Differential expression of miRNAs from extracellular vesicles in chronic graft-versus-host disease: A preliminary study

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D – writing the article; E – critical revision of the article; F – final approval of the article

Advances in Clinical and Experimental Medicine, ISSN 1899–5276 (print), ISSN 2451–2680 (online) *Adv Clin Exp Med*. 2023;32(5):539–544

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Funding sources
This work was supported by the National Science Centre (Kraków, Poland) (grant No. 2018/31/B/NZ2/03065) and European Cooperation in Science & Technology under the COST Action CA17138 (Integrated European Network on Chronic Graft Versus Host Disease: EUROGRAFT) (https://www.gvhd.eu).

Conflict of interest
None declared

Received on July 15, 2022
Reviewed on August 17, 2022
Accepted on September 13, 2022

Published online on November 18, 2022

Abstract

Background. Chronic graft-versus-host disease (cGvHD) is a complex disorder that typically manifests after allogeneic hematopoietic stem cell transplantation (HSCT). It is a major cause of non-relapse mortality, which makes finding biomarkers associated with its occurrence a priority. Recent studies increasingly indicate that microRNAs (miRNAs, short regulatory RNA molecules) can be used as biomarkers of various disorders. They can circulate in patients' bodies encapsulated within extracellular vesicles (EVs).

Objectives. To identify miRNAs associated with the occurrence of cGvHD in EVs isolated from the plasma of patients after allogeneic HSCT.

Materials and methods. We performed global miRNA expression profiling in a pilot cohort of 3 cGvHD cases and 4 non-cGvHD patients without disease symptoms 90 days after the transplantation (control group).

Results. The 2 groups were naturally clustered according to their miRNA profiles using unsupervised hierarchical clustering analysis. We identified 3 miRNAs that were differentially expressed in the cGvHD patients compared to the non-cGvHD patients. The levels of hsa-miR-630 and hsa-miR-374b-5p were lower in the cGvHD patients: 4.1-fold ($p = 0.002$) and 2.7-fold ($p = 0.044$), respectively. In contrast, the levels of hsa-miR-29c-3p were 5.8-fold higher ($p = 0.004$).

Conclusions. Our results suggest that miRNA profiles from plasma EVs may act as markers of cGvHD onset.

Key words: allogeneic HSCT, extracellular vesicles, miRNA profiling, chronic GvHD

Cite as
Łacina P, Crossland RE, Wielińska J, et al. Differential expression of miRNAs from extracellular vesicles in chronic graft-versus-host disease: A preliminary study. *Adv Clin Exp Med*. 2023;32(5):539–544. doi:10.17219/acem/155373

DOI
10.17219/acem/155373

Background

Graft-versus-host disease (GvHD) is a common complication after allogeneic hematopoietic stem cell transplantation (HSCT), which is the main therapeutic procedure for patients with hematologic malignancies, such as leukemias, lymphomas and multiple myeloma. Graft-versus-host disease is driven by the presence of donor-derived T cells and manifests itself in one of the two forms: acute (aGvHD) or chronic (cGvHD). Chronic graft-versus-host disease is one of the most significant long-term complications in patients after HSCT, being the leading cause of non-relapse mortality.[1] The disease significantly reduces the quality of life for post-transplant patients and is associated with various other comorbidities.[2] Since up to 50% of patients develop cGvHD following HSCT,[2] it is important to identify suitable prognostic markers for the earlier detection of the disease, particularly biomarkers that are easily detectable in biological fluids.

Short-circulating RNA molecules, known as microRNAs (miRNAs), have been increasingly studied during the last decade as potential biomarkers of various diseases. They are small (22–25 nucleotides) non-coding molecules that downregulate genes by binding to the 3'UTR region of their mRNA.[3] The miRNAs are present in many biological fluids, including plasma and serum.[4,5] They are known to be very stable and resistant to RNases and detrimental storage conditions. Specific changes in miRNA expression patterns can be associated with different diseases.[6] Their stability, robustness and presence in easily collected fluids, such as plasma, have made them promising candidates for biomarkers of disease.[7] The miRNAs are mostly found either bound to protective proteins or inside extracellular vesicles (EVs), which shield them from the environment.[8]

