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ZIP4 is a Novel Diagnostic and Prognostic Marker in Human Pancreatic Cancer: A Systemic Comparison Between EUS-FNA and Surgical Specimens

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Abstract

Aberrant expression of a zinc transporter ZIP4 in pancreatic ductal adenocarcinoma (PDAC) has been shown to contribute to tumor progression and is a potential target for individualized therapy. The overall objective of this study was to determine whether ZIP4 could serve as a novel diagnostic and prognostic marker in human PDAC, and if it can be assessed by minimally invasive sampling using endoscopic ultrasound guided fine needle aspiration (EUS-FNA). Immunohistochemistry was performed to compare ZIP4 expression in the PDAC samples obtained from EUS-FNA and matched surgical tumors (parallel control). Samples were reported by sensitivity, specificity, and predictive values, all with 95% confidence intervals (CI). A total of 23 cases with both FNA and surgical specimens were evaluated. We found that ZIP4 was significantly

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CONFLICT OF INTEREST

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over-expressed in tumor cells from both sets of samples. The sensitivity, specificity, positive predictive value (PPV), and negative predictive value (NPV) of ZIP4 for the diagnosis of PDAC were 72.9%, 72.5%, 76.1%, and 69.0% in EUS-FNA samples, and were 97.9%, 65.4%, 83.9%, and 94.4% in surgical specimens, respectively. The association between the positive rate of ZIP4 expression in FNA and surgical samples is statistically significant ($P=0.0216$). Both the intensity and percentage of ZIP4 positive cells from the surgical samples correlated significantly with tumor stage ($P=0.0025$ and $P=0.0002$). ZIP4 intensity level in FNA samples was significantly associated with tumor differentiation and patient survival. These results indicate that EUS-FNA is capable of non-operative detection of ZIP4, thus offering the potential to direct pre-operative detection and targeted therapy of PDAC.

Keywords

Pancreatic cancer; prediction; prognosis; survival; ZIP4

INTRODUCTION

With more than 80% of initial diagnoses presenting either as a locally advanced or metastatic disease, pancreatic ductal adenocarcinoma (PDAC) is the 10th most prevalent cancer and the 4th most common cause of cancer deaths in the United States. Its poor prognosis can be attributed to its late detection and consequent lack of surgical resectability, which is currently the only curable therapy [1]. Therefore, there is an urgent need to develop better methods to accurately diagnose PDAC earlier in order to start treatment at a curable stage. Endoscopic ultrasound (EUS) is considered to be the most sensitive technology in detecting small PDACs [2, 3]. However, the diagnosis of PDAC, especially in the presence of chronic pancreatitis, still remains difficult. The challenge is to develop more sensitive and specific molecular markers that are only present in malignant cells, thereby enabling surgeons to better evaluate and advise patients of the relative risks and benefits of the surgical intervention. For locally advanced or metastatic PDAC, pre-operative or palliative chemoradiotherapy is often employed. Advances in individualized therapy require not only pre-operative pathological confirmation, but also the ability to perform molecular classification of tumors.

ZIP4, a zinc transporter encoded by the *SLC39A4* gene, plays an important role in zinc homeostasis in mammalian cells. Mutations in this gene cause a rare human genetic disorder acrodermatitis enteropathica [4, 5]. Previous studies have shown that ZIP4 was upregulated in PDAC compared with chronic pancreatitis and surrounding normal tissues. Overexpression of ZIP4 promoted tumor growth and metastasis in multiple murine models [6]. Moreover, silencing of ZIP4 by shRNA inhibited pancreatic cancer cell proliferation, migration, invasion, and tumor growth, and significantly increased the survival rate of nude mice with orthotopic xenografts [7]. Given ZIP4's potential as a novel target for cancer therapy, it is important to examine the ZIP4 expression at an early stage of human PDAC. To date, human ZIP4 expression levels have only been evaluated in resected tumor tissues, but these specimens are not always available when treatment decisions need to be made, such as in a pre-operative setting or patients with unresectable disease. Thus, for early detection

purpose, it is important to validate the ZIP4 expression in routinely available clinical materials, such as tissue specimens obtained through minimally invasive methods including the endoscopic ultrasound guided fine-needle aspiration (EUS-FNA), to see whether ZIP4 can serve as a sensitive diagnostic marker.

