Serum PIWI-Interacting RNAs piR-020619 and piR-020450 Are Promising Novel Biomarkers for Early Detection of Colorectal Cancer
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ABSTRACT

Background: Early diagnosis can significantly reduce colorectal cancer deaths. We sought to identify serum PIWI-interacting RNAs (piRNAs) that could serve as sensitive and specific noninvasive biomarkers for early colorectal cancer detection.

Methods: We screened the piRNA expression profile in sera from 7 patients with colorectal cancer and 7 normal controls using small RNA sequencing. Differentially expressed piRNAs were measured in a training cohort of 140 patients with colorectal cancer and 140 normal controls using reverse transcription quantitative PCR. The identified piRNAs were evaluated in two independent validation cohorts of 180 patients with colorectal cancer and 180 normal controls. Finally, the diagnostic value of the identified piRNAs for colorectal adenoma (CRA) was assessed, and their expression was measured in 50 patients with lung cancer, 50 with breast cancer, and 50 with gastric cancer.

Results: The piRNAs piR-020619 and piR-020450 were consistently elevated in sera of patients with colorectal cancer as compared with controls. A predictive panel based on the two piRNAs was established that displayed high diagnostic accuracy for colorectal cancer detection. The two-piRNA panel could detect small-size and early-stage colorectal cancer with an area under the ROC curve of 0.863 and 0.839, respectively. Combined use of the two piRNAs could effectively distinguish CRA from controls. Aberrant elevation of the two piRNAs was not observed in sera of patients with lung, breast, and gastric cancer.

Conclusions: Serum piR-020619 and piR-020450 show a strong potential as colorectal cancer-specific early detection biomarkers.

Impact: The field of circulating piRNAs could allow for novel tumor biomarker development.

Introduction
Colorectal cancer is currently the third most common malignancy worldwide with over 1.4 million new cases and over 680,000 deaths annually (1). The prognosis of colorectal cancer largely depends on the TNM stage at the time of diagnosis, with the 5-year survival rate decreasing from about 90% in early-stage disease (stages 0–II) to 12–13% in metastatic disease (stage IV; ref. 2). Unfortunately, colorectal cancer is usually asymptomatic at the early stage. Consequently, most patients are diagnosed at the advanced stage, when tumor invasion and dissemination have already occurred and operative curative resection cannot be performed. Therefore, early detection is crucial for improving the outcome of patients with colorectal cancer.

Colonoscopy is the gold standard for diagnosing colorectal cancer, but its invasiveness, high cost, and complicated bowel preparation makes it inappropriate for large-scale screening (3). Fecal occult blood tests and stool DNA tests are both noninvasive assays, but the former has low sensitivity and specificity and the latter is expensive (3).

Blood tumor marker detection has been advocated for cancer screening because it is simple, economical, and noninvasive. However, commonly used colorectal cancer biomarkers, such as carcinoembryonic antigen (CEA) and carbohydrate antigen 19-9 (CA19-9), are inadequate for early diagnosis because of their poor sensitivities (3). Recent studies have shown abnormal noncoding RNA profiles in the serum/plasma of patients with colorectal cancer compared with healthy people (4, 5). Some researchers have attempted to develop early diagnostic biomarkers for colorectal cancer from serum/plasma long noncoding RNAs (lncRNA) and miRNAs. However, serum/plasma lncRNAs are prone to degradation (6), substantially influencing measurement accuracy. Serum/plasma miRNAs are more stable than serum/plasma lncRNAs, but the use of miRNAs identified as tumor biomarkers has some limitations. First, their performance for early detection of colorectal cancer is still unsatisfactory (7). Second, often times four to five serum/plasma miRNAs are used in combination to perform a diagnosis (8, 9), which can be financially burdensome to patients and requires extensive work from clinical laboratories. Finally, most of the colorectal cancer serum/plasma miRNA biomarkers are discovered on the basis of a small study population from a single medical center, and their clinically diagnostic value requires further verification (9). Thus, it is urgently necessary to develop stable, sensitive, specific, and cost-effective circulating biomarker assays for...
early detection and screening of colorectal cancer in asymptomatic populations.

