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REVIEW

The ins and outs of microRNAs as biomarkers in liver disease and transplantation

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Summary

Ongoing research is being conducted in the field of transplantation to discover novel, noninvasive biomarkers for assessment of graft quality before transplantation and monitoring of graft injury after transplantation. MicroRNAs (miRNAs) are among the most promising in this field. MiRNAs are small noncoding RNAs that function as important regulators of gene expression in response to cellular stress and disease. An advantage that makes miRNAs attractive candidates for biomarker research is their fast release from cells in response to stress and injury, which can occur via different routes. In the context of liver transplantation (LT), noninvasive measurement and stability of extracellular miRNAs in blood, bile, and graft perfusates has been linked to cell-type specific injury and early graft outcome following LT. Furthermore, specific intrahepatic miRNA expression patterns have been associated with graft survival and recurrent disease, like hepatitis C virus-related fibrosis and hepatocellular carcinoma. Therefore, miRNAs with strong predictive value and high sensitivity and specificity might be successfully applied to assess hepatic injury and to diagnose (recurrent) liver disease before, during and after LT. In this review, the current features and future prospects of miRNAs as biomarkers in and out of the liver are discussed.

Introduction

Liver transplantation (LT) remains as the only curative treatment for end-stage liver disease. Both short- and long-term patient and graft survival, however, remain far from satisfactory, despite substantial advances in immunosuppressive therapy and surgical techniques [1,2]. The increased heed for use of marginal donors because of allograft shortage, transplantation of recipients with increasingly higher MELD scores, and recurrence of liver disease are major factors that are negatively influencing outcome following LT [2]. Ongoing research is conducted in the field of transplantation to discover novel, noninvasive biomarkers for assessment of graft quality before transplantation and monitoring graft injury after transplantation. MicroRNAs (miRNAs) are among the more promising in this field.

MiRNAs are a class of newly discovered small noncoding RNAs, which serve as important regulators of post-transcriptional gene expression and as such control many cellular processes [3]. They exert down-regulating effects by preventing translation of messenger RNA (mRNA) into functional proteins. Increasing evidence establishes the important role of miRNA expression in physiological as

well as pathophysiological processes, including tissue injury and repair [4–11].

Although the gene-regulating function of miRNAs is complex and far from fully unraveled, their unique features make them attractive candidate biomarkers for prognostic and diagnostic purposes in liver disease and LT. Profiles of miRNAs that are expressed by various cell types, such as hepatocytes, cholangiocytes and endothelial cells, allow for the study of cell-type specific injury or stress [12–14]. Moreover, in response to injury, cell-type specific miRNAs can be released into the circulation and other body fluids via different routes, which has been demonstrated by multiple studies [13–18]. Surprisingly, these extracellular miRNAs remain fairly stable, despite the abundance of RNA degrading enzymes [7,19–23].

For LT, both miRNA expression patterns in tissue (the Ins) and miRNA release into serum, bile, and graft preservation solutions (the Outs) have been linked with complications that form major threats for patient and graft survival. These include severe ischemia–reperfusion injury (IRI), acute rejection, hepatitis C virus (HCV) reinfection, and recurrence of hepatocellular carcinoma. MiRNAs with strong predictive value and high sensitivity and specificity might be successfully applied to assess graft quality and monitor graft function during different phases of clinical LT. Moreover, they could be valuable contributors to existing decision-making models like the donor risk index and the Milan criteria, which are currently used for the selection of, respectively, suitable donors and recipients in order to optimize graft and patient survival.

In this review, we discuss the recent literature with special attention toward the use of miRNAs as biomarkers to assess graft quality in LT, to monitor graft function shortly after LT, and for diagnosis of recurrent disease after LT. Emphasis is put on the biological relevance of miRNAs in response to cell stress and the associated release of miRNAs from cells.

