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Elevated heat shock proteins in bipolar disorder patients with hypothalamic pituitary adrenal axis dysfunction

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Abstract

Background: Heat shock proteins (HSP) might be useful as biomarkers for bipolar disorder (BD) which would be clinically valuable since no reliable biomarker for BD has so far been identified. The purpose of this study was to assess the heat shock proteins CPN10, CPN60, and CPN70 as potential biomarkers of BD.

Methods: The study included 100 BD patients recruited from a hospital during 2012 and 2013. The study also included 94 healthy controls. Among the BD patients, 33 had abnormal hypothalamic-pituitary-adrenal (HPA) axis activity. Blood samples were obtained from the patients and controls. The chemiluminescence method, mass spectrometry, and flow cytometry were used for analysis.

Results: The BD patients compared with the controls had a significantly lower level of CPN10 and significantly higher levels of CPN60 and CPN70. The BD patients with abnormal HPA axis activity had a significantly lower level of CPN60 compared with the normal HPA axis activity group of BD patients. The CPN60 level significantly inversely correlated with adrenocorticotropic hormone (ACTH) level in patients with bipolar depression and in patients with bipolar hypomania, and CPN70 significantly correlated with ACTH level in patients with bipolar depression and hypomania.

Conclusions: Our findings suggest that the heat shock proteins CPN10, CPN60, and CPN70 might have potential as biomarkers for BD and CPN60 blood level might distinguish patients with abnormal HPA axis activity from those with normal HPA axis activity.

Abbreviations: ACTH = adrenocorticotropic hormone, BD = bipolar disorder, CORT = cortisone, CPN10 = chaperones 10 (aka HSP10), CPN60 = chaperones 60 (aka HSP60), CPN70 = chaperones 70 (aka HSP70), HPA = hypothalamic-pituitary-adrenal, HSP = heat shock proteins, NMNAT = nicotinamide mononucleotide adenylyl transferases, SAA = serum amyloid A protein.

Keywords: adrenocorticotropic hormone, cortisone, CPN10, CPN60, CPN70, flow cytometry, mass spectrometry

1. Introduction

Bipolar disorder (BD) is an affective disorder that has been associated with an increased risk of a variety of medical conditions including cardiovascular disease, cerebrovascular disease, and metabolic syndrome.[1] It also appears there may be a relationship between BD and systemic alterations (e.g., oxidative stress, markers of inflammation, damaged DNA).[1]

An association has been observed between BD and abnormal activity of the hypothalamic-pituitary-adrenal (HPA) axis, which plays an important role in the response to stress, although the role of HPA axis activity abnormalities in the pathophysiology of BD remains unclear.[2–10] It is noteworthy that a recent meta-analysis revealed a significant association between BD and both cortisol and adrenocorticotropic hormone (ACTH).[2] With regard to BD pathophysiology, an interesting finding is that mitochondrial function impairment has been found to be related to pathogenesis of mood disorders in a study of mice in which anxiety- and depressive-like states were induced by chronic injections of corticosterone.[11] The subsequent injection of an agent that improves mitochondrial function prevented anxiety- and depressive-like behavior. In another study of mice that may relate to BD pathophysiology, the animals were exposed to unpredictable chronic mild stress.[12] Behavioral analyses indicated depressive changes and corticosterone level was increased. Altered proteins were identified by proteomic analysis and the findings also included overproduction of reactive oxygen species and changes in cytoskeleton protein and antioxidant enzymes,

One way in which cellular homeostasis is maintained during stress is through increased levels of molecular chaperones or heat shock proteins (HSP). When there is a stress response, heat shock transcription factors are activated which induce transcription of HSP.[13] Originally, it was believed that HSP only had intracellular functions but evidence has been accumulating that

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they also have extracellular functions.[14] The most widely studied molecular chaperones have been CPN10 (also called HSP10), CPN60 (also called HSP60), and CPN70 (also called HSP70).[14] Heat shock proteins are well known to play a role in the folding of proteins but they also appear to have immune functions.[15] It has been proposed that these molecules do not directly affect the immune system but rather the effects are due to molecules that are chaperoned by the HSP.[15] It should also be noted that nicotinamide mononucleotide adenylyl transferases (NMNAT) play a part in the stress response acting as chaperones.[16] It has been found that NMNAT have neuroprotective properties, especially with regard to axon degeneration and injury. It was reported in a study using Drosophila that there is a neuroprotective variant of NMNAT and an alternative splicing mechanism is used as a switch for regulation.[17]

