

MicroRNAs as biomarkers for graft-versus-host disease following allogeneic stem cell transplantation

Ciprian Tomuleasa^{1,2} · Shigeo Fuji³ · Andrei Cucuianu^{1,2} · Markus Kapp⁴ ·
Valentina Pileczki¹ · Bobe Petrushev¹ · Sonia Selicean¹ · Alina Tanase⁵ · Delia Dima² ·
Ioana Berindan-Neagoe^{1,6} · Alexandru Irimie^{1,7} · Hermann Einsele⁴

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Abstract Allogeneic hematopoietic stem cell transplantation (HCT) is a well-established treatment for many malignant and non-malignant hematological disorders. As frequent complication in up to 50 % of all patients, graft-versus-host disease (GVHD) is still the main cause for morbidity and non-relapse mortality. Diagnosis of GVHD is usually done clinically, even though confirmation by pathology is often used to support the clinical findings. Effective treatment requires intensified immunosuppression as early as possible. Although several promising biomarkers have been proposed for an early diagnosis, no internationally recognized consensus has yet been established. Here, microRNAs (miRs) represent an interesting tool since miRs have been recently reported to be an important regulator of various cells, including immune cells such as T cells. Therefore, we could assume that miRs play a key role in

the pathogenesis of acute GVHD, and their detection might be an interesting possibility in the early diagnosis and monitoring of acute GVHD. Recent studies additionally demonstrated the implication of miRs in the pathogenesis of acute GVHD. In this review, we aim to summarize the previous reports of miRs, focusing on the pathogenesis of acute GVHD and possible implications in diagnostic approaches.

Keywords Stem cell transplantation · Biomarkers · MicroRNAs · Graft-versus-host disease

Introduction

Allogeneic hematopoietic stem cell transplantation (HCT) is an established treatment for many malignant and non-malignant hematological disorders. More than 30,000 HCTs are currently performed each year in the Western world [1–3]. The first allogeneic HCT was performed more than 50 years ago. Ever since, this technique is used for the treatment of various disorders that include congenital or acquired bone marrow failure, as well as hematological malignancies such as leukemias, lymphomas, or multiple myelomas [4, 5]. Cure of hematological malignancy is believed to rely on exploiting the graft-versus-leukemia (GVL) effects by allogeneic immune cells in addition to the effects by conditioning regimen [6, 7].

Many studies have used a myeloablative total body irradiation (TBI)-based preparative regimen for HCT [8, 9]. There was a high rate of regimen-related toxicity and treatment-related mortality with this approach, particularly in adults [10, 11]. The introduction of reduced-intensity conditioning (RIC) regimens has led to a decrease in regimen-related mortality and allows also treatment of older patients or patients with comorbidities [12]. Gooley et al. [13] have compared the outcomes of 1993–1997 versus 2003–2007 and reported a

✉ Ciprian Tomuleasa
ciprian.tomuleasa@umfcluj.ro

¹ Iuliu Hatieganu University of Medicine and Pharmacy, Bulevardul 21 Decembrie 1918 Nr. 73, 400124 Cluj Napoca, Romania

² Department of Hematology, Ion Chiricuta Oncology Institute, Bulevardul 21 Decembrie 1918 Nr. 73, 400124 Cluj Napoca, Romania

³ Division of Hematopoietic Stem Cell Transplantation, National Cancer Center Hospital, Tokyo, Japan

⁴ Division of Hematology, Universitätsklinikum Würzburg Medizinische Klinik und Poliklinik II, Würzburg, Germany

⁵ Department of Stem Cell Transplantation, Fundeni Clinical Institute, Bucharest, Romania

⁶ Department of Functional Genomics, Ion Chiricuta Oncology Institute, Cluj Napoca, Romania

