Apoptotic RBM38, TUSC2 and ANO1 Proteins ใน Human Serum Proteome มีความสัมพันธ์กับภาวะโฮโมซิสเตอีนสูงในเลือด

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บทคัดย่อ

ภาวะโฮโมซิสเตอีนสูงในเลือด เป็นหนึ่งในปัจจัยเสี่ยงต่อการเกิดโรคหลอดเลือดหัวใจ เนื่องจากสามารถชักนำให้เกิดภาวะเซลล์ของกล้ามเนื้อหลอดเลือดตายได้ (apoptosis) ในปัจจุบันการศึกษาทางด้านโปรติโอมิกส์มีการนำมาใช้กันอย่างแพร่หลายในงานวิจัยทางด้านวิทยาศาสตร์และทางคลินิก งานวิจัยนี้เป็นการศึกษาเบื้องต้นที่ใช้เทคนิคทางโปรติโอมิกส์ โดยมีวัตถุประสงค์เพื่อศึกษาความแตกต่างของโปรตีนที่แสดงออกที่เกี่ยวข้องกับการเกิดโรคหลอดเลือดหัวใจในผู้ที่มีภาวะโฮโมซิสเตอีนสูงในเลือด 3 ระดับ ใช้ chemiluminescense immunoassay สำหรับวัดระดับโฮโมซิสเตอีนในเลือด ศึกษาการแสดงออกของโปรตีนในซีรัมด้วยเทคนิค gel electrophoresis และ LC/MS-MS วิเคราะห์ปริมาณโปรตีนด้วย DecyderMs software เปรียบเทียบความแตกต่างของโปรตีนในรูปของ Venn diagram ด้วย Jvenn และจัดกลุ่มของโปรตีนด้วย Panther software ทำนายความสัมพันธ์ระหว่างโปรตีนและสารโมเลกุลอื่นโดย STITCH โปรแกรมเวอร์ชั่น 5.0 ผลการศึกษาพบว่า มีโปรตีนเฉพาะ 8 ชนิดที่มีการพบในปริมาณสูง (up-regulated) ในกลุ่มคนที่มีภาวะโฮโมซิสเตอีนสูงในเลือด ได้แก่ MUC-19, LPAR1, MAPO2, GPALPP1, FECH, TUSC2, RBM38 และ ANO1 ประเด็นสำคัญพบว่า RBM38, TUSC2 และ ANO1 เป็นโปรตีนที่พบเฉพาะในกลุ่มที่มีโฮโมซิสเตอีนในเลือดสูงกว่า 50 μmol/L ที่มีสัมพันธ์กับโรคหลอดเลือดซึ่งเกี่ยวข้องกับความผิดปกติในขบวนการตายของเซลล์ ข้อมูลเชิงลึกที่ได้จากการศึกษานี้จะช่วยเพิ่มเติมความรู้ความเข้าใจในการเปลี่ยนแปลงของโปรตีนภายหลังการแปลงรหัสที่มีความสัมพันธ์กับความผิดปกติของระดับโฮโมซิสเตอีน และความเสี่ยงของการเกิดโรคหลอดเลือดหัวใจได้

คำสำคัญ : ภาวะโฮโมซิสเตอีนสูงในเลือด โปรตีโอมิกส์ เซลล์ตาย (apoptosis)

***ผู้รับผิดชอบบทความ**

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Apoptotic RBM38, TUSC2 and ANO1 Proteins in Human Serum Proteome Are Associated with Hyperhomocysteinemia

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Abstract

Hyperhomocysteinemia (HHcy) is known as an independent risk factor for cardiovascular diseases. It can induce apoptosis of vascular smooth muscle cells. Currently, proteomic has been widely applied in scientific and clinical researches. This research is a preliminary study that used proteomic technique for comparative protein expressions with focusing on the molecular basis of CVD in HHcy condition. The aim of this study was to identify protein profiles between control (non-HHcy) group and three groups of HHcy. Chemiluminescense immunoassay was used for determination of plasma homocysteine levels. Protein expressions in human serum were determined and analyzed by gel electrophoresis and LC/MS-MS technique. Proteins were quantified by using DeCyderMS analysis software. Jvenn was used for comparing protein lists with venn diagram. Function and class of protein were demonstrated by using PANTHER software. The interaction of chemical and protein was predicted by using STITCH V.5.0. Eight unique proteins were up-regulated in hyperhomocysteinemia groups, including MUC-19, LPAR1, MAPO2, GPALPP1, FECH, TUSC2, RBM38 and ANO1. Notably, RBM38, TUSC2, and ANO1 found in subjects with plasma homocysteine levels more than 50 μmol/L were discovered and linked to CVD risks via apoptotic signaling pathways. This study provided better insight into the interaction between expressed proteins profiles after post-translational modification which may resulted in induction of physiological alteration that may related to homocysteine-induced CVD.

