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Original Article

Methylated genes *p16* and *ppENK* for diagnoses of pancreatic cancer: systematic review and meta-analysis

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Abstract: Aims and background: The aims of the study were to induce methylated genes, p16 (INK4 a/MTS-1/CDKN2) and ppENK (preproenkephalin), associated to pancreatic cancer and evaluate whether the application of methylated genes in pancreatic cancer diagnosis is valuable and effective. Methods: The American Association for Cancer Research, Medline, Science Citation Index, Google (scholar), National Center for Biotechnology Information (NCBI), China National Knowledge Infrastructure (CNKI) and U.S. National Library of Medicine were searched to identify reports related to methylated gene detection in pancreatic cancer. Data were statistically combined using a random-effect meta-analysis basing on the intention-to-treat principle. Strict selection and exclusion criteria were determined, and odds ratios (ORs) with 95% confidence intervals (CIs) were applied to assess the strength of associations. Basing on the heterogeneity test among studies, a fixed or random effect model was selected. Meanwhile, publication bias was estimated using funnel plots in RevMan. Results: A total of eight studies, involving 583 participants, were included in this meta-analysis. Hereinto, five of the overlapping tests researched on the methylation of *p16* in 237 participants; six of these tests were on the methylation of *ppENK* in 344 participants. The specificity in both detections of *p16* and *ppENK* were higher than the sensibility. Meanwhile, the available summary receiver operating characteristic (SROC) curves indicated that it is more efficacious to apply detection of *ppENK* methylation for diagnosis of pancreatic cancer. Conclusions: The current evidence suggests that the methylated *ppENK* was a better marker than *p16* in the diagnosis of pancreatic cancer with a higher sensitivity and specificity. This result might provide basis for clinical diagnosis of pancreatic cancer.

Keywords: Methylated gene, methylation, pancreatic cancer, diagnoses, *p16*, *ppENK*

Introduction

Pancreatic cancer, a serious disease resulting from both epigenetic and genetic diathesis [1, 2], causes 38,460 deaths of the 45,220 newly estimated cases in 2013 [3]. Large improvements in survival have been for many kinds of cancers, while pancreatic cancer is excepted [3]. Emerging evidence shows that screening first-degree relatives of individuals with family medical history in pancreatic cancer can identify non-invasive precursors of this malignant disease. As for the poor response of pancreatic cancer to most chemotherapeutic agents, surgical resection at present offers the only curable chance for those with malignant disease localized to the pancreas. However, 80-85% of patients present with advanced unresectable disease. Despite of the developments in detection and management of pancreatic cancer, the five-year survival rate was only 4% approximately [4]. Considering chemotherapy and radiotherapy have largely failed to improve the survival rate of patients with pancreatic cancer significantly, identifying better diagnostic markers of pancreatic neoplasia becomes a considerable interest in researchers. Effective markers not only helps improve the diagnosis of pancreatic cancer in early stages so that more patients are able to undergo surgical resection, but also could be used potentially for prognosis of patients at high risk of developing pancreatic cancer through identifying precancerous lesions. A wealth of information on gene expression, DNA methylation, and proteomics alterations that occur in pancreatic cancers has been identified as diagnostic markers of pancreatic cancer.

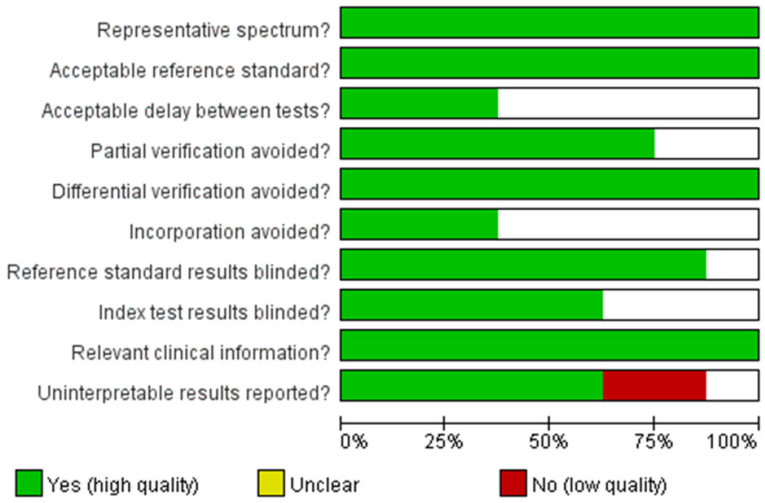

Figure 1. Methodological quality graph.

