This is a pre- or post-print of an article published in
Schneider, B., Nagel, S., Ehrentraut, S., Kaufmann, M.,
Meyer, C., Geffers, R., Drexler, H.G., MacLeod, R.A.F.
Neoplastic MiR-17~92 deregulation at a DNA fragility
motif (SIDDD)
(2012) Genes Chromosomes and Cancer, 51 (3), pp. 219-228.

Neoplastic *MiR-17~92* Deregulation at a DNA Fragility Motif (SIDD)

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Running Title: Neoplastic *MiR-17~92* Deregulation

Supported by: The José Carreras Leukemia Research Fund

ABSTRACT

Chromosomal or mutational activation of *BCL6* (at 3q27) typifies diffuse large B-cell lymphoma (DLBCL) which in the germinal center subtype may be accompanied by focal amplification of chromosome band 13q31 effecting upregulation of *miR-17~92*. Using long distance inverse (LDI)-PCR, we mapped and sequenced six breakpoints of a complex *BCL6* rearrangement t(3;13)(q27;q31)t(12;13)(p11;q31) in DLBCL cells which places *miR-17~92* antisense within the resulting *ITPR2-BCL6* chimeric fusion gene-rearrangement. *MIR-17~92* members were upregulated ~15 fold over controls in a copy number independent manner consistent with structural deregulation. *MIR17HG* and *ITPR2-BCL6* were, despite their close configuration, independently expressed, discounting antisense regulation. *MIR17HG* in t(3;13)t(12;13) cells proved highly responsive to treatment with histone deacetylase inhibitors implicating epigenetic deregulation, consistent with which increased histone-H3 acetylation was detected by chromatin immunoprecipitation near the upstream *MIR17HG* breakpoint. Remarkably, 5/6 DNA breaks in the t(3;13)t(12;13) precisely cut at Stress-Induced DNA Duplex Destabilization (SIDD) peaks reminiscent of chromosomal fragile sites, while the sixth lay 150 bp distant. Extended SIDD profiling showed that additional oncomiRs also map to SIDD peaks. Fluorescence in situ hybridization (FISH) analysis showed that 11/52 (21%) leukemia-lymphoma (L-L) cell lines with 13q31 involvement bore structural rearrangements at/near *MIR17HG* associated with upregulation. As well as fuelling genome instability, SIDD peaks mark regulatory nuclear-scaffold matrix attachment regions open to nucleosomal acetylation. Collectively, our data indict a specific DNA instability motif (SIDD) in chromosome rearrangement, specifically alterations activating *miR-17~92* epigenetically via promoter hyperacetylation, and supply a model for the clustering of oncomiRs near cancer breakpoints.

INTRODUCTION

Recurrent chromosome rearrangements targeting the B-Cell Lymphoma 6 (*BCL6*) gene characterize DLBCL, occurring in ~40% cases (Pasqualucci et al., 2003) and in ~10% of follicular lymphomas (Akasaka et al., 2003). *BCL6* translocations direct expression of this germinal center (GC)-specific B-cell transcriptional repressor to quell TP53 signaling activated by physiological chromosome breakage (Phan and Dalla-Favera, 2004). Canonical *BCL6* translocations capture active promoter regions yielding hybrid mRNAs translating unaltered protein (Knezevich, 2007) with breakpoints clustered within the 4 Kbp major breakpoint region (MBR) comprising the non-coding exon 1 and the adjacent part of intron 1 (Ye et al., 1993). Non-canonical breakpoints undergo structural *BCL6* deregulation yielding unaltered mRNA/protein and chiefly comprise an alternative breakpoint region (ABR), located 245-285 Kbp upstream (Butler et al., 2002), together with additional instances scattered in between (Schneider et al., 2008).

MicroRNAs (miR) target mRNAs, mostly within their 3'-UTR to effect mRNA degradation or translational inhibition (Lau and MacRae, 2009). OncomiRs undergo transcriptional deregulation via copy number alterations (CNA) (Ventura and Jacks, 2009). Copy number independent oncomiR deregulation is restricted to the single instance of miR-125b in myeloid and B-cell hematopoietic neoplasias (Bousquet et al., 2008; Chapiro et al., 2010). Thus, although miRs are frequently located near recurrent cancer breakpoints (Calin et al., 2004), support for the widely held link between cancer breakpoints and miR dysregulation rests on CNA rather than specific structural changes like other cancer genes.