Keywords: Hyperhomocysteinemia, Proteomics, Apoptosis

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Introduction

Proteomics is one of the new era of omics studies involving whole protein expressions profiles in cells together with quantify and identify the protein product of all genes. Proteomics is able to provide better understanding in molecular basis insight of diseases and to identify biological marker, which may be a specific indicator in different conditions. Previous studies of proteomics and homocysteine in animal model reported some significant alteration of protein expression. A study of proteome in rat off spring related to maternal food intake during pregnancy showed that protein expressions in homocysteine cycle was altered among rat groups with different levels of homocysteine intake[1]. Experimental studies revealed association between protein expressions and genetic variations which involved in homocysteine metabolism such as cystathionine β-synthase (CBS)[2] and paraoxonase1 (PON1) in induced mouse to hyperhomocysteinemia condition[3]. There was a report with alteration of apolipoprotein A-I in patients carrying MTHFR C677T mutations in Italian population[4]. A quantitative reduction of apolipoprotein A-I in mutant individuals with hyper-homocysteinemia was associated with an increased risk of cardiovascular diseases. Notably, they highlighted the novel association among genetic factor, homocysteine levels and alteration of protein expression. However, previous proteomic studies of hyper-homocysteinemia in human biofluids related homocysteine (e.g., serum, plasma, urine, and saliva) are very limited. To our knowledge, this is a first preliminary study to investigate serum protein expression in Thai men with hyperhomocysteinemia.

Well-known risk factors of cardiovascular disease include age, genetic, lifestyle pattern and health conditions such as hypertension, hyperlipidemia and hyperhomo-cysteinemia. Homocysteine is an amino acid, which results from methionine utilization in cells. The normal range of total plasma homocysteine in adult with fasting is usually in 5-15 μmol/L, and its level greater than 15 μmol/L is defined as hyperhomocysteinemia[5]. Elevated level of homocysteine can induce oxidative stress by disruption of anti-oxidant enzymes. Simultaneously, it also initiates endothelial cell injury through increased arterial stiffness by decreased nitric oxide (NO) bioavailability[6,7]. Another mechanism of hyperhomocysteinmia induced abnormal molecular function involves apoptosis signaling pathway via endoplasmic reticulum stress (ER stress). ER stress can lead to alter function/ structure of proteins

and disturb lipid metabolism, which may lead to cardiovascular diseases. Moreover, homocysteine play role in apoptosis via P53 mediated pathway or methyl transferase inhibition. A large number of genes related to apoptotic were reported such as Bax, Bcl-2, Casp 12, Casp 3, Cav 3, Cdk2, P38, P53 and Pkc with modulated by elevated level of homocysteine. In addition, homocysteine was associated with regulation of gene expressions, which involved in various metabolic diseases[8]. According to evidences from previous studies, the strong interaction between hyperhomocysteinemia and risk of cardiovascular diseases via apoptotic signaling pathway has been shown. This study hypothesized that elevated homocysteine levels may associate with alteration of apoptotic proteins expressions in human serum and possibly relate to increased risk of cardiovascular diseases. Therefore, this research aims to investigate the different expression of human serum proteome between control and hyperhomocysteinemia groups by using proteomic technique.

Materials and Methods

Subjects: Human serums were obtained from the Electricity Generating Authority of Thailand study (EGAT cohort study) in 2013, which has been described previously[9]. This study recruited Thai male, aged 50-70 years. Subjects with diseases related to abnormal homocysteine metabolism e.g., liver disease, renal disease, and cancer were excluded. Subjects were categorized into non-hyper-homocysteinemia group (non-HHcy/control group; plasma total homocysteine level ≤15 μmol/l) or hyperhomocysteinemia group (HHcy/case group; plasma total homocysteine level > 15 μmol/l). This study was approved by the Ethic Committee on Human Right Related to Research Involving Human Subjects, Faculty of Medicine Ramathibodi Hospital, Mahidol University (MURA2017/116).

Study design: Data of general characteristics and clinical parameters, including body mass index (BMI), plasma homocysteine levels, low-density lipoprotein levels (LDL, high-density lipoprotein levels (HDL, HbA1c, and fasting blood glucose were completely collected. Moreover, physical examinations and specific medical assessment were performed in this study. According to NCEP ATP III criteria for metabolic syndrome, cut point of blood pressure were applied at greater than or equal to 130/85 mmHg[10]. Based on the rational of this pilot study, we further investigated subjects with intensively matched age and other general

characteristics, genetic background of MTHFR gene and some components of metabolic syndrome. A control group with normal level of Hcy were 2 cases (from 20 subjects) with plasma Hcy level of <15 µmol/L, BMI 18.5-22.9 kg/m^2, LDL-cholesterol <130 mg/dL and blood pressure <130/85 mmHg. For hyperhomocysteinemia group, they were subdivided as shown in the following;

- Case group I (N=2 selected from 20 cases): plasma Hcy level of 15-30 µmol/L, and no metabolic syndrome.
- Case group II (N=3 selected from 20 cases): plasma Hcy level of 30-50 µmol/L, BMI > 22.9 kg/m^2, and two metabolic syndrome components
- Case group III (N=3 selected from 20 cases): plasma Hcy level of >50 µmol/L, BMI>22.9 kg/m^2, two metabolic syndrome components and cardio-ankle vascular index (CAVI). Two cases showed CAVI values more than 9.0 with indicative diagnosis of suspected arteriosclerosis.

