MicroRNA expression changes in lymphoblastoid cell lines in response to lithium treatment

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Abstract

Lithium (Li) is commonly used in the treatment of bipolar disorder (BD). However, the molecular mechanism of its action is not completely understood. MicroRNAs (miRNAs), a class of small RNA species are recognized as important regulators in post-transcription gene expression. To explore the role of miRNAs in Li’s action, we quantitatively analysed the expression patterns of 13 miRNAs in 20 lymphoblastoid cell lines (LCLs) with or without Li treatment in culture. Using paired t statistics in the analysis, we identified significant changes in seven of the 13 miRNAs tested in LCLs sampled at treatment day 4 (p < 0.05). Four of the seven significant miRNAs, miR-34a, miR-152, miR-155, and miR-221 consistently changed expression in the same LCLs at a longer treatment time-point, day 16 (Bonferroni p < 0.05). Interestingly, miR-221 and miR-34a also changed expression in rat hippocampus in response to Li treatment (Zhou et al. 2008), although in the opposite direction. We focused on the predicted target mRNAs of miR-221 and miR-34a, and identified 29 and ten targets that were strongly and inversely correlated to expression with the two miRNAs, respectively. Our results suggest that miRNAs are excellent candidates for the study of the molecular basis of Li’s treatment action in cell systems such as lymphocytes given limited access to the human brain.

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Key words: Lithium, post-transcription regulation, target gene.

Introduction

MicroRNAs (miRNAs) are endogenous non-protein-coding RNA species. These molecules of ~21–23 bases are important regulators in post-transcriptional gene expression (Bartel, 2004; Filipowicz et al. 2008; Kim & Nam, 2006). Current estimates indicate that >30% of all mammalian protein-coding messenger RNA (mRNA) species are potential targets of miRNAs (Filipowicz et al. 2008). Some miRNAs are believed to target hundreds, even thousands, of mRNAs; on the other hand a single mRNA may be regulated by multiple miRNAs (Filipowicz et al. 2008). Target mRNAs may be regulated by miRNA-mediated template cleavage or translation repression (Chekanova & Belostotsky, 2006; Eulalio et al. 2008; Filipowicz et al. 2008).

Currently, more than 700 human miRNA species are catalogued in the miRBase (Griffiths-Jones et al. 2008). Expression profiling of multiple normal tissue and cell types in humans has shown that many miRNA species are ubiquitously expressed, and only a small number of miRNAs account for most of the differences between cell lineages and tissues (Landgraf et al. 2007; Liang et al. 2007). In addition, several recognizable features have been identified, such as miRNAs localized in the same genomic cluster have correlated abundance patterns among tissues; intronic miRNAs and their host genes have correlated expression patterns. Interestingly, clustering analysis using the expression patterns of 345 miRNAs showed that brain, peripheral blood mononuclear cells (PBMC), thymus, adrenal gland, and testes tissues form a unique cluster separate from other tissues (Liang et al. 2007). In
particular, a distinct pattern of eight miRNAs are shared between brain and PBMC (Liang et al. 2007). Analysis of known miRNA targets suggests that their protein products are involved in neuronal development, cell fate decision, cell differentiation and proliferation, haematopoietic differentiation, apoptosis, and organogenesis (Ambros, 2004; Kato & Slack, 2008). Because of the importance of miRNAs in cellular process and function, there is enormous interest in the investigation of the role of miRNAs in disease aetiology, treatment response, and progression. Indeed, miRNAs have also been implicated in disease pathogenesis such as cancer (Calin & Croce, 2006; Fabbri et al. 2007; Thomson et al. 2006), Parkinson’s disease (Kim et al. 2007; Wang et al. 2008), and schizophrenia (Beveridge et al. 2008; Burmistrova et al. 2007; Hansen et al. 2007; Perkins et al. 2007). A recent publication shows that some miRNAs are commonly regulated in rat hippocampus treated with Li and VPA (Zhou et al. 2008). However, in humans, miRNA expression in response to lithium (Li) treatment, the most effective medication for bipolar disorder (BD) (Baldessarini et al. 2003; Baldessarini et al. 2006; Cade, 1949; Dunner, 1998; Guzzetta et al. 2007), has not been explored. Here we report a subset of miRNAs that are significantly regulated in lymphoblastoid cell lines (LCLs) in response to Li treatment.

