epigenetic regulation, post-transcriptional regulation and post-translational regulation (30). These studies indicated that piRNAs have diverse functions and complex mechanisms in the tumor context. Although above studies have preliminarily elucidated the roles of piRNAs in a number of tumor types, the majority of studies only focused on a specific piRNA and did not depict the overall changes of piRNAs in tumors, which is not sufficient to fully understand the complex function of piRNAs in tumors.

In the present study, to improve the understanding of the biological function of piRNAs in CRC, the differences in piRNA expression profiles between CRC tissues and adjacent non-tumor tissues were compared using second-generation deep sequencing for small RNAs. Subsequently, the results were validated by reverse transcription-quantitative polymerase chain reaction (RT-qPCR). The potential clinical utility of CRC-associated piRNAs were also assessed by analyzing the association of piRNAs with clinicopathological features of patients with CRC. To the best of our knowledge, the present results provided, for the first time, an overall change of piRNA expression profiles in CRC and an outlook into clinical applications of piRNAs as a therapeutic target.

Materials and methods

Patients and samples. A total of 83 fresh CRC tissues and matched adjacent non-tumor tissues were collected from patients with CRC who underwent surgery from January 2016 to January 2018 at the Second Hospital of Hebei Medical University (Shijiazhuang, China). The diagnosis for CRC and histological evaluation were conducted by an experienced pathologist. All patients exhibited no primary tumors in other sites and did not receive chemoradiotherapy or biological therapy prior to surgery. Tissues were placed in RNAstore reagent (Tiangen Biotech Co., Ltd., Beijing, China) and then stored at -80˚C after being resected from patients. Of the 83 pairs of samples, 3 pairs were used for deep sequencing for small RNAs, and the remaining 80 pairs were used for validation. The clinicopathological characteristics of patients with CRC for small RNA sequencing analysis and RT-qPCR validation analysis are listed in Tables I and II, respectively. Written informed consent was obtained from the recruited patients, and the present study was approved by the Ethics Committee of the Second Hospital of Hebei Medical University.

Small RNA library construction, sequencing and data analysis. Total RNA was extracted from 3 pairs of CRC tissues and matched adjacent non-tumor tissues using TRIzol® reagent (Invitrogen; Thermo Fisher Scientific, Inc., Waltham, MA, USA). The concentration and quality of RNA were assessed by a NanoDrop ND-1000 spectrophotometer (NanoDrop Technologies; Thermo Fisher Scientific, Inc.) and an Agilent 2100 Bioanalyzer (Agilent Technologies, Inc., Santa Clara, CA, USA). Following passing the quality control tests, total RNAs from 3 CRC tissues and 3 matched adjacent non-tumor tissues were pooled separately in equal quantity (0.4 μg) to generate two sample pools [CRC (T) pool and adjacent non-tumor (N) pool] and then were sent to Beijing Genomics Institute (Shenzhen, China) for library preparation and sequencing. Briefly, small RNAs of 18-40 nt in length were purified from total RNA by size fractionation using 15% PAGE and sequentially ligated to 5' and 3' adaptors, followed by RT-PCR amplification to produce sequencing libraries using the TruSeq Small RNA Sample Prep Kit (Illumina, Inc., San Diego, CA, USA) according to the manufacturer's protocol. PCR products were gel-purified and sequenced using Illumina HiSeq 2000 (Illumina, Inc.). Clean reads were obtained by removing low-quality reads and adaptor sequences from raw reads. Subsequently, the length distribution of the clean reads and common and specific sequences between these two samples were summarized. Small RNA reads were aligned with piRNABank (http://pirnabank.ibab.ac.in/) to screen and annotate piRNAs using Bowtie (31). To identify differentially-expressed piRNAs between CRC samples and adjacent non-tumor samples, expression levels of each piRNA were normalized using the following formula: Normalized expression=actual piRNA count/total count of clean reads x 1,000,000. piRNAs with normalized expression values <1.0 in both samples were removed. piRNAs with fold change ≥2.0 (log2 ratio ≥1.0 or ≤-1.0) were considered as differentially expressed.

RT-qPCR of piRNA. Candidate differentially-expressed piRNAs were further validated in 80 pairs of CRC and adjacent non-tumor tissues by RT-qPCR. Briefly, total RNAs, including small RNAs, were isolated from tissues using a miRcute miRNA Isolation kit (Tiangen Biotech Co., Ltd.) and then reverse transcribed with a miScript Plant RT kit (Qiagen GmbH, Hilden, Germany), which is a kit specifically designed for small RNAs with the 2'-O-Me modification at their 3' end. RT-qPCR was performed in triplicate with specific forward primers and universal reverse primers using a miScript SYBR® Green PCR kit (Qiagen GmbH), according to the manufacturer's protocols. The reverse transcription process was performed in two steps of ligation reaction catalyzed

Table I. Clinicopathological characteristics of patients with colorectal cancer for small RNA sequencing.