The role of HSP in the pathogenesis of BD has been suggested by studies on animals and clinical studies. In a study on rats, proteome differential profiling was carried out after the rats were administered 2 antidepressant agents, venlafaxine and fluoxetine, which are monoamine reuptake inhibitors, for 2 weeks.[18] The protein patterns were obtained from hippocampal cytosolic extracts. Thirty-three protein spots were modulated compared with the controls for both drugs and HSP10 was among these 33 proteins. A study has been carried out on patients with BD to investigate what role heat shock factors and HSP might have in BD and their possible interactions with glucocorticoid receptor signaling alterations in BD.[13] Activity of the HPA-axis is regulated by glucocorticoids through binding to intracellular glucocorticoid receptor.[13,19] In the study, several methods were used to analyze lymphocytes from the BD patients and controls and the results suggested that the increased interaction in BD between glucocorticoid receptor and HSP70 as well as other observations might provide insight for understanding the role of these factors in BD pathogenesis.[13]

Stress-responsive proteins have been proposed for serving as biomarkers in a number of specific diseases. HSP70 has been found to be elevated in patients with chronic heart failure.[20,21] In patients with biliary atresia HSP90 was reported to be down-regulated and more altered than any other protein studied.[22] A cross-sectional study of patients with ulcerative colits found that HSP70 is induced in polymorphonuclear cells and that this HSP70 induction is correlated with anxiety and depression.[23]

Because the findings of a number of studies including proteomic studies indicate the likelihood of HSP playing a role in the pathogenesis of BD, there is a possibility that HSP might be useful as biomarkers for BD, which would be clinically valuable since no reliable biomarker for BD has so far been identified. Therefore, the aim of this preliminary study was to assess HSP as potential candidates for in vivo biomarkers for patients with BD, including BD patients with abnormal HPA axis activity.

2. Methods

2.1. Participants

This was a case-control study. Inpatients and outpatients were recruited from Beijing Anding Hospital between January 2011 and April 2014. Healthy controls were blood donors and hospital workers who received routine physical examination.

The inclusion criteria for the patients were the following: age 18 to 55 years; bipolar disorder, schizophrenia, or other types of affective disorders diagnosed according to the diagnostic criteria of the Diagnostic and Statistical Manual of Mental Disorders (DSM-IV) 4th version; not treated with antipsychotics within the prior month. Patients were excluded if they had a history of organic diseases of the central nervous system or other physical illnesses (such as liver dysfunction and kidney dysfunction); were addicted to alcohol; had a history of drug abuse; or their blood sample was not suitable for examination due to hemolysis, piarhemia, or defibrinated blood.

For the controls, the inclusion criteria were the following: age 18 to 55 years; results of routine physical examination and routine blood tests, blood chemistry tests, and endocrine examination were normal; no history of severe diseases or drug abuse.

Bipolar disorder was diagnosed according to the diagnostic criteria in the International Classification of Diseases (10th edition) (ICD-10). The analysis included 57 BD patients, 27 males and 30 females. Their ages ranged from 18 years to 50 years. The course of disease ranged from 3 months to 12 years (median: 2.2 years). The patients were divided into 2 groups, abnormal BD and normal BD, based on HPA-axis activity. The abnormal BD group included 33 patients, 18 males and 15 females, with abnormal levels of ACTH and/or cortisone (CORT). The normal BD group included 26 patients, 13 males and 13 females, with ACTH and CORT levels within the normal range (reference range of ACTH: 0–37 pg/mL; reference range of CORT: 5–25 μg/dL (AM)). This study was approved by the hospital ethics committee. All patients provided written informed consent for all procedures performed.

2.2. Collection of blood samples

2.2.1. Collection of serum samples. Fasting blood (5 mL) was collected at 6 to 8 AM and transferred into a vacuum tube without anticoagulant. After 1 hour, centrifugation was performed at 2000 g for 20 minutes at room temperature. The supernatant was harvested and aliquoted into Eppendorf tubes (500 μL/tube). The serum was stored at −80°C for later analysis. All the serum samples were thawed before being used for MALDI-TOF mass spectrometry.

2.2.2. Collection of plasma samples. Fasting blood (3 mL) was collected at 6 to 8 AM and transferred into an EDTA-3K anticoagulated tube, followed by centrifugation at 1500 g for 5 minutes. The plasma was collected and stored at −80°C for further use.

