A 9 series microRNA signature differentiates between germinal centre and activated B-cell-like diffuse large B-cell lymphoma cell lines

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Abstract. The microRNAs are endogenous, non-coding RNAs that play key roles in a range of pathophysiological processes by up- or down-regulating gene expression. Recent studies have shown that some microRNAs have oncogenic or tumour suppressor activity. Diffuse large B-cell lymphoma (DLBCL) is an aggressive non-Hodgkin's lymphoma with a heterogeneous biology, which has impeded the clinical assessment of patients. The currently-used clinically-based IPI provides useful information for treatment decision making, but has limited predictive power. Recent immunohistochemical approaches have identified two different prognostic groups: the more indolent germinal centre (GC)- and the higher risk activated B-cell (ABC)-like phenotypes. Although useful, prediction based on immunophenotype has limitations. The present study uses microRNA profiling and a number of well-characterised B-cell lymphoma cell lines to identify microRNA signatures that are correctly assigned to the DLBCL prognostic subgroups and distinguish DLBCL from other more indolent lymphoma, including follicular lymphoma (FL). MicroRNA microarray analysis was based on miRBase version 12.0 and analysis was performed using an unsupervised hierarchical clustering model. Discriminatory microRNAs were validated by qRT-PCR. We identified a 9 microRNA signature that discriminated between ABC- and GC-like DLBCL. This included 3 newly identified microRNAs, not previously associated with DLBCL and predicted to target genes that are de-regulated in lymphoma. DLBCL was distinguished from FL by 4 microRNAs and a total of 18 microRNAs were identified that differentiated between all lymphoma and control populations. Most of the discriminatory microRNAs have been reported previously to be known oncomiRs or act as tumour suppressors. In conclusion, the present study identified a microRNA signature that correctly classified GC and ABC phenotypes in DLBCL cell lines. This signature has yet to be assessed for prediction in clinical samples.

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Introduction

Diffuse large B-cell lymphoma (DLBCL) is a malignancy of large transformed B lymphocytes. It is the most common non-Hodgkin's lymphoma (NHL), accounting for approximately 40% of all NHL (1). DLBCL can arise from small B-cell lymphoma, marginal zone lymphoma and follicular lymphoma (FL) and in such cases, it is referred to as transformed DLBCL (2,3). This aggressive malignancy of mature B-cells is a clinically heterogeneous disease, which may present in primary lymph nodes or in extra-nodal sites (4).

The heterogeneous nature of DLBCL has impacted on patient's response to treatment and resulted in highly variable disease outcome. This observed variability is most likely due to diverse genetic abnormalities, molecular and biological heterogeneity and different clinicopathological features (5). This highlights the need to evaluate the prognostic significance of specific molecular, genetic and epigenetic factors and to identify new parameters that provide more accurate prognostic information. Indeed, numerous biological markers and genes have now been studied and linked to aspects of tumour biology. Recently, gene expression array technology and immunohistochemical studies have been successful in defining at least two molecular subgroups, with different prognoses, namely; germinal centre (GC)- and activated B-cell (ABC)-like DLBCL (6-9). The GC-like phenotype has been associated with a more indolent disease, whereas ABC-like predicts for poorer outcome (8). However, in clinical terms, this distinction remains unclear and some patients with an indolent phenotype may present or develop aggressive disease. This is most likely due to the underlying molecular heterogeneity and may highlight the existence of additional

genetic control. Recent studies have provided evidence to suggest involvement of small interfering RNAs and microRNAs in the regulation of gene expression (10-12). Dysfunctional expression of gene-regulatory molecules may have implications in disease development, including cancer.

The microRNAs are endogenous, naturally occurring, non-coding RNAs that play key regulatory roles in a diverse range of pathways, including development, cell proliferation, differentiation and apoptosis. Since their discovery in 1993 (13), a fundamental role for microRNAs has been uncovered in small RNA-guided post-transcriptional regulation of gene expression. These 18-24 nucleotide single-stranded RNAs form a complex with associated proteins known as the RNA-induced silencing complex (RISC) and bind to complimentary sites in the 3' UTR of messenger RNAs (mRNA) (14). The result is up-regulation (15) or translational repression (12), either with or without mRNA degradation (12).

A number of findings early on in the history of microRNAs suggested their potential role in human cancer. MicroRNA genes are frequently located at fragile sites or cancer-associated genomic regions (16) and cancer susceptibility loci that influence the development of solid tumours (17). In 2006, Calin *et al* directly associated the de-regulation of miR-15 and miR-16 expression in the development of B-cell chronic lymphocytic leukemia (B-CLL) (18).

Recent microRNA expression profiling studies have been successful in stratifying cancers more accurately than the traditional gene expression profiles and have also provided signatures that relate to diagnosis, or reflect tumour-specific developmental stages (19). With respect to lymphoma, microRNA signatures that are lymphoma-specific and discriminate between the DLBCL immunophenotypes have produced variable findings. Using cell lines of GC and ABC phenotypes, Malumbres *et al* identified a 9 microRNA signature that differentiated between these subgroups (20). Similarly, Lawrie *et al* reported that miR-21, -155 and -221 were significantly over-expressed in ABC-like cell lines compared to the GC type (21). More recently, two independent studies have reported predictive microRNA signatures for patients with DLBCL against those with FL (22,23) and also against normal lymph node B-cells (22). However, consensus between these studies is lacking. With respect to studies that used clinical material, the reported variability may be due to the use of large numbers of clinical samples which were most likely biologically and molecularly very heterogeneous.

