Biol Blood Marrow Transplant 25 (2019) e375–e386

Biology of Blood and Marrow Transplantation

journal homepage: www.bbmt.org

Review

MicroRNAs as Potential Diagnostic, Prognostic, and Predictive Biomarkers for Acute Graft-versus-Host Disease

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Article history:
Received 15 June 2019
Accepted 6 August 2019

Keywords:
aGVHD
MicroRNAs
Biomarkers
Prognostic
Predictive

A B S T R A C T

Successful treatment of various hematologic diseases with allogeneic hematopoietic stem cell transplantation is often limited due to the occurrence of acute graft-versus-host disease (aGVHD). So far, there are no approved molecular biomarkers for the diagnosis and prediction of aGVHD at the clinical level due to our incomplete understanding of the molecular biology of the disease. Various studies have been conducted on animal models and humans to investigate the role of microRNAs in aGVHD pathogenesis to implicate them as biomarkers and therapeutic targets. Because of their high stability, tissue specificity, ease of measurement, low cost, and simplicity, they are excellent targets for biomarkers. In this review, we focused on microRNA expression profiling studies that were performed recently in both animal models and human cases of aGVHD to identify diagnostic and predictive biomarkers for this disease. The expression pattern of microRNAs can be specific to cells and tissues. Because aGVHD affects several organs, microRNA signatures in target tissues may help to understand the molecular pathology of the disease. Identification of organ-specific microRNAs in aGVHD can be promising to categorize patients for organ-specific therapies. Thus, microRNAs can be used as noninvasive diagnostic tests in clinic to improve prophylaxis, predict incidence and severity, and reduce morbidity.

INTRODUCTION

Allogeneic hematopoietic stem cell transplantation (allo-HSCT) or allogeneic bone marrow transplantation (allo-BMT) is the only effective treatment for many malignant and non-malignant hematologic diseases. Despite the widespread use of this technique, successful hematopoietic stem cell transplantation (HSCT) treatment is possible when HLAs are fully matched between recipients and donors [1]. Only one third of transplantations occurs between HLA-matched siblings, and the rest is performed between individuals who are HLA-matched unrelated [2].

Graft-versus-host disease (GVHD) is the most common cause of post-transplantation mortality and morbidity, which occurs in 70% and 30% to 50% of allo-HSCT recipients in matched-unrelated and matched-related donors, respectively [3]. Historically, if GVHD occurred during the first 100 days of allo-HSCT, it had been called acute GVHD (aGVHD), and after 100 days, it was known as chronic GVHD (cGVHD). In 2005, based on the National Institutes of Health consensus, patients with GVHD were reclassified according to clinical manifestation instead of time. Accordingly, the aGVHD category is defined in the absence of a diagnostic or distinct characteristic of cGVHD and includes classic aGVHD occurring within the first 100 days of the transplant and another new class, late aGVHD, which occurs after 100 days with aGVHD characteristics observed. cGVHD consists of 2 categories: classic cGVHD, in which there are no aGVHD characteristics, and cGVHD overlap, in which the aGVHD and cGVHD characteristics appear together [4]. They have distinct pathologic processes. The incidence of aGVHD and cGVHD in HSCT recipients is 50% and 70%, respectively [5,6].

Donor immune cells may be the cause of GVHD by identifying and attacking the host tissues such as liver, skin, and gastrointestinal (GI) tract in immunocompromised allogeneic recipients. Thus, aGVHD is characterized by the death of epithelial cells in the skin, liver, and GI tract. Donor allogeneic T cells target tumor cells and organ recipients [7]. Tissue damage

Financial disclosure: See Acknowledgments on page e384.

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https://doi.org/10.1016/j.bbmt.2019.08.004
1083-8791/

increases the release of inflammatory cytokines, such as IL-1, IL-6, and TNF-α, resulting in the activation of antigen-presenting cells (APCs). APCs include host hematopoietic cells such as B cells, dendritic cells, Langerhans cells, nonhematopoietic cells, and donor hematopoietic cells [8]. Host hematopoietic cells are likely to play the biggest role in initiating aGVHD. Following the antigen presentation by APCs along with costimulatory signaling, donor T cells become active. Active and differentiated T cells (effector T cells) migrate to target tissues in GVHD and cause tissue destruction and damage through cellular processes (perforin/granzyme) or inflammation (IFN-γ, TNF-α, and IL-1) [3]. The acute form of GVHD is thought to be mainly mediated by Th1 and the immune response Th17, whereas its chronic form is mediated mainly by Th2-type responses [7]. The pathogenesis of cGVHD depends on the polarization of $CD4^+$ T cells toward Th1, Th2, Th17, and follicular helper T cells [6].

Innate immunity is responsible for the onset of aGVHD. The tissue damage in the GI tract causes release of lipopolysaccharides, which activate the innate immune receptor, including Toll-like receptors (TLRs), and produces inflammatory cytokines such as IL-1, TNF-α, and IL-12 [9]. Both donor $CD4^+$ T cells and donor $CD8^+$ T cells play an important role in the pathogenesis of GVHD. It is feasible that $CD8^+$ T cells are primarily activated by recipient hematopoietic APCs, whereas $CD4^+$ T cells can also be activated by nonhematopoietic cells in the GI tract [7]. The diagnosis of aGVHD, especially in early stage, is crucial to prolong the patients' survival rate. Unfortunately, there has been little progress reported in the treatment of patients with steroid-refractory aGVHD, and thus the outcome is poor [10].

Biomarkers can be used effectively in noninvasive diagnosis, prognosis, and therapeutic guidance along with identifying complications after allo-HSCT. According to the National Institutes of Health consensus, biomarkers are classified into 4 categories: diagnostic, prognostic, predictive, and response to treatment [11]. Diagnosis and prognosis in aGVHD depend on the presence of clinical symptoms and pathologic findings, and no distinct laboratory tests predict the risk of aGVHD, patient survival, and response to treatment [12].

PROTEIN BIOMARKERS IN ACUTE GVHD

Numerous genomic and proteomic biomarkers affect the risk of aGVHD and cGVHD. The most common genetic variations in humans are single-nucleotide polymorphisms (SNPs). Thus far, several SNPs have been identified in various genes such as *IL-6*, *UDP-glucuronosyltransferase 2B17*, *TNF-α*, *IFN-γ*, *IL-23 receptor*, nucleotide-binding oligomerization domain-containing protein 2 (*NOD2*), and *IL-10*, which could affect the risk of GVHD [13-15]. Increased expression of *TNF-α* in lethal aGVHD has been reported in both humans and mice. *TNF-α* SNP (rs361525G>A) has increased the mortality risk in 30% of patients with grade II to IV aGVHD [16]. Increased *TNF-α* level during transplant and early post-transplantation activates host macrophages and stimulates donor cells in aGVHD [17].

