bjh research paper

MicroRNA signatures characterize diffuse large B-cell lymphomas and follicular lymphomas

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Received 14 December 2007; accepted for publication 4 March 2008

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Summary

MicroRNAs (miRNA, miR) are negative regulators of gene expression that play an important role in diverse biological processes such as development, cell growth, apoptosis and haematopoiesis, suggesting their association with cancer. Here we analysed the expression signatures of 157 miRNAs in 58 diffuse large B-cell lymphoma (DLBCL), 46 follicular lymphoma (FL) and seven non-neoplastic lymph nodes (LN). Comparison of the possible combinations of DLBCL-, FL- and LN resulted in specific DLBCL- and FL-signatures, which include miRNAs with previously published function in haematopoiesis (*MIRN150* and *MIRN155*) or tumour development (*MIRN210*, *MIRN10A*, *MIRN17-5P* and *MIRN145*). As compared to LN, some miRNAs are differentially regulated in both lymphoma types (*MIRN155*, *MIRN210*, *MIRN106A*, *MIRN149* and *MIRN139*). Conversely, some miRNAs show lymphoma-specific aberrant expression, such as *MIRN9/9**, *MIRN301*, *MIRN338* and *MIRN213* in FL and *MIRN150*, *MIRN17-5P*, *MIRN145*, *MIRN328* and others in DLBCL. A classification tree was computed using four miRNAs (*MIRN330*, *MIRN17-5P*, *MIRN106a* and *MIRN210*) to correctly identify 98% of all 111 cases that were analysed in this study. Finally, eight miRNAs were found to correlate with event-free and overall survival in DLBCL including known tumour suppressors (*MIRN21*, *MIRN127* and *MIRN34a*) and oncogenes (*MIRN195* and *MIRNLET7G*).

Keywords: microRNA, lymphoma, diffuse large B-cell lymphoma, follicular lymphoma.

Diffuse large B-cell lymphoma (DLBCL) is the most frequent lymphoma in adults worldwide, accounting for 30% to 40% of lymphoid neoplasms (The Non-Hodgkin's Lymphoma Classification Project, 1997). The diversity in clinical presentation and outcome, as well as the pathological and biological heterogeneity, suggest that DLBCL comprises several disease entities that may require different therapeutic approaches. Gene-expression profiling has identified three major subgroups of DLBCL, termed germinal center B-cell-like DLBCL (GCB-DLBCL), activated B-cell-like DLBCL (ABC-DLBCL), and primary mediastinal DLBCL (PMBCL) (Alizadeh *et al*, 2000; Rosenwald *et al*, 2002; Rosenwald *et al*, 2003; Savage *et al*, 2003). These three subgroups of DLBCL are associated with a widely disparate clinical outcome with 5-year survival rates of 59%, 30% and 64% in patients with GCB-DLBCL, ABC-DLBCL and PMBCL, respectively (Alizadeh *et al*, 2000; Rosenwald *et al*, 2002; Rosenwald *et al*, 2003). In addition, GCB-DLBCL is characterized by frequent *REL* amplifications, *BCL2* translocations and ongoing somatic hypermutation of the immunoglobulin genes (Lossos *et al*, 2000; Huang *et al*, 2002; Rosenwald *et al*, 2002). In contrast, ABC-DLBCL and PMBCL have a constitutive activation of the nuclear factor κB (NF-κB) pathway that they require for survival, which is not a feature of GCB-DLBCL (Davis *et al*, 2001; Rosenwald *et al*, 2003; Savage *et al*, 2003; Lam *et al*, 2005).

First published online 5 June 2008
doi:10.1111/j.1365-2141.2008.07237.x

MiRNAs are an abundant class of small non-coding RNAs which modulate the expression of their target mRNAs at the post-transcriptional level (Valencia-Sanchez *et al*, 2006; Jackson & Standart, 2007). Mature miRNAs are 19- to 25-nucleotide long molecules cleaved from 70- to 100-nucleotide hairpin pre-miRNA precursors (Bartel, 2004). The precursor is cleaved by cytoplasmic RNase III Dicer into a 22-nucleotide miRNA duplex: one strand of the short-lived duplex is degraded, whereas the other strand serves as mature miRNA (Hutvágner *et al*, 2001; Ketting *et al*, 2001; Lee *et al*, 2003, 2004; Yi *et al*, 2003; Bohnsack *et al*, 2004; Cai *et al*, 2004; Lund *et al*, 2004; Maniataki & Mourelatos, 2005; Kim & Nam, 2006). In animals, single-stranded miRNA binds through partial sequence homology to the 3'untranslated region (3'UTR) of the target mRNAs, and causes either block of translation or, less frequently, mRNA degradation (Lewis *et al*, 2005; Rajewsky, 2006). At present, 711 miRNAs have been identified in the human genome, most of them evolutionary conserved in mice and rats (http://microrna.sanger.ac.uk/). The discovery of this class of genes has identified a new layer of gene regulation mechanisms, which play an important role in development and in various cellular processes, including apoptosis, cell differentiation and proliferation (Lee *et al*, 1993; Wightman *et al*, 1993; Brennecke *et al*, 2003; Xu *et al*, 2003; Chen *et al*, 2004; Chan *et al*, 2005; He *et al*, 2005).

Recently, many miRNAs have been found to be involved in cancer, acting either as oncogenes or tumour suppressors (He *et al*, 2005; Johnson *et al*, 2005; Voorhoeve *et al*, 2006). In particular, *MIRN155* has been shown to be up-regulated in B-cell lymphomas and the induction of up-regulation results in B-cell malignancy in mice (Metzler *et al*, 2004; Eis *et al*, 2005; Costinean *et al*, 2006; Lawrie *et al*, 2007). Many other reports have described altered expression of miRNAs in cancer samples compared to their healthy counterparts, suggesting that these small RNAs could represent novel clinical and prognostic markers (Mattie *et al*, 2006; Pallante *et al*, 2006; Yanaihara *et al*, 2006).

In this report, follicular lymphoma (FL), as a tumour of germinal centre origin, was compared with GCL-DLBCL and non-GCB-DLBCL. We present specific DLBCL and FL miRNA signatures. Similarities between lymphoma profiles were identified and entity-specific miRNA expression was singled out. An outstanding role for *MIRN155* in lymphoma development could be confirmed. A classification tree was developed that correctly identified 98% of all DLBCL-, FL- and LN cases. Finally, a correlation between miRNA expression levels and outcome (event-free and overall survival) was found in a multivariate analysis.

