Commentary

Significance of microRNA-based biomarkers for pancreatic cancer

Sukhwinder Kaur[1], Shiv Ram Krishn[1], Satyanarayana Rachagani[1], Surinder K. Batra[1,2,3]

[1]Departments of Biochemistry and Molecular Biology; [2]Fred and Pamela Buffett Cancer Center; [3]Eppley Institute for Research in Cancer and Allied Diseases, University of Nebraska Medical Center, Omaha, Nebraska 68198-5870, USA

Correspondence to: Surinder K. Batra, PhD. Departments of Biochemistry and Molecular Biology; Fred and Pamela Buffett Cancer Center; Eppley Institute for Research in Cancer and Allied Diseases, University of Nebraska Medical Center, Omaha, Nebraska 68198-5870, USA.
Email: sbatra@unmc.edu.

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Pancreatic cancer (PC) is an extremely aggressive malignancy with one of the worst prognoses of all cancers. Currently, it is the tenth most commonly diagnosed malignancy and fourth leading cause of cancer-related deaths in the United States. The National Cancer Institute (NCI) in the United States estimates that 48,960 Americans will be newly diagnosed with this cancer in 2015, of which 40,560 deaths due to this lethal malignancy. Among clinical cases, 74% of patients die within the first year of diagnosis, and ~94% of patient deaths occur within 5 years of diagnosis. Alarmingly, the changing demographics and average annual percentage alterations in incidence and death rate suggest that PC will surpass breast, prostate, and colorectal cancers and will be the second leading cause of cancer related deaths by 2030 (1). Even after intense research on PC, the mortality rate has been unaltered/increased whereas, death rates for other malignancies are reducing over past decades, placing it among the most lethal malignancies. Major barriers to the better prognoses of PC are late detection, inherent resistance to chemotherapy, radiotherapy and relapse of the disease.

To date, the successful curative option for PC is only surgical resection. In patients with the localized disease with no lymph node or extra-pancreatic metastasis, complete surgical resection provides a 5-year survival rate of 30-75% (2-4). Unfortunately, till date >80% of PC patients are clinically diagnosed when the primary tumor is unresectable and has metastasized to distant organs (5). Considering the sporadic nature of PC, the major challenges in early diagnosis is to identify non-invasive or minimally invasive diagnostic technology that possesses high sensitivity (SN), specificity (SP) and the ability to discriminate the real disease (PC precursor lesions and resectable neoplasms) from confounding risk groups including pancreatitis, benign pathologies (acute biliary obstruction, common bile duct stone, cholelithiasis) and non-malignant benign cystic neoplasms (serous pancreatic cystic neoplasms).

Among non-invasive serological markers, the Gold-standard CA 19-9 has multiple concerns including aberrant expression in various benign conditions (pancreatitis, cirrhosis, acute cholangitis), absence in 5-10% of the Caucasian population, poor to moderate SN (69-98%) and SP (46-98%) in detecting PC, and the observation that only 65% of patient with resectable PC have elevated levels of this biomarker (6). Several biochemical markers of various types (7,8) have been tested with the intent of increasing the likelihood of diagnosis in high-risk patients prior to the onset of symptoms (8). Among these markers PC driver genes mutational analyses, inflammatory serological signatures, circulating tumor cells, autoantibodies, epigenetic markers and microRNAs (miRNAs) are well studied. Unique miRNA expression profiles have been observed in various gastrointestinal cancers including PC at different stages, suggesting their potential as diagnostic biomarkers (9). In addition, dysregulated miRNA expression patterns exists in tumor tissues, plasma, sputum and exosome samples of patients; therefore carries high potential to serve as minimally invasive screening and novel tools for diagnostic evaluations (10). Further, due to their small size, high stability, and ease of profiling/detection in the serum, miRNAs are being projected as promising tools for the early diagnosis of PC (11). Comprehensive analyses of PC miRNAs have been carried out in the past (12-14), however, inconsistent reports from the past studies prompted Xu *et al*.

to identify miRNAs differentially expressed in PC patients in comparison to benign and malignant control groups.

