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Review

MicroRNAs in Diffuse Large B-Cell Lymphoma: Implications for Pathogenesis, Diagnosis, Prognosis and Therapy

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Abstract. *Diffuse large B-cell lymphoma (DLBCL) is the most commonly-occurring type of non-Hodgkin lymphoma and is considered a curable disease in at least 50% of patients. Considering that the disease represents a heterogeneous group of tumors, recent efforts using gene expression profiling have identified two subgroups, with significantly different response rates to standard immunochemotherapy. Nevertheless, multiple factors in the pathogenesis of this disease remain unclear and continue to be the focus of further research. MicroRNAs are small non-coding RNA molecules that regulate gene expression at the post-transcriptional level. The role of microRNAs in cancer initiation and progression has been demonstrated in multiple types of solid cancers and hematological malignancies such as lymphomas. MicroRNAs also have diagnostic potential, and therapeutic of microRNAs targeting is actively pursued. This review provides an overview on the role of microRNAs in the diagnosis of diffuse large B-cell lymphoma, their role in molecular pathogenesis and hence their prospective role in implementing novel future treatment options.*

Diffuse large B-cell lymphoma (DLBCL) is the most common form of adult lymphoma, accounting for 30 to 40% of newly-diagnosed non-Hodgkin lymphoma (NHL) (1). DLBCL represents a heterogeneous group of tumors with a high variance of genetic abnormalities, clinical features, response to treatment, and prognosis (2). An immunochemotherapy regimen, consisting of rituximab, cyclophosphamide, doxorubicin, vincristine, and prednisone (R-CHOP) has been established as the standard treatment of patients with DLBCL (3). With this therapy, even in advanced stage, DLBCL is considered a curable disease. Despite improvements in therapy, disease in approximately one-third of patients with advanced-stage DLBCL is refractory to therapy or will relapse (4).

Historically, clinicians and investigators have relied on prognostic schemes that imply clinical risk factors to predict for risk of disease progression, relapse and death of patients with DLBCL. One of the most commonly used schemes of rating, the International Prognostic Index (IPI) for lymphomas, developed in the 1990s, remains a robust clinical prognostic index for aggressive lymphomas. It involves five features: age, tumor stage, serum lactate dehydrogenase (LDH) concentration, performance status and number of extranodal disease sites. The IPI distinguishes four risk groups with different 5-year overall survival, ranging from 26-73% (5). With the application of rituximab, a revised IPI (R-IPI) has been introduced, and showed superior prediction in outcome of patients with DLBCL treated with standard immunochemotherapy. The R-IPI identifies three distinct prognostic groups, with a very good 4-year overall survival (OS) 94%, good (OS 79%), and poor (OS 55%) outcome, respectively (6). Nevertheless, despite using this risk stratification tool, a large group of patients remains poorly-characterized and presents with an unfavorable course of disease despite a good prognostic index.

Gene expression profiling has defined two molecular subgroups, namely a germinal center (GCB) or activated B-cell (ABC)-like subtype, which reflect the cell of origin (COO) of different lymphoid maturation stages (7). These profiling signatures, independent of IPI stratification, have prognostic

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advantages in determining patient outcomes following standard immunochemotherapy: The 5-year survival rate is 60% for patients with the GCB subtype and 40% for patients with the ABC subtype (8). A differentiation between these subtypes can also be achieved by immunohistochemistry and provides a further stratification tool for a COO classification (9, 10). Nevertheless, even by applying these newer prognostic tools, there still remain numerous patients with difficult predictable clinical course. More refined differentiation methods to screen for patients with poor prognosis are necessary to adapt targeted therapy for these patients.

MicroRNAs

MicroRNAs are 19-24-nucleotide-long non-coding RNAs that regulate gene expression through sequence complementarity with the target genes, mostly 3'-untranslated regions of target genes (11). MicroRNAs are transcribed as long primary transcripts, which are then converted into precursor microRNAs in the nucleus by the RNAse III enzyme DROSHA (12). Exportin 5 protein arranges the transfer of precursor microRNAs from the nucleus to the cytoplasm. Further processing *via* cutting of the hairpin-structure of precursor microRNAs by a second RNAse III enzyme, called DICER, leads to a microRNA duplex (13). The double-stranded microRNAs unwind and the functional strand is loaded onto the RNA-induced silencing complex enzyme, which guides the microRNA to its complementary sequence on the messenger RNA (14). Dependent on the complementarity, the binding results in either direct RNA degradation or inhibition of protein translation (15).