⁷ Department of Surgery, Ion Chiricuta Oncology Institute, Cluj Napoca, Romania

statistically significant decrease in day 200 of non-relapse mortality by 60 %, as well as of the overall non-relapse mortality by 52 %, a relapse or progression of malignancy by 21 %, and an overall mortality by 41 %. Thus, a significant reduction of death related to allogeneic stem cell transplantation (SCT) and an improved long-term survival were achieved in the last decade. A review by Koh et al. identified a median non-relapse mortality (NRM) of approximately 45 % in SCT patients treated with myeloablative conditioning ($N=6$ studies) as compared with 15 % with reduced-intensity/non-myeloablative conditioning ($N=5$ studies) ($p=0.002$) [14]. In allogeneic HCT, graft-versus-host disease (GVHD) is still the main cause of morbidity and non-relapse mortality, which affects up to 50 % of patients, and accounts for up to 15 % of allogeneic HCT-related deaths [15, 16]. Acute GVHD is usually diagnosed within the first 3 months after allogeneic HCT and affects mainly the skin, with around 80 % of symptoms, as well as the gastrointestinal tract and the liver. Usually, cutaneous manifestations occur first, approximately when the leukocytes engraft. Sometimes, these lesions are even the first manifestation of a hyperacute GVHD, before engraftment. A maculopapular rash starts on the palms or soles and spreads on the whole surface of the body. In severe cases, blisters ulcerate and a toxic epidermal necrolysis mimics a Stevens-Johnson syndrome. The pathological finding of skin GVHD manifestation includes lymphocytic exocytosis, perivascular lymphocytic infiltration, dyskeratotic epidermal keratinocytes, and apoptosis at the base of crypts [17, 18]. Hepatic damage includes cholestasis due to blocking of the bile canaliculi, leading to hyperbilirubinemia. The differential diagnosis that includes a veno-occlusive disease, viral hepatitis, and drug-induced liver failure is sometimes difficult because it is often hard to obtain a specimen for the pathological diagnosis. In terms of gastrointestinal GVHD, patients complain of symptoms that include cramping, abdominal pain, hematochezia, ileus, and nausea or vomiting. Differential diagnosis should exclude *Clostridium difficile* gastroenterocolitis, cytomegalovirus enteritis, ulcers, or even post-chemotherapy enteritis. Bone marrow-related GVHD is accompanied by cytopenia, usually thrombocytopenia, as well as hypogammaglobulinemia [19–21].

Diagnosis of GVHD is usually done clinically, even though an anatomopathological confirmation is often performed. Although several promising biomarkers have been identified recently, no internationally recognized consensus has yet been established [22, 23]. Such biomarkers include trappin-2, peptidase inhibitor-3, or skin-derived anti-leukoproteinase, all elevated in dermatological GVHD [24, 25]. Patients with gastrointestinal manifestations have increased levels of regenerating islet-derived 3 α protein. Serum markers that can be useful also include IL-2 receptor alpha, hepatocyte growth factor, or even IL-8. The usefulness of these markers should be explored further in the future studies [26–29].

The Michigan group has published important data on potential biomarkers for acute GVHD that can predict treatment outcome. In a paper by Levine et al. [30], they have conducted a multicentric, four-arm, phase 2 clinical trial in which they have developed a six-protein biomarker panel correlated with the most important clinical outcomes at day 28 post-therapy non-response, as well as mortality at day 180 from onset. This paper was preceded by two other seminal papers. In one of them, published a few months before by Harris et al. [31], they have studied gastrointestinal and hepatic GVHD and observed that three biomarkers are elevated in liver GVHD. These biomarkers were regenerating islet-derived 3 α (REG 3 α), hepatocyte growth factor (HGF), and cytokeratin fragment 18, but even though the study included 954 HCT recipients, the three-marker panel was unable to distinguish GVHD from hyperbilirubinemia caused by other causes. This paper comes as a continuation of the work by Ferrara et al. [32] that correlated REG 3 α with the clinical stage and histological grade of gastrointestinal GVHD, in order to improve risk stratification of transplant patients.

Still, as these papers clearly state, all the described biomarkers so far are incomplete and inaccurate for an efficient differential diagnosis between GVHD and other conditions such as hyperbilirubinemia or even chronic inflammation from other causes. Thus, a new diagnostic panel should be proposed, based on more disease-specific and tissue-specific biomarkers. As recent years have brought microRNAs as potential building blocks of future diagnosis, these short non-coding RNAs might also be used for the differential diagnosis of acute GVHD.

MicroRNAs (miRs) are recently reported to be an important regulator of various cells, including immune cells such as T cells [33]. Therefore, we could assume that miRs play a key role in the pathogenesis of acute GVHD. Recent studies demonstrated the implication of miRs in the pathogenesis of acute GVHD [34, 35]. Consequently, in this review, we aim to summarize the previous reports of microRNAs, focusing on the pathogenesis of acute GVHD.

Overview of microRNAs

miRs are small non-coding RNAs of 19–24 nucleotides in length [36]. miRs are synthesized from primary microRNAs in two stages by the action of Drosha in the nucleus and Dicer in the cytoplasm [37, 38]. Drosha is a class 2 RNase III enzyme responsible for initiating the processing of miRs by interacting with the RNA-induced silencing complex (RISC) to induce cleavage of complementary messenger RNA (mRNA). This protein works together with Dicer, which further cleaves double-stranded RNA (dsRNA) and pre-microRNA (pre-miRNA) into short double-stranded RNA fragments called small interfering RNA and microRNA,

respectively. Up-to-date research strongly supports the role for miRs in the regulation of crucial processes such as cell proliferation, apoptosis, development, differentiation, and metabolism [39]. By extension, the role of miRs in a variety of solid cancers has been investigated [40]. These studies strongly suggest that miRs are critical regulators of cancer homeostasis, including cell cycle regulation [41], proliferation [42], invasion, and metastasis [43]. Lastly, miRs recently emerged as attractive therapeutic targets [44].