Patients	Sex	Age	Tumor location	Differentiation	T stage[a]	Lymph node metastasis	AJCC stage[a]
1	Male	52	Rectum	Moderately	T2	No	I
2	Male	75	Rectum	Poorly	T4	Yes	III
3	Female	62	Colon	Poorly	T3	No	II

[a]AJCC 7th edition of T, nodes and metastasis staging system. T, tumor; AJCC, American Joint Committee on Cancer.

Table II. Correlation of piRNA expression with clinicopathological characteristics of patients with colorectal cancer.

Clinicopathological characteristics	No. (%)	piR-18849 fold change	P-value	piR-19521 fold change	P-value	piR-17724 fold change	P-value
All cases	80 (100)						
Sex			0.235		0.849		0.647
Male	46 (57.50)	2.27 (1.38-6.11)		2.03 (1.20-3.58)		1.25 (0.43-5.64)	
Female	34 (42.50)	3.10 (1.98-6.06)		2.10 (1.47-3.39)		1.83 (0.72-3.17)	
Age			0.194		0.388		0.614
≥60	49 (61.25)	3.27 (1.74-6.88)		2.17 (1.42-3.61)		1.52 (0.56-4.15)	
<60	31 (38.75)	2.33 (1.45-4.45)		2.00 (1.38-3.27)		1.64 (0.42-3.24)	
Tumor location			0.725		0.296		0.121
Colon	39 (48.75)	3.15 (1.78-6.08)		2.23 (1.47-3.75)		0.97 (0.44-2.74)	
Rectum	41 (51.25)	2.84 (1.37-6.08)		2.06 (1.29-2.96)		1.91 (0.62-6.89)	
Differentiation			0.001		0.001		0.329
Well or Moderately	56 (70.00)	2.13 (1.35-4.73)		1.87 (1.12-2.60)		1.36 (0.45-3.22)	
Poorly	24 (30.00)	4.87 (2.64-7.99)		3.22 (1.80-7.89)		1.97 (0.59-8.07)	
T stage[a]			0.794		0.618		0.321
T1 or T2	16 (20.0)	2.86 (1.75-7.98)		2.03 (1.40-2.97)		2.35 (1.06-3.77)	
T3	33 (41.25)	2.84 (1.46-4.61)		2.07 (1.45-3.39)		1.15 (0.40-3.56)	
T4	31 (38.75)	3.35 (1.59-6.94)		2.09 (1.21-3.68)		1.21 (0.50-3.15)	
Lymph node metastasis			0.043		0.441		0.735
No	50 (62.50)	2.32 (1.45-4.86)		2.05 (1.18-3.10)		1.65 (0.53-3.55)	
Yes	30 (37.50)	4.58 (1.81-7.28)		2.10 (1.48-4.24)		1.28 (0.43-3.59)	
AJCC stage[a]			0.249		0.417		0.501
I	14 (17.50)	2.86 (1.94-9.54)		2.18 (1.38-3.07)		1.98 (0.93-3.90)	
II	35 (43.75)	1.96 (1.32-4.45)		1.79 (1.16-3.04)		1.20 (0.42-3.24)	
III or IV	31 (38.75)	4.44 (1.81-7.14)		2.14 (1.48-4.36)		1.36 (0.44-3.31)	

Data are presented as n (%) or median with interquartile range. [a]AJCC 7th edition of T, nodes and metastasis staging system. T, tumor; AJCC, American Joint Committee on Cancer.

by ligase and reverse transcription reaction catalyzed by reverse transcriptase. RT-qPCR was performed on an ABI StepOne™ real-time PCR System (Applied Biosystems; Thermo Fisher Scientific, Inc.). Amplification conditions were pre-denaturation at 95˚C for 15 min, followed by 40 cycles of denaturation at 94˚C for 15 sec, annealing at 55˚C for 30 sec and extension at 72˚C for 30 sec. The expression of piRNAs was normalized against U6 small nuclear RNA levels and calculated using the $2^{-\Delta Cq}$ method (32). Specific primers used in RT-qPCR are detailed in Table III.

Statistical analysis. Normally distributed data were expressed as the mean ± standard deviation and non-normally distributed data were expressed as median with interquartile range. Non-normally distributed data were analyzed using the Wilcoxon signed rank test (when paired) or Mann-Whitney U test (when unpaired). The correlation of piRNA expression with T stage and American Joint Committee on Cancer stage (7th edition) (33) was analyzed using Spearman's correlation analysis. $P<0.05$ was considered to indicate a statistically significant difference. Statistical analysis was

performed using SPSS software version 17.0 (SPSS, Inc., Chicago, IL, USA).