In a recent study by Hyvarinen et al. [18], 122 SNPs related to GVHD were studied in 492 HLA-matched sibling HSCT donor-recipient pairs from the population of Finland and Spain after transplantation. Despite the heterogeneity of SNPs in the 2 populations, most GVHD-related markers, including *TNF*, *IL-10*, *NOD2*, *IL-23R*, *IL-1*, and *TLR9* genes, played a remarkable role in host responses to microbial antigens. The researchers that the functional effect of these SNPs could regulate expression of *IFN-γ*, *IL-1β*, and *IL-6* cytokines.

Various protein biomarkers have been studied for aGVHD, and nearly all have been validated (Table 1). Biomarkers may have putative physiologic functions such as innate immune activation (TNF-α, IL-6, and *CRMF44*), T cell-mediated cytotoxicity (IL-33, IL-2ra, TNF-R1, IL-8, IL-7, and suppressor of tumorigenicity 2 [ST2]), endothelial damage (angiopoietin 2 [Ang2], vascular endothelial growth factor (VEGF)-A, ST2, Ang2/VEGF ratio, and regenerating islet-derived 3 α [REG3α]), dysbiosis (*Picobirnavirus*), and poor wound healing (REG3α, hepatocyte growth factor, microRNAs, calprotectin, and REG3α) during pretransplant or before or after aGVHD onset [19]. Donor T cells are responsible for tissue damage in the host. Some host factors, such as host-circulating IL-33, affect the activation of donor T cells. Thus, IL-33 could be a predictive biomarker for aGVHD in a pretransplant stage [20]. ST2, the receptor of IL-33, as a prognostic biomarker at the onset of aGVHD treatment has strong association with steroid-resistant patients [21].

Porkholm et al. [22] showed that in pediatric patients, the serum level of Ang2 and VEGF correlated with clinical status. The level of Ang2 in both pre- and post-transplant was associated with intestinal aGVHD and skin aGVHD, respectively, whereas the level of VEGF in pre- and post-transplant was associated with skin and intestinal aGVHD, respectively. Citrulline is an amino acid produced by small intestine enterocytes with no hepatic metabolism. The low level of citrulline at pretransplant is associated with high risk of grade II to IV aGVHD [23]. REG3α protein is an antimicrobial agent secreted by Paneth cells against a gram-positive bacterium. In 1 study, the level of REG3α, cytokeratin fragment 18, and hepatocyte growth factor in lower gastrointestinal (LGI) GVHD were compared with non-GVHD diarrhea. Although the concentration of all 3 markers was significantly higher in LGI GVHD, REG3α distinguished LGI GVHD from non-GVHD diarrhea better than the 2 other markers [24].

There are several drawbacks to the application of protein biomarkers at the clinical level in aGVHD. First, increasing the level of these biomarkers often occurs during inflammation as a result of bacterial infections and sinusoidal obstruction syndrome [25,26]. Studies on protein biomarkers often exclude other inflammatory factors in patients [27]. An increase in the level of soluble interleukin-27 receptor α in patients with aGVHD, sinusoidal obstruction syndrome, or sepsis has been reported [25]. Therefore, it is not clear that the increase in soluble interleukin-27 receptor α level is due to infection or aGVHD. Xiao et al. [27], by measuring the levels of microRNA signature in aGVHD after HSCT, showed that the difference in microRNA expression was related to aGVHD, which could not be identified in other conditions, such as sepsis. Second, the methods for identifying protein biomarkers, compared with quantitative PCR, have a higher cost, with more complexity and lower sensitivity [26].

MICRORNAS

MicroRNAs (miRNAs), with a length of approximately 22 to 25 nucleotides, are small noncoding RNAs that, by binding to 3′ untranslated regions in the target mRNA, regulate gene expression at the post-transcriptional level. MicroRNAs play an important role in many diseases, such as GVHD [39]. Recently, miRNAs have been identified in body fluids as noninvasive biomarkers in several diseases, such as GVHD [40]. Although proteins are more informative than miRNAs, but they are more stable in body fluids, with less complexity. Besides, they are tissue specific and can be easily measured by using quantitative PCR techniques [12].

So far, many miRNAs have been reported as GVHD biomarkers, with a high heterogeneity (Table 2). Several factors have been identified to explain the heterogeneity of miRNAs,

Table 1
Protein Biomarkers for aGVHD

Name	Biomarker Type	Role in aGVHD	Reference
Plasma biomarkers			
ST2	Predictive	Patients with ST2 values had treatment-resistant GVHD.	[21]
	Prognostic	Increase on day 7 after HCT was associated with high risk for lethal GVHD and NRM.	[28]
sTNFR1, ST2, TIM3, and IL-6	Diagnostic, prognostic	In patients before starting treatment, sTNFR1, TIM3, and IL-6 predicted III to IV GVHD grades. Plasma sTNFR1 and ST2 predicted 1-year NRM. Plasma levels of sTNFR1, ST2, TIM3, and IL-6 were associated with more severe GVHD and NRM.	[29]
AREG	Prognostic	Elevated AREG concentrations were associated with a worsening response to steroids, OS, and NRM.	[30]
Elafin	Prognostic	Increased plasma levels of elafin in patients after cyclophosphamide/fludarabine-based nonmyeloablative allotransplantation were associated with aGVHD.	[31]
CD30	Diagnostic	Increased plasma levels of CD30 and its expression on T cells were associated with severe aGVHD.	[32]
Serum biomarkers			
SF	Prognostic	Increased pretransplantation SF was significantly correlated with worse OS, PFS, and higher risk of NRM, as well as low incidence of cGVHD without effect on aGVHD.	[33]
CRP	Prognostic	Elevated pretransplantation serum CRP was correlated with worse OS, increased risk of aGVHD, and NRM.	[34]
B7 family	Prognostic	Increased post-transplantation serum B7H1/B7H3 serum in haplo-HSCT patients was associated with grade III to IV aGVHD.	[35]
sIL-27Rα	Prognostic	The probability of grade II to IV aGVHD and NRM was significantly higher in patients with low serum levels of sIL-27Rα compared with patients with high serum levels of sIL-27Rα.	[36]
Albumin	Prognostic	Patients with SR aGVHD score and hypoalbuminemia ($<$35 g/L) had a significantly lower OS and NRM.	[37]
GI biomarkers			
REG3α	Diagnostic	REG3α distinguished lower GI GVHD from non-GVHD diarrhea.	[24]
Skin biomarkers			
Elafin	Diagnostic	Elafin expression in the skin was associated with cutaneous aGVHD diagnosis.	[38]
Skin and intestinal biomarkers			
Ang2	Diagnostic	The level of Ang2 in both pre-and post-transplant was associated with intestinal aGVHD and skin and aGVHD, respectively.	[22]
VEGF	Diagnostic	The level of VEGF in the pretransplant was associated with skin aGVHD and in post-transplantation correlated with intestinal aGVHD.	[22]

HCT indicates hematopoietic cell transplantation; NRM, nonrelapse mortality; AREG, amphiregulin; OS, overall survival; PFS, progression-free survival; SF, serum ferritin; CRP, C-reactive protein; sIL-27Rα, soluble interleukin-27 receptor α; SR, standard risk.

including the variety of body fluids to be analyzed, the type of miRNA detection platform, sampling time points, diversity in patient populations, sample preparation, and miRNA technical normalization methods [41]. To date, there are no diagnostic, prognostic, predictive, or response to treatment biomarkers for aGVHD at the clinical application level [27].