MiRs are either released by cells protected from the surrounding environment in a variety of vesicles or are associated in complexes with proteins. The package through which cells communicate with each other in the human body by using miRs is extremely important, and exosomes were the first extracellular vesicles shown to contain miRs [45–48].

Even if membrane release into the extracellular space has been known and described by Black et al. some years ago [49], only recently it has been proven that microvesicles released by cells are indicative to the cell type or its function. This statement was proven in a previously published paper by our group, in which we described that malignant liver epithelial cells contain a cell-specific microRNA-based signature, released into the human bile through exosomes [50]. These microstructures play an important role in cell-to-cell transfer and communication and are thought to have a key role in both physiological and pathological processes, being found in all body fluids and binding only to selected targets [51]. There are various types of such vesicles, based on their different origin, biogenesis, and function. Endosomes and lysosomes undergo a physiological process similar to exocytosis. This will lead to the insertion on the plasma membrane of certain receptors that are also found in the endoplasmic reticulum [52]. Two different intracellular regions are considered to be the main sources of vesicle biogenesis: the cell plasma membrane blebs including the lipid rafts, as well as the network of endosomal reticulum [53, 54].

Based on their size, the major vesicle populations may be exosomes, microvesicles, or apoptotic bodies [55]. Extracellular vesicles are depicted as unique “messenger” used in cell-to-cell communication and mediate the trafficking of various molecules that are traditionally regarded as either insoluble or cell-associated. Such molecules include various membranes, cytoplasmic or nuclear proteins, as well as nucleic acids [56]. Various researchers worldwide have isolated RNA from circulating cancer cells [57] or from body fluids [58]. If we consider that RNA is easily degraded and has a short half-life if unprotected in serum [59], we could deduce that the majority of the cell-free RNA species is harbored in different exosomes or similar microvesicle fractions. miRs may also be encapsulated by protein complexes in the blood [60, 61]. The vesicles are very stable and protect the cell-free RNA even when stored in the freezer for many years and give researchers considerable benefits when performing retrospective analyses.

MicroRNA biogenesis and function

The importance of RNA molecules in regulating gene expression was first suggested by Jacob and Monod decades ago [62], but for a long time, this natural process was limited to the control of mobile elements such as plasmids or transposons and isolated endogenous bacterial small RNAs in *Escherichia coli*. A major step forward was done when the first identification of miRs in nematodes [63] in the 1990s, and since then, more than 1500 miRs have been described, being estimated to regulate the expression of more than half of the human genome [64–67].

Most genes that encode miRs are located in introns and share promoters as well as other regulatory loops with exons [68, 69], but some of them are located between protein-coding genes, having their own promoter, and in this way are able to form clusters of miR genes organized in polycistronic primary transcripts [70]. These transcripts are generated either by RNA polymerase II or III [71, 72] before this transcript is processed by Drosha and its cofactor DGCR8 in the nucleus of the cell and formed a 60–90-nucleotide stem-loop structure named pre-miRNA [73]. Afterwards, the pre-miR is transported by exportin-5 to the cytoplasm using RanGTP and further processed by the ribonuclease III endonuclease Dicer [74, 75]. This is how a 20–23-base RNA complex appears. At this point, the strand could be either incorporated into the RNA-induced silencing complex or degraded [76]. Dicer functions by releasing the double-stranded miR product [77, 78], and often, only one strand of miR is incorporated into a multicomponent protein complex, called the RISC, which contains the very important Argonaute proteins [79]. These proteins have key functions in RNA interference and different miR pathways [80] and form together with a single-stranded small RNA the core of RISC, thus regulating embryonic development, cell differentiation, and stem cell maintenance [81]. A RISC-connected miR may bind to the 3'-UTR of the cognate mRNA and then coordinate the cleavage or the translational repression of various target mRNAs. In this way, it is provided the post-transcriptional control of gene expression. The synthesis and function of microRNAs are depicted in Fig. 1.