In the present study, we also used microRNA microarray technology to re-assess microRNA expression and identify lymphoma-specific microRNA signatures, signatures that differentiate between GC- and ABC-like DLBCL and between DLBCL and the more indolent FL phenotypes. To achieve this, we used a number of well-defined B-cell lymphoma cell lines and a microRNA profiling system that uses the highest number of gene sets currently available.

Materials and methods

Cell lines and controls. The cell lines studied comprised 2 FL (SC-1 and WSU-FSCCL) and 8 DLBCL (SU-DHL-5, OCI-Ly19, SU-DHL-4, SU-DHL-10, HBL-1, SU-DHL-8, NU-DHL-1 and HLY-). All cell lines were obtained from the DSMZ collection (Braunschweig, Germany) with the exception of HBL-1 and HLY-1, which were kindly donated by Professor Gatter (LRF Lymphoma Antigens Group, John Radcliffe Hospital, Oxford, UK). All cell lines were cultured in RPMI-1640 supplemented with 10-20% v/v heat-inactivated foetal calf serum (FCS) (Sera Lab Ltd.), 2 mM L-glutamine (Gibco, UK), 100 IU/ml penicillin (Gibco) and 100 μg/ml streptomycin (Gibco), according to the repositories guidelines. Cultures were incubated at 37˚C in a 5% CO_2 humidified atmosphere. $CD19^+$ negatively-selected B-cell total RNA (pooled from 3 unrelated donors) served as the normal healthy B-cell control population (AllCells LLC, USA).

Immunophenotyping of cell lines. In order to perform immunohistochemical (IHC) staining on DLBCL cell lines, loose preparations of ~10^7 cells were formalin fixed and embedded in FFPET blocks. These were then cut for streptavidin-biotin (SAB) based indirect IHC, exploiting 3,3'-diaminobenzidine (DAB) as the chromagen and using mouse anti-human mono-clonal antibodies against CD10, BCL-6 and MUM1. IHC was performed using the automated Ventana BenchMark® immunostaining system. Full details of conditions were described previously (24). All DLBCL cell lines were segregated on the basis of their immunophenotype and classified as either GC-like or ABC-like, based on the algorithm proposed by Hans *et al* (8). Immunohistochemical staining was graded both in terms of intensity on a three-point scale (1+ to 3+) and the overall percentage of malignant cells showing reactivity (1-100%). Sections were reviewed independently by two histopathologists.

RNA extraction. All total RNA was extracted by TRIzol® using ~10^7 cells per cell line and its quality assessed on an Agilent 2100 Bioanalyzer (Agilent, UK), as well as by gel electrophoresis. All samples had RIN values of >8.0 and clear gel bands at 4 kb (28 s), 2 kb (18 s) and 25 bp (microRNAs).

MicroRNA expression by microarray analysis. MicroRNA microarray analysis was carried out by Miltenyi Biotec (Miltenyi Biotec Headquarters, Bergisch Gladbach, Germany) using miRXplore technology. This was based on miRBase version 12.0, containing 1860 mature microRNA sequences, from multiple species (Hu, Mo, Rat, Viral). The Universal microRNA Reference (UR) control pool was synthesised by Miltenyi Biotec and was labelled with Cy3 (e-570 nm), whilst the test samples (i.e. cell lines and $CD19^+$ B-cell control RNAs) were stained with Cy5 (e-670 nm). Each solid phase test array incorporated probes in quadruplicate, mismatch controls (n=13), positive controls (n=36), hybridization controls (n=5) and calibration controls (n=18). Data capture was facilitated using ImaGene Software (BioDiscovery Inc, CA) and analysed using PIQOR™ software (Miltenyi Biotec).

Unsupervised hierarchical cluster analysis. Microarray analysis was performed using an unsupervised hierarchical clustering model, employing GeneSpring GX10 v10.0 software (Agilent Technologies Inc.). Multiple data sets were analysed, filtering between 75 and 100%, using Cy5/Cy3 median normalised data (10 cycles) as the primary input.

Cluster analysis incorporated a 100% return on selected gene sets (n=420 species) and the CD19+ control B-cells were treated as 3 separate entities, as these were derived from 3 un-related donors (see Cell lines and controls). The distance metric used was Euclidean and Euclidean Square and the linkage was centroid/average.

MicroRNA expression by relative quantitative real-time PCR. In order to validate array results by RT-PCR, total RNA samples were treated with DNA-*free*™ (Ambion, USA) to remove any potential residual trace DNA contamination. All mature microRNA-specific qRT-PCR reactions were carried out using TaqMan® microRNA assays (Applied Biosystems, USA) according to the manufacture's instructions. Reverse transcription reactions were each seeded with 10 ng of total RNA and cycling conditions were as follows; 30 min 16˚C, 30 min 42˚C, 5 min 85˚C, hold 4˚C. Cell line cDNA samples were then amplified by qRT-PCR in triplicate using the LightCycler 480 platform (Roche, UK). Each reaction comprised 20 μl of reaction mix, inoculated with 5 μl of 1:10 diluted cDNA and cycling conditions were as follows; 1 cycle activation (10 min 95˚C), 45 cycles PCR (10 sec 95˚C, 1 min 60˚C then 1 sec 72˚C) and 1 cycle cool (30 sec 40˚C). Expression levels were calculated using the E-method (25), producing a normalised ratio (N.R) for all target samples. The reference gene employed was RNU48 (Applied Biosystems), as this was found to give the most consistent expression levels across the cell lines used in this study. A negative control was incorporated into every run, in which cDNA inoculate was replaced by nuclease free PCR-grade water (Roche).