MicroRNAs as master regulators of cellular stress

Approximately 30% of all human genes are believed to be regulated by miRNAs, of which over 1000 types have been identified to be expressed by different cells. A distinct set of miRNAs were found to be expressed by hepatocytes and cholangiocytes of the liver, including miR-30a,[1] miR-30c, miR-30e, miR-122, miR-133a, miR-148a, miR-191, miR-192, miR-194, miR-198, miR-200c, miR-222, miR-296, miR-710, and miR-711 [15,24–28]. The most abundantly expressed miRNA in liver tissue is miR-122 [12,28]. This miRNA has been shown to be an important regulator of cholesterol metabolism [29], iron homeostasis [30] and as a crucial host factor for hepatitis C virus (HCV) infection and replication [31,32].

General miRNA-induced gene regulation is a two-way process that is able to respond rapidly to specific cellular needs, especially under circumstances of cellular stress where they play a central role [33]. Not only do miRNAs regulate gene expression, they are sometimes also regulated themselves by stress signals such as NF-kB and p53 during, for instance, inflammation and DNA damage [34]. Furthermore, miRNAs have been shown as important mediators of metabolic stress, for example, during hypoxia, hyperglycemia, hypertriglyceridemia & hypercholesterolemia, and caloric restriction [35]. Furthermore, this regulation due to repression by miRNAs is a reversible process. For instance, the mRNA for cationic amino acid transporter 1 (CAT-1), which is normally repressed by miRNA-122, is relieved from repression during cellular stress (amino acid deprivation) to allow increased CAT-1 protein formation by translation of pre-existing mRNA [33], suggesting an important role for miRNAs as regulators of cellular stress.

Circulating miRNAs, their release and their extracellular stability

The presence of tissue-specific, extracellular miRNAs in the circulation has made them an important subject for noninvasive biomarker research. Already in the early 1970s, it was reported that, beyond expectation, intact free stable RNA could be found in the blood circulation, suggesting that such RNAs had to be relatively resistant to degradation by RNases [36]. More recently, circulating miRNAs have also been demonstrated to exert unexpected stability. Even after prolonged times at room temperature and after repeated cycles of freezing and thawing, miRNAs in serum, plasma, and graft perfusate samples remained insensitive from degradation [7,13,14,19]. But miRNAs can also be detected in other body fluids, including amniotic fluid, breast milk and colostrum, bronchial lavage, cerebrospinal and peritoneal fluid, bile, saliva, tears, urine, pleural fluid, and seminal fluid [17,18,37], suggesting protection against degradation.

The general observation is that stability of circulating miRNAs exceeds the stability of circulating mRNA. This has been attributed to either packing of miRNAs in small particles or their association with (lipo)protein complexes, protecting miRNAs from RNase activity. According to the literature, the largest portion of circulating miRNAs in

[1]Under a standard nomenclature system, names are assigned to experimentally confirmed miRNAs before publication of their discovery. The prefix "miR" is followed by a dash and a number, the latter often indicating order of naming. For example, mir-123 was named and likely discovered prior to mir-456. Species of origin is designated with a three-letter prefix, e.g., hsa-miR-123 is a human (Homo sapiens). MiRNAs with nearly identical sequences except for one or two nucleotides are annotated with an additional lower case letter. For example, miR-123a would be closely related to miR-123b (Source Wikipedia).

plasma or serum is present in a protein-bound form. They have been shown to bind to the Ago2 protein in particular, which is a catalyzing component in the RNA-induced silencing complex (RISC) [22,38]. The involvement of proteins in stabilizing extracellular miRNAs has been demonstrated in serum samples treated with proteinase K, which results in degradation of proteins and subsequently diminished stability of extracellular miRNAs [22]. The exact mechanism by which miRNA–protein complexes are formed and excreted in the setting of LT is unknown. Furthermore, what the faith of liver-derived miRNAs is once they have been released from cells is currently unknown. One interesting hypothesis is that released miRNAs can be taken up by cells inside or outside the liver and thereby remotely regulate gene expression in recipient cells [39]. However, this hypothesis requires further research in order to demonstrate a biological role of extracellular miRNAs.