**Materials and methods**

**LCLs**

We used 20 LCLs derived from bipolar I disorder (BD I) family members in the present study. These 20 LCLs, 10 BD individuals and 10 corresponding discordant unaffected siblings, were selected and matched by gender from the University of Michigan Depression Center Prechter Bipolar repository. All subjects provided informed consent for their material to be used in genetic studies, and the protocols were approved by the local IRB. All 20 cell lines were cultured under identical conditions, i.e. grown in RPMI-1640 media supplemented with 12% FBS, 2 mM l-glutamine, and 1% ampicillin/streptomycin in an incubator set at 37 °C with 5% constant CO₂. When the number of cells reached 1 x 10⁶/ml, we seeded cells from each cell line at 2 x 10⁶ cells/ml concentration in new T25 flasks. Two identical groups were created, and all cell lines were grown in the same environment except for the addition of 1 mM LiCl in the media of the treatment group. We sampled the cell cultures at three treatment time-points: days 4, 8, and 16. The samples were stocked as cell pellets at –80 °C until RNA extraction for TaqMan assays.

**Total RNA isolation**

We isolated total RNA from the cell pellets from each cell line with TRizol reagent according to the protocols from the manufacturer (Invitrogen, USA). RNA samples were then treated with the RNase-free DNase kit (cat. no. 79254, Qiagen, USA), and further cleaned with the RNeasy MinElute Cleanup kit (Qiagen) according to the manufacturer’s recommended protocols: purification of miRNA from animal cells using the RNeasy Plus Mini kit and RNeasy MinElute Cleanup kit (Qiagen). RNA quantity was determined on a ND-1000 spectrophotometer (NanoDrop Technologies Inc., USA). We also loaded 1 μg RNA on a 1% agarose gel for electrophoresis to examine for any potential visible DNA contamination. This procedure produces high-quality total RNA with A₂₆₀/A₂₈₀ ratio in the range of 1.8–2.0, and maximally maintains the integrity of RNA species as measured by an Agilent 2100 Bioanalyzer (USA).

**Quantitative analysis of miRNA expression**

We used TaqMan assays (Holland et al. 1991) to quantify the expression levels for selected miRNA species. We purchased commercially available quantitative real-time reverse transcription PCR (qRT–PCR) reagent kits from Applied Biosystems Inc. (ABI; USA), including TaqMan assays for selected human miRNA species and a control assay (RNU6B), TaqMan MicroRNA Reverse Transcription kit (Part no. 4366596), and TaqMan Universal PCR Master Mix (Part no. 4324018). All TaqMan assays were performed using a two-step procedure according to the supplier’s manual (Pat no. 4364031 Rev. B, ABI). First, we performed single-stranded cDNA synthesis from total RNA using the TaqMan MicroRNA Reverse Transcription kit. Briefly, for each 7 μl of reverse transcription (RT) master mixture, we combined 0.15 μl of 100 mM dNTPs, 1 μl of MultiScribe™ reverse transcriptase, 1.5 μl of 10 × RT buffer, 0.19 μl of RNase inhibitor, and 4.16 μl of nuclease-free water. Then the 7 μl RT master mixture was combined in a fresh tube with 5 μl total RNA (10 ng) and 3 μl RT primer (specific to each TaqMan assay), and gently mixed. The RT reactions were then performed on an iCycler thermal cycler (Bio-Rad, USA) programmed to incubate the reactions at 16 °C for 30 min, 42 °C for 30 min, and 85 °C for 5 min. The synthesized cDNA was diluted 10 times with H₂O and stored in a –20 °C freezer for qRT–PCR analysis.

Second, we carried out TaqMan qRT–PCR assays according to the assay developer’s recommended conditions (ABI) on a 7900HT Fast Real-Time PCR
false results. The other two (miR-24a, miR-144) were potentially introduced because there was only one base difference between these two sequences, and we were concerned that TaqMan probe cross-hybridization might introduce false results. The other two (miR-24a, miR-144) were not included because no ‘off the shelf’ TaqMan assays were available at ABI.

Second, in a preliminary miRNA microarray experiment (Lee et al. 2008) we identified a number of miRNAs that showed nominally significant changes in LCLs from BD subjects (n=7) compared to normal controls (n=7). We then used TaqMan assays to validate 10 of the nominally significant miRNAs from our microarray analysis, but unfortunately failed to validate the expression differences of the selected 10 miRNAs (H. Chen et al., unpublished observations). We included these 10 miRNAs in the current Li experiment because they were expressed in both brain and LCL, and hypothesized that culturing of LCLs with Li would identify expression differences relevant to the biology of Li.

