Investigating the Targets of MIR-15a and MIR-16-1 in Patients with Chronic Lymphocytic Leukemia (CLL)

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Abstract

Background: MicroRNAs (miRNAs) are short, noncoding RNAs that regulate the expression of multiple target genes. Deregulation of miRNAs is common in human tumorigenesis. The miRNAs, MIR-15a/16-1, at chromosome band 13q14 are down-regulated in the majority of patients with chronic lymphocytic leukaemia (CLL).

Methodology/Principal Findings: We have measured the expression of MIR-15a/16-1, and 92 computationally-predicted MIR-15a/16-1 target genes in CLL patients and in normal controls. We identified 35 genes that are deregulated in CLL patients, 5 of which appear to be specific targets of the MIR-15a/16-1 cluster. These targets included 2 genes (BAZA and RNF41) that were significantly up-regulated (p<0.05) and 3 genes (RASSF5, MKK3 and LRIG1) that were significantly down-regulated (p<0.05) in CLL patients with down-regulated MIR-15a/16-1 expression.

Significance: The genes identified here as being subject to MIR-15a/16-1 regulation could represent direct or indirect targets of these miRNAs. Many of these are good biological candidates for involvement in tumorigenesis and as such, may be important in the aetiology of CLL.

Introduction

Chronic lymphocytic leukemia (CLL) is the most common adult leukemia in the Western world. It is a heterogeneous disease associated with a highly variable clinical course [1]. A key feature of CLL is cytogenetic instability, with chromosomal abnormalities occurring in around 80% of cases [2]. While the molecular aetiology of CLL remains largely undetermined, specific recurrent chromosomal aberrations have been well described and serve as independent prognostic indicators for disease progression and survival [2]. Deletion of chromosome 13q is the most frequent chromosomal aberration in CLL, occurring in approximately 50% of patients [2]. The deletion of chromosome band 13q14 has also been reported in a variety of other malignancies [3–5], demonstrating the importance of this region in tumorigenesis. There has been wide speculation that the 13q14 region harbours tumour suppressor gene(s) involved in the aetiology of these diseases [6,7]. Various candidate tumour suppressor genes within the minimal deleted region (MDR) at 13q14 have been investigated, yet studies have consistently failed to detect any pathogenic mutations [8–10]. There remains, therefore, a need to identify alternative mechanisms that may influence the development of CLL.

There is increasing evidence for the involvement of microRNAs (miRNAs) in tumorigenesis [11–15]. MicroRNAs are small, non-coding RNAs that mediate the expression of target genes through sequence-specific base pairing with target messenger RNA (mRNA) [16]. Target gene expression is regulated by the degradation of the mRNA or more commonly, through blocking translation [17–19]. Deregulation of miRNAs has been implicated in human tumorigenesis and many miRNAs are located in genomic regions involved in cancer [20].

Two miRNAs, MIR-15a and MIR-16-1, are located at chromosome band 13q14 and are down-regulated in the majority of patients with CLL [21]. These genes induce apoptosis through the negative regulation of the anti-apoptotic gene BCL2 [22]. As such, down-regulation of MIR-15a/16-1 has been associated with the pathogenesis of CLL, although this remains controversial [23].

Each miRNA has the potential to mediate the expression of many target genes [19]. It is therefore possible that MIR-15a/16-1 may regulate the expression of genes, other than BCL2, which may be important in the development of CLL. Indeed, a recent study combining experimental and bioinformatic data identified a MIR-15a/16-1 gene signature in leukaemic cells [24]. The aim of this study was to examine the expression patterns of computationally-predicted targets of MIR-15a/16-1 to identify further novel candidate genes involved in the aetiology of CLL.

Materials and Methods

Ethics Statement

Ethical approval was granted by the North and East Devon Local Research Ethics Committee and all patients provided
available on request. Each 25
Biosystems, Foster City, USA). Probe and primer sequences are
were performed in triplicate on the ABI 7900HT Prism (Applied
resulting cDNA samples were of adequate quality. PCR reactions
two samples to be run in duplicate per card.

