Non-invasive detection of pancreatic cancer by measuring DNA methylation of Basonuclin 1 and Septin 9 in plasma

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To the Editor: Pancreatic cancer (PC), a deadly malignancy with an overall 5-year survival rate of 5% to 15%, is ranked as the seventh leading cause of cancer death in the world in spite of its low occurrence rate.[1,2] Early detection appears to be the most effective approach to improve the overall survival of patients with PC. However, the difficulty in early detection of PC is a lack of specific symptoms and reliable biomarkers. Currently, carbohydrate antigen 19-9 (CA 19-9) is a serum biomarker that is widely used in PC detection. However, 10% of patients with PC cannot produce CA 19-9 and serum CA 19-9 is frequently absent in patients with early-stage cancer. Furthermore, CA 19-9 is often found to be elevated in benign conditions or in other cancers, making its utility limited. Therefore, it is important to identify new diagnostic biomarkers to improve PC detection.

Recently, it has been reported that PC is characterized by multiple genetic and epigenetic changes.[3] One of these epigenetic modifications is DNA hypermethylation which can be detected in plasma-derived cell-free DNA, thus making it an ideal biomarker for early detection and staging of cancer. Recent studies have also demonstrated that the tumor suppressor genes Basonuclin 1 (*BNC1*) and Septin 9 (*SEPT9*) might play a role in PC. In particular, *BNC1* and *SEPT9* silencing by promoter methylation in cancer have been suggested to be a useful diagnostic biomarker in cancer.[4,5] Therefore, in the present study, we aimed to measure *BNC1* and *SEPT9* DNA methylation levels in PC for both tissue and plasma samples in order to evaluate their diagnostic utility as biomarkers. All procedures performed in this study involving human participants were in accordance with the Ethical Standards of the Institutional and/or National Research Committee and with the 1964 *Helsinki Declaration* and its later amendments or comparable ethical standards. This study was approved by the Peking Union Medical College Ethics Committee (No. HS-1328).

Patient plasma samples were obtained from individuals with PC ($n = 57$), pancreatic intraepithelial neoplasia (PanIN) ($n = 14$), and benign disease ($n = 44$, 15 cases of pancreatitis and 29 benign tumors) before treatment, confirmed by post-operative pathology at the department of basic surgery in Peking Union Medical College Hospital from January 2017 to February 2018. As many as 53 plasma samples were obtained from healthy volunteers to serve as normal controls (NC). In the PC group, a total of eight pairs of PC and matched adjacent normal tissues were collected.

Standard sample collection, storage and transportation, DNA extraction, bisulfite conversion, and quantitative methylation-specific polymerase chain reaction (PCR) were performed manually using the Diagnostic Kit for *SEPT9* Gene Methylation Assay (BioChain [Beijing] Science and Technology, Inc., Beijing, China). The manufacturer's instructions were followed carefully. For quantification of *BNC1* and *SEPT9* methylation in tissues, the cycle threshold (CT) method was used, with normalizing of the CT values for the indicated genes to the CT values of ACTB relative to a methylated reaction sample.

In this study, we initially assayed the methylation levels of *BNC1* and *SEPT9* in primary pancreatic tumor and adjacent normal tissues ($n = 8$, stage I or II). The results showed that *BNC1* and *SEPT9* methylation levels in all eight tumor tissues were elevated in comparison to the matching adjacent normal tissues [Figure 1A and 1B]. We then assayed the *BNC1* and *SEPT9* methylated DNA in cell-free DNA of plasma by methylation-specific PCR in PC, PanIN, benign disease, and NC. The CT value was set