Results

Common and specific reads analysis. To evaluate the overall differences of small RNAs in CRC and adjacent non-tumor tissues, the common and specific reads were analyzed between the CRC (T) library and adjacent non-tumor (N) library, including the number of unique reads (types of reads) and total reads. Small RNA sequencing yielded 570,231 unique reads with 114,209,517 total reads from these two libraries. Among these unique reads, only 67,866 (11.90%) unique reads were shared by the two libraries, whereas 297,988 (52.26%) and 204,377 (35.84%) unique reads were specific in the T library and N library, respectively (Fig. 1A). These specific reads were determined to not be abundant, with only 0.51% of total reads in the T library and 0.30% of total reads in the N library (Fig. 1B). These data indicate that CRC tissues and adjacent non-tumor tissues have diverse small RNA profiles and that specific small RNAs exhibit low expression levels.

Length distribution of small RNAs. In general, the length of small RNAs ranged from 18-35 nt, and the peak of length distribution was beneficial to identify the classes of small RNAs. miRNAs were concentrated in 21 or 22 nt, whereas piRNAs were concentrated in 26-32 nt (34). As depicted in Fig. 2, in the N and T libraries, the lengths of small RNAs were clustered in two ranges: 18-24 and 30-34 nt, and the most abundant cluster was 18-24 nt with a 22 nt peak point, the canonical length of miRNAs. The percentage of reads within the 18-24 nt cluster demonstrated no notable difference between these two libraries (82.64% vs. 95.19%), indicating that the abundance of miRNAs was not significantly changed in CRC. However, an increased 30-34 nt peak, primarily comprised of piRNAs, was determined in the T library compared with the N library (14.69% vs. 3.01%), indicating that the piRNA pathway is activated in human CRC.

piRNA annotation. To analyze the differentially expressed piRNAs between CRC and adjacent non-tumor tissues, piRNAs were first annotated and screened by mapping clean reads to the piRNA database. The results demonstrated that 367 unique reads in the N library and 423 unique reads in the T library, accounting for 0.13% and 0.12%, respectively, were aligned with piRNA sequences. Although the proportion of unique piRNA reads indicated no notable changes between these two libraries, the counts of total piRNA reads increased from 42,999 (0.07%) in the N library to 75,946 (0.15%) in the T library (Table IV), indicating that the overall expression of piRNAs was increased in CRC tissues, compared with adjacent non-tumor tissues, and further implying that piRNAs may be implicated in colorectal tumorigenesis.

Differentially-expressed piRNAs between CRC and adjacent non-tumor tissues. Comparison of the expression levels of piRNAs in CRC and adjacent non-tumor tissues is beneficial for understanding the roles of piRNAs in the pathogenesis of CRC. In the present study, a total of 367 unique piRNA reads in the N library and 423 unique piRNA reads in the T library belonged to 141 registered piRNAs. Among these piRNAs, 87 piRNAs were excluded due <1.0 normalized expression values in both libraries. Therefore, 54 piRNAs were selected for further differential expression analysis. As depicted in Fig. 3, a total of 35 differentially-expressed piRNAs were identified, of which 33 piRNAs were upregulated in CRC tissues, whereas only 2 piRNAs were downregulated in CRC tissues. These differentially-expressed piRNAs are detailed in Table V.

Table III. Primer sequences for reverse transcription-quantitative polymerase chain reaction analysis.

Name	Sequence (5'-3')
hsa-piR-19521	GAGTAGAGTGCTTAGTTGAACAG
hsa-piR-18849	TTTGGCAATGGTAGAACTCACAC
hsa-piR-17724	TTCCGTAGTGTAGTGGTTATCAC
U6 snRNA	CTCGCTTCGGCAGCACATA

piR, Piwi-interacting RNA; snRNA, small nuclear RNA.

RT-qPCR validation. Since the vast majority of piRNAs were upregulated in CRC, the focus was on these upregulated piRNAs and the top three upregulated piRNAs (piR-18849, piR-19521 and piR-17724) were selected for further validation in 80 matched pairs of CRC and adjacent non-tumor tissues by RT-qPCR. The relative expression levels of these three piRNAs, calculated using the $2^{-\Delta Cq}$ method, are depicted in Table VI. Consistent with the results from small RNA sequencing, the RT-qPCR results demonstrated that the expression levels of piR-18849, piR-19521 and piR-17724 were consistently increased in CRC tissues, compared with adjacent tissues ($P<0.05$; Fig. 4). Among these three piRNAs, piR-18849 was expressed at the lowest level but had the most notable difference between these two sets of samples. Notably, the fold changes from the RT-qPCR results were less than those from small RNA sequencing, which may be due to the increased sensitivity of deep sequencing.