In addition to the protein-bound form, a smaller portion of miRNAs is transported in small particles like exosomes, microvesicles, and apoptotic bodies [20–23]. All these particles contain a lipid layer surrounding the miRNAs cargo to protect their content. Apoptotic bodies are released by cells during programmed cell death and are relatively large in size compared to microvesicles and exosomes. Microvesicles again are larger in their size compared to exosomes and are released from living cells by bleb formation of the lipid layer. The smallest particles known to carry miRNAs are exosomes. These small particles are produced in endosomes and are released from cells by fusing with the lipid cell membrane [20–23]. Recent studies have already shown that genetic exchange, and even transmission of HCV, through exosomes is possible [20,39]. Hypothetically, these small vesicles could be involved in signal transduction and intercellular communication mediated by miRNA exchange.

Finally, stable forms of extracellular miRNAs have recently been found in association with high-density lipoproteins (HDL) and low-density lipoproteins (LDL) [23,40]. The exact method of binding between miRNAs and lipoproteins is not understood. Some studies suggest that this association occurs within the circulation, where miRNAs are picked up by lipoproteins, rather than packaged in HDL and LDL particles in the cell [23,40]. A summary of all routes of cellular miRNA release is illustrated in Fig. 1.

For most miRNAs found in circulation, it appears that excretion is caused by a selective and active mechanism of controlled release rather than a passive or coincidental leakage [22]. *In vitro* studies show differences in ratios of intracellular miRNAs and their release through small particles; some miRNAs were effectively excreted, while others were retained completely by the same cells, suggesting selective packaging and excretion mechanisms [20,41,42]. In case of lipoprotein-associated miRNAs, it was shown that levels varied in certain diseases, underlining their potential as biomarkers [23,43]. This specific controlled release further strengthens the hypothesis that released miRNAs are involved in regulatory, pathophysiologic mechanisms.

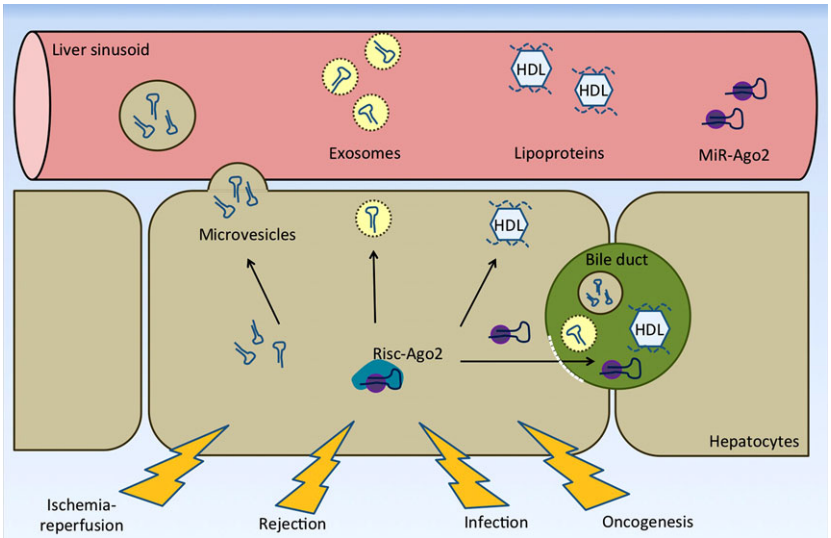

Figure 1 Mechanisms of miRNA release from (injured) cells. Mature miRNAs inside the cell cytoplasm are bound to the RISC–argonaute2 complex. Cell stress induced by, for instance, ischemia–reperfusion, infection, rejection, and oncogenesis, can cause active release or secretion of miRNAs into the circulation. Extracellular circulating miRNAs have been found in vesicles and smaller exosomes or bound to lipoproteins (HDL and LDL) and argonaute2. Recently, microRNAs were also described to be released from the liver into bile during. (Not included in this figure; miRNA release through apoptotic bodies).

