Plasma Micro-RNA Alterations Appear Late in Pancreatic Cancer

Oskar Franklin, MD, PhD,* Pär Jonsson, PhD,† Ola Billing, PhD,* Erik Lundberg, MD, PhD,* Daniel Öhlund, MD, PhD,* Hanna Nyström, MD, PhD,* Christina Lundin, MSc,* Henrik Antti, PhD,‡ and Malin Sund, MD, PhD‡

Objectives: The aim of this research was to study whether plasma micro-RNAs (miRNA) can be used for early detection of pancreatic cancer (PC) by analyzing prediagnostic plasma samples collected before a PC diagnosis.

Background: PC has a poor prognosis due to late presenting symptoms and early metastasis. Circulating miRNAs are altered in PC at diagnosis but have not been evaluated in a prediagnostic setting.

Methods: We first performed an initial screen using a panel of 372 miRNAs in a retrospective case-control cohort that included early-stage PC patients and healthy controls. Significantly altered miRNAs at diagnosis were then measured in an early detection case-control cohort wherein plasma samples in the cases were collected before a PC diagnosis. Carbohydrate antigen 19–9 (Ca 19–9) levels were measured in all samples for comparison.

Results: Our initial screen, including 23 stage I-II PC cases and 22 controls, revealed 15 candidate miRNAs that were differentially expressed in plasma samples at PC diagnosis. We combined all 15 miRNAs into a multivariate statistical model, which outperformed Ca 19–9 in receiver-operating characteristic analysis. However, none of the candidate miRNAs, individually or in combination, were significantly altered in prediagnostic plasma samples from 67 future PC patients compared with 132 matched controls. In comparison, Ca 19–9 levels were significantly higher in the cases at <5 years before diagnosis.

Conclusion: Plasma miRNAs are altered in PC patients at diagnosis, but the candidate miRNAs found in this study appear late in the course of the disease and cannot be used for early detection of the disease.

Keywords: blood samples, early detection, micro-RNA, miRNA, pancreatic cancer

(Ann Surg 2018;267:775–781)

Plasma Micro-RNA Alterations Appear Late in Pancreatic Cancer

This study was funded by The Swedish Research Council (2011-3089 for M.S., 537-2013-7277 for D.O.), the Swedish Cancer society (110679 and 120135 for M.S.) the Swedish Society of Medicine (SLS-591551 for D.O.), the County Council of Västerbotten (M.S. and D.O.), Cancer research foundation in northern Sweden (AMP15-793 for D.O.), JC Kempe Memorial Foundation Scholarship Fund (O.F.), and Grants from the medical faculty at Umeå University (223-1828-13 O.F.). O.F., P.J., O.B., and M.S. planned the study and the study design. O.F., D.O., E.L., H.N., and M.S. collected and interpreted patient data from hospital charts. O.F., O.B., C.L., performed the experimental procedures. O.F., P.J., and H.A. performed data analysis. O.F, O.B., P.J., and M.S. wrote the manuscript. All authors read and critically reviewed the intellectual content of the final text, and gave their final approval of the text.

Supplemental digital content is available for this article. Direct URL citations appear in the printed text and are provided in the HTML and PDF versions of this article on the journal’s Web site (www.annalsofsurgery.com). This is an open-access article distributed under the terms of the Creative Commons Attribution-Non Commercial-No Derivatives License 4.0 (CCBY-NC-ND), where it is permissible to download and share the work provided it is properly cited. The work cannot be changed in any way or used commercially without permission from the journal.

Reprints: Oskar Franklin, MD, PhD, Department of Surgical and Perioperative Sciences/Surgery, Umeå University, 901 85 Umeå, Sweden. E-mail: oskar.franklin@umu.se.

Copyright © 2017 The Author(s). Published by Wolters Kluwer Health, Inc.

ISSN: 0003-4922/17/26704-0775
DOI: 10.1097/SLA.0000000000002124

From the †Department of Surgical and Perioperative Sciences, Umeå University, Sweden; and ‡Department of Chemistry, Umeå University, Sweden.