Patients
Thirteen patients with CLL under the care of the Haematology
Unit at the Royal Devon and Exeter Hospital were enrolled in this
study. Each patient had an established diagnosis of CLL based on
current World Health Organization (WHO) classification guide-
lines [25]. Patients had a history of persistent lymphocytosis
>5 x 10⁹/l and an immunophenotypic profile typical of CLL [26].
The majority of patients (12/13) had received treatment for their
disease prior to involvement in this study. Of the 13 CLL patients
included in this study, 7 (54%) had a deletion at 13q14 confirmed
by fluorescence in situ hybridisation (FISH) and multiplex ligation-
dependent probe amplification (MLPA) (data not shown). Five
normal control samples from healthy volunteers were also
obtained for this study.

RNA Extraction
Total RNA was extracted from whole blood using the MirVana
miRNA Isolation kit (Ambion/Applied Biosystems, Foster City,
USA), according to the manufacturer’s instructions. RNA
concentrations were measured using a ND-1000 spectrophotom-
eter (NanoDrop Technologies, Wilmington, USA).

MicroRNA-15a/16-1Expression
MicroRNA-15a/16-1 expression was determined for each sample
using the TaqMan MicroRNA assays kit (Applied Biosystems,
Foster City, USA), according to the manufacturer’s instructions.
The protocol involves real-time quantification of microRNAs by
stem-loop RT-PCR, as previously described [27]. All PCR
reactions were performed in triplicate. Crossing points (Ct) were
determined for each miRNA and an endogenous control gene (beta
glucorinidase (GUSB). The relative abundance of each miRNA
transcript was then determined using the comparative Ct method
[28]. Down-regulated miR-15a/16-1 expression in CLL patients
was defined as a ≥50% reduction in the expression levels of either
MIR-15a or MIR-16-1 when compared with the average MIR-15a
and MIR-16-1 expression levels in the normal control subjects.

Tiled Low Density Array (TLDA) Analysis
Reverse Transcription. Total RNA (1 µg) was reversed
transcribed in 50 µl reactions using the TaqMan Reverse
Transcription kit (Applied Biosystems, Foster City, USA),
according to the manufacturer’s instructions. PCR amplification
of the endogenous GUSB gene was performed to ensure that the
resulting cDNA samples were of adequate quality. PCR reactions
were performed in triplicate on the ABI 7900HT Prism (Applied
Biosystems, Foster City, USA). Probe and primer sequences are
available on request. Each 25 µl reaction included 5 µl universal
master mix, (no AMPerase) (Applied Biosystems, Foster City
USA), 5.0 µM probe, 20 µM each primer and 2 µl of cDNA. PCR
cycles were 50°C for 2 minutes, 95°C for 10 minutes followed by
60 cycles of 95°C for 15 seconds and 60°C for 1 minute. The
cDNA was considered adequate quality when GUSB amplification
was observed in all three reactions with minimal difference
between crossing points.

TLDA. TLDA allows for the simultaneous detection of the
expression of 384 genes per card. In the present study, TLDA
cards were configured into four identical 96-gene sets, allowing
two samples to be run in duplicate per card.

The web-based programmes TargetScan [http://www.targetscan.
org/]) and PicTar [http://pic.tar.mdct-berlin.de/) were used to predict
potential targets of MIR-15a/16-1 for inclusion on the TLDA cards.
TargetScan predicts biological targets of miRNAs by searching for
the presence of conserved 8mer and 7mer sites that match the seed
region (nucleotides 2-7 at the 5’ end segment of the MiRNA) of
each miRNA [29], while PicTar is a sophisticated algorithm that
predicts miRNA targets by searching for pair-wise alignments that
are conserved across species [30]. Genes were selected for inclusion
on the TLDA cards if the following criteria were satisfied: (1) the
genes were predicted as targets of MIR-15a/16-1 by both
computational programmes (TargetScan and PicTar), and (2)
Searches with Pubmed showed good biological evidence for
potential involvement in tumorigenesis (Eg. proto-oncogenes,
tumour-suppressor genes, transcription factors or genes involved
in cell cycle regulation). Each 96-gene set included 92 predicted
targets of MIR-15a/16-1 as well as four endogenous control genes;
18S, ACTB, GUSB and B2M.

