Insulin-like growth factor 1 (IGF-1) expression is up-regulated in lymphoblastoid cell lines of lithium responsive bipolar disorder patients

Alessio Squassina a, *, Marta Costa a, Donatella Congiu a, Mirko Manchia b, c, Andrea Angius d, Valeria Deiana e, Raffaella Ardati e, Caterina Chillolli e, Giovanni Severino a, Stefano Calza f, Maria Del Zompo a, e

a Section of Neuroscience and Clinical Pharmacology, Department of Biomedical Sciences, University of Cagliari, Monserrato, Cagliari 09042, Italy
b Department of Psychiatry, Dalhousie University, Halifax, Nova Scotia B3J 2E2, Canada
c Department of Pharmacology, Dalhousie University, Halifax, Nova Scotia B3H 4R2, Canada
d Laboratory of Genomics, CRS4, Pula, Cagliari 09010, Italy
e Unit of Clinical Pharmacology of the University Hospital of Cagliari, Cagliari 09100, Italy
f Department of Biomedical Sciences and Biotechnologies, University of Brescia, Brescia 25123, Italy

ARTICLE INFO

Article history:
Received 28 March 2013
Received in revised form 11 April 2013
Accepted 11 April 2013

Keywords:
Bipolar disorder
Lithium response
Lymphoblasts
Lithium treatment in vitro
Genome wide expression

ABSTRACT

Bipolar disorder (BD) is a debilitating psychiatric disease characterized by alternating episodes of mania and depression. Among mood stabilizers, lithium is the mainstay for the treatment of BD, with approximately one-third of patients showing remission from episode recurrence. While there is evidence suggesting genetic load for lithium response in BD, its molecular underpinnings are still not completely understood. To identify genes potentially involved in (or correlated with) lithium response, we carried out a genome-wide expression analysis on lymphoblastoid cell lines (LCLs) from 10 BD patients responders (R) and 10 non-responders (NR) to lithium. We compared expression levels of the two groups and tested whether in vitro lithium treatment had different effects in LCLs of R compared to NR. At basal, 2060 genes were differentially expressed between R and NR while no genes were differentially regulated by lithium in the two groups. After pathway analysis based on the 2060 genes, 9 genes were selected for validation with qRT-PCR. Eight genes were validated in the same sample of LCLs while only insulin-like growth factor 1 (IGF-1) was significantly over-expressed in R compared to NR in the same sample as well as in an independent sample comprised of R and NR (sample 1, fold change = 1.94; p = 0.005; sample 2, fold change = 2.21; p = 0.005). IGF-1 was also significantly over-expressed in R but not in NR when compared to a sample of non-psychiatric controls. Our findings suggest that IGF-1 may be involved in lithium response, supporting further investigation on its potential as a biomarker.

© 2013 The Authors. Published by Elsevier Ltd. Open access under CC BY-NC-ND license.
1. Introduction

Bipolar disorder (BD) is a severe and debilitating psychiatric disease characterized by extreme changes in mood and behavior. It has a prevalence of 0.8–1.2% in the general population [1–3] and constitutes a major cause of disability and premature mortality [4].

Among mood stabilizers, lithium is considered the first line treatment for acute episodes and maintenance of BD. After more than 50 years of use there is general consensus that at least 30% of patients treated in naturalistic settings shows long-term stabilization of symptoms [5–7]. However, 50–60% of treated patients have insufficient clinical response and require adjunctive or alternative mood stabilizing therapies [8]. In addition, lithium has a range of common and rare side effects that can have a substantial impact on long-term treated patients [9].

In this context, research has focused on the identification of predictors of lithium response in BD. Clinical studies have shown that lithium responders have distinct clinical features compared to non-responders, such as an episodic clinical course, family history of BD and absence of rapid cycling [10]. Moreover, response to lithium appears to be familial, [11] suggesting that genetic predisposition may constitute a key factor.