Circulating microRNAs as noninvasive biomarkers for liver injury in a nontransplant setting

Current research concerning circulating miRNAs as biomarkers has mainly focused on liver disease and liver failure prior to transplantation. This has encouraged further investigation of miRNAs as biomarkers in the setting of LT, though the total number of published studies for this field is still limited. Markers for liver disease, however, could be relevant for predicting or diagnosing recurrent disease after LT. Therefore, this paragraph discusses relevant studies regarding circulating miRNAs in a nontransplant setting (Table 1).

Globally, viral hepatitis is one of the most important indications for LT. A study by van der Meer *et al.* [44] demonstrated that serum levels of previously described hepatocyte-abundant miR-122 and miR-192 are increased in HCV-infected patients. Interestingly, these miRNAs were also able to identify patients with normal transaminase levels during active HCV infection. In patients with HCV infection and nonalcoholic fatty liver disease (NAFLD), not only miR-122 but also miR-34a and miR-16 levels were found to be increased in serum compared to controls [45]. These levels of miR-122 and miR-34a correlated with liver enzyme levels and histological fibrosis stage and inflammation activity in both HCV and NAFLD patient groups. Roderburg *et al.* [46] showed lower serum levels of miR-29 in mice and humans with liver fibrosis compared to healthy controls and that serum levels of miR-571 were closely correlated with the stage of disease during alcoholic or HCV-induced liver cirrhosis [47]. The findings from these studies indicate a higher sensitivity of serum miRNAs compared with conventional transaminases in screening liver injury, and the potential of miRNAs as biomarkers for monitoring fibrosis and severity of cirrhosis.

Studies by other groups show that miR-885-5p[2] is significantly increased in serum of patients with hepatocellular carcinoma (HCC), liver cirrhosis, and hepatitis B virus (HBV) infection compared to controls but does not differentiate between the different types of liver disease [48]. Similarly, levels of miR-21, miR-122, and miR-223, which are commonly deregulated in HCC tissue, were increased in serum of patients with HCC compared to healthy controls, but also in patients suffering from chronic viral hepatitis without known HCC [49]. This illustrates the problem that some serum miRNAs can only differentiate patients with liver injury from healthy controls, but not specify the nature of the injury.

In contrast, a different study shows that serum miRNAs could specifically identify HBV infection. Serum levels of miR-25, miR-375, and let-7f clearly differentiated between patients with combined HBV infection and concurrent HCC from healthy controls and patients with only HBV or HCV infection. Serum levels of miR-375 achieved specificity and sensitivity of, respectively, 96% and 100% for predicting HCC, making it a useful marker for HCC in HBV-infected patients [50]. A comparable study in three independent cohorts identified a different set of circulating miRNAs (miR-122, miR-192, miR-21, miR-223, miR-26a, miR-27a, and miR-801) that provided high diagnostic accuracy for predicting HCC in HBV-infected patients [51]. Li *et al.* [52] found that increased serum levels of miR-221 correlated with HCC tumor size, cirrhosis, tumor stage, and diminished patient survival by 2.5 times, suggesting its prognostic usefulness.

In general, these studies demonstrate the potential of miRNAs as predictive, diagnostic, and prognostic biomarkers in liver diseases, which are common indications for LT, with higher sensitivity and specificity compared to transaminases. However, small sample sizes and the lack of prospective studies make most current miRNA biomarkers still premature. This is indicative of the limitations of current biomarker discovery research. Whether miRNAs as biomarker could be utilized in the clinical setting of LT therefore remains to be determined. However, the strong correlation of specific miRNAs with the degree of histological inflammation, fibrosis, and cirrhosis suggests that they could prove useful for various purposes in LT, such as screening for (and thus early treatment of) recurrent disease, identification of specific post-transplant complications, and safe tapering of immunosuppressive drugs to minimize side effects.