In this study, we have already performed the pooled samples which can be used as a powerful strategy to compensate for limited amounts of samples or high biological variation. Pooling of samples in proteomics experiments may help overcome resource constraints when many individuals are analyzed.

Plasma homocysteine measurement: Participants were asked for fasting before collecting blood sample. Plasma homocysteine level was measured by electrochemiluminescence immunoassay technique with COBAS (C800, Roche.)

Determination of protein concentration: Bradford assay was used for measurement of serum protein concentrations. Briefly, Bradford reagent was added to a well-plate along with samples with triplicate determination. The absorbance at 595 nm was read after 5 min of incubation by Rayto Rt-2100c microplate reader. Amount of protein were calculated by using a standard curve of protein standard which was diluted accordingly to various concentration.

SDS-PAGE: The 10 µg proteins samples were separated by 12.5% SDS-PAGE through two layers of gel, including 12.5% separating gel and 4% stacking gel. Proteins samples were loaded and performed by using Atto AE-6540 with constant Amps power supply (40 mA) for approximately 1-2 hours. After electrophoresis, protein bands were visualized by using silver nitrate solution and subsequently scanned with imaging densitometer as described in previous study[11].

In-solution digestion: The samples were subjected to the in-solution digestion and purified to remove buffer and salts. Briefly, an aliquot of 2 µl (4 µg of protein) were added into low-binding tubes. Protein samples were reduced by addition of 5 µl of 10 mM dithiothreitol (DTT) in 10 mM ammonium bicarbonate (Ambic) and 5 mM DTT (final concentration). Then, the samples were spun down at 10,000 g for 1 minute and incubated at 56°C for 1 hour. Protein samples were alkylated with 10 µl of iodoacetamide (IAA) and incubated at room temperature in the dark for 1 hour. Next, 10 µl of trypsin solution (2 ng/µl) was added into each sample tubes and incubated at 37°C overnight. The digested samples were dried by SpeedVac. The dried samples were re-dissolved by addition of 42 µl formic acid and centrifuged at 10,000 rpm for 10 minutes. The peptides were provided in vials for liquid chromatography-tandem mass spectrometry (LC-MS/MS) analysis.

LC-MS/MS: The purified peptides were analyzed by Ultimate 3000 Nano/Capillary LC System (Dionex Ltd., UK) coupled to a Hybrid quadrupole Q-Tof impact II™ (Bruker Daltonics GmbH, Germany) equipped with a Nano captive spray ion source. The 500 nl of the purified bioactive peptide was subjected to the trapping column (Thermo Scientific, PepMap100, C_{18}, 300 µm i.d.×5 mm), using full loop injection. The samples were separated on analytical column (PepSwift Monolithic Nano Column, 100 µm × 5 cm i.d.). A linear gradient for elution method was used to elute peptides into the mass spectrometer, mobile phases A and B at a constant flow rate of 1 µl/min which mobile phase A was 0.1% formic acid in water and mobile B was 0.1 % formic acid in 80 % acetonitrile. The gradient condition was performed as follows; the mobile phase gradients were conditioned as follows: equilibrated period, 0-4.0 min: 15% B, separation period, 4.01-14.0 min: 15– 60% B, washing period, 14.01-16.00 min: 95% B and re-equilibration period 16.01-20.00 min, 15% B.

Protein identification and database analysis: Proteins were quantified by using DeCyder MS Differential Analaysis software (DeCyderMS, GE Healthcare)[12, 13]. Consequently, the data were submitted to the Mascot software (Matrix Science, London, UK[14] and searched against the NCBI database. Database interrogation was; taxonomy (*Homo sapiens*); enzyme (trypsin); variable modifications (carbamidomethyl, oxidation of methionine residues); mass values (monoisotopic); protein mass (unrestricted); peptide mass tolerance (1.2 Da); fragment mass tolerance (±0.6 Da),

peptide charge state (1+, 2+ and 3+) and max missed cleavages (3). Protein levels were presented in log2 value.

