Published in final edited form as:
Br J Haematol. 2011 June ; 153(6): 753–757. doi:10.1111/j.1365-2141.2011.08662.x.

MIR29B regulates expression of *MLLT11 (AF1Q)*, an *MLL* fusion partner, and low *MIR29B expression* associates with adverse cytogenetics and poor overall survival in AML

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Summary

MLLT11, an *MLL* fusion partner, is a poor prognostic biomarker for paediatric acute myeloid leukaemia (AML), adult normal cytogenetics AML, and adult myelodysplastic syndrome. *MLLT11* is highly regulated during haematopoietic progenitor differentiation and development but its regulatory mechanisms have not been defined. In this study, we demonstrate by transfection experiments that *MIR29B* directly regulates *MLLT11* expression *in vitro*. *MIR29B* expression level was also inversely related to *MLLT11* expression in a cohort of 56 AML patients ($P < 0{\cdot}05$). AML patients with low *MIR29B*/elevated *MLLT11* expression had poor overall survival ($P = 0{\cdot}038$). Therefore, *MIR29B* may be a potential prognostic biomarker for AML patients.

Keywords

AML prognostic marker; *MLLT11*; *MLL* fusion gene; *MIR29*; AML survival

The *MLLT11* gene, located on chromosome *1* band *q21*, was initially identified as a mixed-lineage leukaemia (*MLL*) fusion partner from acute myeloid leukaemia (AML) patients whose leukaemic cells carried a *t(1; 11) (q21; q23)* chromosomal abnormality (Tse *et al*,

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†This work was jointly supported by the ASCO Career Development Award/The John Hartford Foundation/T. Franklin Williams Scholar Program from the Association of Specialty Professors (WT).

Author contributions

YX, ZL, MJ, JB, JP, and JVT designed and conducted experiments. ACT: analysed microarray data and performed statistical analysis data. KDB, GH, CJ, and JC: analysed data and participated in manuscript writing. LTB, WT designed experiments and wrote manuscript.

1995). *MLLT11* expression is highly regulated in normal lineage committed haematopoietic progenitor cells (HPC) (Tse *et al*, 1995). Elevated *MLLT11* expression is widely seen in acute myeloid and lymphoid leukaemias (Tse *et al*, 1995). We have consistently shown that high *MLLT11* expression is a poor prognostic biomarker for paediatric AML (Tse *et al*, 2004), adult normal cytogenetics AML (NC-AML) (Strunk *et al*, 2009), and adult myelodysplastic syndrome (MDS) (Tse *et al*, 2005). However, the underlying mechanism(s) of *MLLT11*, regulation in normal and abnormal haematopoiesis remain unclear.

MicroRNAs are small *RNA*s that play important roles in the post-transcriptional regulation of genes, such as oncogenes and tumour suppressor genes that are mostly related to cell differentiation. They are located throughout the genome but are often found at fragile sites (Ambros, 2001). We hypothesized that *MLLT11* expression may be regulated by micro-RNAs and the *MLLT11* 3′-UTR, like other genomic regions, is involved in the development of cancers. Through web-based search and analysis, we found and confirmed that *MIR29B*, a member of the *MIR29* family was the strongest candidate to potentially regulate *MLLT11*. In this report, we demonstrate that *MIR29B* specifically interacts with the *MLLT11* 3′-UTR and directly regulates *MLLT11* expression and may be a coordinate biomarker with *MLLT11* in myeloid leukaemias.

Materials and methods

MicroRNA and gene expression profile from AML patients, human control samples, and statistical analysis

The information, sample collection, and preparation have been previously described (Li *et al*, 2008) for the 56 patients included in this study. As there is molecular heterogeneity within specific cytogenetic groups that can lead to variation in overall survival (OS), we grouped these patients' OS according to their *MIR29B* expression levels instead of cytogenetics. Clinical variables across the groups were compared by using the chi-square or a two-sided Fisher's exact test for categorical variables. *P* values < 0·05 were considered as statistically significant. OS was calculated by using the method of Kaplan–Meier, and log-rank test was used to assess the differences between survival curves.

Computational predictions of microRNA binding

The web-based "TargetScanHuman" (http://www.targetscan.org/vert_50/) and miRGen (http://www.diana.pcbi.upenn.edu/cgi-bin/miRGen/v3/Targets.cgi#Results) were used to identify microRNA candidates that may potentially regulate *MLLT11*. The MIR29A/B/C required binding sequence matches of 100% in the *MLLT11* 3′UTR.

Cell lines and growth conditions

Two human lung cancer cell lines, H157 and SKMES1, and a leukaemic cell line REH were chosen because they have higher *MLLT11* expression and acceptable transfection efficiency. Cell lines were cultured in RPMI 1640 medium supplemented with 10% fetal bovine serum at 37°C and 5% CO_2.