Methods

Tissue acquisition

The study was conducted in accordance with the Helsinki declaration. Patients were eligible if they had previously untreated, biopsy-confirmed aggressive non-Hodgkin's lymphoma according to the World Health Organization criteria. Tissue samples of 58 DLBCL were included in this study. These patients were treated within the NHL-B1 and NHL-B2 trials of the German High-Grade Non-Hodgkin's Lymphoma Study Group (Pfreundschuh *et al*, 2004a,b). Seven lymph nodes (LN) were included as non-neoplastic lymphatic tissue. These included LN with only minor reactive changes. 46 samples of low grade FL were retrieved from the files of the pathology departments (UKS-H, Campus Luebeck and Semmelweis University, Budapest). All FL samples were grade 1 and 2 tumours and no transformation in DLBCL was noted.

RNA extraction, reverse transcription and Real-Time polymerase chain reaction (PCR) quantification

Total RNA was extracted from four 20 μm sections of formalin-fixed paraffin-embedded tissues using the RecoverAll kit (Ambion, Austin, Texas, USA) according to the manufacturer's protocol. RNA concentrations were subsequently quantified using a NanoDrop Spectrophotometer (NanoDrop Technologies, Wilmington, Delaware, USA). MiRNA quantification took place in two steps: (*i*) miRNA-specific reverse transcription (RT); (*ii*) quantitative PCR using diluted miRNA-specific cDNA as a template.

cDNA was synthesized from total RNA using gene-specific stem-loop primers according to the TaqMan MicroRNA Assay protocol (PE Applied Biosystems, Foster City, California, USA). Each 15 μl RT-reaction contained 10 ng of total RNA, 3 μl stem-loop RT primer, 1·5 μl 10 × RT buffer, 0·15 μl of dNTPs (100 mM), 1 μl MultiScribe reverse transcriptase (50 U/μl) and 0·188 μl RNase Inhibitor (20 U/μl) (TaqMan MicroRNA Reverse Transcription Kit, PE Applied Biosystems) and 4·162 μl nuclease-free water (Ambion, Austin, Texas, USA). They were incubated in a T1 Thermocylcer (Biometra, Goettingen, Germany) using a 96-well format and the following settings: 30 min at 16°C, 30 min at 42°C, 5 min at 85°C and held at 4°C. The resulting cDNA was then diluted 1:15 using nuclease-free water (Ambion). Real-time PCR was performed using an Applied Biosystems 7900HT Fast Real-Time PCR System. The 20 μl PCR included 1·33 μl diluted RT product, 1× TaqMan Universal PCR master mix and 2 μl of primers and probe mix of the TaqMan MicroRNA Assay protocol (PE Applied Biosystems). The reactions were incubated in a 96-well optical plate (PE Applied Biosystems) at 95°C for 10 min, followed by 40 cycles of 95°C for 15 s and 60°C for 1 min. The Ct was determined using default threshold settings.

Follicular lymphoma miRNA-profiles were measured on the LightCycler® 480 Instrument (Roche, Basel, Switzerland). The following program was used: (*i*) Enzyme Activation: 95°C 10 min; (*ii*) Amplification (45 cycles): 95°C 15 s (ramp: 4·4°C/s, analysis mode: quantification), 60°C 1 min (ramp: 2·2°C/s); (*iii*) Cooling: 40°C 30 s (ramp: 2°C/s). The detection format was set to 'Mono Colour Hydrolysis Probe' and the second derivative maximum method was used for absolute

quantification. In order to demonstrate that miRNA profiles obtained from the LightCycler® 480 and the 7900HT instrument are highly similar, all seven LN profiles were measured and analysed on both real-time machines. Confidence intervals (95%) for a paired t-test comparing both PCR techniques include 134 miRNA species in the null hypothesis value of zero difference and no significant differential expression after correction for multiplicity.

Immunohistochemical staining

Immunohistochemical (IHC) stainings were performed according to a standard three-step immunoperoxidase technique with diaminobenzidine as chromogen.

Fifteen different IHC stainings were performed on every DLBCL case previously analysed (*BCL6*, *CD10*, *MUM1*, *IgM*, *BCL2*, *CD138*, *Ki-B5*, *Ki-B3*, *CD23*, *OPD4*, *HLA-DR*, *IgD*, *CD38*, *Ig light chain* κ and *light chain* λ). The following antibodies were used: mouse monoclonal anti-BCL6 (1:10, Dako, Glostrup, Denmark), rabbit polyclonal anti-CD10 (1:10, Dako), mouse monoclonal anti-MUM1 (1:100, Dako), rabbit polyclonal anti-IgM (1:500, Dako), mouse monoclonal anti-BCL2 (1:50, Biocarta, Hamburg, Germany), mouse monoclonal anti-OPD4 (1:50, Dako), mouse monoclonal anti-HLA-DR (1:200, Dako), rabbit polyclonal anti-IgD (1:30, Dako), rabbit polyclonal anti-κ (1:1000, Dako) and rabbit polyclonal anti-λ (1:1000, Dako). All secondary antibodies and all reagents were purchased from Dako.

Statistical analyses

Except outcome analysis, all analyses are based on standard statistical functions available from the R-language (http://www.R-project.org).

Data preprocessing

Quantile normalisation was used to normalise the miRNA profiles (modified from Bolstad *et al*, 2003; as implemented in the R-package *limma*). Values of Ct = 40 (saturation) were deleted before this step and added again afterwards.

Differential miRNA expression

Differential expression was tested both on a global profile level (Goeman *et al*, 2004) and for all single miRNA species using Welsh two-sided *t*-tests. Multiplicity was corrected taking a False Discovery Rate (FDR) approach (Benjamini *et al*, 2001). miRNAs were called *differentially expressed* for $FDR < 0{\cdot}05$ and $\Delta Ct \geq 1{\cdot}5$.

Unsupervised data mining

MiRNA profiles correlation structures were analysed using principal component analyses. Scores plots for profile data were colour-coded using the sample type information [LN, FL, DLBCL (GCB and non-GCB)].