Ethan Franklin, MD, PhD,‡ Pär Jonsson, PhD,† Ola Billing, PhD,* Erik Lundberg, MD, PhD,* Daniel Öhlund, MD, PhD,* Hanna Nyström, MD, PhD,* Christina Lundin, MSc,* Henrik Antti, PhD,‡ and Malin Sund, MD, PhD‡

Pancreatic cancer (PC) patients have an extremely poor prognosis since the vast majority present with metastatic disease at diagnosis. Moreover, cure is rare even for patients with early-stage disease who undergo surgery with a curative intent, and half of them die within two years.1,2 Improvement in diagnostics and treatment of PC has not been comparable to other cancer forms, and despite its low prevalence, PC is predicted to become the second most common cause of cancer-related death within 4 years.3 Sensitive biomarkers facilitating earlier diagnosis of PC are needed, as the most commonly used PC biomarker, carbohydrate antigen 19–9 (Ca 19–9), lacks sufficient accuracy for early detection of PC.4

Micro-RNAs (miRNAs) are single stranded, noncoding RNA species of ~22 nucleotides length that participate in post-transcriptional gene regulation. In cancer, miRNAs can act as tumor suppressors or oncogenes by post-transcriptional regulation of genes involved in carcinogenesis.5 miRNAs are surprisingly stable in blood samples, due to high resistance to both temperature changes and endogenous RNase activity.6 This makes them suitable as blood-based biomarkers and several studies have highlighted the potential of circulating miRNAs as biomarkers for various cancer forms, including PC.7 Schultz et al8 screened for >700 miRNAs and validated two whole blood miRNA panels that in combination with Ca 19–9 could accurately differentiate PC patients from controls. Recently, Xu et al9 showed that plasma-miR-486-5p performed equally well as Ca 19–9 in discriminating PC patients from healthy controls. However, all previous studies have examined circulating miRNA levels in patients with an established PC diagnosis, and therefore provide no information on whether miRNA levels are altered before clinical symptoms appear, and thus if they are useful as early detection markers. miRNAs are differentially expressed in PC tissue compared with normal pancreas10–13 and corresponding alterations are also evident in premalignant pancreatic intraepithelial neoplastic lesions (PanINs),14 indicating that miRNA expression changes appear early in PC carcinogenesis. PC is predicted to develop from the initial PanIN lesion into metastatic disease in a time span over 10 years,15 giving a possible window for early detection using miRNAs.

We hypothesized that early miRNA changes in PC might aid in early detection of PC, and thus aimed to find plasma-miRNAs that are altered years before a clinical PC diagnosis.

METHODS

Ethics Statement

All subjects taking part in the study provided written informed consent. The study was approved by the regional research ethics board of northern Sweden and conducted in accord with the ethical standards of the Helsinki Declaration of 1975.

Screening Cohort (Samples Collected Before Surgery)

We retrospectively reviewed the hospital charts for patients who underwent pancreatic surgery for PC between the years 2008...
and 2014 at Umeå University Hospital, Sweden. Blood samples were collected from all patients before surgery, and ethylenediaminetetraacetic acid (EDTA) plasma was stored at −80 °C in a prospectively maintained research biobank. We included patients with histopathologically confirmed pancreatic ductal adenocarcinoma at tumor-node-metastasis (TNM) stage I-II with an available preoperative plasma sample. Cases were only included if an age- and sex-matched healthy control was available. Case samples were randomly assigned to matched control samples, collected from patients who either underwent endoscopy without malignant findings or elective surgery for a nonmalignant disease. Controls with a previous history of cancer were excluded.

The following patient characteristics were extracted from hospital charts: age at diagnosis, sex, first clinical sign, preoperative staging (resectability) according to the 7th edition of the American Joint Committee on Cancer staging (AJCC), TNM stage according to AJCC, histopathological grade, tumor size, patient survival, and serum conjugated bilirubin levels (SBR). We only included SBR measurements made on the same day as collection of the plasma samples used for miRNA analysis.