Association between the expression of piRNAs and clinicopathological features. To clarify the function of the three piRNAs in the onset and progression of CRC, the correlation of their expression with clinicopathological characteristics of patients with CRC was analyzed. As depicted in Table II, the expression of piR-18849 was positively correlated with lymph node metastasis potential and negatively correlated with the degree of tumor differentiation; additionally, CRC with poor differentiation and high lymph node metastasis had significantly increased levels of piR-18849 ($P<0.05$). The expression of piR-19521 was only negatively correlated with the degree of tumor differentiation ($P=0.001$). However, piR-17742 expression levels did not correlate with any clinicopathological features.

Discussion

It is well known that ncRNAs, as key molecules regulating gene expression, are widely distributed in various tissues (35-37). Aberrant expression of ncRNAs is associated with numerous

Table IV. Number of unique reads and total reads aligned to piRNA sequences in the N and T libraries.

Categories	N library		T library	
	Unique reads (%)	Total reads (%)	Unique reads (%)	Total reads (%)
piRNA	367 (0.13)	42,999 (0.07)	423 (0.12)	75,946 (0.15)
Other	271,876 (99.87)	62,488,846 (99.93)	365,431 (99.88)	51,601,726 (99.85)
Total	272,243 (100)	62,531,845 (100)	365,854 (100)	51,677,672 (100)

Data are presented as n (%). N, adjacent non-tumor; T, colorectal cancer; piRNA, Piwi-interacting RNA.

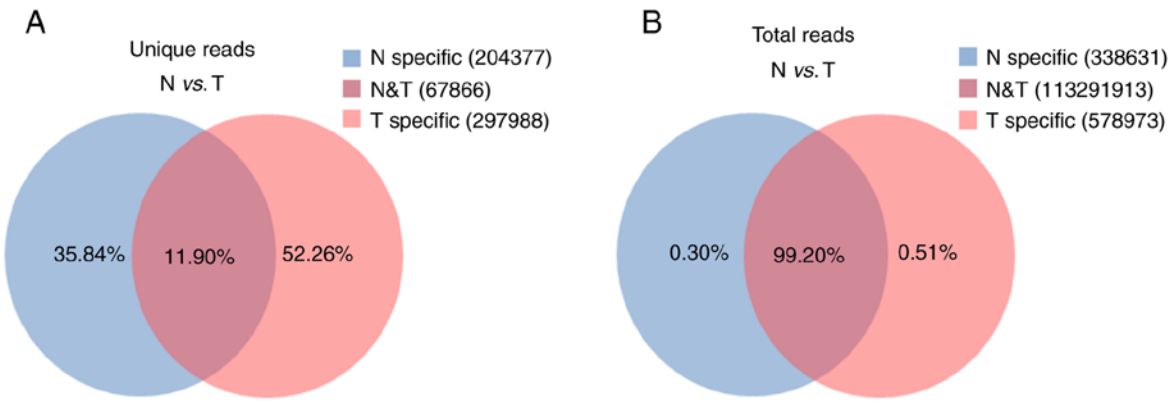

Figure 1. Common and specific reads between the N and T libraries. Venn diagrams visualize the common and specific (A) unique reads and (B) total reads in the N and T libraries. N, adjacent non-tumor; T, colorectal cancer.