Extracellular vesicles are lipid bilayer structures of varying sizes that can transport various molecules, such as proteins, lipids, carbohydrates, and nucleic acids, including miRNAs.[9] They are released by cells to mediate intercellular communication and can release their contents into another cell by fusing with its cell membrane. Extracellular vesicle content can differ markedly between different body fluids. Blood EVs show slightly different contents depending on the fluid they are harvested from (serum or plasma).[10] The impact of EVs differs depending on the content of their cargo. However, they have been shown to be implicated in many processes, and they may be involved in the development of various pathological disorders.[11] Extracellular vesicles may also be involved in the modulation of the immune response and complications arising after HSCT.[12]

While many biomarkers, mostly proteins, have been described in aGvHD, there is a need for cGvHD markers.[13] Earlier studies have shown that the miRNA signature in both serum and plasma can be a marker for aGvHD.[3,14] Previous studies have also revealed that EVs can affect the development and symptoms of cGvHD in a mouse model.[15] Current multiplex methods, such as NanoString nCounter® technology, facilitate the analysis of the full spectrum of differentially expressed miRNAs. While sets of miRNAs differentially expressed in aGvHD are already established,[16] to date, there have been no studies focusing on miRNAs in cGvHD.

Objectives

In this study, we aimed to profile 798 highly conserved human miRNAs in EVs isolated from the plasma of a group of post-HSCT patients to establish a panel of miRNAs from EVs differentially expressed in cGvHD patients.

Materials and methods

A total of 7 patients were investigated, including 4 patients without cGvHD 90 days after the transplantation (the control group) and 3 cGvHD patients who presented with extensive symptomatology. Their characteristics are presented in Table 1. All patients were transplanted either at the Department of Haematology, Blood Neoplasms and Bone Marrow Transplantation, or the Department of Paediatric Bone Marrow Transplantation, Oncology and Haematology (Wroclaw Medical University, Poland). The study was approved by Bioethics Committee of Wroclaw Medical University, Poland on July 1, 2019 (approval No. 561/2019). Written informed consent was obtained from the participants, and the study was performed in compliance with the Declaration of Helsinki.

Plasma was separated from whole blood samples through centrifugation and stored at –80°C. Extracellular vesicles were precipitated from 2 mL of plasma samples using the Total Exosome Isolation Kit (Thermo Fisher Scientific, Waltham, USA), following the supplier's instructions. Total RNA was isolated from the resuspended EVs using the Total Exosome RNA and Protein Isolation Kit (Thermo Fisher Scientific), following the manufacturer's recommendations. For further analysis, the isolated RNA samples were concentrated to 25 µL using Amicon Ultra-0.5 Centrifugal Filter Units (Merck Group, Darmstadt, Germany). The RNA was quantified using a Bioanalyzer and RNA 6000 pico kit (Agilent Technologies, Santa Clara, USA). Any variation in RNA recovery between the samples was compensated for by the use of NanoString endogenous controls.

The miRNA profile was analyzed in 3 µL of concentrated total RNA samples using the nCounter® Human v3 miRNA Expression Assay Kit (NanoString Technologies, Inc., Seattle, USA), following the supplier's protocols. The code set included 798 microRNAs, 5 mRNA housekeeping controls (*ACTB*, *B2M*, *GAPDH*, *RPL19*, and *RPLP0*), 6 ligation controls, 8 negative controls, and 6 positive controls.

The output raw data obtained from nCounter® miRNA profiling were normalized using nSolver Analysis Software

Table 1. Characteristics of patients

Patient No.	Diagnosis	Age	Sex	Donor (SIB/HAP/MUD)	Transplant material	Conditioning regimen	aGvHD grade	Viral infections EBV/CMV/other	cGvHD	Fig. 1#
1	AML	22	F	HAP	BM	RIC	I (skin)	no/yes/no	no	non-cGvHD I
2	AML	58	F	MUD	PBPC	RIC	I (skin)	no/yes/no	no	non-cGvHD II
3	MDS	63	M	MUD	PBPC	RIC	no	no/yes/no	no	non-cGvHD III
4	PMF	56	M	MUD	PBPC	MAC	no	no/yes/no	no	non-cGvHD IV
5	AML	61	F	MUD	PBPC	RIC	I (skin)	yes/yes/no	extensive lung, liver	cGvHD I
6	MDS/AML	14	F	MUD	PBPC	RIC	I (skin), IV (gut)	no/no/no	extensive skin	cGvHD II
7	AML	18	F	SIB	BM	MAC	III (liver)	no/no/BKV cystitis	extensive liver	cGvHD III