**Prediagnostic Cohort (Samples Collected Before Diagnosis)**

Prediagnostic plasma samples were derived from a biobank associated with the ongoing population-based Västerbotten Intervention Program (VIP). VIP was launched in 1985 as a primary prevention project for reducing cardiovascular disease in the Swedish county of Västerbotten. Besides offering routine health examinations, participants are asked to donate plasma samples and take part in a large prospective research cohort. Since 1987, the project has covered the entire county, which in 2007 included 258,000 inhabitants. Participation rates have varied between 48% and 67%.16 We included VIP participants who were diagnosed with PC between January 1990 and February 2009, and where EDTA plasma samples collected before the diagnosis date were available. Each case was matched with two healthy controls from the same biobank. Controls were matched by sex, age at sampling, and sampling date (±3 months). Previous history of cancer was an exclusion criterion for both cases and controls. The resulting cohort was randomly split in half into a training set and a validation set. Patient characteristics for all cases were extracted from hospital charts.

**miRNA Isolation**

miRNA isolation was performed using the Qiagen miRNeasy serum/plasma kit according to the manufacturer’s instructions (Qiagen, Hilden, Germany). One hundred microliters of thawed and centrifuged plasma was mixed with 500 µL of QIAzol Lysis Reagent and 0.5 µL of spike-in miRNAs for quality control measurements (Exiqon spike-in kit UniSp2, UniSp4, UniSp5; Exiqon, Vedbaek, Denmark). RNase-free water was run in parallel as a negative control. Sample hemolysis can affect plasma miRNA levels due to hemolysis between the patterns. Hence, in a strong model, controls will be assigned with a lower Ct value than cases and controls will be assigned with a higher Ct value than cases.

**miRNA Expression by Real-time Quantitative PCR (RT qPCR)**

miRNA isolates were shipped on dry ice to Exiqon (Exiqon, Denmark) for subsequent complementary DNA (cDNA) synthesis and RT qPCR. Matched samples were assayed on the same plate, but the match order was randomized. The case/control designation was blinded and all samples were analyzed in duplicates. Samples were reverse transcribed into cDNA using miRCURY LNA Universal RT microRNA PCR. cDNA was then mixed with Exilent SYBR Green mastermix (Exiqon, Vedbaek, Denmark) and RT qPCR was performed on Exiqon miRCURY LNA Universal RT microRNA PCR Human Panel I (V.4, Exiqon, Vedbaek, Denmark) Human Panel I (V.4), with primers covering 372 validated miRNAs. On the basis of the screening results from the patient cohort, we custom-designed a new qPCR panel to be used in the prediagnostic cohort. cDNA amplification, melting points, and raw Ct value acquisition was performed on a Roche LightCycler 480 (Roche Diagnostics, Rotkreuz, Switzerland). Samples had to pass quality control measurements of spike-ins (UniSp2, −4, and −5) before final analysis. Reactions with poor amplification efficacy or multiple melting points were excluded as well as Ct values within 5 Ct values of the negative control (RNase-free water). miRNAs detected in <20 cases or controls were excluded from statistical analysis.

**Sample Hemolysis Assessment**

Sample hemolysis can affect plasma miRNA levels due to miRNA contamination from red blood cells.17 We therefore measured hemolysis in all samples before isolation, using a previously described spectrophotometric method.18 Samples with hemoglobin levels above 25 g/L were excluded. We also controlled for hemolysis by calculating the cycle threshold (Ct) ratio between miR-451 and miR-23a. A Ct ratio > 7 was considered indicative of sample hemolysis.19

**Plasma Ca 19–9 Measurement**

Plasma Ca 19–9 was measured in preoperative samples using the MILLIPLEX MAP Kit and the WideScreen Human Cancer Panel I for the prediagnostic samples (Merck KGaA, Darmstadt, Germany) according to the respective manufacturer’s instructions. Fluorescence intensities were measured on a Bio-Plex 200 System (Bio-rad, Hercules, California, USA). Results were compared with kit standards using 5-parameter logistic regression. Samples with Ca 19–9 levels below the lowest limit of detection were assigned a value equal to 50% of the lower detection limit.

**Statistics**

We used Exiqon GenEx 6 Software (Exiqon, Denmark), STATA 12.1 (College Station, Texas, USA), and Prism 5 (GraphPad Software, La Jolla, California, USA) for the statistical analysis. Multivariate statistical analysis was performed using Simca-p 14 (MKS Data Analytics Solutions, Umeå, Sweden). Raw miRNA Ct values were first normalized using the global mean method and then compared using Student’s t test. False discovery rate for miRNA alterations was controlled at 5% by calculating Benjamin-Hochberg corrected P values.21 The delta mean of normalized Ct values for cases and controls were log2-transformed to calculate the fold change. Other variables were compared using Student’s t test for continuous data and Chi-square test for categorical data using P < 0.05 as cutoff level for significance. The correlation between miRNAs and SBR were analyzed using Pearson’s r.