The majority of pharmacogenetic studies of lithium response have investigated genes putatively involved in the mechanism of action of lithium or in the etiology of BD while only two genome-wide association analysis have been performed so far [12]. These studies provided promising findings for a number of genes, but replication in independent samples and functional investigations are needed to further explore and clarify their role in lithium response. Other studies have focused on gene and protein expression measurements in peripheral tissues such as fresh blood or lymphoblastoid cell lines (LCLs) sampled from BD patients characterized for lithium response. Evidence from these studies suggested that altered protein levels of brain derived neurotrophic factor (BDNF) [13] and expression of Synapsin II (SYN2) gene [14,15] may be involved in lithium response or serve as peripheral markers.

Genome-wide investigation of gene expression in complex phenotypes represents a valuable tool in pharmacogenomics as it has the potential to provide functional-based hypothesis for further investigation. This approach is of particular importance in pharmacogenetic studies on drugs that, as lithium, are still the object of investigation to shed light on their mechanism of action. To date, only one study carried out a genome-wide expression analysis in peripheral tissues from BD patients characterized as responders (R) and non-responders (NR) to lithium [16]. However, the definition of response in these patients was based on variations in scores of the Hamilton Depression Rating scale during an eight-week open-label trial. Therefore, findings from this study are hardly comparable with the literature of lithium pharmacogenetics, as these studies used for the most part patients characterized prospectively or retrospectively for response to maintenance treatment with lithium.

Here we report the findings of the first genome-wide expression study in BD patients who were characterized as R and NR to maintenance treatment with lithium. We measured expression levels in LCLs from these patients and compared the two groups. We also tested whether in vitro treatment with lithium differentially affected the expression patterns in R compared to NR. The top genes selected from the microarray analysis were tested for validation by quantitative Real-Time PCR (qRT-PCR) in the same sample of LCLs as well as in an independent sample of BD patients characterized for lithium response. The aim of our study was to identify genes involved in lithium response and that could constitute new potential biomarkers.

2. Materials and methods

2.1. Subjects

Twenty patients with a diagnosis of BD type I (BDI) were selected from an existing BD sample recruited at the Lithium Clinic of the Clinical Psychopharmacology Centre of the University Hospital of Cagliari, Italy. Details of the clinical characterization and assessment of lithium response have been previously published [17]. Briefly, a lifetime consensus diagnosis according to Diagnostic and Statistical Manual of Mental Disorders (IV) (DSM-IV) criteria was obtained using personal semi-structured interviews [18] and a systematic review of patients’ medical records. Clinical response to lithium maintenance treatment was assessed using the “Retrospective Criteria of Long-Term Treatment Response in Research Subjects with Bipolar Disorder”, as described previously [11,21,22]. Briefly, the scale measures the degree of improvement in the course of treatment (score A) weighted against a number of clinical factors considered relevant in determining whether or not the improvement observed is due to lithium treatment (score B). The degree of response for each patient is quantified with a score from 0 to 10 (total score), obtained by subtracting the score B from the score A. Patients with total score (TS) equal to 7 or higher are considered responders (R) to treatment.

Of the 20 patients included in this study, 10 were R and 10 were NR, the latter defined as subjects with a TS equal to 0. A second sample set of 12 BDI patients (6R and 6 NR, Table 1) was selected to test for replication the identified genes. The expression of genes replicated in the independent sample was also measured in LCLs from non-psychiatric controls, comprised of 8 subjects recruited from blood donors screened with direct interview for the absence of personal or family history of any psychiatric disorder. The independent sample and the control groups were matched for age and sex with the microarray sample and did not differ for any of the variables tested (Table 1). The study was approved by the ethics committee of the Teaching Hospital of Cagliari (Italy) and informed written consent was obtained from all participants.

2.2. Cell cultures

Lymphoblastoid cell lines were established from fresh blood by transforming lymphocytes with Epstein–Barr virus (EBV) following standard procedures [23]. At the reaching of the confluence state, each cell line was adequately prepared for storage in liquid nitrogen.