AML – acute myeloid leukemia; PMF – primary myelofibrosis; MDS – myelodysplastic syndrome; F – female recipient; M – male recipient; SIB – HLA-matched sibling donor; HAP – haploidentical family member; MUD – matched unrelated donor; BM – bone marrow; PBPC – peripheral blood progenitor cells; RIC – reduced intensity conditioning; MAC – myeloablative conditioning; aGvHD – acute graft-versus-host disease; cGvHD – chronic graft-versus-host disease; EBV – Epstein–Barr virus; CMV – cytomegalovirus; BKV – BK virus; # reflects sample description in Fig. 1.

v. 2.5 (NanoString Technologies, Inc.) with code set content normalization based on the top 100 miRNAs with geometric mean and standard flagging limits. Additionally, fold change expression differences between the groups of patients compared in the study were calculated based on normalized count data.

Statistical analysis was performed according to the procedures designed at the Haematological Sciences Department of Newcastle University (UK), as described by Crossland et al. in 2017.[3] In short, various R statistical packages were used within RStudio software (RStudio, Boston, USA) in order to generate volcano plots ("ggplots") and heatmaps ("glops" and "RColorBrewer") based on an unsupervised clustering approach of the normalized expression counts with a Euclidean (L2 norm) distance measure and "Complete" as the agglomeration method.

Gene analysis and pathway prediction for the identified miRNAs were performed based on the method described by Lou et al.[17] The miRNet database (http://www.mirnet.ca/), which uses data from 11 different databases, was used to identify potential gene targets of miRNAs obtained from NanoString.[18] Subsequently, the target genes were used to construct protein–protein interaction networks in the STRING database (http://string-db.org),[19] separately for miRNAs upregulated and downregulated in cGvHD. Hub genes were then identified using Cytoscape software (v. 3.7.2; https://cytoscape.org/)[20] and used in KEGG pathway enrichment analysis performed in the Database for Annotation, Visualization and Integrated Discovery (DAVID) (https://david.ncifcrf.gov/).[21]

Results

Out of the 798 miRNAs tested, 73 had sufficiently high levels above background after normalization in at least 2 samples and were subsequently included in a further analysis. The unsupervised hierarchical clustering analysis was able to separate samples according to their disease status (cGvHD compared to non-cGvHD). Three miRNAs were significantly differentially expressed in cGvHD compared to non-cGvHD; miR-374b-5p and miR-630 were downregulated, while miR-29c-3p was upregulated (Table 2 and Fig. 1).

Potential target genes for the 3 miRNAs were identified using miRNet. A total of 254 potential target genes were found for upregulated hsa-miR-29c-3p, while 76 and

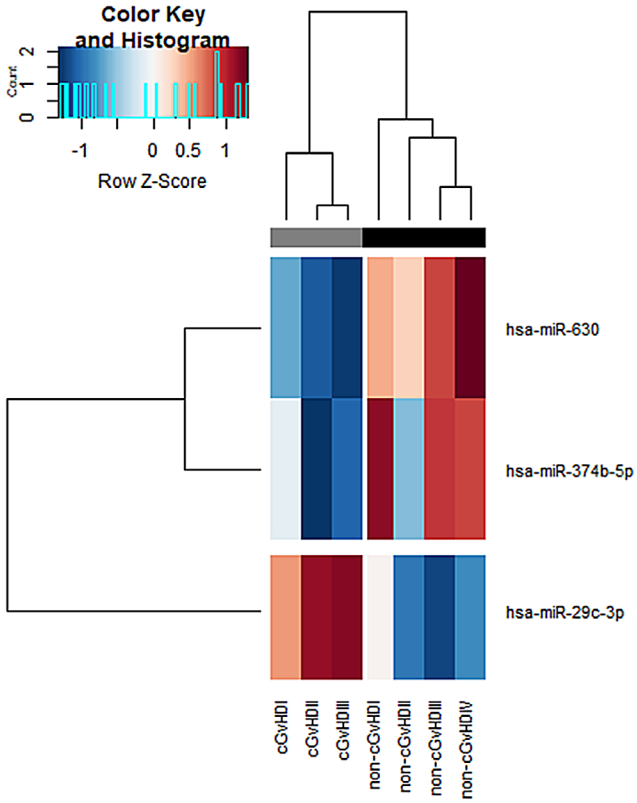

Fig. 1. Fold change in plasma levels of microRNAs (miRNAs) between chronic graft-versus-host disease (cGvHD) and non-cGvHD patients. Hierarchical clustering of differentially expressed miRNAs is shown based on normalized digital expression counts