In this study, we compared the immunohistochemistry staining of the cytology samples from EUS-FNA with the surgical samples (control). In addition, we evaluated the correlation between ZIP4 expression in EUS-FNA and resected specimens with the clinical characteristics of PDAC to determine whether ZIP4 can serve as a potential diagnostic and prognostic marker of PDAC.

MATERIALS AND METHODS

Human Tissue Collection

Twenty three cases of histologically confirmed PDAC from 2004–2006 in Mayo Clinic were included in this study. All patients had both pre-operative EUS-FNA and surgical resection of pancreatic lesions. The slides of surgically resected PDAC, with corresponding pre-operative EUS-FNA cytology specimens were carefully reviewed to make sure there were sufficient cellular materials for immunohistochemistry (IHC) in both samples. Only cases with abundant cells in both surgical and FNA samples were included. Cases with chemotherapy or radiotherapy prior to tissue sampling (within 5 years) were excluded. This study was carried out with the approval of the Institutional Review Board. EUS samples were obtained using standard methods for tissue acquisition. Briefly a 19–22g needle was inserted into the tumor under EUS guidance. Suction was applied and the needle passed to and fro for approximately 30 seconds. Each aspirated sample was expressed onto 2 glass slides for thin smear cytology (one for DiffQuick, one for Papanicolaou) and the remainder placed in formalin. An on-site cytologist examined each DiffQuick stain and FNA was repeated until the cytologist confirmed an adequate sample; typically 3 passes. The formalin fixed material was spun into a cell pellet, paraffin embedded (FFPE), and cut for standard H&E staining. The Papanicolaou smear and H&E stained slides were examined by a staff pathologist for a final diagnosis.

Immunohistochemistry Staining

All the tissue blocks, including the FFPE cell pellet were de-identified and were processed to create slides approximately 5 microns thick. These paraffin embedded samples were deparaffinized, re-hydrated, blocked by 5% BSA, incubated with human ZIP4 antibody (1: 300 dilution in 5% BSA), then stained by the Vectastain Elite ABC kit and colorized by DAB according to the standard IHC staining protocol. The expression levels of ZIP4 by IHC in specimens obtained by EUS-FNA and the corresponding surgically resected PDACs (parallel control) were compared. Staining intensity (0, no staining; 1+, weak; 2+, moderate; 3+, strong) and percentage of positive cells (negative, less than 10%; 1+, 10–25%; 2+, 26–75%; 3+, greater than 75%) were assessed. Positive staining was defined as more than 10% of cells with a minimum of 1+ intensity. To avoid artificial effects, the areas with poor morphology or margins of the sections were excluded. Samples were reported in terms of sensitivity, specificity, positive predictive value, negative predictive value with 95%

confidence intervals (CI). Archived data from these patients were obtained from the Mayo Clinic pancreatic cancer bank. The correlation between ZIP4 intensity and percentage of positive cells in resected or FNA IHC slides and the patients' age, gender, tumor size, tumor site, tumor stage, pathological grade (differentiation), and survival were assessed.

Statistical Analysis

The Fisher's exact test was used to (1) assess the relationship of ZIP4 expression in FNA and surgical samples and (2) the relationship of ZIP4 expression with clinicopathological variables. The proportional odds model was used to analyze ZIP4 expression as an outcome. The Cox proportional hazard model was used to investigate the relationship between ZIP4 expression and patient survival, with ZIP4 as a risk factor. A *P*-value of <0.05 was considered statistically significant.

RESULTS

Characterization of the PDAC Patients in this Study

A total of 23 PDAC patients with both resection specimens and sufficient EUS-FNA cell blocks (CBs) were included. There were 11 male and 12 female patients with a medium age of 69 years old, ranging from 47 to 85. Most tumors were over 40 mm in size, and the majority of AJCC staging were IIB (16/23), with only 4 cases in stage IIA and 3 in stage III. The tumor differentiation was graded as 1 (well), 2 (moderate), or 3 (poor). Table 1 illustrates the detailed clinicopathologic characteristics of the patients examined in this study.