Circulating miRNAs Signature for Prediction of aGVHD

Several studies have evaluated the expression of specific miRNAs in post-HSCT to identify biomarkers for diagnosis or prognosis or to determine the outcome of patients with aGVHD [27,29,39,41]. However, many miRNAs or panels have the potential to be used as all 3 diagnostic, prognostic, or predictive biomarkers for aGVHD.

CIRCULATING MICRORNAS AS DIAGNOSTIC AND PROGNOSTIC BIOMARKERS OF AGVHD

MicroRNAs can potentially be used as noninvasive diagnostic tests in the clinic to improve prophylaxis, predict the incidence and severity, and reduce morbidity. Xiao et al. [27] reported 6 miRNAs (miR-30a, miR-93*, miR-155, miR-199a-3p, miR-377, and miR-423), which had a high expression in plasma of patients with aGVHD compared with non-GVHD. Four of these miRNAs (miR-93*, miR-199a-3p, miR-377, and miR-423) were elevated about 2 weeks before the onset of aGVHD, which could predict the likelihood of developing aGVHD. In a prognostic cohort study by Crossland et al. [42], an increase in serum levels of all 4 miRNAs was replicated. In addition, the same group validated the increase of miR-93*, miR-199, and miR-423 expression at the onset of aGVHD in another independent diagnostic cohort study. They elucidated that miR-93*, miR-199, and miR-423 had roles in tissue damage, tissue repair, regulation of cell proliferation, and inflammation, although their specific functions in aGVHD have not yet been determined. In fact, increased expression of circulating miRNAs may be initiated by the inflammatory reaction with a conditioning regimen and a transplantation procedure. Subsequently, tissue damage and the cytokine storm caused by alloreactive T cells from the donor lead to maintenance of increased expression of miRNAs in patients with aGVHD. Conversely, in patients with non-aGVHD, inflammatory reaction is reduced, which may diminish the expression of miRNAs [42]. The expression levels of miR-93*, miR-199, and miR-423 have been associated with the incidence and severity of aGVHD

Table 2
MicroRNA Biomarkers for aGVHD

Types of MicroRNA	Study Population (n)	Assay	Body Fluid Sources	Role in aGVHD	Reference
miR-153-3p	70	qRT-PCR	Plasma	Increased plasma levels of miR-153-3p in patients at +7 days after transplant were associated with aGVHD. MiR-153-3p was involved in the pathogenesis of aGVHD through the inhibition of indoleamine-2,3-dioxygenase. The injection of miR-153-3p in aGVHD mice resulted in improved aGVHD and survival.	[93]
miR-155	64	qRT-PCR	Serum	Elevated serum miR-155 was correlated with the severity of aGVHD. In addition, serum IL-9, IL-17, and IFN-γ levels increased in patients with aGVHD with high levels of miR-155. See Figure 1a for the role of miR-155 in aGVHD pathogenesis.	[83]
MiRNAs 194 and 518f	24	qRT-PCR-based high-throughput miRNA array	Plasma	The level of both microRNAs in patients with aGVHD was significantly higher than patients without aGVHD. Increasing expression of miRNAs 194 and 518f could be detected after allo-HSCT and before the onset of aGVHD. The target genes identified for miR-194 and miR-518f were located in the MAPK signaling, STAT pathway and NF-κB pathways; the role of these pathways in aGVHD pathogenesis was known. EGR-1 transcription factor was one of the target genes of miR-518f that regulated the expression of genes involved in neutrophil and monocyte adhesion, chemotaxis, and inflammation. SOCS2 was one of the target genes of miR-194 that was a negative regulator of the cytokine receptor signaling via the JAK/STAT pathway.	[39]
miR-503-5p	38 30	qRT-PCR-based high-throughput miRNA array	Skin Plasma	The expression level of miR-503-5p in both serum and skin was inversely correlated with improved OS. The overexpression of miR-503-5p was significantly positively associated with stages II to IV in skin aGvHD. MiR-503-5p negatively regulated expression of the CD40 gene, thereby inhibiting T cell activation.	[94]
miR-34a	38 30	qRT-PCR-based high-throughput miRNA array	Skin Plasma	The overexpression of miR-34a levels was significantly positively associated with severe aGVHD. The expression of c-MYC and TP53 target genes was correlated with cutaneous aGVHD stage and expression of miR-34a.	[94]
miR-146a, miR-155	54	qRT-PCR	Peripheral blood	miR-146a and miR-155 showed significant decreased levels of expression in patients who developed aGvHD (grades I-III) compared with non-aGVHD. MiR-155 and miR-146a regulated the SPI1 gene in early stages of inflammation in aGVHD. There was a negative correlation between the expression of both the microRNAs and their target gene (SPI1) in patients with aGVHD.	[91]
miR-128	10	qRT-PCR-based TaqMan low-density array	Plasma	The overexpression of miR-128 levels was significantly associated with onset of late-onset GVHD. The target genes (NEK2, BMI1, RXRA, FBXW7, SREBF1, ABCG1, TGFBR1, DCX, ABCA1, WEE1, RELN, and SREBF2) of this miRNA were involved in the immune system and inflammation.	[95]
MiRNA panel (miR-30a, miR-93*, miR-155, miR-199a-3p, miR-377, miR-423)	196	qRT-PCR-based high-throughput miRNA array	Plasma	The overexpression of miR-93*, miR-155, miR-199a-3p, miR-377, and miR-423 levels was significantly positively associated with more severe aGVHD. Increased plasma levels of the miR-93*, miR-199a-3p, miR-377, and miR-423 were correlated with poor OS.	[27]
miR-423, miR-199, miR-93*, miR-377	81	qRT-PCR	Serum	Increasing the expression of these miRNAs in the serum was associated with the severity of aGVHD and poor OS.	[42]
	168				[27]

(*continued*)

Table 2 (*Continued*)