The detection of aberrantly methylated genes in the pancreatic juice of patients with pancreatic cancer has been indicated as promising diagnostic strategy [5, 6]. Numerous genes such as *p16* [7] and *ppENK* (preproenkephalin) [6] have been identified aberrantly methylated in a high proportion of pancreatic cancers and can be detected with methylation-specific polymerase chain reaction (MSP), which makes such genes potentially attractive for early detection. Indeed, there are many researches on the detection of these methylated genes in pancreatic juice or plasma samples of patients with pancreatic diseases. However, review on the sensibility or significance of these genes in the utilization of diagnosis is rare. Taking that a single study is short of the power to reach a reliable conclusion, we performed a meta-analysis on these eligible studies to evaluate the sensibility of methylated genes in pancreatic cancer diagnosis, which would make sense in the clinical application.

Materials and methods

Information sources and search strategy

A search of the literature was conducted for studies that reported the detection of methylated genes in pancreatic cancer. The American Association for Cancer Research, Medline (US National Library of Medicine, Bethesda, MD), Science Citation Index, Google (scholar), National Center for Biotechnology Information (NCBI), and China National Knowledge Infrastructure (CNKI) were searched to identify articles published in the field of methylated genes detection for pancreatic cancer. The keywords used in literature searches included the following: methylated gene; aberrant methylation; diagnose of pancreatic cancer, *p16* or *INK4 a/MTS-1/CDKN2*, and *ppENK* or preproenkephalin. We restricted the selection on published language to English or Chinese. The PubMed option "Related Articles" for each study was applied to retrieve additional potentially relevant articles, while all kinds of pancreatic cancers were set in cancer group.

Eligibility criteria

The inclusion criteria were the following: (1) association study with a case-control or cohort design; (2) the study identified methylation of *p16/INK4a/MTS-1/CDKN2* or *ppENK* (preproenkephalin) in pancreatic cancer; (3) in the case of multiple publications from the same study group, the most complete and recent results were used; (4) participant number was larger than 10; (5) blind method was applied; (6) the published time was from 2000 till now. Reports of duplicated studies were excluded by examining the author list, parent institution, sample size and results.

Exclusion criteria were: (1) overlapping data; (2) not case-control studies; (3) only relevant to oncotherapy; (4) review, abstracts, animal studies or letter.

Data extraction

Two reviewers independently screened titles and abstracts for potential eligibility and then assessed the quality of the included studies according to the full texts for final eligibility. The methodological quality of the trials was assessed based on the Cochrane Collaboration Risk of Bias Tool (CCRBT) [8] (**Figure 1**). Data from the included trials was extracted independently by another two authors for quantitative analyses, and any disagreement was subsequently resolved by discussion. The primary information about author, published time, and nationality of participants was collected. The