Establishment of stable transfectants of green fluorescent protein (GFP), GFP- MLLT11 3′-UTR (GFP-A3U) and GFP- MLLT11 3′-UTR mutation at the MIR29 binding site (GFP-A3U-Mutant)

H157, SKMES1, and REH cells were transfected with 10 nmol/l miRIDIAN *MIR29B* mimic or miRIDIAN mimic negative control (Dharmacon, Chicago, IL, USA), using Lipofectamine2000 Transfection Reagent (Invitrogen, Carlsbad, CA, USA). H157 cells were transfected with *GFP*, *GFP-A3U* and *GFP-A3U*-mutant (mis-matched at 2-nucleotides,

S2B) by using Lipofectamine2000 transfection reagent. After transfection, neomycin-resistant clones were selected in the presence of 900 μg/ml G418 sulfate (Mediatech, Manassa, VA, USA). GFP-positive cells were sorted by flow cytometry. Cells were imaged under the Nikon eclipse Ti microscope using the NIS-Elements image system.

RNA isolation, cDNA synthesis, quantitative reverse transcription polymerase chain reaction (qRT-PCR), and Western blotting

Total RNA isolation, cDNA synthesis, qRT-PCR, and cell lysates were performed as previously reported (Tse *et al*, 2004). qRT-PCR was used to determine the *MLLT11*, *GFP*, and *B2M* (internal control) expression. *Corresponding* MLLT11 protein levels in three tested cell lines were confirmed by Western blot using the rabbit monoclonal anti-MLLT11 *(Epitomics, Burlingame, CA, USA)*. Samples were tested in triplicate fashion. Primers for each gene amplification were as follows:

MLLT11 Forward: 5′-GCACTCCCTCCATCTTTGGA-3′

MLLT11 Reverse: 5′- CAGCTCCGACAGATCCAGTTC-3′

GFP Forward: 5′-CGACAAGCAGAAGAACGGCATCAA-3′

GFP Reverse: 5′-AACTCCAGCAGGACCATGTGAT-3′

B2M Forward: 5′-ATGAGTATGCCTGCCGTGTGA-3′

B2M Reverse: 5′-GGCATCTTCAAACCTCCATG-3′

Construction of GFP reporter vectors

The *MLLT11* 3′-UTR was subcloned into *pEGFP-C1* (Clontech, Mountain View, CA, USA) at the EcoRI/BamHI sites. Mutation of the *MIR29B* binding site in the *MLLT11* 3′UTR was created by the Stratagene QuickChange method with the following primers:

MLLT11-3UTR (forward): 5′-TACTGTGTTGGTGGTTCGGATGAATCTG-3′

MLLT11-3UTR (reverse): 5′-CAGATTCATCCGAACCACCAACACAGTA-3′

Results and discussion

Through using a web-based scoring system, *MIR29* was identified as a potential regulator of *MLLT11* expression. This observation was further supported by our subsequent finding that *MLLT11* and *MIR29A/B/C* expression were inversely related in the tested cell lines and AML patient samples. We chose to study *MIR29B* in this report because only *MIR29B* expression had significant predictive power for OS in a cohort of AML patients reported here. It was confirmed that most of the 56 AML patients with elevated *MLLT11* expression had depressed *MIR29B* expression (Fig 1A). The prevalence of elevated *MLLT11* expression among this cohort of AML patients (66%) was similar to our previous reports (Tse *et al*, 2004; Strunk *et al*, 2009). To test whether *MIR29B* could specifically and directly regulate *MLLT11* expression by interacting with the *MLLT11* 3′-UTR, *MIR29B* was transfected into 3 cell lines (H157, SKMES1, and REH) and found to knockdown *MLLT11* mRNA and MLLT11 protein (Fig 1B, C). This regulatory relationship between *MIR29B* and *MLLT11* was direct and specific because the knockdown effect on *MLLT11* mRNA and MLLT11 protein could be ablated by mutation of the *MIR29B* binding site in the *MLLT11* 3′-UTR (Fig 1D).

We next wanted to determine whether *MIR29B* would offer the same prognostic power as the high *MLLT11* expression quartile in AML patients (Strunk *et al*, 2009). It was found that 15 patients with low *MIR29B* expression (2·6-fold), corresponding to the highest quartile of

high *MLLT11* expression, had significantly poorer OS ($P < 0{\cdot}05$, Fig 2) and a stronger trend toward an association with adverse cytogenetics ($P = 0{\cdot}06$) compared to the remaining AML patients. Therefore, low *MIR29B* expression could be a similar poor prognostic marker as high *MLLT11* expression and adverse cytogenetics in AML patients as we reported (Tse *et al*, 2004; Strunk *et al*, 2009), and *vice versa*. Notably among 56 AML patients, there was only one patient with −*7q*, one of the coding regions for *MIR29B*, who had very low *MIR29B* expression but very high *MLLT11* expression. This indicative case demonstrated that expression of mature *MIR29B* in AML might probably come from the chromosomal locus *7q23*. The observation on this specific case potentially provides a hint that deletion of the tumour suppressive *MIR29B* coding region may be mechanistically responsible for the pathogenesis of MDS/AML patients with −*7*q/monosomy *7*.

