The Proteomic Analysis of Pancreatic Exocrine Insufficiency Protein Marker in Type 2 Diabetes Mellitus Patients

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Abstract. Type 2 Diabetes Mellitus (T2D) is the vast majority case of diabetes. Patient with T2D is at higher risk for developing acute or chronic pancreatitis. Prolonged hyperglycemia results in damages to tissue, which also causes dysfunctions of some organ systems, including enzyme or hormone secretions. Commonly, dysfunction or insufficiency of pancreatic exocrine is evaluated by increasing activity of serum pancreatic enzyme, such as amylase and lipase. Although incidence of pancreatitis was found in Indonesian T2D, the pathogenic mechanism still unclear. The aim of this study was to characterize the marker protein that indicated the correlation of pancreatic exocrine insufficiency with progression of T2D. Proteomic analysis using LC-MS/MS was used in identification and characterization of protein marker which indicates insufficiency pancreatic exocrine. First step, protein profile was analyzed by SDS-PAGE methods using serum sample of T2D compared with normal or healthy control, as negative control, and pancreatitis patients, as positive control. Protein with 18 kDa was found as a candidate protein marker which indicated the pancreatic exocrine insufficiency in T2D. The further identification of that protein using LC-MS/MS showed 4 peptide fragments. In silico analysis of the peptide fragment indicated the correlation of pancreatic exocrine insufficiency with progression of T2D was METTL10 – methyltransferase like protein-10.

Keyword: T2D, pancreatic exocrine, protein, marker

1. Introduction

Type 2 Diabetes Mellitus (T2D) is the vast majority case of diabetes. The main pathophysiological feature of T2D is impaired insulin secretion and insulin resistance [1]. There are multiple defects insulin secretion and signal transduction in Type 2 DM which may effect in synthesis of enzymes and their release from pancreatic exocrine. Although the endocrine system seems to be involved in pathophysiological diabetes, exocrine functions is proposed to be involved as well, since exocrine system is close to endocrine system. Any process that diffusely injures the pancreas, such as pancreatitis, trauma, infection, and pancreatic carcinoma, mainly disturb the exocrine system, but the endocrine system could also be affected and causes diabetes. Diabetes due to that pancreatic exocrine disease is categorized in Type 3 DM, according to American Diabetes Association (ADA) 2012. Prevalence of pancreatitis has been increasing rapidly. Some research showed that alcoholism, consumption of healthcare resources, and using anti-diabetic therapies can increase the risk factor of pancreatitis. Our recent study has demonstrated the increasing incidence of pancreatitis in Indonesian T2D patients [2, 3].

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including enzyme or hormone secretions. Commonly, dysfunction or insufficiency of pancreatic exocrine is evaluated by increasing activity of serum pancreatic enzyme, such as amylase and lipase. Although incidence of pancreatitis was found in Indonesian T2D, the pathogenic mechanism still unclear. The aim of this study was characterization of the marker protein that indicated the correlation of pancreatic exocrine insufficiency with progression of T2D.

2. Research Methods
The study was approved by institutional Human ethics committee (Medical Faculty, Brawijaya University) and informed consent was obtained from all participant.

2.1. Subjects
This study included 67 subjects having general check-up in Central Laboratory of Dr. Saiful Anwar Hospital for a period of six months that meet the inclusion criteria. The inclusion criterion of them were males and females with type 2 diabetes mellitus with or without complications or any comorbid condition like hypertension, coronary artery disease, and also non diabetic healthy individual as the controls. All the subjects, including the controls, were fully informed about the study and their voluntary informed consents were taken. The subjects were categorized into four groups according to the criteria established by The ADA in 2012. Based on ADA criteria, the subjects were categorized into four groups including: healthy controls (n = 21), prediabetes (n = 10), diabetes (n=10), and prediabetes or diabetes with exocrine insufficiency (n=26).

2.2 Methods:
Clinical identification of normal or healthy control, prediabetes, and diabetes were classified according to ADA criteria. The diagnostic criteria of diabetes were assessed according to ADA, i.e. subjects with a fasting plasma glucose > 126 mg/dL and/or 2 h plasma glucose level > 200 mg/dL and/or HbA1c > 6.5 % were considered to have diabetes; subjects with a fasting plasma glucose 100 to 125 mg/dL (IFG) or 2 hour plasma glucose level 140 to 199 mg/dL (IGT) or HbA1c 5.7 to 6.4% were considered to have increased risk for diabetes (prediabetes); subjects with a fasting plasma glucose < 110 mg/dL or 2 hour plasma glucose level < 140 mg/dL or HbA1c < 5.6 % were regarded as having normal glucose tolerance (NGT) [4]. The exocrine insufficiency was assessed according to increasing of amylase and or lipase level. The considered reference range of normal amylase was 30 - 110 U/L and lipase was 30 - 210 U/L. Fasting venous blood was collected from all of subjects, it was centrifuged (at 1500 g for 15 min). The separated plasma was used to assay the Hba1c. Hba1c was measured with ion-exchange high-performance liquid chromatography using an automated analyzer (BioRad D10). Amylase and lipase activity were measured by photometric enzymatic method. The amylase activity was assayed using BioAssay Systems’ QuantiChromTM α- Amylase Assay Kit (DAMY-100). Lipase activity was assayed using BioAssay Systems’ QuantiChromTM Lipase Assay Kit (DLPS-100).

