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Proteomic Analysis of Malignant Ascites From Patients With Pancreatic Ductal Adenocarcinoma

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Abstract. *Background/Aim: Pancreatic ductal adenocarcinoma (PDAC) is an aggressive malignant tumor. Research using an innovative research approach is needed to identify effective biomarkers or therapeutic targets for PDAC. We aimed to identify proteins related to the peritoneal dissemination of PDAC. Materials and Methods: We performed proteomic analysis using ascites samples from patients with advanced PDAC and peritoneal dissemination and patients with liver cirrhosis (LC). Proteins specific to PDAC were identified in comparison to the findings for ascites from patients with LC as a control group. Results: In total, 336 proteins were identified in ascites from patients with PDAC. We identified 18 specific proteins in ascites from patients with advanced PDAC. Among these proteins, CD13, lymphatic vessel endothelial hyaluronan receptor 1, ficolin-3, and V-set and immunoglobulin domain containing 4 were the most frequently detected. In addition, these 18 proteins could be classified into four categories: extracellular matrix, immunity, metabolism, and others. Conclusion: The identified proteins could be informative for developing treatment strategies for patients with PDAC and peritoneal dissemination.*

Pancreatic cancer is the fourth-leading cause of cancer-related death (1, 2), and approximately 90% of cases are pancreatic ductal adenocarcinoma (PDAC) (3). Patients with PDAC have poor prognoses because of the lack of symptoms in the early stage and difficulty in early detection in most cases (4). Despite pancreatic resection, the 5-year survival rate is only 20% (4). To improve the prognosis of patients with PDAC, identification of new biomarkers and therapeutic targets is urgent.

Currently, the prognosis of patients with PDAC has been improved by surgery combined with chemotherapy or chemoradiotherapy as neoadjuvant or adjuvant therapy (5, 6). These treatments are effective for some patients; however, chemotherapy or chemoradiotherapy have adverse effects in other patients in addition to being ineffective (7). This is not limited to pancreatic cancer because cancer cells are highly diverse and have different characteristics, even in the same cancer (8). Therefore, it is essential to examine the characteristics of each pancreatic cancer on the basis of the degree of progression and patient factors as well as the characteristics of cancer cells before starting treatment to improve outcomes.

CA19-9 is a well-documented and validated serum biomarker associated with pancreatic cancer (4). To identify more useful biomarkers and therapeutic targets, several studies have examined gene mutation or DNA methylation (9). However, gene mutation and DNA methylation are not always reflected by protein expression (10). Because protein expression determines the phenotype of cancer, proteomics is very important in cancer research. Proteomics can provide valuable information related to the molecular mechanism of cancer development and progression, therapeutic targets, and biomarkers (11). Accumulating evidence has illustrated that proteomics is a useful technology for identifying therapeutic targets in cancers (12). In PDAC, a previous study has identified 20 proteins utilizing three ascites samples from

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patients with advanced pancreatic cancer (13). However, the study did not perform a direct comparison with non-malignant ascites.

The present study aimed to identify protein biomarkers specific to advanced PDAC by utilizing proteomics and ascites from patients with advanced PDAC and peritoneal dissemination. We identified 18 proteins specific to advanced PDAC by comparing the results with those of ascites from patients with liver cirrhosis (LC). In addition, we classified these proteins on the basis of their functions and roles in cancer.

Materials and Methods

Proteomics. Kanamecho Hospital provided ascites from patients with intractable ascites during cell-free and concentrated ascites reinfusion therapy. Samples were cryopreserved at −80˚C. We evaluated ascites from 10 patients with PDAC and 3 patients with LC as controls.

Proteomics was conducted as described in previous reports (14, 15). Proteins were separated by 10% SDS-PAGE (#2331830, ATTO, Tokyo, Japan) until they were 1 cm from the well and then stained with GelCode™ Blue Safe Protein Stain (#24594, Thermo Fisher Scientific, Waltham, MA, USA) for in-gel digestion. Protein-containing gel was excised and cut into approximately 1-mm pieces. Proteins in the gel pieces were reduced with DTT (#P2325, Thermo Fisher Scientific), alkylated with iodoacetamide (#A39271, Thermo Fisher Scientific), and digested with trypsin and lysyl endopeptidase (Promega, Madison, WI, USA) in a buffer containing 40 mM ammonium bicarbonate (pH 8.0) overnight at 37˚C. The resultant peptides were analyzed using an Advance UHPLC system (AMR/Michrom Bioscience, Radnor, PA, USA) coupled to a Q-Exactive mass spectrometer (Thermo Fisher Scientific), and the raw mass spectrum data were processed using Xcalibur (Thermo Fisher Scientific). Raw data from liquid chromatography coupled to tandem mass spectrometry were compared against the SwissProt database restricted to *Homo sapiens* using Proteome Discoverer version 1.4 (Thermo Fisher Scientific) with Mascot search engine version 2.5 (Matrix Science, London, UK). A decoy database comprising either randomized or reversed sequences in the target database was used for false discovery rate (FDR) estimation, and the Percolator algorithm was used to evaluate false positives. The results were filtered using the criterion of a global FDR of 1% to ensure a high confidence level.

