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การเปลี่ยนแปลงแบบแผนการแสดงออกของโปรตีนในผู้ป่วยไทยที่มีภาวะซึมเศร้ารุนแรงที่มีการตอบสนองต่อยา Fluoxetine แตกต่างกัน

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บทคัดย่อ

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บทนำ : ถึงแม้ว่ายารักษาโรคซึมเศร้า (antidepressant) จะเป็นหนทางเลือกแรกสำหรับผู้ป่วยภาวะซึมเศร้ารุนแรง (major depressive disorder; MDD) แต่ผู้ป่วยแต่ละรายมีการตอบสนองต่อยาดังกล่าวแตกต่างกัน ดังนั้นการพยากรณ์ความสามารถในการตอบสนองต่อยาล่วงหน้าจึงมีความสำคัญ ผู้วิจัยได้ทำการวิเคราะห์การเปลี่ยนแปลงแบบแผนการแสดงออกของโปรตีนในพลาสมาของผู้ป่วยภาวะซึมเศร้ารุนแรงคนไทยที่ได้รับยา Fluoxetine เป็นครั้งแรก ด้วยเทคนิค 2D gel electrophoresis ผลการวิเคราะห์พบโปรตีน 1 ชนิดที่มีแบบแผนการแสดงออกแตกต่างกันในระหว่างกลุ่มผู้ป่วยภาวะซึมเศร้ารุนแรงที่มีการตอบสนองอย่างเร็วต่อ Fluoxetine (fast-response; FR) กับกลุ่มผู้ป่วยภาวะซึมเศร้ารุนแรงที่ไม่ตอบสนองต่อยาดังกล่าว (non-response; NR) ซึ่งจากการศึกษาโครงสร้างของโปรตีนดังกล่าวด้วยเทคนิค Matrix Assisted Laser Desorption/ionization Time of Flight Mass Spectrometry (MALDI-TOF MS) สามารถระบุได้ในเบื้องต้นว่า คือ α1-antitrypsin โปรตีนชนิดนี้อาจสามารถใช้เป็น biomarker ตัวหนึ่งสำหรับการพยากรณ์ความสามารถของผู้ป่วยในการตอบสนองต่อ Fluoxetine **วัสดุและวิธีการ :** ผู้ป่วยคนไทยที่มีภาวะซึมเศร้ารุนแรง จำนวน 15 ราย อาศัย HAM-D score ในการตรวจชี้ภาวะการตอบสนองต่อ Fluoxetine และแบ่งผู้ป่วยออกเป็น 2 กลุ่ม คือ กลุ่มที่มีการตอบสนองอย่างรวดเร็ว (จำนวน 10 ราย) และกลุ่มที่ไม่ตอบสนอง (จำนวน 5 ราย) เตรียมพลาสมาจากตัวอย่างเลือดของผู้ป่วยที่เก็บก่อนการรักษาและระหว่างการรักษาด้วย Fluoxetine ทำการวิเคราะห์โปรตีนในพลาสมาตัวอย่างด้วยเทคนิค 2D gel electrophoresis แล้วนำมาเปรียบเทียบ จากนั้นทำการสกัดแยกโปรตีนที่สนใจและตรวจชี้ด้วยเทคนิค MALDI-TOF MS **ผลการศึกษา :** จากการเปรียบเทียบแบบแผนโปรตีนบนเจล พบว่า α1-antitrypsin เป็นโปรตีนที่มีการแสดงออกแตกต่างกันในระหว่างกลุ่มผู้ป่วย FR กับผู้ป่วย NR โดยพบ α1-antitrypsin ในผู้ป่วย FR ทุกราย ในขณะที่แทบไม่พบเลยในผู้ป่วย NR หลังการรักษาด้วย Fluoxetine การแสดงออกของโปรตีนดังกล่าวในผู้ป่วย FR ยังคงเดิม ในขณะที่หยุดยั้งในผู้ป่วย NR **สรุปผล :** การแสดงออกของ α1-antitrypsin ในผู้ป่วย FR แตกต่างจากผู้ป่วย NR เช่นเดียวกัน การได้รับยา fluoxetine ก็ทำให้การแสดงออกของโปรตีนชนิดนี้ในผู้ป่วย FR แตกต่างจากผู้ป่วย NR สิ่งนี้อาจส่งผลถึงความสามารถในการตอบสนองต่อ flouxetine ที่แตกต่างกันของผู้ป่วยทั้งสองกลุ่ม ดังนั้น α1-antitrypsin น่าจะเป็น biomarker ตัวหนึ่งในพลาสมาที่สามารถใช้ในการพยากรณ์การตอบสนอง/การต้านยารักษาโรคซึมเศร้ากลุ่ม SSRI โดยเฉพาะ fluoxetine ของผู้ป่วยภาวะซึมเศร้ารุนแรง