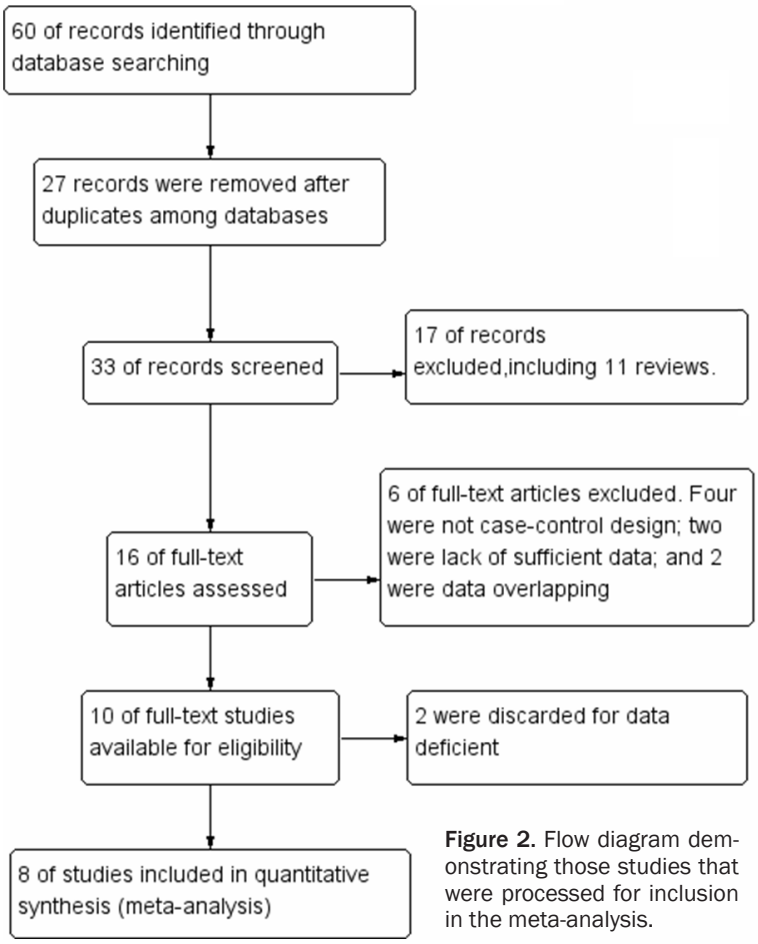

Figure 2. Flow diagram demonstrating those studies that were processed for inclusion in the meta-analysis.

quantitative data included the patient characteristics, such as average age, sample size, value of true positivity (TP), true negativity (TN), false positivity (FP) and false negativity (FN). Chronic pancreatitis patients were summed together with normal patients as control group. Patients with all kinds of pancreatic adenocarcinoma were set as pancreatic cancer case.

Review authors' judgements about each methodological quality item presented as percentages across all included studies.

Statistical analysis

Heterogeneity was explored using a Chi-square test, and the quantity of heterogeneity was measured using the I^2 statistic with Review Manager. $P \leq 0.10$ or $I^2 \geq 50\%$ suggest that there is heterogeneity and random-effect model should be chosen. In experimental group, samples with detected gene methylation were considered as "event"; while in control group, the normal tissues without gene methylation were taken as "event". Trails in the eight studies were subgrouped basing on the involving methylated gene (*p16* and *ppENK*).

With the extracted value of true positivity (TP), true negativity (TN), false positivity (FP) and false negativity (FN), sensitivity and specificity of each trail were obtained. In condition that there were no heterogeneity among trails in each subgroup, sensitivity and specificity, positive and negative likelihood ratio, as well as diagnostic odds ratio could be pooled analyzed directly. Meanwhile, Summary receiver operating characteristic (SROC) curves comparing methylated *p16* and *ppENK* markers for detection of pancreatic cancer versus normal and pancreatitis.

The possible publication bias was assessed using funnel plots, in which an asymmetric plot suggests a possible publication bias [9]. All statistical tests were performed with RevMan version 5.1 (Nordic Cochrane Centre, Copenhagen, Denmark). *P* values smaller than 0.05 for any test was considered to be statistically significant.