ZIP4 was Overexpressed in Surgical Specimens of PDAC

ZIP4 was mainly stained in the cytoplasm of tumor cells (Fig. 1) and significantly overexpressed in tumor cells compared to the surrounding normal tissues. The intensity of ZIP4 staining and the portion of positive cells were shown in Tables 2 and 3. The majority of the PDAC tissues (15/25) showed strong intensity of ZIP4 staining, and most of them carried a percentage of positive staining greater than 75%. According to our definition of positive staining, all 23 of the surgical specimens investigated were positive for ZIP4 staining (Table 4).

ZIP4 was Overexpressed in the Majority of the EUS-FNA Specimens

The IHC staining of ZIP4 in EUS-FNA samples were shown in Fig. (2). Similar to surgical specimens, ZIP4 was significantly overexpressed in tumor cells, and most of the samples investigated possessed a staining intensity of moderate or strong (Table 2). Seventy four percent of the FNA samples had more than 10% of cells with positive staining (Table 3). The overall percentage of ZIP4 positive staining in EUS-FNA samples was 74% (16/23, Table 4).

The association of ZIP4 expression intensity between FNA and surgical samples was statistically significant (Table 2, *P*=0.0226), and the association of the percentage of ZIP4 positive cells between FNA and surgical samples was also statistically significant (Table 3, *P*=0.0086). When the intensity and percentage of ZIP4 positive cells are taken together, the

association between the positive rate of ZIP expression in FNA cell blocks and surgical tissues was statistically significant (Table 4, $P=0.0216$).

ZIP4 Can Serve as a Novel Diagnostic Marker for PDAC in Surgical and FNA Specimens

Using ZIP4 for PDAC diagnosis in pre-operative EUS-FNA samples with a 95% confidence interval and adjacent normal cells as an internal negative control, the sensitivity, specificity, positive predictive value (PPV), and negative predictive value (NPV) for diagnosis of PDAC were 0.729 (0.529, 0.866), 0.725 (0.506, 0.872), 0.761 (0.539, 0.912), and 0.690 (0.454, 0.871), respectively (Table 5). In contrast, the sensitivity and NPV of ZIP4 for PDAC diagnosis in surgical specimens were 0.979 (0.828, 0.998) and 0.944 (0.585, 1.000), while the specificity and PPV were 0.654 (0.389, 0.849) and 0.839 (0.652, 0.950), respectively.

ZIP4 Expression was Correlated to the Patients' Clinical and Pathological Features

We compared ZIP4 expression to the patients' clinical characteristics. In the surgical samples, both the intensity and percentage of ZIP4 positive cells were correlated with PDAC tumor stage ($P=0.0025$ and $P=0.0002$, respectively). As shown in Table 6, there were no strong staining (intensity: 3+) in stage IIA samples, but 93% (14/16) had 3+ staining in stage IIB samples. In contrast, there were 50% (2/4) weak staining in stage IIA and no weak staining in IIB samples. In FNA samples, the majority of ZIP4 strong staining were found in stage IIB and stage III with only 10% (1/10) in stage IIA (Table 6). ZIP4 intensity level in FNA samples was significantly associated with tumor differentiation ($P=0.023$ and $P=0.026$ for grade 2 and 3, respectively). For survival analysis with ZIP4 expression, age, gender as risk factors, we found that after adjusting the other covariates, the increase of FNA intensity from level 0 to level 1 is significantly associated with increased risk of death ($P<0.001$). Similarly, the increase of FNA intensity from level 1 to level 2 is strongly associated with increased risk of death ($P=0.016$).