MicroRNAs play a role in various biological processes including cancer development. Genes coding for microRNAs are frequently located at fragile sites or genomic regions, typically associated with cancer (16). In 2002, Calin *et al.* identified for the first time a direct association of de-regulation in miR-15 and miR-16 expression, and the development of chronic lymphocytic leukemia (17). Following the initial findings, many other microRNAs have been identified which participate in cancer development in various tumor types (18). Additionally, many research groups explored the potential clinical application of microRNAs as diagnostic or therapeutic tools for patients with cancer (19-26). The aim of the present review is to provide a deeper insight into the role of microRNAs in the pathogenesis of DLBCL, describing their potential as diagnostic and prognostic markers, and to delineate their feasibility as future therapeutic targets.

MicroRNAs and the Pathogenesis of DLBCL

In DLBCL, microRNAs, involved in hematopoiesis and lymphomagenesis, were identified (27-29). Understanding their exact biological function continues to be a huge challenge in this specific field, as one microRNA can target multiple mRNAs and *vice versa* multiple microRNAs can influence the same mRNA molecule, making a prediction of the various effects of a particular microRNA often difficult (30). Nevertheless, the pathogenetic effects of some microRNAs in the pathogenesis of DLBCL are well-characterized. Kim *et al.* demonstrated that miR-125a and miR-125b constitutively activate the nuclear factor kappa-light-chain-enhancer of activated B-cells (NF-kB) pathway, one of the most de-regulated pathways in DLBCL pathogenesis. They found that miR-125a and miR-125b, are frequently gained or overexpressed in DLBCL, target tumor necrosis factor, alpha-induced protein 3 (TNFAIP3), a negative regulator of NF-kB, and consequently enhance NF-kB signaling (31). By ectopic expression and inhibition of these two microRNAs in cell lines, they demonstrated their oncogenic role in lymphomagenesis.

The miR-17-92 cluster, encoding for six different microRNAs, is located at chromosome 13q31-q32, a region frequently amplified in GCB DLBCL (32). This cluster of microRNA contributes to survival of early B-cell progenitors, and thus adds to lymphomagenesis by repressing anti-apoptotic genes (33).

MiR-155, one of the most studied microRNAs, is accumulated 10- to 30-fold in several types of B-cell lymphoma, including DLBCL, compared to normal B-cells (34). In addition, miR-155 expression levels are higher in the ABC- compared to the GCB subtype. In mucosa-associated lymphoid tissue (MALT) lymphoma, an increase of miR-155 was invariably associated with a suppression of the pro-apoptotic gene tumor protein p53-inducible nuclear protein 1 (TP53INP1) (35). As a proapoptotic stress-induced p53 target gene, *TP53INP1* induces cell-cycle arrest and apoptosis when overexpressed. MiR-155 also targets human germinal-center associated lymphoma (HGAL), a new prognostic biomarker that is indispensable for germinal center formation, immunoglobulin gene class-switch recombination, and somatic hypermutation. By mediating the effects of IL-6, it interacts with the cytoskeleton and cellular motility and migration in lymphoma (36, 37). *Via* quantitative genomics, Huang *et al.* identified phosphatidylinositol 3-kinase (PIK3R1), a negative regulator of the phosphatidylinositol 3-kinase (PI3K-AKT) pathway, as a direct target of miR-155 (38). Costinean *et al.* generated Eμ-mmu-miR155 transgenic mice, developing a lymphoproliferative disease resembling human acute lymphatic leukemia or high-grade lymphoma when overexpressing miR-155 (39). This strongly suggests that miR-155 is directly implicated in the initiation and progression of these diseases. Furthermore, because of the disease's polyclonal character, miR-155 could be the downstream target of signal transduction pathways activated in cancer (39). A further target, bone morphogenetic protein (BMP)-responsive transcriptional factor mothers against decapentaplegic

Table I. *microRNAs associated with lymphomagenesis*.