Results

Characteristics of eligible studies

Relevant publications were retrieved from databases (PubMed, Google scholar, and CNKI). As shown in **Figure 2**, a total of 33 relevant publications were adopted through reading records. Among them, 17 publications were excluded, including 11 reviews and 6 qualitative investigations. After scanning full-text articles, 6 were excluded (4 were not case-control design and 2 were data overlapping). Afterwards, two papers [10, 11] were discarded because of insufficient data when extracting data. Finally, 8 papers were identified for data extraction and assessment [1, 2, 6, 12-16]. Five of the trials in all

Table 1. Characteristics of all included studies in the meta-analysis

NO.	Author	Year	Case-control	Sample size	Average age (years)	Methylation		Method	Sample type
						ppENK	*p16*		
1	Fukushima N	2003	Resected pancreatic ductal adenocarcinoma	33	64.4	30	6	Methylation-Specific PCR (MSP)	Pancreatic juice
			Normal pancreatic ductal epithelium	7	59.6	0	0		
2	Klump	2003	Pancreatic carcinoma (PCA)	37	70.2	-	16	Methylation-Specific PCR (MSP)	Pancreatic secretion
			Chronic pancreatitis (CP)	14	51.6	-	0		
			Pancreatic disease (NAD)	6	-	-	0		Tissue
3	Lixin Y	2013	Pancreatic cancer tissues	31	-	28	-	Methylation-Specific PCR (MSP)	Cell line
			Normal pancreatic tissues	32	-	0	-		Tissue
4	Fukushima N	2002	Pancreatic ductal adenocarcinomas	15	-	14	4	Methylation-Specific PCR (MSP)	Pancreatic juice
			Nonneoplastic pancreatic epithelia	28	-	0	0		
5	Ohtsubo K	2006	Pancreatic carcinoma	26	62.4	12	-	Methylation-Specific PCR (MSP)	Pure pancreatic juice
			Intraductal papillary mucinous neoplasms	15	-	4	-		
			Chronic pancreatitis	20	-	1	-		
6	Park J	2012	Pancreatic cancer (PC)	16	63.9 ± 9.8	4	5	Methylation-Specific PCR (MSP)	Tissue
			Chronic pancreatitis (CP)	13	49.5 ± 11.8	2	2		
7	Takashi	2000	Pancreatic carcinomas	36	-	-	5	Methylation-Specific PCR (MSP)	Tissue
			Pancreatic cell lines	9	-	-	3		
			Normal pancreata	14	-	-	0		
8	Takashi	2001	Pancreatic adenocarcinomas	75	-	70	-	Methylation-Specific PCR (MSP)	Cell line
			Chronic pancreatitis	5	-	1	-		
			Normal pancreata	15	-	0	-		Xenograft

