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β-actin as a loading control for plasma-based Western blot analysis of major depressive disorder patients

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Short title: β-actin as a plasma-based Western blot LC in MDD

Subject category: Immunological Procedures
Abstract

Western blot analysis is a commonly utilized technique for determining specific protein levels in clinical samples. For normalization of protein levels in Western blot, a suitable loading control is required. On account of its relatively high and constant expression, β-actin has been widely employed in Western blot of cell cultures and tissue extracts. However, β-actin’s presence in human plasma and this protein’s putative role as a plasma-based loading control for Western blot analysis remain unknown. In this study, an enzyme-linked immunosorbent assay was used to determine the concentration of β-actin in human plasma, which is 6.29 ± 0.54 ng/ml. In addition, the linearity of β-actin immunostaining and loaded protein amount was evaluated by Western blot, and a fine linearity (R²=0.974 ± 0.012) was observed. Furthermore, the expression of plasma β-actin in major depressive disorder subjects and healthy controls was compared. The data revealed no statistically significant difference between these two groups. Moreover, the total coefficient of variation for β-actin expression in the two groups was 9.2 ± 1.2%. These findings demonstrate that β-actin is present in human plasma and may possibly be used as a suitable loading control for plasma-based Western blot analysis in major depressive disorder.

Key words: β-actin, loading control; Western blot; major depressive disorder; plasma

Abbreviations used: HC, healthy controls; LC, loading control; HDRS, Hamilton Depression Rating Scale; CV, coefficient of variation.
Introduction

The development of global proteomic profiling since the mid-1990s has enhanced the discovery of new diagnostic biomarkers for various disease states [1]. As the field has advanced, the experimental methods involved have become increasingly sophisticated. This improved technological sophistication has enabled proteomic analysis of complex protein mixtures such as clinical samples. Currently, protein expression in clinical samples is quantitatively analyzed either by two-dimensional gel electrophoresis (2-D SDS-PAGE) followed by mass spectrometry (MS) or directly through gel-free MS [2]. Despite these technical achievements, the quantitization of protein expression in clinical samples still requires improvement [3, 4].

The MS-based orthogonal validation technique involving Western blot analysis shows promise as a semi-quantitative analytic method of measuring protein expression in clinical samples [5, 6], and may eventually serve as a platform for proteomic-based study across a wide variety of disease states. Major depressive disorder (MDD) is a serious widespread mental illness with a lifetime prevalence of 16% [7], but remains underdiagnosed due to the lack of empirical laboratory-based tools. This situation makes MDD an excellent disease candidate for applying the MS-based orthogonal validation technique with Western blot analysis.

A previous study by our group adopted a proteomic approach to examine alterations in protein expression within the hippocampus of a rat depression model. This work revealed several proteins that may be associated with the neurogenesis of depression [8]. Furthermore,
our previous proteomic investigation on plasma samples from MDD subjects has identified several key proteins that may serve as diagnostic biomarkers for MDD.

The MS-based orthogonal validation technique with Western blot analysis may enable semi-quantitation of protein expression associated with MDD. In order to advance this line of investigation, a rigorous Western blot analysis of plasma from MDD subjects is required. In Western blot analysis, an appropriate loading control (LC) is crucial to the accurate assessment of protein expression [9, 10, 11].

β-actin is a well-known housekeeping protein with high conservation that participates in a wide variety of eukaryotic cellular functions including muscle contraction, amoeboid movement, cytokinesis and mitotic division. β-actin has already been widely used as a LC in Western blot of cell cultures and tissue extracts. However, β-actin’s presence in human plasma and this protein’s putative role as a plasma-based LC for Western blot analysis remain unknown.

In this study, the feasibility of using β-actin as a plasma-based LC for Western blot analysis in MDD was assessed. This examination should facilitate future studies on the application of the MS-based orthogonal validation technique with Western blot analysis to MDD research.

Materials and Methods

Subjects

Six patients with MDD and six sex- and age-matched healthy controls (HC) were selected for the current study. Written informed consents were acquired from all subjects recruited for this
study. The Ethical Committee of Chongqing Medical University reviewed and approved the protocol of this study and the procedures for sample collection.

