MicroRNA-21 and -155 are induced by a widely expressed mutation in MyD88(L265P) in Waldenstrom's Macroglobulinemia.

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ABSTRACT

INTRODUCTION

MicroRNA dysregulation has been described in Waldenstrom's Macroglobulinemia (WM), an indolent non-Hodgkin's lymphoma characterized by the accumulation of IgM secreting lymphoplasmacytic cells (LPC). Whether microRNA dysregulation represents a primary oncogenic event, is reactive to an oncogenic event, or is related to the stage of B-cell differentiation remains to be clarified. As part of these efforts, we sought to delineate aberrant microRNAs which emerge by direct comparison of WM LPC with memory B-cells, from wherein the WM clone is believed to have emerged. To further these efforts, we also sought to clarify whether microRNA dysregulation is related to the presence of a somatic mutation in MYD88 (L265P), a widely expressed mutation in WM patients, which also induces NF-kB and STAT3 activation via IRAK 1/4 (Nature 2011, 470:115-119).

PATIENTS AND METHODS

CD19+ selected bone marrow cells from WM patients and healthy donors were isolated by immunomagnetic sorting. Healthy donor B-cells (CD19+) were also isolated from peripheral blood, and further sorted using a memory B-cell (CD19+ CD27+) isolation kit (Miltenyi, Auburn CA). MicroRNA profiling was performed using Taqman low-density arrays. Taqman stem-loop Real-Time PCR was used for validation of MicroRNA changes. MYD88 L265P expressing BCWM.1 and MWCL-1 cells were used for lentiviral transduction experiments. Down stream inhibitory studies were conducted using an IRAK 1/4 kinase inhibitor (407601, EMD, Gibbstown, USA) and a MyD88 dimerization inhibitor (IMGENEX). STAT3 and IκBα phosphorylation were detected by Western blot using phospho-specific antibodies.

RESULTS

MicroRNA profiling was performed using bone marrow LPC from 11 WM patients, and compared to bone marrow total B cells from 7 healthy donors. Validation of the microRNA profiling in 25 additional patients for a total of 36 patients showed that miR-21, -155, -29c and 339-5p were increased, and miR-126, and -145 were decreased in WM bone marrow LPC (Table 2). When WM LPC were directly compared to healthy donor memory B-cells, only miR-21 and -155 remained significantly higher in WM LPC (Figure 1). To investigate whether miR-21 and -155 are impacted by the MYD88 L265P mutation, we next performed lentiviral knock-down experiments in BCWM.1 and MWCL-1 cells. Levels of miR-21 and -155 decreased following knockdown of MYD88 (Figure 2). In addition, miR-21 but not -155 decreased following treatment of BCWM.1 and MWCL-1 cells with an IRAK 1/4 kinase inhibitor potentially signifying differences in downstream activation of miR-21 and -155 by MYD88 L265P (Figure 3).

Since it has been shown that MYD88 L265P can lead to IL6 secretion and activation of STAT3 (Nature 2011, 470:115-119), we also assessed the potential autocrine role of this pathway on miR-21 expression in WM. We therefore investigated the effect of the IRAK1/4 and MyD88 inhibitors on IL6 secretion and STAT3 phosphorylation in MCWM.1 and MWCL-1 cell lines (Figure 4). Overexpression of MYD88 L265P and IL6 stimulation led to enhanced Micro-RNA-21 expression (Figure 5&6).

RESULTS

Table 1. Study population and design

	TLDA Cohort	Validation Cohort
population size	11	36
Male/Famale	11 Male 0 Famale	26 Male 10 Famale
Previous Treated (%)	45% (5/11)	66% (24/36)
Age (Years)	72 (49-81)	72 (49-88)
β-2 Microglobulin (mg/L)	2.8 (1.7 -5.2)	3.35 (1.7 -12.1)
Serum IgM (mg/dl)	3,330 (202 -6,110)	3,255 (516 -8,490)
Bone Marrow Infiltration (%)	40% (5% -80%)	70% (5% -90%)

Table 2: Summary of miRNA array and validation.