Circulating microRNAs as noninvasive biomarkers in liver transplantation

As mentioned earlier, outcome after LT has improved considerably over the last decades, but patient and graft survival and quality of life could still be improved [14,53–56]. Outcome after LT is often compromised as a result of various causes such as inadequate graft selection and consequent primary nonfunction or delayed graft function, recurrence of disease, ischemic cholangiopathy, and lifelong usage of immunosuppressive drugs and its complications [2,55,57,58]. The need for noninvasive biomarkers to monitor graft quality before, during, and after LT therefore remains. Despite this need, only a limited number of studies have been conducted so far in the field of LT, which results are summarized in Table 2.

[2]When two mature microRNAs originate from opposite arms of the same pre-miRNA, they are denoted with a -3p or -5p suffix. In the past, this distinction was also made with 's' (sense) and 'as' (antisense). When relative expression levels are known, an asterisk following the name indicates an miRNA expressed at low levels relative to the miRNA in the opposite arm of a hairpin. For example, miR-123 and miR-123* would share a pre-miRNA hairpin, but more miR-123 would be found in the cell (Source Wikipedia).

Table 1. A summary of the literature is given of identified miRNAs and their potential as biomarkers of liver injury in a nontransplant setting.

Manuscript	Medium	miRNAs	Description
van der Meer *et al.* [44]	Serum	miR-122 miR-192	Sensitive detection of liver injury by miRNAs even when transaminases are low in HCV-infected patients
Cermelli *et al.* [45]	Serum	miR-16 miR-34a miR-122	Increased levels in patients with HCV infection and NAFLD Positive correlation of miR-122 and miR-34a with liver enzyme levels and histological fibrosis stage and inflammation activity
Roderburg *et al.* [46]	Serum	miR-29	Lower circulating levels in patients with liver fibrosis
Roderburg *et al.* [47]	Serum	miR-571	Levels closely correlated with disease stages during alcoholic or HCV-induced liver cirrhosis
Gui *et al.* [48]	Serum	miR-885-5p	Increased levels in patients with HBV, HCC, and liver cirrhosis
Xu *et al.* [49]	Serum	miR-21 miR-122 miR-223	Elevated levels in patients with HCC but also in patients with chronic hepatitis
Li *et al.* [50]	Serum	let-7f miR-25 miR-375	Differentiation between HBV-infected patients with concurrent HCC and healthy controls and patients with only HBV or HCV infection Specificity of 96% and sensitivity of 100% for predicting HCC with miR-375
Zhou *et al.* [51]	Serum	miR-21 miR-26a miR-27a miR122 miR-192 miR-801	Combined miRNA profile with high diagnostic accuracy for predicting HCC in HBV-infected patients
Li *et al.* [52]	Serum	miR-221	Elevated levels correlated with HCC tumor size, cirrhosis, tumor stage, and significantly diminished patient survival by 2.5 times

Table 2. A summary of the literature is given of identified miRNAs and their potential as biomarkers of liver injury in a transplant setting.

Manuscript	Medium	miRNAs	Description
Farid *et al.* [13]	Peritransplant liver tissue and post-transplant serum	miR-122 miR-148a miR-194	Reduction of miR-122 and miR-148a in liver tissue negatively correlated with length of ischemia time Correlation of serum miR-122, miR-148a, and miR-194 levels with transaminases Early detection and quick response of miR-122 and miR-148a during acute rejection and its treatment
Verhoeven *et al.* [14]	Pretransplant graft perfusates	miR-30e miR-122 miR-148a miR-222 miR-296	Profiles of combined cholangiocyte- and hepatocyte-derived miRNAs predictive for development of post-transplant ischemic cholangiopathy.
Hu *et al.* [59]	Plasma and portal lymphocytes	miR-122 miR-146a miR-192	Increased plasma levels of all miRNAs during acute rejection Specific higher expression of miR-146a in portal lymphocytes.
Lankisch *et al.* [61]	Post-transplant bile	miR-517a miR-892a miR-106a*	Elevated in bile after development of ischemic cholangiopathy after liver transplantation.