Jvenn diagram was applied for counting and comparing lists of proteins in each group[15]. It is an integrative tool for computing and identifying the number of specific or intersection proteins among groups. Jvenn displays the data as venn diagram and two statistic chart for evaluating homogeneity of the lists size and comparing the compactness of multiple venn diagram. Moreover, proteins were classified according to its function, which related to protein class and biological process at the level of the cell or organism by using Protein ANalysis THrough Evolutionary Relationships or the PANTHER classification system (available at http://www.pantherdb.org)[16]. The PANTHER classifications are the result of bioinformatics algorithms. Lastly, the identified proteins were submitted to The Search Tool for Interacting Chemicals (STITCH) (http://stitch.embl.de) for predicting interactions between proteins and small molecules[17]. STITCH is a database of known and predicted interaction between chemical and proteins. Interaction sources in STITCH are derived from five main sources, including genomic context predictions, high-throughput lab experiments, (conserved) co-expression, automated text mining and previous knowledge databases.

Results

SDS-PAGE analysis: Serum protein concentrations were determined by using Bradford assay. Sodium dodecyl sulfate polyacrylamide gel electrophoresis (SDS-PAGE) analysis was performed for separating proteins of 10 different groups. Protein (10 μg) were loaded and visualized with silver staining as shown in Figure 1. Differential protein expressions according to various molecular weights (kDa) of each sample were observed. The differences in the protein bands with molecular mass 17-30 kDa were observed between pooled control group and hyperhomocysteinemia groups. Total proteins in serum samples were digested and analyzed by LC-MS/MS.

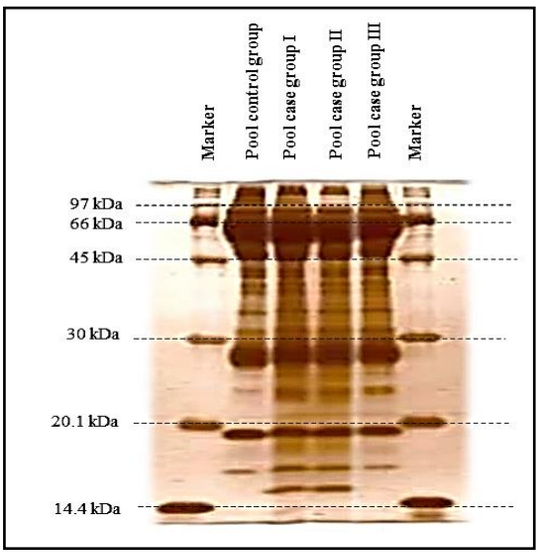

Figure 1. SDS-PAGE protein profiles of human serum presented sample in the pooled of control and case groups.

Protein classification: A total of 625 differentially expressed proteins were classified by Panther program and presented into the categories of biological process and protein class as shown in Figure 2. Most proteins involved in the cellular process (27.6%) and metabolic process (23%) when classified by the biological process (Figure 2A). In addition, these proteins were further categorized by the class of protein that mainly distributed in nucleic acid binding (18.9%), hydrolases (13.9%), enzyme modulators (8.8%) and transcription factors (8.4%) (Figure 2B).