Multivariate projection analysis to evaluate miRNA combinations was carried out using orthogonal projections to latent structure-discriminative analysis (OPLS-DA).22,23 OPLS-DA regresses the miRNA levels against a binary “dummy vector” carrying the sample class information, in this case patients = 1 and controls = 0, in the search for systematic patterns of miRNAs related to discrimination between the patterns. Hence, in a strong model, controls will cluster around 0 and patients around 1. A 7-fold cross validation of the analysis was performed to estimate the predictive ability of the models.24 Briefly, a model was created using 67% of the observations...
and the remaining 1/7 were predicted by the model. This was repeated 7 times so that all samples were predicted once. The predictive ability was evaluated by cross-validated analysis of variance (CV-ANOVA).25

Receiver-operator characteristic (ROC) curves were generated for the significantly altered miRNAs; the combination of all significant miRNAs and Ca 19–9 and the area under the curve (AUC) was calculated to compare the discriminative performance.

RESULTS

Study Cohorts

Screening Cohort

Twenty-three PC patients and 22 controls were included in our miRNA screening cohort (Fig. 1A). The clinical characteristics of the cases and controls are summarized in Table 1 and Supplementary Table 1, http://links.lww.com/SLA/B163. All samples passed hemolysis testing.

Prediagnostic Cohort

Eighty-one individuals who later developed PC (cases), with a prediagnostic plasma sample available, were randomized to the prediagnostic training cohort (Fig. 1B). During miRNA isolation, 13 case samples and four controls were excluded due to sample hemolysis or insufficient sample volume. The four excluded controls were replaced with controls matched to the excluded cases. Two additional controls were excluded after RT qPCR analysis; one due to low miRNA yield and the other due to uncertain sample identity. The final cohort thus consisted of 67 PC patients and 132 matched controls. The clinical characteristics are summarized in Table 2.

Plasma miRNAs are Altered in Patients at Diagnosis

To identify candidate miRNAs that were altered at PC diagnosis, we analyzed a panel of 372 miRNAs by RT qPCR in the screening cohort. Patients and controls separated somewhat in principle component analysis based on all detectable plasma miRNAs (n = 233), although with a considerable overlap between the groups (Supplementary Figure 1, http://links.lww.com/SLA/B163). One hundred ninety miRNAs (51%) were expressed in cases or controls and were thus included in the statistical analysis. No miRNA displayed an on/off pattern, meaning that none were solely detected in either cases or controls.

TABLE 1. Clinical Characteristics of the Screening Cohort

| Variables                          | Cases (n = 23) | Controls (n = 22) |
|------------------------------------|---------------|------------------|
| Age, y, Mean (95% CI)              | 63.6 (60.3–66.9) | 61.9 (58.8–64.9) |
| Sex                                | 12/11         | 12/10            |
| First clinical sign                |               |                  |
| Jaundice, n (%)                    | 17 (73.9%)    |                  |
| Abdominal pain, n (%)              | 3 (13.0%)     |                  |
| N.A., n (%)                        | 3 (13.0%)     |                  |
| TNM stage                          |               |                  |
| Stage I A–IB, n (%)                | 7 (30.4%)     |                  |
| Stage IIA–IIB, n (%)               | 16 (69.6%)    |                  |
| Tumor grade                        |               |                  |
| Grade 1, n (%)                     | 3 (13.0%)     |                  |
| Grade 2, n (%)                     | 14 (60.9%)    |                  |
| Grade 3, n (%)                     | 4 (17.4%)     |                  |
| N.A., n (%)                        | 2 (8.7%)      |                  |
| Tumor size, cm                     | 2.5 (1.5–7)   |                  |
| Survival, mo                       | 21 (4–50)     |                  |
| N.A. indicates not available in hospital charts, TNM, tumor-node-metastasis. | |