For each patient sample, a reaction mix of 400 µl was prepared,
including 200 µl 2x TaqMan universal master mix (no AMPerase)
(Applied Biosystems, Foster City, USA), 160 µl dH2O and 40 µl
cDNA template. Aliquots (100 µl) of the reaction mix were added
to the appropriate sample loading ports on the TLDA card. The
cards were centrifuged twice for 1 minute at 12,000 rpm to allow
distribution of the reaction mix to each of the reaction wells. PCR
amplifications were performed on the ABI 7900HT platform
(Applied Biosystems, Foster City, USA). Cycling conditions were
50°C for 2 minutes, 94.5°C for 10 minutes followed by 40 cycles of
97°C for 30 seconds and 57.9°C for 1 minute. The expression of
each gene was measured in duplicate for each patient.

Analysis of gene expression
The abundance of each transcript in the TLDA analysis is
directly proportional to the point in the reaction where the signal
appears above the background fluorescence levels. This is termed
the crossing point (Ct). Differences in the abundance of two targets
are therefore directly proportional to the differences in their
crossing points (ΔCt). We calculated the difference between the
crossing point (ΔCt) for each test target (Ct<sup>test</sup>) compared with the
average crossing point of four endogenous control genes (18S, ACTB,
GUSB and B2M; G<sup>endogenous</sup>) in each sample. The level of
each transcript relative to that of the endogenous control levels for
given any sample could then be calculated from the equation
2<sup>-ΔΔCt</sup> [28] where ΔΔCt is the ΔCt value of the test transcript in
that sample (ΔCt<sup>test</sup>) normalised to a reference transcript which
was taken to be the average levels of BCL2 in normal controls
(ΔG<sup>endogenous</sup>).

Gene Ontology Analysis
The Gene Ontology (GO) categories of genes that were
differentially expressed in CLL patients and normal controls were
analysed with the ‘PANTHER Classification System’ GO browser
tool. (http://www.panttherdb.org/).

Analysis of Adenylate Uridylate-Rich Elements (ARE)
Deregulated targets of the MIR-15a/16-1 cluster were analysed
for the presence of AU-rich elements which have been reported to
mediate mRNA stability (ref). Analysis was performed using the
ARE-mRNA database version 3.0 (ARED; http://rc.kfshrc.edu.
.sa/ared/).

Protein Expression Analysis
Where possible, protein expression analysis of the putative MIR-
15a/16-1 target genes in CLL was investigated using the ‘The
Human Protein Atlas’ bioinformatics tool (http://www.proteinatlas.org/).

Statistical Analysis
Statistical analysis was performed using SPSS 11.0 (SPSS, Inc., Chicago, IL, USA). The comparison of target gene expression between different patient groups was performed using the Mann-Whitney U test. A P value of <0.05 was considered statistically significant.

Results

MicroRNA-15a/16-1 Expression
MicroRNA-15a/16-1 expression was down-regulated in the majority of CLL patients (10/13, 77%), including each patient with an identified deletion at 13q14. In the majority of cases, down-regulation of MIR-15a/16-1 correlated with chromosome 13q14 deletion status (Table 1), but in three cases (CLL 03, CLL 11 and CLL 13) the MIR-15a/16-1 cluster was down-regulated in the absence of an identifiable 13q14 deletion.

Assessment of Sample Quality for TLDA Analysis
RNA was extracted and reverse transcribed for all subjects as described above. GUSB was successfully amplified in triplicate in all 18 cases (data not shown), indicating that the cDNA was of adequate quality to perform TLDA analysis.

Micro RNA Target Prediction
A total of 99 and 145 potential gene targets were predicted by the web-based programmes (TargetScan and PicTar) for MIR-15a and MIR-16-1 respectively. Of these putative targets, 92 genes with a high likelihood for involvement in tumorigenesis were selected for inclusion on the TLDA cards.