microRNA	Target	Method	Notes	References
Pathogenesis				
miR-125a,b	TNFAIP3 (A20)	qPCR	Overexpression in DLBCL	31
miR-17-92				32,33
miR-155	TP53INP1, HGAL, PIK3R1, SMAD5		Overexpression in DLBCL, in ABC higher than a in GCB, initiation of ALL and/or high grade lymphom	34-41
miR-34	FOXP1		Tumour-suppressive	42,43
Diagnosis				
miR-125b	-	Microarray	Up regulated in DLBCL	25
miR-155	-	Microarray, qPCR	Up regulated in DLBCL, lost in Burkitt lymphoma, in ABC higher than in GCB, expression in serum up regulated	25, 27, 28, 48-50, 53
miR-221	-	Microarray, qPCR	Overexpression in ABC	49
miR-21	-	qPCR	Overexpression in ABC, expression in serum up regulated, up regulated in CSF	49, 53, 55
miR17-92	-	Microarray, qPCR	Overexpression in ABC	48
miR-210	-	qPCR	Expression in serum up regulated	53
miR15a	-	qPCR	Expression in serum up regulated	54
miR16-1	-	qPCR	Expression in serum up regulated	54
miR-29c	-	qPCR	Expression in serum up regulated	54
miR-34a	-	qPCR	Expression in serum down regulated	54
miR-19	-	qPCR	Up regulated in CSF	55
miR-92a	-	qPCR	Up regulated in CSF	55
Prognosis				
miR-21	-	Microarray, qPCR	High levels correlate with better OS	49
miR-155	-	-	No prognostic impact	49,56
miR-18a	-	qPCR	High levels correlate with better OS	57
miR-181a	-	qPCR	High levels correlate with better OS	57
miR-222	-	qPCR	High levels correlate with better OS	57
Therapy				
miR-155	SHIP1	qPCR	Withdrawing leads to tumour repression	59
miR-15a	Cyclin D1	qPCR	Reinduce apoptosis	60
miR-16	Cyclin D1	qPCR	Reinduce apoptosis	60

TNFAIP3: Tumor necrosis factor, alpha-induced protein 3; qPCR: quantitative real-time polymerase chain reaction; DLBCL: Diffuse large B-cell lymphoma; TP53INP1: Tumor protein p53-inducible nuclear protein 1; HGAL: human germinal-center associated lymphoma; PIK3R1: phosphatidylinositol 3-kinase; SMAD5: Mothers against decapentaplegic homolog 5; ABC: activated B-cell like; GCB: germinal center B-cell; ALL: acute lymphocytic leukemia; FOXP1: Forkhead box protein P1; CSF: cerebrospinal fluid; OS: overall survival; SHIP1: src homology 2 containing inositol 5-phosphatase.

homolog 5 (SMAD5), was inhibited and disrupted in its activity by miR-155 overexpression (40). Overexpression rendered DLBCL resistant to the growth-inhibitory effects of both transforming growth factor (TGF-β1) and BMPs, *via* defective induction of *p21* and impaired cell-cycle arrest. Jiang and Aguiar further dissected the role of miR-155 in modulating this pathway (41): In DLBCL cell lines and a miR-155 knock-out mouse model, they demonstrated that levels of the transcription factor SMAD5 are elevated in mature B-lymphocytes, which display an increased sensitivity to TGFβ1 characterized by suppression of retinoblastoma protein (RB) phosphorylation and a more pronounced G_0/G_1 cell-cycle arrest (41).

MiR-34, a pro-apoptotic and growth-suppressive microRNA executes these functions *via* the TP53 pathway (42). Another tumor-suppressive effect attributed to miR-34a is the de-regulation of its target forkhead box protein P1 (FOXP1), leading to blocked proliferation of DLBCL (43) (Table I).

MicroRNAs in the Diagnosis of DLBCL

Today, the diagnosis of DLBCL is a histological task, supplemented by immunohistochemistry and, if available, gene expression profiling (44). The addition of a microRNA profile to the diagnosis of lymphoma, especially for sub-typing, could represent an important novel future tool and consequently, in uncertain cases microRNA profiling has attended special interest within the diagnostic procedure.

The extensive effort emerging in this research field is well-demonstrated by Jima *et al*., who elucidated the complete small RNA transcriptome of normal and malignant

B-cells through deep sequencing of 31 normal and malignant human B-cell samples that comprise the entire spectrum of B-cell differentiation and their common malignant phenotypes (45). They were able to evaluate the expression of 333 known microRNAs, and to further measure the expression of 286 candidate novel microRNAs in normal and malignant B-cells. Since their study in 2010, every year, hundreds of new microRNAs are identified and added to the analysis (46).