The *miR-17~92* cluster comprising (hsa)-*miR-17/18a/19a/20a/19b-1/92a-1* (5'-to-3') in chromosome band 13q31 is hosted by the non protein-coding gene *MIR17HG*, alias *C13orf25* (Ota et al., 2004; He et al., 2005). Focal 13q31 amplifications targeting *MIR17HG/miR-17~92* upregulation are characteristic of the GC-DLBCL subtype (Lenz et al., 2008). Its mRNA targets include *E2F1*, *PTEN*, *BIM* or *CDKN1A/p21* (O'Donnell et al., 2005;

Xiao et al., 2008; Inomata et al., 2009; Ventura and Jacks, 2009) mediating cell cycle progression and apoptosis. *MIR17HG* expression is regulated by transcription factors *MYC* (O'Donnell et al., 2005) and E2F - the latter inhibited by some miRs of the cluster to form a negative feedback loop (O'Donnell et al., 2005; Sylvestre et al., 2006). Although pathways regulating *miR-17~92* are incompletely documented, a key role for epigenetics, both as effectors and effectors is emerging (Fabbri and Calin, 2010).

Here we identify and characterize the mode of action of a specific DNA signature linking DNA fragility to structural oncomiR activation by a *BCL6* translocation in DLBCL cells.

MATERIALS AND METHODS

Cells and Treatments

Validated cell lines from the DSMZ repository (detailed in Drexler, 2001; 2010) were cultured as recommended (www.dsmz.de). SU-DHL-16 is a GC-DLBCL cell line established from a male patient at relapse (Elenitoba-Johnson et al., 2003). CA-46 is from Burkitt lymphoma (BL), and NC-NC is a B-lymphoblastic cell line (lcl) from a healthy donor. For histone deacetylase (HDAC)-inhibitor treatment cells were seeded at 10^6 /ml and incubated with 1.25 μ M suberoylanilide hydroxamic acid (SAHA), or equivalent DMSO vehicle for 24 h, 48 h and 72 h. For pulse-treatment cells were washed after 24 h, medium replenished and harvested at 72 h for RNA purification and reverse transcription.

Genomic Analysis

Cytogenetic analyses were conducted as described (MacLeod et al., 2007). Imaging was performed using a Zeiss Axioplan (Oberkochen, Germany) microscope with a-Planapochromat 40x and 100x objectives configured to a Spectral Karyotyping system (ASI Ltd, Migdal Haemek, Israel). Bacterial artificial chromosome (BAC) and fosmid clones were

obtained from BACPAC Resources, Children`s Hospital, Oakland, CA or the Sanger Centre (Cambridge, UK) and DNA extracted and fluor-labeled (MacLeod et al., 2007).

Rapid amplification of cDNA ends (RACE) transcript analysis (Frohman et al., 1988) was performed according to the manufacturer`s protocol (Invitrogen, Darmstadt, Germany).

Primer sequences are available from the authors.

LDI-PCR (Willis et al., 1997) was performed as detailed in Schneider et al. (2011). Briefly, 1 µg DNA was digested with 30-50 units restriction enzyme tailored to breakpoints. For SU-DHL-16 (see Suppl. Fig. 1) these were as follows; BP1-4, EcoRI; BP5, VspI; BP6, Bsp1407I (Fermentas, St. Leon-Rot, Germany). Digested DNA was purified using QIAquick gel extraction kit (Qiagen, Hilden, Germany) and religated to form circular fragments with 5 U T4 DNA ligase (Fermentas) overnight at 2-8 °C in a total volume of 80 µl, followed by heat-inactivation at 65 °C for 20 min. Five µl (62.5 ng) DNA were used as template for LDI-PCR performed using the PCR Extender System (5Prime, Hamburg, Germany) according to the manufacturer`s protocol. PCR products were analyzed on a 1.2% agarose gel and discrepant bands excised, purified with QIAquick gel extraction kit (Qiagen) and sequenced. Primer sequences are obtainable on request.

Sequencing coordinates refer to the HG18 release.

SIDD Analysis

Stress Induced DNA Duplex Destabilization (SIDD) analysis predicts the probability of double strand breakage under superhelical strain (Benham, 1996;1997; Bi and Benham, 2004). The algorithm calculates the energy required for each base pair to separate in a given sequence context. Analyses were performed with the SIDD online tool (<http://benham.genomecenter.ucdavis.edu/sibz/>). For each breakpoint the fusion and both corresponding wild type sequences (for breakpoint nomenclature see Suppl. Fig. 1) were analyzed. Sequences comprised 5 Kbp centered on breakpoints. Default parameters were used throughout.