2.3. Measurement of plasma CPN60 and CPN70 concentrations

Plasma concentrations of CPN60 and CPN70 were measured using commercially available solid phase sandwich enzyme-linked immunosorbent assay (ELISA) kits (Enzo Life Sciences, New York), following the manufacturer's instructions. Intra-assay coefficients of variation were <5%, and interassay coefficients of variation were <13%.

2.4. Detection of ACTH and CORT

The chemiluminescence method was employed to detect ACTH and CORT. Sequential immunoassay was used to detect ACTH and the competitive method was used to detect CORT. Detection kits were purchased from Tianjin Depu Biotech and Medical Products Co, Ltd, China. A fully automated chemiluminescence immunoassay analyzer (IMMULITE 1000; Malvern, PA) was

used. The intrabatch coefficient of variation for ACTH and CORT was <10%.

2.5. Mass spectrometry of serum proteins

2.5.1. Sample preparation. The serum stored at −80°C was thawed on ice and then centrifuged at 10,000 rpm for 20 minutes. Then, 10 μL of supernatant was mixed with 20 μL of U9 buffer (9 mol/L urea, 20 g/L CHAPS, 10 g/L DTT, 50 mmol Tris-HCl, pH 9.0), followed by incubation at 4°C for 30 minutes under constant shaking. Then 370 μL of 100 mmol/L sodium acetate buffer (pH 4.5) was added.

2.5.2. Activation of WCX nano-magnetic beads. Weak cationic exchange (WCX) nano-magnetic bead suspension (50 μL) was washed with 100 μL of sodium acetate buffer twice (5 minutes for each).

2.5.3. Capture of serum proteins with WCX nano-magnetic beads. One hundred microliters of prepared serum were added to activated WCX nano-magnetic beads, followed by incubation for 1 hour under constant shaking. The mixture was placed on a magnetic separation plate, and the supernatant was removed 2 minutes later. Then, 100 μL of sodium acetate buffer was used to wash the beads twice (5 minutes for each). Next, 10 μL of 0.5% (v/v) TFA was used to elute the beads for 5 minutes. Subsequently, 5 μL of eluent was mixed with SPA solution of equal volume, followed by loading of the mixture (2 μL). After air-drying, the protein compositions were detected by using PBSII-c matrix-assisted laser desorption/ionization time-of-flight (MALDI-TOF) mass spectrometry (BioRad, Hercules, CA) and protein fingerprinting was delineated.

2.5.4. Quality control and repeatability. One serum sample for quality control was randomly added during each microarray (serum sample was provided by the Beijing Deyi Clinical Laboratory Medical Center). The mean coefficient of variation of the protein peak was <15%.

2.6. Flow cytometry of CPN10 on peripheral blood mononuclear cells

Fresh anticoagulated peripheral blood (2 mL) was slowly added onto lymphocyte separation solution (2 mL), followed by centrifugation at 400 g for 20 minutes. The middle layer (lymphocyte layer) was collected and transferred into a new tube, followed by addition of PBS. After centrifugation at 300 g for 5 minutes, cells were resuspended in PBS and the cell density was adjusted to 10^7 cells/mL. Then, 100 μL of cell suspension was added to a tube for flow cytometry, followed by addition of 500 μL of fixation solution. After incubation at 4°C for 30 minutes, centrifugation was carried out at 300 g for 5 minutes, and the supernatant was removed. Then, 2 mL of PBS was added, followed by centrifugation at 300 g for 5 minutes. The supernatant was removed, and 2 mL of membrane permeabilization solution was added, followed by centrifugation at 300 g for 5 minutes. The supernatant was removed, and 0.25 μg of CPN10 antibody was added into the cell suspension, followed by incubation at 4°C for 30 minutes. After addition of 2 mL of membrane permeabilization solution, centrifugation was performed at 300 g for 5 minutes. The supernatant was removed, and cells were washed again. The supenatant was removed, and the remaining 100 μL of cell suspension was mixed with 0.25 μg of Goat anti-Rabbit IgG (H&L) Alexa Fluor488 secondary antibody, followed by incubation in the dark for 30 minutes at 4°C. After addition of 2 mL of membrane permeabilization solution, centrifugation was carried out at 300 g for 5 minutes. The supernatant was removed, and 2 mL of PBS was added, followed by centrifugation at 300 g for 5 minutes. After addition of 0.5 mL of PBS for washing, cells were stored at 4°C for flow cytometry. At least 20,000 mononuclear cells were counted. Cells were circled to exclude cell debris and adherent cells. The green fluorescence intensity was measured. The CPN10 positive rate and the mean Alexa Fluor488 fluorescence intensity of the whole cell population were determined according to the isotype group.