Statistical analyses. In order to analyse the microarray data, Statistical Analysis of Microarrays (SAM)/one way ANOVA (GeneSpring) was applied to identify discriminatory microRNAs between sub-sets of lymphoma, and between lymphoma groups and the normal control. The selected test was one-way ANOVA, the p-value computation was asymptotic (p=0.05) and the Benjamini-Hochberg multiple testing correction algorithm was applied.

MicroRNA microarray data and qRT-PCR results were tested for positive correlation using the Spearman's correlation test. Filtered $\log_2$ microarray values and N.R RT-PCR results were used for analysis.

Results

Immunophenotyping of DLBCL cell lines. All DLBCL cell lines were immunophenotyped as GC-like or ABC-like, as defined by the Hans classifier (8). GC-like cell lines defined in this manner comprised; SU-DHL-5, OCI-Ly19, SU-DHL-4 and SU-DHL-10, whilst ABC-like cell lines included: HBL-1, HLY-1, SU-DHL-8 and NU-DHL-1 (data not shown).

Microarray-based analysis of microRNA expression

GeneSpring GX10 unsupervised hierarchical cluster analysis. Initially, all cell lines and all entities (n=926) were analysed. However, subsequently this analysis was restricted to include only those entities with complete return and no missing data (n=420) thus, retaining optimum data load, whilst not excessively over restricting the spectrum of entities returned. For cluster analysis, variation of the distance metric did not affect the results.

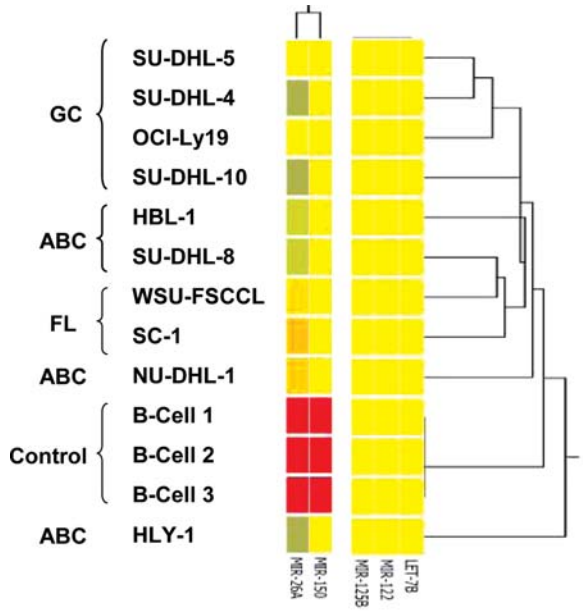

Figure 1. GeneSpring unsupervised hierarchical cluster analysis of the B-cell lymphoma cell lines and CD19+ normal B-cell control.

The GeneSpring hierarchical cluster analysis, incorporating 100% return, segregated each of the GC cell lines together. Amongst the ABC cell lines, the SU-DHL-8 segregated closely to the more indolent FL (Fig. 1). Interestingly, the ABC cell line HLY-1 showed a microRNA signature quite distinct from all other cell lines, expressing a number of γ-herpesvirus-associated microRNAs.

Discriminatory microRNA analysis. Statistical analysis (GeneSpring) revealed 18 discriminatory microRNAs (Table I) with a false discovery rate (FDR) set at 0%. Of these, 5 were microRNAs of the miR-17-92 cluster (miR-17, miR-19A, miR-19B, miR-20A, miR-92A) and three of the discriminators (miR-20B, miR-92B, miR-106A) were from the miR-106A-92 cluster (26).

MicroRNA expression is distinct between GC- and ABC-like DLBCL cell lines and both are different from normal CD19+ B-cells. Based upon the immuno-phenotype, patients with DLBCL can be segregated into GC- and ABC-like DLBCL, presenting with distinct outcome characteristics (8). Our discriminatory analysis identified a signature of 9 microRNAs that could differentiate between GC- and ABC-like DLBCL immunophenotyped cell lines and all of these microRNAs were expressed at a higher level in ABC- than GC-like cell lines (Table I, Figs. 2A and 4A). Of the 9 microRNAs identified, 4 were members of the miR-17-92 cluster (miR-17, miR-19B, miR-20A and miR-92A) and one, miR-106A, has been described as a paralog of miR-17-5, belonging to the miR-106a-92 cluster, localised to chromosome X (26).

Comparing DLBCL cell lines to the CD19+ B-cell controls, 9 microRNAs were identified that could discriminate between ABC-like DLBCL and the controls (Table I, Figs. 2B and 4B). The microRNAs miR-17, miR-106A and