Being evolutionarily conserved among remotely related species and being essential in cell physiology [82], it can repress gene expression by decreasing the levels of mRNA or it may act as a mediator through deadenylation, decapping, or by exonucleolytic digestion of the messenger RNA [83, 84]. Recent data has proven that miRs themselves undergo regulation via post-transcriptional modification. One such example is the altering of specificity as adenosine deaminases acting on RNA (ADARs) are able to catalyze adenosine-to-inosine transitions in dsRNA substrates, a process also known as RNA editing [85]. Because inosine behaves just like guanosine and base pairs with cytidine, the editing process may alter base pairing specificity. One important reference to

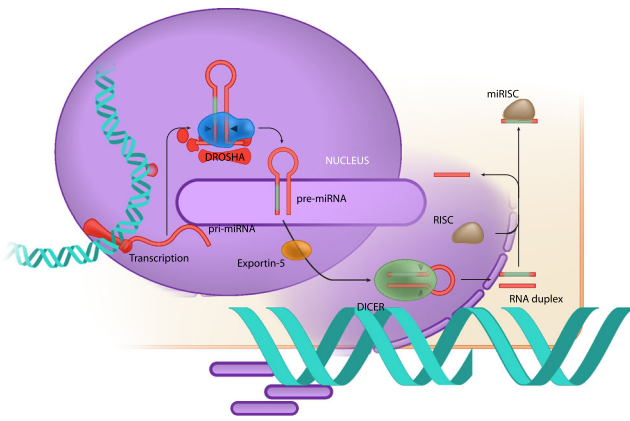


Fig. 1 MicroRNA synthesis. Genes that encode miRs are located in introns. The transcripts are generated either by RNA polymerase II or III before processing by Drosha and its cofactor DGCR8 in the nucleus of the cell. Afterwards, the pre-miR is transported by exportin-5 to the cytoplasm using Ran GTP and further processed by the ribonuclease III endonuclease Dicer. Afterwards, the RNA-induced silencing complex (RISC)-connected small non-coding RNA binds to the 3'-UTR of the cognate messenger RNA and then coordinates the cleavage or the translational repression of various target messenger RNAs

support the above statements is the paper of Yang et al. [86], who have proven that editing of pri-miR-142 is able to inhibit Drosha cleavage, raising a series of questions about the link between editing and the processing of some microRNAs.

MicroRNA dysregulation in leukemia

Before the discovery of miRs, tumorigenesis was thought to be caused by the alteration of protein-coding oncogenes and of tumor-suppressor genes [87, 88]. Once identified in B cell chronic lymphocytic leukemia (CLL), miRs' function as either oncogenes or tumor-suppressor genes was confirmed in different types of malignancies [89–92]. Usually, miR can change cancer cell's behavior because it alters the levels of gene expression for both mature and precursor gene sequences when compared with the corresponding healthy tissue [93]. In a tumor, miR global expression profile can be influenced by three mechanisms. The first mechanism is when a miR acts in a cancer-associated genomic lesion [94] such as loss of heterozygosity in a tumor-suppressor gene or amplification in an oncogene. The second mechanism is the epigenetic dysregulation of miR expression, such as DNA hypomethylation, CpG hypermethylation, or histone modification [95–97]. The third mechanism is related to the abnormalities in miR processing genes or proteins. A failure of Drosha processing step may explain the downregulation of miRs in primary tumors [98] and is linked to a reduced post-operative surgical and low tumor differentiation grade, all leading to a bad prognosis [99, 100]. miR species interfere with the phenotype and behavior of the cancer cell by influencing its cell cycle,

invasion and metastasis potential, as well as the stem-like tumor-initiating pool.

For example, chronic myelogenous leukemia (CML) is overwhelmingly caused by a common cytogenetic abnormality, with over 95 % of cases having a balanced translocation t(9;22)(q34;q11) known as the Philadelphia chromosome, which produces a chimeric BCR-ABL protein with tyrosine kinase activity. CML is associated with upregulation of the MYC-regulated miR cluster miR-17-92 through an indirect and unknown mechanism. Also, CMLs express lower levels of miR-10a, miR-150, and miR-151 and elevated levels of miR-96 [101]. Bueno et al. showed that miR-203, which directly targets BCR-ABL for downregulation, is lost in many cases of BCR-ABL-related leukemias by genomic alteration instability and CpG methylation, which potentially disables a key negative feedback pathway [102]. Their results were confirmed by San Jose-Eneriz et al., who have found a distinct miR signature in analyses of 8 patients with imatinib-resistant CML, with 18 downregulated and 1 upregulated miRs [103]. In the case of acute lymphocytic leukemias (ALL), including T- or B-lineage ALL, various cytogenetic features have been reported such as t(12;21), t(8;14), t(2;8), or t(8;22). Cytogenetics also includes MYC-related translocations, TAL1, t(1;19) E2A-PBX, 11q23 translocations (MLL), or t(9;22) translocations (BCR-ABL) [104, 105]. Zanette et al. first characterized miR expression abnormalities in ALL by pooling samples of 7 patients and grouping the abnormalities according to the cell lineage compared with CD19+ normal cells [106]. The miR-17-92 cluster was upregulated to a lesser extent and was shown to repress E2F1 in T-lineage ALL. Mi et al. later on have performed a high-throughput bead-based profile comparing B-lineage ALL to AML and normal bone marrow cells (mononuclear and CD15+) [107]. They have compared the miR signatures of 18 ALL samples, all with 11q23 translocations, against 54 AML samples that had a broad spectrum of cytogenetic abnormalities and found differential expression of 27 miRs in ALL, with 6 upregulated and 21 downregulated. These reports confirmed that miR-128a/b is upregulated, attributed to epigenetic dysregulation of CpG island methylation.