TABLE 2. Clinical Characteristics of the Prediagnostic Cohort (Training Set)

| Variables                          | Cases (n = 67) | Controls (n = 132) |
|------------------------------------|---------------|------------------|
| Age at sampling, y Mean (95% CI)   | 54.8 (53.1–56.5) | 54.8 (53.6–56.0) |
| Age at diagnosis, y Mean (95% CI)  | 63.6 (61.6–65.5) |                  |
| Sex                                |               |                  |
| Men, n (%)                         | 24 (35.8%)    | 49 (37.12%)      |
| Women, n (%)                       | 43 (64.2%)    | 83 (62.9%)       |
| TNM stage at diagnosis             |               |                  |
| Stage IA-IB, n (%)                 | 3 (4.5%)      |                  |
| Stage IIA-IB, n (%)                | 5 (7.5%)      |                  |
| Stage III, n (%)                   | 13 (19.4%)    |                  |
| Stage IV, n (%)                    | 46 (66.7%)    |                  |
| Tumor grade at diagnosis           |               |                  |
| Grade 1, n (%)                     | 1 (1.5%)      |                  |
| Grade 2, n (%)                     | 18 (26.9%)    |                  |
| Grade 3, n (%)                     | 10 (14.9%)    |                  |
| N.A., n (%)                        | 38 (56.7%)    |                  |
| Surgical treatment                 |               |                  |
| Curative resection                 | 8 (11.9%)     |                  |
| Palliative surgery                 | 11 (16.4%)    |                  |
| None                               | 48 (71.6%)    |                  |
| Time from sampling to diagnosis, y Median (range) | 8 (0.4–18.8) |                  |
| N.A. indicates not available in hospital charts, TNM, tumor-node-metastasis. | |

© 2017 The Author(s). Published by Wolters Kluwer Health, Inc.
miRNA Case / Ctrl FC P FDR (P)

miR-574–3p Up 1.5 0.00008 0.0149
miR-885–5p Up 3.9 0.00013 0.0115
miR-144–3p Down 0.4 0.00014 0.0080
miR-130b–3p Up 1.5 0.00199 0.0083
miR-34a–5p Up 2.2 0.00021 0.0073
miR-24–3p Up 1.2 0.00488 0.0121
miR-106b–5p Down 0.8 0.00060 0.0134
miR-22–5p Up 1.4 0.00067 0.0131
miR-451a Down 0.5 0.01125 0.0221
let-7d–3p Up 1.3 0.00201 0.0323
miR-451a Down 0.5 0.01125 0.0221
let-7d–3p Up 1.3 0.00201 0.0323
miR-101–3p Down 0.7 0.00244 0.0360
miR-26a–5p Down 0.6 0.00257 0.0350
miR-197–3p Down 0.7 0.00244 0.0360
miR-24–3p Up 1.2 0.00048 0.0121
miR-34a–5p Up 2.2 0.00021 0.0073
miR-885–5p Up 3.9 0.00013 0.0115
miR-574–3p Up 2.5 0.00199 0.0083

TABLE 3. Fifteen Significantly Altered miRNAs in Plasma Samples From PC Patients at Diagnosis

TABLE 4. Fold Changes and Corresponding P Values of the 15 Candidate miRNAs in the Prediagnostic Cohort

Significant P values in bold. Ctrl indicates controls; FC, fold change; P, Student t test P (not corrected for false discovery rate).

**Plasma miRNAs Are Not Altered Before Diagnosis**

Having established that none of our 15 candidate miRNAs were significantly correlated with SBR, we investigated if they could be used to predict a future PC diagnosis. First, we assessed candidate miRNA alterations in the prediagnostic samples independent of time before diagnosis. This clearly showed that none of the 15 miRNAs that were found significantly altered at the time of diagnosis differed before diagnosis (Table 4). We reasoned that one explanation for this negative result could be the time differences in our prediagnostic cohort, where time of sampling varied from 3 months to 18 years before PC diagnosis. We therefore divided the prediagnostic cohort into three groups: 1) >10 years, 2) 5 to 10 years, and 3) <5 years before diagnosis. At <5 years before diagnosis (3 months to 4.9 years), the P value for miR-24-3p was <0.05 and miR-106b was close to being significant, although none passed false discovery rate testing. Of note, most miRNAs had case/control fold changes close to 1 (Table 4).