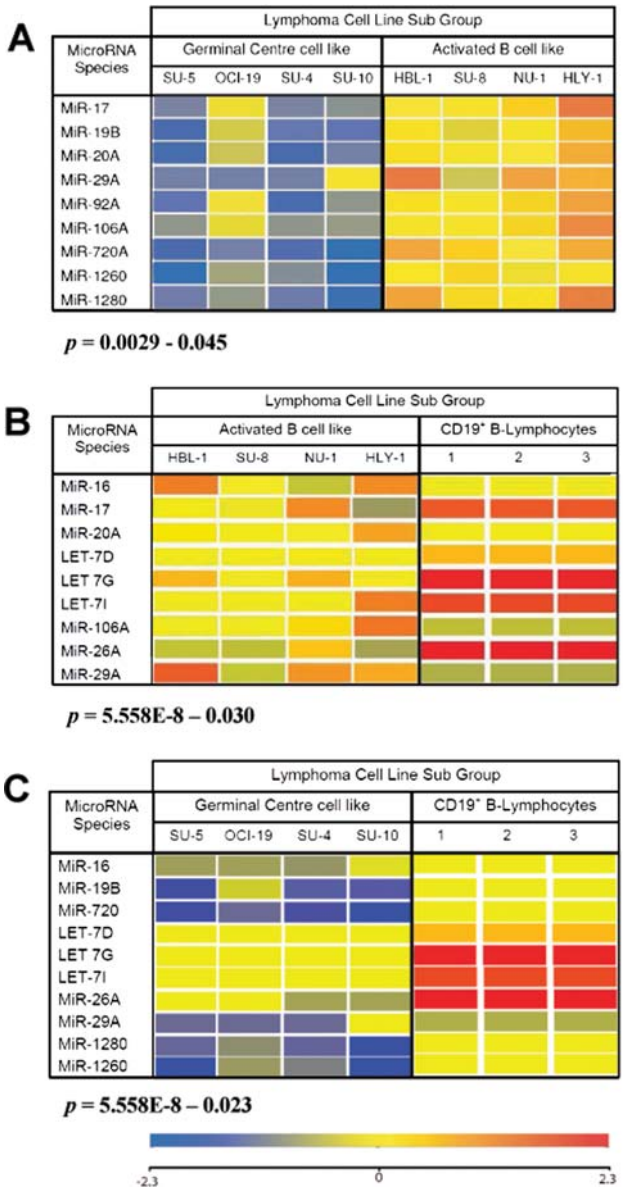

Figure 2. Heat maps to show the discriminatory microRNA signatures. MicroRNA signatures that differentiate between (A) GC- and ABC-like DLBCL, (B) ABC-like and normal CD19+ B-cells and (C) GC-like and normal CD19+ B-cells. Expression was measured by microarrays (see Materials and methods). Values shown are median of 4 replicates ($p<0.05$).

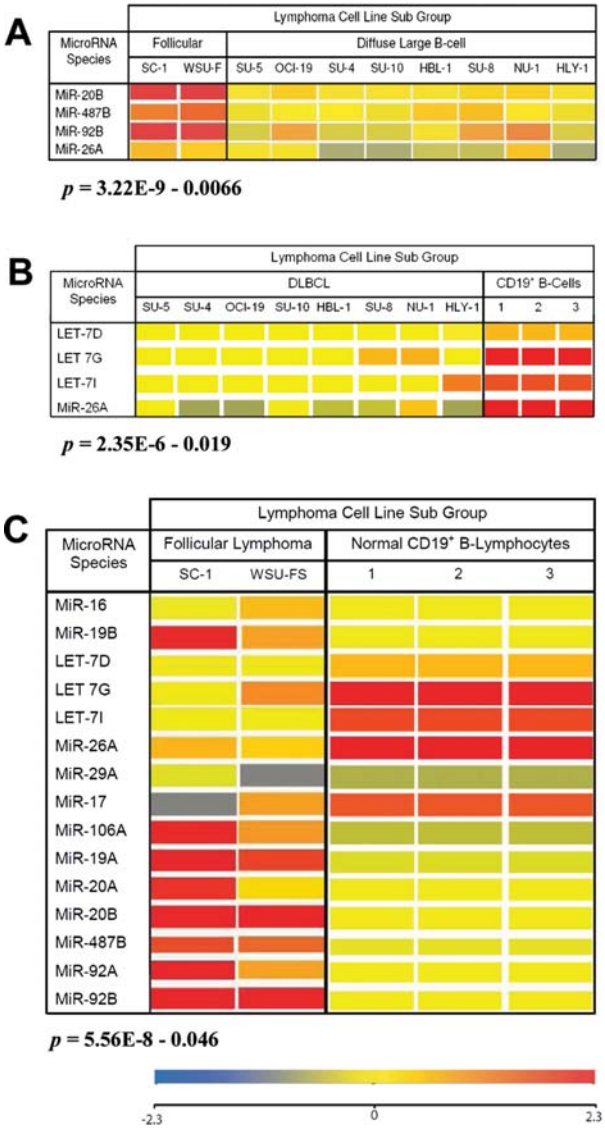

Figure 3. Heat maps to show the discriminatory microRNA signatures. MicroRNA signatures that differentiate between (A) FL and DLBCL, (B) DLBCL and normal CD19+ B-cells and (C) FL and normal CD19+ B-cells. Expression was measured by microarrays (see Materials and methods). Values shown are median of 4 replicates ($p<0.05$).

miR-20A were expressed at higher levels in ABC-like DLBCL cell lines compared to the control samples, whilst miR-16, miR-29A, miR-26A, LET-7D, LET-7G and LET-7I were over-expressed in normal CD19+ B-cells (Fig. 4B). A 10 microRNA signature discriminated GC-like from the CD19+ controls (Table I, Figs. 2C and 4C) and all of these were expressed at lower level in the GC-like cell lines than the CD19+ controls (Fig. 4C).

MicroRNA expression varies between DLBCL and FL and both are different from normal CD19+ B-cells. A separate analysis was performed, based on grouping ABC-like and GC-like DLBCL as one entity. These results identified 4 microRNAs that were differentially expressed between DLBCL and FL (miR-20B, miR-487B, miR-92B and miR-26A) (Table I, Figs. 3A and 4D). Two of these (miR-20B and miR-92B) are members of the miR-106A-92 homologous cluster. Moreover, analysis identified 4 microRNAs that differentiated between DLBCL and normal CD19+ B-cells, including LET-7D, LET-7G, LET-7I and miR-26A (Table I, Figs. 3B and 4E).

