Research Article

Cancer Prevention Research

Salivary microRNAs Show Potential as a Noninvasive Biomarker for Detecting Resectable Pancreatic Cancer

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Abstract

Early surgery is vital in the treatment of pancreatic cancer, which is often fatal. However, there is currently no useful noninvasive biomarker to screen for pancreatic cancer. Studies have documented that many salivary molecules can be used to detect systemic diseases. We investigated whether salivary miRNAs are useful biomarkers for detecting resectable pancreatic cancer. Using an Agilent microarray, salivary miRNAs were profiled from saliva samples of 8 patients with resectable pancreatic cancer and 8 healthy controls. Candidate biomarkers identified in the profiles were subjected to validation using quantitative PCR and an independent sample set of 40 patients with pancreatic cancer, 20 with benign pancreatic tumors (BPT), and 40 healthy controls. The validated salivary miRNA biomarkers were evaluated within three discriminatory categories: pancreatic cancer versus healthy control, pancreatic cancer versus BPT, and pancreatic cancer versus noncancer (healthy control + BPT). miR-3679-5p showed significant downregulation in the pancreatic cancer group within the three categories ($P = 0.008$, 0.007, and 0.002, respectively), whereas miR-940 showed significant upregulation in pancreatic cancer ($P = 0.006$, 0.004, and 0.0001, respectively). Logistic regression models combining the two salivary miRNAs were able to distinguish resectable pancreatic cancer within the three categories, showing sensitivities of 72.5%, 62.5%, and 70.0% and specificities of 70.0%, 80.0%, and 70.0%, respectively. Salivary miR-3679-5p and miR-940 possess good discriminatory power to detect resectable pancreatic cancer, with reasonable specificity and sensitivity. This report provides a new method for the early detection of pancreatic cancer and other systemic diseases by assessing salivary miRNAs. *Cancer Prev Res; 8(2); 165–73. ©2014 AACR.*

Introduction

Pancreatic cancer is one of the most frequently fatal cancers worldwide. Because of its insidious onset, aggressive development, and lack of effective screening methods, the prognosis of pancreatic cancer is dismal, with a 5-year survival rate of only 5% (1). Surgical resection is presently the only effective therapy for pancreatic cancer. For patients with stage I pancreatic cancer, the 5-year survival rate can reach 50% after surgery (2, 3). Accordingly, early diagnosis and surgery are vital for treating pancreatic cancer. To date, carbohydrate antigen 19-9 (CA19-9) is the only biomarker related to pancreatic cancer for which FDA-cleared diagnostics exist (4). However, due to the high false-positive and -negative rates, it is not recommended for pancreatic cancer screening. Thus, there is an urgent need to find a noninvasive biomarker for screening early pancreatic cancer.

Saliva is considered to represent the body's health, reflecting various conditions, such as cancer and infectious and systemic diseases. A large blood supply flows into the salivary glands; thus, saliva is a terminal product of blood circulation. Most compounds found in blood are also present in saliva, such as DNA, RNA, protein, drugs, and viruses. In the United States, commercial products for saliva-based diagnosis of drug abuse and HIV infection and for assessing hormone levels and various toxicology issues have been approved by the FDA and are in widespread use (5). Studies have demonstrated that evaluation of salivary transcripts, proteins, metabolites, and other molecules can detect oral cancer (6–8), breast cancer (9, 10), lung cancer (11), ovarian cancer (12), Sjögren syndrome (13), and other oral and systemic diseases (14, 15).

Salivary mRNA (16) and microbiota (17) may also possess discriminatory power for resectable pancreatic cancer. Aberrantly expressed miRNAs are associated with the onset and development of various cancers; thus, they may be biomarkers with good diagnostic power for detecting cancers (18). Several miRNAs have been found to be aberrantly expressed in pancreatic cancer tissue,

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Note: Supplementary data for this article are available at Cancer Prevention Research Online (http://cancerprevres.aacrjournals.org/).

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plasma, and serum (19). Moreover, salivary miRNAs have been reported as useful for detecting oral cancer (20, 21), Sjögren syndrome (22), and esophageal cancer (23, 24). On the basis of these findings, we hypothesized that salivary miRNAs may also possess discriminatory power for pancreatic cancer. We conducted this study to assess that hypothesis.

Materials and Methods

Subject selection

From January 2011 to July 2013, saliva samples from 48 patients with resectable pancreatic cancer and 20 with BPT were collected at Guangdong General Hospital, The First Affiliated Hospital of Sun Yat-sen University and Sun Yat-sen University Cancer Center (Guangzhou City, Guangdong Province, China). All patient histopathology results were confirmed by pathology after surgical tumor resection, and no concurrent oral, systemic, or infectious diseases, such as hepatitis or systemic lupus erythematosus, were diagnosed. Patients with a diagnosis of other malignancies and those receiving chemotherapy and radiotherapy before saliva collection were excluded.

Pancreatic cancers were deemed resectable or borderline resectable according to the National Comprehensive Cancer Network: Practice Guidelines in Oncology (v.2.2010; ref. 25). Details are presented in Supplementary Material S1. Cancer staging was based on The American Joint Committee on Cancer: Seventh Edition on Cancer Staging (26). Details are presented in Supplementary Material S2. Of the patients with BPT, 11 underwent tumor resection, and the histopathology results were pathologically confirmed after surgery. The other 9 patients with BPT only underwent biopsies, and their histopathology results were confirmed. On the basis of negative results from health examinations, including chest X-rays, oral examinations, abdominal ultrasounds, fecal occult-blood testing, and blood cancer biomarker measurements (AFP, CEA, CA19-9), saliva samples from 48 age-, gender-, and ethnicity-matched healthy individuals were obtained from the Physical Examination Center of East Ward, Guangdong General Hospital (Guangzhou City, Guangdong Province, China).