**A Multivariate Statistical Model of Candidate miRNAs Separates Cases and Controls at Diagnosis but not Before**

Multiple miRNAs have been shown to act synergistically in gene regulation and previous studies have combined several miRNAs to better separate PC patients from controls. We therefore hypothesized that a combination of our 15 candidate miRNAs might perform better to detect early alterations than single miRNAs alone. To test this, we generated a multivariate statistical model on the basis of candidate miRNAs. At diagnosis, the model clearly separated cases from controls, a separation that was consistent and significant after cross validation (Fig. 2B). However, the model failed to separate cases from controls at any time point before diagnosis, although a tendency to separation was noted <5 years before diagnosis (Fig. 2A). The poor group separation before diagnosis was independent of TNM stage and sex (Supplementary Figure 4, http://links.lww.com/SLA/B163).
By comparison, Ca 19–9 levels were significantly altered < 5 years before diagnosis, although only three of these cases presented with levels above 37 U/mL, which is the standard clinical cutoff for Ca 19–9.4 Interestingly, Ca 19–9 levels increased the closer the sampling date was to diagnosis (Figs. 2C, D, Supplementary Table 4, http://links.lww.com/SLA/B163).

To compare discriminative performance, we constructed ROC curves for the miRNA model and Ca 19–9. Our miRNA model outperformed Ca 19–9 at diagnosis, but at all time points before PC diagnosis, both the miRNA model and Ca 19–9 performed poorly in discriminating cases from controls (Fig. 3). In the light of the poor performance of miRNAs in the training set of the prediagnostic cohort, we refrained from further analysis in the validation set.

DISCUSSION

Circulating levels of miRNAs are altered in many different cancer forms and various miRNAs and miRNA-combinations have been suggested as potential biomarkers of disease.7 In PC, there is a pressing need for early detection biomarkers, as patients are generally asymptomatic until metastatic disease has developed.1

In the first study of its kind, we evaluated the potential of miRNAs in early PC detection by analyzing candidate miRNAs in plasma samples collected before a PC diagnosis. Although we did identify miRNAs that were altered at diagnosis, they were not suitable for early detection of PC. Early detection performance was unaffected by stratifications for both time to diagnosis and TNM stage. miR-24 was significant < 5 years before diagnosis using a permissive significance level at 0.05, but the fold change was minimal and nonsignificant after correcting for false discovery rate, indicative of a false-positive finding.

Nonetheless, we identified 15 miRNAs that were associated with PC at the time of diagnosis and a multivariate model based on these miRNAs at different time intervals before diagnosis and (D) at diagnosis.

FIGURE 2. Differences between cases and controls over time. A to B shows the estimated (upper panels) and cross-validated (lower panels) multivariate statistical model (OPLA-DA) of the 15 candidate miRNAs. A, Prediagnostic samples at different time intervals before diagnosis and (B) at diagnosis. Each dot represents an individual. In a strong model, the cases would cluster around 1 and controls around 0. CV-ANOVA P values are derived from the cross validation. C to D shows boxplots of Ca 19–9 levels in (C) prediagnostic samples at different time intervals before diagnosis and (D) at diagnosis.
FIGURE 3. Receiver-operating characteristics (ROC) curves plotting the sensitivity and false-positive rate (1 – specificity) for the cross-validated multivariate model of 15 miRNAs and Ca 19–9 at (A) different time intervals in relation to diagnosis and (B) at diagnosis.
5. Di Leva G, Garofalo M, Croce CM. MicroRNAs in cancer. *Ann Rev Pathol.* 2014;9:287–314.

6. Mitchell PS, Parkin RK, Kroh EM, et al. Circulating microRNAs as stable blood-based markers for cancer detection. *Proc Natl Acad Sci U S A.* 2008;105:10513–10518.

7. Jarry J, Schadendorf D, Greenwood C, et al. The validity of circulating microRNAs in oncology: five years of challenges and contradictions. *Mot Oncol.* 2014;8:819–829.

8. Schultz NA, Dehlendorff C, Jensen BV, et al. MicroRNA biomarkers in whole blood for detection of pancreatic cancer. *JAMA.* 2014;311:392–404.