Real Time PCR Analysis of Gene Expression and Genomic Copy Number

For measuring expression of *MIR17HG* hosting *miR-17~92*, total RNA was purified using RNeasy Mini Kit (Qiagen) and reverse transcribed with Superscript II RT-Kit (Invitrogen) and random hexamer primers (Invitrogen). Analysis was performed using quantitative PCR running on an ABI 7500 Sequence Detection System (Applied Biosystems, Darmstadt, Germany). For master-mix 2X Immomix (Bioline, Luckenwalde, Germany) was used with ROX reference dye (Invitrogen) and SYTO-82 (Invitrogen) to stain double stranded DNA (1.6 μ l 1:100 dilution per 25 μ l reaction). Primer pairs were designed with Primer-3 software (<http://frodo.wi.mit.edu/primer3/>) and set within discrete regions of *MIR17HG* before (exon 1, 5' and 3' miR-coding regions) and after (5' and 3' intron 3, exon 4) the genomic break in SU-DHL-16. TATA box binding protein (TBP) served as endogenous control, while the B-lymphoblastoid cell line NC-NC which expresses *MIR17HG* inconspicuously served as normalization control (calibrator), set to unity to yield expression values using the $2^{-\Delta\Delta C_t}$ formula.

For mature miRs *miR-17/18a/19a/20a/19b-1/92a-1*, small RNA species were purified with the miRvana Kit (Ambion, Austin, TX). For quantification, TaqMan Assays for each miR were purchased (Applied Biosystems). Each kit contains a specific RT primer and a primer pair/TaqMan probe combination for amplification and detection. Reverse transcription was performed with TaqMan MicroRNA Reverse Transcription Kit (Applied Biosystems) using the RT primers multiplexed for all six miRs and the endogenous control gene RNU6B. qPCR was performed using 2X MasterMix (Applied Biosystems) and the primer/probe combinations. Expression values with respect to the calibrator NC-NC were calculated using the $2^{-\Delta\Delta C_t}$ formula.

Analysis of other genes was also performed by qPCR using SYTO-82 based detection. In contrast to the analyses described above, values were normalized to the endogenous control TBP only, as *ITPR2-BCL6* fusion RNA is unique to SU-DHL-16, and *BCL6* is

expressed but weakly in NC-NC. Expression values were calculated by using the formula $2^{-\Delta\Delta Ct}$. Primer sequences are available on request.

HG-U133 Plus 2.0 Expression arrays (Affymetrix, High Wycombe, UK) were used for quantification of gene expression by microarray profiling. Heatmaps and similarity trees were constructed using CLUSTER (v.2.11) and TREEVIEW (v.1.60) (<http://rana.lbl.gov/EisenSoftware.htm>).

Genomic copy number analysis was performed using DNA purified using High Pure Template Purification Kit (Roche, Mannheim, Germany). Quantification was performed by qPCR with the LINE1 repetitive element as endogenous control and cell line NC-NC as calibrator. Primer pairs were those used for expression analysis of *MIR17HG*, without exon 1.

Western Analysis

Proteins obtained from cell lysates were transferred semi-dry onto nitrocellulose membranes (Bio-Rad, Munich, Germany) and blocked with 5% dry milk powder dissolved in PBS. The following antibodies were used: E2F1, PTEN, ERK1/2 (Santa Cruz Biotechnology, Heidelberg, Germany) and biotinylated secondary antibody (GE Healthcare, Little Chalfont/UK). Blots were incubated with primary antibody solutions for 2h at room temperature and washed with PBS. For detection of antibody binding we used streptavidin-biotinylated horseradish peroxidase complex (GE Healthcare) and the Western Lightning ECL (Perkin Elmer, Waltham, MA).

Chromosome Immunoprecipitation (ChIP) Analysis

For precipitation of histone H3-acetylated chromatin an anti-histone H3-Ac specific antibody was used (Millipore, Schwalbach, Germany). Control reactions were performed using an unspecific antibody of the same isotype (Santa Cruz) or no antibody, to show non-specific heavy chain DNA or agarose bead binding. All reactions were performed according to the

manufacturer's protocol. Precipitates were analyzed by real time-PCR using 3 µl of precipitate for each reaction and 50 ng of genomic SU-DHL-16 DNA as positive control and calibrator. Genomic PCR sequences are available from the authors. Signal intensities were normalized to the weakest signal which was set to unity.