Types of MicroRNA	Study Population (n)	Assay	Body Fluid Sources	Role in aGVHD	Reference
miR-586	98	qRT-PCR	Plasma	A significant increase in the expression of miR-586 expression on day 7 after transplantation was observed in patients with GVHD compared with non-GVHD. MiR-586 may cause aGVHD by reducing the expression of target genes (KCTD12, TNRC6B, SFPQ, SLC12A2, and FAM129A).	[58]
miR-411	18	qRT-PCR-based TaqMan	Serum	A significant reduction in miR-411 was observed in the onset of aGVHD. Moreover, with aGVHD recovery, there was no significant difference in expression of this miRNA.	[60]
miR-489, miR-671-3p, and miR-28-5p	18 54	qRT-PCR-based TaqMan. Validation and blinded testing of the diagnostic model for aGVHD.	Serum Plasma	The level of miR- 489 and miR-671-3p at aGVHD onset was significantly higher than the non-GVHD control, whereas miR-28-5p was downregulated in patients with aGVHD. All 3 miRNAs had high specificity and sensitivity. These microRNAs have important roles in tissue damage, tissue repair, and inflammation.	[60]
miR-29a	19	Small RNA sequencing	Serum	miR-29a was significantly upregulated in aGVHD onset compared with non-aGVHD. Serum miR-29a was also elevated as early as 2 weeks before time of diagnosis of aGVHD compared with time-matched control subjects. MiR-29a via TLR-7 and TLR-8 activated the NF-κB pathway and secreted the anti-inflammatory cytokines of IL-6 and TNF-α in dendritic cells. MiR-29a induced maturation and migration of dendritic cells and proliferation of alloreactive T cells.	[68]
MicroRNA panel (miR-20a, miR-146a, miR-181a, miR-374-5p, miR-30b-5p, miR-15a, miR-19a, miR-18a, miR-19b, and miR-451a)	NanoString (n = 12) Diagnostic cohorts (n = 42) Prognostic cohort (n = 47)	NanoString microRNA expression Profiling qRT-PCR-based TaqMan	Serum	In a diagnostic cohort study, expression of miR-15a and miR-20a was significantly increased, whereas expression of miR-30b-5p, miR-146a, miR-181a, and miR-374-5p was significantly decreased in aGVHD. In a prognostic cohort study, 6 of the miRNAs (miR-18a, miR-19a, miR-19b, miR-20a, miR-146a, and miR-451) before the onset of the disease had a significant increase in aGVHD compared with non-aGvHD. The pathway analysis of these miRNAs showed that their targeted genes are involved in inflammatory or hematologic diseases and inflammatory responses.	[41]

qRT-PCR indicates quantitative reverse transcription polymerase chain reaction.

[42]. All 3 miRNAs have the potential to be used as diagnostic, prognostic, and predictive biomarkers. Therefore, simultaneous application of several biomarkers increases the sensitivity and specificity of the test.

MiR-423 causes cellular growth by regulating G1/S via p21Cip1/Waf1 in hepatocellular carcinoma [43]. It is hypothesized that the progression of the G1/S phase of the cell cycle is important for the activation of cells that are involved in the pathogenesis of aGVHD [42]. Caveolin 2 is a target gene for miR-199, which regulates cell growth and apoptosis. Caveolin 1 and 2 have a similar structure and are commonly expressed simultaneously [44]. An increased expression of caveolin 1 was reported in human and murine T cells after allo-HSCT. Cav-$1^{-/-}$ donor T cells, in comparison with controls, could cause a lower intensity of aGVHD and a greater number of Treg cells. In fact, caveolin 1 regulates the signaling pathways of TCR, TGF-β, and Treg differentiation [45]. It has also been shown that mTOR is regulated by miR-199 in malignant cells [46]. The overexpression of miR-199 leads to mTOR inhibition and a less severe aGVHD disease. Thus, miR-199 may play a protective role in aGVHD [42]. The inhibition of mTOR with rapamycin has been shown to inhibit the differentiation of Th17 pathogenic cells and to increase the production of Treg immunosuppressive cells [47]. The low Th17/Treg ratio has been associated with the severity of the clinicopathologic grade of GVHD, apoptosis of epithelial cells, and the level of expression of Fas, TNF, and TNF receptor [48].

MiR-93*, associated with downregulation of multiple stem cell regulatory genes, including *EZH1*, *STAT3*, *AKT3*, *SOX4*, and *JAK1*, induces mesenchymal epithelial transition in breast cancer cells [49]. *JAK1* inhibition in mice following aGVHD resulted in survival improvement, decreased histopathologic grading, decreased effector T cell proliferation, and decreased serum proinflammatory cytokine production [50]. On the other hand, STAT3 plays an important role in T cell alloactivation. Inhibition of STAT3 activation with nifuroxazide has stopped the development of aGVHD and caused a significant reduction in lethal aGVHD in mice [51]. The miR-377, by targeting cyclin-dependent kinase 6 [50], which has been shown to have a high expression in GVHD, stops cell proliferation [52].

Recently, the association of circulating miRNAs with aGVHD etiology and their application as diagnostic and prognostic biomarkers has been investigated. Circulatory miRNAs can be used as potential biomarkers due to the noninvasive nature of sample collection. In a prospective study, mature circulating miRNA expression profiles were studied using a NanoString nCounter technology to detect aGVHD after allo-HSCT. Sixty-one miRNAs had a different expression in patients with aGVHD compared with non-aGVHD. Ten of these miRNAs (miR-20a, miR-146a, miR-181a, miR-374-5p, miR-30b-5p, miR-15a, miR-19a, miR-18a, miR-19b, and miR-451a) were selected to a diagnostic cohort study based on high fold change or inflammatory or immune response or the previous aGVHD relationship. Expression of miR-15a and miR-20a was significantly increased, whereas expression of miR-30b-5p, miR-146a, miR-181a, and miR-374-5p was significantly decreased in aGVHD. In the same study, in a prognostic cohort, 10 miRNAs were investigated on day 14 post-HSCT before the onset of the disease. Six of them (miR-18a, miR-19a, miR-19b, miR-20a, miR-146a, and miR-451) showed a significant increase in expression level in patients who developed aGVHD compared with non-aGVHD [41]. Therefore, due to the altered expression of these circulating body fluid miRNAs, they may play a role in the pathology and onset of aGVHD and can be used as diagnostic and prognostic biomarkers.

It has been shown that miR-451 directly inhibits the expression of *MYC* and indirectly inhibits the PI3K/AKT pathway, which regulates several major events in the inflammatory response to infection and damage [53-55]. The miR-17-92 cluster contains miR-17, miR-18a, miR-19a, miR-19b-1, miR-20a, and miR-92a, which play a major role in T cell differentiation, autoimmune diseases, and proinflammatory functions [56,57]. A study using murine models of allo-BMT showed that miR-17-92 expression on donor T cells induced GVHD. In addition, miR-17-92 played an important role in $CD4^+$ T cell proliferation, activation and survival, as well as induction of Treg differentiation and Th2 inhibition. On the other hand, $CD8^+$ T cells were migrated to target organs in the GVHD. Furthermore, by using antagomir to block miR-17 or miR-19b, the production of IFN-γ and alloreactive T cell proliferation were inhibited, and survival in mice with GVHD was improved [57].