In addition to IncRNAs and miRNAs, PIWI-interacting RNAs (piRNAs), with a length of 26 to 30 nucleotides, are an important class of noncoding RNAs with increasingly demonstrated involvement in human malignancies (10). More than 30,000 piRNAs have been identified in the human genome, a number far exceeding than that of miRNAs (~2,000; refs. 10, 11). Dysregulated piRNA expression has been detected in multiple tumor tissue types, contributing to the proliferation, migration, invasion, stemness-maintenance, and drug-resistance of tumor cells (12–14). Tumor cells can release piRNAs into the extracellular space in the form of free, protein-binding or exosome-enclosed molecules, which can travel to serum, plasma, saliva, and other body fluids (11, 15, 16). The piRNAs in body fluids can be more resistant to oxidation and degradation than miRNAs because of the distinctive 2′-O-methyl modification at their 3′-ends (16, 17). It has been demonstrated that piR-57125 remains extremely stable in serum and plasma samples regardless of repeated freeze-thaw cycles or long-term incubation at room temperature (18). In light of these characteristics, circulating piRNAs should be considered as a novel potential source of noninvasive biomarkers for early cancer detection.

In this study, for the first time, we performed a large-scale, bi-center investigation to identify serum piRNA markers that can effectively distinguish patients with colorectal cancer, especially early-stage (TNM stages I–II) patients with colorectal cancer, from normal controls. Moreover, the expression of piRNA markers was measured in serum samples of patients with CRA, and lung, breast, and gastric cancers to further evaluate their diagnostic potential.

Materials and Methods

Study design

The study consisted of four phases. The first phase was a discovery phase in which 14 serum samples from 7 patients colorectal cancer with TNM I to II stages and 7 normal controls were collected and subjected to small RNA sequencing to explore differentially expressed piRNAs. The piRNAs with log (fold change) $>3$ or $<-3$, $P$ value $<0.01$, and $Q$ value $<0.001$ were identified as differentially expressed piRNAs between patients and controls.

The second phase was a training phase in which the differentially expressed piRNAs identified in phase I was further tested in an independent cohort of 140 patients with colorectal cancer and 140 normal controls using reverse transcription quantitative PCR (RT-qPCR). The piRNAs with cycle threshold (Ct) values $\leq 30$ in over 95% samples, fold change $>2$, and $P$ value $<0.01$ were selected as potential markers. Then, a predictive piRNA panel was constructed from the potential markers based on the logistic regression model for differentiation of patients and controls.

The third phase was a validation phase in which the diagnostic performance of the piRNA panel was evaluated in two independent cohorts containing 180 patients with colorectal cancer and 180 normal controls from two different medical centers. In this phase, the expression of the piRNAs in the panel was also measured in 12 paired serum samples from patients with colorectal cancer before and one month after surgery.

The fourth phase was an external validation phase in which the expression of piRNA markers in the serum samples of 40 patients with CRA was examined, and their performance to distinguish CRA from control individuals was assessed. In addition, the expression of piRNA markers in the serum samples of 50 patients with lung cancer, 50 with breast cancer, and 50 with gastric cancer was assayed.

Study population

The patients with colorectal cancer and control individuals in the first and second phases were from the Affiliated Hospital of Inner Mongolia Medical University. In the third phase, the 100 patients with colorectal cancer and 100 control individuals in validation cohort 1 were from the Affiliated Hospital of Inner Mongolia Medical University, and the 80 patients with colorectal cancer and 80 control individuals in validation cohort 2 were from the Affiliated People’s Hospital of Inner Mongolia Medical University. The 12 patients with colorectal cancer donating paired serum samples before and after surgery were from the Affiliated People’s Hospital of Inner Mongolia Medical University.

In the fourth phase, the patients with CRA, lung cancer, and breast cancer were from the Affiliated People’s Hospital of Inner Mongolia Medical University, and the gastric cancer patients were from the Affiliated Hospital of Inner Mongolia Medical University. All the lesions were diagnosed by histopathology analysis and the cancers were staged according to the TNM system of the International Union against Cancer. The involved patients had not received any anticancer treatments such as surgery, chemotherapy, radiotherapy, or cytotherapy before serum collection. Serum CEA and CA19-9 levels were measured in the Medical Laboratories of the two hospitals, with the cut-off values being 5 ng/mL for CEA and 27 U/mL for CA19-9. All the control individuals were recruited from the physical examination departments of the two hospitals, and were confirmed to have no indication for cancer in the physical examinations, no history of any malignancy, or no diagnosis of a benign neoplasm in the colorectum. Individuals with pulmonary and gastrointestinal chronic inflammation, as well as abnormal heart rate, blood pressure, and body temperature were excluded from the control group. The distribution of age and gender in the control group was similar to that in colorectal cancer group. All the patients and controls were of the same ethnicity. Demographic and clinical features of the patients and controls have been described in Supplementary Tables S1 and S2. Written informed consent was obtained from all participants. This study was conducted in accordance with the Declaration of Helsinki and approved by the Medical Ethics Committee of Inner Mongolia Medical University.