Results

Specific proteins in ascites from patients with PDAC. We first compared the identified proteins in ascites from patients with PDAC with those from patients with LC. Because LC is the most common non-malignant disease that causes ascites, we excluded the proteins contained in ascites from patients with LC to identify proteins specific for malignant tumors. Immunoglobulins and keratin were also excluded. All LC samples were obtained from male patients with a median age of 59 years (range=55-77 years), and PDAC samples were obtained from three male (30%) and seven female (70%) patients with a median age of 77 years (range=49-88 years, Table I). One case of PDAC involved bloody ascites.

Table I. *Background of PDAC ascites samples*.

Exp. No.	Age	Gender	Disease
#1	55	M	LC
#2	77	M	LC
#3	59	M	LC
#4	78	F	PDAC
#5	71	F	PDAC
#6	56	F	PDAC
#7	64	F	PDAC
#8	79	M	PDAC
#9	83	M	PDAC
#10	49	F	PDAC
#11	88	M	PDAC
#12	76	F	PDAC
#13	80	F	PDAC

LC: Liver chirrosis; PDAC: pancreatic ductal adenocarcinoma.

We detected 336 proteins in ascites from patients with PDAC, and 187 proteins were detected in multiple PDAC samples. Of these, 169 proteins were common to ascites from both patients with PDAC and those with LC. Eventually, we identified 18 specific proteins in ascites from patients with PDAC (Figure 1). The rates of positivity differed among the proteins, and CD13, lymphatic vessel endothelial hyaluronan receptor 1 (LYVE1), ficolin-3, and v-set and immunoglobulin domain containing 4 (VSIG4) were the most frequently observed in ascites from patients with advanced PDAC, each being detected in five (50%) samples (Figure 1).

The functions and roles of specific proteins in ascites from PDAC. Next, we classified these 18 proteins on the basis of their functions or roles (Figure 1) as follows: extracellular matrix (ECM, n=6, 33%), immunity (n=3, 17%), metabolism (n=3, 17%), and others (n=6, 33%, Figure 2). LYVE1, thrombospondin-4 (TSP-4), and versican core protein were categorized as ECM-related proteins, and ficolin-3, VSIG4, and C4b-binding protein β chain (C4BPB) were classified as immunity-related proteins by proteomics utilizing ascites from patients with advanced PDAC (Figure 1).

Discussion

Several studies have assessed surgery plus chemotherapy or chemoradiotherapy in the treatment of PDAC, but the postoperative prognosis remains poor. New therapeutic targets are needed to develop more effective treatment strategies and improve patient prognosis. In this study, using proteomics and ascites from patients with PDAC or LC, we identified 18 specific proteins in malignant ascites from

