Low expression of microRNA-146b-5p and microRNA-320d predicts poor outcome of large B-cell lymphoma treated with cyclophosphamide, doxorubicin, vincristine, and prednisone

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Summary
Although diffuse large B-cell lymphoma (DLBCL) encompasses a biologically and clinically diverse set of diseases, increasing evidence has pointed to an important role of microRNAs (miRs) in the pathogenesis of DLBCL. We report here that low expression of miR-146b-5p and miR-320d is associated with poor prognosis of DLBCL patients treated with the standard cyclophosphamide, doxorubicin, vincristine, and prednisone (CHOP) regimen and that this is related to the inhibitory effect of these miRs on DLBCL cell proliferation. Analysis of a retrospective cohort of 106 primary nodal DLBCL samples from patients who were treated with CHOP showed that, when the median survival period (40.8 months) was used as the cutoff point, miR-146b-5p and miR-320d were expressed at lower levels in DLBCLs with poor prognosis. Indeed, whereas low expression of miR-146b-5p was correlated with reduced progression-free survival, low expression of miR-320d was associated with decreases in both progression-free survival and overall survival. Moreover, low expression of miR-146b-5p was correlated with reduced progression-free survival, low expression of miR-320d was associated with decreases in both progression-free survival and overall survival. Moreover, miR-146b-5p and miR-320d were expressed at significantly lower levels in DLBCLs with the MYC (8;14) translocation. Functional studies demonstrated that overexpression of miR-146b-5p or miR-320d inhibited DLBCL cell proliferation, whereas knockdown of miR-146b-5p or miR-320d promoted proliferation of DLBCL cells. Taken together, these results suggest that low expression of miR-146b-5p and miR-320d may be predictive of compromised responses of a subset of DLBCL patients to treatment with the CHOP regimen and that restoration of these miRs may be useful to improve the therapeutic efficacy of CHOP.

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1. Introduction
Diffuse large B-cell lymphoma (DLBCL) is the most common type of non-Hodgkin lymphoma in adults [1,2]. However, approximately half of DLBCL patients cannot be
cured with the standard cyclophosphamide, doxorubicin, vincristine, and prednisone (CHOP) regimen or the rituximab plus CHOP (R-CHOP) regimen [3,4]. This is closely associated with the heterogeneity of DLBCLs both biologically and clinically [5]. DLBCLs can be divided into at least 2 subgroups: germinal center B-cell (GCB) type and nongerminallike (non-GCB) type; yet even within each subgroup, there are still wild variations in molecular characteristics [6]. Apart from the International Prognostic Index (IPI) [7], there is currently no consensus on biomarkers predictive of responses of DLBCL patients to treatment. Further understanding of molecular mechanisms responsible for the heterogeneity of DLBCLs is therefore urgently needed.

MicroRNAs (miRs) regulate gene expression in a sequence specific manner through binding to 3’UTRs of mRNAs, either targeting the transcripts for degradation or blocking their translation [8,9]. MiRs can potentially affect multiple steps of the pathogenesis of cancer by regulating a variety of target genes. In DLBCL, a number of miRs have been demonstrated to play roles in the disease development, progression, and responses to treatment [10]. In particular, miR-21 regulates the sensitivity of DLBCL cells to the CHOP regimen [11], whereas miR-181a and miR-222 have been proposed to be independent predictors of the outcome of DLBCL patients in response to treatment with R-CHOP [12]. Moreover, the increased expression of miR-155 in DLBCL cells may have a diagnostic value; and elevated miR-34a may be of prognostic significance [13,14].

In this study, we have examined the expression of miRs in a cohort of DLBCL samples from 106 patients with varying clinicopathological characteristics who were treated with the CHOP regimen. We report here that miR-146b-5p and miR-320d are expressed at lower levels in DLBCL patients with poor prognosis and that low expression of these miRs is an independent predictor of compromised responses of DLBCL patients to treatment with CHOP. In addition, we show that both miR-146b-5p and miR-320d have an inhibitory effect on DLBCL cell proliferation.