Serum preparation and RNA extraction

Five milliliters of fasting venous blood from each individual was drawn before breakfast and sampled in a coagulating tube. Within 1 hour, the tube was centrifuged at 4,000 rpm for 10 minutes at 4°C to obtain serum. Then, 1 mL aliquots of the serum were transferred to 1.5 mL RNase-free tubes and centrifuged at 12,000 rpm for 10 minutes at 4°C to remove cell debris. The serum samples were then transferred to RNase-free tubes and stored at −80°C until use.

Total RNA was extracted from serum samples using the mirNeasy Serum/Plasma Kit (Qiagen) following the manufacturer’s instructions. For small RNA sequencing, the quantity and integrity of RNA were assessed with the Qubit 2.0 (Life Technologies) and Agilent 2100 bioanalyzer (Agilent Technologies). For RT-qPCR, the quantity and purity of RNA was assessed with the SpectraMax QuickDrop spectrophotometer (Molecular Devices).

Small RNA sequencing

Fourteen libraries were prepared from the serum RNA of 7 patients and 7 controls according to the procedure described previously (19), and sequencing was conducted on the BGISEQ-500 platform.
Potential biomarkers which were practical and convenient to identify the sera of patients with colorectal cancer compared with normal were found aberrantly upregulated and six piRNAs downregulated in lower than 0.05 indicating statistical significance.

Discovery of differentially expressed piRNAs

During the discovery phase, 14 small RNA libraries established from the serum samples of 7 patients with colorectal cancer and 7 controls were subjected to small RNA sequencing. The sequenced from the serum samples of 7 patients with colorectal cancer and 7 controls were subjected to small RNA sequencing. The sequenced reads had a 5' uridine bias in piRNAs. Sequence analysis of the annotated piRNAs revealed a strong propensity for a 5' uridine bias in piRNAs.

Relative expression levels of piRNAs were calculated by the 2^-ΔΔCT method, normalizing to the internal control. The piRNA-specific primers were designed and synthesized by Sangon Biotech Shanghai Co., Ltd.

Statistical analysis

Expression levels of the analyzed serum piRNAs in patients and normal controls were compared using the nonparametric Mann–Whitney test. The diagnostic performance of the piRNAs for discriminating patients from controls was evaluated by receiver-operating characteristic (ROC) curve analysis. The difference of the area under the ROC curve (AUC) was assessed with Medcalc 18.2.1. The comparison of gender distribution, sensitivity, and specificity was performed using Pearson’s chi-squared test. The difference in age between patients with colorectal cancer and controls was analyzed by unpaired unequal variance t test. Paired samples before and after the surgery were analyzed using two-tailed Wilcoxon test. Logistic regression analysis was performed with Statistical Package for the Social Sciences version 18.0. All tests of significance were two-tailed, with a P value lower than 0.05 indicating statistical significance.

Results

During the discovery phase, 14 small RNA libraries established from the serum samples of 7 patients with colorectal cancer and 7 controls were subjected to small RNA sequencing. The sequenced reads contained on average 29,480,448 ± 2,283,578 raw reads and 24,208,284 ± 1,517,389 clean reads. More than 98.9% of the clean reads had a Q value ≥20. In the clean reads of each sample, 563,551,725 ± 68,838,132 bases passed the filter, of which over 98.9% had a Q value ≥20, indicating a high quality of the obtained data. Mapping the data to the piRNABank, 179,454 ± 58,935 reads were annotated, being a proportion of 0.74 ± 0.27% of the clean reads. The piRNAs with low quality, with poly A, without 3' primer or shorter than 18 nucleotides. The obtained clean reads were mapped against piRNABank (http://pirnabank.ibab.ac.in/request.html) with Bowtie 2 allowing up to one mismatch per read. Differentially expressed piRNAs were screened using the DEGseq R package.