Fulci et al. have also attempted to discriminate between T cell lineage and B cell lineage in ALL [108] and did a clustering analysis of several adult ALL subgroups (B- or T-ALL without known molecular abnormalities or those involving E2A, BCR-ABL, or MLL-AF4). Unsupervised cluster analysis showed distinct expression patterns for each subgroup, whereas a supervised cluster analysis showed that several genes were differentially regulated between T-ALL and B-ALL. T-ALL was characterized by miR-148, miR-151, and miR-424 overexpression, whereas B-ALL was characterized by miR-425-5p, miR-191, miR-146, miR-128, miR-629, and miR-126 overexpression. All this data suggests that miR signatures that are distinctly expressed in different ALL

subtypes, but the relevance of these classifications to prognosis also remains to be determined.

Several high-throughput studies have classified acute myeloid leukemia (AML) according to cytogenetic abnormality [109, 110]. Jongen-Lavrencic et al. analyzed 215 AML samples by multiplex real-time PCR for 206 miRs [111]. They have distinguished clustering of miRs between different cytogenetic subgroups and molecular aberrations, whereas supervised analysis revealed broad signature patterns for cytogenetic abnormalities and common mutations. Garzon et al. used microarrays to analyze 122 pre-treatment AML patients and validated their results with an additional 60 patients. Signatures were also developed for abnormal karyotype and normal karyotype AML, and an overall survival and event-free survival were shown to negatively correlate with high levels of miR-199a and miR-191. Promyelocytic leukemia-retinoic acid receptor α (PML-RAR α) is another common molecular abnormality in AML, specifically acute promyelocytic leukemia (APL) as a result of the t(15;17)(q22;q12) [104]. Currently, AML with PML-RAR α fusions are treated with all-trans-retinoic acid and arsenic trioxide to induce differentiation by dissociating PML-RAR α from repressive histone deacetylase (HDAC) complexes and promoting PML-RAR α degradation. Jongen-Lavrencic et al. described a miR signature for t(15;17) characterized by upregulation of miR-382, miR-134, miR-376a, miR-127, miR-299-5p, and miR-323 [112].

Although cytogenetically abnormal AMLs occur in a sizable percentage of cases, approximately half of all AMLs are cytogenetically normal [113]. Common mutations that affect both cytogenetically normal and abnormal AMLs add an additional layer of complexity. Blum et al. examined normal karyotype AML (NK-AML) against abnormal karyotype AML to discern a signature that is common among NK-AMLs but does not distinguish between NK-AML and AMLs with defined cytogenetic abnormalities [114]. In a seminal paper, Marcucci et al. examined the miR expression profiles of high-risk cytogenetically normal AMLs with FLT3-ITD mutations and/or NPM1 mutations. In their study, patient samples were divided into a training group, which provided the initial signature, and a validation group of similarly characterized patient samples. miR-181a/b correlated with favorable outcomes, whereas miR-124, miR-128-1, miR-194, miR-219-5p, miR-220a, and miR-320 correlated with poor outcomes [115].

This hypothesis is just now starting to be investigated in various hematological transplant centers, but the proof-of-concept is similar in transplant immunology. In addition, the role of miRs is being assessed in the transplantation of solid organs. Zhang et al. [116] have used a cohort of 18 lung transplant patients and have used a whole-genome gene profiling in order to explore the role of the very same miRs and find any potential new biomarkers. This is because in the case of lung transplant, the 5-year post-transplant survival is very

low due to chronic rejection. The American group has proven that a large number of genes are dysregulated post-transplant and confirmed that scientists are on the right track in order to come up with new therapeutic targets, as well as diagnostic tools for transplant complications. Kidneys are transplanted very often in Europe, the USA, and Japan. Wilflingseder et al. [117] have used epigenetics in order to investigate acute cellular rejection, antibody-mediated rejection, and delayed graft function in 65 patients. The Austrian physicians have found that these mechanisms can be discriminated from the control group of protocol biopsies through the use of serum miRs. miRs were reported to be involved in angiogenesis, apoptosis, and transforming growth factor (TGF)- β signaling, all relevant in post-transplant organ ischemia.