คำสำคัญ : ยารักษาโรคซึมเศร้า, Fluoxetine, ภาวะซึมเศร้ารุนแรง, โปรตีโอมิค, การแยกโปรตีนแบบสองมิติ

Abstract

Dynamic protein expression profiles in Thai major depressive disorder with different responses to Fluoxetine

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Introduction : Although pharmacotherapy is the primary choice for medical management of major depressive disorder (MDD), response to antidepressants is marked by inter-individual variability. Therefore, early predictors of the response are of important significant clinical value. We first report on dynamic proteomic analysis of the plasma in Thai patients with MDD who

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differently response to fluoxetine. Two-dimensional (2D) gel electrophoresis of plasma revealed that fast-response patients differed from non-response patients in one protein, and it was preliminary confirmed by Matrix Assisted Laser Desorption/ionization Time of Flight Mass Spectrometry (MALDI-TOF MS) analysis as α1-antitrypsin. This protein could be a peripheral biomarker candidate for early predict treatment response to the antidepressant. **Material and method :** Fifteen MDD patients were examined. Psychopathology was assessed and the patients were classified into fast-response (FR; n=10) and non-response (NR; n=5) groups by means of the Hamilton Depression Rating Scale (HAM-D) (see Table 1 for details). Plasma was collected before the pharmacological treatment and during the medication. Proteins in the plasma were analyzed and compared by 2D gel electrophoresis. Spot of the candidate protein was extracted from gel and verified by MALDI-TOF MS. **Results :** By comparing the protein pattern on gel, the protein corresponding to α1-antitrypsin was found as a difference between FR and NR. While it was present in all FR, it was absence in most NR. After received fluoxetine, the expression of this protein was not changed in FR but be inhibited in NR. **Conclusion :** α1-antitrypsin was differently expressed in FR in comparison with NR. The exposure to fluoxetine was also differently induced changes in the inflammation protein in these two MDD groups, which became apparent in response to the antidepressant treatments. This inflammatory protein could be a candidate biomarker for early prediction of the response/non-response to SSRIs particularly fluoxetine.

Keywords : Antidepressant, Fluoxetine, Major depressive disorder, Proteomic, 2D gel electrophoresis

Introduction

Major depressive disorder (MDD) is a prevalent and devastating mental illness the pathophysiological processes and the molecular correlates underpinning the abnormalities of which are incompletely understood. Multiple interdependent genetic and environment have been suggested to risk factors (Sullivan *et al.*, 2000), which alone is neither necessary nor sufficient for the disorder to develop. MDD is the fourth most important cause of loss in disability-adjusted life years (Longone *et al.*, 2008). Pharmacotherapy is the primary choice for medical management of MDD, and many antidepressants include selective serotonin reuptake inhibitors (SSRIs) as well as serotonin and norepinephrine reuptake inhibitors are most prescriptions (Nutt *et al.*, 2006; Cipriani *et al.*, 2009; for review see Carrasco and Sandner, 2005). However, response to antidepressants is variable. Only half of MDD respond to a first antidepressant medication, and about 30% to 37% do not reach remission after several treatment trials (Rush *et al.*, 2006; Warden *et al.*, 2007; Hennings *et al.*, 2009).