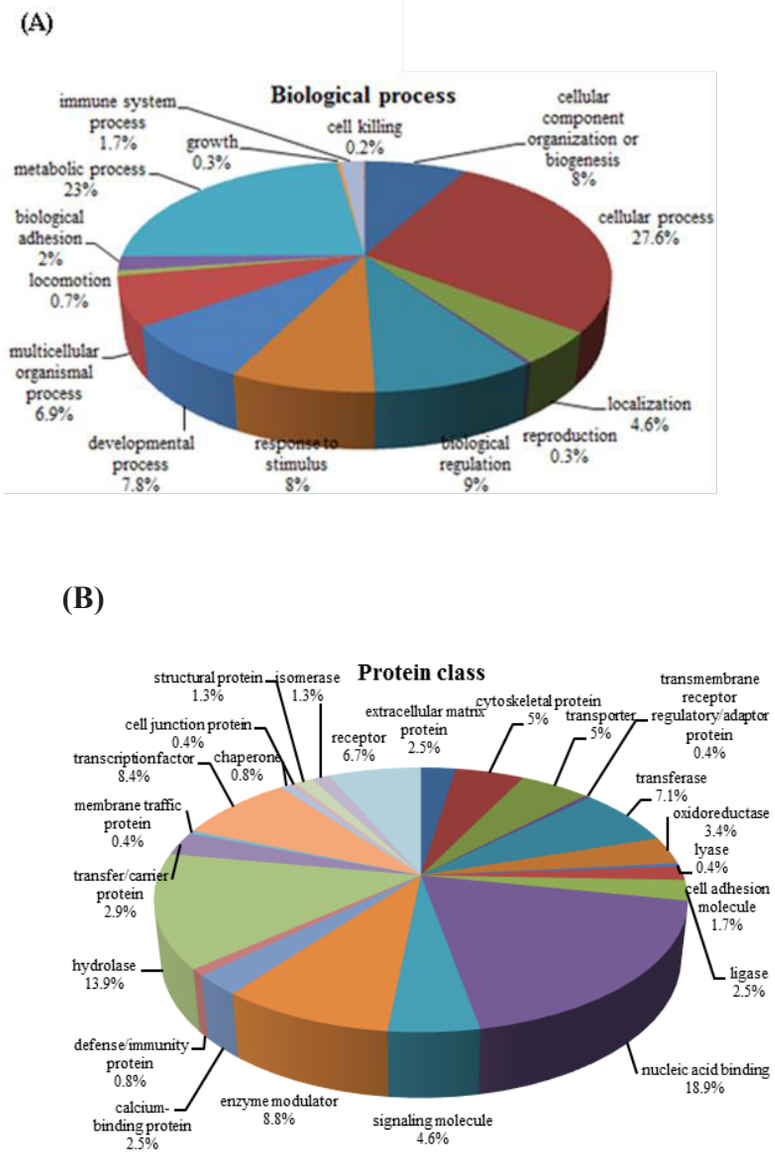

Figure 2. Classification of total proteins by their functions in the categories of (A) biological process and (B) protein class.

Interaction of unique proteins and homocysteine in different groups: Detection of unique proteins in control group (normal homocysteine levels) and hyperhomocysteinemia group were presented as Jvenn diagrams (Figure 3) . Jvenn computes the intersection count number for each element and subsequently displays the chart. The jvenn statistics exhibited that the different homocysteine groups generate gene list with different sizes (minimum 649 from case group III – maximum 685 from case group II) and most of these were shared between groups. The overlapping protein of all four datasets was found to be 625, which was

located in central area of venn diagram. The specific list of protein to only one dataset will be considered as the unique protein. Name and functions of the unique proteins were subsequently identified by UniProt. For control group, double homeobox A was the only 1 unique protein detected. Group of mild hyperhomocysteinemia (case group I) exhibited two specific proteins. Five and six unique proteins were found in case group II and III, respectively. Of total 17 gi numbers of uniques from venn diagram, only 9 unique proteins could be identified by UniProt. The identification of protein markers in human serum with different levels of homocysteine and their functions were summarized in Table 1. By using STITCH 5.0 for further analysis, only 5 out of 9 proteins are reported that they are indirectly related to homocysteine. Homocysteine interact with anoctamin 1, ferrochelatase, tumor suppressor candidate 2, and RNA binding motif protein 38. The results showed that these proteins expressions were induced in high homocysteine group (group III, > 50 µmol/L, with high BMI and CAVI > 9). Homocysteine can also interact with lysophosphatidic acid receptor 1. This protein level is upregulated in the group of moderate hyperhomocysteinemia (group II, 30-50 µmol/L).

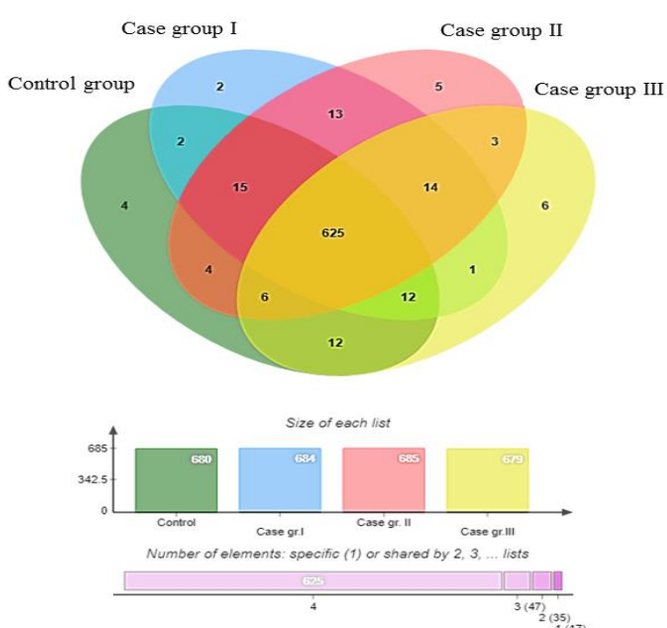

Figure 3. Venn diagram presented the unique proteins detected in four groups (control group, case group I, II and III). The specific lists of gene included 4, 2, 5 and 6 for control, case group I, II and III, respectively. The vertical chart displayed the number of elements in each list for checking the homogeneity of the lists size. The horizontal chart showed the number of specific or shared elements.