Differentially expressed genes in patients with CLL and Normal Controls
Real-time PCR amplification detected the expression of 80/92 (87%) target genes and all four endogenous control genes in both the CLL and the normal control patient cohorts. TLDA analysis identified 35 genes that were differentially expressed (p<0.05) between the CLL and normal control patients (Figure 1). Of these genes, 27 (77%) were up-regulated and 8 (23%) were down-regulated in the patients with CLL compared with the normal controls.

The GO of the differentially expressed genes in CLL patients and normal controls was assessed with the ‘PANTHER Classification System’ (http://www.pantherdb.org/). The most frequently represented GO categories included: (1) transcription factor, (2) cell cycle and (3) signal transduction. Other significant GO categories represented included; apoptosis, inhibition of apoptosis, nucleic acid metabolism, regulatory molecule and kinase.

Genes specifically deregulated by the down-regulation of the MIR-15a/16-1 cluster
Of the 92 gene targets assessed, TLDA analysis identified 5 (5%) that may be specifically deregulated by the down-regulation of the MIR-15a/16-1 cluster. Of these 5 differentially expressed genes, 2 (BAZ2A and RNF41) were up-regulated (Figure 2) and 3 (RASSF5, M KK3, and LRIG1) were down-regulated (Figure 3) in CLL patients with low levels of MIR-15a/16-1 expression. The putative MIR-15a/16-1 targets were investigated for the presence of AU-rich elements, which have been implicated in regulation of mRNA stability. Of the 5 identified targets, 3 (BAZ2A, RNF41 and LRIG1) contained AREs in their 3’ UTR.

Protein expression analysis of the putative MIR-15a/16-1 target genes
Protein expression data from ‘The Human Protein Atlas’ (http://www.proteinatlas.org/) bioinformatics tool was available for RNF41 and MKK3. This data provided evidence for the up-regulation of RNF41 in B-CLL, since immunohistochemistry analysis demonstrated weak-moderated expression of RNF41 in B-CLL samples, while peripheral blood mononuclear cells from healthy blood donors showed negative-weak expression of the protein. MKK3 protein expression data showed that malignant lymphoma tissues typically exhibited weak cytoplasmic immunoreactivity or were negative. Protein expression analysis in CLL subjects for the BAZ2A, RASSF3 and LRIG1 genes is currently unavailable and represents an interesting area for future research.

Discussion
We have investigated the expression patterns of 92 computationally-predicted targets of MIR-15a/16-1 in 13 patients with CLL and 5 normal controls using TLDA analysis. We identified 35 genes that are differentially regulated in patients with CLL compared with normal controls and 5 genes which may be specifically regulated by the MIR-15a/16-1 cluster at chromosome band 13q14. These genes may be important in the aetiology of CLL and as such, provide interesting targets for future studies.

A comparison of the expression profiles of CLL patients and normal controls identified 35 differentially regulated genes (Figure 1), the majority of which (77%) were up-regulated in the CLL patient group. Gene ontology analysis demonstrated that many of the differentially regulated genes were transcription factors, cell cycle-related genes or genes involved in signal transduction. Although not specifically regulated by the MIR-15a/16-1 cluster, these deregulated genes may represent important contributors to the process of leukaemogenesis.

The 5 genes specifically regulated by MIR-15a/16-1 expression included 2 genes (BAZ2A and RNF41) that were significantly up-regulated in CLL patients with low MIR-15a/16-1 expression (Figure 2). These genes may represent direct biological targets of
the MIR-15a/16-1 cluster. Further experimental work including luciferase reporter gene assays or mutagenesis of predicted miRNA binding sites may be useful to examine these possibilities further. A further 3 genes (RASSF5, MKK3 and LRIG1) were expressed at significantly lower levels in CLL patients with down-regulated MIR-15a/16-1 expression (Figure 3). These genes may be indirect targets of MIR-15a/16-1, their expression perhaps being repressed by another, as yet unidentified, direct target of the MIR-15a/16-1 cluster.