For the present study, LCLs were thawed and regrown in RPMI-1640 medium, supplemented with 15% fetal bovine serum, 1% penicillin/streptomycin, 1% L-glutamine 200 mM and 1% sodium pyruvate 100 mM (Sigma–Aldrich, St. Louis, MO, USA). Once LCLs reached the required cell count (6–9 × 10⁶ cells), two equivalent aliquots were transferred into separate flasks, one containing medium with 1 mM lithium chloride (LiCl) and the other one containing a drug-free medium. Each flask was cultured at 37 °C in a humidified incubator with 6% CO₂. After 1 week of treatment, in which continuous presence of 1 mM LiCl was warranted, cells were harvested for RNA isolation.

2.3. RNA extraction and microarray procedures

Total RNA was extracted from cell pellets using TRIreagent solution (Ambion, Austin, TX) and quantified with a NanoDrop ND–1000 spectrophotometer (Thermo Fisher, Waltham, MA, USA). Quality was considered adequate when the A260/280 ratio was in the range of 1.8–2.0. RNA integrity was checked by the Agilent 2100 Bioanalyzer (Agilent, Santa Clara, CA, USA) using a RNA 6000 Nano Chip
Table 1
Demographic and experimental-relevant information of the samples studied.

<table>
<thead>
<tr>
<th>Microarray sample</th>
<th>Independent sample</th>
</tr>
</thead>
<tbody>
<tr>
<td>Subjects (M/F)</td>
<td>R</td>
</tr>
<tr>
<td></td>
<td>10 (5/5)</td>
</tr>
<tr>
<td>Age at sampling, yr ± SD</td>
<td>46.6 ± 17.5</td>
</tr>
<tr>
<td>LCL frozen storage, yr ± SD</td>
<td>8.8 ± 4.7</td>
</tr>
<tr>
<td></td>
<td>NR</td>
</tr>
<tr>
<td></td>
<td>p</td>
</tr>
<tr>
<td></td>
<td></td>
</tr>
</tbody>
</table>

R = responders, NR = non-responders, yr = years.

Fig. 1. Analysis flow. LCLs = lymphoblastoid cell lines, R = responders, NR = non-responders, Li = lithium, FDR = false discovery rate, FC = fold change.
2.5. Statistics

2.5.1. Microarray data analysis

An analysis flow is reported in Fig. 1. GeneChip data quality control was performed using Expression Console Software (Affymetrix, CA, USA). Raw data were preprocessed based on the Robust Multi-array Average algorithm (RMA) for normalization and summarization [24]. Data were filtered to remove duplicated or missing Entrez IDs. Genes were tested for differential expression between R and NR both at basal and after in vitro lithium treatment using linear models. Significance was defined based on a false discovery rate (FDR) threshold of 5% [25]. The analyses were performed using the Bioconductor [26] and the limma [28] packages implemented in R software [27].

We carried out pathway analysis using genes showing statistically significant difference in the comparison of R versus NR. The FDR threshold of 5% allowed us to include the largest possible number of genes at this step. This approach was employed to implement a functional-based interpretation of findings for selecting genes to be validated. Pathways were identified based on the hypergeometric test for over-representation of specific terms in the Kyoto Encyclopedia of Genes and Genomes (KEGG) within the selected list of genes.

Genes to be validated with qRT-PCR were selected from the list generated by KEGG analyses according to the following criteria: (a) genes comprised in pathways significantly over-represented in the comparison of R versus NR and (b) with fold change (FC) of the difference between R and NR ≥ 1.20 or ≤ 0.80.

2.5.2. qRT-PCR data analysis

Relative expression levels were measured by means of the comparative Ct method (ΔΔCt) using GAPDH as endogenous control. Fold changes of the difference between R and the other groups were calculated with \(2^{-\Delta\Delta Ct}\) equation. Differences between R and NR were analyzed with the Mann–Whitney U-test. ANOVA with Tukey's multi comparison test was used to compare the expression of R, NR, and controls. The significance threshold was set at \(p < 0.05\). Statistical tests were carried out using SPSS statistical software package v20 (SPSS, Inc., Chicago, IL, USA) and GraphPad Prism version 5.00 for Windows, GraphPad Software, San Diego, CA USA.