Supervised analyses

Partial Least Squares regression discriminance analysis (PLS-DA) was performed as a supervised learning method to find combinations of miRNAs differentiating the different tumour types as opposed to the LN samples. The work flow was as follows: First, in a cross-validation approach we sought to determine if PLS regression using different numbers of latent variables (i.e. linear combinations of miRNA profiles) arrived at acceptable classification results. As a quality measure, we computed the error of prediction normalized with the variance of the variable in question (Q2) (Eriksson *et al*, 1999). From these results, the optimal number of latent PLS components was assessed. Second, via permutation analysis, we assessed the significance of the PLS-DA based on the distribution of Q2-values for 100 permutated response vectors. Third, candidate miRNAs with exceptional high contributions to the combination optimized for covariance with the response were selected using the variable importance plot (VIP) score (Chong & Jun, 2005). Candidates for combinatorial differentiation of the tumour types under investigation could be filtered out by comparing lists of differential expression against VIP-scores.

A classification tree was trained on a prefiltered set of miRNA profiles. MiRNA profiles used for this analysis were *MIRN9*, *MIRN301*, *MIRN320*, *MIRN149*, *MIRN150*, *MIRN155*, *MIRN145*, *MIRN330*, *MIRN92*, *MIRN338* – as taken from top of the lists of differential expression between the three sample groups (LN, FL, DLBCL) in this study (Fig 1). The classification tree was calculated with cross-entropy as measure for node impurity. A tree is grown by binary recursive partitioning using Ct values and choosing splits from the set of miRNAs described above. The split which maximizes the reduction in impurity is chosen, the data set split and the process repeated. Splitting continues until the terminal nodes are too small or too few to be split.

Outcome analysis

The data of 53 patients were available for outcome analysis. From 153 miRNAs 51 with a range ≤ 3 Ct were excluded. The median for each of the miRNAs (group 0: <median, group 1: ≥median; except for *MIRN21*: group 0: ≤median, group 1: >median) was applied as the cut-off points. Event-free survival (EFS) was defined as the time from the beginning of therapy to either disease progression, initiation of additional (off-protocol) or salvage therapy, relapse or death. Overall survival (OS) was defined as time from the beginning of therapy to death for any cause. EFS and OS were estimated according to Kaplan and Meier (Kaplan & Meier, 1958). In a univariate analysis logrank tests were performed and miRNAs with $P < 0{\cdot}1$ were included in the

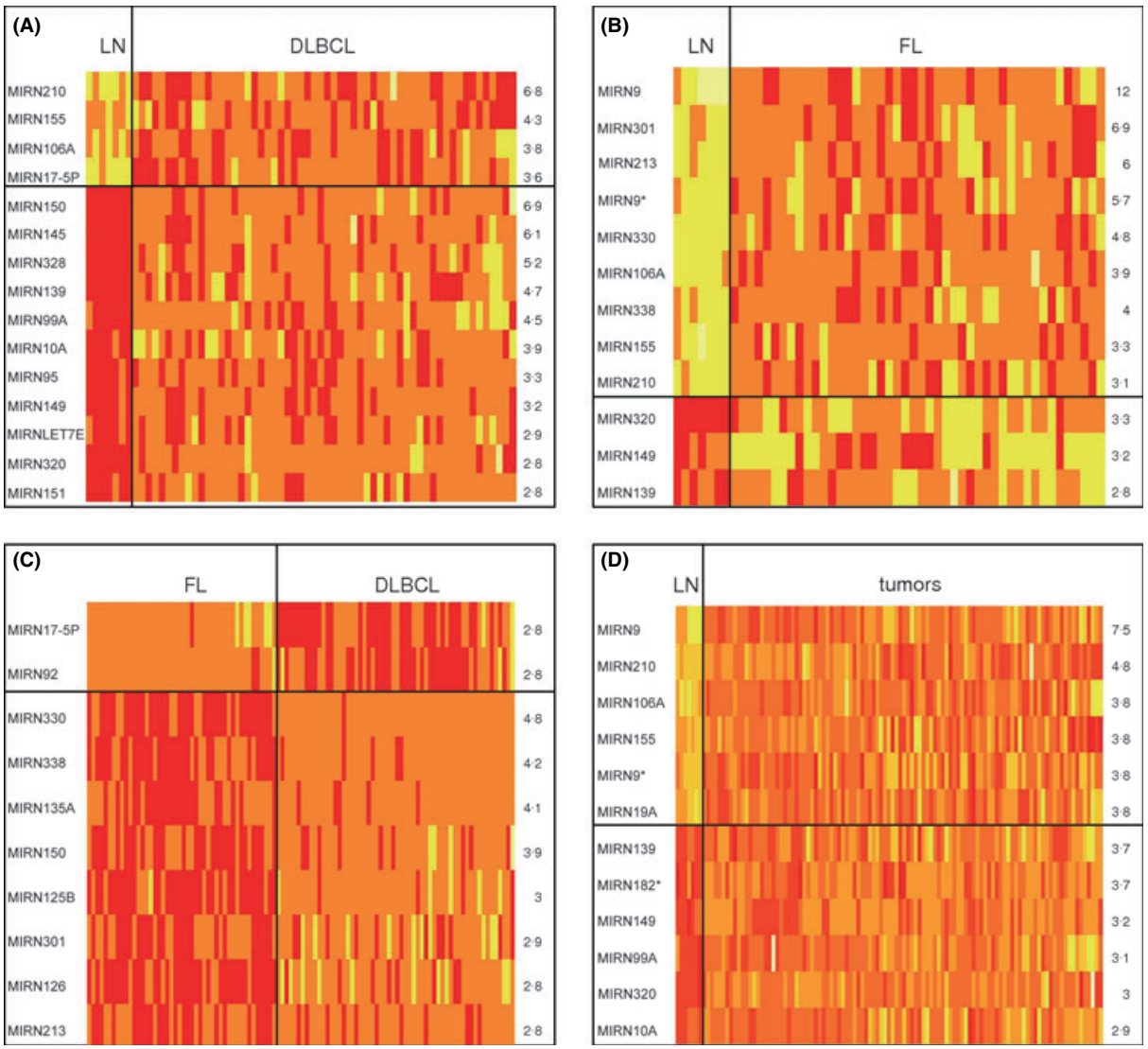


Fig 1. Differentially expressed miRNAs in DLBCL and FL. The four heatmaps display cases in columns and miRNA species in rows. High expression is indicated in red, week expression is yellow. MiRNA profiles have been scaled to unit variance for this representation. However, numbers to the right of the panels show the true 'fold change' of differential expression (up-regulation on the top, down-regulation on the bottom). (A) Comparison of lymph nodes (LN) *versus* DLBCL and (B) FL. (C) Comparison of FL and DLBCL. (D) Comparison of LN and tumours.

multivariate analysis. Proportional hazard models for each of the selected miRNAs separately were fitted adjusted for the prognostic factors of the International Prognostic Index (age > 60 years, lactate dehydrogenase [LDH] > normal, Eastern Cooperative Oncology Group [ECOG] performance score > 1, stage III/IV and extranodal involvement > 1). For miRNAs with *P*-values <0·05 for EFS or OS within the Coxmodels, the relative risk with 95% confidence interval (CI) is shown in a forest plot. Because of the explorative character of the analysis and the small number of patients the *P*-values were not adjusted for multiple comparisons. Outcome analyses were performed with the Statistical Package for the Social Sciences (SPSS) v. 15.0.