Institutional review boards or ethics committees from all participating institutes approved the study protocol. All participants provided written informed consent for their information to be stored in the hospital database and used for research.

Brief demographic information regarding the subjects is presented in Supplementary Table S1. More detailed information is presented in Supplementary Material S3.

Saliva collection

Saliva was obtained before the resection of the pancreatic cancers or the benign tumors. Subjects were asked to refrain from eating, drinking, smoking, and oral hygiene procedures for at least 2 hours before collection. To stimulate glandular salivary flow, subjects received a citric acid solution to apply to the surface of the tongue with a cotton swab for 5 seconds every 30 seconds. The citric acid stimulation continued at 30-second intervals during the entire collection procedure. Up to 3 mL of saliva from each subject was collected in a 50 mL centrifuge tube. Saliva samples were centrifuged (3,000 × *g*, 15 minutes, 4°C) to spin down the exfoliated cells, and the supernatant was transferred into microcentrifuge tubes, followed by further centrifugation (12,000 × *g*, 10 minutes, 4°C) to completely remove cellular components. Samples were then stored at −80°C until use (27).

Quantification of salivary miRNAs

The mirVana PARIS Kit (Life Technologies) was used to isolate total RNA from 1.2 mL saliva supernatant sample, according to the manufacturer's protocol. Finally, RNA was eluted in 30 μL preheated nuclease-free water (95°C) and stored at −80°C until use. The reverse transcription reaction was first carried out with 11 μL mixture containing 2 μL RNA extract, 2 μL RT primer (Ribo), and 7 μL nuclease-free water. The 11 μL mixture was incubated at 70°C for 10 minutes and in ice for 2 minutes. Next, 5 μL RT buffer, 2 μL dNTP (2.5 mmol/L), 0.5 μL RNase inhibitor (40 U/μL), 0.5 μL reverse transcriptase (200 U/μL; all from TaKaRa), and 6 μL nuclease-free water was added to the 11 μL mixture. The reverse transcription reaction continued at 42°C for 60 minutes, 70°C for 10 minutes, and then terminated at 4°C. cDNA solution (3 μL) was amplified using 9 μL SYBR Premix Ex Taq (TaKaRa), 2 μL miRNA forward primer, 2 μL miRNA reverse primer, 0.5 μL ROX 2, and 3.5 μL nuclease-free water in a final volume of 20 μL. Quantitative PCR (qPCR) reactions were run using an ABI 7500 Real-Time PCR System (Life Technologies), and the reaction mixtures were incubated at 95°C for 2 minutes, followed by 45 cycles of 95°C for 5 seconds and 60°C for 32 seconds. After completion of the PCR cycles, melt curve analysis was performed to validate the generation of the expected PCR products. Each sample was analyzed in triplicate. The expression levels of each miRNA were normalized to that of U6 snRNA (20, 21, 28). All expression levels were calculated using the $2^{-\Delta\Delta Ct}$ method (29).

Study design

This study consisted of a discovery phase and a verification phase, followed by an independent validation phase (Supplementary Fig. S1). Of the 116 samples, 8 pancreatic cancer samples and 8 healthy control samples were randomly selected for the discovery phase. The salivary miRNA profiles from the 16 salivary samples were detected using the Human miRNA Microarray, release 19.0, 8 × 60 K (Agilent). In total, 2006 mature miRNA sequences were assembled and integrated into our miRNA microarray design. Candidate biomarkers identified from the microarray study were first verified using qPCR on the discovery sample set (8 cancers and 8 healthy controls). The selection criteria for candidate biomarkers were as follows. First, a two-tailed *t* test was used for comparison of average miRNA expression signal intensities between the pancreatic cancer and control groups. After obtaining estimates and the *P* values of each probe set, we corrected the *P* values for the false discovery rate (FDR). The critical α level of 0.01 was set for statistical significance. Second, only those miRNAs that exhibited at least a 4-fold change were selected for further analysis. Third, we excluded probe sets on the array that were assigned as "Absent" (A) in all samples from the two groups. Accordingly, 10 miRNAs were selected. The raw microarray data have been uploaded to the GEO database (http://www.ncbi.nlm.nih.gov/geo), access number GSE53325. Normalized microarray data are presented in Supplementary Material S4.

In the verification phase, the expression levels of the 10 selected miRNAs were verified preliminarily using qPCR and the discovery sample set. The results showed that two miRNAs (miR-3679-5p and miR-940) showed significant differential expression between the two groups.

In the validation phase, the two verified miRNA biomarkers, miR-3679-5p and miR-940, were assayed by qPCR in the independent sample set of 40 pancreatic cancer, 20 BPT, and 40

healthy controls. The validated biomarkers were evaluated within four discriminatory categories: pancreatic cancer versus healthy control, pancreatic cancer versus BPT, pancreatic cancer versus noncancer (healthy control + BPT), and BPT versus healthy control. The patients with BPT were included in the validation to determine whether the candidate biomarkers could also differentiate patients with malignant pancreatic tumors from those with benign ones, which is difficult to discriminate from early pancreatic cancer using imaging radiology and clinical manifestations such that misdiagnoses can easily occur. For example, it is usually very difficult to differentiate pancreatic cancer from local chronic pancreatitis.

Statistical analysis

miRNA expression levels and ages were compared using the Mann–Whitney *U* test. Genders were compared using the χ^2 test. Receiver-operating characteristic (ROC) curves were used to evaluate the discriminatory power of each miRNA for differentiating between patients and controls. The correlation between serum total bilirubin and CA19-9 or validated miRNAs was analyzed using the Spearman correlation test. The validated salivary biomarkers were constructed into binary logistic regression models (separately for the comparisons of the three discriminatory categories), and step-wise backward model selection was performed to determine the final combinations of biomarkers. The predicted probability for each subject was obtained and used to construct ROC curves. An optimal cut-off value is needed for the ROC curve to define the discriminatory power. When the Younden index (Younden index = sensitivity + specificity −1) reaches the maximum value, the corresponding cut-off value will yield the highest sum of sensitivity and specificity. Statistical analyses were performed using the SPSS software (ver. 13.0).