Of most important diagnostic relevance is the differentiation between normal lymphatic tissue and the presence of a lymphoid neoplasm. Lawrie *et al*. identified 40 differentially-regulated microRNAs in B-cell lymphoma samples, compared to normal B-lymphocyte subsets (25). Using the 20 most de-regulated microRNAs, they were able to predict the malignant nature of the samples with a success rate of 99%. This microRNA signature also included miR-125b and miR-155, which were significantly up-regulated in DLBCL (25).

A further research focus is the distinction between the various lymphoma subtypes. Di Lisio *et al*. proposed a model of 128 microRNAs enabling the discrimination of various lymphoid malignancies, including Burkitt lymphoma, chronic lymphocytic leukemia, DLBCL, follicular lymphoma (FL), marginal zone lymphoma, and mantle cell lymphoma. For the distinction between DLBCL and Burkitt lymphoma, often a twilight zone, a signature of 19 microRNAs including miR-155, was sufficient to discriminate these entities with 93% accuracy (28).

Another point of interest is the discrimination between *de novo* DLBCL and transformed FL, which is indistinguishable in histology, but of eminent importance for prognosis (47). Lawrie *et al*. addressed this issue by identifying a cluster of 14 microRNAs, relevant for this distinction, however only in a small cohort of 16 samples of transformed FL (25).

By immunohistochemistry and gene expression profiling, the presence of two distinct subtypes of DLBCL, reflecting the cellular origin, has been revealed (7, 9). Several recent reports focused on the differentiation of these subtypes by applying a microRNA signature (25, 27, 48, 49). In 2007, Lawrie *et al*. performed a microarray analysis of 225 microRNAs in four different DLBCL cell lines (48). They were able to identify miR-155, miR-221, and miR-21 as being more highly expressed in ABC compared to GCB cell lines. Confirmation was performed in primary 49 DLBCL samples. In a later study, Culpin *et al*. broadened the sample cohort, as well as the amount of individual microRNAs used for calculation and established a series of nine differentially-expressed microRNAs being able to differentiate between the two subtypes (49). Interestingly, none of the above-mentioned microRNAs detected by Lawrie *et al*., but four microRNAs of the miR-17-92 cluster were among the nine discriminative microRNAs (49). In two recent publications, miR-155 was confirmed to be differentially de-regulated in DLBCL subtypes, in conjunction with a series of five and eight microRNAs, respectively (27, 50).

In most of the studies mentioned above, the specimens were derived from frozen tumor samples. Formalin-fixed paraffin-embedded tissue (FFPET) is often more widely-available in clinical practice. Culpin *et al*. demonstrated the equality of FFPET and frozen samples results with respect to microRNA expression measurements (51).

Beyond the recognition of novel markers for histopathological diagnosis, some studies suggest the evaluation of serum microRNA expression levels as a non-invasive method for rapid diagnosis or monitoring of minimal residual disease (52). The expression level of three DLBCL-associated microRNAs (miR-155, miR-210, and miR-21) was shown to be significantly higher in serum derived from DLBCL patients compared to normal control sera (53). In another study, a total of seven microRNAs was analyzed in serum samples from patients with DLBCL and healthy controls (54): The expression levels of miR-15a, miR-16-1, miR-29c, and miR-155 were significantly elevated, whereas miR-34a was significantly down regulated in DLBCL sera samples, suggesting a potential future tool for this non-invasive diagnostic (54). The diagnosis of primary central nervous system lymphoma (PCNSL), so far diagnosed by brain biopsy, microRNAs have also been shown to play an important role as disease markers in the cerebrospinal fluid (CSF). Three microRNAs, namely miR-21, miR-19, and miR-92a, showed a significant presence in the CSF of patients with PCNSL. Diagnostic accuracy for these miRNAs in diagnosing PCNSL was high, with 95.7% sensitivity and 96.7% specificity (55) (Table I).

In summary, regarding the above-mentioned analyses, miRNA evaluation in routine diagnostics could significantly improve diagnostic precision and contribute to a more personalized and targeted treatment approach.

MicroRNAs in the Prognosis of DLBCL

Studies suggest that microRNAs, besides their usefulness in improving diagnosis, also possess a prognostic potential for cancer patients.