The FL group was distinguishable from the CD19+ controls by a series of 15 microRNAs (Table I, Fig. 3C). The expression levels of the microRNAs within this signature were varied. Six of 15 microRNAs were expressed at a lower level in FL compared to the CD19+ control (miR-16, miR-26A, miR-29A, LET-7D, LET-7G and LET-7I) and 9 were expressed at a higher level in FL than the CD19+ control (miR-17, miR-19A, miR-19B, miR-20A, miR-20B, miR-92A, miR-92B, miR-106A and miR-487B) (Fig. 4F). Of the latter, 5 were members of the miR-17-92 cluster (miR-17, miR-19A, miR-19B, miR-20A and miR-92A). Three of the four remaining microRNAs that were expressed at a higher

Table I. MicroRNAs that discriminate between the various lymphoid cell lines and normal CD19+ B-cells.

ABC vs control	GC vs control	ABC vs GC	ABC vs FL	GC vs FL	FL vs control	DLBCL vs FL	DLBCL vs control
miR-16	miR-16				miR-16		
miR-17		miR-17	miR-17	miR-17	miR-17		
			miR-19A	miR-19A	miR-19A		
	miR-19B	miR-19B	miR-19B	miR-19B	miR-19B		
miR-20A		miR-20A	miR-20A	miR-20A	miR-20A		
				miR-20B	miR-20B	miR-20B	
miR-26A	miR-26A		miR-26A		miR-26A	miR-26A	miR-26A
miR-29A	miR-29A	miR-29A	miR-29A		miR-29A		
		miR-92A	miR-92A	miR-92A	miR-92A		
			miR-92B	miR-92B	miR-92B	miR-92B	
miR-106A		miR-106A	miR-106A	miR-106A	miR-106A		
	miR-720	miR-720		miR-720			
	miR-1260	miR-1260		miR-1260			
	miR-1280	miR-1280		miR-1280			
			miR-487B	miR-487B	miR-487B	miR-487B	
miR-LET-7D	miR-LET-7D				miR-LET-7D		miR-LET-7D
miR-LET-7G	miR-LET-7G				miR-LET-7G		miR-LET-7G
miR-LET-7I	miR-LET-7I				miR-LET-7I		miR-LET-7I
9	10	9	10	12	15	4	4

Table II. MicroRNAs that are common between the various lymphoma cell lines and normal CD19+ B-cells.

ABC vs control	GC vs control	ABC vs GC	ABC vs FL	GC vs FL	FL vs control
		miR-16	miR-16	miR-16	
	miR-17				
miR-19A	miR-19A	miR-19A			
miR-19B					
	miR-20A		miR-20A		
miR-20B	miR-20B	miR-20B			
		miR-26A		miR-26A	
miR-27B	miR-27B	miR-27B	miR-27B	miR-27B	miR-27B
				miR-29A	
miR-30C	miR-30C	miR-30C	miR-30C	miR-30C	miR-30C
miR-92A	miR-92A				
miR-92B	miR-92B	miR-92B			
	miR-106A				
miR-150	miR-150	miR-150	miR-150	miR-150	miR-150
miR-223	miR-223	miR-223	miR-223	miR-223	miR-223
miR-342-3P	miR-342-3P	miR-342-3P	miR-342-3P	miR-342-3P	miR-342-3P
miR-487B	miR-487B	miR-487B			
miR-720			miR-720		miR-720
miR-1260			miR-1260		miR-1260
miR-1280			miR-1280		miR-1280
miR-LET-7C	miR-LET-7C	miR-LET-7C	miR-LET-7C	miR-LET-7C	miR-LET-7C
		miR-LET-7D	miR-LET-7D	miR-LET-7D	
		miR-LET-7G	miR-LET-7G	miR-LET-7G	
		miR-LET-7I	miR-LET-7I	miR-LET-7I	
15	14	15	14	12	9