Up Regulated miRNA

	TLDA Results		Validation	
miRNA	Fold change	p-Value	Fold Change	p-Value
hsa-miR-21	3.3	0.035	6.6	0.06
hsa-miR-29c	3.2	0.003	4.6	<0.001
hsa-miR-155	5.5	0.082	5.4	0.004
hsa-miR-339-5p	2	0.006	1.8	0.04

Down Regulated miRNA

	TLDA Results		Validation	
miRNA	Fold change	p-Value	Fold Change	p-Value
hsa-miR-9*	-3.9	0.001	-1.6	0.55
hsa-miR-126	-21.5	0.006	-38.5	0.001
hsa-miR-145	-198.5	<0.001	-33.3	<0.001
hsa-miR-886-5p	-3.0	0.004	-1.9	0.15

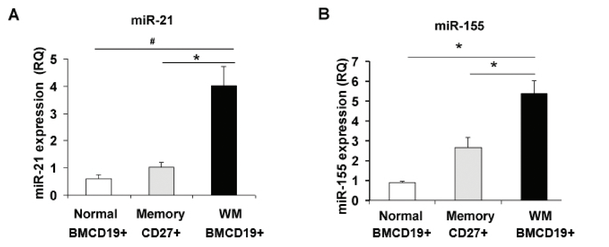

Figure 1: miR-21 (1A) and miR-155 (1B) expression in normal bone marrow CD19+ cells, CD19+ CD27+ Memory B cells, and WM bone marrow CD19+ cells. WM cells and normal bone marrow cells were isolated by immunomagnetic sorting. Memory B cells were isolated from peripheral blood using a memory B-cell (CD19+ CD27+) isolation kit . n=7 for normal BMCD19+ cells, n=9 for memory B cells, n=36 for WM cells. #p=0.06 and *p<0.05.

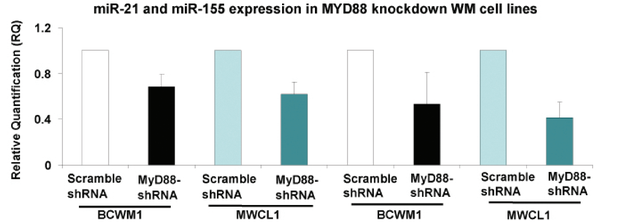

Figure 2: miR-21 and miR-155 are decreased in WM cells with MYD88 knockdown. MYD88 knockdown was performed by lentiviral transduction of GFP tagged MYD88 shRNA or scramble shRNA in BCWM.1 and MWCL-1 cells. 7 days after lentivirus transduction, green fluorescent cells were sorted out by flow and lyzed in Qiazol for RNA. miR-21 and miR-155 expression were quantified by Taqman stem-loop reverse transcription followed by realtime PCR. Signals were normalized to RNU48 expression.

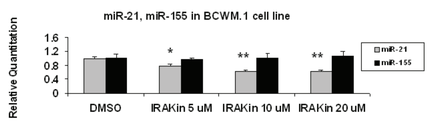

Figure 3: miR-21, but not miR-155 is decreased in WM cell lines treated with IRAK1/4 inhibitor. A. BCWM.1 cells were treated with IRAK1/4 inhibitor (Calbiochem) at indicated concentration. 48hrs after treatment, miR-21 and miR-155 expression levels were determined by Taqman qPCR. Expression was normalized to expression of RNU48. B. pri-miR-21 expression levels in BCWM.1 cells with 18S as internal controls. C. miR-21 and miR-155 expression in MWCLL-1 cells. Data represent the mean ± SD, n=3 *p<0.05 ,**P<0.001 vs DMSO