Third, we elected to test one additional miRNA, miR-181b, following Zhou et al. (2008) not only because of its significant change in response to Li, but also because of its implication in schizophrenia (Beveridge et al. 2008), and therefore arguably related to BD. Expression changes of miRNAs in response to Li may contribute to the molecular basis of Li’s action in BD. This speculation is evidenced by some miRNAs that have been implicated in neuropsychiatric disorders (Beveridge et al. 2008; Burmistrova et al. 2007; Hansen et al. 2007; Kim et al. 2007; Perkins et al. 2007; Wang et al. 2008).

To determine the effect of gender and disease status on the expression of the 13 miRNAs, we performed analysis of variance (ANOVA) on the quantitative expression data, but did not identify any statistically significant changes between male and female samples, or between BD subjects and their discordant siblings. Therefore, we included all 20 samples in each group to test for significant changes induced by Li treatment. There is a possibility that the sample may not be homogeneous. However, clustering analysis using genome-wide gene (mRNA) expression profiles on the same LCLs did not separate the BD samples from corresponding siblings (H. Chen et al., unpublished observations). Since we did not identify significant miRNA changes in LCLs of BD subjects compared to corresponding siblings, our data indicate that Li’s effect on miRNA expression may be larger than the natural difference between bipolar subjects and controls. A larger sample size will be necessary to rule out the role of these miRNAs in the aetiology of BD. This speculation is evidenced by some miRNAs that have been implicated in neuropsychiatric disorders (Beveridge et al. 2008; Burmistrova et al. 2007; Hansen et al. 2007; Kim et al. 2007; Perkins et al. 2007; Wang et al. 2008).

### Table 1. TaqMan-based qRT–PCR analysis of a subset of miRNAs in 20 LCLs treated with Li for 4 d (LiD4) and the same 20 LCLs without treatment (nLiD4)

<table>
<thead>
<tr>
<th>miRNA ID</th>
<th>FC (LiD4/nLiD4)</th>
<th>t test p value</th>
<th>Corrected p value</th>
</tr>
</thead>
<tbody>
<tr>
<td>miR-221</td>
<td>1.43</td>
<td>0.000073</td>
<td>0.000945</td>
</tr>
<tr>
<td>miR-152</td>
<td>1.48</td>
<td>0.000405</td>
<td>0.005271</td>
</tr>
<tr>
<td>miR-15a</td>
<td>1.32</td>
<td>0.002684</td>
<td>0.034895</td>
</tr>
<tr>
<td>miR-494</td>
<td>0.69</td>
<td>0.003625</td>
<td>0.047131</td>
</tr>
<tr>
<td>miR-155</td>
<td>1.27</td>
<td>0.012045</td>
<td>n.s.</td>
</tr>
<tr>
<td>miR-181c</td>
<td>1.28</td>
<td>0.023387</td>
<td>n.s.</td>
</tr>
<tr>
<td>miR-34a</td>
<td>1.23</td>
<td>0.023917</td>
<td>n.s.</td>
</tr>
<tr>
<td>miR-605</td>
<td>1.17</td>
<td>0.083604</td>
<td>n.s.</td>
</tr>
<tr>
<td>miR-17-3p</td>
<td>0.94</td>
<td>0.346810</td>
<td>n.s.</td>
</tr>
<tr>
<td>miR-148a</td>
<td>1.07</td>
<td>0.535045</td>
<td>n.s.</td>
</tr>
<tr>
<td>miR-200C</td>
<td>0.98</td>
<td>0.739029</td>
<td>n.s.</td>
</tr>
<tr>
<td>miR-181b</td>
<td>1.01</td>
<td>0.939922</td>
<td>n.s.</td>
</tr>
<tr>
<td>miR-513</td>
<td>0.27</td>
<td>0.941175</td>
<td>n.s.</td>
</tr>
</tbody>
</table>

FC, Expression fold change; LCLs, lymphoblastoid cell lines; n.s., not significant.