RESULTS

Genomic Alterations

Cytogenetic analysis of SU-DHL-16 revealed a t(3;13)(q27;q31)t(12;13)(p11;q31) which sandwiches *MIR17HG* at 13q31 between *BCL6* at 3q27 and *ITPR2* at 12p11 (Fig. 1a/b). LDI-PCR (coordinates and sequences in Suppl. Table 1) placed the 3q27 breakpoint at 188 966 488 bp, 20.3 Kbp upstream of *BCL6* exon 1, i.e. outside canonical MBR and ABR, while the adjoining 13q31 breakpoint mapped to 90 795 961 bp, 2 114 bp upstream of *MIR17HG*. Sequencing the reciprocal derivative fragment showed a second 13q31 breakpoint within *MIR17HG* just downstream of miR-92a at 90 802 364 bp (Fig. 1c). Extended LDI-PCR analysis revealed a complex structure with rearranged and partially inverted sequences covering 3'-*MIR17HG* fused to *BCL6*. The segment encoding *miR-17~92* was translocated ~10 Kbp downstream and juxtaposed to chromosome 12 inside intron 13 (26 713 613 bp) of the inositol 1,4,5-triphosphate receptor type 2 (*ITPR2*) gene (Fig. 1c, Suppl. Fig. 1).

3'-RACE of *MIR17HG* showed truncation just beyond miR-92, 230 bp upstream of the genomic break. 3'-RACE starting inside exon 13 of the *ITPR2* gene showed fusion to chromosome 3 upstream of, and in opposition to, *BCL6*, while the intervening chromosome 13 material encoding *miR-17~92* revealed by LDI-PCR had been spliced out, thus forming an intron (Fig. 1d). Open reading frame analysis from the translation start site in *ITPR2* exon 1 showed a stop-codon shortly after the fusion breakpoint, generating truncated *ITPR2* comprising amino acids encoded by exons 1 to 13, plus 27 bp from the chromosome 3 moiety.

Wider Cytogenetic Involvement of *MIR17HG*

We investigated *MIR17HG* translocations by FISH in other cancer cell lines with 13q31 involvement (Fig. 1e). Rearrangements (CNA or structural) were found in 20/52 (38%) hematopoietic and 0/14 solid tumor cell lines (Fig. 1f), evenly split between CNA (9/52), and structural rearrangements in 11/52, including three combined with CNA. Examples are shown in Suppl. Fig. 2. Our data suggest that *MIR17HG* is non-randomly rearranged in different L-L, e.g. all 6 BL displayed *MIR17HG* CNA with 3/6 structurally rearranged as well. *MIR17HG* amplification was also seen in 3/12 B-cell lymphomas and 2/15 acute myeloid leukemia (AML) cell lines. Two out of four Hodgkin cell lines and 4/15 AML showed structural *MIR17HG* rearrangements. Collectively our findings show that significant 13q31 involvement targets *MIR17HG* deregulation in L-L and that rearrangements may be clinically stratified by configuration.

MIR17HG was actively expressed in rearranged cell lines CA-46, KM-H2, L-540, MEG-01, MEGAL, MONO-MAC-1, NAMALWA, as well as SU-DHL-16 (Fig. 1g). While remaining discordancies between Rq-PCR and microarray expression await clarification - that in SU-DHL-16 was attributable to the rearrangement itself as discussed below in Gene Expression.

Genomic Fragility

Chromosomal fragile sites (CFS) preferentially cleave under genotoxic stress. However, aphidicolin-induced breakage at *BCL6*, *ITPR2* and *MIR17HG* (none at common CFS) was not detected in SU-DHL-16 (not shown). To investigate an alternative clastogenic endpoint, SIDD analysis was performed computationally at all 12 breakpoint coordinates. Remarkably, SIDD profiling showed that wild type sequences at 5/6 breakpoints (BP) precisely cut SIDD peaks, while the sixth lay 150 bp distant (Fig. 2), providing compelling evidence for a direct association between DNA fragility and oncomiR rearrangement (see Suppl. Fig. 1 for nomenclature, Suppl. Table 1 for sequences, and Suppl. Fig. 3 for long-range comparison data).