RT-qPCR

Complementary DNA was reverse-transcribed from 100 ng total RNA with the miRcute Plus miRNA First-Strand cDNA Kit (Tiangen). Real-time PCR was performed on an ABI PRISM 7500 Sequence Detection System (Applied Biosystems) using the miRcute Plus miRNA qPCR Kit (Tiangen). The PCR reactions were incubated at 95°C for 15 minutes, followed by 40 cycles of 94°C for 20 seconds and 60°C for 34 seconds, and then ramped from 60 to 95°C to obtain melting curves. After the cycling, melting curve analyses and electrophoreses on agarose gels were conducted to evaluate the specificity of the PCR products. The Ct values of three candidate internal controls were analyzed with NormFinder to identify an internal control. Relative expression levels of piRNAs were calculated by the 2^-ΔΔCT method, normalizing to the internal control. The piRNA-specific primers were designed and synthesized by Sangon Biotech Shanghai Co., Ltd.

Evaluation of the upregulated piRNAs during the training phase

The nine upregulated piRNAs discovered in phase I was further tested on an independent training cohort of 140 patients with colorectal cancer and 140 normal controls. Among the nine piRNAs, piR-000330, piR-022421, piR-017458, and piR-001169 had Ct values greater than 30 in over 5% of samples. Both piR-019544 and piR-017723 exhibited similar expression levels between patients with colorectal cancer and normal controls, piR-020619, piR-020450, and piR-020814 were confirmed to be upregulated in patients with colorectal cancer compared with normal controls (Supplementary Table S5; Fig. 1A). The diagnostic performance of piR-020619, piR-020450, and piR-020814 was evaluated by a ROC curve analysis, and the AUCs were 0.871 (sensitivity = 84.29%, specificity = 76.43%), 0.841 (sensitivity = 81.43%, specificity = 75.00%), and 0.680 (sensitivity = 58.57%, specificity = 72.86%), respectively (Supplementary Table S6; Fig. 1B).

Establishment of a predictive colorectal cancer piRNA panel

A stepwise logistic regression model to estimate the risk of being diagnosed with colorectal cancer that included piR-020619, piR-020450, and piR-020814 was applied to the training cohort. piR-020619 and piR-020450 turned out to be significant predictors of colorectal cancer risk. The predicted probability of being diagnosed with colorectal cancer from the logit model based on the two-piRNA panel was calculated using the following equation: logit(P) = -3.793 + 0.592 × piR-020619 + 0.498 × piR-020450. The calculated probability was used to construct an ROC curve to evaluate the diagnostic performance of the piRNA panel. The panel yielded an AUC of 0.879, which was larger than those for CEA (0.657, P < 0.0001) and CA19-9 (0.557, P < 0.0001), a sensitivity of 86.00% [higher than those for CEA (84.29%), specificity = 76.43%], 0.841 (sensitivity = 81.43%, specificity = 75.00%) and 0.680 (sensitivity = 58.57%, specificity = 72.86%), respectively (Supplementary Table S6; Fig. 1C).

Validation of the piRNA panel

During the validation phase, patients with colorectal cancer also displayed higher levels of serum piR-020619 and piR-020450 than did normal controls (Fig. 2). The parameters estimated from the training phase were used to predict the probability of being diagnosed with colorectal cancer in the two independent validation cohorts. In validation cohort 1, the piRNA panel demonstrated an AUC of 0.883 [larger than those for CEA (0.658, P < 0.0001) and CA19-9 (0.596, P < 0.0001)], a sensitivity of 86.00% [higher than those for CEA (40.00%, P < 0.0001) and CA19-9 (27.00%, P < 0.0001)], and a sensitivity of 86.00% [higher than those for CEA (84.29%), specificity = 76.43%], 0.841 (sensitivity = 81.43%, specificity = 75.00%) and 0.680 (sensitivity = 58.57%, specificity = 72.86%), respectively (Supplementary Table S6; Fig. 1B).
In the training cohort, the piRNA panel demonstrated an AUC of 0.920 [similar to those for CEA (93.00%, $P = 1.0000$) and CA19-9 (95.00%, $P = 0.4503$; Fig. 2A; Supplementary Table S6)]. In validation cohort 2, the piRNA panel demonstrated an AUC of 0.913 [larger than those for CEA (0.681, $P < 0.0001$) and CA19-9 (0.625, $P < 0.0001$)], a sensitivity of 88.75% [higher than those for CEA (42.50%, $P < 0.0001$) and CA19-9 (28.75%, $P < 0.0001$)], and a specificity of 93.75% [similar to those for CEA (93.75%, $P = 0.4795$) and CA19-9 (96.25%, $P = 0.4795$; Fig. 2B; Supplementary Table S6)]. When the data from the two cohorts were processed in a mixed-up manner, similar results were obtained (Fig. 2C; Supplementary Table S6).