Table 2. MicroRNAs (miRNAs) found to be differentially expressed between patients with chronic graft-versus-host disease (cGvHD) and non-cGvHD controls

miRNA	fold change	p-value
hsa-miR-29c-3p	5.83	0.004
hsa-miR-374b-5p	–2.71	0.045
hsa-miR-630	–4.13	0.002

234 were found for downregulated hsa-miR-630 and hsa-miR-374b-5p, respectively (3 genes, *APOL6*, *LRIG3* and *ATXN1*, were shared between the up- and downregulated miRNAs). The genes obtained from miRNet were then used to construct protein–protein interaction networks and identify their hub genes based on the degree of connectivity. The 10 hub genes linked to the upregulated hsa-miR-29c-3p were: *VEGFA*, *GAPDH*, *PTEN*, *JUN*, *MMP2*, *SIRT1*, *ITGB1*, *CDC42*, *COL1A2*, and *COL1A1*. The 10 hub genes linked to the downregulated miRNAs were: *AKT1*, *CCND1*, *VEGFA*, *PPP2CA*, *GSK3B*, *SP1*, *SMURF2*, *YY1*, *SNAI2*, and *YAP1* (Fig. 2). Subsequent KEGG pathway enrichment analysis revealed that the targets of the upregulated hsa-miR-29c-3p were enriched in many pathways, including those associated with focal adhesion ($p < 0.001$) and leukocyte transendothelial migration ($p = 0.009$). On the other hand, the targets of the downregulated miRNAs were enriched in pathways associated with transforming growth factor beta (TGF-β) signaling ($p = 0.004$) and various cancers ($p < 0.05$).

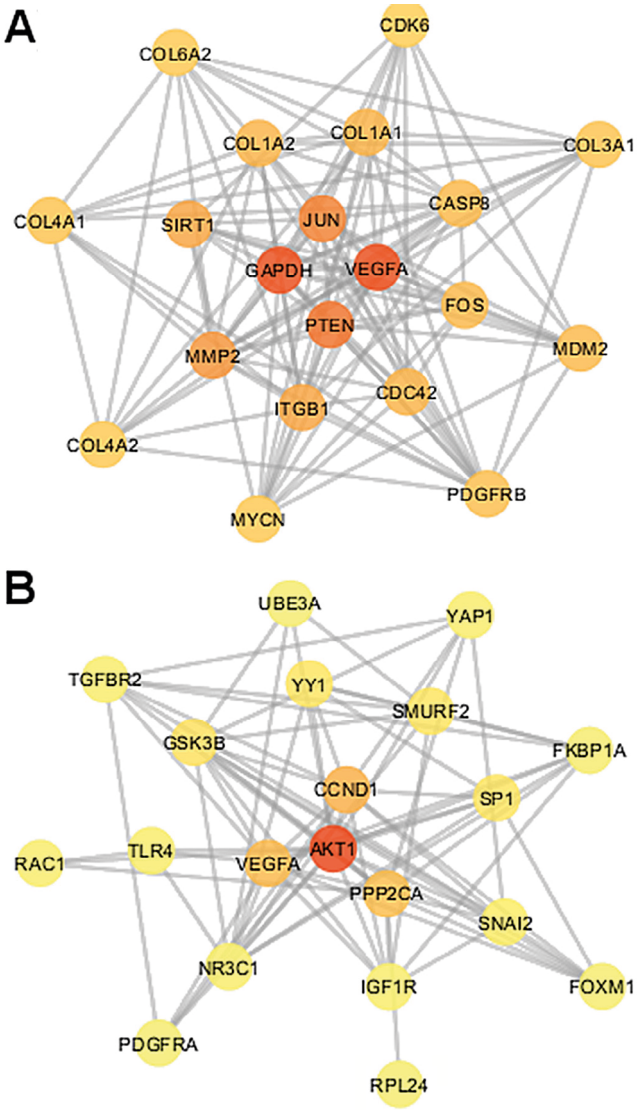

Fig. 2. Hub genes in the protein–protein interaction network. Twenty hub genes with the highest degree of connectivity in the protein–protein interaction networks of (A) upregulated microRNA (miRNA) target genes and (B) downregulated miRNA target genes. Data were analyzed using the method described by Lou et al.[17]