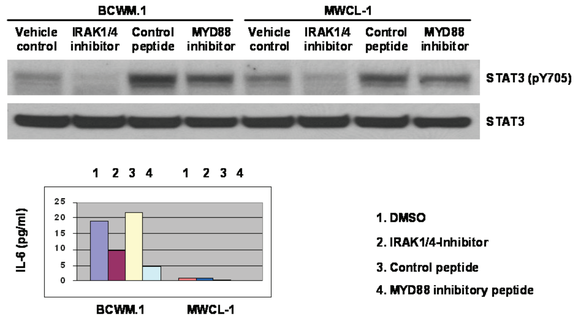

Figure 4: Stat3 phosphorylation and IL6 secretion were decreased in WM cell lines treated with IRAK1/4 inhibitor or MYD88 homodimerization inhibitor. BCWM.1 and MWCL-1 cells were treated with IRAK1/4 inhibitor (10uM) and MYD88 homodimerization inhibitor (100uM, IMGENEX) for 24hrs. A. cell lysates were immunoblotted for STAT3(pY705) and total STAT3. B. IL6 secretion was measured in the supernatant of BCWM.1 and MWCL-1 cells by ELISA.

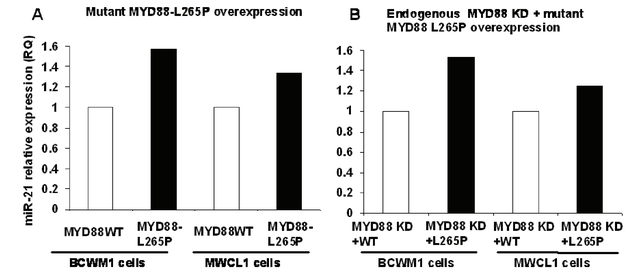

Figure 5: miR-21 expression in BCWM.1 cells and MWCL-1 cells with the overexpression of mutant MYD88-L265P. Two types of MYD88L265P mutant lentiviral constructs were transduced. A. Overexpression of wild type (WT) or mutant MYD88L265P using GFP tagged lentiviral vectors. B. Overexpression of GFP tagged lentivial vectors containing both MYD88 shRNA targeting MYD88 3'UTR (untranslated region) and either WT or mutant MYD88-L265P. 7 days after transduction, green fluorescent cells were sorted out by flow and lyzed in Qiazol for RNA. miR-21 expression were quantified by Taqman stem-loop reverse transcription followed by realtime PCR.

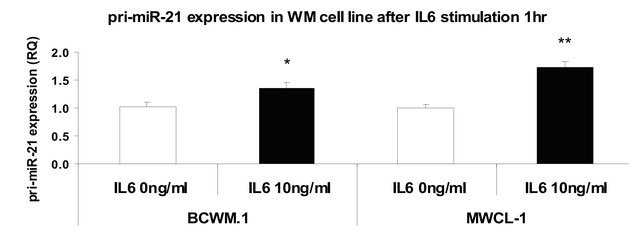

Figure 6: pri-miR-21 expression is increased in IL6 stimulated WM cell lines. BCWM.1 and MWCL-1 cell lines were incubated with IL6 (10ng/ml) 1 hour before RNA extraction and determination of pri-miR-21 expression by Taqman qPCR. Expression was normalized to expression of 18s. Data represent the mean ± SEM, n=6. *P<0.05, **p<0.001 vs 0 ng/ml of IL6.

SUMMARY

1. MicroRNA expression varies based on which population of healthy donor B-cells are compared to WM LPC. Since memory B-cells more accurately approximate the precursor cell in WM, direct comparison of WM LPC to healthy donor memory B-cells was undertaken which revealed a different profile vs. unselected CD19+ B-cells.
2. When comparison of WM microRNA expression to memory B-cells was made, only increased expression of microRNA-21, and -155 was observed.
3. Levels of miR-21 and miR-155 decreased following knockdown of MYD88 in WM cells.
4. The data also suggest that miR-21 is regulated by mutant MyD88-L265P, likely through the IL-6-Stat3 pathway.

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