Selective serotonin reuptake inhibitors (SSRI) are a class of antidepressants that act at the neuronal synapse to block the reuptake of serotonin (5-hydroxytryptamine; 5-HT) which is lower level in MDD brain. Blocking of the serotonin reuptake allows the neurotransmitter to stimulate receptors on the postsynaptic neuron for a longer duration of time. Many SSRIs share common pharmacokinetic characteristics including linear and dose-proportional pharmacokinetics, steady-state plasma concentrations within 1 week of daily dosing, and clearance through hepatic or renal mechanisms (Heikkinen *et al.*, 2003).

Fluoxetine is a common SSRI that was first used more than ten years ago, and is at present the only registered SSRI for the treatment of MDD in children over 8 years (Kapornai and Vetró 2008). The target of fluoxetine is 5-HT neurotransmitter system, which is known to play a key role in brain development through its role in the connective organization of the nervous system which includes control of proliferation, differentiation, migration, cell death, synaptogenesis and dendritic pruning (Gaspar *et al.*, 2003; Homberg *et al.*, 2010; Whitaker-Azmitia *et al.*,

1996). Its long-term side-effects are reported in adulthood (Benmansour *et al.*, 1999; Cipriani *et al.*, 2007; Mourilhe and Stokes, 1998; Racagni and Popoli, 2008; Schule, 2007), and in the perinatal period (Alwan and Friedman, 2009; Borue *et al.*, 2007; Morrison *et al.*, 2005; Oberlander *et al.*, 2006; Olivier *et al.*, 2011). In addition, treatment with fluoxetine during the juvenile period is believed to have a stimulatory effect on adult hippocampal neurogenesis (Navailles *et al.*, 2008) and on neuroplasticity in the visual system following retinal lesions (Bastos *et al.*, 1999).

The treatment of MDD is highly cost in part because it takes long for patients to recover from the illness. On average, at least 4 weeks are needed to attain response, and 6 weeks to attain remission during treatment with an initial antidepressant, however, remission can take 12 weeks or longer (Trivedi *et al.*, 2006a,b). Therefore, more predictors for response and remission in advance with high accuracy are required. Proteomics analysis by 2D gel electrophoresis allows simultaneous separation and identification of many hundreds of proteins. It is an effective method to identify the molecular changes associated with diseases and disorder including depression (for reviews see D'Aquanno *et al.*, 2007; Filiou *et al.*, 2011). There have been many published studies comparing the proteomics in experimental animals or postmortem brain tissue in MDD (Brunner *et al.*, 2005; Kim and Kim, 2007) with have been primarily focused on genetic analyses and certain brain circuits (Geracioti et al, 1997; Carpenter et al, 2004; Sullivan et al, 2006; Tanis *et al.*, 2007; Jokinen et al, 2008; Altar *et al.*, 2009). Even though the peripheral markers for psychiatry disorders have been explored for many years, most are on to predict the treatment efficacy, which generally requires knowing in advance whether or not depressive disorder patients are responding to the drugs. In addition, the specific biomarker(s) for prediction of an individual patient's responses to a particular antidepressant have not been identified. With human body, plasma is an ultimate source of biomarker discovery since it is the most comprehensive proteome which represent to all body tissues and to both physiological and pathological processes (Anderson and Anderson, 2002). In this research, dynamic protein expression profiles in plasma of MDD were analyzed and compared with an attempt to identify protein biomarker(s) for predict clinical responsiveness to fluoxetine.

Objective

To identify protein marker(s) for predicting clinical responsiveness of MDD to fluoxetine by 2D gel analysis of plasma collected, before and during the treatment, from the patients with different responsiveness to the antidepressant.

Meterials and Methods

Blood collection and plasma preparation

Plasma samples were prepared from whole blood of 15 Thai MDD, collected before treatment with fluoxetine (week 0) and during the treatment (week 4), with a standard protocol. All participants had provided written informed consent to take part in the study, and were classified into FR (n=10) and NR (n=5) according to HAM-D score (Table 1). The plasma supernatant was aliquot and kept at -20°C until use.