In the study, "Plasma miRNAs effectively distinguish patients with pancreatic cancer from controls", published in November issue of *Annals of Surgery*, Xu *et al*., evaluated the efficacy of plasma miRNAs profiles to distinguish PC patients from healthy and chronic pancreatitis (CP) controls in a multicenter, three-phase study (15). The authors for the first time identified miRNAs differentially expressed in pancreatic neuroendocrine tumors (PNET) and other pancreatic tumors (OPT) including (serous or mucinous cystadenomas, solid pseudo-papillary tumors, intraductal papillary mucinous neoplasms or epithelial cysts). Initial discovery phase with 3 pooled samples from seven patients with PC, six patients with CP, and five healthy volunteers identified 29 miRNAs that were specifically dysregulated in the patients with PC compared to the controls [healthy controls (HC) and CP] using two reference internal controls (U6 and miR-16). Fifteen miRNAs (miR-106b-3p, miR-1233*, miR-1271-5p, miR-1285-3p, miR-15b-3p*, miR-181c-5p, miR-26b-3p, miR-30d-3p, miR-335-3p*, miR-454-5p*, miR-589-3p, miR-616-3p, miR-663b, miR-664-3p, and miR-744-3p) were significantly dysregulated in patients with PC compared to HC on normalization to both U6 and miR-16. Further, nineteen miRNAs (miR-1233*, miR-127-3p, miR-15b-3p*, miR-19a-3p, miR-26a-1-3p, miR-296-5p, miR-335-3p*, miR-339-5p, miR-361-3p, miR-378a-5p, miR-454–5p*, miR-545-3p, miR-579, miR-584-5p, miR-589-3p*, miR-629-5p, miR-645, miR-7-5p, and miR-938) were differentially expressed in PC cases in comparison to CP (* text represent miRNA common to HC and pancreatitis group). Further, a preliminary validation of the efficacy of above mentioned 29 miRNAs combined with four additional miRNAs of potential diagnostic value for pancreatic cancer and identified in earlier studies (miR-126-3p, miR-19b-3p, miR-486-5p, and miR-942) was performed in a small cohort comprising of PC (n=29), CP (n=16) and HC (n=31). Intriguingly, out of multiple miRNAs dysregulated during the discovery phase, only hsa-miR-181c-5p was significantly upregulated in PC cases compared with CP, when normalized using both U6 and miR-16 during preliminary validation in the small patient cohort. Further, in comparison to HC, multiple miRNA (hsa-miR-126-3p, hsa-miR-1271, hsa-miR-1285, hsa-miR-19b-3p, hsa-miR-296-5p, hsa-miR-486-5p, hsa-miR-663B, hsa-miR-7-5p, hsa-miR-942) were upregulated for the U6 reference control, however except for hsa-miR-486-5p, their upregulation did not hold true when miR-16 was utilized as internal control. Interestingly, to minimize the risk of false-positive results, Xu *et al*. used both U6 and miR-16 to normalize plasma miRNA levels during the discovery and preliminary validation phases. However, the data from two different reference genes were not found to be consistent across various test sets. As miR-16 dysregulation is observed in various malignancies, its utility as a reference gene for normalization seems to be quite inappropriate for identifying diagnostically important panel of circulating miRNAs in such an important and critical study set (16). Under this scenario, it would have been interesting to delineate an internal reference control in three tired study set and evaluate miRNAs biomarker performance using the resampling set. As normalization is critical to control variations in the extracted RNA yield, reverse-transcription yield, efficiency of amplification and overall dictates the reliability of qPCR, rigorous efforts should have been focused on normalization while conducting the present study. For lack of consensus on reference miRNAs, multiple normalization *miRNA* genes, average Cq method and the external RNA spike-in control method could have provided improved diagnostic panel for differentiating PC cases from control groups.

During validation phase using large set of samples, Xu *et al*., validated the diagnostic performance of thirteen miRNAs (miR-106b-3p, miR-126-3p, miR-1271, miR-1285, miR-19b-3p, miR-26b-3p, miR-296-5p, miR-486-5p, miR-663B, miR-7–5p, miR-938, miR-942, and miR-181c-5p) that were significantly dysregulated when normalized using both U6 and miR-16 during preliminary validation. The sample set comprised of PC (n=156), CP (n=57), HC (n=65), PNET (n=27) and OPT (n=58). The miR-486-5p strongly discriminated PC from HC, with AUC values of 0.861 [95% confidence interval (CI), 0.808-0.904; P<0.0001] as well as CP cases with AUC values of 0.706 (95% CI, 0.639-0.766; P<0.0001). Interestingly, miR-486-5p matched with the diagnostic efficacy of CA19.9 in differentiating PC cases from CP and HC. However, it could not differentiate PC from PNET and OPT. Here it is noteworthy that, miR-486-5p was included in the study on the basis of its diagnostic utility observed earlier by Ali *et al*. while profiling MicroRNA of diagnostic needle aspirates from patients with PC (17). Additionally, AUC of 0.754 (95% CI, 0.691-0.811; P<0.0001) was observed for miR-938 to differentiate PC from CP. Further, only miR-938 exhibited AUC of 0.618 (95% CI, 0.549-0.683; P=0.0063) for differentiating patients with PC from patients with OPT. The miR-938 also emerged in the pool of miRNAs differentially regulated