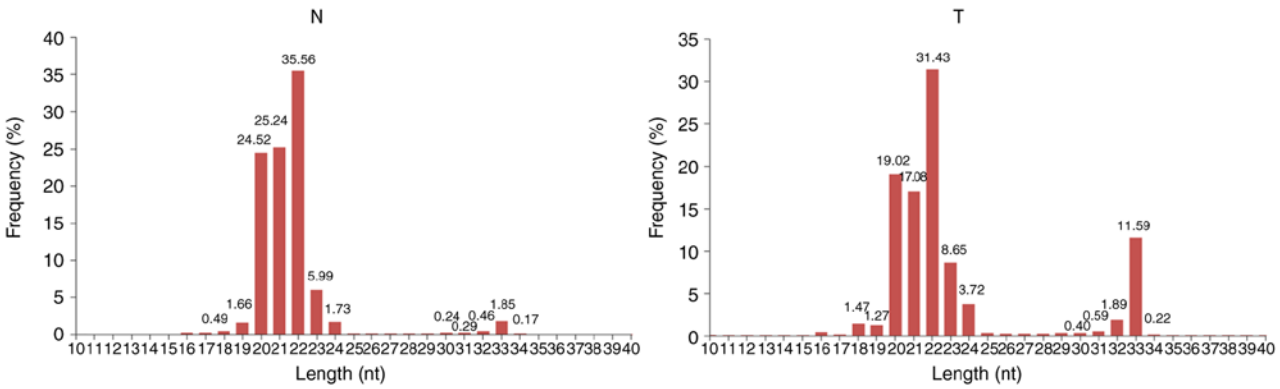

Figure 2. Length distribution of small RNAs. Bar graphs represent the percentage of small RNAs of different lengths from the N and T libraries. N, adjacent non-tumor; T, colorectal cancer.

human disorders (38-40). A large number of studies demonstrated that lncRNAs and miRNAs are implicated in a variety of tumor types and may serve as potential therapeutic targets or diagnostic markers for these tumor types, including CRC (41-44). The rapid development of the second-generation deep sequencing technology has provided an unprecedented platform to comprehensively analyze non-coding transcriptomes as well as reveal a number of novel non-coding transcripts in various tissues, organs and disease models. Furthermore, second-generation sequencing has high sensitivity, which can avoid some minor differentially-expressed RNAs being missed. In view of the advantages of second-generation sequencing and our shortage of funds, studies from Huang *et al* (45), Wang *et al* (46) and Zhang *et al* (47) were referred to and only 3 pairs of CRC tissues and adjacent tissues were used for deep sequencing of small RNAs (≤40 nt). The present data demonstrated that only 11.90% of small RNAs were shared by CRC and adjacent tissues, indicating that the expression patterns of small RNAs in CRC and adjacent tissues were notably different. These observations further implied that small RNAs may be involved in colorectal carcinogenesis.

To date, the expression patterns of miRNAs and lncRNAs in tumors have been extensively investigated (48,49). However, piRNAs, as a class of newly identified small ncRNAs, and the expression patterns of piRNAs in tumors remain largely unknown, and the field remains in its infancy. Numerous studies