Study candidates were enrolled in the psychiatric center of the First Affiliated Hospital at Chongqing Medical University. A structured clinical interview assessing the relevant DSM-IV criteria was used to diagnose those candidates with a single depressed episode [12], and the 17-item version of the observer-rated Hamilton Depression Rating Scale (HDRS) was applied to define the severity of their depression [13]. Only depressed candidates with HDRS scores of greater than 17 were recruited into the study. Study candidates with one or more confounding factors, such as physical or mental disorders, were excluded.

HC were recruited from the medical examination center in the First Affiliated Hospital, Chongqing Medical University. HC who presented with a history of DSM-IV Axis I or Axis II diagnosis, systemic medical illness requiring treatment as well as neurological disorders were excluded from the present study. Clinical characteristics and details of clinical data of the subjects are shown in Table 1.

Sample preparation

Venous blood samples from MDD patients and HC were collected in 10 ml EDTA coated plastic tubes. The plasma was immediately separated by centrifugation at 3000 rpm for 15 min. 150 μl plasma aliquots were individually stored at -80°C until analysis. The total protein content was quantified by the Bradford method, according to the manufacturer’s instructions (Bio-Rad Laboratories).

Expression levels of β-actin in human plasma
Three samples from the MDD group and three samples from the HC group were randomly chosen. The concentration of β-actin in the plasma was measured by means of a Human β-actin ELISA Kit (BlueGene Biotechnology, Shanghai, China) according to the manufacturer’s instructions. The kit contained a 96-well test plate, standards of known β-actin concentrations, a β-actin antigen, a monoclonal anti-β-actin, a β-actin HRP conjugate, wash buffers, and a substrate solution. Plasma samples as well as standards of known concentrations were loaded into the appropriate wells on the antibody-precoated microtiter plate; 100 μl of each plasma sample or standard and 50 μl of conjugate were pipetted into each well. Plates were covered and incubated at 37°C for 1 h after mixing of the solutions. The wells were then washed, and 50 μl of the substrate for the enzyme was added to each well. After incubation for 15 min at 25°C, the wells were washed three times to remove unbound substrates. For each well, 50 μl of the stop solution was added and properly mixed.

OD at 450 nm was immediately measured utilizing an ELISA microplate reader. Consequently, a calibration curve was constructed using OD values corresponding to each concentration of the standard, and the OD values of the samples were converted into β-actin concentrations. All results were expressed in micrograms per milliliter.

Effects of loading error and protein transfer on β-actin expression

Previously described procedures [14] were used to verify that any observed variability in the β-actin expression level was not due to loading error and/or protein transfer effects. Two samples from the MDD group as well as two from the HC group were randomly chosen, and six lanes were loaded with the same sample. The β-actin intensity of each lane from the same
sample was then analyzed by Western blot. The loading error was evaluated via the coefficient of variation (CV) value.

As for the Western blot procedure, the plasma samples were diluted with 0.1 M phosphate buffered solution (PBS) and Laemmli buffer (5×) (10% (W/V) SDS, 50% (v/v) glycerol, 50 mM dithiotreitol (DTT), 500 mM Tris-HCl (pH 6.8), and 0.5% (w/v) traces of bromophenol blue) to a protein concentration of 2 μg/μl and then heated at 99°C for 5 min. Samples (5 μg/lane) were run on a 10% SDS-PAGE gel, and then the protein was transferred to polyvinylidene fluoride (PVDF) membranes (Millipore, Billerica, MA, USA). Then, the membranes were blocked in a 5% (w/v) skimmed milk solution for 1 h at room temperature and incubated overnight at 4 °C with primary rabbit polyclonal anti-β-actin (1:3000, bioworld). After three 30-min washings in 100mM Tris-HCl buffer, pH 7.5, with 150mM NaCl and 0.05% Tween 20 (TTBS buffer), the membranes were incubated at 37 °C for 60 min with horseradish peroxidase-conjugated anti-rabbit IgG and washed three times for 30 min with TTBS buffer. Then, the membranes were developed with ECL reagents and the chemiluminescence signal was imaged using a ChemiDoc XRS (Bio-Rad, Hercules, CA, USA). The immunoblots were quantified using Quantity One software (Bio-Rad, Hercules, CA, USA).