2. Materials and methods

2.1. Patients

A retrospective cohort of 106 primary nodal DLBCL samples from patients who attended the Department of Hematology of Shanxi Cancer Hospital and underwent the treatment with the CHOP regimen between 2001 and 2010 was recruited into this study. The patients were followed up quarterly for the first year, semiannually for the next 2 years, and annually thereafter. Detailed clinicopathological information of these patients is shown in Table 1. All the DLBCL samples were reviewed for diagnostic accuracy by 2 independent pathologists. Thirty reactive hyperplasia lymph node tissues were also obtained from the Department of Hematology of Shanxi Cancer Hospital that were used as controls. The study was approved by the Medical Ethics Committee of Shanxi Province Cancer Hospital. Informed consent was obtained from each patient at the time of the first visit according to the Declaration of Helsinki [15].

2.2. Cell culture and reagents

Human DLBCL cell lines were kindly provided by professor Jiang Zhu (Ruijin Hospital, Shanghai Jiao Tong University School of Medicine), professor Tong Zhao (Nanfang Medical University), and professor XiaoYan Zhou (Cancer Hospital, Shanghai Fudan University), including DB, SUDHL-4, RJ-Ly2, OCI-Ly1, and OCI-Ly8 (GCB type), and RJ-Ly1 and OCI-Ly10 (activated B-cell-like, ABC type). They were cultured in RPMI-1640 medium (HyClone, Logan, UT) supplemented with 15% fetal bovine serum (Gibco, Carlsbad, CA) at 37°C in a humidified air atmosphere containing 5% CO2.

2.3. Immunohistochemistry and fluorescence in situ hybridization analysis

Immunohistochemical staining was performed on formalin-fixed, paraffin-embedded (FFPE) tissue sections using the EnVision method for CD3, CD10, CD20, Bcl-6, and

<table>
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* P < .05 was considered statistically significant.
MUM-1 (Maixin-Bio, Fuzhou, China). According to Hans’ algorithm, all patients were assigned to the GCB or non-GCB group [16]. Interphase fluorescence in situ hybridization was also performed on FFPE tissues to detect translocations of the MYC gene using break-apart assays for MYC and the dual-color, dual-fusion translocation probes LSI MYC/IGH (from Abbott/Vysis, Downers Grove, IL). Briefly, after overnight hybridization and subsequent washing, the slides were analyzed using a Leica TCS SP5 Laser Scanning Confocal Microscope (Leica, Wetzlar, Germany).

2.4. Cell proliferation and apoptosis

Cell Counting Kit–8 (CCK-8, Dojindo, Kumamoto, Japan) was used to determine the number of viable cells. Briefly, cells were plated onto 96-well plates (1 × 10⁴ cells per well). Quantitation of apoptotic cells was carried out using Annexin V/7-aminooactinomycin D staining according to the manufacturer’s instruction (KeyGEN, Nanjing, China). Stained samples were analyzed using a FACscan flow cytometer (Becton Dickinson, San Jose, CA) equipped with FlowJo software.

2.5. MiRNA expression profiling

MiR expression profiling was carried out using the Agilent Human miR Microarray 16.0 (CapitalBio, Beijing, China) according to the manufacturer’s instruction. This array contains 15,024 probes and can recognize 939 miRs. Total RNA was extracted using a miR isolation kit (Ambion). Fluorescein-labeled miRs were used for hybridization on each miR microarray chip. The chips were scanned using ScanArray Express laser scanner (Packard Bioscience, Covina, CA), and the images were analyzed using LuxScan 3.0 software (CapitalBio). The filtered results were subject to cluster software (Cluster 3.0) and TreeView analysis (Stanford University) for unsupervised hierarchical clustering by genes and arrays. Metric (distance) and linkage were set as Euclidean distance and average, respectively.