2.7. Statistical analysis

Mean and standard deviation were calculated for CPN10. Box plots were illustrated for data on the level of CPN60 and CPN70, which were composed of the 1st quartile (P_{25}, the bottom of box), the 3rd quartile (P_{75}, the top of box), and median (the line in the box) due to skewed distributions. Independent *t* test was carried out to examine CPN10, and Mann–Whitney *U* test was performed to test the differences in CPN60 and CPN70, between patients with and without normal HPA axis activity. The correlations between CPN10, CPN60, CPN70, ACTH, and CORT were examined using Spearman rank correlation. A P value $< .05$ was considered statistically significant. All statistics were 2-sided and performed using SPSS statistical software (version 22.0, IBM Corp, Armonk, NY).

3. Results

For our previously published pilot study,[24] we recruited 40 patients with primary BD, 44 with schizophrenia, and 43 healthy controls. We used weak cationic exchange nano-magnetic beads for capturing serum proteins. We also used a MALDI-TOF-MS for detecting the protein expression profile. To analyze the data, Biomarker Wizard 3.1 and Biomarker Patterns Software 5.0 were used for screening of differentially expressed proteins. Then the diagnosis model of BD was established, and then further validated in a blinded manner. In the BD group and schizophrenia group, there were 36 differentially expressed proteins as compared with the control group ($P < .05$). Of these proteins, 17 differentially expressed proteins ($P < .01$) were selected, one of which had the M/Z of M10847.90, for the establishment of a linear model. The sensitivity and specificity were 95% and 91%, respectively, in the diagnosis of BD. Then, 76 patients were randomly recruited to validate this model. The results showed that the accuracy rate was 88.5% for BD, 88.9% for other mental diseases, and 87.0% for healthy controls with this model. After separation and purification, the 3 proteins (M10847, M11357, and M15887) were identified as chaperonin 10 (CPN10), serum amyloid A protein (SAA), and hemoglobin β chain (Figure S1, http://links.lww.com/MD/C311). Then, a protein fingerprinting technique integrating MALDI-TOF-MS and WCX nano-magnetic beads was employed to detect the profile of serum proteins with small molecular weight. The results showed that the peak of a protein with M/Z of 10847.9 in BD patients with abnormal HPA axis activity was down-regulated markedly when compared with patients with normal HPA axis activity.

For our present study, data on CPN10, CPN60, and CPN70 were obtained from different populations. For CPN10, data from 41 patients and 35 controls were used for analysis. For CPN60 and CPN70, data from 59 patients and 59 controls were used for analysis.

In the normal control subjects, the CPN10 level was significantly higher than in the patients ($P = .036$) (Fig. 1A). However, their CPN60 and CPN70 levels were significantly lower compared with the patients ($P = .009$ and $P < .001$, respectively) (Fig. 1B and C).