MiR-21, already established as differentiator between the GCB and ABC subtypes, also plays a role as a prognostic indicator (48). High expression levels were found to be associated with longer relapse-free survival in patients with DLBCL in a multivariate Cox analysis (48). The other microRNAs identified in that study, used for predicting COO-subtypes (miR-155 and miR-221) showed no prognostic impact. A confirmatory study by Jung *et al*. demonstrated no correlation in their cohort of 129 DLBCL patients of expression level miR-155 and OS (56). Interestingly, a marked trend towards a better survival for

patients with the ABC subtype with high expression of miR-155 was found.

Alencar *et al*. identified three (miR-18a, miR-181a, and miR-222) out of 11 microRNAs as being independent predictors of outcome in DLBCL (57). They integrated these microRNAs in a combined model, including the IPI and a 6-gene mortality predictor score, and demonstrated the predictive power of these microRNAs on the 5-year OS and progression-free survival (PFS) (57). Interestingly, miR-155 was also analyzed in this study and again was not associated with prognostic significance.

A further predictive model was created by Montes-Moreno *et al*. (58) for 36 patients by employing a microarray to identify differentially-expressed microRNAs, based on OS: 57 microRNAs were correlated positively or negatively with overall OS, none of them were able to discriminate between the ABC and GCB subtype. A set of nine microRNAs was further evaluated in the entire test group of 240 patients. With these data, a microRNA expression-based model for prediction of OS and PFS was applied. Low expression levels of these nine microRNAs were found to be significantly associated with better OS and PFS. Further accuracy could be improved by the combination of the microRNA-based model and the IPI score (58).

MiR-155 perfectly reflects the difficulties frequently occurring in the analysis of microRNAs. Various studies found a de-regulation of miR-155 in lymphoma samples (25, 28, 49) but, because of discordant findings, a final assessment about its role as prognostic parameter is still a matter of an ongoing debate (56, 57) (Table I).

To summarize, overwhelming evidence suggests that microRNAs are a valuable tool to accurately predict therapy response. Using multi-microRNA expression models instead of a single marker, the predictability can significantly be improved. Further, incorporation into already existing predictive models like the IPI should also be encouraged.

MicroRNAs in (Future) Therapy

Identifying the role of various microRNAs in the pathogenesis of lymphomas has raised the question of their use in therapies. In general, two ways exist of employing microRNA targets for therapeutic use: the down-regulation of oncogenic microRNAs by for instance antagomiRs, or the replacement of suppressed microRNAs, important for normal cell development (26). In a mouse model, Babar *et al*. demonstrated a rapid regression of lymphadenopathy in mice with established lymphoma by suppressing miR-155 (59). They further demonstrated that tumor regression was partly caused by apoptosis and that nanoparticle delivery of anti-miR-155 embedded in polylysine-conjugated peptide nucleic acids (PNAs) inhibited miR-155 expression *in vitro*.

Since microRNAs are down-regulated in the majority of lymphoma cases, microRNA replacement therapy appears to be a promising treatment approach. Although no data about microRNA replacement in DLBCL are available, promising results exist for chronic lymphocytic leukemia. Therein, the correction of down-regulated miR-15a and miR-16 induced apoptosis *in vitro* and led to a restoration of cell cycle control by arresting cells in the G_1 phase (60) (Table I).

The paramount importance of microRNAs in the treatment of human disease has been recently demonstrated in patients with hepatitis C (HCV) infection in whom miravirsen, an anti-miR-122 component, showed a prolonged dose-dependent reduction in HCV RNA levels (61). Nevertheless, many questions related to microRNA-based treatment approaches are still unanswered and requires a large amount of continuous research. One challenge is certainly the evaluation of the safety of microRNA therapeutics: The potential immune response, toxicity or unexpected side effects, related to the fact that one microRNA can affect hundreds of target genes is still the focus of research. Also the successful delivery of the agent to the target tissues or overcoming tissue barriers remains unacknowledged yet (62).

In conclusion, microRNAs are a promising novel field of research, providing interesting insights into the pathogenesis and diagnosis of lymphomas. In the future even targeted treatment approaches in DLBCL could be accomplished by influencing expression levels of oncogenic or tumor suppressive microRNAs. However, in the meantime further efforts are needed to precisely elucidate the miRNAs' role in the development and progression of DLBCL in order to create targets for future therapy.

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