The diagnosis of aGVHD after transplantation is often delayed. The aGVHD biopsy is performed when the clinical signs are severe and other pathologic markers are absent. Diagnosis of aGVHD is often perplexed by complications such as infections. The miR-586 level decreases in the onset of grade I to II aGVHD. In contrast, when patients with aGVHD or non-GVHD have infections, the level of this miRNA is elevated. Increasing the expression of miR-586 may occur due to cytokine storms as a result of some infections [58]. Furthermore, a significant increase in the expression of miR-586 expression on day 7 after transplantation has been observed in patients with GVHD compared with non-GVHD. Hence, miR-586 may contribute to the development of aGVHD and can be considered a diagnostic and predictive biomarker and a potential novel therapy target in aGVHD. The miR-586 mechanism is unknown, but it is assumed that patients with high expression of miR-586 have reduced expression in target genes, which may have an inhibitory effect on aGVHD. The target genes for miR-586, such as *KCTD12*, *TNRC6B*, *SFPQ*, *SLC12A2*, and *FAM129A*, have been predicted by bioinformatics [59]. Many studies have focused on plasma miRNAs to have diagnostic advantages such as noninvasiveness, high sensitivity and specificity, inexpensiveness, and accuracy [58].

CIRCULATING MICRORNAS AS PREDICTIVE BIOMARKERS OF AGVHD

The rate of mortality, recurrence, and poorer diagnosis in patients with grade II to IV aGVHD is higher than non-GVHD. Thus, the identification of biomarkers is necessary to predict the incidence, development, and diagnosis of GVHD after transplantation. Zhang et al. [60] identified a significant reduction in miR-411 at the onset of aGVHD. Moreover, with aGVHD recovery, there was no significant difference in the expression of this miRNA. As a result, miR-411 could be used as an indicator of aGVHD monitoring, although its molecular mechanism has not yet been determined. The same group surveyed a diagnostic model for 3 miRNAs (miR-489, miR-671-3p, and miR-28-5p). The level of miR-489 and miR-671-3p at aGVHD onset was significantly higher than the non-GVHD control, whereas miR-28-5p was downregulated in patients with aGVHD. The diagnostic model for miR-489, miR-671-3p, and miR-28-5p was 0.841, 85.71%, and 83.33% for the area under the receiver operating characteristic curve, positive predictive value, and negative predictive value, respectively. In addition, the predictive model for miR-374a and miR-26b, which could predict an increased risk for 1 to 2 weeks before the onset of aGVHD, was 0.722, 76.19%, and 69.70% for area under the receiver

operating characteristic curve, positive predictive value, and negative predictive value, respectively [60]. Thus, miRNA panels (miR-671-3p, miR-489, miR-28-5p, miR-374a, and miR-26b) may be used as diagnostic and predictive biomarkers for grade II to IV aGVHD.

Gimondi et al. [39] reported a significant increase in the expression of miR-194 and miR-518f in patients with aGVHD compared with non-aGVHD. Interestingly, overexpression of both miRNAs was detectable before the onset of aGVHD in patients who later developed aGVHD. Pathway prediction analysis suggested that both these miRNAs may regulate pathways, important in the pathogenesis of aGVHD [39]. *EGR-1 transcription factor* is one of the target genes of miR-518f that regulates the expression of genes involved in neutrophil and monocyte adhesion, chemotaxis, and inflammation [61,62]. Moreover, *SOCS2* is one of the target genes of miR-194, which is a negative regulator of cytokine receptor signaling via the JAK/STAT pathway [39]. In addition, Ingenuity Pathway Analysis for these miRNAs identified pathways of TGFβ signaling, Wnt/β-catenin signaling, and TLR signaling as canonical pathways that may play a role in aGVHD pathogenesis [39,63,64]. In general, there is currently no diagnostic and prognostic test for aGVHD, but the identification of biomarkers to predict aGVHD in early allo-HSCT increases the likelihood of success in treatment and life expectancy in patients.

Zhang et al. [60] also demonstrated that miR-671-3p, miR-489, and miR-28-5p have important roles in tissue damage, tissue repair, and inflammation. Myeloid differentiation primary response gene 88 (Myd88) is an important cytosolic adaptor protein in the TLR signaling pathway, which acts as a target of miR-489 [65]. MiR-489 stimulates the secretion of various cytokines via the RAS-MAPK pathway [66]. MyD88 and TLR contribute to the progress of all GVHD phases. Oral administration of TJ-M2010-5 as an inhibitor of MyD88 has been shown to improve the inflammatory environment, reduce apoptosis, and increase tissue repair in the target organs of GVHD. Thus, it inhibits lethal GVHD [67].

There is little information about the function of miRNAs in aGVHD. A recent study showed that miR-29a was significantly upregulated in the onset of aGVHD compared with non-aGVHD. Serum miR-29a was also elevated as early as 2 weeks before the time of diagnosis of aGVHD compared with time-matched control subjects [68]. Furthermore, the use of miR-29a as a predictive biomarker for aGVHD should be investigated in large cohort studies.

It has been shown that miR-29a via TLR-7 and TLR-8 binds to dendritic cells and activates the nuclear factor (NF)-κB pathway and secretes the proinflammatory cytokines of IL-6 and TNF-α. Using the murine model of aGVHD, it was elucidated that the downregulation of this miRNA with locked nucleic acid anti-miR-29a resulted in a clinically significant improvement in the survival and reduction of aGVHD severity, without impairment of graft-versus-leukemia effects [68]. The TLR signaling pathway activates MyD88-dependent transcription factors, including IRF7 and NF-κB [69]. MiR-29a stimulation causes translocation of both NF-kB-p65 and IRF7 phosphorylated into the cellular nucleus [68]. In addition to miR-29, so far, the function of several important miRNAs in the mechanism of aGVHD has been investigated.

MIR-146A AND MIR-155 IN AGVHD

Because both miR-155 and miR-146a are involved in innate and adaptive immunity, we briefly reviewed the association of both miR-146a and miR-155 with aGVHD.