Gene Expression

mRNA expression at six coding regions of *MIR17HG* flanking the breakpoint was quantified in SU-DHL-16 and other L-L cell lines (Fig. 3a). Before the breakpoint, SU-DHL-16 showed ~5-fold overexpression across exon 1 and both 5′-/3′-miR coding regions equalling or surpassing CA-46 (BL) with ~3- to 4-fold overexpression, while, excepting KMS-12-BM (multiple myeloma), other cell lines barely hit control levels. In contrast, all three *MIR17HG* regions behind the breakpoint were nearly silent in SU-DHL-16 (explaining silencing of a main Affymetrix transcript), contrasting with CA-46 where upregulation matched the forward region. In remaining lines, 3′-*MIR17HG* expression resembled 5′-regions.

Analysis of mature miRs showed that all 6 members of the *miR-17~92* cluster were overexpressed (13- to 18-fold) in SU-DHL-16, about thrice CA-46 levels despite comparable pri-miR expression, while - again excepting KMS-12-BM where expression overlapped CA-46 - remaining cell lines resembled normal controls (Fig. 3b). In KMS-12-BM which carries moderate CNA affecting 13q2-3 (www.sanger.ac.uk; this study) *miR-17~92* upregulation, rivalling CA-46, is also present. In contrast to pri-miR, neoplastic expression of mature forms surpassed NC-NC control cells, probably attributable to processing differences.

Copy number analysis by genomic qPCR of *MIR17HG* showed ca. two- to four-fold amplification in CA-46 (matching expression levels of pri-miR and mature-miRs) consistent with the cytogenetic findings (Suppl. Fig. 2), while copy numbers in SU-DHL-16 remained unaltered (Fig. 3c). Collectively, these data show that *MIR17HG* translocation effects transcriptional truncation sparing the *miR-17~92* coding region in SU-DHL-16. And, while upregulation of both *MIR17HG* and its constituent miRs in CA-46 reflects genomic amplification, that in SU-DHL-16 is copy number independent, highlighting the underlying genomic rearrangement.

As well as *MIR17HG/miR-17~92*, additional relevant gene transcripts were measured (Fig. 3d). Not unexpectedly, *BCL6* was highly expressed in SU-DHL-16 (38-fold TBP), while in

CA-46, like other BL cells, overexpression was also detected (16-fold). *ITPR2-BCL6* fusion RNA was modestly but uniquely expressed in SU-DHL-16 (0.1-fold TBP), while full length *ITPR2* was universally well expressed (2.6- to 5.6-fold) as seen in most B-cell tumor lines (not shown). The *miR-17~92* activator *MYC* was highly expressed across the board: SU-DHL-16 (9.5-fold), CA-46 (31-fold) and NC-NC (9.3-fold), albeit uncorrelated with mature miR expression (Fig. 3d).

***MiR-17~92* Targets**

Western analysis confirmed downregulation of PTEN and E2F1 in SU-DHL-16 (Fig. 3e). At the expression array level, *PTEN* and E2F1 mRNA in SU-DHL-16 matched other DLBCL (not shown) consistent with posttranscriptional control. Another *miR-17~92* target *CDKN1A* (p21) showed weak mRNA expression in both high *miR-17~92* cell lines SU-DHL-16 and CA-46 (respectively, 0.07-fold and 0.01-fold TBP), but generous expression in the *miR-17~92*-inconspicuous cell line NC-NC (4.3-fold) (Fig. 3d). Micro-RNAa may act at either translational or transcriptional levels, the latter mechanism predominating in mammals (Guo et al., 2010). Gene ontology profiling of SU-DHL-16 against other GC-DLBCL cells (<http://www.broadinstitute.org/gsea/>) showed significant depletion of several mRNAs targeted by miR-17/19 (Suppl. Table 2). Thus, *miR-17~92* activation is both functionally and contextually appropriate in SU-DHL-16 cells.

***MiR-17~92* Deregulation**

ITPR2-BCL6 fusion transcript might plausibly effect regulation of *MIR17HG* expression via an antisense mechanism. To investigate this model, transcription was modulated by HDAC-i. Time course experiments showing treatments with SAHA are summarized in Fig. 4a, where *ITPR2-BCL6* and *MIR17HG* responded contrastingly. While exposure to SAHA for 24 h prompted upregulation of both *ITPR2-BCL6* (6.3-fold) and *MIR17HG* (3.3-fold), after further incubation *ITPR2-BCL6* had regressed to control levels by 72 h, while *MIR17HG* maintained progressive upregulation throughout, reaching 8-fold after 48 h, and 20-fold by 72 h.