2.3. Identification and characterization of protein marker of pancreatic exocrine defect in T2D
The protein profile of serum samples of all the groups (normal as negative control, patients with pancreatitis as positive control, pre-diabetes, T2D, and T2D with high amylase and/or lipase levels) were performed according to the methods described by Laemly with minor modification. The protein profiles were separated by 12.5% of separating gel with a 5% stacking gel. Then, the gel was stained with silver staining methods to visualize the protein profile.

Based on the protein band pattern resulted, band that was expressed in positive control (pancreatitis) but not in negative control (normal control) will be obtained 1 band. Otherwise, the same molecular weight of protein that express in positive control could be also express in T2D serum sample, thus, that band was suspected as a protein marker candidate of pancreatic exocrine defect.

Then the production process was conducted by SDS-PAGE with sample of serum pancreatitis patients that contained protein marker candidate on majority of electrophoresis gel wells. After the
silver nitrate staining was carried out, subsequent preparations were performed to isolate the target protein according to the Bringan method.

Protein samples were digested with trypsin, and peptides extracted according to standard techniques [5]. Peptides were analyzed by electrospray ionization mass spectrometry using the Ultimate 3000 Nano HPLC system (Dionex) coupled to a 4000 QTRAP mass spectrometer (Applied Biosystems). Tryptic peptides were loaded onto a C18 PepMap100, 3 μm (LC Packings) and separated with a linear gradient of water/acetonitrile/0.1% formic acid (v/v). Spectra were analysed to identify proteins of interest using Mascot sequence matching software (Matrix Science) with Ludwig NR database. Database: Ludwig NR Taxonomy: Homo sapiens (human).

3. Result and Discussion
3.1. Protein characters T2D sera
According to the previous study, we found that more than 50% T2D serum sample showed the pancreatic exocrine insufficiency condition [2]. The condition was showed by increasing level of serum amylase and or lipase. In order to explore protein marker for pancreatic exocrine insufficiency beside amylase and lipase marker analysis that commonly applied, electrophoresis SDS-PAGE was conducted as the first step of the exploration process. It was shown that 18 kDa peptide band appeared in positive controls and some T2D samples but was not obtained in negative control (Figure 1). The 18 kDa protein is suspected to be the candidate of expected protein marker.

![Figure 1](image)

Figure 1. The protein profile of serum sample of T2D patients, pre-diabetic, normal as negative control and pancreatitis patient as positive control, separated in 1D-GE with 12.5% polyacrylamide gel and visualized by silver staining method.
The protein 18 kDa was identified further using LC-MS/MS peptide analysis. Spectra pattern and peptide fragmentation sequence of LC-MS/MS analysis of the 18 kDa protein band analyzed using Mascot sequence matching software (Matrix Science) is presented in Figure 2. The further analysis of the peptide was identified as cDNA FLJ50670 that very similar to RNA polymerase I-specific transcription initiation factor RRN3, U2AF1 U2 small nuclear RNA auxiliary factor 1 isoform, METTL10 Methyltransferase-like protein 10, and FAM19A3 isoform 2 of protein FAM19A3. The LC-MS/MS identification fragment was also confirmed by a Blast test to match the peptide sequences present with the peptide database in the National Centre for Biotechnology Information (NCBI) with a summary of the results as presented in Table 1. There are 2 peptides with high protein sequence coverage (10% and 18%) and approximating calculated molecular weight that estimated from electrophoregram data (18 kDa). Blast test results indicate that the protein has a similarity level of 100% with FAM19A3 protein isoform 2 of FAM19A3 and METTL10 protein methyltransferase-like protein 10 [8].
Table 1. Peptide identification result of protein marker candidate using LC-MS/MS

| Character                                                                 | cDNA FLI50670, highly similar to RNA polymerase I-specific transcription factor RRRN3 | U2AF1 U2 small nuclear RN auxiliary factor 1 isoform | METTL10 methyltransferase-like protein 10 | FAM19A3 isoform 2 of protein FAM19A3 |
|---------------------------------------------------------------------------|--------------------------------------------------------------------------------------|------------------------------------------------------|------------------------------------------|-------------------------------------|
| Amino acid fragment from LC-MS/MS                                          | R.LP GGAASSSSAV KT.L                                                               | R.GRGGGGGGGGG GGR.E                                  | MSSGADGGGAAVAAR SDK.G                    | R.AKPSCVDLLL AAHCAR.R                |
| % identity with protein database                                          | 99                                                                                   | 100                                                  | 100                                      | 100                                 |
| Calculated molecular weight                                                | 70364                                                                                   | 27855                                             | 11340                                     | 18537                               |
| Predicted Pi                                                              | 5.63                                                                                   | 8.87                                               | 4.76                                      | 9.48                                |
| Protein sequence coverage                                                 | 2                                                                                     | 6                                                   | 18                                       | 10                                  |
| The number of amino acid sequence                                          | 621                                                                                   | 240                                               | 104                                      | 169                                 |
| Fragment location in the amino acid sequence                               | 10-23                                                                                  | 210-224                                            | 1-19                                     | 82-98                               |