MicroRNA dysregulation in GVHD

MiRs are dysregulated in a wide variety of diseases, including GVHD and autoimmune diseases, especially in systemic lupus erythematosus (SLE), rheumatoid arthritis (RA), and multiple sclerosis (MS). As most autoimmune disease are female-predominant, sex hormones are considered to be involved in miR response to inflammation, as estrogen has been proven to link miRs to both T cells and B cells. Some studies examined miR expression in RA synovial tissue and fibroblasts. Stanczyk et al. reported increased miR-155 and miR-146a expression in RA synovial fibroblasts compared to those in osteoarthritis (OA) patients [118]. Furthermore, miR-155 expression was higher in RA synovial tissue compared to OA synovial tissue. Interestingly, miR-155 expression was higher in RA synovial fluid monocytes compared to RA peripheral blood monocytes. Enforced expression of miR-155 in RA synovial fibroblasts revealed matrix metalloproteinase 3 (MMP-3) as a potential target of miR-155, suggesting that miR-155 may modulate downstream tissue damage.

The regulation by miRs of human genes has an enormous potential, as the identification of various miR expression patterns in autoimmune diseases and an accurate understanding of their role in disease pathogenesis offers not only the potential to develop new molecular diagnostic markers but also the possibility of new gene therapy strategies for treating SLE as well as other inflammatory autoimmune diseases [119–122].

In a recently published paper on the mechanism of action of regulatory T lymphocytes in the prevention of GVHD, Liu et al. [123] have used a xenogenic GVHD murine model and have proven that miR-15a/16 were important in modulating the suppressive function of regulatory T cells via inhibition of forkhead box P4 (FOXP3) and cytotoxic T lymphocyte A4 (CTLA4) expression. The intervention to control the level of miR-15a/16 might be a way to control the level of FOXP3, which leads to an improvement of GVHD. The exact mechanism of action of umbilical cord blood (CB)-derived

regulatory T cells (Tregs) in the prevention of GVHD remains unclear, but the p53 pathways have been shown to be correlated to the regulation of miR-15a/16 expression. In order to explore the clinical relevance of miRs in T cell function, Xie et al. [124] have recruited 64 patients who underwent allogeneic SCT. Twenty five of them were diagnosed with acute GVHD, 20 of them with chronic GVHD, and 19 of them did not develop this illness at all. Real-time RT-PCR was used as a tool to assess the expression of miR-155, miR-214, and miR-326. miR-155 was found to be upregulated in acute GVHD, in correlation with higher levels of interferon (IFN) gamma, IL-9, and IL-17, suggesting that these miRs might be used as a biomarker for acute GVHD diagnosis, and be a potential target of acute GVHD treatment. From the candidate miRs of the two-arm study (acute GVHD and chronic GVHD), miR-214 and miR-326 showed no significant difference regarding GVHD between the investigated groups, but miR-155 correlated with the severity of acute GVHD, but not with chronic GVHD. Thus, miR-155 might be a possible tool for a targeted therapy of acute GVHD.

Until recently, most of the work has focused on proteins and polypeptides as potential and specific biomarkers to determine GVHD, as shown by the work of Weissinger et al. [125, 126], as well as by the data of Rezvani et al. [127] or of Shaiegan et al. [128]. However, with the discovery of miRs, the attention was directed towards them. Since miR expression is disease-specific, it might be easy to differentiate between normal and a certain disease type. Several microRNA species are downregulated in a certain tissue, but then get upregulated in case that specific tissue will develop a malignancy. This is the case of miR-21, that is, differentially expressed in gastric cancer in comparison with the normal stomach tissue [129]. We have confirmed this hypothesis by proving that miR-21 inhibits the tumor suppressor effects of programmed cell death protein 4 (PCDP4) and phosphatase and tensin homolog (PTEN) [130].

A specific plasma signature of miRs for acute GVHD was determined by Xiao and coworkers after analyzing the blood of 196 patients that underwent allogeneic HCT. In the plasma of patients with acute rejection and healthy donors, a difference between the expression of six miRs was found. Among these miRs, the level of miR-423 was higher, suggesting that it is related to the development of acute GVHD, by being involved in the regulation of inflammation [131]. A series of miRs were identified to be significantly expressed higher in the blood of acute GVHD patients compared to the ones that underwent liver or lung transplant, therefore demonstrating that miR expression is disease- or organ-specific. One third up to half of the SCT patients develop GVHD, and current therapy is, unfortunately, often very unsuccessful because of the severity of the disease. Thus, finally, diagnosis of GVHD II or a diagnosis before the onset of clinical symptoms would be of great help in the clinic. In the abovementioned paper of

Xiao et al., a four-miR-based diagnostic panel predicts the probability of acute GVHD with an area under the curve of 0.80. This signature was not identified in the plasma of lung transplant or sepsis non-transplant patients, but is useful in the prediction or early diagnosis of GVHD, as well in its staging.