In addition, the expression of piR-020619 and piR-020450 was detected in 12 paired serum samples from patients with colorectal cancer before and 1 month after surgery. Both the piRNAs showed decreased expression levels in postoperative samples (Fig. 3).

**Logistic regression analysis of the piRNA panel**

Next, we performed a stepwise binary logistic regression analysis to evaluate whether the diagnostic usefulness of the piRNA panel was affected by clinical features of colorectal cancer cases, including age, gender, tumor location, TNM stage, lymph node metastasis, and distant metastasis. The results showed that the piRNA panel...
Figure 2.
Diagnostic performance of piR-020619 and piR-020450 for colorectal cancer in the validation phase. A, In validation cohort 1, levels of serum piR-020619 and piR-020450 were significantly elevated in patients with colorectal cancer compared with normal controls \((P < 0.0001)\); the two-piRNA panel displayed significantly higher performance than CEA and CA19-9 for colorectal cancer detection \((P < 0.0001)\). B, In validation cohort 2, levels of serum piR-020619 and piR-020450 were also significantly elevated in patients with colorectal cancer compared with normal controls \((P < 0.0001)\); the two-piRNA panel also displayed significantly higher performance than CEA and CA19-9 for colorectal cancer detection \((P < 0.0001)\). C, When the data from the two cohorts were mixed together, the analyses gave similar results.
remained a strong predictor of colorectal cancer regardless of these subgroupings of the patients in the training and validation cohorts (Supplementary Table S7).

Diagnostic performance of the piRNA panel for small colorectal tumors

There were 71 patients with colorectal cancer with tumors ≤ 3 cm in the training and validation cohorts of this study. We assessed the performance of the piRNA panel in differentiating the small-tumor patients from normal controls. The piRNA panel presented an AUC of 0.863, which was larger than those for CEA (0.587, P < 0.0001) and CA19-9 (0.548, P < 0.0001). The sensitivity for the panel was 81.69%, higher than those for CEA (23.94%, P < 0.0001) and CA19-9 (15.49%, P < 0.0001). The specificity for the panel was 90.94%, similar to those for CEA (93.44%, P = 0.0991) and CA19-9 (94.06%, P = 0.0051; Fig. 4A; Supplementary Table S8). Among the 71 patients with small tumor lesions, 54 patients were CEA negative and 60 patients were CA19-9 negative. The piRNA panel was able to distinguish the CEA-negative and CA19-9-negative patients from normal controls, as evidenced by an AUC of 0.844 (sensitivity = 77.78%, specificity = 90.94%) and 0.855 (sensitivity = 80.00%, specificity = 90.94%), respectively (Fig. 4B and C; Supplementary Table S8).

Diagnostic performance of the piRNA panel for early-stage colorectal cancer

There were 173 patients with early-stage (TNM stages I to II) colorectal cancer in the training and validation cohorts of this study. We further assessed the performance of the piRNA panel in differentiating the patients with early-stage colorectal cancer from normal controls. The piRNA panel exhibited an AUC of 0.839, which was larger than those for CEA (0.595, P < 0.0001) and CA19-9 (0.536, P < 0.0001). The piRNA panel had a specificity of 76.79%, which was higher than those for CEA (25.60%, P < 0.0001) and CA19-9 (13.10%, P < 0.0001), and had a sensitivity of 90.94%, which was similar to those for CEA (93.44%, P = 0.0991) and CA19-9 (94.06%, P = 0.0551; Fig. 4D; Supplementary Table S8). Among the 173 early-stage patients, 125 were CEA negative and 146 were CA19-9 negative. The piRNA panel was also able to distinguish the CEA-negative and CA19-9-negative patients from normal controls, with an AUC of 0.839 (sensitivity = 76.80%, specificity = 90.94%) and 0.828 (sensitivity = 74.66%, specificity = 90.94%), respectively (Fig. 4E and F; Supplementary Table S8).