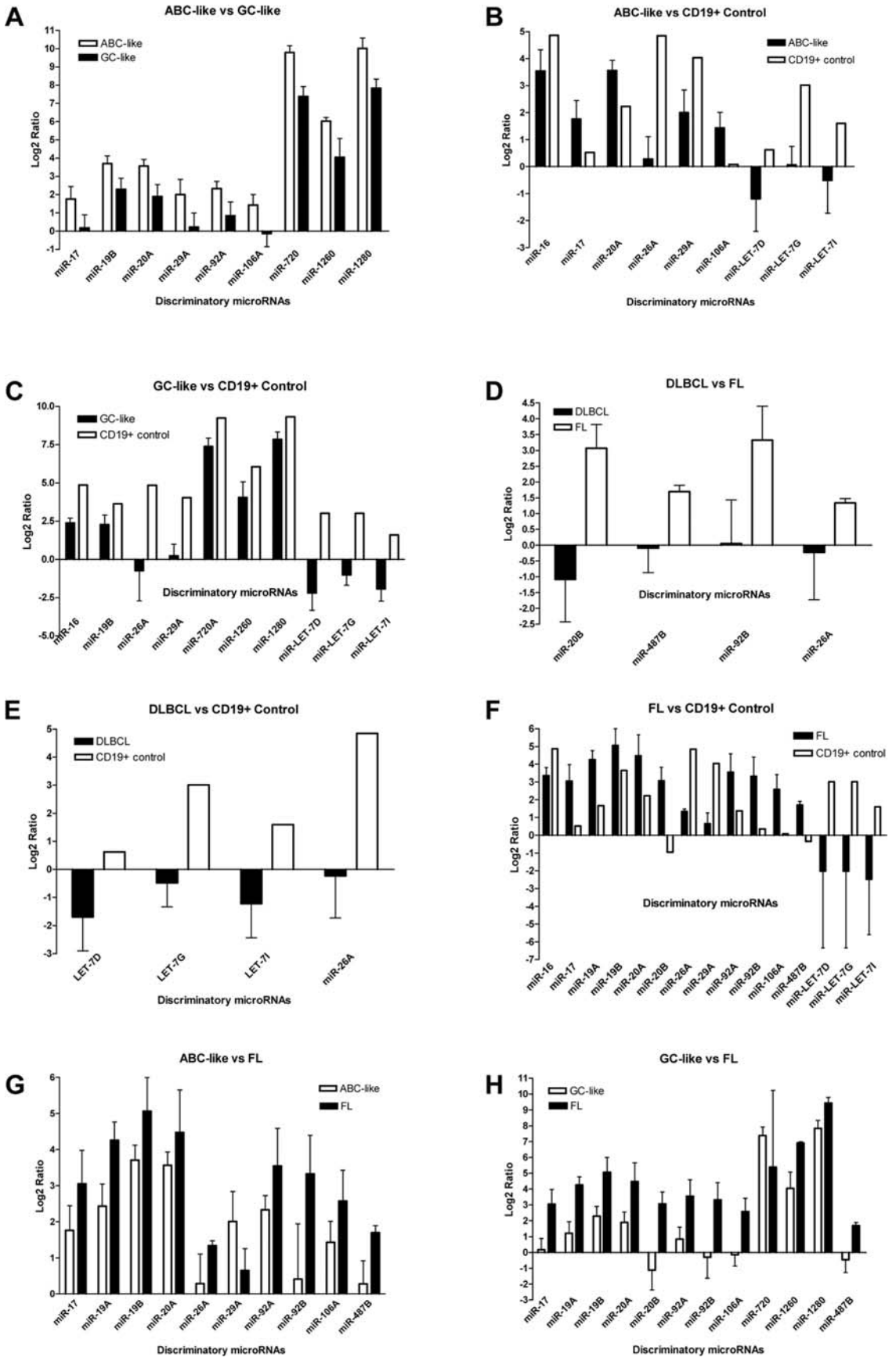

Figure 4. Microarray-based comparison of expression levels of discriminatory microRNAs in the various lymphoma cell lines and normal CD19+ B-cells. Discriminatory microRNAs were identified by statistical analysis of microarrays (SAM)/one way ANOVA (GeneSpring). Each bar represents mean (± SD) expression levels of each microRNA in the corresponding cell lines or normal CD19+ B-cells. Expression is based on $\log_2$ expression ratios (cell lines relative to the universal reference). With respect to CD19+ B-cells, the RNA was pooled from 3 unrelated donors but for the expression analysis, this was treated as 3 separate entities.

Table III. Validation of microarray-based microRNA expression by qRT-PCR.[a]

MicroRNA	Array vs qRT-PCR	
	r^2-value[b]	p-value
miR-17	0.3129	0.0586
miR-19B	0.6143	**0.0293**
miR-20	0.2570	0.0925
miR-92	0.3644	**0.0377**
miR-29A	0.2964	0.0673
miR-106A	0.4447	**0.0178**

[a]MicroRNA expression levels that discriminate between ABC-like and GC-like DLBCL as assessed by microarray analysis were correlated with expression determined by qRT-PCR in all samples. Statistical analysis was based on array filtered $\log_2$ data and RT-PCR normalised ratios (NR). Bold p-values indicate those significant at the 95% level, whilst the remaining p-values represent those significant at the 90% level. [b]Spearman correlation.

level in FL were miR-17-92 homologues (miR-20B, miR-92B and miR-106A).

ABC-like DLBCL could be differentiated from FL by 10 microRNAs (Table I) and all of these microRNA were expressed in a higher level in FL, with the exception of miR-29A which was expressed at a higher level in ABC-like DLBCL (Fig. 4G). Twelve microRNAs distinguished GC-like DLBCL from FL (Table I) and once again, with the exception of miR-720, these microRNAs were expressed at a higher level in the FL than the GC-like (Fig. 4H). Looking at the identified discriminators, miR-16, miR-LET-7D, miR-LET-7G and miR-LET-7I were identified as discriminatory between all lymphoma (ABC-like, GC-like and FL) and CD19$^+$ control groups (Table I).

Common microRNA analysis. We also used GeneSpring to identify common microRNAs between the same groups as above using the same parameters (Table II). This analysis revealed a set of 24 microRNAs that are common between the 6 groups analysed, including the same 5 microRNAs of the miR-17-92 cluster (miR-17, miR-19A, miR-19B, miR-20A and miR-92A) that were identified as being discriminatory.

Of the microRNAs identified, miR-150, miR-27B, miR-30C, miR-223, miR-342-3P and miR-LET-7C were expressed by all B-cells (malignant and non-malignant), miR-16, miR-LET-7D, miR-LET-7G and miR-LET-7I were common between all lymphoma groups (ABC-like, GC-like and FL) and miR-92A was common between all DLBCL and control groups (Table II).

The ABC-like vs GC-like microarray-based discriminatory signature was verified by qRT-PCR. The differential expression of microRNAs that distinguishes between ABC-like and GC-like DLBCL was confirmed by qRT-PCR. We found a positive and significant correlation between microRNA expression by microarray-technology and qRT-PCR (Table III). However, in some cases, the p-values were approaching significance. The microRNAs -720A, -1260 and -1280 were not tested due to lack of appropriate primers for these human microRNAs at the time this study was performed.