In an earlier study by our group, a diminished expression of hepatocyte-abundant miR-122 and miR-148a in allograft tissue during LT was shown to significantly correlate with the length of graft ischemia time. At the same time, serum levels of these miRNAs increased and correlated with traditional markers of liver injury after LT. Furthermore, during episodes of histologically proven acute cellular rejection, miRNAs rised earlier compared to transaminases during injury and normalized more rapidly after treatment, showing that miRNAs are promising candidates for very early detection of liver injury after transplantation [13]. More recently, these findings were confirmed in a rat model, showing plasma levels of miR-122, miR-146a, and miR-192 to be significantly increased during acute rejection. Interestingly, the researchers suggest miR-146a to be more specific in detecting of acute rejection because this miRNAs was

higher abundant in portal lymphocytes within the liver, compared to levels of miR-122 and miR-192 that were assumed to represent more general hepatic injury [59].

More recent work from our team shows that pretransplant perfusates, that are used for cold storage of liver allografts, contain stable extracellular miRNAs originating from hepatocytes (miR-122, miR-148a) as well as cholangiocytes (miR-30e, miR-222, miR-296). Profiles of these miRNAs were independent predictors for the development of ischemic cholangiopathy after LT. The proof of the concept that miRNAs could be used as early biomarkers already before graft implantation to predict graft quality could be a valuable feature for the selection of allografts in the future [14,60].

Not only blood or perfusates, but also measurement of miRNAs in bile can be of use after LT. Very recently, Lankish *et al.* [61] showed that bile levels of miR-517a, miR-892a, and miR-106a* were increased in patients with ischemic cholangiopathy and could distinguish between ischemic cholangiopathy and other causes for biliary obstructions. In particular for biliary complications, miRNA composition in bile rather than serum might better reflect ongoing injury of cholangiocytes [62].

Although miRNA biomarkers clearly have potential for clinical application in the setting of LT, the number of studies on this topic should be expanded as their numbers are limited. During transplantation, miRNAs in perfusates can be used for diagnostic, and in the future also maybe for therapeutic, purposes. Hence, not only selection of good-quality grafts might benefit, but also alleviating ischemia–reperfusion injury might be an option once the biology of miRNAs has been unraveled. Furthermore, detection of circulating miRNAs in bile and serum can be equally useful post-transplantation follow-up, such as monitoring for recurrent disease.

Tissue miRNA expression patterns and Hepatitis C recurrence after liver transplantation

Recurrence of disease is the most important cause of graft loss after LT, and its prevention could lead to decreased need for re-LT and significant improvement of outcome after LT. One major determinant for patient and graft survival after LT is the recurrence of HCV infection of the graft. Several studies investigated whether miRNA profiles in liver tissue in recipients can be used to predict the severity and time to develop fibrosis caused by recurrence of HCV and whether microRNAs can monitor response to antiviral therapy.

One study investigated slow versus fast progressing fibrosis in recipients with recurrent HCV after LT. Recipients with slow progression of liver fibrosis at 12 months after LT (Ishak score <F2) showed up-regulated expression of miR-146a, miR-19a, miR-20a, and let-7e in graft liver biopsies compared to recipients with fast progression (Ishak score ≥F2 at 12 months) [63]. In addition, the investigators were also able to distinguish fast progressing HCV reinfection from acute cellular rejection using miRNAs, which can usually be clinically challenging after LT but is essential as therapies for both conditions differ significantly.

A similar study compared miRNA expression between nonprogressors (Knodell fibrosis score F0-F1) and progressors (F3-F4) in liver allograft tissue biopsies that were collected during clinical recurrence of HCV. In a training set of 27 recipients, a profile of nine differentially expressed miRNAs was identified of which seven could be validated successfully in an independent set of recipients. In particular, miR-155 and miR-30c were, respectively, up- and down-regulated in progressors and were described as key-regulator miRNAs for the development of fibrosis through ingenuity pathway analysis [64]. Why these two comparable studies do not identified common miRNAs is unclear.