Further evidence for the deregulation of the target genes we identified in CLL was obtained from protein expression data (http://www.oriteubatkas.org/). This analysis confirmed the up-regulation of RNF41 in B-CLL subjects. It also demonstrated weak or negative MKK3 protein expression in malignant lymphoma tissues, consistent with our data. The functions of the identified MIR-15a/16-1 target genes are discussed further below. Protein data were not available for the remaining three targets we identified. This represents an interesting area for future work.

BAZ2A is a member of the bromodomain family of genes which function as integral components of chromatin re-modelling complexes [31]. They are believed to play a role in the chromatin-dependent regulation of transcription. Interestingly, putative BAZ2A deregulation has been implicated in a paediatric case of pre-B acute lymphoblastic leukemia (ALL) in which a cryptic rearrangement between 12p13 and 12q13 generated a fusion of ETV with an intronic sequence of BAZ2A [32]. The authors do acknowledge, however, that the leukaemogenic impact of putative BAZ2A deregulation remains undetermined at present [32].

RNF41 is an evolutionarily conserved RING finger-containing ubiquitin ligase. It has been speculated that RNF41 is involved in the aetiology of haematological malignancies [33]. The gene resides at chromosome band 12q13, a locus that frequently demonstrates aberrations associated with acute myeloid leukemia (AML) or non-Hodgkin’s lymphoma (NHL) [33]. Additionally, the gene is differentially expressed in foetal and adult haematopoietic stem cells and progenitors [33], suggesting that it may be involved in cell lineage commitment and differentiation [33]. A recent study demonstrated that over-expression of RNF41 in a murine multipotent haematopoietic progenitor cell line (EML) attenuated erythroid and myeloid differentiation in response to the cytokines erythropoietin (EPO), interleukin-3 (IL-3) and retinoic acid [34]. This response resulted from RNF41-specific regulation of cytokine receptor levels [34]. Further studies are required to determine whether other haematopoietic cytokine receptors are regulated by RNF41 and whether the gene additionally influences haematopoietic progenitor cell differentiation into lymphoid lineages.

RASSF5 is a member of the RAS association domain family. It can act as a tumour suppressor by inducing apoptosis and delaying cell cycle progression in different cancer cell lines [35]. The gene is epigenetically silenced in a variety of human cancers by CpG island promoter hypermethylation [36,37]. Interestingly, miRNAs can themselves act as epigenetic modifiers by the post-transcriptional regulation of chromatin modifying enzymes [38].

The mitogen-activated protein (MAP) kinase pathways mediate the transduction of extracellular signals via protein phosphorylation cascades. Three distinct MAP kinase pathways have been defined; (1) extracellular-signal-related kinases (ERKs), (2) the c-Jun N-terminal kinases (JNKs) and (3) p38 stress-activated protein kinases (p38 MAPKs) [39]. MKK3 is one of the upstream activator kinases for the p38 MAPK pathway. A recent study demonstrated that the p38 MAPK pathway, including MKK3, is constitutively activated in B-CLL cells but not their normal peripheral B-cell counterpart [40]. The constitutive p38 MAPK pathway activation results in

![Figure 1. Differentially expressed genes in patients with CLL and normal controls.](http://www.oriteubatkas.org/)

Bar graph representing the difference in relative gene expression in CLL patients compared with normal control patients. The dark grey bars above the x-axis represent genes that are significantly up-regulated in CLL patients compared with normal controls. Light grey bars below the x-axis represent genes that are significantly down-regulated in CLL patients when compared to normal controls. P-values; * represents <0.05, and ** represents <0.01
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up-regulation of matrix metalloproteinase-9 (MMP-9) [40], a critical factor in tumour angiogenesis and tumour homing. Elevated serum levels of MMP-9 are associated with an unfavourable prognosis for patients with CLL [41]. Our study identified significantly lower levels of MKK3 expression in CLL patients with down-regulated MIR-15a/16-1. This is consistent with CLL patients harbouring chromosome 13q14 deletions, and hence MIR-15a/16-1 down-regulation, displaying a more favourable prognosis [2].