Table 1 Classification of Thai MDD according to the responsiveness to fluoxetine by HAM-D score

	HAM-D score			
Category	**week 0**	**week 4**	**week 8**	**week 12**
fast response [FR]	>=18	reduce **more** than 1/2 of week **0**	reduce **more** than 1/2 of week **4 AND** score<8	score<8
none response [NR]	>=18	>=18	>=18	>=18

2D gel electrophoresis

Total protein concentration of plasma samples was determined by Bradford assay (Bradford, 1976), using bovine serum albumin as standard.

For 2D gel electrophoresis the Ettan IPGphor 3 (GE Healthcare) was used for the first dimension and the Mini-PROTEIN Tetra Cell (BioRad Laboratories) for the second dimension SDS gel electrophoresis. An aliquot of plasma (180 μg of total protein) was diluted in rehydration buffer with IPG buffer (7 M urea, 2 M thiourea, 4% CHAPS, 60 mM DTT, 0.5% IPG buffers, pH 4-7 (GE Healthcare) and 0.002% bromophenol blue to a final volume of 130 μL. IPG strip, pH 4-7 gradient, was rehydrated in the plasma solution mixture overnight at room temperature prior isoelectro-focalization. IEF was carried out at 20°C as followings: 300 V for 0.3 h, 1000 V for 0.3 h, 5000 V for 1.20 h, 5000 V for 0.25 h and 100 V for at least 0.3 h. Thereafter, the strip was then subjected to reduction in an equilibration buffer (50 mM Tris–HCl, pH 8.8, 6 M urea, 30% glycerol and 2% SDS) containing 1% DTT and followed by an alkylation in the same buffer but containing 2.5% iodoacetamide for 15 min each at room temperature. Further separation in the second dimension was processed by SDS-PAGE in a 12.5% gel. The electrophoresis was carried out at 25 mA/gel for 1 h, and then at 60 mA/gel as described by Richard *et al.*, 2007. After electrophoresis, proteins were stained with colloidal Coomassie Blue and scanned with a densitometer (Imagescanner, GE Healthcare). Image analysis was carried out with the ImageMaster 2D Platinum 7.0 software (GE Healthcare).

In-gel Protein Digest and Mass Spectrometry Analysis

Protein spot of interest was excised from the gel using an edge-cut sterile pipette tip. Destaining, drying, and tryptic digestion of gel spots was carried out according to a standard protocol. For MALDI-TOF MS analysis, sample was prepared by a dried droplet method on a 600-mm AnchorChip MALDI Target (Bruker Daltonics). α-cyano-4-hydroxycinnamic was dissolved in 30% (v/v) acetonitrile and 70% (v/v) 0.1% trifluoroacetic acid to saturation. The matrix preparation was diluted 10-fold in a 2:1 ethanol : acetone solution. An aliquot of sample was spotted onto the AnchorChip sample target, followed by an addition on top the spot with matrix solution. The peptide calibration standard (Bruker Daltonics) was used for instrument calibration. Peptide mass fingerprints were acquired using a mass window between 0 and 100 kDa.

Protein Identification

MALDI-TOF MS data analysis was carried out with Biotools software (Bruker Daltonics). For protein identification, the database search program Mascot (Matrix Science, London, UK) was applied to identify proteins according to their tryptic mass fingerprints. The search was carried out against NCBI non-redundant and human protein database. Mascot search was performed with peptide mass tolerance ±1.2 Da, fragment mass tolerant ±0.6 Da, and three possible tryptic miscleavages. Chemical modifications were fixed carbamidomethylation modification for cysteine and oxidation for methionine residues. Protein hits that were significant according to the Mascot score ($P<0.05$) were accepted. All protein hits were further verified by reviewing the position of the spot with regard to molecular weight and isoelectric point (pI) on the 2D-gel image.

Results

No significant differences between FR and NR were found regarding psychometric scales for severity of depression. Aaverage HAM-D scores on week 0 of FR and NR was 21.8 ± 3.5 and 24.8 ± 4.5, respectively. Image analysis of colloidal Coomassie-stained 2D gels resulted in over 100 spots matched between these two MDD groups with one being highly significant different. MALDI-TOF MS analysis of the excised gel spot identified α1-antitrypsin. Figure 1A and 2A show representative Coomassie-stained gels of the plasma samples, which collected before medication with fluoxetine, from FR and NR patients with differentially expressed protein indicated. After medication for 4 weeks, this protein spot was still detected in FR, but absence in NR. Figure 1B and 2B show representative

Coomassie-stained gels of the plasma samples from a FR and a NR, respectively, which were collected before the medication and after received fluoxetine for 4 weeks.