Results

MiRNAs differentially expressed between DLBCL and FL

By comparing miRNA profiles of seven LN and 58 DLBCL, we identified 15 differentially expressed miRNAs (Fig 1A). Four miRNAs (*MIRN210*, *MIRN155*, *MIRN106A*, *MIRN17-5P*) were expressed at a significantly higher level in DLBCL than in normal tissue. In contrast, the remaining 11 miRNAs (*MIRN150*, *MIRN145*, *MIRN328*, *MIRN139*, *MIRN99a*, *MIRN10a*, *MIRN95*, *MIRN149*, *MIRN320*, *MIRN151*, *MIRNLET7E*) were expressed at a significantly lower level in DLBCL. Overexpression of *MIRN210* was highest (7-fold)

and expression of MIRN150 was most strongly reduced (7-fold).

Similarly, comparison of the miRNA profiles of seven LN and 46 cases of FL identified 12 differentially expressed miRNAs (Fig 1B). Nine miRNAs (*MIRN9*, *MIRN301*, *MIRN213*, *MIRN9**, *MIRN330*, *MIRN106A*, *MIRN338*, *MIRN155*, *MIRN210*) were significantly up- and three miRNAs (*MIRN320*, *MIRN149*, *MIRN139*) down-regulated in the lymphoma samples. Expression of *MIRN9* was most strongly increased (12-fold) and of *MIRN320* most strongly decreased (3-fold). Interindividual differences of *MIRN9*/*MIRN9** expression was noticed to be particularly high in DLBCL, FL and LN (up to 32-fold).

Comparing DLBCL and FL with another (Fig 1C), two miRNAs (*MIRN17-5P*, *MIRN92*) were overexpressed and eight miRNAs (*MIRN330*, *MIRN338*, *MIRN135A*, *MIRN150*, *MIRN125B*, *MIRN301*, *MIRN126*, *MIRN213*) were down-regulated in DLBCL.

Identifying miRNAs of general importance in lymphoma genesis

To identify miRNAs with a general role in lymphoma genesis, 20 miRNAs that showed the strongest differential expression between DLBCL and FL were removed and obtained a new list of miRNAs with highly similar expression of the remaining 137 miRNA species in both lymphoma entities. This new list was then used for a 'lymphoma *versus* reactive lymphatic tissue' comparison (Fig 1D) and taking a PLS-DA approach. A VIP was computed in order to identify miRNAs that might play a general role in lymphoma development (Fig 2A). Amongst others, *MIRN155*, *MIRN210*, *MIRN106A*, *MIRN149* and *MIRN139* most strongly contribute to differences in miRNA-profiles of lymphomas and reactive lymphatic tissue (Fig 1D). 'Leave one out' (LOO) cross validation supported the validity of the identified miRNAs. The hit rate was determined to be 96% (Fig 2B).

Using miRNAs for DLBCL subgroup identification

In order to assign the 58 cases of DLBCL the GCB and non-GCB subgroups, we analysed IHC stains of CD10, BCL6 and MUM1 were analysed (Hans *et al*, 2004). 25 cases were assigned to GCB-DLBCL and non-GCB-DLBCL, respectively. Eight cases with unclear GCB-status, due to non-evaluable IHC stains, were removed from further analysis.

It was now possible to address the four entities, a) reactive LN, b) FL, c) GCB-DLBCL and d) non-GCB-DLBCL. Principal components analysis indicated that miRNA profiles created separate groups for LN, FL and DLBCL (Fig 3A, green, red and blue dots, respectively). However, GCB- and non-GCB-DLBCL cases (Fig 3A, light blue dots and dark blue dots, respectively) appeared to be hard to separate. MiRNAs important for discriminating GCB *versus* non-GCB cases were filtered using a t-test approach. Nine miRNAs were identified as candidates for differential expression taking into account an FDR cut-off of