MiR-155 was also associated with poor prognosis in patients with acute GVHD, and it was shown to be involved in the upregulation of the tumor necrosis factor α (TNF- α) which is a cytokine associated with the severity of acute GVHD, the activation of T cells, and the ongoing inflammation and tissue damage [33]. Therefore, miR-155 is considered to play an important role in the pathogenesis and modulation of GVHD. It has a high potential in being used as a biomarker. By studying the molecular function and miR-mRNA network relation of miR-155 in regulating gene expression, we can get novel information on the pathophysiology of acute GVHD, thus proving to be a promising tool in developing novel therapeutic targets of GVHD and changing the outcome of HCT.

Xiao et al. [131] have shown that miRs are important in the function of T cells. Other investigators, as is the case of Wang et al., confirm this data on severe acute GVHD in patients that have undergone SCT for Fanconi anemia. They focus on miR-34a for an early diagnosis of apoptosis in the target epithelial cells in the detriment of more classic hallmarks of apoptosis, as TP53 [132]. In the case of miR-34a, its expression was studied for patients that have undergone SCT for Fanconi anemia and was upregulated in the gut of these subjects with grades II–IV in comparison with the ones with grades 0–I. In the case of microRNA-155, it is upregulated in allo-stimulated T lymphocytes and has an important role in regulating the immune response. This miR is also upregulated in intestinal biopsies from GVHD and may potentially be used as a biomarker to early detect intestinal GVHD.

There is currently no specific microRNA pattern for each individual disease even if physicians around the world are investigating the role of each non-coding RNA. Thus, it is expected that in the following few years that a diagnostic panel based on microRNAs is to be proposed and afterwards validated in the clinic. For GVHD, a miR-based panel that can be used for various transplantation protocols would be of great help as it would allow physicians to choose the best treatment in a more individualized manner. Following transplantation, a potential GVHD could be diagnosed before the clinical symptoms appear or at a very early stage. Thus, therapy could be introduced easier and the morbidity and mortality associated with a SCT be reduced. Such a panel, which might include various miRs like miR-155, miR-214, or miR-326, would also be of much help in the case of older individuals that undergo transplantation. Elderly patients are in a more delicate situation, due to their increased comorbidities. But, if a miR-based panel for GVHD would be available, SCT could be TRM of allo-SCT, could potentially be reduced, and could have better outcomes.

Table 1 MicroRNAs dysregulated in leukemia and their respective targets and functions to autoimmune disorders

MicroRNA	Function	Disease	Reference no.
miR-17-92	Upregulated by the MYC cluster	CML	102
miR-203	Dowregulates BCR-ABL	CML	103
miR-17-92	Represses E2F1	ALL	107
miR-382, miR-134, miR-376a, miR-127, miR-299-5p, miR-323	Signature for t(15;17)+APL	APL	113

In the clinic, all these potential benefits this would mean the difference between the success of a transplant and failure, as we could act in a more individualized patient-tailored transplant procedure in accordance to the molecular and epigenetic profile of each case, not according to various physical parameters, that are sometimes misleading.

In a recently published paper by Leonhardt et al. [133], it has been proven that enhanced neoangiogenesis and α v integrin expression correlated with the severity of GVHD in humans. The expression analysis of miRs in the inflamed intestinal tract in mice identified miR-100 as being significantly downregulated, as this microRNA may play a role in blocking inflammatory neovascularization. The main assay was the in vitro cytotoxicity assay, for which effector T lymphocytes were isolated from BALB/c recipients treated with cilengitide or phosphate buffer solution (PBS) as control on day 14 after allo-HCT by depletion of non-CD3⁺ T cells. Afterwards, the cells were cocultured for 6 h at 37 °C with L1210 target cells at the indicated ratio of target/effector cells. Staining was done with annexin V-fluorescein isothiocyanate/propidium iodide (PI), and cytotoxicity was analyzed by flow cytometry. Furthermore, Stickel et al. [134] have investigated the role of miR-146a in modulating the TNF/TNF receptor-associated factor 6 (TRAF6) axis in GVHD. They have found that miR-146a is a negative regulator of donor T cells in GVHD by targeting TRAF. In a paper by the same group,

Stickel and Zeisser [135] have stated that miR-423, miR-199a-3p, miR-93, miR-377, miR-155, and miR-30a are the six miRs most important in the plasma of GVHD patients and could potentially be used for the differential diagnosis of these cases. Thus, especially miR-423, miR-199a-3p, miR-93, and miR-377 could be used to predict the probability of a patient developing GVHD, as the increase in the amount of these potential biomarkers can be documented approximately 16 days before clinical symptoms of GVHD develop. Xie et al. [136] have showed that miR-155 was also elevated in the plasma of patients with GVHD, whereas Wang et al. [137] have identified miR-34 as the candidate biomarker. Ranganathan et al. [33] have found miR-155 to be elevated in the gastrointestinal tract of GVHD patients, in comparison with their normal counterparts, where this miR is almost absent. The functional analysis described by this group used miR-155-deficient murine T cells that once transplanted and caused a less strong GVHD. This was associated with a low expression of CCR5, CCR4, and S1P1, leading to a less intense migration of T cells.