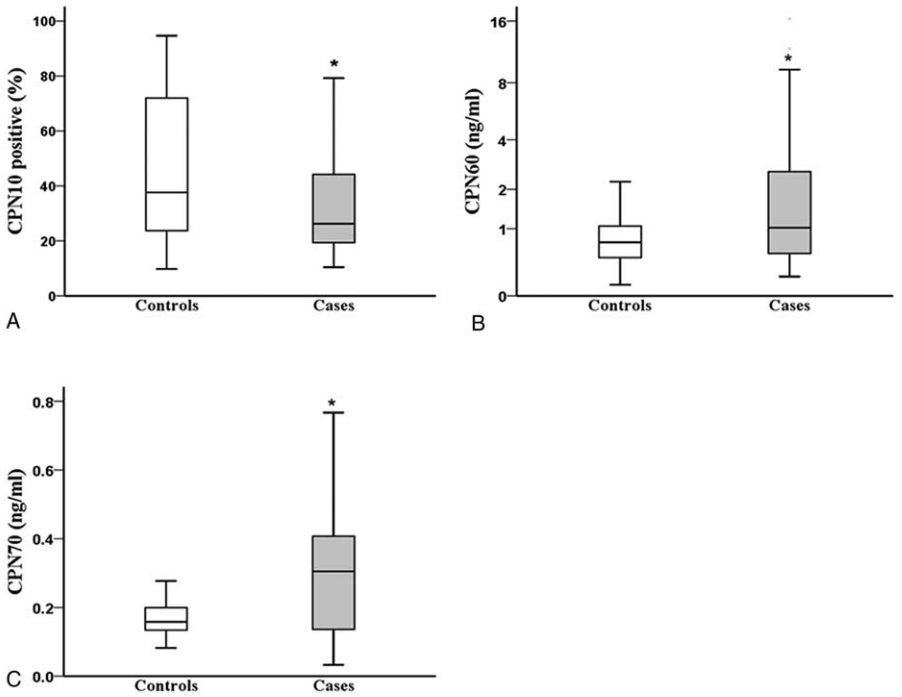

Figure 1. Comparison of level of CPN10 (A), CPN60 (B), and CPN70 (C) between healthy subjects and patients with bipolar disorder. Data are presented as box plots consisting of the first quartile (P_{25}, the bottom of box), the third quartile (P_{75}, the top of box), and median (the line in box). Mann–Whitney *U* test was implemented. Asterisk indicates significant difference between the 2 groups, $P < .05$.

For the comparisons between normal and abnormal HPA activity in the BD group, only CPN60 was found to be significantly lower in the abnormal BD group compared with the normal BD group ($P = .002$); there were no significant differences in CPN10 and CPN70 (Fig. 2).

As for correlations of CPN10, CPN60, and CPN70 with ACTH and CORT (Fig. 3A–F), a significant inverse correlation was found in the patients overall with regard to CPN60; the CPN60 level increased as the ACTH level decreased ($\rho = -0.420$,

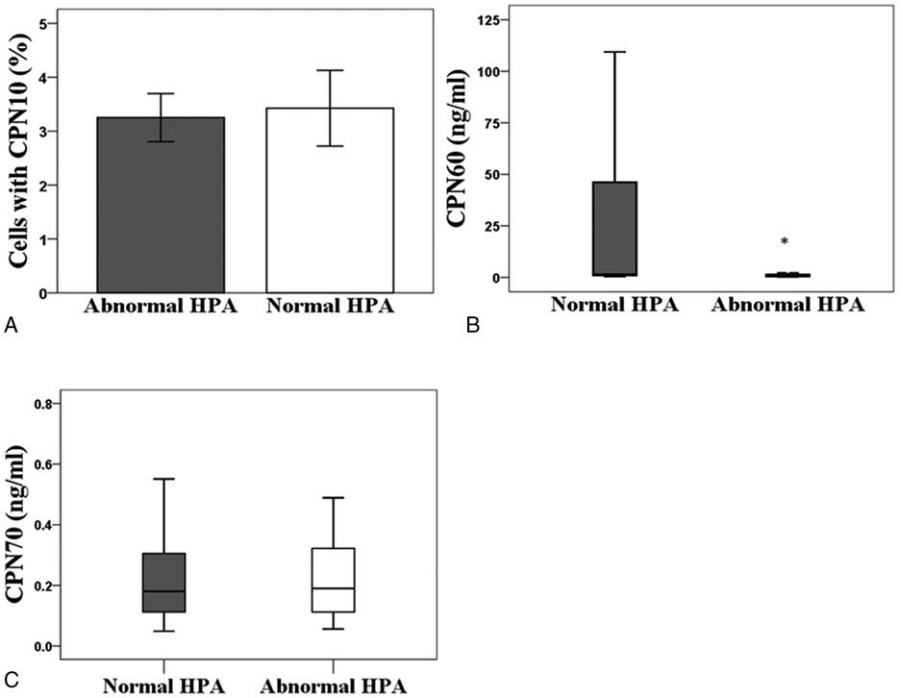

Figure 2. Comparison of Level of CPN10 (A), CPN60 (B), and CPN70 (C) between bipolar disorder patients with abnormal and normal HPA axis activity. Data on CPN10 are shown expressed as mean standard deviation and tested by independent sample *t* test. Data on CPN60 and CPN70 are presented as box plots consisting of the first quartile (P_{25}, the bottom of box), the third quartile (P_{75}, the top of box), and median (the line in box). Mann–Whitney *U* test was implemented. Asterisk indicates significant difference between the 2 groups, $P < .05$.