9. Xu J, Cao Z, Liu W, et al. Plasma miRNAs effectively distinguish patients with pancreatic cancer from controls: a multicenter study. *Ann Surg.* 2016;263:1173–1179.

10. Bloomston M, Frankel WL, Petrocca F, et al. MicroRNA expression patterns to differentiate pancreatic adenocarcinoma from normal pancreas and chronic pancreatitis. *JAMA.* 2007;297:1901–1908.

11. Szafranska AE, Davison TS, John J, et al. MicroRNA expression alterations are linked to tumorigenesis and non-neoplastic processes in pancreatic ductal adenocarcinoma. *Oncogene.* 2007;26:4442–4452.

12. Lee EJ, Guo Y, Liang J, et al. Expression profiling identifies microRNA signature in pancreatic cancer. *Int J Cancer.* 2007:120:1046–1054.

13. Jamieson NB, Morrán DC, Morton JP, et al. MicroRNA molecular profiles associated with diagnosis, clinicopathologic criteria, and overall survival in patients with resectable pancreatic ductal adenocarcinoma. *Clin Cancer Res.* 2012;18:534–545.

14. Yu J, Li A, Hong SM, et al. MicroRNA alterations of pancreatic intraepithelial neoplasias. *Clin Cancer Res.* 2012;8:991–992.

15. Yachida S, Jones S, Bozin I, et al. Distant metastasis occurs late during the genetic evolution of pancreatic cancer. *Nature.* 2010;467:1114–1117.

16. Norberg M, Wall S, Boman K, et al. The Vasterbotten Intervention Programme: background, design and implications. *Glob Health Action.* 2010;3:3.

17. Kirschner MB, Edelman JJ, Kao SC, et al. Impact of hemolysis on cell-free microRNA biomarkers. *Front Genet.* 2013;4:94.

18. Fairbanks VF, Ziaeser SC, O’Brien PC. Methods for measuring plasma hemoglobin in micromolar concentration compared. *Clin Chem.* 1992;38:132–140.

19. Shah JS, Soon PS, Marsh DJ. Comparison of methodologies to detect low levels of hemolysis in serum for accurate assessment of serum microRNAs. *PLoS One.* 2016;11:e0153200.

20. Mesdagh P, Van Vlierberghe P, De Weer A, et al. A novel and universal method for microRNA RT-qPCR data normalization. *Genome Biol.* 2009;10:R64.

21. Benjamini Y, Hochberg Y. Controlling the false discovery rate: a practical and powerful approach to multiple testing. *J Royal Stat Soc B Methodol.* 1995;57:289–300.

22. Bylesjo M, Rantalainen M, Cloarec O, et al. OPLS discriminant analysis: combining the strengths of PLS-DA and SIMCA classification. *J Chemometrics.* 2006;20:341–351.

23. Trygg J, Wold S. Orthogonal projections to latent structures (O-PLS). *J Chemometrics.* 2002;16:119–128.

24. Wold S. Cross-validated estimation of number of components in factor and principal components models. *Technometrics.* 1978;20:397–405.

25. Eriksson L, Trygg J, Wold S. CV-ANOVA for significance testing of PLS and OPLS (R) models. *J Chemometrics.* 2008;22:594–600.

26. Hayat JO, Loew CJ, Assress KN, et al. Contrasting liver function test patterns in obstructive jaundice due to biliary strictures [corrected] and stones. *QJM.* 2005;98:35–40.

27. Srirawindana AK, Srirawindana AM. Pancreatic cancer. *BMJ.* 2014;349:g6385.

28. McGill MR, Jaeschke H. MicroRNAs as signaling mediators and biomarkers of drug- and chemical-induced liver injury. *J Clin Med.* 2015;4:1063–1078.

29. Han Y, Yu Y, Ma P, et al. An allelic series of mir-17 approximately 92-mutant mice uncovers functional specialization and cooperation among members of a microRNA polycistron. *Nat Genet.* 2015;47:766–775.

30. Kojima M, Sudo H, Kawauchi J, et al. MicroRNA markers for the diagnosis of pancreatic and biliary-tract cancers. *PLoS One.* 2015;10:e0118220.

31. Liu J, Gao J, Du Y, et al. Combination of plasma microRNAs with serum CA19-9 for early detection of pancreatic cancer. *Int J Cancer.* 2011;131:683–691.