Treatment with another HDAC-i, trichostatin-A (TSA), yielded similar results (not shown). Control cells showed no upregulation of *MIR17HG* in response to SAHA, while CA-46 responded modestly but transiently. *ITPR2* responded only weakly throughout as did *MYC*, while *BCL6* was transiently upregulated in NC-NC only. Thus, *MIR17HG* upregulation occurs without *ITPR2-BCL6* overexpression. Significantly, among B-/T-lymphoma cell lines, SU-DHL-16 exhibited the greatest sensitivities to SAHA and TSA (Suppl. Fig. 4), perhaps because unmodulated levels are set to spare essential target genes. To check whether upregulation of *MIR17HG* in SU-DHL-16 might, nevertheless, involve “hit-and-run” activation following transient *ITPR2-BCL6* upregulation, cells were pulsed with SAHA for 24 h. After 24 h incubation without SAHA, *MIR17HG* and *ITPR2-BCL6* had reverted to control levels demonstrating the need for continuous exposure (Fig. 4b). Collectively, these findings support the independent regulation of *MIR17HG* and *ITPR2-BCL6*, discounting a straightforward antisense mechanism.

Nevertheless, the exquisite responsiveness of *MIR17HG* in SU-DHL-16 to HDAC-i (Fig. 4a) implies a regulatory role for epigenetic modifications. Treatment with the DNA methyltransferase inhibitor 5-azacytidine (aza), however, failed to modulate *MIR17HG* expression in SU-DHL-16, CA-46, and NC-NC, again contrasting with *ITPR2-BCL6* (Suppl. Fig. 5), focusing attention onto protein modifications. To assay de novo acetylation at genomically altered sites flanking wild type and rearranged *MIR17HG*, ChIP-assays were performed using acetylated-histone H3 antibodies. DNA ChIP fragments were analyzed by real time PCR using primers specific for both fusion and wild-type sequences. This revealed acetylation gains at BP5 (upstream of *MIR17HG*) in SU-DHL-16 where the fusion breakpoint showed 4.8-fold and 12-fold enrichment over wild type 5'- or 3'- component regions, respectively (Fig. 4c). In contrast, acetylation at BP6 (downstream) just undercut (~25%) wild type *MIR17HG*. Collectively these findings highlight upstream hyperacetylation as a plausible regulatory consequence of t(3;13)t(12;13) impacting *MIR17HG/miR-17~92*.

DISCUSSION

We characterized a complex rearrangement t(3;12)t(12;13) in GC-DLBCL cells which targeted *BCL6* and *miR-17~92* for deregulation. t(3;12)t(12;13) cells showed high-level *MIR17HG* expression: however, in contrast to CNA-driven upregulation seen in CA-46, expression of *miR-17~92* in SU-DHL-16 was boosted an additional threefold, implying that structural *MIR17HG/miR-17~92* deregulation embraces both transcription and subsequent processing. The selective upregulation of mature *miR-17~92* members over pri-miR in L-L cells when compared to controls also deserves further investigation in this regard.

Reflecting known patterns of deregulation in these tumors, 21% hematologic but no solid tumor examples carried *MIR17HG* rearrangements. Given that orphan cancer breakpoints at 13q31 are not uncommon (<http://cgap.nci.nih.gov/Chromosomes/Mitelman>), the claim of *MIR17HG* is persuasive when set against rival candidates at this locus (listed by Dessen and Huret, 2011).

A clue to oncomiR deregulation linked to chromosome instability was provided by the observed coincidence of 5/6 breakpoints and SIDD peaks. SIDD sites are reminiscent of CFS, notably FRA7H which is also stabilized by rearrangement with *BCL6* in DLBCL cells (Schneider et al., 2008). Thus, for example, SIDD sites and FRA7H are both AT-rich and ultra-flexible (Mishmar et al., 1998). Although oncomiRs cluster near CFS (Calin et al., 2004), their size - often exceeding 1 Mbp - has hitherto thwarted identification of putative DNA targets therein. SIDD sites are closely associated with bacterial promoters and transcriptional start sites (Wang et al., 2004), together with eukaryotic nuclear scaffold matrix attachment regions which in turn tether chromatin loops to control gene expression via recruitment of acetylated nucleosomes (Martens et al., 2002).