in PC when compared to PNET. Significant differences in the levels of miR-126-3p were also observed in PC cases in comparison to controls groups. Interestingly, in larger sample set multiple miRNAs including, miR-181c-5p, miR-1285 and miR-942 did not achieve very high significance in terms of diagnostic efficacy. Further, both miR-26b-3p and miR-938 were downregulated in PC in accordance to small sample set while in larger sample set their upregulation was observed. Similarly, miR-126-3p, miR-19b-3p, miR-942 was downregulated when normalized with miR-16 in small validation set but were upregulated in PC in the larger validation set.

MiR-126-3p, miR-26b-3p, miR-938, and miR-19b-3p were found to be significantly upregulated in PC when compared to PNET; while miR-181c-5p, miR-19b-3p, miR-26b-3p, and miR-938 were significantly upregulated in PC patients in comparison to OPT cases. In comparison to CA19.9, only miR-486-5p exhibited equivalent performance in differentiating PC cases from HC and CP. It would have been interesting to see whether the diagnostic efficacy of miR-486-5p or combination of other miRNAs based biomarkers add up to the diagnostic efficacy of CA 19-9. However, lack of precisely matched sample set limited the study from this aspect. Further, comparison of comprehensive miRNA analyses between various PC studies identifies only very small sub-set of common miRNAs. Schultz *et al*. in the highly comprehensive study of ~1,494 patients identified 38 miRNAs differentially expressed in PC in comparison to healthy and CP controls. They identified two diagnostic panels with 4 miRNAs in index I (miR-145, miR-150, miR-223, miR-636), and 10 miRNAs in index II (miR-26b, miR-34a, miR-122, miR-126, miR-145, miR-150, miR-223, miR-505, miR-636, miR-885.5p) that distinguished PC cases from controls with the high SN and SP and improved the diagnostic performance of CA 19-9 (13). Interestingly, only miR-126 was the significant diagnostic marker from the study of Schultz *et al*. that is consistent with the current study for differentiating PC from healthy and CP controls. Further, though miR-26b were observed in both the studies; however, it didn't significantly differentiate PC from controls in the present study. Similarly, in the comprehensive study set, Li *et al*. (12) identified miR-24, miR-134, miR-146a, miR-484, miR-628-3p, miR-1825 and miR-1290 has a potential to improve the early detection of PC (12). However, none of these miRNAs emerged as sensitive and specific diagnostic markers in the current study. Even after this comprehensive study the issues faced by Xu *et al*. seems to be inherent to the miRNA field.

Circulating miRNAs have been projected as potential diagnostic markers due to differential dysregulation, high stability and ease of diagnosis. Nevertheless, the observed alterations in circulating miRNAs are mostly sporadic with little consensus among multiple studies carried by different groups; thereby dampening the enthusiasm for their use as biomarkers for clinical utility. These poor overlap of results could appear from the lack of consensus on ideal internal reference controls leading to improper normalization, difference in the ethnicity across various study sets, variations in the sample choice, sample collection process across various institutes, no consensus on the standard operating protocols for their isolation, and pre-analytical and analytical variables causing inaccurate quantification of circulating miRNAs. Further studies with the standard operating protocol will help to clear the dilemma present in the literature regarding the usage of miRNA as diagnostic tools of clinical utility.

Overall, Xu *et al*. did the comprehensive analyses of miRNAs differentially expressed in PC in comparison to healthy, CP, pancreatic neuroendocrine and OPT. Studies validated the performance of newly identified miRNA. Further, the present study validated miR-486-5p and miR-938 as most differential miRNAs for differentiating PC from various control groups. It would be interesting to evaluate the combinatorial efficacy of newly identified miRNAs in differentiating early-stage PC cases from chronic pancreatitis and healthy control groups either alone or in combination with the CA 19-9 in the future. Further, it would be highly important to investigate the diagnostic performance of miRNAs emerging from various groups in the blinded analysis where samples are collected under standard operating protocols.

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Footnote

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