LRIG1 is a member of a family of LRIG genes that encode integral membrane proteins with extracellular/luminal extensions consisting of leucine-rich and immuloglobulin-like domains [42]. LRIG1 interacts with the ErbB receptor tyrosine kinase to negatively regulate EGFR signalling [43]. This regulation is mediated through the recruitment of E3 ubiquitin ligases, resulting in ubiquitylation, internalisation and lysosomal degradation of the ErbB receptors. LRIG1 is a proposed tumour suppressor gene. It localizes at chromosome band 3p14.3, a chromosomal region that is commonly deleted in human cancers. Additionally, LRIG1 is down-regulated in a variety of different tumour cell lines [44] consistent with it being a tumour suppressor gene. It has been hypothesised that the down-regulation of LRIG1 could unleash EGFR signalling which may contribute to the development of various malignancies [45]. Of note, however, LRIG1 expression is up-regulated in some tumours, suggesting that the gene functions as a tumour promoter under certain circumstances [45]. Further studies are required to unravel the functions of the LRIG proteins and to further understand the contribution of LRIG1 dysregulation to human tumorigenesis.

The majority (87/92, 95%) of the computationally-predicted targets investigated in this study were not differentially regulated in
CLL patients with varying levels of MIR-15a/16-1 expression. A possible explanation for this may be that the analysis was performed on mRNA rather than on proteins. Through imperfect pairing with their target mRNAs, some miRNAs can reduce the protein levels of a target gene with minimal variation of the mRNA levels. Alternatively, the lower predictive power of the bioinformatics tools used for miRNA gene target prediction may also have contributed to this finding. Computational algorithms for the prediction of miRNA targets are acknowledged to yield a large number of false-positive hits. TargetScan and PicTar are estimated to have a 22–31% and ~30% false-positive rate respectively [46]. Our data suggests that these figures may under-estimate the false-positive rates associated with these programmes. Use of additional bioinformatics programmes, such as miRanda [http://chiro.ksu.edu/cgi-bin/mirnaviewer/mirnaviewer.pl], in combination may enhance the positive predictive power of these commonly used tools.

The regulation of gene expression is often complex and multifactorial. The removal of one regulatory element, such as MIR-15a/16-1, may be compensated for by the altered expression of other regulatory elements, thus maintaining the normal expression of the target gene. This may also explain why our study identified so few differentially regulated MIR-15a/16-1 targets. Interestingly, the expression patterns of the anti-apoptotic gene BCL2 may support this hypothesis. Cimmino et al. [2005] demonstrated that MIR-15a/16-1 negatively regulate BCL2 [22], although this relationship remains controversial [23]. In the current study, BCL2 was significantly over-expressed in CLL patients compared with normal controls (p = 0.001). The anti-apoptotic gene was also up-regulated in CLL patients with low MIR-15a/16-1 expression compared to those with normal expression levels of the miRNAs, however, this did not reach the level of significance (p = 0.161) probably due to the small sample size in this study. Our data indicates that the regulation of BCL2 may be influenced by MIR-15a/16-1 as well as other regulatory elements, exerting a combinatorial effect.

In conclusion, our work has investigated the expression patterns of computationally-predicted targets of MIR-15a/16-1 in patients with CLL using TLDA analysis. We have identified 35 genes that are deregulated in patients with CLL, and 5 genes that are specifically deregulated by low levels of MIR-15a/16-1 expression. The identified genes are all good biological candidates for involvement in tumorigenesis and as such, may be important in the aetiology of CLL. They provide interesting candidate genes for future studies and may represent possible targets for therapeutic intervention.

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Author Contributions

Conceived and designed the experiments: LWH. Performed the experiments: KH. Analyzed the data: KH. Contributed reagents/materials/analysis tools: CR LWL. Wrote the paper: KH LWL.

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