System (ABI). Each qRT–PCR reaction was performed in 20 μl volume, which contained 10 μl TaqMan Universal PCR Master Mix, 1 μl of 20× TaqMan MicroRNA assay mix, and 9 μl of the 10× diluted single-stranded cDNA product. We used six replicates for each sample. We used SDS2.2.1 software (ABI) for quantification analysis in conjunction with the 2−ΔΔCt method (Holland et al. 1991) using RNU6B as the reference control for normalization. For significance testing, we performed two-tailed paired t tests.

### Results and Discussion

We performed TaqMan assays and quantitatively determined the expression levels of selected miRNAs in LCL cultures with and without LiCl supplement. Since the same LCLs were used in treatment and control cultures, we applied the paired t statistic in data analysis for significance testing. There were 13 miRNAs (Table 1) selected for testing of expression difference in the presence of Li. First, miR-34a and miR-221 were drawn from the list of six miRNAs (let-7b, let-7c, miR-24a, miR-34a, miR-221, miR-144) reported by Zhou et al. at the 46th ACNP Meeting (Zhou et al. 2007) prior to the full publication of their study (Zhou et al. 2008). We did not include let-7b and let-7c because there was only one base difference between these two sequences, and we were concerned that TaqMan probe cross-hybridization might introduce false results. The other two (miR-24a, miR-144) were
Among the 13 miRNAs tested, we identified seven of them expressed differentially in LCLs treated with Li for 4 d [fold change in expression (FC) >20%, \( p<0.024 \)], and four of the seven miRNAs (three increased and one decreased in expression), remained statistically significant after a Bonferroni stepdown correction for the 13 multiple tests (Table 1). We then focused on the analysis of these seven miRNAs in cells from day 8 and day 16 sampling points (Table 2). Relative to the non-treated parallel LCLs, four of the seven miRNAs tested were significantly regulated in the samples treated for 16 d; and none of them showed significant changes in the samples treated for 8 d. However, one miRNA (miR-221) approached a nominally significant change in the day 8 sample (FC = 1.25, \( p=0.0546 \)). The fold changes and significant \( p \) values for the seven miRNAs at all three time-points are given in Table 2. The inconsistency of day 8 data with that of days 4 and 16 may imply that there are several phases of action of Li, or indicate that miRNA expression change varies in the initial phase preceding the efficacy of Li in time.

The two miRNAs, miR-221 and miR-34a initially reported by Zhou et al. (2007), were down-regulated consistently in two independent samples of rat hippocampus in response to Li treatment (Zhou et al. 2008). We found that the expression of miR-221 and miR-34a were consistently up-regulated by Li in LCLs at two consecutive time-points. This discrepancy indicates a tissue-specific difference in response to Li treatment. At present, there are no documented direct comparisons between LCL and brain for miRNA expression changes in response to Li treatment. In terms of gene (mRNA) expression analysis, it has been shown that the LIM gene was up-regulated in post-mortem brains but down-regulated in LCLs from BD subjects compared to normal controls (Iwamoto et al. 2004). Clearly further work is necessary to compare and possibly correlate expression patterns of various species of mRNA and miRNAs in peripheral (and accessible) tissue with that of the central nervous system. The purpose of which is to explore the value of such peripheral expression patterns as biomarkers for disease and treatment outcome.

Focusing on the analysis of the changes in mRNA species induced by Li (H. Chen et al., unpublished observations), we found significantly inverse co-regulation (cut-off \( r < -0.3 \)) in 29 and 10 predicted target mRNAs for miR-221 and miR-34a, respectively (Table 3). Gene ontology (GO) database search using the EASE algorithms (Hosack et al. 2003) revealed that five genes (AP2A1, AP2B1, CD2AP, EIF1, and VCL) from the 39 targets are significantly enriched for three GO-defined biological processes, i.e. macromolecular complex assembly, protein complex assembly, and cellular component assembly. Here, we demonstrated a novel approach to understand cellular processes in response to Li treatment at the level of mRNA expression. Although we tested only 13 miRNAs, four of them (miR-34a, miR-152, miR-155, miR-221) were consistently up-regulated at treatment time-point days 4 and 16. We anticipate that Li may induce expression changes in a substantial number of miRNAs \textit{in vivo} and/or \textit{in vitro}.

Several hypotheses can be proposed based on our current results. First, Li induced miRNA expression changes leading to post-transcriptional regulation, which resulted in changes in the transcript abundance...
of target genes or in the levels of their protein products (Bartel, 2004). Therefore, identification of target mRNAs and determination of the targets’ physical transcript and protein product levels are important for further study of the mechanism of Li’s action through miRNA-regulated gene expression.