32. Ali S, Almhamma K, Chen W, et al. Differentially expressed miRNAs in the plasma may provide a molecular signature for aggressive pancreatic cancer. *Am J Transl Res.* 2010;3:28–47.

33. Baeuerle A, Keller A, Costello E, et al. Diagnosis of pancreatic ductal adenocarcinoma and chronic pancreatitis by measurement of microRNA abundance in blood and tissue. *PLoS One.* 2012;7:e34151.

34. Ganepola GA, Rutledge JR, Suman P, et al. Novel blood-based microRNA biomarker panel for early diagnosis of pancreatic cancer. *World J Gastrointest Oncol.* 2014;6:22–33.

35. Liu R, Chen X, Du Y, et al. Serum microRNA expression profile as a biomarker in the diagnosis and prognosis of pancreatic cancer. *Clin Chem.* 2010;56:610–618.

36. Hamada S, Satoh K, Miura S, et al. mir-197 induces epithelial-mesenchymal transition in pancreatic cancer cells by targeting p120catenin. *J Cell Physiol.* 2013;228:1255–1263.

37. Qazi AM, Grudyn O, Semaan A, et al. Restoration of E-cadherin expression in pancreatic ductal adenocarcinoma treated with microRNA-101. *Surgery.* 2012;152:704–711. discussion 11–13.

38. Liu R, Zhang H, Wang X, et al. The miR-24-2 lab pathway promotes tumor growth and angiogenesis in pancreatic carcinoma. *Oncotarget.* 2015;6:43831–43482.

39. Batchu RB, Grudyn OV, Qazi AM, et al. Enhanced phosphorylation of p53 by microRNA-26a leading to growth inhibition of pancreatic cancer. *Surgery.* 2015;158:981–986. discussion 6–7.

40. Deng J, He M, Chen L, et al. The loss of mir-26a-mediated post-transcriptional regulation of cyclin E2 in pancreatic cancer cell proliferation and decreased patient survival. *PLoS One.* 2013;8:e76450.

41. Luo ZL, Luo HJ, Fang C, et al. Negative correlation of ITCH E3 ubiquitin ligase andmicroRNA-106b dictates metastatic progression in pancreatic cancer. *Oncotarget.* 2016;7:14747–14845.

42. Sureban SM, May R, Lightfoot SA, et al. DCAMKL-1 regulates epithelial-mesenchymal transition in human pancreatic cells through a miR-200a-dependent mechanism. *Cancer Res.* 2011;71:2328–2338.

43. Volinia S, Calif G, Liu CG, et al. A microRNA expression signature of human solid tumors defines cancer gene targets. *Proc Natl Acad Sci U S A.* 2006;103:2257–2261.

44. Papaconstantinou IG, Manta A, Gazouli M, et al. Expression of microRNAs in patients with pancreatic cancer and its prognostic significance. *Pancreas.* 2013;42:67–71.

45. Ali S, Saleh H, Sethi S, et al. MicroRNA profiling of diagnostic needle aspirates from patients with pancreatic cancer. *Br J Cancer.* 2012;107:1354–1360.

46. Nolen BM, Brand RE, Prosser D, et al. Prediagnostic serum biomarkers as early detection tools for pancreatic cancer in a large prospective cohort study. *PLoS One.* 2014;9:e894928.

47. Meyers JR, Wu C, Clish CB, et al. Elevation of circulating branched-chain amino acids is an early event in human pancreatic adenocarcinoma development. *Nat Med.* 2014;20:1193–1198.

48. LaConti JJ, Shivapurkar N, Preet A, et al. Tissue and serum microRNAs in the prediagnostic serum biomarker panel for early detection of pancreatic cancer. *World J Gastrointest Oncol.* 2014;6:22–33.

49. Chang TC, Wentzel EA, Kent OA, et al. Transactivation of miR-34a by p53 dependent mechanism. *Oncotarget.* 2015;6:9811–9816. discussion 6–7.

50. Cote GA, Gore AJ, McElvy SD, et al. A pilot study to develop a diagnostic test for pancreatic ductal adenocarcinoma based on differential expression of select miRNAs in plasma and bile. *Ann J Gastroenterol.* 2014;109:1942–1952.