Protein FAM19A3 was predicted have MW 18,537 Da, pI (isoelectric point) of 9.48 Da [6, 7, 9-11]. According to Table 2, protein FAM19A3 was not expressed in pancreas. Protein FAM19A3 known as Chemokine-like protein TAFA-3. Protein FAM19A3 or TAFA protein that contain conserved cysteine residue that was dominant expressed in brain. The FAM19A3 protein was the FAM19A3 gene expression that demonstrated the highest associated with Type 1 DM [12, 13].

The other identified protein was METTL10 (Methyltransferase like protein 10). METTL10 was predicted have MW 11,340 Da, pI 4.76. In silico analysis of METTL10 have pI of 5.77, MW 31.830.02 Da [6, 7, 9, 10, 14]. The differences between in-silico analysis and LC-MS/MS combined with Mascot software was supposed because there was conformational changing or protein sub unit splitting in electrophoretic process. According to the Table 2, METTL10 was highly expressed in almost all of body tissue, included pancreas and kidney. METTL10 was the enzyme that catalyzed methylation reaction of S-adenosyl-L-methionine (SAM or AdoMet). Product methylation of S-adenosyl-L-methionine (SAM or AdoMet) is S-adenosyl-L-homocysteine (AdoHcy) and other methylated biomolecules [15]. Increasing total concentration of Homocystein (tHcy) and methionine intermediate product (AdoMet and AdoHcy) sometimes correlate with kidney dysfunction and also cardiovascular disease that is common complication in T2D (15–17). Besides, overexpression of methyltransferase in methylation reaction was found in pancreas, especially in ductal, exocrine and islet cell, also found in early stage Pancreatic Carcinoma [18]. Pancreatic Carcinoma is one of the disease that indicated defect in exocrine pancreas. According to the Figure 1, protein 18 kDa that was showed in pancreatitis (positive control) but also found in some sample of T2D, so we can suppose that METTL10 (Methyltransferase like protein 10) was the protein marker candidate of insufficiency exocrine pancreas and correlated with progression of T2D.
Table 2. The local expression of Protein FAM19A3 dan METTL-10 in organ [6, 7]

| Organ                                | Expression Level |
|--------------------------------------|------------------|
|                                      | FAM19A3 | METTL-10 |
| Liver and pancreas                   | ++++     | ++       |
| Gallbladder                          | +++      |          |
| Pancreas                             | +++      |          |
| Gallbladder                          | +++      |          |
| Oral mucosa                          | +++      | ++       |
| Salivary gland                       | +++      | ++       |
| Stomach                              | +++      |          |
| Duodenum                             | +++      |          |
| Small intestine                      | +++      |          |
| Appendix                             | +++      |          |
| Colon                                | +++      |          |
| Rectum                               | +++      |          |
| Kidney                               | +++      | +++      |
| Urinary bladder                      | ++       | +++      |
| Testis                               | +++      | +++      |
| Epididimis                           | +++      | ++       |
| Prostat                              | ++       |          |
| Seminal vesicle                      | +++      | ++       |
| Breast                               | +++      |          |
| Vagina                               | ++       |          |
| Cervix, uterine                      | ++       | +++      |
| Endometrium                          | +++      | ++       |
| Fallopian tube                       | +++      | ++       |
| Ovarium                              | +++      |          |
| Placenta                             | +++      |          |
| Skin                                 | ++       | +++      |
| Adipose tissue                       |          |          |
| Skeleton tissue                      |        |          |
| Smooth muscle                        |        |          |
| Soft tissue                          |        |          |
| Bone marrow                          |          |          |
| Limphatic tissue                     |        |          |
| Tonsil                               | ++       |          |
| Spleen                               |          |          |
| Cerebral cortex                      | +++      |          |
| Hippocampus                          | +++      |          |
| Lateral ventricle                    | +++      |          |
| Cerebellum                           | +++      |          |
| Thyroid tissue                       | +++      |          |
| Parathyroid tissue                   | ++       |          |
| Adrenal tissue                       | +++      | ++       |
| Nasopharynx                          | +++      |          |
| Bronchus                             | +++      |          |
| Lungs                                | +++      |          |
| Heart                                | +++      |          |

+  = very weak
++ = weak
+++ = medium
++++ = strong

4. Conclusion
The current study has highlighted the proteomic analysis using LC-MS/MS to identify and characterize protein marker which indicated insufficiency pancreatic exocrine. A protein with 18 kDa has been suggested as a candidate protein that shown the pancreatic exocrine insufficiency in T2D. In addition, further identification of that protein using LC-MS/MS showed 4 peptide fragments. In silico analysis of the peptide fragment supported that pancreatic exocrine insufficiency correlated with progression of T2D.

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