DISCUSSION

Recent studies have demonstrated that zinc transporters and zinc homeostasis play important roles in various cancers, such as pancreatic, liver, breast, prostate, and brain cancer [8–12]. We have recently shown that ZIP4 is the only mammalian zinc transporter that was significantly upregulated in PDAC and played a critical role in cancer progression [13]. Currently, ZIP4 expression in FNA samples of human PDAC and its correlation with the patients' characteristics are not known. Thus, in the present study we compared the expression of ZIP4 in FNA and corresponding surgical tissue samples of PDAC. The associations between ZIP4 expression and tumor characteristics (including tumor grade, stage, and patient overall survival time) were also assessed. We found that ZIP4 in PDAC was up-regulated both in the surgical PDAC specimens and in the corresponding FNA cell blocks compared with adjacent non-tumor tissues. The ZIP4 expression levels in FNA samples were closely related to those in surgical tissues, and ZIP4 expression in surgical tissues had a positive correlation with tumor stage. The ZIP4 intensity level in FNA samples was significantly associated with tumor differentiation and patient survival.

ZIP4 is a membrane bound glycoprotein that mediates a variety of biological processes through a unique signaling cascade in PDAC. However, whether ZIP4 can serve as a diagnostic and prognostic marker in PDAC has not been investigated. In this study, we have shown that ZIP4 was overexpressed in all 23 surgical PDAC tissues compared to adjacent non-tumor tissues. The sensitivity and NPV of ZIP4 in surgical specimens for the diagnosis of PDAC were almost 100% (0.979 and 0.944, respectively). Other studies have shown that NEDD9, MTA2, and transgelin were elevated in resected PDAC tissues compared with their paired adjacent non-cancerous tissues [14–16], suggesting the potential of those markers in early detection of PDAC. In the present study, we also assessed ZIP4 expression levels in the EUS-FNA samples, obtained pre-operatively with minimal invasion, and found it was overexpressed in most cases of PDAC. The sensitivity, specificity and accuracy for PDAC diagnosis with pre-operative EUS-FNA were all above 72%. In a previous study, p53 was found to be overexpressed in PDAC patients. The EUS-FNA diagnostic test statistics for PDAC were similar as our results: 76% sensitivity, 91% specificity and 79% accuracy [17]. These results indicate that ZIP4 could serve as a novel and promising molecular marker for the diagnosis of PDAC with pre-operative and minimally invasive EUS-FNA.

Current practice to diagnose PDAC could be accomplished by EUS-FNA with a cytological modality which is minimally invasive with low cost and by far the best method for obtaining tissue diagnosis as well [18, 19]. The diagnostic accuracy of EUS-FNA cytology could be as high as 90% [3]. However, the diagnostic yields for PDAC are markedly decreased in the presence of chronic pancreatitis even with the EUS-FNA cytology because there are some similarities between PDAC and chronic pancreatitis with limited samples obtained from EUS-FNA [20, 21]. Molecular marker testing applied to EUS-FNA samples is a very encouraging approach to help increase the diagnostic yields for PDAC [22]. Very few studies have been done to compare the molecular marker profiling in the surgical tissues and FNA samples in PDAC. In a recent study, S100A6 expression was found to be significantly elevated in resected PDAC tissues and FNA specimens by real-time PCR, moreover, the expression level in surgical samples was significantly correlated with that in EUS-FNA samples, indicating that S100A6 has potential diagnostic value [23]. Our study, for the first time, has shown that ZIP4 protein level was significantly elevated in both surgical and FNA specimens, moreover, both the intensity and percentage of positive cells were closely correlated between FNA cell blocks and corresponding surgical tissues, providing strong evidence for ZIP4 as a novel marker to help improve the diagnostic yields of PDAC.

We recently reported that high ZIP4 expression was significantly associated with a higher grade of glioma and shorter overall survival, which makes it a promising prognostic marker for gliomas [8]. In this study, we found that a higher expression level of ZIP4 in surgical tissue, including intensity and percentage of positive cells, was correlated with a higher tumor stage. In a similar study, high MTA2 expression in surgical PDAC tissue was correlated with poor tumor differentiation, TNM stage, and lymph node metastasis [15]. Conversely, another study demonstrated low expression of mixed lineage kinase domain-like protein (MLKL) was associated with decreased overall survival in patients with resected pancreatic cancer and decreased recurrence-free and overall survival in the subset of patients with resected tumor who received adjuvant chemotherapy [24]. In pre-operative FNA samples, we found that a high ZIP4 intensity level was significantly associated with poor

histological differentiation of the tumor. Adjusting for age and gender, high ZIP4 expression intensity in FNA samples was significantly associated with a high risk of death and poor overall survival.