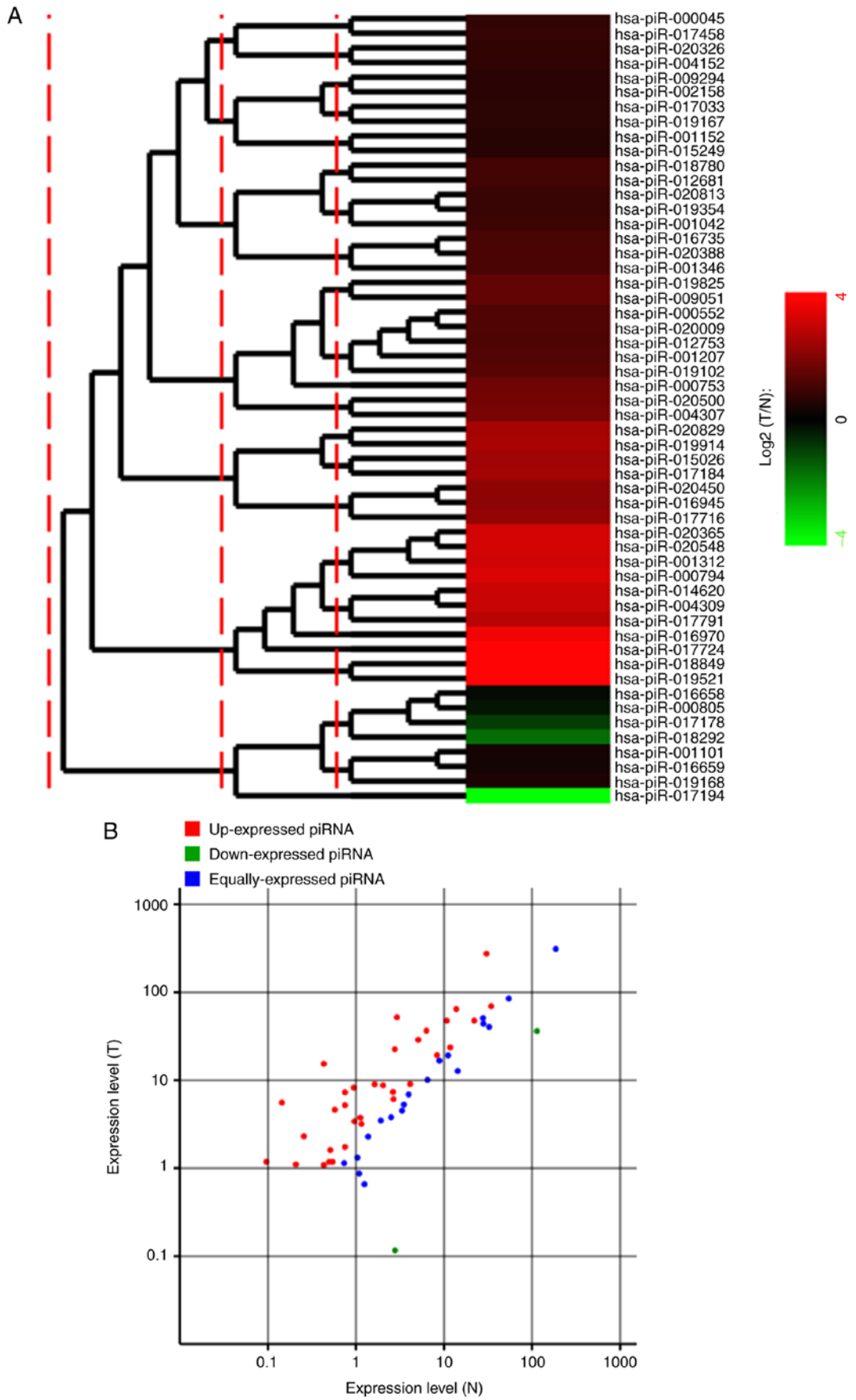

Figure 3. Differential expression analysis of piRNAs. (A) Hierarchical clustering represnts the relative expression levels of piRNAs in the N and T libraries by deep sequencing. The color scale indicates the relative expression levels of piRNAs in the T library vs. the N library: Red, higher expression; green, lower expression; and black, equal expression. (B) Scatter plot represents the normalized expression levels of piRNAs in the N and T libraries by deep sequencing. The red dots in the plot represent upregulated piRNAs, the green dots represent downregulated piRNAs and blue dots represent piRNAs not differentially expressed in the T library vs. the N library. N, adjacent non-tumor; T, colorectal cancer; piRNA, Piwi-interacting RNA.

Table V. Differentially expressed piRNAs between colorectal cancer and adjacent non-tumor tissues by small RNA sequencing.

piR-name	N-normalized expression	T-normalized expression	Log2(T/N)	Regulation
hsa-piR-18849	0.1439	5.5730	5.28	Up
hsa-piR-19521	0.4318	15.3838	5.15	Up
hsa-piR-17724	2.9265	52.0728	4.15	Up
hsa-piR-16970	0.0960	1.1804	3.62	Up
hsa-piR-794	0.7516	7.2952	3.28	Up
hsa-piR-20365	30.4165	275.2446	3.18	Up
has-piR-20548	0.2559	2.3027	3.17	Up
hsa-piR-1312	0.9435	8.2434	3.13	Up
hsa-piR-4309	2.7666	22.5436	3.03	Up
hsa-piR-14620	0.5757	4.6248	3.01	Up
hsa-piR-17791	0.7516	5.2053	2.79	Up
hsa-piR-19914	6.3168	36.5922	2.53	Up
has-piR-20829	5.1014	28.8132	2.50	Up
hsa-piR-17184	1.6312	8.9787	2.46	Up
hsa-piR-15026	0.2079	1.1030	2.41	Up
hsa-piR-17716	13.8010	64.4379	2.22	Up
hsa-piR-20450	10.7625	47.6028	2.15	Up
hsa-piR-16945	2.0310	8.7465	2.11	Up
hsa-piR-4307	0.9595	3.4057	1.83	Up
hsa-piR-20500	1.1194	3.7540	1.75	Up
hsa-piR-753	0.5117	1.6061	1.65	Up
hsa-piR-9051	2.6387	7.3726	1.48	Up
hsa-piR-19825	1.1514	3.1929	1.47	Up
hsa-piR-19102	0.4318	1.0836	1.33	Up
hsa-piR-1207	0.4957	1.1804	1.25	Up
hsa-piR-552	0.7516	1.7416	1.21	Up
hsa-piR-20009	8.3318	19.2927	1.21	Up
hsa-piR-12753	2.6706	6.1148	1.20	Up
hsa-piR-1346	4.1259	9.0368	1.13	Up
hsa-piR-20388	0.5437	1.1804	1.12	Up
hsa-piR-16735	22.1007	47.7189	1.11	Up
hsa-piR-12681	34.3825	69.5271	1.02	Up
hsa-piR-18780	11.7700	23.6079	1.00	Up
hsa-piR-18292	113.846	36.2632	-1.65	Down
hsa-piR-17194	2.7826	0.1161	-4.58	Down

N, adjacent non-tumor; T, colorectal cancer; piRNA, Piwi-interacting RNA.