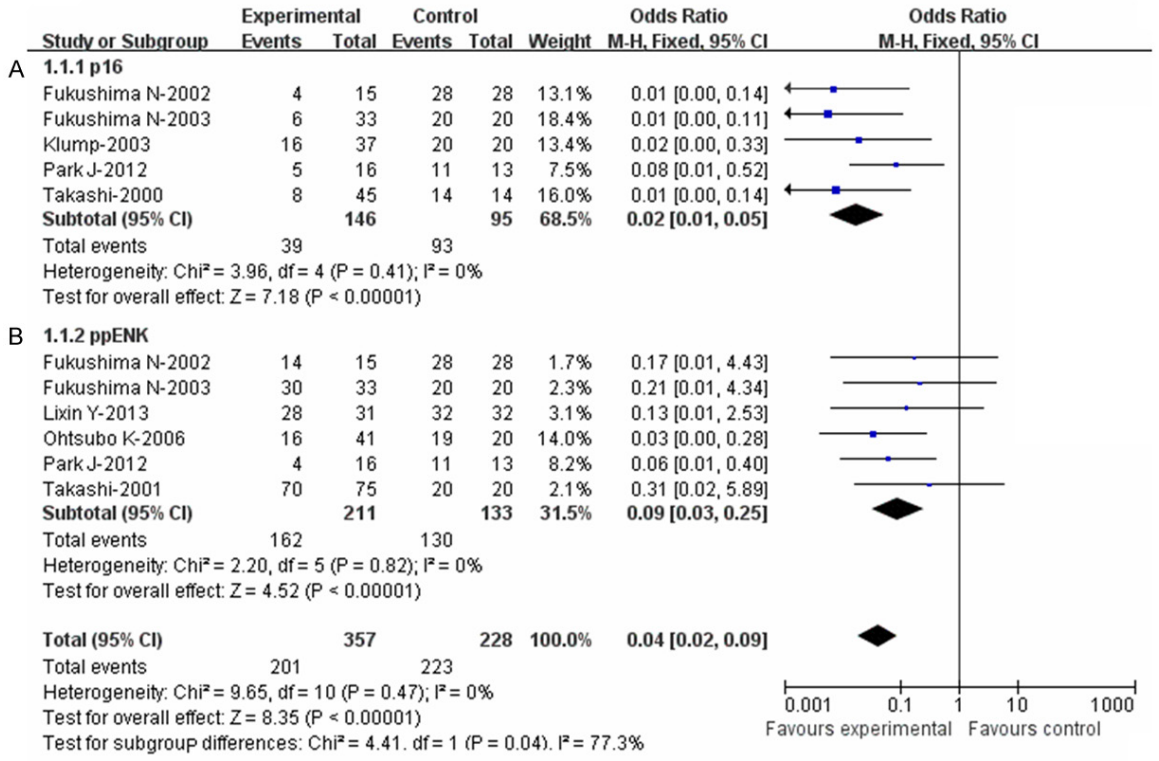

Figure 3. Forest plot of association between the methylation genes in patient and control samples. A. Forest plot of association between the methylated p16 in patient and control samples; B. Forest plot of association between the methylated ppENK in patient and control samples.

papers were related to the detection of methylated *p16*; six of the trials were associated with *ppENK*. **Table 1** listed the available characteristics of the eight papers.

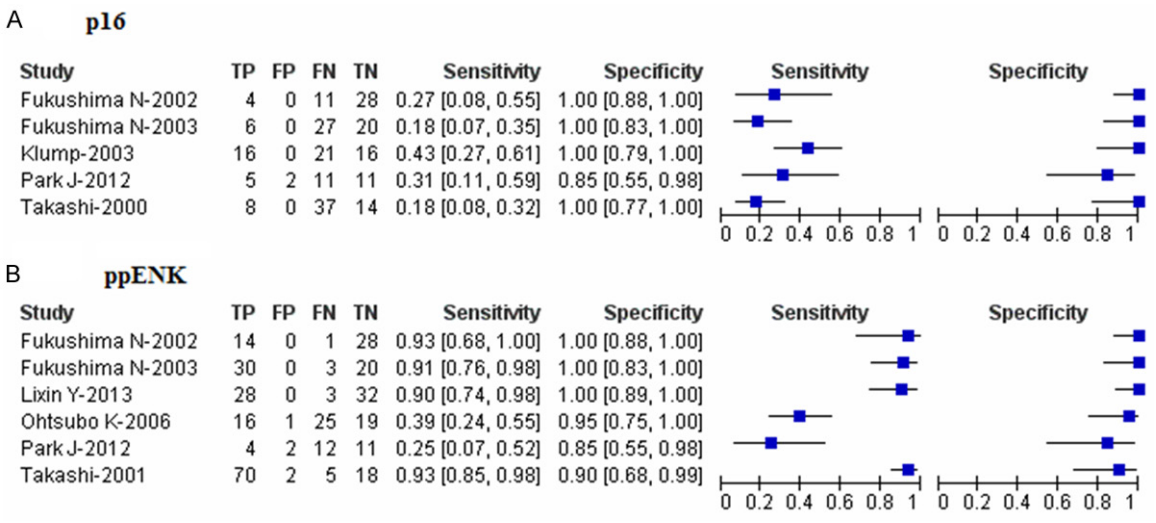

Figure 4. Forest plot of the sensitivity and specificity in detection of methylated p16 and ppENK. A. Forest plot of the sensitivity and specificity in detection of methylated p16; B. Forest plot of the sensitivity and specificity in detection of methylated ppENK.