Second, there is evidence that Li regulated the expression of genes involved in the transcription machinery, such as the activator protein 1 family genes FOS, FOSB, and JUND in the brain (McQuillen et al. 2007) and LCLs (H. Chen et al., unpublished observations). The altered expression of transcription

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**Table 3.** Inverse correlation of expression changes in targets predicted by miRanda or TargetScan treatment with the changes of corresponding miRNAs in response to lithium

<table>
<thead>
<tr>
<th>miRNA</th>
<th>Symbol</th>
<th>( r^a )</th>
<th>miRanda score</th>
<th>TargetScan score</th>
<th>( p ) value( ^b )</th>
<th>FC( ^c )</th>
</tr>
</thead>
<tbody>
<tr>
<td>miR-34a</td>
<td>CDK5RAP1</td>
<td>−0.709191722</td>
<td>18.1925</td>
<td>n.a.</td>
<td>0.008731604</td>
<td>0.9683044</td>
</tr>
<tr>
<td></td>
<td>PLEKHC5</td>
<td>−0.56578046</td>
<td>16.6637</td>
<td>0.024409779</td>
<td>0.9871394</td>
<td></td>
</tr>
<tr>
<td></td>
<td>AIFM1</td>
<td>−0.54936926</td>
<td>17.6244</td>
<td>0.002365642</td>
<td>0.932673</td>
<td></td>
</tr>
<tr>
<td></td>
<td>ARPC4</td>
<td>−0.543646712</td>
<td>16.0927</td>
<td>0.014907286</td>
<td>0.9252348</td>
<td></td>
</tr>
<tr>
<td></td>
<td>TEX264</td>
<td>−0.50727484</td>
<td>15.9185</td>
<td>0.004776969</td>
<td>0.9401515</td>
<td></td>
</tr>
<tr>
<td></td>
<td>VCL</td>
<td>−0.48227557</td>
<td>n.a.</td>
<td>−0.13</td>
<td>0.000251854</td>
<td>0.9186163</td>
</tr>
<tr>
<td></td>
<td>TMEM55A</td>
<td>−0.427098928</td>
<td>15.9017</td>
<td>0.021254545</td>
<td>0.9871394</td>
<td></td>
</tr>
</tbody>
</table>

| miR-221 | STARD3NL | −0.617528613 | 17.0656 | n.a. | 7.30136 \( \times 10^{-4} \) | 0.875465 |
| | EIF1 | −0.56199565 | 17.4044 | 1.0736197 | 0.9544306 |
| | ZDHHC11 | −0.54157049 | 17.3494 | 0.794208 \( \times 10^{-4} \) | 0.9151195 |
| | YWHAG | −0.531232549 | 17.3494 | 0.09018329 | 0.8428797 |
| | SFRS3 | −0.524833531 | 15.9606 | 0.000157089 | 0.890522 |

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**FC,** Expression fold change; n.a., not available.

\( ^a \) Correlation coefficient.

\( ^b \) Student’s \( t \) test of mean changes in targets in lymphoblastoid cell lines (LCLs) induced by lithium treatment for 4 d.

\( ^c \) Mean fold changes in LCLs induced by lithium treatment for 4 d are shown.
factors can lead to increased or decreased expression of downstream genes including miRNAs. Therefore, transcription factors as miRNA targets are excellent candidate genes for genetic variant identification and association studies in determining the genetic basis of Li’s responsiveness.

Third, miRNAs response to Li treatment may serve as biomarkers for the understanding of different treatment outcomes in individual patients. In clinical practice, a lag-time of 2–3 wk is typical before the treatment takes effect. However, a substantial number of patients do not respond to this medication. Therefore, identification of reliable biomarkers in peripheral tissues (due to limited access to the brain) such as PBMCs or LCLs to predict treatment outcome is critical to the success in medication management.

The understanding of the molecular basis of Li’s treatment action remains incomplete. We identified four miRNAs that changed expression in LCLs in response to Li treatment. We also showed significant changes in mRNA targets are inversely correlated with the changes of miR-221 and miR-34a. Our results may suggest co-regulation of miRNA and corresponding mRNA targets as a model for Li action.

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Statement of Interest
M.G.M. has served as a consultant and received honoria from Wyeth & Jansen Pharmaceuticals in the past 12 months.

References