To obtain positive and negative predictive values of the models of biomarker combinations, tree-based classification models, classification and regression tree (CART), were constructed with the validated miRNA biomarkers as predictors. CART fits the classification model by binary recursive partitioning, in which each step involves searching for the predictor variable that results in the best split of the cancer versus the control groups. In this way, the parent group containing all samples was subsequently divided into cancer and control groups. Our initial tree was pruned to remove all splits that did not result in sub-branches with differing classifications.

Simulation studies were conducted to test the magnitude of the bias introduced by model selection using multiple biomarker models. Briefly, we first permuted the group identities for the subjects using the cancer versus noncancer (healthy control and/or BPT) comparison. For each marker, we used the *t* test to compare the permuted groups, and then built a logistic regression model with the permuted group identities as the outcome and using stepwise selection with the two most significant biomarkers. For each of the resulting multiple marker models, we estimated the prediction accuracy by computing the areas under the curves (AUC). This process was iterated 1,000 times. The set of AUC values form an unbiased permutation distribution for the true model AUC and correct for biases generated by the model selection and coefficient estimation process.

CART and simulation studies were performed using the "R" software (ver. 3.0.2). A two-tailed $P < 0.05$ was considered statistically significant.

Results

Comparison of salivary miRNA profiles between pancreatic cancer and healthy controls

According to the microarray results, a total of 2,006 mature miRNAs were assayed. According to the selection criteria, 10 miRNAs were selected and further assessed as follows: miR-4433-5p ($P = 0.005$, fold change = 65.075), miR-4665-3p ($P < 0.001$, fold change = 27.845), miR-940 ($P = 0.001$, fold change = 16.260), miR-1273g-3p ($P < 0.001$, fold change = 0.054), miR-3676-5p ($P = 0.002$, fold change = 0.187), miR-3679-5p ($P = 0.001$, fold change = 0.177), miR-3940-5p ($P = 0.002$, fold change = 0. 231), miR-4327 ($P = 0.001$, fold change = 0.079), miR-4442 ($P < 0.001$, fold change = 0.103), miR-5100 ($P = 0.001$, fold change = 0.162). These *P* values were corrected for the false discovery rate. According to the fold change, the first three miRNAs listed showed significant upregulation in pancreatic cancer, while the latter seven showed the opposite trend. Hierarchical clustering was performed to categorize the 16 profiled subjects in the discovery phase according to the expression signals of the 10 miRNAs from the microarray using covariance values and the complete linkage clustering method included in the MeV_4_9_0 software. The results showed that the patients with pancreatic cancer ($n = 8$) and healthy controls ($n = 8$) could be classified into distinct groups, preliminarily indicating the discriminatory power of these salivary miRNAs. A heatmap of the 10 verified miRNA was built based on the microarray data (Fig. 1).

Verification and independent validation of salivary biomarkers

In the verification phase, expression levels of the 10 miRNAs were preliminarily verified using qPCR with the discovery sample set. The results showed that miR-3679-5p was significantly downregulated ($P = 0.027$) and miR-940 significantly upregulated ($P = 0.027$) in pancreatic cancer. The deregulation patterns (up-/downregulation) of these candidate biomarkers were consistent with those from microarray analysis. The other eight miRNAs (miR-1273g-3p, miR-3676-5p, miR-3679-5p, miR-3940-5p, miR-4327, miR-4433-5p, miR-4442, miR-4665-3p, miR-5100, and miR-940) did not show significant differences between the two groups (*P* values = 0.067, 1.000, 0.753, 0.674, 0.916, 0.753, 0.674, and 0.529, respectively).

In the validation phase, the two verified miRNA biomarkers, miR-3679-5p and miR-940, were assayed using qPCR with the independent sample set of 40 pancreatic cancer, 20 BPT, and 40 healthy controls. The results showed that in the comparison of pancreatic cancer versus healthy controls, miR-3679-5p showed significant downregulation ($P = 0.008$) and miR-940 significant upregulation ($P = 0.006$) in pancreatic cancer. For miR-3679-5p and miR-940, the AUCs after constructing ROC curves were 0.673 and 0.680, the sensitivities for pancreatic cancer detection were 82.5% and 90.0%, and the specificities were 45.0% and 40.0%, respectively.

In the comparison of pancreatic cancer versus BPT, miR-3679-5p showed significant downregulation ($P = 0.007$) and miR-940 significant upregulation ($P = 0.004$) in pancreatic cancer. For miR-3679-5p and miR-940, the AUCs were 0.716 and 0.729, the sensitivities for pancreatic cancer detection were 90.0% and 62.5%, and the specificities were 45.0% and 75.0%, respectively.