Almost equally striking was the repositioning of *miR-17~92*, sandwiched antisense within the *ITPR2-BCL6* fusion mRNA. *ITPR2-BCL6* fusion mRNA and *MIR17HG/miR-17~92* were, despite their intimate and provocative association, independently regulated, discounting

antisense control of the latter. In another oncogenic 3'-deletion, that involving CCND1, truncation and mutation reportedly increase tumorigenicity (Wiestner et al., 2007), with loss of miR-16-1 regulatory binding sites proposed as the underlying mechanism (Chen et al., 2008). However, scrutiny of the *MIR17HG* truncation failed to reveal regulatory miR, pumilio or FBF post-transcriptional repressor sites. This, together with the conspicuous absence of recurrent activating deletions or mutations of 3'-*MIR17HG*, tells against activational truncation. Instead, ChIP experiments suggested that t(3;12)t(12;13) deregulates *miR-17~92* by increasing histone acetylation at its upstream breakpoint near the *MIR17HG* promoter.

Taken together, our findings suggest that SIDD alterations can promote oncomiR deregulation via chromatin modification and support the wider effector role for epigenetic micro-RNA activation proposed recently (Fabbri and Calin, 2010).

ACKNOWLEDGMENTS

The authors thank Drs. Sonja Eberth and Hilmar Quentmeier for comments and criticisms, Mrs. SriLaxmi Kalavalapalli for additional technical help, and donors of cell lines used in this study.

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FIGURE LEGENDS

Figure 1. Characterization of *MIR17HG* Breakpoints and Related Transcripts:

(a) Shows chromosome painting with probes for chr. 3 (red) and 12 (green) and G-banding analysis (inset) of t(3;12)(q27;p11) where arrows show breakpoints. Note q-arm duplication of der(12). (b) FISH shows rearrangement within *miR-17~92* fosmid 8960A3 (gold) with BACs 632M13 (red) covering *BCL6* at 3q27, and 13K14 (green) covering *ITPR2* at 12p11. Material from the *MIR17HG* region was inserted near the *BCL6* and *ITPR2* fusion. (c) Shows key breakpoints mapped by LDI-PCR at 3q27, 12p11 and 13q31 (x2) (broken lines). The proximal 3q27 break lay at 188 966 488 bp, 20,317 bp upstream of *BCL6*, between MBR and ABR. The 12p11 break lay at 26 713 361 bp, inside intron 13 of *ITPR2*. At 13q31 two breaks were detected at 90 795 961 and at 90 801 989 bp, respectively, 2 114 and 2 841 bp upstream of the *MIR17HG* transcription startpoint, with another 13q31 break flanking *miR-17~92* at 90 801 989 juxtaposing the 12p11 breakpoint. (d) 3'-RACE of *MIR17HG* yielded transcript truncated shortly after miR-92, 230 bp upstream of the genomic breakpoint. 3'-RACE starting within the *ITPR2* gene showed fusion of exon 13 with a non-annotated RNA starting 20 760 bp upstream of *BCL6*. (e) Genomic map shows RP11 BAC (clear boxes) and fosmid (black) clones used to locate breakpoints. Structural breakpoints are shown as horizontal lines (broken for simple rearrangements, solid when combined with amplification) to reflect actual mapping precision. (f) Chart summarizes *MIR17HG* FISH results on 52 hematopoietic and 14 solid tumor cell lines with peri-13q31 involvement. Note contrasting incidences in hematopoietic (38%) and solid tumors (0%) and in particular, conspicuous genomic *MIR17HG* amplification in Burkitt and other non-Hodgkin lymphomas. (g) Shows expression of *MIR17HG* determined by Rq-PCR relative to NC-NC B-Icl (columns) and by microarray profiling (circles). Rq-PCR data for NAMALWA, and microarray data for MEG-01,

MONO-MAC-1, NC-NC were unavailable. Primer location (grey box) below *miR-17~92* is shown inset. Abbreviations: AML, acute myeloid leukemia; BCP-ALL, B-cell precursor acute lymphoblastic leukemia; BL, Burkitt lymphoma; B-Icl, B-lymphoblastic cell line; B-NHL, B-cell non-Hodgkin lymphoma; cen(tromeric); cen(tromeric); CML, chronic myeloid leukemia; HL, Hodgkin lymphoma; MM/PCL, multiple myeloma and plasma cell leukemia; natural killer cell lymphoma (NKL) tel(omeric).