Table VI. Relative expression levels of the top three upregulated piRNAs in colorectal cancer.

	Relative expression level		
Name	Adjacent tissues	Colorectal cancer tissues	P-value
hsa-piR-18849	0.02 (0.01-0.04)	0.06 (0.03-0.12)	<0.001
hsa-piR-19521	0.10 (0.04-0.17)	0.19 (0.09-0.30)	<0.001
hsa-piR-17724	0.63 (0.24-1.39)	0.92 (0.34-2.33)	<0.05

Data are presented as median with interquartile range. piRNA, Piwi-interacting RNA.

demonstrated that a number of piRNAs are dysregulated in tumor types, including gastric cancer, multiple myeloma and bladder cancer, and these piRNAs are also involved in the onset and progression of these tumor types (25,28,29). However, the majority of these studies only focused on a particular piRNA or profiled piRNA expression patterns in tumor cell lines, but not in tumor tissues. Based on the current research status, the differences in piRNA expression patterns in CRC and adjacent non-tumor tissues were investigated using deep sequencing. The present results demonstrated that only low proportions of unique piRNA reads (0.12% vs. 0.13%) and total piRNA reads (0.15% vs. 0.07%) were identified in both the N and T libraries, indicating that the types of piRNAs were few and that their expression levels were also low in CRC and adjacent

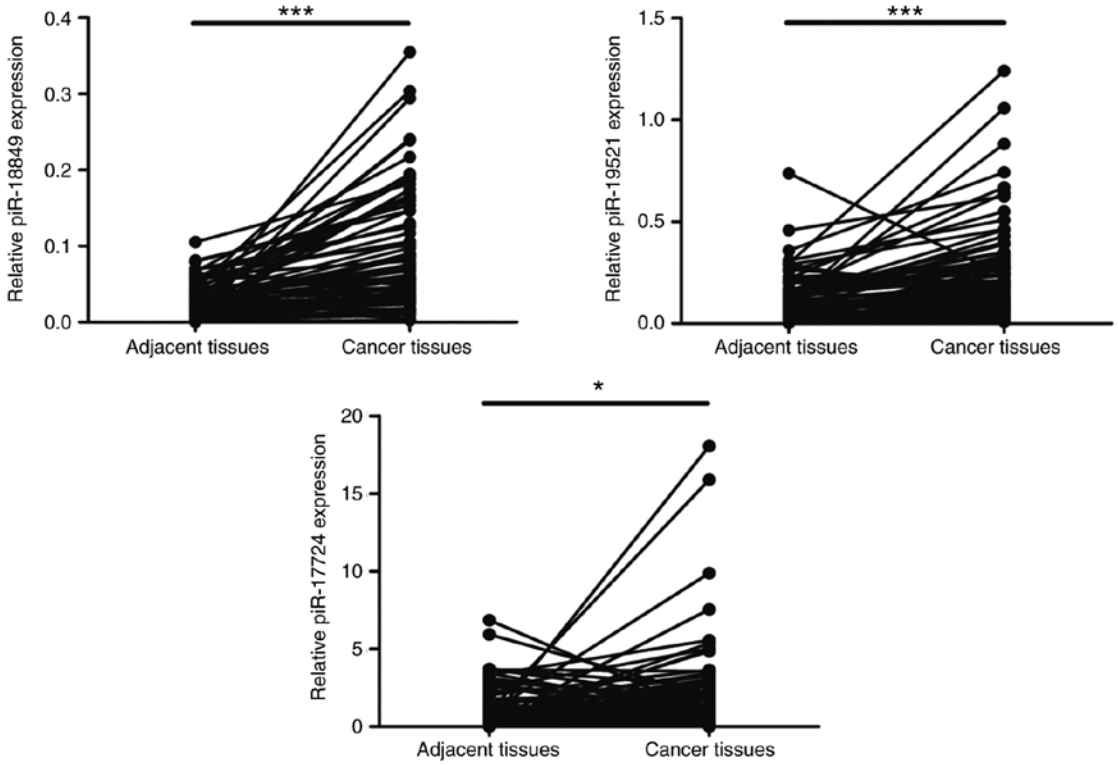

Figure 4. Relative expression levels of the three upregulated piRNAs in CRC and adjacent tissues. The expression levels of piR-18849, piR-19521 and piR-17724 normalized to U6 small nuclear RNA were assessed by reverse transcription-quantitative polymerase chain reaction using the $2^{-\Delta Cq}$ method in 80 pairs of CRC and matched adjacent tissues. *P<0.05 and ***P<0.001 vs. adjacent tissues. piRNA, Piwi-interacting RNA.

non-tumor tissues. Data from Yang *et al* (50) demonstrated that in normal human testis tissues, 25,845 unique reads corresponding to 1,051,404 total reads were matched to known piRNA sequences. Another study by Girard *et al* (18) identified 52,099 piRNAs in human testes. The aforementioned data indicate that despite piRNAs being expressed in somatic cells, including normal somatic cells and malignant cells, there are fewer types and their expression is reduced in somatic cells, compared with germ cells. The results were expected since piRNAs and Piwi are known to be germ cell-specific and serve key roles in germ cell development, stemness maintenance, meiosis and spermatogenesis (51).