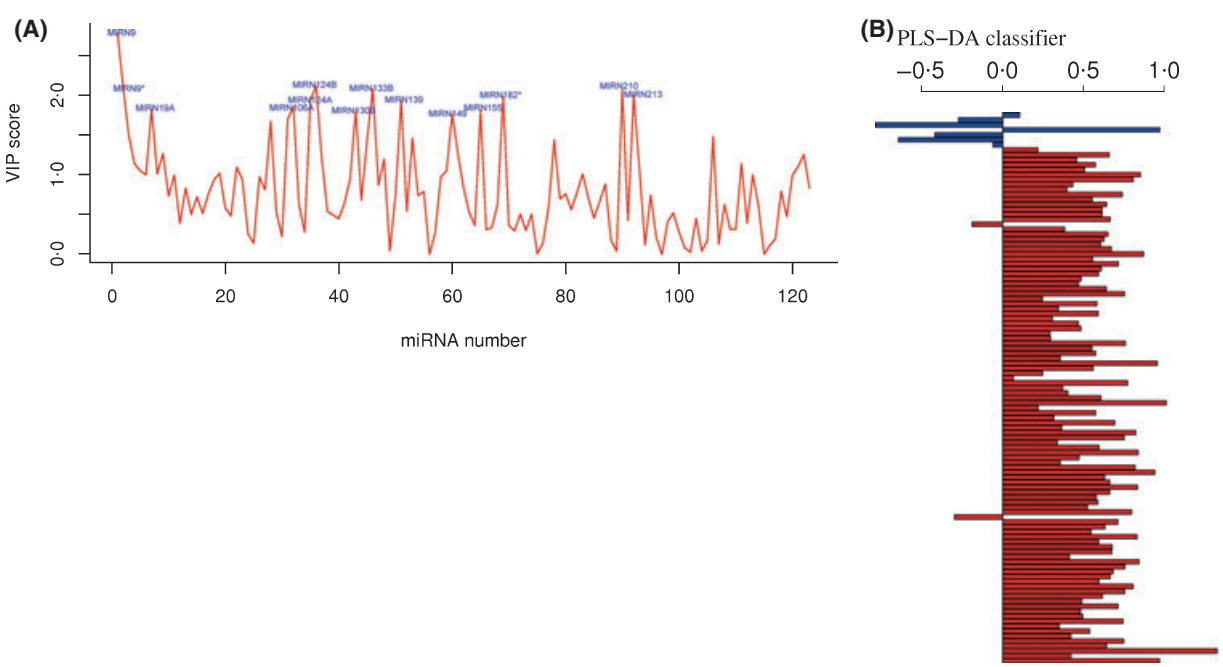
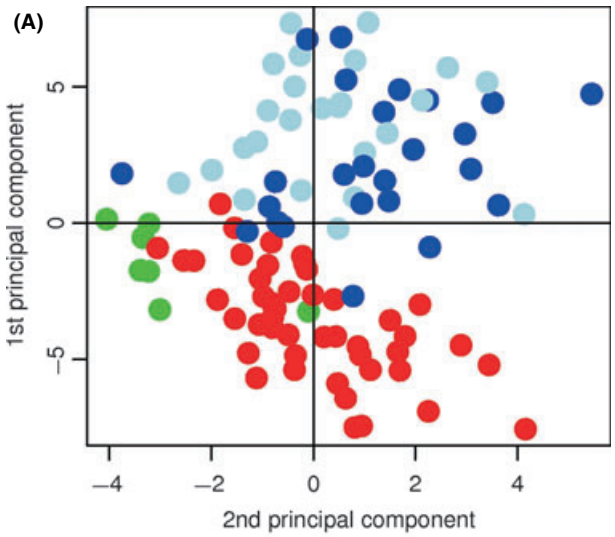


Fig 2. Lymphoma specific (DLBCL and FL) miRNA signatures. Differentially expressed miRNAs that are characteristic for lymphoma miRNA profiles compared to normal tissue of origin (LN). (A) Variable importance plot (VIP) highlights the miRNAs that most strongly contribute to differences between lymphoma and normal haematopoietic tissue (blue). (B) A 'leave one out' (LOO) cross validation estimates the validity of the miRNA candidates identified via VIP score. The hit rate is 96% (lymph nodes are depicted in blue, lymphoma are shown in red).



(B)

miRNA	FDR	deltaCT	KI-1	KI-2
MIRN27B	0·109	−0·743	−1·277	−0·21
MIRN28	0·057	−1·059	−1·721	−0·397
MIRN129	0·033	−1·242	−1·907	−0·577
MIRN133A	0·087	−2·218	−3·73	−0·706
MIRN133B	0·17	−1·909	−3·429	−0·389
MIRN138	0·114	−1·202	−2·087	−0·317
MIRN151	0·021	−0·934	−1·4	−0·468
MIRN155	0·181	1·272	0·228	2·317
MIRN199B	0·036	−1·497	−2·355	−0·638

Fig 3. Differentially expressed miRNAs comparing GCB- *versus* non-GCB-DLBCL subgroups. GCB- and non-GCB cases were determined by IHC staining as published (Hans *et al*, 2004). (A) Principle components analysis (PCA) of miRNA profiles shows clustering of DLBCL (blue), FL (red) and LN (green). No apparent clustering is evident when comparing GCB- *versus* non-GCB-DLBCL cases (light and dark blue, respectively). (B) Table of candidates for differential expression for GCB- *versus* non-GCB-DLBCL subgroups, for a FDR (Storey & Tibshirani, 2003) up to 20%.

20% (Fig 3B). Interestingly, *MIRN155* was identified as one of the discriminating markers, as previously reported (Lawrie *et al*, 2007).

Four miRNAs accurately diagnose DLBCL and FL

The minimal number of miRNAs that can be used to accurately diagnose and discriminate DLBCL and FL cases needed to be determined. *MIRN330*, *MIRN17-5P*, *MIRN106A* and *MIRN210* were the most discriminatory miRNAs. The classification tree (Fig 4A) showed that expression of *MIRN330* alone (Ct ≤ 38·6) could separate FL from DLBCL and non-neoplastic LN with high confidence (40 of 46 cases), while erroneously assigning one additional case of DLBCL to this group. Similarly, using expression results of three additional miRNAs [*MIRN17-5P* (Ct < 32·17), *MIRN106A* (Ct < 33·45) and *MIRN210* (Ct < 33·66)] correctly grouped 57 of 58 cases of DLBCL, six of seven LN and all (46 of 46) FL. In summary, the classification tree, shown in Fig 4(A), utilized miRNA expression results of four miRNAs to discriminate DLBCL, FL and LN with an overall accuracy of 98% (109 of 111 cases).

MiRNAs with prognostic capability in DLBCL

The Characteristics of the patients with diagnosis DLBCL are shown in Table I. The median age was 63 years, with 3- and 5-year OS rates of 71·2% and 66·9%, respectively. The median follow-up time was 72 months.