Figure 2. SIDD Values of Breakpoints and Wild Type Sequences:

Graphs show the SIDD probability values plotted +/-200 bp of breakpoints (vertical lines). Central graphs show fusion rearrangements flanked by wild type component sequences. Note placement of breakpoints either within (BP-1/2/3/4/6), or near (BP5), SIDD peaks, and the tendency of rearrangements, excepting BP6, to reduce wild-type peaks indicating stabilization by chromosome rearrangement.

Figure 3. Gene Expression Analysis:

a) Shows expression of *MIR17HG* (pri-miR) upstream and downstream of the genomic breakpoint (broken line) normalized to control NC-NC cells. Note upregulation in SU-DHL-16 limited to regions upstream of the genomic breakpoint, unlike other cell lines: CA-46 (Burkitt lymphoma) upregulated throughout, and KMS-12-BM (multiple myeloma), L-540 (Hodgkin lymphoma), OCI-M2 (acute myeloid leukemia), and SU-DHL-10 (DLBCL) which remained low throughout. (b) Expression analysis of mature miRs in SU-DHL-16 and other cell lines with *MIR17HG* copy numbers (Suppl. Fig. 2) varying as follows: CA-46 (significantly amplified); KMS-12-BM (slightly amplified); L-540; OCI-M2; SU-DHL-10 (normal). Note expression in SU-DHL-16 and KMS-12-BM in excess of copy number while upregulation in CA-46 matches amplification. Note also upregulation of *miR-17~92* in L-L cells over control.

(c) To validate FISH data, genomic copy number analysis was performed in triplicate by qPCR on SU-DHL-16 and CA-46, confirming genomic amplification in the latter only. (d) Absolute expression relative to TBP showed upregulation of *BCL6* in SU-DHL-16 consistent with the genomic rearrangement. Moderate upregulation in CA-46 is also typical of BL cells.

Figure 4. Epigenetic Analyses:

HDAC-i treatments with SAHA were used to modulate gene expression in SU-DHL 16 and comparison/control cell lines CA-46 and NC-NC. (a) Expression of *BCL6*, *ITPR2*, *ITPR2-BCL6*, *MIR17HG*, and *MYC* were quantified after 24 h, 48 h and 72 h SAHA treatment. Note progressive high-level (20x) upregulation of *MIR17HG* in SU-DHL-16 from 24 h to 72 h, contrasting with weaker transient upregulation of *ITPR2~5'-BCL6* peaking (6x) at 24 h, thereafter regressing to below control levels by 72 h. Thus *MIR17HG* and *ITPR2-BCL6* appear to be under independent control despite their intimate genomic configurations. Data show means of three independent replicates. (b) Pulse SAHA treatments showed a general need for HDAC-i to maintain upregulation of both *MIR17HG* and *ITPR2-BCL6*, inconsistent with “hit-and-run” antisense regulation of the former by the latter. The column chart shows the relative expression of SAHA treated cells with respect to the vehicle controls. Data show means of three independent replicates. (c) Chromatin immunoprecipitation (ChIP) assays compare histone acetylation in SU-DHL-16 cells at BP5 and BP6 flanking *MIR17HG*. Note increased acetylation at BP5 in fusion, over wild-type components, while both wild type and fused 3' regions of *MIR17HG* are highly acetylated at BP6 unlike wild type *ITPR2*. Thus, chromosomal rearrangement effects selective hyperacetylation of upstream *MIR17HG*. ChIP analysis of SU-DHL-16 cell lysates was performed twice using ChIP-Kit (Millipore).

CDKN1A was downregulated in both *miR-17~92* upregulated cell lines consistent with mRNA targeting thereby. *MYC* expression in CA-46 exceeded that in SU-DHL-16 which matched NC-NC. (e) Representative western blot shows protein expression of E2F1 and

PTEN in three DLBCL cell lines with *BCL6* rearrangement: RC-K8, RI-1, and SU-DHL-16 (For *MIR17HG* expression see Fig. 1g). Note downregulation of PTEN in SU-DHL-16 which alone shows *miR-17~92* upregulation. ERK1/2 served as loading control. Quantitative data show means ($\pm$ se) of 3 independent replicates.