Discussion and Conclusion

Hereto, we first report on proteomic analysis of the plasma in Thai MDD with fast- and non-responsiveness to fluoxetine. No significant differences between these two MDD groups were found regarding psychometric scales for severity of depression.

The only biological marker that differed between FR (n=10) and NR (n=5) was the protein corresponding to α1-antitrypsin in the plasma detected by 2D gel electrophoresis. Since α1-antitrypsin is an inflammatory marker that functions in immune system and was recently reported up-regulated in MDD patients compared with non-depressed subjects (Papakostas *et al.*, 2011), it could conceive that the different responsiveness to fluoxetine or other SSRI of MDD may be related to immune system.

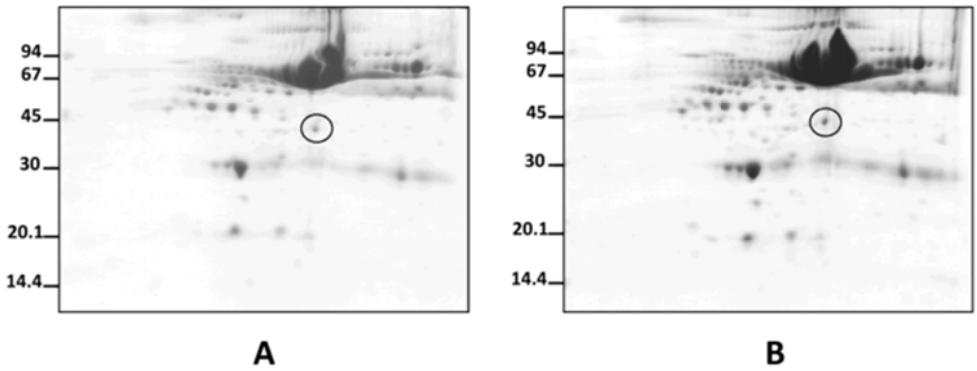

Figure 1 Representative colloidal Coomassie-stained 2D gel image of plasma from a FR collected at week 0 (A) and week 4 (B). The presence of the protein spot corresponding to α1-antitrypsin, as indicated by circles, were detected in both gels.

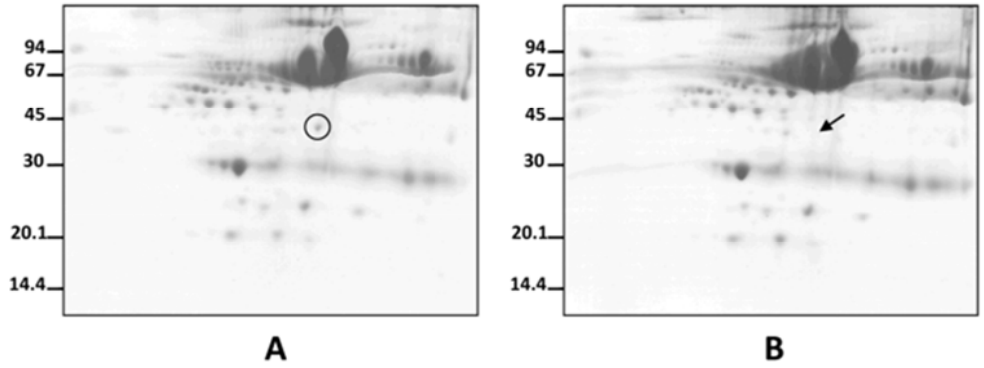

Figure 2 Representative colloidal Coomassie-stained 2D gel image of plasma from a NR collected at week 0 (A) and week 4 (B). The presence of the protein spot corresponding to α1-antitrypsin was detected at week 0 as indicated by a circle, but absence at week 4 as indicated by an arrow.

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