Diagnostic performance of piR-020619 and piR-020450 for CRA

To evaluate the diagnostic performance of piR-020619 and piR-020450 for precancerous colorectal cancer lesions, we measured the expression of the two piRNAs in the sera of 40 patients with CRA, and observed an elevated expression compared with control individuals (Fig. 5). ROC curve analyses revealed that piR-020619 and piR-020450 could discriminate patients with CRA from normal controls, with an AUC of 0.701 (sensitivity = 82.50%, specificity = 55.90%) and 0.689 (sensitivity = 67.50%, specificity = 72.50%), respectively. Combining the two piRNAs resulted in an increased AUC of 0.779 with 72.50% sensitivity and 76.60% specificity (Fig. 6).

Serum piR-020619 and piR-020450 levels in patients with lung, breast, and gastric cancers

The expression levels of piR-020619 and piR-020450 in the sera of patients with lung, breast, and gastric cancer were similar to those of normal controls (Fig. 5), suggesting that the two piRNAs could serve as colorectal cancer-specific early diagnostic biomarkers.

Discussion

PIWI-interacting RNAs were first discovered in D. melanogaster testis tissue in 2001, and were initially thought to be expressed solely in gonadal tissues, where they function to safeguard the germline genome against transposon-induced mutations. Recent studies demonstrate extensive expression of piRNAs in various human somatic tissues, where they play an important role in maintaining cellular homeostasis by silencing transposable elements, keeping normal DNA methylation and histone modifications, controlling mRNA stability, modulating transcription factor activity, as well as regulating protein phosphorylation and protein-protein interactions (20–25).

Dysregulated piRNA expression leads to amplification and mobilization of transposable elements, hypermethylation or hypomethylation of genomic DNA, and disruption of normal histone modifications (26–30). These abnormalities further cause a variety of genomic instability events such as insertion mutations, gene amplifications, and chromosomal rearrangements (31). Because genomic instability is a major driving force of carcinogenesis and cancer evolution (32), some piRNAs may be dysregulated at very early phases of cancer development. In this study, for the first time, we discovered that expression of serum piR-020619 and piR-020450 significantly decreased after surgical removal of the tumors.
Figure 5.
Expression of piR-020619 and piR-020450 in sera of normal controls, and patients with CRA, stage I to II CRC, stage III to IV colorectal cancer, lung cancer, breast cancer, and gastric cancer. The levels of serum piR-020619 and piR-020450 in normal controls were significantly lower than those in patients with CRA, stage I to II colorectal cancer, and stage III to IV colorectal cancer (P < 0.05), whereas they were similar to those in patients with lung, breast, and gastric cancers (P > 0.05).

Figure 4.
Diagnostic performance of the two-piRNA panel for small-size and early-stage colorectal cancer in the combined training and validation cohorts. A, The two-piRNA panel displayed significantly higher performance than CEA and CA19-9 for detection of small-size colorectal cancer (P < 0.0001). B, The ROC curve of the two-piRNA panel for detection of CEA-negative small colorectal cancer (AUC = 0.844). C, The ROC curve of the two-piRNA panel for detection of CA19-9-negative small colorectal cancer (AUC = 0.855). D, The two-piRNA panel displayed significantly higher performance than CEA and CA19-9 for detection of early-stage colorectal cancer (P < 0.0001). E, The ROC curve of the two-piRNA panel for detection of CEA-negative early-stage colorectal cancer (AUC = 0.839). F, The ROC curve of the two-piRNA panel for detection of CA19-9-negative early-stage colorectal cancer (AUC = 0.828).
colorectal cancer compared with normal controls, suggesting that the aberrant expression of the two piRNAs occurs early in the precancerous stages of colorectal cancer. Activation of the two piRNAs may contribute to the pathological process of colorectal cancer initiation, and their abnormal upregulation in sera can serve as a sensitive indicator for early colorectal cancer detection.