In the comparison of pancreatic cancer versus noncancer (healthy control + BPT), miR-3679-5p showed significant

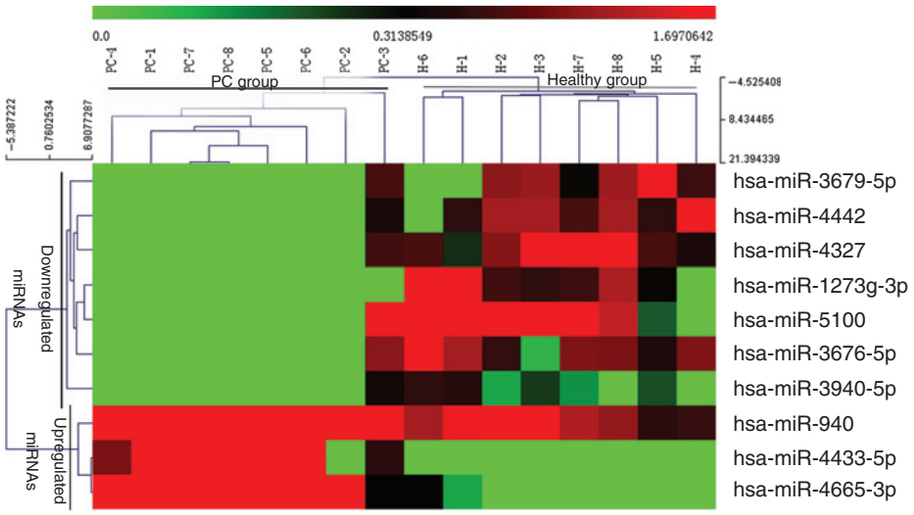

Figure 1.
Heatmap of the 10 qPCR-verified miRNAs (3 upregulated and 7 downregulated) based on the microarray data. Hierarchical clustering was performed using covariance values and the complete linkage clustering method included in the MeV_4_9_0 software. Pancreatic cancer patients ($n = 8$) and healthy controls ($n = 8$) could be classified into distinct groups, preliminarily indicating the discriminatory power of these salivary miRNAs. hsa, *Homo sapiens*; PC, pancreatic cancer; H, healthy control.

downregulation ($P = 0.002$) and miR-940 significant upregulation ($P = 0.001$) in pancreatic cancer. For miR-3679-5p and miR-940, the AUCs were 0.688 and 0.696, the sensitivities for pancreatic cancer detection were 85.0% and 90.0%, and the specificities were 45.0% and 41.7%, respectively.

In the comparison of BPT versus healthy controls, miR-3679-5p and miR-940 showed no significant difference between the two groups. The *P* values were 0.627 and 0.363, respectively.

Detailed qPCR data are presented in Supplementary Material S3. The results are summarized in Table 1, and ROC curves are presented in Fig. 2.

Prediction model building and biomarker combination analysis

The two validated salivary biomarkers (miR-3679-5p and miR-940) were used to construct binary logistic regression models (separately for comparisons of the three discriminatory categories), and step-wise backward model selection was performed to determine the final combinations of biomarkers.

Supposing that the expression levels of miR-3679-5p and miR-940 are X1 and X2, respectively, then in the pancreatic cancer versus healthy control comparison, the prediction model obtained was logit (P1) $= -0.22 - 0.382$ X1 $+ 0.301$ X2. The model was examined using a likelihood test, and $\chi^2 = 18.690$ and $P < 0.001$, indicating that the model showed statistical significance. According to the Hosmer–Lemeshow test, $P = 0.581$, indicating that the model fit the data well. The expression levels of miR-3679-5 and miR-940 were combined in the model, and a new ROC curve was constructed. The results yielded an AUC of 0.750 with a sensitivity and a specificity of 72.5% and 70.0%, respectively, for pancreatic cancer detection. According to the CART, the positive predictive value (PPV) of the model combining the two miRNA biomarkers for pancreatic cancer detection was 78.9%, and the negative predictive value (NPV) was 76.2%.

In the category of pancreatic cancer versus benign pancreatic tumors, the prediction model obtained was logit (P2) $= 0.52 - 0.452$ X1 $+ 0.386$ X2. The model was examined by a likelihood test, and $\chi^2 = 18.497$ and $P < 0.001$, indicating that the model showed statistical significance. According to the Hosmer–Lemeshow test, $P = 0.821$, indicating that the model fit the data well. The logistic regression model yielded an AUC of 0.781 with a sensitivity and specificity of 62.5% and 80.0%, respectively, for pancreatic cancer detection. According to the CART, the PPV was 83.7% and the NPV 76.5%.

In the category of pancreatic cancer versus noncancer, the prediction model obtained was logit (P3) $= -0.624 - 0.389$ X1 $+$ 0.322 X2. The model was examined by a likelihood test, and $\chi^2 =$ 25.561 and $P < 0.001$, indicating that the model showed statistical significance. According to the Hosmer–Lemeshow test, $P = 0.828$, indicating that the model fit the data well. The logistic regression model yielded an AUC of 0.763 with a sensitivity and a specificity of 70.0% and 70.0%, respectively, for pancreatic cancer detection. According to the CART, the PPV was 82.4% and the NPV 81.9%.

To test whether the logistic models is the result of data overfitting, simulation studies for the prediction models of the three discriminatory categories were conducted, and all resulted in $P <$ 0.001 for the AUCs. Thus, even after accounting for model selection and data overfitting with multiple markers, the validated biomarker sets have significantly more discriminatory power to detect pancreatic cancer than would be expected by chance.

The discriminatory power of the biomarker combination for resectable pancreatic cancer by logistic regression model and CART is shown in Table 2 and Figs. 3 and 4.

Comparison between serum CA19-9 levels in pancreatic cancer and BPT

According to international criteria, the upper limit of serum CA19-9 for healthy people is 37 U/mL. In this study, 22 of 48 patients with pancreatic cancer showed CA19-9 levels exceeding this limit, indicating that serum CA19-9 showed a sensitivity of 45.8% (22/48) for the detection of resectable pancreatic cancer. In the BPT group, 10 cases exceeded and 10 were below this limit, indicating that CA19-9 showed specificity of 50% in differentiating between benign and malignant pancreatic tumors. Because healthy controls were included based on their normal serum CA19-9 levels, no analysis was made on the CA19-9 specificity for the detection of healthy controls.