2.6. Quantitative reverse transcriptase polymerase chain reaction

Quantitation of the expression of miR-320d and miR-146b-5p by quantitative reverse transcriptase polymerase chain reaction (qRT-PCR) was carried out using the TaqMan MicroRNA Assays kit (Applied Biosystems) according to the manufacturer’s instructions. Five microliters of total RNA from each sample was reverse transcribed using the TaqMan MicroRNA Reverse Transcription kit (Applied Biosystems). qRT-PCR were carried out in final volumes of 10 µL using the ABI Sequence Detection System Instrument and software (Applied Biosystems). Amplifications were initiated with 10-minute incubation at 95°C followed by 40 cycles at 95°C for 15 seconds and 60°C for 60 seconds. To normalize the expression levels of miRs, we used U6 RNA as an internal control. qRT-PCR was performed in triplicate, including no-template controls. The relative expression of each miR was calculated from the following equation: relative expression = 2^−ΔΔCt, where Ct is the threshold cycle for a sample and ΔCt = (CtmiRNA)mean − (CtU6)mean.

2.7. Lentiviral transduction

The expression plasmids for miR-146b-5p and miR-320d were created by PCR amplification with human genomic DNA as templates. The primers are as follows: hsa-miR-320d: 5’-GGGCCCGCTCTAGACTCGAGATATTTG-3’, hsa-miR-146b-5p: 5’-GGGCCCGCTCTAGACTCGAGATATTTTGCATGTCGCTATGTG-3’, hsa-miR-146b-5p shRNA: 5’-AGCCTATGGAATTCAGTTCTCA-3’. The PCR product of miR-146b-5p and miR-320d was cloned into GV259 vector carrying the green fluorescent protein (GFP) gene (pGV259-GFP, Genechem, Shanghai, China). A universal sequence (PSC-NC: TTACTCGAAGCTCTACGT) was used as a negative control for RNA interference. All constructs were confirmed by DNA sequencing. To produce lentivirus, these 3 plasmid DNAs were individually transferred into 293 T cells using pHelper1.0, pHelper2.0 packaging construct, and Lipofectamine Plus reagent (Invitrogen) according to the manufacturer’s protocol. For transduction assay, OCI-Ly8, DB, and OCI-Ly10 were seeded into 24-well plates (1 × 10⁵ cells per well) and transduced with different lentiviral vectors at a multiplicity of infection of 50. Three days after transduction, GFP expression was detected to calculate the transduction efficiency by fluorescence-activated cell sorting (FACS). Five days after transduction, FACs-sorted GFP+ transduced cells were diluted to 10,000 cells per milliliter; and about 3000 cells (0.3 mL) were plated on 6 Petri dishes (10 cm) in which colonies were scored at 7 and 15 days. qRT-PCR was performed to determine miR-146b-5p and miR-320d efficiency.

2.8. Statistics

The miR genes showing significant expression changes (1.5-fold change) and false discovery rate = 0 were considered to be significantly expressed in different prognostic samples. The Mann-Whitney test was used to analyze the differences in miR expression between the patients and controls. Up- and downregulation groups were based on miR expression below or above the corresponding median. Survival curves were estimated using the product-limit method of Kaplan-Meier and were compared using the log-rank test. Univariate and multivariate analyses were performed. Multivariate regression analysis according to the Cox proportional hazards regression model, with overall survival (OS) or progression-free survival (PFS) as the dependent variables, was used to adjust for the effects of the miRs expression. Student t test was used to compare test groups with negative control ones. P value of <.05 was considered to be statistically significant.
3. Results

3.1. MiR-146b-5p and miR-320d are downregulated in DLBCLs from patients with poor prognosis

A retrospective cohort of primary nodal DLBCLs from 106 patients who attended the Department of Hematology at Shanxi Cancer Hospital between 2001 and 2010 was included in this study. The clinicopathological characteristics of these DLBCLs and patients are summarized in Table 1. The median OS of the patients after treatment with the standard CHOP regimen was 40.8 months. Among the DLBCLs, 47 were the GCB type (44.3%); and the remaining 59, the non-GCB type (55.7%) (Fig. 1A). Whereas 15 DLBCLs of the non-GCB type displayed the t(8;14) MYC translocation, this was not detected in those of the GCB type (Fig. 1B).