Table 1: Unique proteins were identified by UniProt in each group and the relative fold changed of protein expressions were compared with control group

Group	No.	gi number	Protein names	Mass (Da)	Function	Fold changed
Non-hyperhomocysteinemia (control)	1.	gi|160380653	Double homeobox protein A (DUXA)	885.4	DNA-binding, regulation of transcription	-
Case group I	1.	gi|1035664363	Mucin 19 (MUC19)	1391.6	Mucin family members are glycoproteins that have tandem repeats which are extensively O-glycosy-lated. The structural features of mucin proteins are responsible for the gel-like properties of mucus	18.1
Case group II	1.	gi|26454626	Lysophosphatidic acid receptor 1 (LPAR1)	3184.3	G-protein coupled receptor, lipid binding	18.6
	2.	gi|74746565	O^6-methylguanine-induced apoptosis 2 (MAPO2)	1749.6	Apoptosis	10.4
Case group III	1.	gi|7920153	GPALPP motifs-containing protein 1 (GPALPP1)	2003.0	Unknown	17.6
	2.	gi|62897941	Ferrochelatase (FECH)	1003.7	Heme biosynthetic process	10.5
	3.	gi|7531123	Tumor suppressor candidate 2 (TUSC2)	654.3	Cell cycle, inflammatory response	9.9
	4.	gi|215273895	RNA-binding motif protein 38 (RBM38)	1512.6	RNA-binding, cell cycle, cell differentiation	9.2
	5.	gi|158259637	Anoctamin-1 (ANO1)	881.7	Intracellular calcium activated chloride channel activity	7.4

Discussion

Hyperhomocysteinemia is an independent risk factor for cardiovascular diseases. The promoting effect of homocysteine on vascular dysfunction has been considered as one of the important pathological bases of atherosclerosis. However, the various mechanism induced by homocysteine remains unclear. The present research used proteomic techniques to initially analyze the protein changes in this clinical event. In this study, we identify the alteration of serum proteome in subjects with hyper-homocysteinemia (Table 1).

Based on these results of protein identifications in different groups, one unique protein (Mucin 19) from serum of subjects with mild level of homocysteine (15-30 μmol/L) was markedly induced. Mucin 19 (MUC19) is a glycoproteins localized to salivary mucous cells. Alterations of salivary expression of MUC19 affecting the oral microbiota may in turn influence the intestinal microbiota and immune homeostasis, leading to CVD risk[18]. Homocysteine may contribute to pyroptosis (caspase 1-dependent programmed cell death) and changes gut microbiome.

In other point of view, lysophosphatidic acid receptor 1 (LPAR1) and O^6-methylguanine induce apoptosis 2 (MAPO2) were unique proteins that up-regulated in moderate hyper-homocysteinemia group (30-50 μmol/L). The impact of HHcy over different cell signaling pathways including G-protein coupled receptor (GPCR) and consequent cellular outcomes are increasingly realized. GPCRs are very important in modulation of cell structure, cell microenvironment, and cell responses to various stimuli. Receptor of lysophosphatidic acid (LPAR1) is one of the GPCRs and play role in LPA pathways related to CVD risks. LPA acts as phospholipid messenger with multi-functions and mainly produced by activated platelets. Therefore, blood LPA level has been targeted to be an ideal molecular marker for reflecting platelet activation state[19]. In addition, homocysteine causes a multitude of adverse effects that disrupt vascular cell homeostasis via activating apoptosis by ROS release and ER stress[20]. For O^6-methylguanine-induced apoptosis 2 (MAPO2), it has involved in the induction of cellular apoptosis[21]. Upstream DNA damage pathway with increased apoptotic proteins is involved in triggering apoptosis vascular smooth muscle cell and this consequence can hasten plaque rupture. Increased both of two proteins level suggested greater homocysteine level and greater risks of CVD.

In group of very high homocysteine levels (> 50 μmol/L), up-regulation of five unique proteins were observed, including GPALPP motifs-containing protein 1 (GPALPP1 or KIAA1704), ferrochelatase (FECH), tumor suppressor candidate 2 (TUSC2, RNA-binding protein 38 (RBM38) and anoctamin-1 (ANO1).

GPPALPP1 gene encodes the protein in human cell as lipopolysaccharide-specific response protein 7 (LSR7). Up-regulation of this protein was observed in U937 cells when treated with nicotin. However, the function of this protein was not yet well understood but likely related to inflammation, immune responses[22]. For ferrochelatase (FECH), it is an enzyme which involves in heme biosynthesis. Heme is an essential prosthetic group for hemoproteins involved in numerous cardiovascular processes, including oxygen transport (hemoglobin), oxygen storage (myoglobin), oxygen metabolism (oxidases), antioxidation (peroxidases, catalases), and electron transport (cytochromes). Another important issue is heme function in the liver which is the synthesis of cytochrome P450 enzymes. This enzyme system are required for liver detoxification under different conditions. It is also recognized that heme accumulation caused by up-regulation of important

enzymes, such as FECH can lead to cellular damage through altering membrane permeability and increasing oxidative stress and is associated with abnormal cardiovascular functions[23].