The relationship between expression of single miRNAs and survival prognosis was examined using a multivariate Cox regression analysis including factors of the International Prognostic Index (IPI) (Table I). MiRNAs with significance level $P < 0{\cdot}1$ for EFS or OS in a univariate analysis (EFS: *MIRN19A*, *MIRN29A*, *MIRN92*, *MIRN127*, *MIRN195*, *MIRN222*, *MIRNLET7G*; OS: *MIRN21*, *MIRN23A*, *MIRN27A*, *MIRN142-5P*, *MIRN182**, *MIRN296*; both: *MIRN34A*, *MIRN125A*, *MIRN186*) were used for multivariate analysis. Based on this, eight miRNAs (*MIRN19A*, *MIRN21*, *MIRN23A*, *MIRN27A*, *MIRN34A*, *MIRN127*, *MIRN195* and *MIRLET7G*) with *P*-values <0·05 for EFS or OS were identified (Fig 5A). Only one miRNA, *MIRN127*, was found to significantly influence EFS and OS (Fig 5B). In a multivariate analysis (Fig 5A) patients with low expression of *MIRN127* (Ct ≥ 37·73) had a poor prognosis for OS (RR = 4·3, 95% CI, 1·2–15·3, $P = 0{\cdot}023$) and EFS (RR = 4·9, 95% CI, 1·7–14·1, $P = 0{\cdot}003$). The remaining seven miRNAs were significantly correlated with only one of them. Patients with down-regulated *MIRN21*, *MIRN23A*, *MIRN27A* and *MIRN34A* expression levels had inferior OS. In contrast, EFS was influenced by low expression levels of *MIRN19A* (shorter EFS), *MIRN195* and *MIRNLET7G* (longer EFS, respectively). A poor OS was most strongly correlated with decreased expression of *MIRN21* (RR = 4·5, 95% CI, 1·4–14·0, $P = 0{\cdot}010$) and *MIRN27A* (RR = 4·6, 95% CI, 1·5–13·6, $P = 0{\cdot}007$). In addition to *MIRN127*, EFS was most strongly influenced by *MIRNLET7G* and *MIRN19A*. A reduced expression level of *MIRNLET7G* was contributed to significantly longer EFS (RR = 0·2, 95%, 0·1–0·6, $P = 0{\cdot}002$) whereas a reduced expression level of *MIRN19A* correlated with significantly shorter EFS (RR = 4·2, 95%, 1·5–11·8, $P = 0{\cdot}005$).

Discussion

Prior studies have shown that a small subset of miRNAs may define tumour entities better than microarray expression data from thousands of mRNAs (Lu *et al*, 2005). The present study used a qRT-PCR based method to characterize signatures for 157 miRNAs in DLBCL and FL. We found that miRNA expression differentiated both lymphoma entities from normal tissue and DLBCL from FL. In addition, a small number of miRNAs were differentially expressed in DLBCL as well as in

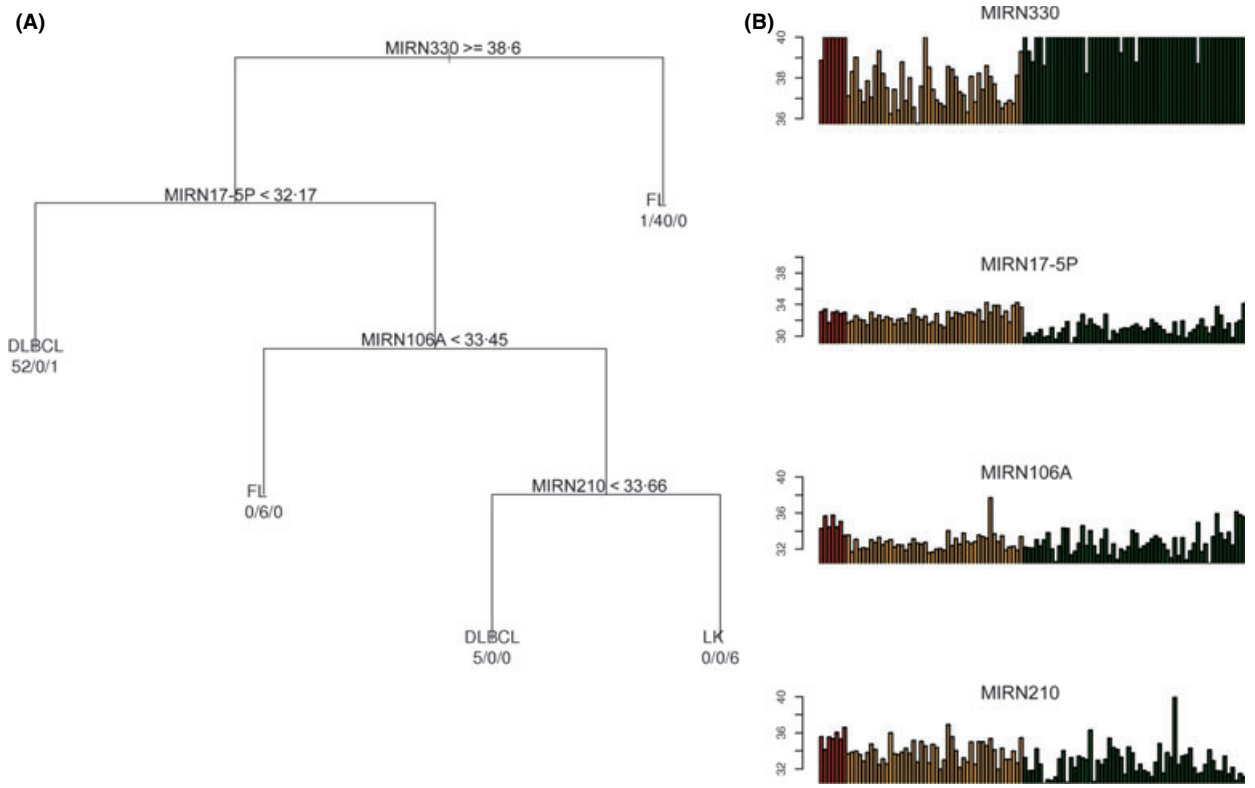


Fig 4. Classification tree of DLBCL, FL and LN, correctly identifying 98% of all cases analysed. (A) Expression of *MIRN330* with a Cp < 38·6 placed 87% of all FL in one clade and wrongly addressed one case of DLBCL and none of the LN analysed. Similarly, *MIRN17-5P*, *MIRN106A* and *MIRN210* also contribute to identify lymphoma and LN. (B) Diagrams in which the expression levels of *MIRN330*, *MIRN17-5P*, *MIRN106A* and *MIRN210* are depicted for each individual case (LN in red, FL in yellow and DLBCL in green).

FL compared to normal tissue. Furthermore, a number of individual miRNAs were associated with clinicopathological factors. This study formed a basis for developing miRNA expression signatures as diagnostic and prognostic tools for DLBCL and FL and also adds to our understanding of the role of miRNAs in cancerogenesis.