Discussion

The present study identified a 9 microRNA signature that discriminated between GC- and ABC-like DLBCL. Three members of the signature are newly discovered microRNAs which have not been associated with lymphoma before this investigation and are predicted to target genes known to be de-regulated in DLBCL. The study also reports discriminatory signatures for DLBCL and FL and for lymphomas compared with normal CD19$^+$ B cells.

We performed a microRNA microarray analysis using 1860 mature microRNAs and miRXplore technology on well characterised DLBCL (ABC/GC), FL cell lines and normal peripheral blood CD19$^+$ B-cells. GeneSpring GX10 analysis incorporated a 100% return on selected data sets and resulted in 420 microRNAs finally being analysed.

Using this analysis, we identified a 9 microRNA signature that differentiated between ABC-like and GC-like DLBCL. Of these, 4 microRNAs, namely miR-17, 19B, 20A and 92A, are members of the C13orf25-encoded miR-17-92 cluster, a set of microRNAs that are known to be up-regulated in DLBCL (27), as well as other solid cancers (28). These microRNAs were all expressed at a higher level in ABC-like cell lines, which is in keeping with the more aggressive immunophenotype DLBCL. In addition, miR-106A, which is located within the miR-106A-92 cluster and shares high sequence homology with miR-17-92 (26), was also discriminatory. Expression of this microRNA was higher in the ABC-like cell lines. MiR-106A is up-regulated in a number of cancers (28-30) and one of its predicted target is BCL-6 (TargetScan), a transcription factor that governs BCL-6 protein expression. Down-regulation of expression of this protein identifies ABC-type DLBCL according to Han's classifier (8). Moreover, expression levels based on microarray analysis were significantly associated with those assessed by qRT-PCR.

Another discriminatory microRNA of the signature includes miR-29A which was over-expressed in all ABC type cell lines compared with GC type. However, its expression surprisingly, was even higher in normal CD19$^+$ cells. miR-29A has been reported to act in a tumour suppressor capacity (31,32) or as an oncogene (33) depending upon the cellular context. The findings in the present study would support an oncogenic role of miR-29A for the DLBCL cell lines but a tumour suppressor activity regarding normal B-cells.

The remaining 3 discriminatory microRNAs, miR-720A, miR-1260 and miR-1280 were also expressed at a higher level in ABC type cell lines compared with the GC phenotype. All three microRNAs have recently been identified (34,35) and the present study is the first to implicate these microRNAs in lymphoma. MiR-720A is believed to inhibit the cellular Dicer from effective processing in a mouse system (36), while miR-1260 and miR-1280 are predicted to target BCL-2, BCL-6 and cyclin D1 (CCDN1) (37), respectively, all of which are known to be associated with lymphoma.

Comparing microRNA expression of ABC-like and GC-like with normal CD19$^+$ B-cells, the discriminatory signa-

tures of both entities included up-regulated microRNAs of the known oncomiR miR-17-92 (27) as well as down-regulated microRNAs recognised as tumour suppressors, including miR-26A (38,39) and members of the the LET-7 family (40-43). Thus, these signatures correctly predicted the prognostic significance assigned to the immunophenotype, especially that of the ABC-type.

Recent studies have also used microRNA microarray profiling to identify lymphoma-specific signatures and signatures that differentiate between DLBCL prognostic subgroups. By contrast to the findings of the present study, Lawrie *et al* (21) identified miR-21, miR-155 and miR-221 as discriminatory between ABC and GC with higher expression seen in ABC-type cell lines and also in patient material. miR-21 and miR-155 were confirmed to differentiate between ABC- and GC-like cell lines by another group (20). However, these microRNAs were not identified as discriminatory in the present study. This was most likely due to technical differences including sample preparation, array platforms and the control population used. Moreover, all three studies used a variety of cell lines with little or no overlap; one ABC and one GC type was common between the present investigation and Malumbres *et al* (20) and 2 GC types were used by both Lawrie *et al* and this study (21). Also, it is worth mentioning that the total number of microRNAs analysed in the present study was significantly higher than the previous two investigations (20,21) (420 vs 225 and 217, respectively) thus, including many more microRNAs which are differentially expressed between the DLBCL subgroups.

In contrast, Roehle *et al* using patient tissue were unsuccessful in identifying a signature that could differentiate between ABC and GC DLBCL. Recently, Li *et al* identified 16 microRNAs that could effectively discriminate DLBCL into 3 unique subgroups that were unrelated to GC/ABC classifications (44). These authors were unable to distinguish GC- and ABC-like subgroups in 53 primary DLBCL tumours using the Malumbres 9 microRNA signature (44). By contrast, more recently, Lawrie *et al* using clinical samples have identified a discriminatory signature (23) that includes microRNAs which they previously reported as discriminatory (21), which correctly predicted lymphoma subtype and disease outcome (23).

Taken together, the above findings further highlight the molecular heterogeneity within DLBCL of the same immunophenotype (in both clinical samples and cell lines) and also the lack of consensus between the various studies, most likely the result of the number of microRNAs being analysed, technical issues and due to the underlying molecular heterogeneity.

Regarding differentiation between DLBCL and FL, the present study identified a 4 microRNA signature that discriminated between these groups. The signature includes expression of miR-20B, miR-26A, miR-92B and miR-487B all of which were expressed at a lower level in DLBCL. MiR-20B and miR-92B are derived from the miR-106a-92 cluster, which is highly homologous to the oncogenic miR-17-92 (26). MiR-20B has been reported to be over-expressed in a number of solid cancers (29,45) and recent studies suggest that miR-92B may act in a tumour suppressor capacity, by inhibiting translation of the oncogenic PRMT5 (46). Our results are in agreement with these findings, as the higher expression of miR-92B observed in FL compared to DLBCL support a tumour suppressor role for this microRNA. However, this role needs to be confirmed in functional assays.