Relationship between serum CA19-9 and total bilirubin in pancreatic cancer and BPT

Bilirubinemia can influence CA19-9 expression. A good example is that obstructive jaundice contributes to a high false-positive

Table 1. Discriminatory power of salivary miR-3679-5p and miR-940 for the detection of resectable pancreatic cancer (PC)

	PC versus healthy control					PC versus BPT					PC vs. noncancer (healthy control + BPT)				
miRNA	***P***	**AUC**	**Sensitivity**	**Specificity**	**Fold change**	***P***	**AUC**	**Sensitivity**	**Specificity**	**Fold change**	***P***	**AUC**	**Sensitivity**	**Specificity**	**Fold change**
miR-3679-5p	0.008	0.673	82.5%	45.0%	2.162↓	0.007	0.716	90.00%	45.00%	4.708↓	0.003	0.688	85.0%	45.0%	3.012↓
miR-940	0.006	0.680	90.0%	40.0%	2.586↑	0.004	0.729	62.50%	75.00%	3.020↑	0.001	0.696	90.0%	41.7%	2.716↑

rate for CA19-99 (30, 31). In patients with pancreatic cancer ($n = 48$) and BPT ($n = 20$), the correlation between serum total bilirubin and CA19-9 was analyzed using Spearman correlation test within three categories: pancreatic cancer, BPT, and benign/malignant pancreatic tumor (pancreatic cancer + BPT). The findings showed no significant correlation in pancreatic cancer ($P = 0.904$), although a significant correlation was found in BPT ($P < 0.001$, correlation coefficient = 0.806). Regardless of whether the pancreatic tumor was benign or malignant, no significant correlation was seen ($P = 0.146$).

Correlation between salivary miRNAs and serum total bilirubin

To test whether serum bilirubin influenced the expression of miR-3679-5p and miR-940, as it influences CA19-9, the correlation between the expression levels of the two salivary miRNAs and serum total bilirubin was analyzed using Spearman test within three categories: pancreatic cancer, BPT, and benign/malignant pancreatic tumor (pancreatic cancer + BPT). The results showed that in pancreatic cancer ($n = 48$) both miR-3679-5p and miR-940 showed no significant correlations ($P = 0.768$ and 0.359, respectively) with serum bilirubin, in BPT ($n = 20$) there were also no significant correlations ($P = 0.494$ and 0.767, respectively), and in the category of benign/malignant pancreatic tumor ($n = 68$), there were also no significant correlations ($P = 0.900$ and 0.102, respectively).

Discussion

To our knowledge, this is the first report to profile salivary miRNAs from patients with resectable pancreatic cancer using microarray analysis. According to qPCR, logistic regression, and CART models, salivary miR-3679-5p and miR-940 were potentially useful biomarkers, with good discriminatory power for differentiating pancreatic cancer from healthy controls and BPT. The sensitivities and specificities ranged from 60% to 80%, which were higher than those of the currently used biomarker for pancreatic cancer detection, CA19-9 (in this study, the sensitivity and specificity of CA19-9 for resectable pancreatic cancer detection were only 45.8% and 50%, respectively). In addition, by building a logistic regression model, the combination of miR-3679-5p and miR-940 enhanced the discriminatory power. Furthermore, according to the CART models, the combination of the two biomarkers also showed high PPVs and NPVs for pancreatic cancer detection. miR-3679-5p and miR-940 could also discriminate pancreatic cancer from BPT with good sensitivity and specificity, which may help in differentiating pancreatic malignant tumors from benign tumors in clinical practice. In the discriminatory category of BPT versus healthy controls, the expression levels of the two miRNAs showed no significant differences between the two groups, indicating that the two miRNAs could be deregulated only when pancreatic cancer developed. Due to the insidious development and frequently fatal nature of pancreatic cancer, early diagnosis and treatment are vital for improving the prognosis in patients with pancreatic cancer. Saliva collection is simple and noninvasive. This study provides a new method for the early detection of pancreatic cancer using a noninvasive screening method, and it also provides a new prospect of using salivary miRNAs as biomarkers to potentially detect other systemic diseases.

CA19-9 was detected in the colorectal cancer cell line SW1116 by Koprowoski and colleagues in 1979. CA19-9 is associated with