3. Results

3.1. Microarray data and pathway analysis

A total of 2060 genes were differentially expressed between R and NR at FDR < 5%; 1095 genes were up-regulated and 965 were down-regulated in R. At the same FDR threshold, no genes were differentially influenced by in vitro lithium treatment in R and NR. KEGG analysis based on the 2060 genes showed that 20 pathways were significantly over-represented in R compared to NR (Table 2). These pathways comprised 230 genes, of which 120 had a FC > 1.20 or < 0.80: 68 were up-regulated and 44 down-regulated in R. A complete list of the 230 differentially expressed genes is reported in Table 2 of the supplementary material. Phosphatidylinositol-4,5-bisphosphate 3-kinase, catalytic subunit gamma (PIK3CG; FC = 2.52; FDR = 0.0005) was the most significantly over-expressed gene in R compared to NR, while phospholipase D1, phosphatidylycholine-specific (PLD1) was the most under-expressed (FC = 0.31; FDR = 0.016).

Supplementary material related to this article found, in the online version, at http://dx.doi.org/10.1016/j.jphrs.2013.04.004.

4. Discussions

In this study we conducted a genome-wide expression profiling of lymphoblasts from BD patients responders and non-responders to maintenance treatment with lithium. Among the genes validated with qRT-PCR, only IGF-1 was significantly over-expressed in R.

Table 2

| Pathway analysis with KEGG: comparison of responders versus non-responders. |
|--------------------------|--------|-----|
| Comparison of basal expression levels | OR    | p   |
| Protein processing in endoplasmic reticulum | 1.927  | 0.001 |
| B cell receptor signaling pathway | 2.408  | 0.001 |
| Inositol phosphate metabolism | 2.629  | 0.002 |
| Prostate cancer | 2.152  | 0.002 |
| Pancreatic cancer | 2.291  | 0.003 |
| Acute myeloid leukemia | 2.430  | 0.003 |
| Colorectal cancer | 2.340  | 0.003 |
| Thyroid cancer | 2.997  | 0.007 |
| Chronic myeloid leukemia | 2.011  | 0.010 |
| Antigen processing and presentation | 1.977  | 0.013 |
| Phosphatidylinositol signaling system | 1.903  | 0.018 |
| Osteoclast differentiation | 1.626  | 0.022 |
| Liposome | 1.634  | 0.023 |
| Endocytosis | 1.461  | 0.026 |
| Citrate cycle (TCA cycle) | 2.437  | 0.028 |
| SNARE interactions in vesicular transport | 2.275  | 0.029 |
| Other glycan degradation | 3.096  | 0.032 |
| Pathways in cancer | 1.335  | 0.032 |
| Viral myocarditis | 1.824  | 0.032 |
| Folate biosynthesis | 4.536  | 0.034 |

The significance threshold was \(p < 0.05\). R = responders, NR = non-responders, OR = odds ratio.

3.2. Validation with qRT-PCR

Nine genes were selected for validation among the 120 genes based on the largest FC in up- or down-regulation. The list of these genes together with their KEGG pathways is reported in Table 3. These genes included: PIK3CG, interleukin 2 receptor, alpha (IL2RA), insulin like growth factor 1 (IGF-1), major histocompatibility complex, class II, DQ beta 1 (HLA-DQB1), par-6 partitioning defective 6 homolog gamma (PARD6G), PLD1, ms-related tyrosine kinase 1 (PLT1), cyclin A1, (CCNA1) and heat shock 70 kDa protein 1A/1B (HSPA1A/1B). Eight genes were validated by qRT-PCR in the same sample of LCLs used for the microarray study (Table 4). Insulin like growth factor 1 (IGF-1) was validated in both the microarray and the independent sample, being significantly over-expressed in R compared to NR in both samples (sample 1: FC = 1.94; \(p = 0.005\); sample 2: FC = 2.21; \(p = 0.005\)). We therefore compared IGF-1 expression levels of R and NR with healthy controls. IGF-1 was significantly over-expressed in R (FC = 1.63; \(p = 0.004\); adjusted \(p = 0.029\)) but not in NR (FC = 0.68; \(p = 0.105\); adjusted \(p = 0.410\); Fig 2).