MiR-146a is essential for Treg cell function [70,71]. *IRAK1*, *TRAF6*, *IRF5*, and *STAT1* are the target genes of this miRNA. It regulates TLR and cytokine signaling via a downregulation of *IRAK1* and *TRAF6* in a negative feedback loop [72]. Increasing the expression of miR-146a results in a decrease in the level of *IRF5* and *STAT1* at the molecular and protein levels [70]. It has been shown that the deficiency of miR-146a in Treg cells in mice was associated with overexpression and activation of STAT1, which leads to IFN-γ mediated autoimmunity [71]. Moreover, miR-146a$^{-/-}$ mice had a high expression level of STAT1 and IFN-γ in the melanoma microenvironment [73]. In lymphoid tissues, donor cells express a lower level of PD-1 expression and a high level of PD-L1 and CD80. PD-L1/CD80 interaction in lymphoid tissues leads to the development of T cell and graft-versus-leukemia effects. In contrast, in the GVHD target tissues, the T cells express a low level of CD80 and a high level of PD-1, but host tissues express a high level of PD-L1. PD-L1/PD-1 interaction results in anergy/exhaustion and apoptosis, resulting in T cell tolerance and prevention of GVHD [74]. Both IRAK1 and TRAF6 act as adapter proteins in the NF-κB activation pathway. In addition, NF-κB activation has been shown to cause overexpression of miR-146a, which in turn downregulates NF-κB through IRAK1 and TRAF6 suppression in a negative feedback loop [75,76]. In mice receiving miR-146a$^{-/-}$ T cells, elevated GVHD severity, reduced survival, and increased levels of TRAF6 and TNF-α were reported [75]. The elevation of TRAF6 levels increases NF-κB activity and enhances TNF-α production. Thus, miR-146a reduces the severity of GVHD by inhibiting TRAF6 and lowers transcription of TNF-α. The SNP rs2910164 in hematopoietic cell donors is associated with a reduction in expression of miR-146a and an increase in the risk of severe GVHD [75]. Lipopolysaccharide (LPS) is one of the components of the gram-negative bacteria that is released in response to the GVHD conditioning regimen. LPS has been shown to increase the expression of miR-146a [72].

MiR-146a rs2910164 (G>C) polymorphism in transplant recipient patients, which reduced the expression of miR-146a levels, was highly correlated with aGVHD grades III to IV. The deficiency of miR-146a in host dendritic cells (DCs) in the mouse model increased aGVHD by accelerating the activity of the JAK-STAT/class II transactivator/the major histocompatibility complex class II signaling pathway [77]. Thus, miR-146a, as a negative regulator of the JAK/STAT pathway, reduces aGVHD by decreasing proinflammatory production and cytokine receptors of this pathway.

MiR-155 is essential for function of dendritic cells and B and T lymphocytes [78]. In a study, high expression of miR-155 in T cells of mice with aGVHD after allo-HSCT with intestinal aGVHD symptoms was observed. Mice receiving miR-155-deficient lymphocytes showed a significant reduction in the incidence and severity of lethal aGVHD, whereas lethal aGVHD was increased in mice receiving T cells with high expression of miR-155. The use of anti-miR-155 reduced the severity of aGVHD and prolonged the survival rate [79]. TNF-α levels increased more in transgenic mouse lymphocytes with miR-155 overexpression than in wild-type controls [80]. The miR-155 inhibition with a specific antagomir in endothelial microparticles of mice gave rise to differentiation of T cells toward Treg and Th2 cells and suppression of T cell differentiation toward Th9, Th1, and Th17 cells, which resulted in a decrease in aGVHD manifestation [81]. In addition, the transfer of miR-155-deficient DCs in murine models of allo-HSCT resulted in a reduction in GVHD severity, a decrease in proinflammatory serum cytokines, and an improved survival [82]. It has been

shown that an increased expression of miR-155 in the gut specimens and serum of patients is associated with severe aGVHD [79,83]. The miR-155, with downregulating SOCS1, IL-2 signaling inhibitor, leads to an increase in the level of STAT5 and an increase in IL-2 signaling (Figure 1a). Interestingly, miR-155, by inhibiting the expression of SOCS1, increases the production of proinflammatory cytokines [84]. Previous studies have shown that IFN-β and IFN-γ through TNF-α induce miR-155 expression [85]. Zitzer et al. [86] used a murine model for aGVHD and showed that expression of miR-155 in both conventional $CD4^+$ CD252 T cells and donor $CD8^+$ T cells was necessary for the pathogenesis of aGVHD. Mice receiving miR-$155^{-/-}$ $CD4^+$ T cells and miR-$155^{-/-}$ $CD8^+$ T cells had amelioration in aGVHD and survival rate. They found that expression of miR-155 in donor T cells is necessary for T cell expansion during aGVHD. Furthermore, miR-155 induced the proinflammatory Th1 phenotype and increased TNF-α and IFN-γ release from normal T cells, and the loss of miR-155 in donor T cells impaired the migration of T cells and tissue infiltration. If T cell migration is impaired, tissue damage in aGVHD will be minimized. Hence, miR-155 can serve as a potential target for the treatment and prevention of aGVHD after allo-HSCT [86].

In humans, the severity of intestinal GVHD is associated with the level of neutrophils at the site of GVHD damage [87]. The neutrophil infiltration after allo-HSCT in the intestine has a high dependence on local microbial flora [88]. Neutrophils are contributing via reactive oxygen species to GVHD [87]. A predominant feature of inflammatory bowel disease is the accumulation of neutrophils [89]. In 1 study, a mechanism independent of reactive oxygen species was identified in inflammatory bowel disease in which neutrophils prevented wound healing and resolution of inflammation by releasing microparticles containing proinflammatory miRNAs (miR-155 and miR-23a) [90]. MiR-155 and miR-23a caused double-strand breaks and genomic instability. The targeted inhibition of miR-155 and miR-23a with antisense oligonucleotides in the intestinal epithelial cells reduced the effects of neutrophils and accelerated mucosal healing [90]. Thus, the mechanism of microparticles containing neutrophilic-derived miRNAs may be a new therapeutic potential to prevent intestinal GVHD.

In another study, low expression of miR-155 and miR-146a was associated with aGVHD grades I to III at day +28 post-allo-HSCT. In addition, there was a negative correlation between expression levels of miR-155 and miR-146a with SPI1 mRNA expression [91]. The SPI1 transcription factor (*PU.1*gene) regulates the expression of both miRNAs in hematopoietic stem cells by binding to the regulatory chromatin regions adjacent to the coding loci [92].

MicroRNAs in Cutaneous aGVHD

Frequently, the skin is one of the major target organs damaged in aGVHD [96]. The cutaneous aGVHD consists of 3 stages. In the first stage (stage I), Langerhans cells are activated by the cytokines released from the damaged tissue after conditioning regimen. Stage II involves the identification of the antigens presented in host DCs activated by donor T cells, and in stage III, apoptosis of immature keratinocytes due to the cytokine storm causes skin damage [94].

The expression pattern of miRNAs can be specific to the cell and tissue. Because aGVHD affects several organs, miRNA signatures in target tissues may assist with understanding the molecular pathology of the disease. Based on this objective, the expression pattern of miRNAs in the rat model of aGVHD was compared with control rats in skin, lung, and gut tissues and T cells isolated from the blood and intestines [97]. In the intestines of rats compared with controls, down-expression of miR-743b and miR-345-5p was reported, whereas in the skin, down-expression of miR-326 and overexpression of miR-34b, miR-146a, and miR-155 were indicated. The analysis of miRNAs of T cells in the intestine of aGVHD and control rats showed that 23 miRNAs were dysregulated, but only 2 of these miRNAs were differentially expressed, which were common within the intestinal (miR-345-5p) or skin (miR-326) tissues. In addition, comparing the expression of T cells in the blood and intestinal tissue in aGVHD with control rats indicated that the different expressions of miR-223, miR-99a, miR-326, and miR-345-5p were common in both. There was slight overlap between aGVHD target tissues for expression of miRNAs. Thus, comparing the expression of miRNAs in different tissues indicated that a tissue-specific expression pattern may not be detected with the blood expression profile.