Another study investigated which miRNAs target HCV receptors and relate to HCV infection and response to antiviral therapy after LT. Different from the previous two papers, the investigators did not use gene array analysis for identification of potentially relevant miRNAs, but miRNAs were selected by target prediction software. High viral load at time of HCV recurrence was significantly associated with increased expression of miR-122. Furthermore, in patients with sustained virological response, miR-122 expression significantly increased when recipients responded to antiviral therapy, next to five other miRNAs. Pretreatment profiles in tissue were, however, not predictive for success of antiviral therapy [65].

These identified miRNAs could serve as diagnostic methods, but more importantly, their biological function should be further investigated as this can give vital insight in the process of recurrence of HCV after LT and why its clinical course can differ considerably between recipients. These insights in biological functions will inevitably be useful in recipient and graft matching and the development of novel therapeutic strategies in order to minimize (the effects of) recurrence of HCV.

Tissue miRNA profiles and recurrence of hepatocellular carcinoma after liver transplantation

Another important recurrent disease associated with diminished patient survival after LT is HCC. The Milan criteria, often used for the selection of patients suffering from HCC in need of a LT, have been shown to be only moderately successful in the reduction of recurrence of HCC in recipients following LT [66]. Therefore, studies have been

conducted to investigate the predictive or prognostic value of miRNAs for HCC recurrence after LT.

In a study by Han *et al.*, miRNA gene array analysis in primary HCC liver samples identified 18 miRNAs that were expressed differentially in recipients who developed HCC recurrence ($n = 5$) and recipients who did not ($n = 5$). Six miRNAs with the strongest fold-change, miR-19a, miR-886-5p, miR-126, miR-223, miR-24, and miR-147 were successfully validated in 105 primary HCC samples of the same center and in 50 patients from another transplant center; especially, the combination of all six miRNAs showed high sensitivity and specificity and was demonstrated to be an independent predictor for HCC recurrence in patients transplanted within the Milan criteria as well as outside of the Milan criteria [67]. Based on this multiple-miRNA-based profile, recipients could be divided into having a low-risk signature with a better recurrence-free and overall survival compared to recipients with a high-risk signature. In addition, in another study, high levels of miR-155 in HCC tissue were demonstrated to promote cell invasion resulting in poor overall and recurrence-free survival [68].

The same research group performed further clinical and experimental studies on the correlation between miR-126 and HCC recurrence. A lower expression of miR-126 in primary HCC was associated with an increased incidence of HCC recurrence and impaired patient survival [69]. Moreover, *in vitro* and *in vivo* experiments showed that overexpression of miR-126 could inhibit HCC cell migration and invasion, thereby suppressing HCC metastasis. The involvement of several miRNAs, including miR-96, miR-139-5p, miR-126*, and miR-142-3p, in HCC recurrence was demonstrated by Sato *et al.* [70] in an elaborate study. Patients in this study were all operated within the Milan criteria but received resection as therapy for HCC instead of LT.

Based on these findings, stricter clinical and radiological follow-up can be granted in recipients identified as high-risk patients for recurrence of HCC, so that early identification of recurrence will result in earlier therapeutic intervention, probably resulting in higher quality of life and longer survival.

MicroRNAs and recurrence of other hepatic pathology after liver transplantation

Recurrence of other liver diseases after LT, such as primary sclerosing cholangitis (PSC), primary biliary cirrhosis (PBC), nonalcoholic fatty liver disease (NAFLD), non-HCV viral hepatitis, auto-immune hepatitis (AIH), and a variety of metabolic diseases could also be useful. However, in our search of the literature, currently no studies were found concerning the use of microRNAs after LT in other hepatic diseases, and thus we are unable to report on this topic in this review.

Current challenges and future applications

As discussed earlier, the analysis of miRNAs for biomarker purposes can be performed in many different biomaterials and at different stages of LT. Ideally, liver biopsies should be avoided, as they impose a risk to the patient because of their invasive nature. Much of the earlier described research, however, has used liver biopsies for identification of miRNAs as they are easier to detect in tissue. Detection of miRNAs in bodily fluids and graft perfusion fluid can be cumbersome because of the lack of generally accepted protocols for isolation, detection, and normalization, and of adequate reference genes. Further investigation on technical standards in detecting miRNAs in fluids is thus needed for discovery of new biomarkers. But most importantly, also the verification of earlier identified markers is crucial. Validation of biomarkers is critical before translation of noninvasive or minimally invasive form can be applied in the clinic and can replace existing suboptimal and/or invasive markers. Therefore, it is not expected that in short term, noninvasive diagnostics will replace liver biopsies taken for the purpose of histological assessment.