Table 3

| Genes selected for validation and their pathways according to KEGG analysis. |
|--------------------------|--------|-----|
| Gene          | Pathway                  |
| PIK3CG        | Inositol phosphate metabolism, B cell receptor signaling pathway |
| IL2RA         | Endocytosis               |
| IGF-1         | Prostate cancer           |
| HLA-DQB1      | Antigen processing and presentation |
| PARD6G        | Endocytosis               |
| PLD1          | Endocytosis; pathways in cancer |
| CCNA1         | Cell cycle; pathways in cancer |
| FLT1          | Endocytosis               |
| HSPA1A/1B     | Protein processing in endoplasmic reticulum |

Genes selected for validation were the most differentially expressed members of the reported pathways between the responders and non-responders.
Table 4
Analysis of microarray and qRT-PCR data for basal expression levels in responders and non-responders.

<table>
<thead>
<tr>
<th>Gene</th>
<th>Microarray sample</th>
<th>qRT-PCR</th>
<th>Independent sample</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>GeneChip</td>
<td>FC</td>
<td>p</td>
</tr>
<tr>
<td>FLT1</td>
<td>2.56</td>
<td>10.0 x 10^-5</td>
<td>0.0005</td>
</tr>
<tr>
<td>IL2RA</td>
<td>2.46</td>
<td>0.0007</td>
<td>0.0078</td>
</tr>
<tr>
<td>IGF1</td>
<td>2.41</td>
<td>1.6 x 10^-6</td>
<td>0.0002</td>
</tr>
<tr>
<td>HLA-DQB1</td>
<td>2.06</td>
<td>0.0027</td>
<td>0.0191</td>
</tr>
<tr>
<td>PARD6G</td>
<td>2.41</td>
<td>2.6 x 10^-6</td>
<td>0.0002</td>
</tr>
<tr>
<td>PDE11A</td>
<td>0.31</td>
<td>0.0021</td>
<td>0.0163</td>
</tr>
<tr>
<td>CCNA1</td>
<td>0.37</td>
<td>0.0001</td>
<td>0.0027</td>
</tr>
<tr>
<td>FLT1</td>
<td>0.42</td>
<td>0.0002</td>
<td>0.0041</td>
</tr>
<tr>
<td>HSPA1A/1B</td>
<td>0.56</td>
<td>2.5 x 10^-6</td>
<td>2.3 x 10^-6</td>
</tr>
</tbody>
</table>

*p-Values in bold are significant at p < 0.05; ns = not significant, FC = fold change, FDR = false discovery rate.

**Fig. 2.** Relative expression levels of IGF-1. The difference between responders and non-responders is highly significant (*"Tukey's p < 0.0001), while controls differ significantly from responders ("Tukey's p < 0.05) but not from non-responders (Tukey's p > 0.05). ns, not significant.

Compared to NR in the same sample of patients as well as in an independent sample of patients characterized for lithium response.

IGF-1 belongs to the super-family of insulin like peptides, which also comprises IGF-2 and insulin. It is involved in the modulation of a large number of cellular processes including apoptosis, autophagy, cell metabolism and vesicle trafficking, ultimately leading to the regulation of development, cell differentiation and survival [29].

IGF-1 is locally expressed in all tissues of the body but the circulating form of the protein is mainly produced by liver and pancreas [30]. This peptide crosses the blood-brain barrier but is also highly produced in the brain with the highest expression perinatally in cortex, hippocampus, cerebellum, brainstem, hypothalamus and spinal cord [31]. In the adult brain the synthesis is reduced but the regional distribution remains the same as perinatally. The effects of IGF-1 are primarily mediated by IGF receptor 1 (IGFR1), which triggers the phosphoinositide 3-Kinase/threonine-serine protein kinase B (PI3K/AKT) and the RAS-mitogen-activated protein kinase (MAPK) signaling pathways [32].