The target genes of these miRNAs are involved in the inflammatory network and possibly the production of cytokines in the intestines and the skin [97]. MiR-326 and miR-34b have 2 common target genes, *SNAIL* and *NOTCH*1 [98-100]. *SNAIL* may play a role in regulating the immune response and producing preinflammatory cytokines in response to TGF-β in keratinocytes [101]. *Notch1* signaling plays a role in the development and activation of T cells, increasing the production of inflammatory cytokines and contributing to generation of Th1 and Treg cells [102]. An elevation in the frequency of T cells in the skin of a rat model of severe aGVHD also has been reported [97].

Recently, Atarod et al. [94] studied miRNAs as diagnostic and prognostic biomarkers in cutaneous aGVHD. In this study, after global miRNA expression profiling in skin biopsy specimens from patients with cutaneous aGVHD and normal controls, 8 miRNAs were suggested as candidates for cohort validation study in pre-HSCT skin biopsies, post-HSCT skin biopsies, and normal controls for their correlation with aGVHD. Then, the expression of validated miRNAs was investigated for noninvasive biomarkers in pre-HSCT and post-HSCT serum samples. Results indicated that expression of miR-34a-3p, miR-34a-5p, and miR-503-5p was correlated with aGVHD incidence and the overall survival rate. The expression level of miR-503-5p in both serum and skin was inversely correlated with improved overall survival. In summary, with skin biopsy at the onset of aGVHD, the different expressions of these miRNAs could be used as diagnostic and predictive biomarkers for aGVHD.

It has been shown that miR-503-5p negatively regulates CD40 expression (Figure 1b) [103]. The role of the CD40-CD40 ligand (CD40L) pathway has been studied in GVHD models [104]. Several studies have indicated that the use of the anti-CD40L antibody and the inhibition of the CD40-CD40L pathway induce Tregs and reduce GVHD [105]. The mechanism, by which the overexpression of miR-503 leads to an increase in the incidence of GVHD through reducing the expression of CD40, is still unknown. Clinical trials show Tregs as promising therapeutic properties to prevent GVHD without using immunosuppressive drugs [106,107]. Studies have found that miR-142-3p negatively regulated ATG16L1. MiR-142-3p knockdown mice increased Treg cell expansion, function, and survival by increasing ATG16L1 and the autophagy process. The accumulation of Treg cells protects against lethal GVHD [107].

MiR-34a-5p showed a significant difference in expression level between pretransplant and grades 0 to I and grades II to III in biopsy specimens of patients with cutaneous aGVHD. Interestingly, Atarod et al. [94] observed a significant positive association between p53-positive cells and miR-34a-5p