We initially compared miR expression profiles between randomly sampled, treatment-naive DLBCLs from 6 patients who survived longer (≥40.8 mo, median OS) and those from another 6 patients with shorter survival (<40.8 mo) using a Human miRNA Microarray Kit that contains 15,024 probes and recognizes 939 miRNAs. The results showed that 29 miRNAs were differentially expressed in DLBCLs from patients who had bad prognosis (<40.8 mo) compared to those from patients with good prognosis (≥40.8 mo) (Fig. 2A). Among them were miR-146b-5p and miR-320d that were decreased on average 2.91- and 1.76-fold, respectively. Downregulation of these miRs was subsequently confirmed by use of qRT-PCR in the whole cohort of 106 DLBCLs. Of note, miR-146b-5p and miR-320d were in general expressed at lower levels in DLBCL tissues than the control lymph nodes (Fig. 2B). In addition, they appeared to be reduced in DLBCLs of the non-GCB type, in particular, in those with the MYC t(8;14) translocation (Table 2).

3.2. Low expression of miR-146b-5p and miR-320d predicts compromised responses of DLBCLs to treatment with CHOP

Because all the patients in this cohort were solely treated with the standard CHOP regimen, the finding of downregulation of miR-146b-5p and miR-320d in DLBCLs from patients with poor prognosis suggests that the levels of these miRs may be predictive of responses of DLBCLs to treatment with CHOP. We confirmed this by analyzing the relationship between the levels of miR-146b-5p and miR-320d and OS and PFS of the patients. As shown in Fig. 3A, low expression of miR-146b-5p (<1.87, median expression level) was associated with reduced PFS; and low expression of miR-320d (<1.97, median expression level) was associated with reduction in both OS and PFS. Patients with low expression of miR-146b-5p had a median PFS of 9.4 months compared to those with high expression of the miR (≥1.87), who had a median PFS of 35.5 months. Similarly, patients with low expression of miR-320d had a median PFS and OS of 6.7 months and 15.4 months, respectively, compared to those with high expression of the miR, who had a median PFS of 36 months and a median OS of 43 months. As expected, patients with DLBCLs that displayed the MYC t(8;14) translocation had markedly shorter OS and PFS.

When assessed by the multivariate analysis, it appeared that the reduced expression of miR-320d was an independent predictor of OS relative to other clinicopathological parameters such as IPI, serum lactate dehydrogenase (LDH) levels, the presence of B symptoms, and Arbor stages (Table 3). In addition, the decrease in miR-320d or miR-146b-5p was an independent predictor of PFS (Table 3). Taken together, these results suggest that reduction in the expression of miR-146b-5p and/or miR-320d may be a useful biomarker to predict compromised responses of DLBCLs to treatment with the standard CHOP regimen.

3.3. MiR-146b-5p and miR-320d inhibit proliferation of DLBCL cells

We also examined the expression of miR-146b-5p and miR-320d in a panel of DLBCL cell lines by qRT-PCR. The levels of miR-320d were markedly lower in DLBCL cell lines of the ABC type than those of the GCB type. Whereas the miR-146b-5p level was 10 times lower in DB cells than OCI-Ly8 cells, the level of miR-320d in OCI-Ly10 cells was reduced approximately 4 times compared with OCI-Ly8 cells (Fig. 4A).

To determine the functional significance of miR-146b-5p and miR-320d in DLBCL cell survival and proliferation, we introduced exogenous miR-146b-5p or miR-320d into OCI-Ly8 cells and DB cells by lentiviral transduction (Fig. 4C). The levels of miR-146b-5p and miR-320d in DLBCL cell lines transduced with the miR-expressing constructs were markedly higher than those transduced with the control constructs (Fig. 4B). Overexpression of either miR-146b-5p or miR-320d significantly inhibited proliferation of both OCI-Ly10 and DB cells (Fig. 4D). This was associated with induction of moderate levels of apoptosis. In contrast, lentiviral transduction of miR-146b-5p shRNA into OCI-Ly8 cells enhanced cell proliferation (Fig. 4D). These results indicate that miR-146b-5p and miR-320d play a role in inhibiting DLBCL cell proliferation.