Table 2. Pooled analysis of p16 subgroup and ppENK group

Value	p16	ppENK
True Positivity (TP)	39	162
False Positivity (FP)	2	5
True Negativity (TN)	89	128
False Negativity (FN)	107	49
Sensitivity	0.2671	0.7678
Specificity	0.978	0.9624
PPV	0.9512	0.9701
NPV	0.4541	0.7232
Positive likelihood ratio (LR+)	12.154	20.4227
Negative likehood ratio (LR-)	0.749	0.2413
Prevalence	0.616	0.6134

Quantitative synthesis

As showed in **Figure 3**, meta-analysis of the total studies showed that there was significant difference between methlated *p16* and *ppENK* in the diagnosis of pancreatic cancer (Chi^2 = 4.41; df = 1, P = 0.04; I^2 = 77.3%); while the specificity in both detections of *p16* and *ppENK* were higher than the sensibility. The same conclusion could be perorated from **Figure 4**. Comparing the sensibility, specificity and other parameters of methylation detection in *p16* and *ppENK*, the detection of methylated *ppENK* for diagnosis of pancreatic cancer was far more effective than that of *p16* (**Table 2**). Meanwhile, the available summary receiver operating characteristic (SROC) curves indicated that it is more effective to apply detection of *ppENK* methylation for diagnosis of pancreatic cancer (**Figure 5**).

Tests for publication bias and sensitivity analyses

According to sensitivity analysis, the results showed us that there was a little substantial modification of our estimates after exclusion of individual studies in subgroup *p16* but none in subgroup *ppENK*, indicating that the result of *ppENK* was more stable better than *p16* (data not shown). However, this problem did not badly influent the final conclusion. Publication bias was showed in **Figure 6**.

Discussion

Timely and efficacious diagnosis is always a major issue for cancer, not excepting pancreatic cancer. In previous studies, many genetic and epigenetic alterations occurred during pancreatic tumorigenesis [17]. Few, however, are useful diagnostic markers. Fortunately, a panel of genes was identified aberrantly methylated and silenced in human pancreatic cancer tissues with rare methylation in nonneoplastic pancreas, including *ppENK* and *p16* [1, 6, 18].

Trail studies about the detection of the methylated *ppENK* or *p16* in pancreatic cancer patients versus normal persons or patients with

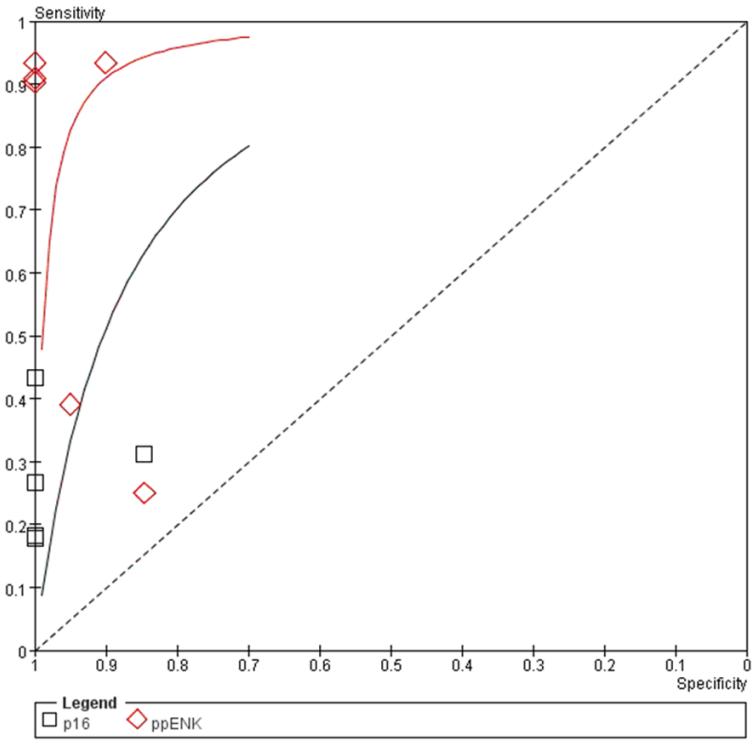

Figure 5. SROC curves of the detection of methylated p16 and ppENK.