The linearity of β-actin staining

To assess the linearity of β-actin staining versus the protein amount loaded per well, gels were loaded with 2.5, 5, 10, 15, 20, 25, and 30μg of total plasma protein from all 12 samples respectively, as previously described [15] with the noted minor modifications. For the Western blot analysis, the samples were electrophoresed and the blots were processed as described above. Linearity was assessed using the correlation coefficient, which described the linear
relationship between the β-actin band intensity and the total amount of plasma protein [15; 16].

**Effect of depression on β-actin protein expression**

Two sets of samples, the depressed group (n=6) and the control group (n=6), were resolved by SDS-PAGE. To normalize for protein content, one set was stained with Coomassie Blue as a LC [17]. Each sample (5 μg/lane) was run on a 10% SDS-PAGE gel, fixed by 40% (v/v) methanol, 10% (v/v) acetic acid, and stained with 0.1% (w/v) colloidal Coomassie Brilliant Blue R-250 for 2h, washed with water for 1 min, and then destained for 2h in 40% (v/v) methanol, 10%( v/v) acetic acid. Gels were scanned and analyzed using Quantity One software (Bio-Rad, Hercules, CA, USA). Meanwhile, the other set was transferred to PVDF membrane [18, 19] and analyzed by Western blot as described above. The variation of β-actin expression in plasma from MDD patients and HC was evaluated by total CV. Triplicate tests were carried out to ensure the robustness of data generated.

**Statistical Analysis**

Data were presented as mean ± SD values. The statistical analyses were carried out using SPSS software. A student t-test was used to determine statistically significant differences between the two groups. All tests were two-tailed. The significance level was set at P<0.05.

**Results**

**Expression levels of β-actin**

The concentration of β-actin in human plasma is 6.29 ± 0.54 ng/ml. Meanwhile, the concentration of total protein in plasma quantified by the Bradford method was 70.74 ± 8.18 mg/ml (Table 2), while the ratio of β-actin to total protein in human plasma is 9.4×10⁻⁸: 1.
Minor effects of loading error and protein transfer on β-actin expression

The mean CV of β-actin expression for the six lanes of the same sample from the two groups was 4.41±1.06% (MDD: 2.99% and 4.78%, HC: 4.37% and 5.51%). This finding illustrates that the effects of loading error and protein transfer in both groups were negligible.

Linear relationship between protein loading amount and β-actin expression

Correlation analysis was performed to analyze the relationship between β-actin expression and the total amount of plasma protein loaded per well. The mean $R^2$ value was 0.974±0.012. As displayed in Fig. 1, the use of β-actin staining in the set-up offered a fine linearity. This result indicates that β-actin can be regarded as a reliable indicator for total plasma protein loading.

Minute impact of depression on β-actin protein expression

The results uncovered a consistent trend of β-actin expression in both the original (Fig. 2A) and repeated (Fig. 2B and C) trials. There was no statistically significant difference in the expression of β-actin between the depressed and control groups (in triplicate: $t=1.287$, $P=0.227$; $t=0.171$, $P=0.870$; $t=0.774$, $P=0.466$). Meanwhile, the total CV of β-actin expression in the two groups was 9.2±1.2%. This finding suggests that the level of β-actin expression in the plasma of MDD patients and HC is comparatively constant.

Discussion

The plasma proteome contains a diverse set of proteins as numerous by-products of cellular signalling continuously filter into the general circulation [20]. As changes in the levels of these plasma proteins can reflect different pathological states (e.g., cardiovascular disease, thrombosis and cancer), the MS-based orthogonal validation technique with Western blot
analysis of the plasma proteome serves as a useful biomarker detection platform in certain diagnostic contexts [21]. However, MDD remains widely underdiagnosed on account of current clinical diagnostic tools. Thus, in order to improve outcomes in depressed populations, it is imperative to develop more empirical laboratory-based diagnostic methods for MDD. Plasma-based Western blot analysis may provide one platform for doing so.

In order to conduct an accurate Western blot analysis, normalization of protein expression levels through a suitable LC is essential to confirming correct protein loading. Despite β-actin's wide use as a LC in Western blot of cell cultures and tissue extracts, there is no reported evidence that β-actin may serve as a proper LC for plasma-based Western blot analysis. The current study illustrates that β-actin exists in human plasma, even if at low concentrations -- the possible reason being that β-actin is released into the extracellular space following cell death by initiating permeabilization of blood vessels, and also via extravasation of plasma proteins [22]. On another hand, our results indicate that β-actin may perform as a suitable LC for plasma-based Western blot analysis in MDD.