The *MIR29* family comprises a group of small non-coding RNAs (*MIR29A/B/C*) that are actively involved in regulating many developmental and differentiation related genes (Ambros, 2001). *MIR29B* is considered a tumour suppressor and has been found to directly regulate *MCL1* (*Mott et al*, 2007), *TCL1A (TCL1)* (Pekarsky *et al*, 2006), and *DNMT3A/B (DNMT3)* (Garzon *et al*, 2009; Takada *et al*, 2009). It is believed that through targeting *p85α* and *CDC42*, *MIR29* can activate *TP53* as part of its biological function as a tumour suppressor (Park *et al*, 2009). *MIR29* also regulates lung (Williams *et al*, 2007), myoblast (Wang *et al*, 2008), and osteoblast (Li *et al*, 2009) differentiation and development that may contribute to a role in lung cancer and rhadomyosarcoma.

Despite that the biological function of *MLLT11* is unclear, we have demonstrated that it is tightly regulated in HPC differentiation and development (Tse *et al*, 1995). We also have consistently shown that elevated *MLLT11* expression is a poor prognostic biomarker for paediatric AML (Tse *et al*, 2004), adult NC-AML (Strunk *et al*, 2009), and adult MDS (Tse *et al*, 2005). Another series of studies also showed similar observations, that naturally elevated *MIR29B* expression appears to associate with certain lower risk AML patients such as those NC-AML patients with *NPM1* mutation (Garzon *et al*, 2008). Given the fact that *MIR29B directly regulates MLLT11* expression, the observations of Garzon *et al* (2008) are consistent with our previous study that high *MLLT11* expression is a poor prognostic marker for NC-AML (Strunk *et al*, 2009). However, our current study further shows that *MIR29B* expression levels may also have OS predictive value for AML patients with different cytogenetics and this observation needs to be confirmed in a larger AML cohort. Biologically, over-expression of pre- *MIR29B* reduces global DNA hypomethylation and restores expression of the hypermethylated tumour suppressors *ESR1* and *CDKN2B* ($p15^{INK4b}$) in AML patients. These changes may explain why these AML patients have better outcomes (Garzon *et al*, 2009). Another group of investigators also observed that the *MIR29* family is down-regulated in AML patients with balanced *11q23* translocations targeting the oncogene *TCL1A*, which represents poor prognosis AML (Pekarsky *et al*, 2006).

Our current study suggests that *MIR29B*, like *MLLT11*, can be a potential prognostic marker for AML. Most importantly the *MIR29* family regulates a group of genes, such as *MCL1*,*TP53*, *DNMT3A/B*, and now *MLLT11* that are related to cell apoptosis, DNA methylation, and differentiation. The *MIR29B* biological phenomenon warrants further clinical investigation to determine whether the signal transduction profiles related to the *MIR29* family and its regulated genes could serve as therapeutic targets for AML patients. This approach alone or in combination with existing targeted apoptotic, methylation, or differentiation therapies might provide novel therapeutic avenues especially for high risk AML patients.