Comparison of miRNA profiles from DLBCL and FL to reactive LN revealed 15 differentially expressed miRNAs in DLBCL and 12 differentially expressed miRNAs in FL. Interestingly, most differentially expressed miRNAs were down-regulated in DLBCL (11 of 15; Fig 1A) whereas the opposite was true for FL, which showed mostly up-regulated miRNAs (nine of 12; Fig 1B). Despite this apparent difference there was a strong overlap of miRNA expression. *MIRN210*, *MIRN155* and *MIRN106A* were up-regulated in both DLBCL and FL as compared to reactive LN. Conversely, expression of *MIRN149* and *MIRN139* was reduced in both entities (Fig 1A and B): It is tempting to speculate that these five miRNAs might be of general importance in the development of lymphatic malignancies. In accordance with our findings, *MIRN155* was previously reported to play a critical role in B-cell maturation and lymphocyte activation (Rodriquez *et al*, 2007; Thai *et al*, 2007). Transgenic mouse studies demonstrated that B-cell targeted expression of *MIRN155* leads to the development of B-cell malignancies (Costinean *et al*, 2006). Further, a number of miRNA profiling studies have shown elevation of *MIRN155* in a wide array of cancers including lymphomas (Van den Berg *et al*, 2003; Eis *et al*, 2005; Kluiver *et al*, 2005; Costinean *et al*, 2006; Volinia *et al*, 2006; Yanaihara *et al*, 2006; Lawrie *et al*, 2007). Recently, a correlation between *MIRN155* and NF-κB expression was found in DLBCL cell lines and patients (Rai *et al*, 2008). Another dysregulated miRNA, *MIRN210*, has been suggested to play an important role in tumour onset as a key regulator of the cell cycle (Giannakakis *et al*, 2007). Even less is known about expression of the other listed miRNAs in cancer, making speculation about their role in lymphoma difficult at this time.

Next, we focussed on miRNAs that showed specific differential regulation in the two lymphoma entities analysed here. In comparison of DLBCL- to FL-profiles, ten miRNAs were differentially expressed. Again, DLBCL was characterized mostly via down-regulation of miRNAs (eight of 10; Fig 1C). Resuming these data, and for the comparison performed in this article (Fig 1A–D), we conclude that expression levels of *MIRN9/9**, *MIRN301*, *MIRN213*, *MIRN330* and *MIRN338* are characteristic for FL, while *MIRN150*, *MIRN17-5P*, *MIRN145*, *MIRN328*, *MIRN99A*, *MIRN10A*, *MIRN95*, *MIRN151* and *MIRNLET7E* add up to specific DLBCL signature. Some of these miRNAs have already been found to play an important role in tumour development. For instance, *MIRN150* – most

Table I. Patient characteristics at initial diagnosis (n = 53).

Characteristic	No. patients	%
Gender		
Male	31	58·5
Female	22	41·5
Median age, range (years)	63 (20–75)	
IPI factors		
Age > 60 years	29	54·7
LDH > N	14	26·4
ECOG > 1	7	13·2
Stage III/IV	26	49·1
Extranodal involvement > 1	12	22·6
IPI score		
0,1	28	52·8
2	11	20·8
3	7	13·2
4,5	7	13·2
Treatment arm		
6 × CHOP-21	15	28·3
6 × CHOP-14	10	18·9
6 × CHOEP-21	14	26·4
6 × CHOEP-14	14	26·4
Event-free survival (rate, 95% CI)		
3-year	54·7% (41·4–68·0)	
5-year	71·2% (58·8–62·4)	
Overall-survival (rate, 95% CI)		
3-year	48·9% (35·4–83·5)	
5-year	66·9% (54·0–79·8)	

IPI, International Prognostic Index; LDH, lactate dehydrogenase; ECOG, Eastern Cooperative Oncology Group; CHOP, cyclophosphamide, hydroxydaunomycin, Oncovin, prednisone; CHOEP, cyclophosphamide, hydroxydaunomycin, Oncovin, prednisone, etoposide.

strongly downregulated in DLBCL – has been described to control B-cell development and is significantly up-regulated in patients with chronic lymphocytic leukaemia (CLL) (Fulci *et al*, 2007; Xiao *et al*, 2007; Zhou *et al*, 2007). This miRNA might have a highly specific role in the development of different lymphatic neoplasias. A significant deregulation of *MIRN10A* in AML and of *MIRN17-5P* and as *MIRN145* is known in various other cancer types (Bandrés *et al*, 2006; Volinia *et al*, 2006; Debernardi *et al*, 2007; Gramantieri *et al*, 2007; Iorio *et al*, 2007; Sempere *et al*, 2007; Slaby *et al*, 2007). Regulation of transcription factor E2F1 – a target of proto-oncogene *MYC* – by *MIRN17-5P* has already been shown (O'Donnell *et al*, 2005). Therefore, it seems to be possible that this miRNA has a general impact on tumour development.

It has been described that cases of the ABC subtype of DLBCL show an inferior prognosis as compared to GCB subtype. Lawrie *et al* (2007) were able to discriminate the GCB- and the non-GCB subgroups of DLBCL using the classifiers *MIRN155*, *MIRN21* and *MIRN221*. In accordance with these results, *MIRN155* was previously reported to be overexpressed in ABC-DLBCL (Eis *et al*, 2005). Although we used a similar approach to that of Lawrie *et al* (2007), we could not confirm these results. Given the number of cases of GCB and non-GCB (25 in each group), it seems remarkable that we were not able to select a combination of miRNA profiles that reliably discriminated between these two subtypes (Fig 3A). This, however, may be caused by the variable reproducibility of IHC stains and interpretations. Our list of candidates for differential expression contained *MIRN155*; however the listing of the other potentially differentially expressed miRNAs comes with a FDR of 20%.