Notably, proteins involved in the apoptosis pathway were observed to be upregulated by high Hcy expression. Tumor suppressor candidate 2 (TUSC2), also known as FUS1, is a novel tumor suppressor gene candidate that has been identified in the 3p21.3 chromosomal region. Activation of FUS1 in normal cells in response to apoptotic stimuli or stress can lead to trigger cytochrome c (Cyt C) release from the inner membrane of mitochondria to the cy-tosol, selectively and directly interacts with Apaf-1. After this stage, it can facilitate downstream Apaf-1-mediated apoptosome assembly, caspase activation, and apoptosis induction[24]. Another protein group is RNA-binding proteins (RBPs) which are master regulators of RNA biogenesis and metabolism. This study found alteration of one kind of RBPs which is RNA-binding motif protein 38 (RBM38). It is a target of the p53 family and modulates p53 expression via mRNA translation. An experimental study demonstrated that overexpression of p53 in endothelial cells was found to reduce the expression of nitric oxide synthase and thrombomodulin, to increase the expression of plasminogen activator inhibitor-1 (PAI-1) and to enhance ex vivo blood coagulation on an endothelial monolayer[25]. Findings from this study also showed that another protein related to Hcy was Anoctamin-1 (ANO1). This protein involves in Ca^{2+}-activated Cl^- channels (CaCCs) and plays role in numerous physiological functions. CaCCs are mediated by intracellular calcium[26]. Activation of ANO1 promotes apoptosis of pulmonary endothelial cells via increased mtROS and p38 phosphorylation, leading to apoptosis[27]. Previous studies confirmed ANO1 activity and expression in smooth muscles cell from various arteries veins[28,29]. There were supported that vasorelaxation of murine and human arteries were induced by ANO1 inhibition. Moreover, the expression of ANO1 was significantly up-regulated in various hypertension models[30, 31].

In this study, we further analyze up-regulated proteins related to increased homocysteine levels with the specific program STITCH version 5.0. This software provides global interactions between proteins and small molecules ae well as an integral part of biological processes in living organisms. In Figure 4, our results from STITCH 5.0 demonstrated a variety of the differentially expressed proteins in hyperhomocysteinemia condition. LPAR1, FECH, TUSC2,

RBM38 and ANO1 proteins indirectly interacted with homocysteine via apoptotic pathways. The possible process linking HHcy and apoptosis is endoplasmic reticulum (ER) stress. Previous study indicated that homocysteine induces ER stress–mediated activation of caspase-3 in endothelial progenitor cells from patients with coronary heart disease. In other words, enhanced ER stress-mediated activation of caspase-3 in endothelial progenitor cells might be involved in hyperhomocysteinemia-associated vascular pathology[32]. A large number of published researches were used to support overall data from this proteomic study. LPA could facilitate endothelial cell death by modulating the redox environment, which dependd on LPAR1-mediated modulation of cell redox state[33]. For TUSC2, it has been shown to induce G1 cell cycle arrest and promote apoptosis[24]. A study in small cell lung cancer (SCLC) exhibited that TUSC2 induces cell death through mechanism related to caspase-dependent apoptosis[34]. Interestingly, TUSC2 expression was negatively regulated by several microRNAs, especially miRNA-378. Previous study revealed that miRNA-378 binded to 3′-UTR of the TUSC2 transcript and repressed its translation, resulting in enhances cell survival and reduce caspase-3 activity[35]. For ANO1, it was another protein presented in group of extremely high homocysteine levels that involved in apoptotic signaling. The association between ANO1 and FAS associated via death domain (FADD) was reported. FADD is an adaptor molecule interacting with many kinds of death receptors and induces apoptosis by caspase-8[36,37]. Noticeably, high levels of FADD and caspase-8 were associated with increased incidence of coronary events[38]. For RBM38, it has been implicated in cell cycle regulation and differentiation. The diverse biological functions of RBM 38 are probably due to its role as an RNA-binding protein to regulate multiple targets. For example, RBM38 regulates mRNA stability of mdm2[39] and p21[40]. In addition, RBM38 can regulate p53 translation by modulating the binding of eIF4E, a translation initial factor, to the p53 mRNA[41, 42]. The importance of p53 is its ability as a transcription factor to regulate a series of downstream target genes, such as p53 induces p21 (WAF1/CIP1) for cell cycle regulation[43]. MAPK can regulate apoptosis through specific phosphorylation of downstream mediators of apoptosis, including the tumor suppressor p53, thus linking cellular stress signaling and regulation of p53 activity[44, 45]. The last finding of unique protein related to homocysteine in group of extremely hyperhomocysteine levels is FECH. It is the enzyme of the heme biosynthetic

process in all cells. Heme modulates enzyme activity of cystathionine-β-synthase (CBS) in response to redox change. CBS is known as pyridoxal phosphate-dependent enzyme that also contains heme b as a cofactor. CBS is the key enzyme in transulfuration pathway for clearance of excessive homocysteine levels to form cystathionine and promotes glutathione synthesis[46]. Glutathione is the important antioxidant, which play critical role for defense mechanism in every cell from HHcy induced oxidative stress.