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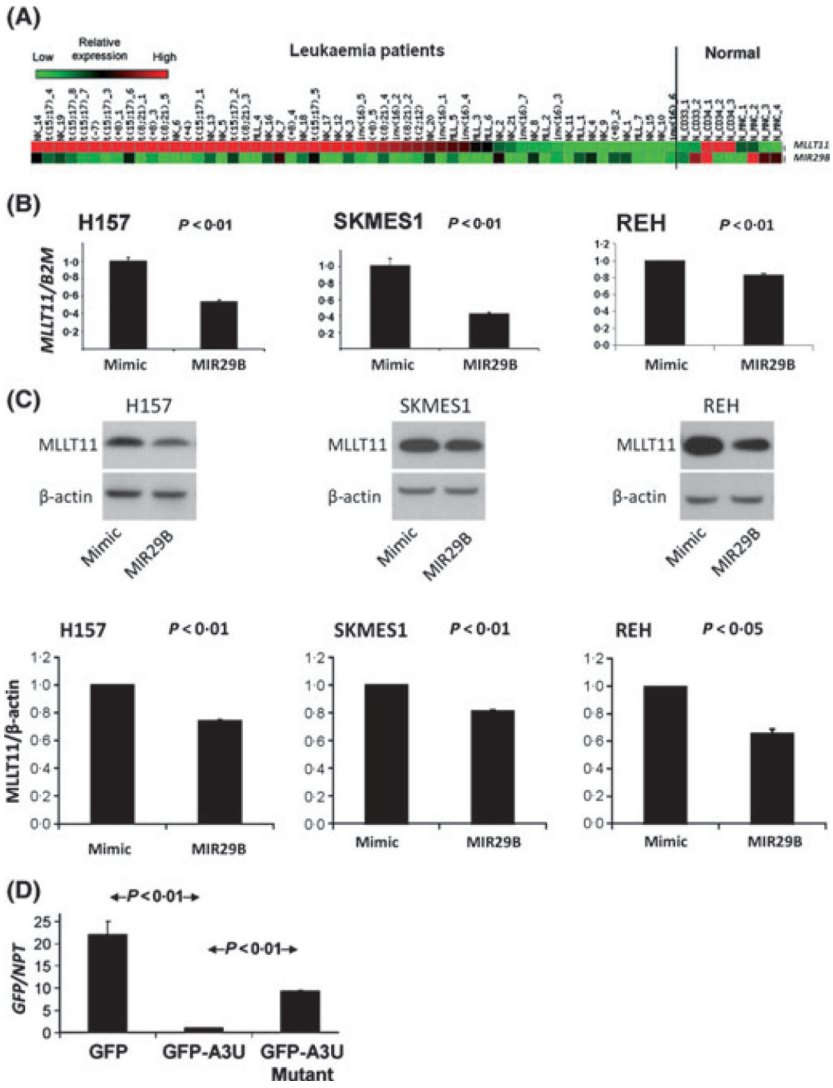

Fig 1.

Low *MIR29B*/high *MLLT11* expression in AML patient samples and demonstration of direct regulation of *MLLT11* by direct interaction of *MIR29B* with the *MLLT11* 3-UTR. (A) *MLLT11* and *MIR29B* expression heat map: columns and rows represent patients and *MLLT11/MIR29B*expression, respectively. Red to green indicates high to low expression of *MLLT11/MIR29B*, respectively. (B) Assessment of expression of *MLLT11* mRNA by qRT-PCR and its response to the transfection of miR-29b: transfection of *MIR29B* into H157 lung cancer cells, SKMES1 lung cancer cells, REH leukaemic cells and a negative control for 24 h, respectively. (C) Assessment of MLLT11 protein level by Western blots in samples corresponding to those of B. (D) *GFP* reporter stable transfectants in H157 cells showing *GFP* empty vector (control), *GFP-A3U* (wild-type), and *GFP-A3U*-Mutant as well as their images taken by the NIS-Elements image system. Assessment of expression of *GFP* mRNA by qRT-PCR normalized to neomycin phosphotransferase (*NPT*) mRNA that also digitally confirmed the image observation. Data in B/C/D are representative of three independent experiments.

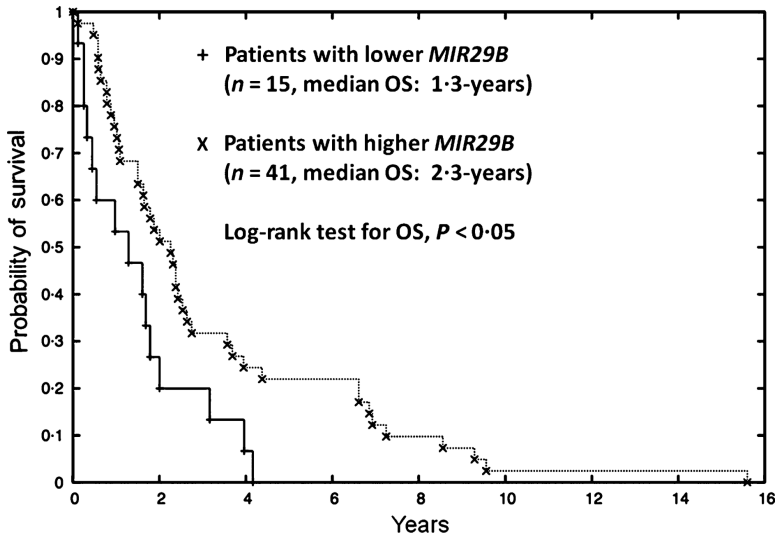

Fig 2.

Lower *MIR29B* expression is a poor prognosis marker for AML patients. Kaplan-Meier survival curves for 56 AML patients are stratified by *MIR29B* expression. AML patients with lower *MIR29B* expression had a strong trend of adverse cytogenetics (<2·6-fold; P = 0·06) and significantly poorer OS (median OS 1·3-year vs. 2·3-year; $P < 0·05$) compared to AML patients with higher *MIR29B* expression (>2·6-fold).