Invasive diagnostic methods, however, do not necessarily always pose a risk. Sometimes, invasively acquired material is already conveniently available because of the nature of the therapy, like tumor tissue that was collected from liver resection specimens [67,68,71]. Though invasive diagnostics in these cases do not pose an additional risk, noninvasive biomarkers could still be useful as the expected prognosis could be known beforehand, and patients be followed-up easier and noninvasively. Another complicating factor of using biopsies as a source for miRNA identification is the fact that biopsies only represent local expression instead of systematic changes. Therefore, using this technique, many interesting miRNAs could be overlooked, and this might also explain the limited overlap in identified miRNAs by the different studies. Moreover, in diseases that tend to have patchy distribution, such as ischemic cholangiopathy, the chances of a sample error are high.

As mentioned earlier, the absence of generally accepted protocols and technologies specifically designed to analyze large amounts of circulating miRNAs at once have significantly hampered research. However, novel technologies now available allow quantification of hundreds of circulating miRNAs at once in a more standardized fashion and have already lead to the discovery of many biomarkers [72–78], thereby opening new possibilities in the setting of transplantation.

Novel, noninvasive biomarkers could be used for earlier detection and treatment of disease possibly preventing the need for transplantation or used for quantifying the response of novel therapies for diseases. During

transplantation, biomarkers will aid in selecting appropriate good-quality allografts [14]. Whereas after transplantation, they could be utilized for individually tailoring the need of immunosuppression, allowing a better balance between effects (prevention of graft rejection) and side effects (long-term nephrotoxicity, infection, and malignancy) [58], or be utilized for early detection of recurrent disease.

The miRNAs discussed in the present study can not only serve as biomarkers but could also give more insight in mechanisms of several clinical entities, such as recurrence of disease or ischemia–reperfusion injury and its repair. This, however, remains difficult, as target prediction of miRNAs is achieved by *in silico* algorithms on the basis of (partial) complementarity, and one unique miRNA usually has many hundreds of potential targets. These targets need to be confirmed through *in vitro* studies, as many predicted targets do not show any regulation by the miRNA expected to regulate [79]. No technique is currently available for mass target verification, which is time consuming, and thus usually a small number of targets are selected on the basis of hypotheses. This inevitably leads to a selection bias in studies and does not give a complete picture of the biology in a certain situation. This currently makes it difficult to quickly relate a certain miRNA to a certain biological function elucidating the pathogenesis.

Another potential role for miRNAs could be their therapeutic appliance. The recent literature implicates that released miRNAs serve as a way of cell-to-cell communication and that they can trigger remote (regenerative) responses following injury and disease [21,80–85]. Studies have already demonstrated the use of antisense, anti-miRNA technology with surprising therapeutic results [11,86]. This application of miRNAs could be used not only for treatment of (recurrent) disease, but possibly also for optimizing allograft quality by treatment of grafts after organ retrieval but prior to transplantation. However, as discussed, actual regulation of targets by miRNAs cannot be calculated reliably, and one should therefore be careful that many other unwanted targets are not affected when applying the miRNAs therapeutically, which can lead to severe side effects.

Finally, when applying miRNAs for diagnostic utility, besides plasma and serum, many other noninvasively obtainable substrates, as mentioned earlier, contain miRNAs, but they have not been investigated thoroughly [14,17,18,37]. All these substrates present possible sources of noninvasive diagnostic possibilities and should be researched. All in all, miRNAs represent a very promising field not only for diagnostic but also for future therapeutic possibilities and therefore extensive research on miRNAs as biomarkers, their role in regulation and pathogenesis, and finally therapeutic appliance is justified and warranted.

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