It should be noted that recent studies have revealed that β-actin functions as a poor LC in some circumstances. For example, when rat spinal cord cells are traumatically injured, the β-actin level is significantly perturbed [14]. Moreover, β-actin is also grossly over-expressed in renal tumors as compared to normal kidney tissue [23]. Taken together, these findings indicate that the use of β-actin as a LC should be discriminantly assessed on a case-by-case basis; the potential variability in certain instances (e.g., tumors [23], trauma [14]) presents a problem when applying β-actin as a LC for plasma-based studies due to cell death or cytolysis that may release β-actin in the plasma.
As to the staining procedure applied in this study, total protein staining by Coomassie blue has been shown to be an effective alternative to high-abundance single-protein controls to eliminate error produced by minute differences in loading [24]. Because Coomassie blue staining acts with high sensitivity and exhibits little staining variability in a protein gel, the total protein in one sample within a stained gel has been historically used as a LC for Western blot analysis [17, 18, 25]. However, there are downsides to this staining method; as compared to antibody detection of β-actin, the Coomassie blue staining procedure requires double sample loading, consumes more time and materials, and is overall far less convenient to implement.

Two limiting factors may adversely affect the conclusions of this study. First, the small sample size is a limiting factor; a larger, more diverse cohort would provide more reliable results. Second, recruited subjects were not excluded for previous or current antidepressant drug use. Although antidepressant therapy has not been reported to affect plasma β-actin expression in MDD patients, this possibility cannot be excluded. Further studies can address these limitations.

In conclusion, β-actin indeed exists in human plasma and may serve as a suitable LC in plasma-based Western blot analysis of MDD patients. Further studies should investigate whether other housekeeping proteins, such as β-tubulin and GAPDH, may also serve as suitable LC for plasma-based Western blot analysis in MDD.

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References


[6] R.R. Tremblay, E. Coulombe, S. Cloutier, C. Brunet, D. Deperthes, G. Frenette, and J.Y. Dube, Assessment of the trypsin-like human prostatic kallikrein, also known as hK2, in the


Figure Legends

Fig. 1. Linearity of β-actin immunostaining from a representative plasma sample.

(A) Plasma dilution series detected with β-actin. (B) Linearity assessed using the square of
the correlation coefficient (R^2). β-actin intensities plotted against total protein loaded per
well.

Fig. 2. Western blot analysis showing β-actin expression in plasma from MDD patients and
HC.

(A) Twelve samples blotted (upper panel). Coomassie Blue-stained gel acting as a LC
(middle panel). Results showing no statistical significant difference in β-actin expression in
plasma from the two groups (lower panel). Data presented as mean ± SD (n=6 per group).

(B,C) Analysis was conducted in triplicate.
A

\[ \beta\text{-actin} \]

\begin{array}{llllllll}
2.5 & 5 & 10 & 15 & 20 & 25 & 30 \\
\end{array}

(ug)

B

\[ R^2 = 0.980 \]

\begin{tikzpicture}
\begin{axis}[
    xlabel=protein per well(ug),
    ylabel=\(\beta\)-actin intensity of the plasma,
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    ymin=5000, ymax=30000,
    xtick={0,5,10,15,20,25,30},
    ytick={5000,10000,15000,20000,25000,30000},
    grid=both,
    legend pos=north west,
]
\addplot[only marks,mark=*] table[x expr=
\thisrow{x}+\thisrow{y},y=\thisrow{y}]
\end{axis}
\end{tikzpicture}

Figure 1
Figure 2
Table caption

Table 1. The performance characteristics of the investigated groups. The characteristics: sample size, sex, age, BMI, HDRS Scores and the patient’s treatment.

Table 2. Plasma $\beta$-actin and total protein levels in MDD and HC groups. The contents of comparison: plasma $\beta$-actin concentration, plasma total protein concentration.
Table 1

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* Treatment with paroxetine, Venlafaxine, mirtazapine and olanzapine

$^a$ Body Mass Index.

$^b$ not applicable.
Table 2

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<th>Plasma total protein concentration (mg/ml)</th>
<th>Sample NO.</th>
<th>Plasma β-actin mean concentration (ng/ml)</th>
<th>Plasma total protein concentration (mg/ml)</th>
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