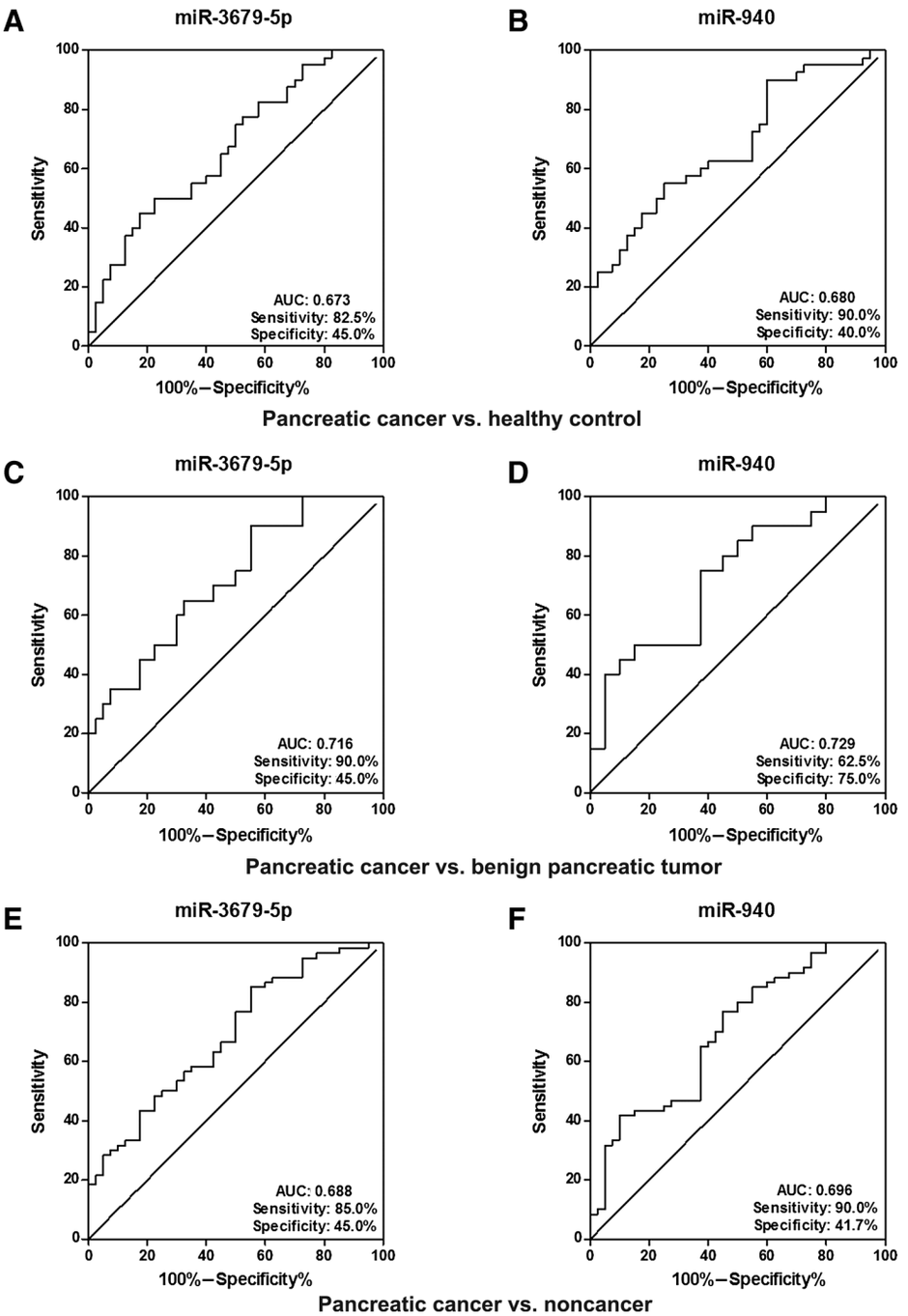

Figure 2.
ROC curve analysis for the discriminatory power of salivary miRNA biomarkers. In pancreatic cancer versus healthy controls, miR-3679-5p showed an AUC of 0.673, with a sensitivity of 82.5% and a specificity of 45.0% (A). The AUC for miR-940 was 0.680, with a sensitivity of 90.0% and a specificity of 40.0% (B). In pancreatic cancer versus benign pancreatic tumors, the AUC for miR-3679-5p was 0.716, with a sensitivity of 90.0% and a specificity of 45.0% (C). The AUC for miR-940 was 0.729, with a sensitivity of 62.5% and a specificity of 75.0% (D). In pancreatic cancer versus noncancer (healthy control + benign pancreatic tumor), the AUC for miR-3679-5p was 0.688, with a sensitivity of 85.0% and a specificity of 45.0% (E). The AUC for miR-940 was 0.696, with a sensitivity of 90.0% and a specificity 41.7% (F).

the Lewis blood group antigens, and only patients with the Le (α−β+) or Le (α + β−) blood groups will express the CA19-9 antigen. Le (α−β−) phenotypes occur in 5% to 10% of the population. Those with Le (α−β−) phenotypes do not express CA19-9 regardless of whether they develop pancreatic cancer or other cancers. CA19-9 has also been detected in the sera of patients with other gastrointestinal tumors, including esophageal, gastric, and biliary cancers. In addition, CA19-9 can also become elevated

Table 2. Discriminatory power of the biomarker combination for detecting resectable pancreatic cancer (PC)

Category	AUC	Sensitivity	Specificity	Positive predictive value	Negative predictive value
PC versus healthy control	0.75	72.50%	70.00%	78.90%	76.20%
PC versus BPT	0.781	62.50%	80.00%	83.70%	76.50%
PC versus noncancer	0.763	70.00%	70.00%	82.40%	81.90%