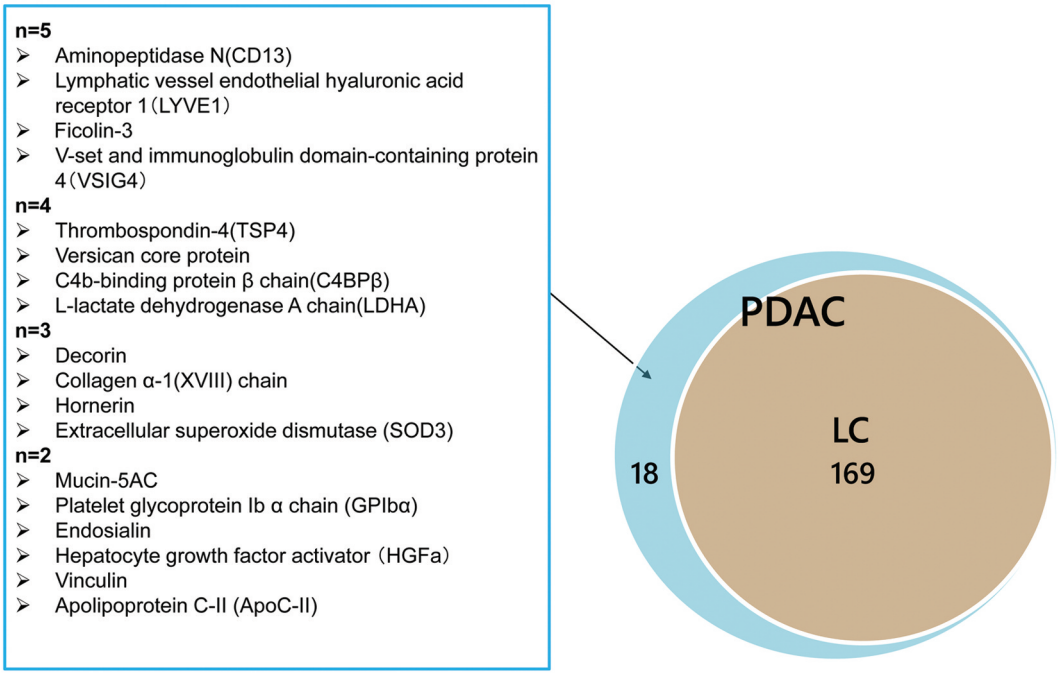

Figure 1. *The identified proteins in PDAC ascites.*

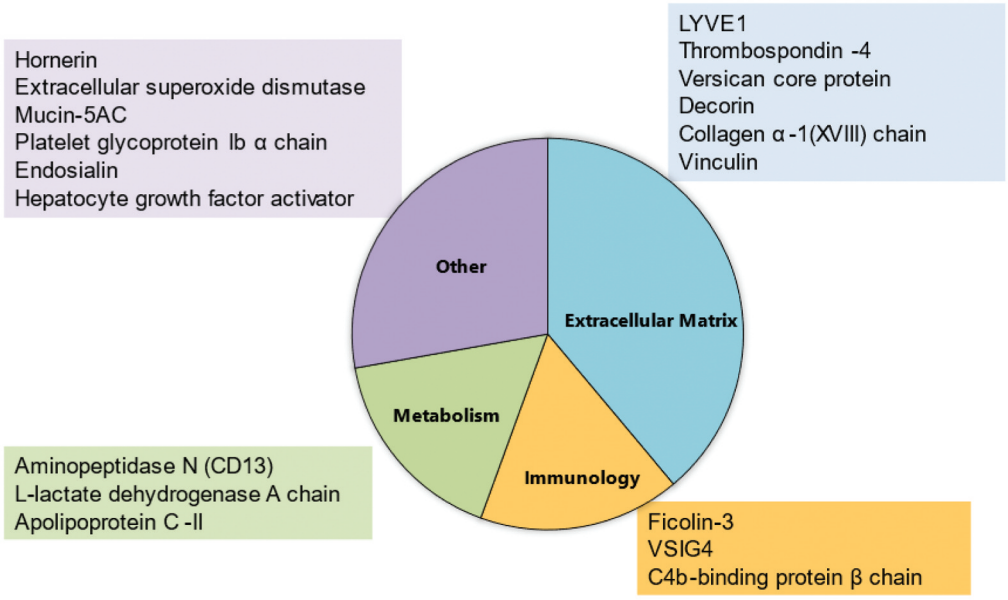

Figure 2. *Functional classification of the identified proteins.*

patients with advanced PDAC and peritoneal dissemination. Our data suggest these proteins can be targets to develop new treatment strategies by clarifying their roles in the progression of PDAC.

Proteomics is a useful technology for analyzing the identity of an organism (16), and it provides valuable information that is directly relevant to protein function in cancer. In pancreatic cancer, proteomics has already been used to detect useful

biomarkers or therapeutic targets (11, 17). Kosanam and colleagues identified 20 proteins utilizing three malignant ascites samples from patients with advanced PDAC (13). To the best of our knowledge, no study has performed proteomics using both ascites from patients with PDAC and non-malignant ascites. In fact, our data revealed that most proteins in ascites from patients with LC overlapped with the proteins identified in patients with PDAC, suggesting that they are not malignancy-specific. Taken together, our results are novel, and provide insights into proteomics in PDAC.