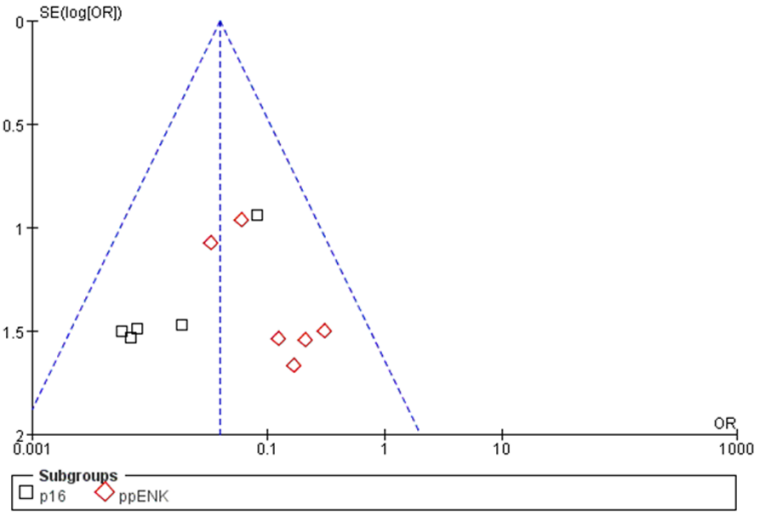

Figure 6. Funnel plot analysis on the detection of publication bias in the meta-analysis.

other disease of pancreas were still not abundant. Searching all available databases, only 16 were related to the methylation of *ppENK* and *p16*, including articles without case-control design and insufficient data. This inadequacy might bring slight bias to this review, instead of a decisive influence to the final result. By the way, considering the narrow available data, we did not limit the sample type for detecting. Papers with regard to case-control tests on the detection of the methylation in *ppENK* and *p16* were accepted and analyzed. Hereinto, four [2, 6, 12, 14] were performed on pancreatic juice; four [1, 13, 15, 16] were on tissues and/or pancreatic cancer cell lines. Due to the narrow data, a further subgroup analysis basing on the sample differences was unfeasible.

Hypermethylations in both *p16* and *ppENK* have been detected in precancerous lesions in the pancreas [2]. It seems that methylation of the *p16* and *ppENK* genes are more frequently detected in patients with localized tumors than those with more advanced tumors. The SROC curves indicated that it was more effective to apply the detection of *ppENK* methylation for diagnosis of pancreatic cancer. Indeed, the *ppENK* gene had been shown to be aberrantly methylated in more than 90% of pancreatic carcinomas [16]. It was reported that the *ppENK* gene encoded the opioid growth factor, which induced apoptosis in lung cancer cell lines, delayed cell cycle progression, and exerted a negative growth regulatory effect on various kinds of cancers, including pancreatic cancer [21]. As an early event in pancreatic carcinogenesis [19, 20], *p16* had been discovered fulfilling all the criteria necessary to be labeled a bona fide tumor-suppressive gene by even the strictest of measures since the last century [22]. However, most of the current studies were on its detection in other cancer patients [23-25], but pancreatic cancer. Recent studies identified *p16*

promoter methylation as a major mechanism of tumor-suppressor-gene silencing [26-28]. However, the *p16* lost function in so many human tumors and the unclear biological role of *p16* in cancer undoubtedly impact on the diagnosis and therapy of many common neoplasms.

In conclusion, despite of the narrow data available, this review compared the sensitivity and specificity in the detection of methylated *p16* versus *ppENK* in samples from pancreatic cancer, normal and other disease of pancreas for the first time. As the result suggested, the methylated *ppENK* was a better marker than *p16* in the diagnosis of pancreatic cancer with a higher sensitivity and specificity. However, more trail studies were definitely in need for more comprehensive reviews.

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Disclosure of conflict of interest

None.

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