Despite the promising results as described above, we are fully aware of the limitation of our current study such as the small sample size. In further studies of ZIP4 expression profile in PDAC, we will expand the sample size by including more surgical and corresponding FNA samples and multiple centers in order to obtain more significant results for better understanding the relationship between ZIP4 and PDAC patients, and to provide more evidence to apply ZIP4 as a diagnostic and prognostic marker for PDAC. In summary, ZIP4 is a promising molecular target in PDAC, and could serve as a novel diagnostic and prognostic marker for PDAC detection and therapy.

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Fig. 1. ZIP4 is overexpressed in PDAC surgical samples

IHC staining of ZIP4 in surgically resected human PDAC tissues with AEC substrate. **(A)** ZIP4 positive staining with surrounding non-tumoral glands as an internal negative control. The upper half is pancreatic ductal adenocarcinoma with strongly positive (3+) ZIP4 staining. The lower half is benign pancreatic tissue including glands, ducts, and stroma which are negative for ZIP4. **(B)** ZIP4 positive staining with 3+ intensity. **(C)** ZIP4 positive staining with 2+ intensity. **(D)** ZIP4 positive staining with 1+ intensity.

Fig. 2. ZIP4 is overexpressed in FNA samples

IHC staining of ZIP4 in EUS-FNA human PDAC samples with AEC substrate. (A) ZIP4 positive staining with 3+ intensity. (B) ZIP4 positive staining with 2+ intensity. (C) ZIP4 positive staining with 1+ intensity. (D) ZIP4 negative staining.

Table 1

Clinical and Pathological Characteristics of the Patients

Characteristic		Number (n=23)	%
Age	<60	5	22%
	60–69	5	22%
	70	13	56%
Gender	Male	11	48%
	Female	12	52%
Tumor size	30 mm	2	9%
	30–40 mm	8	35%
	>40 mm	13	56%
Tumor sites	Head	18	78%
	Tail	3	13%
	Body	2	9%
AJCC Staging	IIA	4	17%
	IIB	16	70%
	III	3	13%
Grade	1	2	9%
	2	12	52%
	3	9	39%
Survival	6 mon	6	31%
	6–12 mon	4	17%
	>12 mon	12	52%

Table 2

ZIP4 Intensity in Surgical and FNA Samples

Surgical		FNA			
		0	1+	2+	3+
1+	3	2	1	0	0
2+	5	1	1	1	2
3+	15	2	1	5	7

Table 3

The Percentage of ZIP4 Positive Cells in Surgical and FNA Samples

Surgical		FNA			
		0	1+	2+	3+
1+	3	2	1	0	0
2+	3	2	0	0	1
3+	17	2	1	6	8

Table 4

ZIP4 IHC Staining in EUS-FNA and Surgical PDAC Specimens

Groups	Positive	Negative
EUS-FNA samples	17 (74%)	6 (26%)
Surgical specimens	23 (100%)	0 (0%)

Table 5

ZIP4's Diagnostic Value for PDAC in EUS-FNA and Surgical Specimens

Groups	Sensitivity (95%CI)	Specificity (95%CI)	PPV (95%CI)	NPV (95%CI)
EUS-FNA	0.729 (0.529, 0.866)	0.725 (0.506, 0.872)	0.761 (0.539, 0.912)	0.690 (0.454, 0.871)
Surgical	0.979 (0.828, 0.998)	0.654 (0.389, 0.849)	0.839 (0.652, 0.950)	0.944 (0.585, 1.000)

Table 6

The Intensity of ZIP4 from Surgical and FNA IHC Samples in Different Stages

Stage	FNA Sample Intensity				Surgical Sample Intensity		
	0	1+	2+	3+	1+	2+	3+
I/A	4	2	1	0	1	2	0
I/B	16	2	2	4	8	0	14
III	3	1	0	1	1	0	2