In the pathway analysis based on genes showing differential expression between R and NR, IGF-1 was a member of the prostate cancer pathway. Other 22 genes ascribed to this pathway were differentially expressed between R and NR in our dataset, though only IGF-1 was included in the validation analysis, being the most significantly altered gene between the two groups. These genes included elements previously shown to be involved in lithium mechanism of action, such as mitogen-activated protein kinase 3 (MAPK3), CAMP responsive element binding protein 1 (CREB1), CREB3, AKT1 and B-cell CLL/lymphoma 2 (BCL2) [33]. Further investigation is needed to explore whether these genes and their products play a role in modulating lithium response in BD.

The possible involvement of IGF-1 in BD is suggested by a number of evidence. IGF-1 gene is located on chromosome 12q23.2, a region previously reported to be in linkage with BD [34–38]. A recent study also reported positive association between IGF-1 polymorphisms and BD [39]. As lithium, IGF-1 is a potent neuroprotective factor, as shown by its beneficial effect in neurodegenerative processes [40,41], an effect that appears to be mediated by the activation of the PI3K/AKT pathway [42]. Numerous studies have also suggested that IGF-1 is involved in ameliorating depression and anxiety [43–45], likely through the stimulation of neurogenesis in the hippocampus [45,46]. Interestingly, neuroimaging studies have shown that hippocampal volumes are reduced in BD patients with limited lifetime exposure to lithium, whereas patients with at least 2 years of exposure had no significant difference with controls [47,48]. Lithium neuroprotection is likely mediated by the prevention of apoptotic-dependent cellular death through the interaction with neurotrophic factors and a large number of signaling pathways, including the PI3K/AKT pathway [49]. The neuroprotective effect of lithium and IGF-1 may also be mediated by their activity on glycogen synthase kinase 3-β (GSK-3β). GSK-3β is a serine-threonine kinase involved in a myriad of cellular processes including apoptosis, cell differentiation and neurogenesis [50]. GSK-3β is one of the most studied targets of lithium and substantial evidence supports that inhibition of GSK-3β is an important mechanism in treatments for mood disorders [51]. Recent evidence showed that both lithium and IGF-1 inhibit GSK-3β in vitro in breast cancer cells through two different mechanisms: IGF1 activates the PI3K/AKT and extracellular signal-regulated kinases (ERK) pathways while lithium directly inhibits GSK-3β through a magnesium-competitive mechanism [52].

In our study, IGF-1 was over-expressed in R compared to NR while lithium treatment in vitro did not influence IGF-1 expression. IGF-1 was also significantly over-expressed in R compared to healthy controls. These findings suggest that an over-expression of IGF-1 may be necessary for lithium to exert its effect and NR may have no sufficient IGF-1 activity to respond properly to lithium treatment. Lithium and IGF-1 may have a synergic effect on GSK-3β inhibition. Interestingly, in our microarray data IGF binding protein 4 (IGFBP4), a member of the IGFBP family involved in the regulation of IGF bioavailability, was under-expressed in R compared to NR (FC = 0.72, FDR = 0.007). IGFBP4 binds to IGF-1 with high affinity partially masking IGF residues responsible for the binding to IGFR1.
Acknowledgments

In conclusion, our study suggests that the over-expression of IGF-1 might be a biological marker of response to long-term treatment with lithium. Further investigation in larger independent samples is warranted to confirm our findings.

Conflict of interest

The authors declare no conflict of interest.

Acknowledgments

We would like to thank Maria Francesca Urru, Marco Marcelli and Franca Scintu for providing technical advice regarding the experimental set-up.


References


suggests pedigree psychiatry affective logy deficits produces of atritic factor-I association.

Genome-wide scan to 1997;275:661–5.


Ewald H, Flint T, Kruse TA, Mors O. A genome-wide scan shows significant linkage between bipolar disorder and chromosome 12q24.3 and suggestive linkage to chromosomes 1p22-21, 4p16, 6q14-22, 10q26 and 16p13.3. Molecular Psychiatry 2002;7:734–44.