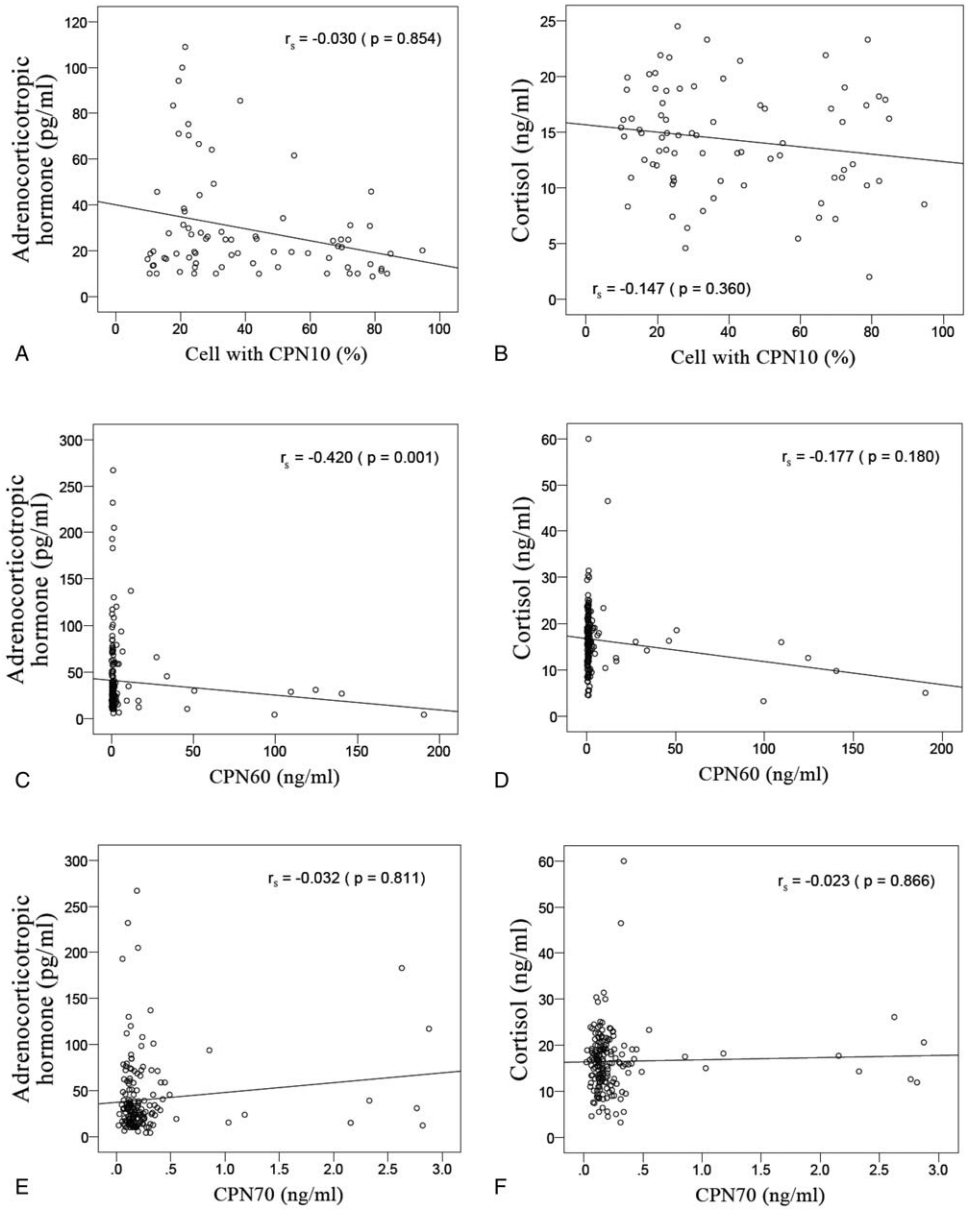

Figure 3. Correlations of CPN10, CPN60, and CPN70 with adrenocorticotropic hormone (ACTH) and cortisone (CORT).

$P = .001$). Also, a significant correlation with CPN60 and ACTH was found in patients with bipolar depression or bipolar hypomania (bipolar depression: $\rho = -0.482$, $P = .037$; bipolar hypomania: $\rho = -0.561$, $P = .030$) (Table 1) and CPN70 had positive correlation with ACTH in bipolar depression and hypomania patients.