Discussion

In this study, we identified a set of miRNAs from plasma EVs (hsa-miR-29c-3p, hsa-miR-374b-5p and hsa-miR-630) that could potentially serve as diagnostic markers of cGvHD. Due to the importance of early diagnosis, various studies have been conducted to identify suitable biological markers of cGvHD, with most of them focusing on serum/plasma proteins. Much less is known about potential non-protein markers. Regarding miRNAs, it has been shown that miR-21 and the miR-17-92 cluster may be of importance in cGvHD.[22,23]

This study is the first to date to profile a wide spectrum of miRNAs differentially expressed in cGvHD EVs. Earlier studies have revealed that EVs can have a major influence on cGvHD.[15,24] The miRNA profiling studies have been conducted on aGvHD patients, although they did not focus on EV-derived miRNAs.[14] While they used different sample material, there was 1 miRNA (hsa-miR-374b-5p) that was differentially expressed in these aGvHD studies and also differentially expressed in our cGvHD EV samples;[14] both were downregulated. Earlier studies demonstrated that miR-374b-5p is associated mainly with nonhematological cancers, being upregulated in some and downregulated in others.[25,26] The miRNA hsa-miR-374b-5p was also shown to modulate the type-I interferon response.[27] The other miRNA we found to be upregulated in cGvHD, miR-630, was likewise reported to be overexpressed in some cancers,[28] while it suppressed the proliferation in others, including the interaction with the TGF-β signaling pathway.[29] Incidentally, TGF-β was found to have a major role in the development of cGvHD.[30,31] This seems to be consistent with our results, which suggest that the miRNAs found to be possibly downregulated in cGvHD may be implicated in TGF-β signaling and cancer pathways. It is, however, not known specifically how the downregulation of the aforementioned miRNAs could influence the onset of cGvHD.

The miRNA identified by our study as potentially upregulated in cGvHD, miR-29c-3p, has also been extensively studied in various other diseases. It was found to be downregulated in acute lymphoblastic leukemia.[32] Furthermore, many studies determined that miR-29c-3p suppresses proliferation, migration and cancer cell migration.[33–36] Likewise, earlier studies established migration-related CD146 expression on T cells to be elevated in patients with cGvHD.[37,38] This seems to be in accordance with the results of our analysis, which indicate that miR-29c-3p may be implicated in leukocyte transendothelial migration.

All the patients in the current study had prior hematological disorders of myeloid lineage, although the group was not entirely homogeneous; most patients had either acute myeloid leukemia or myelodysplastic syndrome, except for 1 non-cGvHD patient who had primary myelofibrosis. Reduced intensity conditioning was used in most patients before transplantation, although 2 patients (1 in each studied group) were administered myeloablative conditioning. For most patients, the transplantation was from a matched unrelated donor. The potential impact of these differences on miRNA profile is difficult to establish. This study compared a group of 3 patients who developed cGvHD with a group of 4 patients without cGvHD, with samples collected 3 months after the transplantation. The number of patients studied was quite low; therefore, the results presented here should be treated with caution and are subject to validation in larger cohorts of patients. Additionally, it should be noted that all 3 of the cGvHD patients and 2 non-cGvHD patients developed prior aGvHD symptoms. These symptoms indicated mild cGvHD (grade I) involving skin in the non-cGvHD group. In the cGvHD group, 1 patient developed grade IV aGvHD in the gut, and another patient developed grade III aGvHD in the liver. This aGvHD occurrence could have influenced our results, although only miR-374b-5p was expressed similarly in our study and in earlier non-EV aGvHD studies. The miRNAs reported earlier as aGvHD serum EV biomarkers did not coincide with the miRNAs detected by us as differentially expressed in cGvHD samples.[7] All patients except 1 were also affected by viral infections (cytomegalovirus (CMV) and BK virus (BKV)). Viruses are known to express their own miRNAs,[39] but little is known about the impact CMV/BKV infections have on host miRNA profiles. Cytomegalovirus infection is known to affect human miRNA expression in its early stage, although a study on infants with congenital CMV did not find any of the 3 miRNAs described in our study to be affected.[40,41]

Limitations

This study has many shortcomings. The most important is the low number of patients analyzed in both groups. Additionally, there are slight differences in the characteristics of the patients (as described in the Discussion section) that could have had an effect on the results.

Conclusions

This study suggests that hsa-miR-29c-3p, hsa-miR-374b-5p and hsa-miR-630 are differentially expressed in plasma EVs from cGvHD patients and may be considered diagnostic markers of this disease. However, due to the very small cohort of patients included and the presence of other potentially confounding factors, this should be regarded as a preliminary study, and its results should be confirmed on a larger group of patients.

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