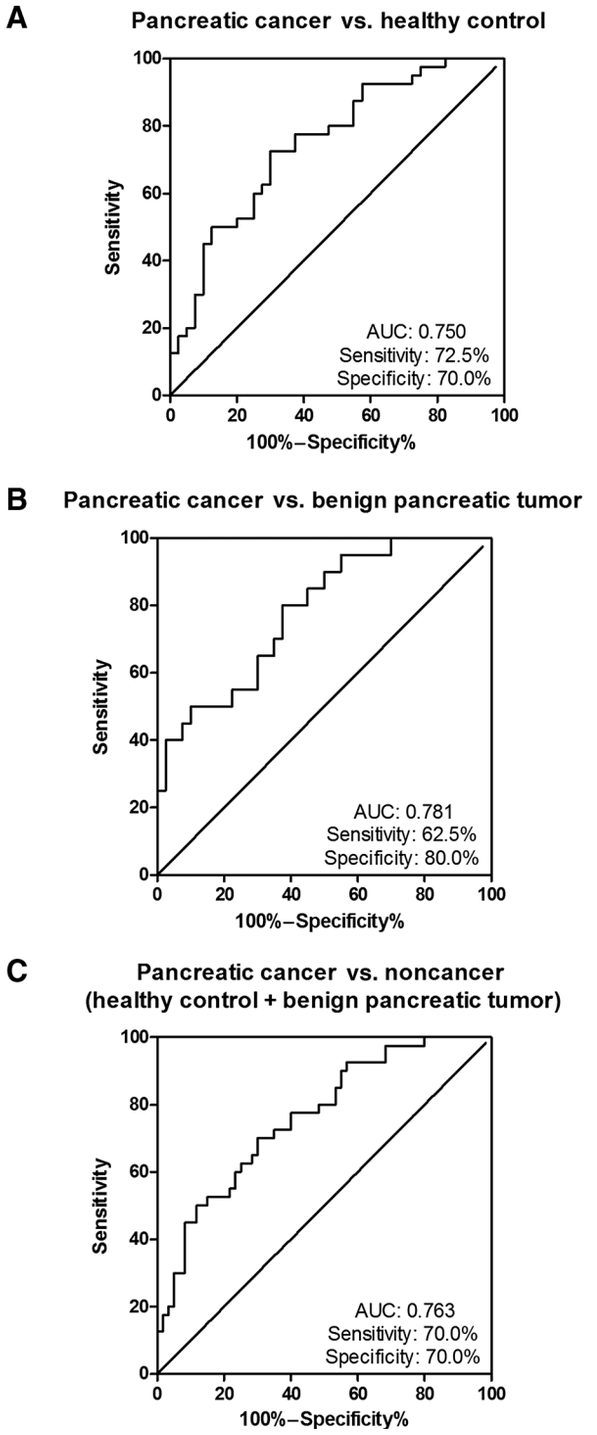

Figure 3.
ROC curve analysis for the discriminatory power of combined salivary miR-3679-5p and miR-940 according to the logistic regression models. In the pancreatic cancer versus healthy controls comparison, the combination showed an AUC of 0.750, with a sensitivity of 72.5% and a specificity of 70.0% (A). In pancreatic cancer versus benign pancreatic tumor comparison, the combination showed an AUC of 0.781, with a sensitivity of 62.5% and a specificity of 80.0% (B). In pancreatic cancer versus noncancer (healthy control + benign pancreatic tumor) comparison, the combination showed an AUC of 0.763, with a sensitivity of 70.0% and a specificity of 70.0% (C).

in other benign diseases, including pancreatitis, renal failure, and mucous cysts. If a patient develops hyperbilirubinemia (serum total bilirubin $\geq$ 17.1 μmol/mL), especially obstructive jaundice, the false-positive rate of CA19-9 increases rapidly (30, 31). In this study, the sensitivity of CA19-9 for the detection of resectable pancreatic cancer was only 45.8%, while the false-positive rate was 50% for differentiating pancreatic cancer from BPT. Satake and colleagues (32) analyzed CA19-9 serum levels in 8,706 individuals with symptoms suspicious of pancreatic cancer, such as weight loss, epigastric pain, and jaundice. Among them, 198 (4.3%) patients had elevated CA19-9 serum levels. Following extensive work-ups, 85 (1.8%) patients were found to have pancreatic cancer, of which 28 (0.4%) were resectable. Kim and colleagues (33) measured CA19-9 serum levels in 70,940 asymptomatic individuals and identified only 4 patients with pancreatic cancer among 1,063 patients with elevated CA19-9 serum levels (>37 U/mL). These authors reported a dismal PPV for CA19-9 of only 0.9%. However, in this study, the PPVs of the two validated biomarkers were >78%. Accordingly, the guidelines of the American Society of Clinical Oncology (ASCO; ref. 34) and the European Guidelines on Tumour Markers (EGTM; ref. 35) do not recommend the use of CA19-9 for screening, diagnosis, or resectability judgments in pancreatic cancer. Taken together, although CA19-9 shows indication for diagnosis of pancreatic cancer, its diagnostic power is limited, especially for resectable pancreatic cancer.

In this study, although serum CA19-9 and total bilirubin levels did not show a significant correlation in pancreatic cancer, a significant positive correlation was observed in BPT ($P < 0.001$, correlation coefficient = 0.806), indicating the limited use of CA19-9 for the differential diagnosis in patients with BPT with jaundice. The detailed mechanism remains unclear. In this study, we not only found that miR-3679-5p and miR-940 may possess better discriminatory power than CA19-9 in detecting resectable pancreatic cancer, but also that they showed no significant correlation with serum bilirubin levels, indicating that their discriminatory power for resectable pancreatic cancer was not affected by serum bilirubin levels. That is, their discriminatory power showed good specificity.

The production of saliva is closely related to that of blood. Almost all molecules found in blood are also present in saliva. These molecules come from the capillary beds in the mouth, and they enter the saliva from the blood circulating in the salivary glands by passing through the spaces between cells by transcellular (passive intracellular diffusion and active transport) or paracellular routes (extracellular ultrafiltration; ref. 36). Thus, in health examinations, saliva may be used in place of plasma/serum to monitor variations of molecules in the blood (28).

Many studies have demonstrated that several salivary molecules, such as transcripts, proteins, and metabolites, can be used for detecting infectious, oral, systemic, and cardiovascular diseases and cancers. The FDA has approved several commercial products for saliva-based diagnoses, increasing the application of salivary diagnostics, now referred to as "salivaomics."

Interestingly, some molecular profiles in saliva differ from those in tissues or blood; for example, some salivary mRNAs cannot be detected in tissues. Gao and colleagues (37) used mouse models of melanoma and non–small cell lung cancer to compare the transcriptomic or mRNA biomarker profiles of