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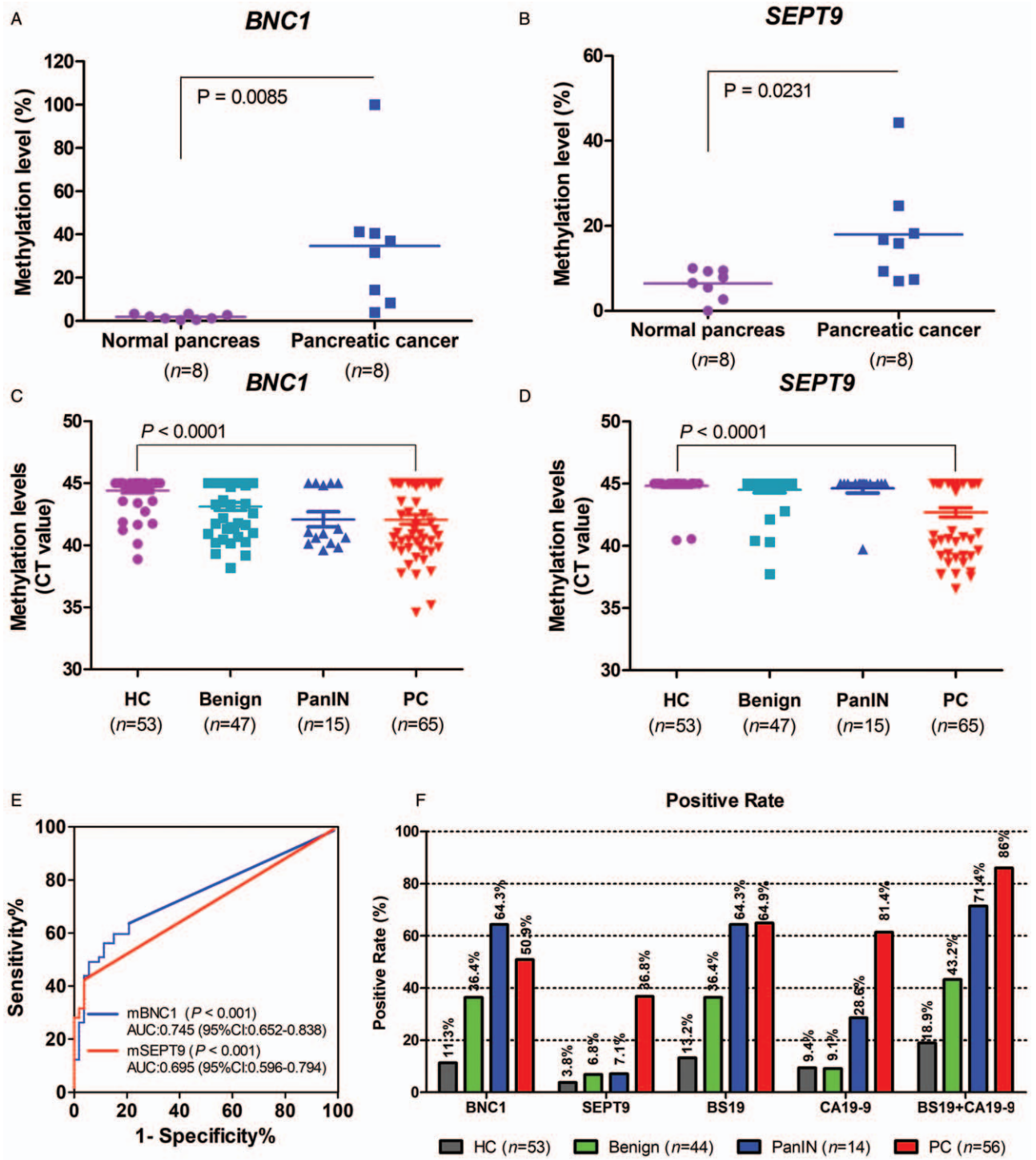

Figure 1: The diagnostic performance of *BNC1* and *SEPT9* methylation for PC. (A and B) The methylation levels of *BNC1* and *SEPT9* were increased in tissues of PC. (C and D) Quantitative PCR showed significantly decreased median CT values of *BNC1* and *SEPT9* methylation in PC ($n = 57$) when comparing HC ($n = 53$) and benign ($n = 44$) ($P < 0.05$, both *BNC1* and *SEPT9*). (E) The ROC curve of plasma *BNC1* and *SEPT9* methylation in the diagnosis of PC from HC. (F) The positive detection rate of plasma *BNC1* and *SEPT9* methylation, CA 19-9 and in combination for all individuals. AUC: Area under the curve; Benign: Benign disease; *BNC1*: Basonuclin 1; CA 19-9: Carbohydrate antigen 19-9; CT: Computed tomography; HC: Healthy control; PanIN: Pancreatic intraepithelial neoplasia; PC: Pancreatic cancer; ROC: Receiver operating characteristic assay; *SEPT9*: Septin 9.

at 45 (the maximal number of PCR cycles in the assay) to establish the limit of detection. We found that the CT values of *BNC1* and *SEPT9* methylation in PC (42.1 ± 0.4 and 42.7 ± 0.4, respectively) were significantly lower than in both NC (44.4 ± 0.2 and 44.8 ± 0.1, respectively, both $P < 0.0001$) and benign disease (43.1 ± 0.3 and 44.5 ± 0.2, $P = 0.0045$ and 0.0003, respectively), suggesting elevated levels of *BNC1* and *SEPT9* methylated DNA in PC plasma [Figure 1C and 1D].