4. Discussion

In this report, we present evidence that miR-146b-5p and miR-320d are expressed at lower levels in DLBCLs from patients with relatively poor prognosis and that low expression of these miRs is an independent predictor of compromised responses of the patients to treatment with the standard CHOP regimen. In addition, we show that both miR-146b-5p and miR-320d have an inhibitory effect on proliferation of DLBCL cells.
Although the recent development of combination chemotherapy has transformed DLBCL from a fatal malignancy to a potentially curable disease, there are wild variations in responses of DLBCL patients to treatment. Virtually only fewer than half of patients can be cured [17]. This is closely related to the molecularly heterogeneous nature of DLBCL cells [18]. Although there is a large body of evidence showing that deregulated expression of miRs contributes significantly to the heterogeneity of DLBCLs, reports on the potential role of miRs in regulating sensitivity of DLBCLs to treatment with CHOP remain sparse [13], although miR-21 has been shown to regulate sensitivity of DLBCL cells to the regimen [11]. Our finding that low expression of miR-146b-5p and miR-320d is associated with poor prognosis of DLBCL patients treated with CHOP irrespective of other well-established clinicopathological parameters, such as IPI, serum LDH levels, the presence of B symptoms, and Arbor stages, strongly suggests that downregulation of these miRs is a potential predictive biomarker of compromised responses of DLBCL patients to the CHOP regimen. However, whether low expression of miR-146b-5p and miR-320d is also predictive of responses of DLBCL patients to other treatment protocols such as R-CHOP remains to be studied.

The impact of reduction in miR-146b-5p and miR-320d responses of DLBCL patients to CHOP is associated with the ability of these miRs to inhibit proliferation of DLBCL cells. This was demonstrated by decelerated proliferation of DLBCL cells when miR-146b-5p or miR-320d was over-expressed. In contrast, knockdown of either of the miRs resulted in increased proliferation of DLBCL cells. However, the exact molecular mechanisms by which these miRs inhibit DLBCL cell proliferation are unknown. This is unlikely due to induction of apoptosis in that overexpression of miR-146b-5p or miR-320d did not trigger significant apoptotic cell death. One possibility is that these miRs, like many others, may regulate key components of signaling pathways that control cell proliferation. For example, miR-125a and miR-125b modulate activation of NF-kB; and miR-155 regulates the PI3K/Akt pathway, thus affecting DLBCL cell proliferation [19,20]. MiR-320a has also been identified as a critical component of the PTEN tumor suppressor axis in stromal fibroblasts [21]. In addition, low expression of miR-
320d is known to correlate with recurrence-free survival of stage II colon cancer patients [22]. Although our results clearly indicated that miR-146b-5p inhibited DLBCL cell proliferation, a recent study has pointed to an oncogenic role of miR-146b-5p in normal and transformed thyroid cells by regulation of SMAD4 [23]. On the other hand, miR-146b-5p can inhibit glioma migration and invasion by targeting matrix metalloproteinase 16 [24]. These findings, along with ours, suggest that miR-146b-5p may have contrasting effects on cell proliferation in a cell type–dependent manner.

Another possibility is that miR-146b-5p or miR-320d may directly regulate the expression of cell cycle regulators such as cyclins and CDKs, which are crucial for the cell cycle progression. These molecules are overexpressed in DLBCL, and their inhibition may contribute to the growth inhibition observed in our experiments. Additionally, miR-146b-5p has been shown to target the oncogene MYC, which is frequently amplified and overexpressed in DLBCL, suggesting a possible mechanism for the suppression of cell proliferation by this miRNA.

### Table 2

<table>
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<th>(P^b)</th>
<th>MYC translocation +/− (^c)</th>
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\(^a\) GCB/non-GCB: the median expression level of miR in GCB/the median expression level of miR in non-GCB group.

\(^b\) \(P\) value < .05 was considered statistically significant.