MiR-26A is also believed to act as a tumour suppressor (38,47) and this property may explain the findings of the present study, in which we observed significantly lower levels of expression in DLBCL and FL compared to normal $CD19^+$ control cells and also down-regulation of its expression in DLBCL cell lines compared with the FL. The significant differences in miR-26A expression between the tumour groups and between normal and malignant cells suggest a role for this microRNA in the pathogenesis of B-cell lymphoma. miR-26A has recently been identified as a potential tumour suppressor, via its ability to attenuate proliferation in *c-MYC*-dependent cells (39). In addition, miR-26A can influence the cell cycle, by repressing cyclins D2 and E2 (38) and also *via* targeting the *EZH2* oncogene, a global regulator of gene expression (39).

Of all the 4 differentially-expressed microRNAs identified in the present study between DLBCL and FL, none were reported as discriminatory in earlier studies carried out by Roehle *et al* (22) and Lawrie *et al* (23). However, both these authors analysed a large number of clinical samples, while B-cell lymphoma cell lines were used in the present study.

In our discriminatory analysis, we found both miR-26A and three members of the LET-family of microRNAs (LET-7D, LET-7G and LET-7I) to differentiate lymphoma groups (DLBCL and FL) from normal $CD19^+$ control cells. Expression levels of these microRNAs were higher in the control B-cells than the lymphoma groups. Similarly, He *et al* reported up-regulation of the expression of the LET family of microRNAs in normal B-cells compared with B-cell lines (27). By contrast, our findings do not confirm the discriminatory signature for DLBCL and FL previously reported by Roehle *et al* (22). However Roehle *et al*, used a significantly smaller number of microRNAs than the present study and clinical samples rather than B-cell lymphoma cell lines. Also, these authors compared tumour (DLBCL and FL) microRNA expression against that of non-malignant inflammatory lymph nodes, while pure $CD19^+$ PB B-cells were used in the present study. In addition, the discriminatory signature reported recently by Lawrie *et al* (23), using clinical samples, did not include any of the miRs reported by Roehle *et al* (22), or identified in the present study.

The role of the LET family and that of miR-26A in lymphoma remains unclear. In humans, the LET-7 has been shown to act as a tumour suppressor, via targeting the human *RAS* oncogene (40-43). Similarly, as explained previously, miR-26A also acts in a tumour suppressor capacity (18,38,39,47). Evaluation of the role of these miRs in B-cell lymphoma pathogenesis merits further investigation.

In addition to their discriminatory activity, some of the microRNAs were identified as common between the various groups. The discriminatory power of these microRNAs was probably the result of their differential expression within the groups. The common microRNAs identified include miR-27B, miR-30C, miR-150 and miR-342-3p. Both miR-150 and miR-30C have been reported to be involved in normal haematopoiesis. The role of miR-150 in mature and resting

B-cell development, via targeting *c-MYB*, is well known (48) and miR-30C is known to be B-cell lineage-specific and regulates *blimp-1* in the GC- to plasma cell transition (49). Although the role of miR-27B and miR-342 in B-cell development remains unclear, miR-27B is reported to act either as a tumour suppressor (50), or as an oncomiR (51).

Apart from common and discriminatory microRNAs revealed by GeneSpring, this analysis also highlighted the molecular heterogeneity on the basis of microRNA expression within the various groups. GeneSpring, as well as Cluster 3.0 analysis viewed by Java™ Tree applet (based on a 75% filtration rate, results not shown), clustered all GC-like DLBCL cell lines together, but segregated the ABC-like, clustering some with FL and normal $CD19^+$ B-cells. These findings indicate that immunophenotypic subgroups may not necessarily be mutually exclusive in terms of microRNA expression profile and may highlight the heterogeneity and continuum between distinctions imposed upon DLBCL. Therefore, some DLBCL cell lines of the 'poor' immunophenotype, on the basis of microRNA profiles may be more indolent or conversely, possess a more 'aggressive' phenotype than that expected based on Han's classifier. Thus, the ABC-type HLY-1 showed differential up-regulation of expression of the miR-17-92 cluster, a known oncomiR (27), and also of miR-106A, -106B, -93, -30D, -15B and -103, which are reported to be up-regulated in a variety of aggressive solid cancers or have oncogenic potential (28,29,31,51-53). In addition, the ABC-like SU-DHL-8 had a more 'indolent' phenotype compared with the other ABC types. Expression levels of miR-21 which has been implicated with more aggressive DLBCL (21) and also of miR-29A known to act as an oncogene (33), were expressed at lower levels in SU-DHL-8 compared with the other ABC types and more in keeping with the expression observed in GC-like and FL cell lines. It is possible therefore that the lower levels of these two microRNAs may partly explain the more indolent microRNA-based phenotype of this cell line.

In conclusion, microRNA profiling in B-cell lymphoma cell lines identifies discriminatory signatures between DLBCL prognostic subgroups, DLBCL vs FL and lymphoma against normal $CD19^+$ PB B-cells. The numbers of microRNAs identified within each signature are manageable for potential use in a clinical setting. However, as it has recently been reported that microRNA signatures of B-cell lymphoma cell lines may differ from those of clinical material (23), we have undertaken additional studies using patient samples to confirm these recent findings and to determine the role of microRNAs in lymphoma pathogenesis and prognosis. Such studies would be of great value in assessing the potential of microRNAs as biomarkers or therapeutic targets.

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