Given the dismal prognosis typically associated with DLBCL, we sought to identify miRNAs that are of prognostic relevance for tumour outcome. For that purpose, a multivariate analysis for EFS and OS was performed. Adjusted for IPI-factors, a group of eight miRNAs were identified (Fig 5A). Reduced expression levels of six miRNAs (*MIRN19A*, *MIRN21*, *MIRN23A*, *MIRN27A*, *MIRN34A* and *MIRN127*) identified poor EFS and/or OS, whereas the opposite was true for the remaining two (*MIRN195* and *MIRNLET7G*). Only *MIRN127* significantly influenced both OS and EFS in a multivariate analysis (Fig 5B). A down-regulated expression level of this miRNA correlates with poor survival prognosis. In accord with our findings, *MIRN127* was previously reported to be methylated in tumour cells and the expression level was inversely proportional to the expression of *BCL6*, a known proto-oncogene (Saito *et al*, 2006). Therefore, it seems possible that *MIRN127* functions as tumour suppressor in the cell. The same effect is already known for p53-regulating *MIRN34A* (He *et al*, 2007; Raver-Shapira *et al*, 2007; Tarasov *et al*, 2007; Tazawa *et al*, 2007; Welch *et al*, 2007). Further, the outstanding role of *MIRN21* in cancer development was confirmed by proving a correlation between low expression level and reduced OS. *MIRN21* is associated with tumour growth, invasion and metastasis by targeting multiple tumour and metastasis suppressor genes, such as *PDCD4, TPM1* and *PTEN* (Chan *et al*, 2005; Cheng *et al*, 2005; Löffler *et al*, 2007; Meng *et al*, 2007; Si *et al*, 2007; Zhu *et al*, 2007; Frankel *et al*, 2008). It is differentially expressed in various cancer types and associated with poor survival and therapeutic outcome by affecting the potencies of a number of anticancer agents (Iorio *et al*, 2005, 2007; Meng *et al*, 2006; Roldo *et al*, 2006; Volinia *et al*, 2006; Fulci *et al*, 2007; Lawrie *et al*, 2007; Lee *et al*, 2007; Lui *et al*, 2007; Slaby *et al*, 2007; Tran *et al*, 2007; Blower *et al*, 2008; Feber *et al*, 2008; Schetter *et al*, 2008). Interestingly, the reduced expression levels of only two miRNAs, *MIRN195* and *MIRNLET7G*, indicated better prognosis. Both of these miRNAs apparently act as oncogenes, confirmed by known overexpression in CLL and colon cancer, respectively (Nakajima *et al*, 2006; Zanette *et al*, 2007). Remarkably, *MIRN155* was not identified as a prognostic marker for survival in the present study, although this miRNA has been described to be exclusively overexpressed in ABC-DLBCL (Eis *et al*, 2005). This subtype was associated with a poor clinical outcome and a 5-year survival rate of only 30% compared with 59% and 64% for GCB-DLBCL and PMBCL, respectively (Alizadeh *et al*, 2000; Rosenwald *et al*, 2002; Rosenwald *et al*, 2003).

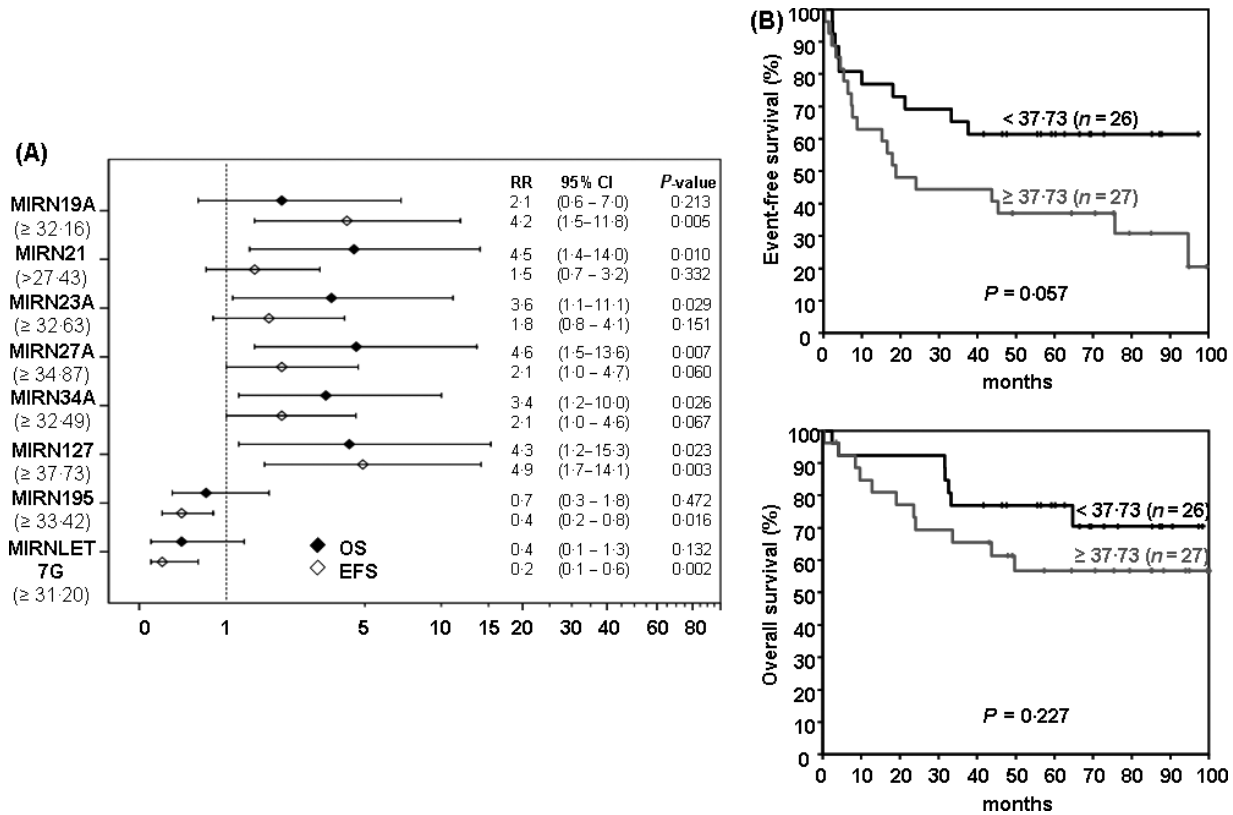


Fig 5. Impact of miRNA expression levels on survival of DLBCL patients. (A) Multivariate Cox regression analysis including IPI factors examined the relationship between expression of single miRNAs and survival prognosis. A forest plot shows the eight miRNAs with prognostic value for event-free (EFS) and/or overall survival (OS). *MIRN127* expression level significantly correlated with both EFS and OS. OS was exclusively influenced by *MIRN21*, *MIRN23A*, *MIRN27A* and *MIRN34A*, EFS by *MIRN19A*, *MIRN195* and *MIRNLET7G*. (RR relative risk, CI confidence interval) (B) Kaplan–Meier estimate of EFS and OS for *MIRN127* expression level in a univariate analysis. Low *MIRN127* expression (Ct ≥ 37·73) was associated with poor outcome.

Additional studies are required to improve our knowledge regarding the role of miRNA expression and cancer development to determine the potential of these small RNAs as either biomarkers or therapeutic targets.

Acknowledgements

This work was supported by the German High-Grade Non-Hodgkin Lymphoma Study Group. We thank Biggi Branke for the skilled and dedicated technical assistance.

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