In autoimmune diseases, a conditional deletion of Dicer influences the normal development of both B and T lymphocytes, and miR-181a plays an important role in the positive selection of T lymphocytes in the thymus, thus preventing autoimmune reactions, as proven by Bernecker et al. [138]. MicroRNA-155 and microRNA-326 are also of key

Table 2 MicroRNAs potentially involved in GVHD and au

MicroRNAs	Function	Pathology	Reference no.
miR-15a/16—murine	Modulates suppressive function of Tregs via inhibition of FOXP3	GVHD	124
miR-15a/16—murine	Inhibits CTLA4 expression on cytotoxic T lymphocytes	GVHD	124
miR-155	Correlated with higher levels of IFN gamma, IL-9, and IL-17	Acute GVHD	125
miR-155	Upregulation of TNF- α	GVHD	133
miR-155	Low expression of CCR5, CCR4, and S1P1	GVHD	33
miR-21	Inhibits the tumor-suppressor effects of PCPD4 and PTEN	GVHD	131
miR-423	Regulation of inflammation	GVHD	132
miR-100	Blocks inflammatory neovascularisation	GVHD	136
miR-146a	Negative regulator of T cells by targeting TRAF	GVHD	137
miR-155	Targets MMP-3 and regulates downstream tissue damage	Collagen diseases	119
miR-155, miR-326—murine	Differentiation of T helper 17 subtype	Autoimmune disorders	141
miR-146	Control of T helper 1 response	Autoimmune disorders	142
miR-181	Positive selection of T lymphocytes	Autoimmune disorders	138

importance as they are involved in the differentiation of T helper cell subtypes and upregulated in multiple sclerosis [139]. In an experimental mouse model of encephalomyelitis, a correlation between these upregulated miRs and Th17 differentiation was demonstrated. In the review of Picascia et al. [140], it is assumed that epigenetic alterations play an important role in the initiation and progression of autoimmune diseases, even though the exact mechanism is still unclear. The authors emphasize the need for further knowledge in the field, as these basic mechanisms of cell fate regulation may provide the tools to control or prevent autoimmune diseases through the use of various targeted drugs. Another potential important small non-coding RNA molecule is miR-146, a negative regulator of T cell activation that is upregulated in Tregs which control the Th1 response [141]. MicroRNA 146a is shown to play an important role in inflammatory signaling, as its expression is strongly induced following challenge of cells with bacterial endotoxin. The prolonged expression has been linked to immune tolerance, implying that it acts as a fine-tuning mechanism to prevent an overstimulation of the inflammatory response [142]. Stem cell transplantation has been the treatment of choice for several other conditions of autoimmune pathophysiology, including MS. Arruda et al. have performed in São Paulo autologous stem cell transplantation for MS patients and have investigated the role of short non-coding RNAs as potential biomarkers for post-transplantation follow-up. The Brazilian physicians have found an increase in exhausted PD-1+ T cells and of suppressive CD8+CD28–CD57+ T cells. CD4+ and CD8+ T cells from MS patients had upregulated miR-16, miR-155, and miR-142-3p and downregulated FOXP3, FOXO1, PDCD1, and IRF2BP2. After transplantation, the expression of FOXP3, FOXO1, PDCD1, and IRF2BP2 increased.

The expression of miR-16, miR-155, and miR-142-3p decreased towards normal levels at 6 months post-therapy, remaining downregulated until the end of follow-up [143]. These miRs, alongside miR-155, have also been found to be overexpressed in different immune cells, as well as in the serum of rheumatoid arthritis [144].

The microRNAs relevant to hematologic malignancies and GVHD are summarized in Tables 1 and 2.

Conclusions

In many cases, diagnosis of diseases with a potential fatal outcome, including graft-versus-host disease, is too late or a very efficient treatment fails due to the lack of specific biomarkers to initiate or monitor treatment. This is also the case for GVHD or disease recurrence after allogeneic HCT. Early detection of all these complications is essential to improve the clinical outcome in patients with GVHD, as grades I–II GVHD have a much better prognosis than grades III–IV.

Further studies to assess the impact of miR dysregulation on the pathogenesis of GVHD are warranted, and prospective studies in which GVHD is treated following the status of microRNAs should be conducted to demonstrate that monitoring of miRNAs to initiate and modify GVHD treatment should be evaluated in prospective trials. miRs are emerging as a promising tool available for the early diagnosis of different diseases, ranging from acute myocardial infarction to sepsis or to various malignancies.

Thus, as GVHD is the main cause of HCT morbidity and mortality, miRs should be evaluated as potential biomarkers for an early diagnosis and thus help to improve the overall survival of patients undergoing SCT.

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