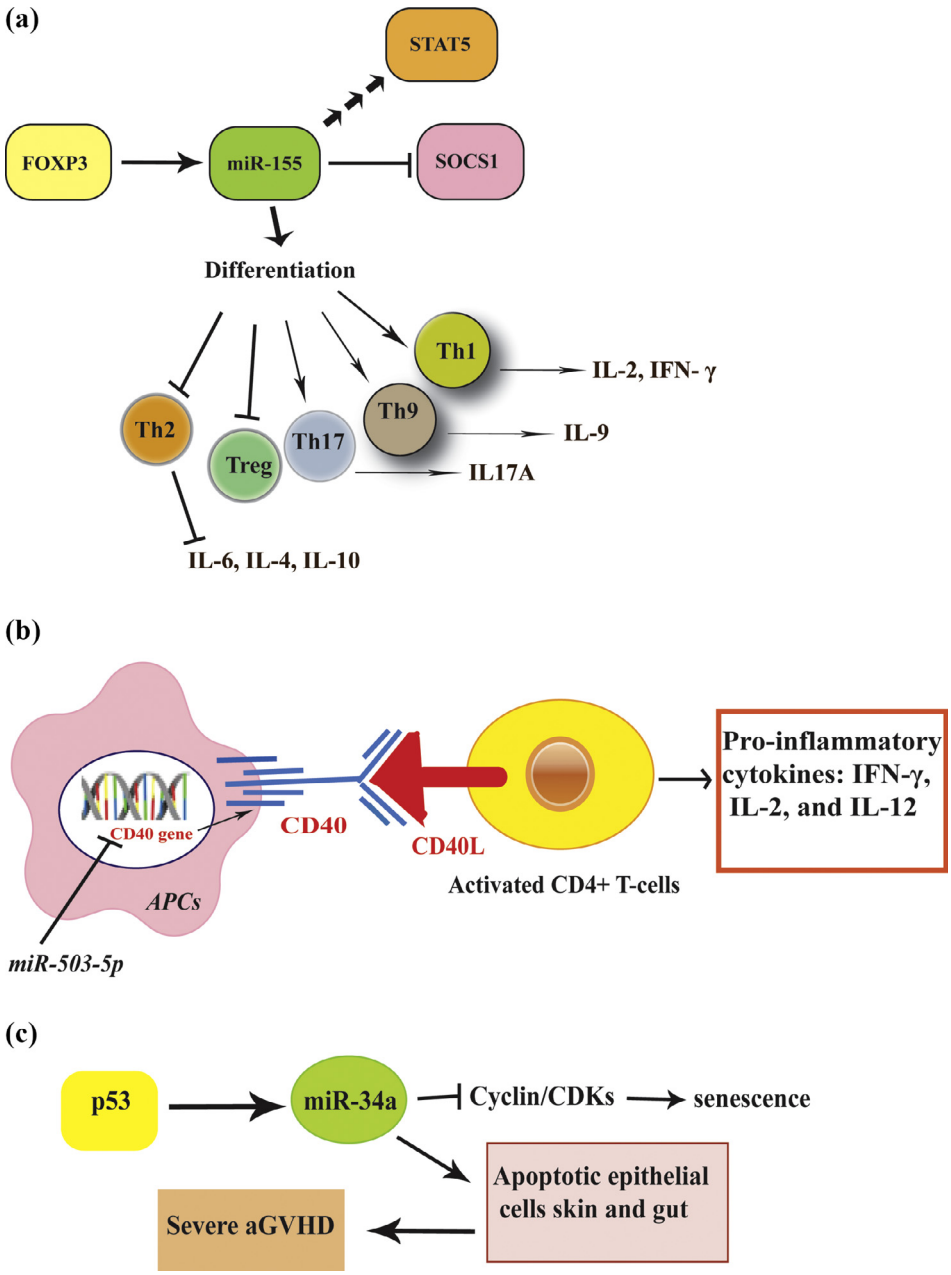

Figure 1. The role of miR-155, miR-503-5p, and miR-34a in aGVHD. (a) FOXP3 positively controls the production of miR-155. The miR-155 inhibits the SOCS1 gene along with an increase in the level of STAT5 transcription factor and decreases the production of proinflammatory cytokines. During aGVHD, Th1, Th9, and Th17 cells play a pathogenic role and Th2 and Treg cells have protective effects. MiR-155 inhibition has been shown to stimulate the production of IL-4, IL-6, and IL-10 cytokines and inhibit the production of IL-2, IFN-γ, IL-9, and IL-17A cytokines. Decreasing Th2 and Treg cells, as well as increasing Th1, Th9, and Th17 cells and proinflammatory cytokines, may be the primary cause of aGVHD in the presence of miR-155. (b) CD40 and its ligand, CD40L, are expressed on the cell APCs and activated $CD4^+$ T cells and a small subset of $CD8^+$ T cells, respectively. Ligation of CD40 to CD40L increases the secretion of proinflammatory cytokines, thereby activating NK and T cells. The overexpression of miR-503 may inhibit expression of the *CD40* gene and activate T cells. In addition, increased expression of miR-503-5p may cause more monocytes to migrate from the blood to the host skin. (c) P53 protein regulates the expression of miR-34a, and in 1 positive feedback loop, miR-34a itself increases the activity of p53. MiR-34a, depending on the type of cell, may lead to inhibition of cell cycle and senescence or induction of apoptosis. Thus, the overexpression of miR-34a levels increases the apoptosis of target epithelial cells in severe aGVHD.

expression. P53 proteins increased the expression of miR-34a, and in 1 positive feedback loop, miR-34a itself increased the activity of p53 [108]. Evaluation of TP53 and miR-34a expression in Fanconi anemia in skin and gut biopsy specimens showed an enhanced expression of miR-34 in patients with severe aGVHD compared with non-aGVHD patients or those who had not undergone transplantation [109], which had a significant association with apoptotic cell numbers. Apoptotic epithelial skin and gut cells were significantly increased in patients with Fanconi anemia with grade II to IV aGVHD in comparison with patients with grade 0 to I Fanconi anemia who had not undergone transplantation. Therefore, the increase of miR-34a levels may be associated with increased apoptosis of target epithelial cells in severe aGVHD (Figure 1c). Identification of organ-specific miRNAs in aGVHD can be promising to categorize patients for organ-specific therapies.

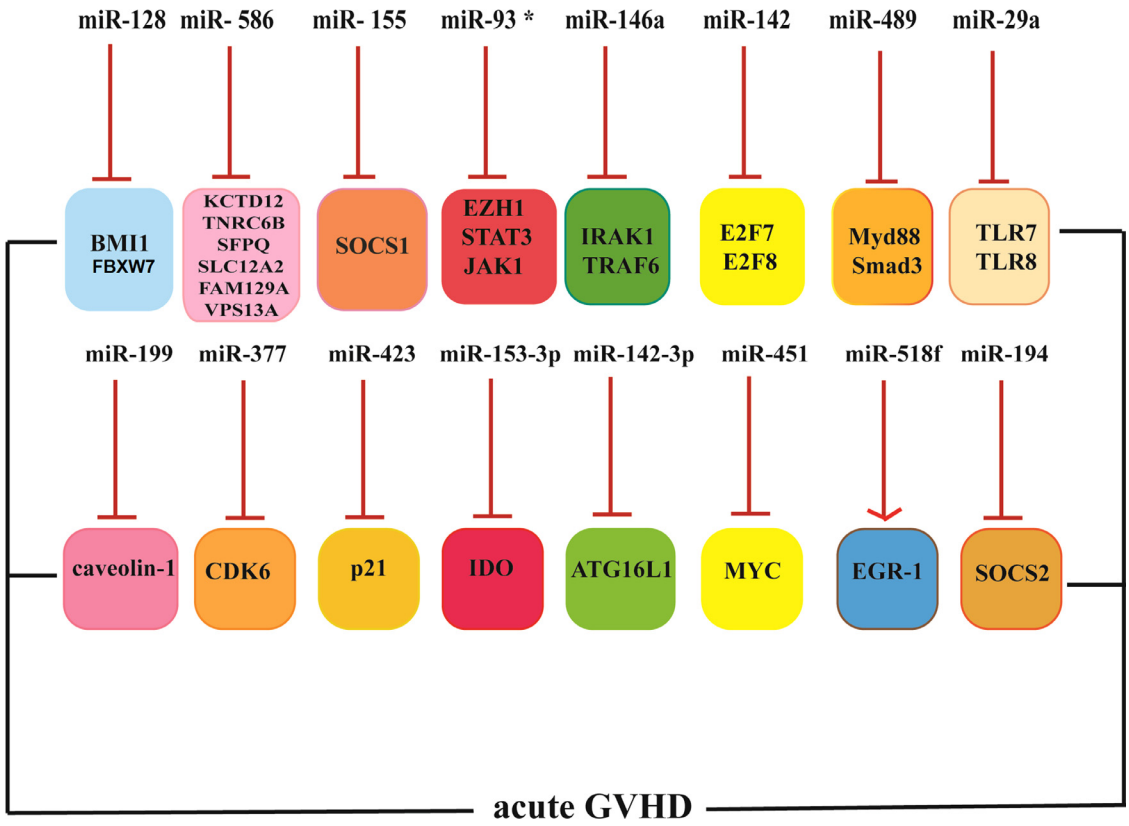

Figure 2. The diagram represents different miRNAs and their target genes. These miRNAs, by inhibiting or activating target genes in numerous cellular pathways involved in the immune system and the production of proinflammatory cytokines, ultimately cause aGVHD.

CONCLUSION

Successful treatment of various hematologic diseases with allo-HSCT is often limited due to the occurrence of GVHD. Recently, several miRNAs have been identified that affect GVHD biology by regulating the target genes involved in inflammation and anti-inflammation (Figure 2). Evidently, miRNAs not only regulate the development of GVHD but also may potentially be used as biomarkers and therapeutic targets. MicroRNAs regulate the lymphopoiesis pathway, including generation, development, and differentiation of B and T lymphocytes and NK cells [110]. Differences in the expression pattern of miRNAs may affect reconstitution of the immune system after allo-HSCT. On the other hand, changes in the expression of immune-related miRNAs may be due to differences in the dosage of myelosuppressive and immunosuppressive drugs during allo-HSCT [95]. Various studies have been conducted on animals and humans to investigate the role of miRNAs in aGVHD pathogenesis so that they can be used as biomarkers and therapeutic targets. Because of their high stability, tissue specificity, ease of measurement, low cost, and simplicity, miRNAs are an excellent target to study [111].

ACKNOWLEDGMENTS

This study is part of a PhD thesis (J.M.) with proposal No. 971083 approved by Mashhad University of Medical Sciences, Mashhad, Iran.

Financial disclosure: The authors have nothing to disclose.

Declaration of Competing Interest: There are no conflicts of interest to report.

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