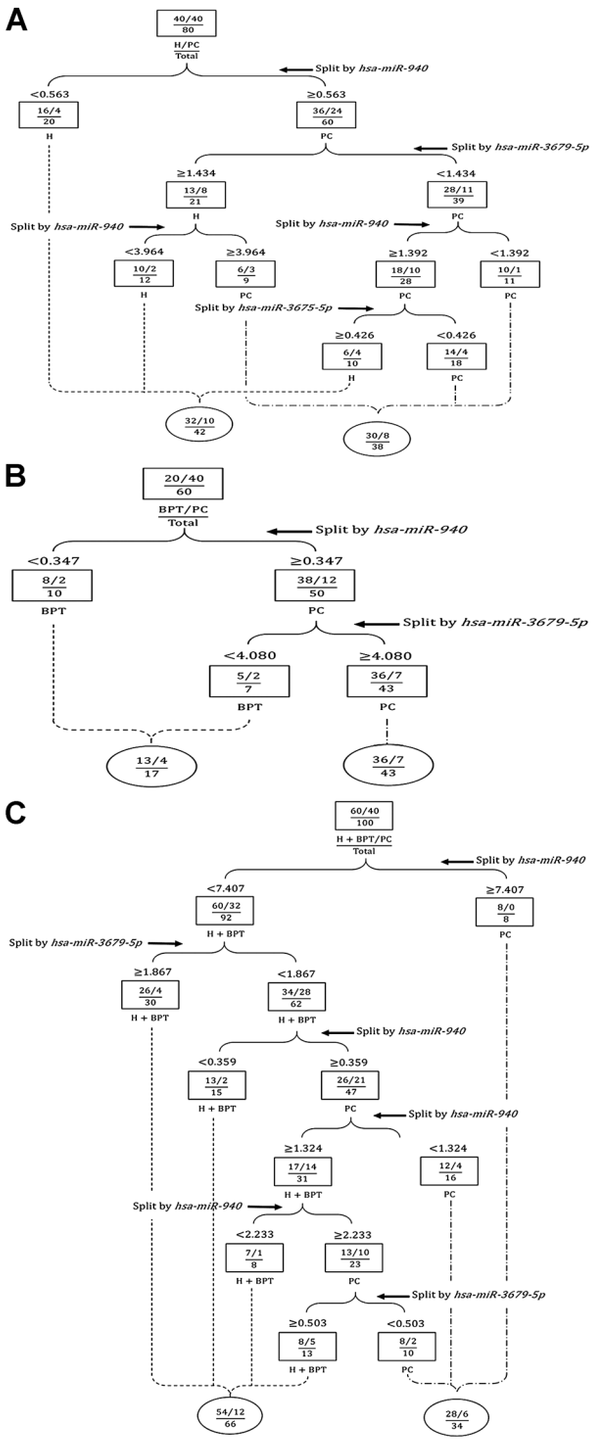

Figure 4.
CART models evaluating the salivary miRNA biomarkers for pancreatic cancer. According to the models, in the pancreatic cancer versus healthy controls comparison, the positive predictive value (PPV) was 78.9% (30/38) and the negative predictive value (NPV) 76.2% (32/42; A). In the pancreatic cancer versus benign pancreatic tumor comparison, the PPV was 83.7% (36/43) and the NPV 76.5% (13/17; B). In pancreatic cancer versus noncancer (healthy control + benign pancreatic tumor), the PPV was 82.4% (28/34) and the NPV 81.8% (54/66) (C).

mice with tumors versus those of control mice. Microarray analysis showed that the transcriptomes of saliva, tissue, serum, and salivary glands were significantly altered in mice with tumors versus controls. However, the transcriptomic profiles of saliva, tissue, serum, and salivary glands did not coincide. Some mRNAs expressed in the saliva of mice were not expressed in tissues, salivary glands, or serum. Of the salivary transcripts, 69.6% were present in the salivary gland, 51.6% in serum, and 67.6% in the tumor (melanoma). Zhang and colleagues (16) speculated that tumors produce mediators (hormones, cytokines, lymphokines) that can regulate activities and gene expression patterns in distal organs (e.g., salivary glands) through the vasculature. Upon contact with the salivary gland, cancer-specific mediators modulate the DNA, RNA, and proteins that are secreted into the saliva. Stimulation of the salivary glands by mediators released from distal tumors plays a significant role in regulating the salivary surrogate biomarker profiles. These constitute cancer-associated salivary gland surrogate biomarkers, which do not necessarily reflect the transcriptome or proteome of the primary tumor.

The number of miRNAs is continuing to increase with time. Over 2,000 miRNAs have now been found in the human body. The miR-3679-5p and miR-940 validated in this study were recently detected for the first time. Their biologic function(s) remain(s) to be investigated.

This study has some limitations. First, miRNAs in tissue, plasma, and saliva from the same patient were not profiled at the same time, and therefore miRNA profiles of tissue, plasma, and saliva could not be compared. Although previous studies showed that salivary mRNA profiles did not overlap with those of tissue or serum (37), the occurrence of this phenomenon for miRNA profiles needs to be explored. Second, reagents and technologies to detect Lewis blood group antigens are not yet readily available in China, so the Lewis blood groups of the subjects could not be determined to assess whether these blood antigens influence salivary miRNAs expression as they do serum CA19-9 expression. Third, no reagent or technology was available to detect salivary CA19-9. Accordingly, no comparison between serum and salivary CA19-9 levels could be made. Finally, the sample size was small, and the subjects were limited to a Chinese Han population. Multicenter studies with larger sample sizes are needed. However, this was a feasibility study, and future prospective studies are warranted. With further research, useful discoveries regarding salivary miRNAs as a biomarker are likely to be made.

Disclosure of Potential Conflicts of Interest

No potential conflicts of interest were disclosed.

Authors' Contributions

Conception and design: Z. Li
Development of methodology: Z. Xie, B. Gong, W. Nie, B. Wu
Acquisition of data (provided animals, acquired and managed patients, provided facilities, etc.): Z. Xie, X. Yin, B. Gong, W. Nie, B. Wu, J. Huang, Z. Zhou
Analysis and interpretation of data (e.g., statistical analysis, biostatistics, computational analysis): Z. Xie, X. Yin, B. Gong, W. Nie, B. Wu, X. Zhang, Z. Li
Writing, review, and/or revision of the manuscript: Z. Li
Administrative, technical, or material support (i.e., reporting or organizing data, constructing databases): X. Yin, B. Gong, W. Nie, B. Wu, X. Zhang, J. Huang, P. Zhang, Z. Zhou
Study supervision: Z. Xie, Z. Li

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