Interplay of homocysteine induced apoptosis-proteomics outcomes may be applicable in facilitating the assessing potential novel molecular targets to reduce cardiovascular risk related with elevated Hcy levels in various human populations. In addition, it can lead to elucidate new mechanisms through which protein functions can be regulated by the redox status with the use of various strategies (such as dietary, lifestyle modification or targeted drugs). However, the limitation of this study is a small sample size in each group for proteomic analysis. Further proteomic studies in large population are needed to validate protein markers and clarify role of different protein expressions related to hyperhomocysteinemia in human serum.

Conclusion

To our knowledge, this is the first preliminary study that applied proteomic techniques for studying protein markers and identified expression profiles in serum with hyperhomocysteinemia condition in Thai men population. In this study, 8 unique proteins were up-regulated in human serum with hyperhomocysteinemia condition, including MUC-19, LPAR1, MAPO2, FECH, GPALPP1, TUSC2, RBM38 and ANO1. Interestingly unique proteins in very high homocystetine level, TUSC2, RBM38 and ANO1 interacted with homocysteine via apoptotic pathways that may indirectly associated to vascular pathology.

Conflicts of Interest

The authors declare that there are no conflicts of interests regarding the publication of this paper.

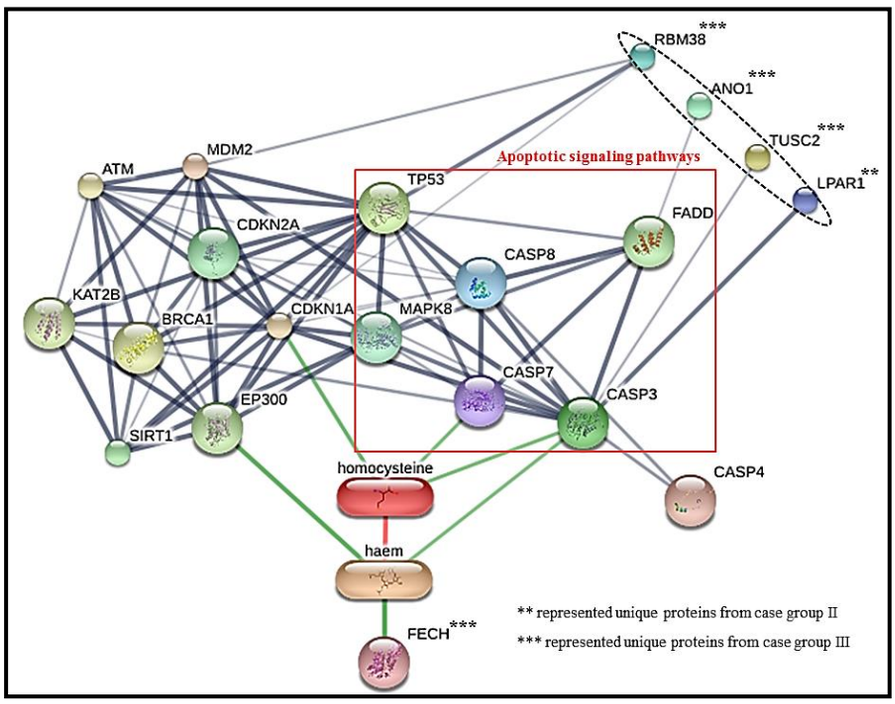

Figure 4. An interaction of homocysteine and unique proteins related to apoptotic pathways (including LPAR1, FECH, TUSC2, RBM38 and ANO1 by using STITCH 5.0. ; LPAR1, lysophosphatidic acid receptor 1; FECH, ferrochelatase; TUSC2, tumor suppressor candidate 2; RBM38, RNA binding motif protein 38; ANO1, anoctamin1; FADD, fas-associated via death domain; CASP3, caspase 3; CASP4, caspase 4; CASP7, caspase 7; CASP8, caspase 8; MAPK8, mitogen-activated protein kinase 8; EP300, E1A binding protein p300; CDKN1A, cyclin-dependent kinase inhibitor 1A; MDM2, mdm2; BRCA1, breast cancer; KAT2B, K(lysine) acetyltransferase 2B; SIRT1, sirtuin 1; Haem, heme b; TP53, tumor protein p53, CDKN2A, cyclin-dependent kinase inhibitor 2A; ATM, ataxia telangiectasia mutated). **, *** represented unique proteins from case group II and case group III, respectively.

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