\(^c\) MYC translocation +/−: the median expression level of miR with MYC translocation/the median expression level of miR without MYC translocation.
Fig. 3  Reduction in miR-146b-5p or miR-320d expression or the MYC t(8;14) translocation is associated with compromised responses of DLBCL patients to treatment with CHOP. A, Kaplan-Meier analysis using the median abundance of miR-146b-5p as the cutoff showing that low expression of the miR is associated with shorter OS (upper panel) and PFS (lower panel) of DLBCL patients after treatment with CHOP. B, Kaplan-Meier analysis using the median abundance of miR-320d as the cutoff showing that low expression of the miR is significantly associated with shorter OS (upper panel) and PFS (lower panel) of DLBCL patients after treatment with CHOP. C, Kaplan-Meier analysis showing that patients with DLBCLs with the MYC t(8;14) translocation had shorter OS (left panel) and PFS (right panel).

Table 3  The low expression of miR-146b-5p and miR-320d is an independent predictor of DLBCL except the low expression of miR-146b-5p for OS

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Abbreviation: M, the median expression level of miR; OS, overall survival; PFS, progression-free survival; CI, confidence interval.

a P value < .05 was considered statistically significant.
**Fig. 4** MiR-146b-5p and miR-320d inhibit proliferation of DLBCL cells. A, left panel: relative abundance of miR-146b-5p in 7 DLBCL cell lines as measured by qRT-PCR. The relative abundance of the miR expression in DB cells was arbitrarily designated as 1. Right panel: relative abundance of miR-320d in 7 DLBCL cell lines was measured by qRT-PCR. The relative abundance of the miR expression in DB cells was arbitrarily designated as 1. The expression of miR-320d was downregulated in DLBCL cell lines of the ABC type than those of the GCB type (*P < .05). B, Overexpression of miR-146b-5p (left panel) and miR-320d (right panel) in DB and OCI-Ly10 cells, respectively. The data shown were mean ± SE of 3 individual experiments (*P < .05). C, Representative microphotographs of DB, OCI-Ly8, and OCI-Ly10 cells transduced with miR-146b-5p–expressing constructs, miR-146b-5p shRNA, and miR-146b-5p–expressing constructs, respectively, that were enriched by FACscan sorting. Scale bar, 100 μm. D, DB (upper panel), OCI-Ly8 (middle panel), and OCI-Ly10 cells (lower panel) were respectively transduced with miR-146b-5p–expressing constructs, miR-146b-5p shRNA, and miR-146b-5p–expressing constructs. Cell proliferation was measured using Cell Counting Kit–8 assays at indicated time points after transduction. The data shown were mean ± SE of 3 individual experiments (*P < .05; **P < .01).
as p21 and p27 [25]. A large number of miRs, such as miR-224, miR-17, miR-663, and miR-221, are known to play a role in regulating cell proliferation through modulating the expression of key regulators of cell cycle progression [26–28].

An interesting finding of this study is that miR-146b-5p and miR-320d are expressed at significantly lower levels in DLBCLs with the MYC t(8;14) translocation. Whether there is a causal relationship between downregulation of these miRs and the MYC translocation need to be further investigated. Deregulated MYC is known to be a poor prognostic factor in DLBCL patients [29]. The MYC protein regulates the expression of a number of miRs, including the miR-17-92 cluster, miR-22, miR-26a, and miR-29b [30,31]. On the other hand, the expression and function of MYC may in turn be regulated by miRs. For instance, miR-196b has been recently shown to down-regulate the over-activated the c-MYC gene in B-cell ALL [32]. It is likely that downregulation of miR-146b-5p and miR-320d in DLBCLs is related to loss of function of the MYC gene. Another notable observation of this study is that miR-320d is expressed at remarkably higher levels in DLBCLs of the GCB type than those of the non-GCB type, but further studies are needed to determine whether the varying expression of miR-320d is of assistance in differentiating DLBCLs of various immunophenotypes.

In summary, we have found in this study that low expression of miR-146b-5p and/or miR-320d may be a useful biomarker to predict compromised responses of DLBCL patients to treatment with the CHOP regimen and that this is associated with the inhibitory effect of these miRs on DLBCL cell proliferation. Prospective studies in larger cohorts of DLBCL samples are warranted to further consolidate the practical significance of low expression of these miRs in decision making in the treatment of DLBCLs.

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References