Peritoneal dissemination is often found in staging laparoscopy, even in resectable pancreatic cancers (18), and it is one of the factors that contributes to poor prognoses. Various mechanisms have been demonstrated to promote peritoneal dissemination, which consist of multiple steps occurring through the coordinated interaction of cancer cells and other cells, such as cancer-associated fibroblasts or immune cells (19). This study illustrated that the most common proteins specific to PDAC were ECM-related proteins. ECM is involved in the migration and proliferation of cancer cells and the formation of metastatic scaffolds (20). Therefore, proteins related to ECM also play a pivotal role in peritoneal dissemination (21, 22). In fact, versican, a large ECM proteoglycan (23), has been reported to be involved in peritoneal mesothelial cell attachment, spheroid formation, and peritoneal tumor formation in mice (21). Versican is highly expressed in pancreatic cancer tissue, and, similar to decorin, it is also associated with the malignant phenotype of pancreatic cancer (24, 25). LYVE1, the receptor for hyaluronan in lymphatic vessel endothelium (26), promotes metastasis *via* lymphatic vessels (27). In addition, mice with ovarian cancer display massive infiltration of CD11b(+)/LYVE1(+) macrophages, which promote lymphatic remodeling by secreting VEGF family ligands, resulting in massive ascites formation (22). In fact, Radon and colleagues identified urinary LYVE1 as a useful biomarker to distinguish patients with early-stage PDAC from healthy individuals (28). High XVIII collagen expression in stroma has been associated with shorter survival in PDAC in prior research (29). To the best of our knowledge, TSP-4, a secreted ECM protein (30), has not yet been studied in PDAC.

In recent years, immunotherapy has attracted attention in clinical practice and in the field of pancreatic cancer (31). Immune cells in malignant ascites can potentially serve as therapeutic targets in advanced cancer. Ficolin-3 is a pattern-recognition molecule with the ability to activate the lectin pathway of complement (32). Serum ficolin-3 levels have been identified as an independent prognostic biomarker for disease-specific and overall survival in patients with esophageal cancer (33). VSIG4 is a potent negative regulator of T-cell responses, and has been suggested to regulate antitumor immunity (34). VSIG4 expression was significantly decreased in HCC tissues and HCC cell lines, and disease-free survival in patients with hepatitis B virus-related HCC and low VSIG4 expression was shorter than that of patients with high VSIG4 expression, which was consistent with the results of bioinformatics analysis (35). C4b-binding protein (C4BP) is a protein complex involved in the complement system where it acts as inhibitor. C4BPB can modulate CD40 to sCD154 interactions by forming a high-molecular-weight multimeric sCD154 and C4BPB complex that suppresses critical intracellular signaling pathways, permitting cell survival without inducing proliferation. Immunohistochemistry has demonstrated the co-localization and enhanced expression of C4BPB and CD40 in human liver cancers (36). To the best of our knowledge, no study has examined the roles of ficolin-3, VSIG4, and C4BPB in PDAC.

Pancreatic cancer cells are characterized by extensively reprogrammed metabolism, which is driven by oncogene-mediated cell-autonomous pathways, the unique physiology of the tumor microenvironment, and interactions with non-cancer cells (37). Aminopeptidase N (CD13) is a widely expressed ectoenzyme with multiple functions (38). CD13 has been reported to be associated with prognostic markers for pancreatic cancer (39). However, the detailed role of CD13 in pancreatic cancer is unknown. Lactate dehydrogenase A, an enzyme that catalyzes the interconversion of pyruvate and lactate, promotes cancer cell invasion, anoikis resistance, and tumor metastasis (40). It has been reported that glycolysis is enhanced in the ascites of patients with malignant lymphoma (41). Apolipoprotein C-II (ApoCII) is involved in lipid metabolism, and serum ApoCII levels independently predict survival and improve the selection of patients with PDAC for pancreaticoduodenectomy (42). ApoCII has also been detected in the plasma of patients with colorectal cancer *via* proteomics (43).

We identified six proteins with function other than extracelluer matrix, immunity and metabolism and the identified proteins have unique characteristics. Platelet-derived glycoprotein Ibα is a platelet surface membrane glycoprotein. Platelets promote the formation of ovarian cancer spheres that express metastasis-initiating cell markers and metastatic protein tissue factor (44). Endosialin (CD248)-expressing pericytes in primary tumors facilitate distant site metastasis by promoting tumor cell intravasation in a cell contact-dependent manner, resulting in elevated numbers of circulating tumor cells (45).

This study has multiple limitations. First, we did not have sufficient patient information such as treatment history, tumor marker expression, or stage. Second, we could not obtain more samples for validation. However, no prior study identified PDAC-specific proteins using patient ascites; therefore, our findings provide new insights in this field. In conclusion, we identified proteins specific to malignant ascites that may be involved in cancer progression. Additional research is needed to validate these proteins and identify new diagnostic and therapeutic targets for PDAC.

Conflicts of Interest

The Authors have no conflicts of interest to disclose in relation to this study.

Authors' Contributions

Conception and design were contributed by FK, TM. Experimental samples were provided by KM. The experiment was performed by NU, TU, KI, and HH. Data analysis and interpretation were contributed by FK, YY and TI. Drafting of manuscript was performed by FK. Critical revision of the manuscript was contributed by TI and HB. All Authors approved the final manuscript.

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