It is notable that although piRNAs had fewer types and lower expression compared with miRNAs in CRC and adjacent tissues, the overall expression levels were notably different between these two types of tissues, indicating that piRNAs are dysregulated in CRC and further supporting that piRNAs are implicated in tumorigenesis (52). These data also indicate that low levels of piRNAs are sufficient to generate notable biological effects, similar to lncRNAs (53).

The signal transduction pathway and epigenetic status in tumors are similar to those in stem cells, indicating that tumors are an aberrant stem-like state (54). Therefore, Piwi and piRNAs that are highly enriched in germline stem cells may also be expressed in tumor cells. Indeed, studies disclosed that Piwi is overexpressed in all detected tumor types, as well as associated with tumor prognosis (55-58). Consistent with the aforementioned data, the present small RNA sequencing results demonstrated that the overall expression levels of piRNAs in CRC were increased compared with adjacent non-tumor tissues. Furthermore, among 35 differentially-expressed piRNAs, 33 piRNAs were upregulated in CRC, indicating that the majority of piRNAs in CRC may serve tumor-driving roles, which is consistent with the role of Piwi in tumors. RT-qPCR further demonstrated that the expression levels of the top three upregulated piRNAs, piR-18849, piR-19521 and piR-17724, were increased in CRC, compared with adjacent non-tumor tissues. Notably, the increased levels of piR-18849 and piR-19521 were significantly correlated with a poorer degree of differentiation. This may be because piRNAs are highly enriched in germline stem cells (51), and tumor cells with a poorer degree of differentiation are more similar to stem cells (59,60). Therefore, as the degree of tumor differentiation decreases, the expression of piRNAs may increase.

In breast cancer, the upregulation of piR-4987 was associated with lymph node metastasis (45). The present study determined that in addition to the degree of tumor differentiation, the overexpression of piR-18849 was also associated with lymph node metastasis in patients with CRC. piRNAs thus may emerge not only as a potential therapeutic target but also as an indicator for prognosis in patients with CRC. However, in the present study, the prognostic value of identified piRNAs could not be verified due to the short follow-up period of patients recruited to the study. Additionally, the specific function of these piRNAs and their roles in the survival or prognosis of patients will be investigated in subsequent studies.

Collectively, to the best of our knowledge, the present study presented, for the first time, global piRNA expression profiles in CRC and adjacent non-tumor tissues by deep sequencing for small RNAs. Based on the small RNA sequencing data, it was determined that the overall expression levels of piRNAs in CRC tissues were increased, compared with adjacent tissues, implying

that piRNAs may be involved in colorectal tumorigenesis. These observations will provide a theoretical basis for piRNA-targeted therapeutic strategies for CRC. However, only 3 pairs of samples were used for deep sequencing in the present study, which may cause a number of notable piRNAs to be omitted due to the limited samples. Another notable question was that the focus was only on those upregulated piRNAs, instead of those downregulated piRNAs. Therefore, determining what causes downregulation of a number of piRNAs in CRC, whether the downregulation of piRNAs is active or passive, and what roles these downregulated piRNAs serve in CRC will help the comprehensive understanding of the function of piRNAs in CRC.

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Availability of data and materials

The datasets used and/or analyzed during the current study are available from the corresponding author on reasonable request.

Authors' contributions

JY, WQ and HQJ conceived and designed the experiments. JY and CGJ conducted the experiments. DXZ, QD and JH recruited patients and collected samples as well as patients' clinico-pathological information. JY, WQ, XLX and XYJ analyzed and interpreted the data. JY drafted the manuscript. HQJ and XYJ revised the manuscript. HQJ supervised the whole project. All authors have read and approved the final manuscript.

Ethics approval and consent to participate

The present study was approved by the Ethics Committee of the Second Hospital of Hebei Medical University (Shijiazhuang, China). All patients provided written informed consent prior to participation in this study.

Patient consent for publication

Not applicable.

Competing interests

The authors declare that they have no competing interests.

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