4. Discussion

The main findings of this study were that BD patients had significantly higher levels of the CPN60 and CPN70 than the controls, that the abnormal HPA axis BD group had significantly lower levels of CPN60 than the normal HPA axis BD group, and

Table 1

Correlations of CPN10, CPN60, and CPN70 with adrenocorticotropic hormone (ACTH) and cortisone (CORT) according to 3 subtypes of bipolar disorder.

	Bipolar depression (n = 19)		Bipolar hypomania (n = 15)		Bipolar depression and hypomania (n = 25)	
	CORT (μg/dL)	ACTH (pg/mL)	CORT (μg/dL)	ACTH (pg/mL)	CORT (μg/dL)	ACTH (pg/mL)
CPN60 (ng/mL)	−0.264 (0.274)	−0.482 (**0.037**)	−0.125 (0.657)	−0.561 (**0.030**)	0.109 (0.603)	−0.191 (0.359)
CPN70 (ng/mL)	−0.167 (0.495)	−0.418 (0.075)	−0.029 (0.919)	−0.307 (0.265)	0.107 (0.612)	0.474 (**0.017**)

Data are presented as Spearman correlation coefficient (*P* value).
Bold value indicates significant correlation, $P < .05$.

that in BD patients CPN60 levels increased as ACTH levels decreased. The results provide additional evidence that HSP are involved in BD and suggest that these proteins potentially could be useful as biomarkers for BD and for distinguishing BD patients with abnormal HPA axis activity from BD patients with normal HPA axis activity. Although biomarkers are commonly used in medicine, the use of biomarkers in psychiatry is a relatively recent development.[25] There is evidence suggesting that in BD there are changes in the serum which occur before there is any indication of symptoms.[25] It seems likely that biomarkers will play a much larger role in the future in psychiatry then is now the case and our findings might eventually contribute to this likely increased role of biomarkers in psychiatry.

There is considerable evidence that increased activity of neurons that release corticotropin-releasing factor plays an important role in depression.[26] Since there are a number of factors that modulate the expression of corticotropin-releasing factors, a study by Wang et al[26] was performed to investigate the expression of these factors in the hypothalamus in depressed patients. Postmortem hypothalamus specimens for depressed patients and controls were compared and it was found that there were abnormal expression levels of several receptors that affect corticotropin-releasing factor neurons. With regard to HSP, no alterations in the expression of CPN70 or CPN90 were observed. This appears to somewhat conflict with our finding that BD patients had higher levels of CPN70 than the controls; however, our study only examined BD patients whereas the study by Wang et al included patients with depression, and we measured blood levels whereas Wang et al used postmortem specimens of hypothalamus.

Understanding the relationship between BD and HPA axis abnormalities has become an important focus of research. One strategy that is being used to further understanding the role of abnormal HPA axis activity in BD has been to obtain proteomic profiles. Stelzhammer et al[27] employed this strategy using postmortem pituitary specimens from BD and major depressive disorder patients. The BD patients were found to have increased levels of a major pituitary hormone, pro-opiomelanocorin. The level of this hormone was not increased in patients with major depressive disorder. Recently, Belvederi Murri et al[2] performed a meta-analysis that included 41 studies to gain further understanding of how HPA axis activity is involved in BD pathophysiology. Associations were found between BD and both ACTH and cortisol whereas there was no association between BD and corticotropin-releasing hormone. The authors suggested that therapeutic targeting of HPA axis dysfunction might prove to be valuable for achieving better outcomes for BD patients.

One of the functions of HSP is to regulate the folding and function of glucocorticoid receptor. To investigate how HSP and heat shock factor, which is involved in HSP transcription, play a role in BD, including how they interact with glucocorticoid receptor, Bei et al[12] studied lymphocytes from BD patients. The patients compared with controls had increased levels of CPN70-glucocorticoid receptor heterocomplex. This observation could be useful for understanding the role of CPN70 in BD. In our study the BD patients had increased levels of CPN70 suggesting that this HSP might have potential value as a biomarker for BD.

A limitation of our study is that we did not statistically compare baseline demographic characteristics between the BD patients and controls. There may have been significant differences between the groups in demographic characteristics although it seems unlikely that would have affected the results.

In summary, our findings in this preliminary study suggest that HSP potentially might be useful as a biomarker of BD and for distinguishing BD patients with abnormal HPA axis activity from BD patients with normal HPA axis activity. Our results may eventually be useful for making an earlier diagnosis and therefore being able to initiate treatment for BD earlier.

Author contributions

Conceptualization: Yuhang Cheng, Zhili Li.
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Writing – review & editing: Yuhang Cheng.

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