To measure the observed test performance, a receiver operating characteristic assay (ROC) curve was generated to calculate the respective areas under the curves (AUCs) with 95% confidence interval. The sensitivity, specificity, positive

Table 1: Performance of plasma *BNC1*, *SEPT9* methylation, and serum CA 19-9 in the diagnosis of pancreatic cancer.

Parameters	AUC	Sensitivity (%) ($n = 57$)		Specificity (%) ($n = 53$)		PPV (%)	NPV (%)	Accuracy (%)
		Value	95% CI	Value	95% CI			
BNC1	0.745	50.9	37.3–64.4	88.7	77.0–95.7	82.9	62.7	69.1
SEPT9	0.695	36.8	24.5–50.7	96.2	87.0–99.5	91.3	58.6	65.5
BNC1, *SEPT9*	0.759	64.9	55.0–78.8	86.8	74.7–94.5	84.1	69.7	75.5
CA 19-9	0.741	61.4	47.6–74.0	90.6	79.3–96.9	87.5	68.6	75.5
BNC1, *SEPT9*, CA19-9	0.836	86.0	74.2–93.7	81.1	68.0–90.6	83.1	84.3	83.6

CI: Confidence interval; AUC: Area under curve; *BNC1*: Basonuclin 1; CA 19-9: Carbohydrate antigen 19-9; NPV: Negative predictive value; PPV: Positive predictive value; *SEPT9*: Septin 9.

predictive value, negative predictive value and accuracy of *BNC1* and *SEPT9* gene methylation for PC diagnosis were then correlated at the determined cut-off value, with $P < 0.05$ being considered statistically significant. The ROC curve analysis showed that the AUC was estimated to be 0.745 and 0.695 for PC patients at the CT cut-off value of 42 and 41 for *BNC1* and *SEPT9*, respectively [Figure 1E]. The sensitivity and specificity were 50.9% and 88.7%, respectively, for *BNC1*, and 36.8% and 96.2%, respectively, for *SEPT9*. The combination of these two genes (*BS19*, *BNC1* plus *SEPT9*) for PC detection improved sensitivity (64.9%) and decreased the specificity (86.8%). *BS19* together with CA 19-9 demonstrated improved diagnostic performance over CA 19-9 alone (AUC = 0.836 *vs*. AUC = 0.741) with significantly improved sensitivity (86% *vs*. 61.4%), but reduced specificity (81.1% *vs*. 90.6%) [Table 1].

BS19 increased detection of patients with PC (14/22) which were negative for serum CA 19-9. For PanIN and benign disease in the pancreas, *BS19* could increase the detection approximately 60% (6/10) and 40% (16/40), respectively, for CA 19-9 negative patients. The sensitivity of detection of PanIN and benign disease was 64.3% and 36.4%, respectively, for *BS19*, higher than that of CA 19-9 (28.6% and 9.1%). *BS19* combined with CA 19-9 showed improved sensitivity (71.4% for PanIN and 43.2% for benign disease) over *BS19* or CA 19-9 alone, which would improve the early diagnosis of PC [Figure 1F]

Nevertheless, DNA methylation of *BNC1* and *SEPT9* in cell-free DNA from plasma has several limitations in PC diagnosis. One limitation is that DNA methylation of *BNC1* and *SEPT9* in plasma can be elevated in approximately one-third of benign pancreatic diseases, such as acute pancreatitis, chronic pancreatitis, or any benign occupation of the pancreas, although such benign diseases may themselves be an important predisposing condition of PC. Another limitation is that DNA methylation of *BNC1* and *SEPT9* in plasma may be elevated in other cancers, such as colorectal cancer, lung cancer, and hepatocellular carcinoma.[5-7] The combination of the current diagnostic protocol may improve the diagnostic performance for PC. However, present results may still be preliminary results because of relatively small sample sizes. Thus, further large scale and prospective studies should be performed to confirm our findings.

In conclusion, the present study indicates that DNA methylation levels of *BNC1* and *SEPT9* are elevated in PC tissues and in plasma-derived cell-free DNA from patients with PC. DNA methylation of *BNC1* and *SEPT9* in plasma cell-free DNA, therefore, has the potential to become useful diagnostic biomarkers for PC. The combination of *BNC1* and *SEPT9* as well as serum CA 19-9 might improve the diagnostic performance for PC.

Declaration of patient consent

The authors certify that they have obtained all appropriate consents for this study. These included the consent by the patient/patient's guardians for their images and other clinical information to be reported in this study. The patient/patient's guardians understand that their names and initials will not be published and due efforts will be made to conceal their identity, but anonymity cannot be guaranteed.

Conflicts of interest

None.

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