Most patients with early-stage colorectal cancer do not present any phenotypic symptoms, and are often missed on routine physical examination. The conventional serum tumor markers CEA and CA19-9 are useful in therapeutic evaluation and recurrence surveillance, but are still inadequate for colorectal cancer screening mainly because of low sensitivity in early-stage patients (3). In our study cohorts, CEA and CA19-9 indeed demonstrated poor performance for early diagnosis of colorectal cancer, as their sensitivities were only 25.60% and 13.10%, respectively. In comparison, the two-piRNA panel established in our study generated a greatly improved sensitivity of 76.79% in detecting early-stage colorectal cancer. In recent years, the abnormally methylated SEPT9 gene has been tested for utility in colorectal cancer screening. However, the reported sensitivities of plasma SEPT9 methylation in diagnosing early-stage patients often vary between over 30% to less than 70% (33–35), which is lower than that for our piRNA panel. Thus, the two-piRNA panel may be a useful noninvasive biomarker for early-stage colorectal cancer screening in asymptomatic populations.

Up to 85% of colorectal cancers originate from adenomatous polyps. Timely recognition and removal of CRA can prevent the benign precancerous lesions from progressing to malignancy. Thus far, few effective blood biomarkers for CRA have been developed. In this study, we found that the expression levels of serum piR-020619 and piR-020450 were higher in patients with CRA than in normal controls, and that combined use of the two piRNAs had significant diagnostic value for CRA with a sensitivity of 72.50%. Therefore, the two piRNAs may be suitable for the early detection of precancerous lesions of colorectal cancer and help reduce colorectal cancer incidence and mortality.

Circulating tumor biomarkers can be organ-specific or nonspecific. Organ-specific biomarkers provide the advantage of directly identifying tumor tissue sites. The serum levels of the tumor markers CEA and CA19-9 can be elevated in multiple malignant conditions, making it difficult to specify the precise lesion location. In this study, the expression of serum piR-020619 and piR-020450 was increased in patients with colorectal cancer but not in patients with lung, breast, and gastric cancers, suggesting a potential of the two piRNAs to serve as colorectal cancer-specific circulating tumor biomarkers. One published study focusing on several types of human normal and tumor tissues discovered that the dysregulation of piRNA expression in tumor tissues was organ-specific (36). This specificity in both sera and tissues of patients with cancer suggests significant value of piRNAs as tumor biomarkers.

The expression levels of serum piR-020619 and piR-020450 significantly decreased after the colorectal tumors were surgically removed, suggesting that the excessive circulating piR-020619 and piR-020450 in the patients might originate from colorectal cancer tissues. They could be overexpressed in colorectal cancer cells and directly promote the cellular malignant behaviors such as proliferation, migration, invasion, stemness-maintenance, or drug-resistance by disrupting the epigenetic regulation of the transposable elements, protein-coding genes, or other noncoding RNAs vital for cancer risk. We will investigate the roles of the two piRNAs in colorectal cancer initiation and development in future work.

In conclusion, serum piR-020619 and piR-020450 are promisingly novel colorectal cancer-specific biomarkers that can effectively detect small colorectal cancer lesions, early-stage colorectal cancer, and CRA. This study not only provides potential tools for colorectal cancer screening in asymptomatic populations, but also implies that circulating piRNAs are a valuable source for developing new noninvasive tumor biomarkers.

**Disclosure of Potential Conflicts of Interest**
No potential conflicts of interest were disclosed.

**Authors’ Contributions**
Conception and design: Z. Wang, Y. Jia, H. Zhao
Development of methodology: W. Xin, Y. Chen, Y. Wu
Acquisition of data (provided animals, acquired and managed patients, provided facilities, etc.): Z. Wang, H. Yang, D. Ma, Y. Mu, L. Feng, J. Liang
Analysis and interpretation of data (e.g., statistical analysis, biostatistics, computational analysis): H. Yang, Y. Mu, X. Tan
Writing, review, and/or revision of the manuscript: Z. Wang, H. Yang, X. Tan
Administrative, technical, or material support (i.e., reporting or organizing data, constructing databases): Z. Wang, D. Ma, Y. Mu, Q. Hao, Y. Jia
Study supervision: H. Zhao

**Image**
Figure 6.
ROC curves of piR-020619, piR-020450, and the combination of piR-020619 and piR-020450 for discriminating CRA from normal controls (AUC = 0.701, 0.689, and 0.771, respectively).
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