Circulating MicroRNAs Do Not Provide a Diagnostic Benefit Over Tissue Biopsy in Patients With Brain Metastases

Michaela Ruckova
Department of Biochemistry, Faculty of Science, Masaryk University
Dagmar Al Tukmachi
Central European Institute of Technology, Masaryk University
Marek Vecera
Central European Institute of Technology, Masaryk University
Tereza Deissova
Central European Institute of Technology, Masaryk University
Marketa Hermanova
First Department of Pathology, St. Anne's University Hospital and Faculty of Medicine, Masaryk University
Michal Hendrych
First Department of Pathology, St. Anne's University Hospital and Faculty of Medicine, Masaryk University
Leos Kren
Department of Pathology, University Hospital Brno and Faculty of Medicine, Masaryk University
Vaclav Vybihal
Department of Neurosurgery, University Hospital Brno and Faculty of Medicine, Masaryk University
Pavel Fadrus
Department of Neurosurgery, University Hospital Brno and Faculty of Medicine, Masaryk University
Hana Valekova
Department of Neurosurgery, St. Anne's University Hospital and Faculty of Medicine, Masaryk University
Radim Jancalek
Department of Neurosurgery, St. Anne's University Hospital and Faculty of Medicine, Masaryk University
Tomas Kazda
Department of Radiation Oncology, Masaryk Memorial Cancer Institute
Martin Smrcka
Department of Neurosurgery, University Hospital Brno and Faculty of Medicine, Masaryk University
Ondrej Slaby
Department of Biology, Faculty of Medicine, Masaryk University

Jiri Sana

jiri.sana@med.muni.cz

Comprehensive Cancer Care Department, Masaryk Memorial Cancer Institute

Research Article

Keywords: brain metastases, circulating microRNAs, liquid biopsies, diagnostic potential, small RNA sequencing

Posted Date: November 1st, 2023

DOI: https://doi.org/10.21203/rs.3.rs-3511107/v1

Additional Declarations: No competing interests reported.

Abstract

Background: Brain metastases (BMs) are frequent and devastating complications of systemic malignancies, necessitating accurate diagnosis and origin identification for effective treatment strategies. Invasive biopsies are currently required for definitive diagnosis, highlighting the need for less invasive diagnostic approaches and robust biomarkers. Circulating microRNAs (miRNAs) have demonstrated potential as sensitive and specific diagnostic biomarkers in various cancers. Thus, our objective was to identify and compare miRNA profiles in BM tissue, cerebrospinal fluid (CSF), and plasma, with a specific focus on liquid biopsies for diagnostic purposes.

Methods: Total RNA enriched for miRNAs was isolated from histopathologically confirmed BM tissues (n=30), corresponding plasma samples (n=30), and CSF samples (n=27) obtained from patients with diverse BM types. Small RNA sequencing was employed for miRNA expression profiling.

Results: Significantly differentially expressed miRNAs were observed in BM tissues, enabling the differentiation of primary origins, particularly breast, colorectal, renal cell carcinoma, and melanoma metastases. The heterogeneity observed in lung carcinomas also manifested in the corresponding BMs, posing challenges in accurate discrimination from other BMs. While tissue-specific miRNA signatures exhibited the highest precision, our findings suggest low diagnostic potential of circulating miRNAs in CSF and blood plasma for BM patients.

Conclusions: Our study represents the first analysis of miRNA expression/levels in a unique set of three biological materials (tissue, blood plasma, CSF) obtained from the same BM patients using small RNA sequencing. The presented results underscore the importance of investigating aberrant miRNA expression/levels in BMs and highlight the low diagnostic utility of circulating miRNAs in patients with BMs.

1 Introduction

Brain metastases (BMs) represent a devastating complication of advanced cancer that affects approximately 30–40% of patients over the course of their illness. The increasing incidence of BMs can be attributed to several factors, such as advancements in cancer screening, diagnosis, and treatment, resulting in longer survival of the patients. In turn, the extended overall survival conceivably increases the likelihood of developing BM over time. Furthermore, the availability of more sophisticated imaging techniques has enabled clinicians to detect metastatic tumors in the brain at earlier stages. Additionally, certain cancer types possess a greater propensity to metastasize to the brain, contributing to the upward trend of BMs incidence (1, 2). BMs frequently arise from lung, breast and renal cell carcinoma, and melanoma. These types of cancer have been identified as common origins of BMs possibly due to their high incidence rates and ability to spread beyond their primary sites, however, almost any cancer can metastasize to the brain, including colorectal cancer (3). Also, primary site of cancer is not detected in up

to 15% of BM patients (4), and although sources vary slightly, they make up a non-negligible subgroup of BMs of unknown primary.

Due to the limitations of currently available methods, BM diagnostics remains challenging. Invasive procedures, such as tumor tissue biopsy, may not always be feasible or practical in all patients due to the location of the metastases or other clinical factors (5). Besides, the accuracy of biopsy depends on the size and location of the metastasis and may not always provide a representative sample of the tumor. Therefore, there is an urgent need for new diagnostic methods that are less invasive and more reliable. Liquid biopsies, especially blood plasma and cerebrospinal fluid (CSF), have emerged as promising alternatives for the diagnosis of many diseases including brain metastases. Liquid biopsies are less invasive than biopsy and can provide real-time information about tumor development and progression. Moreover, they allow for repeated sampling over time, which is particularly relevant for monitoring treatment response and disease recurrence (6).

Circulating microRNAs (miRNAs) are small non-coding RNAs that have been postulated as potential biomarkers providing valuable diagnostic and prognostic information for various diseases since they can be detected in body fluids. Several studies have investigated levels of miRNAs in the cerebrospinal fluid (CSF) of patients with brain tumors. For example, miR-21, which is known to promote tumor cell proliferation and invasion, has been shown to be elevated in the CSF of both brain metastasis and glioblastoma (GBM) patients compared to control samples (7). A previous study investigated CSF miRNA profiles in brain tumor patients and suggested strong potential for these molecules as prognostic and diagnostic biomarkers (8). Other findings outlined that certain miRNAs in the blood plasma have the potential to serve as new biomarkers for GBM and could be valuable in the clinical management of these patients. Specifically, plasma levels of miR-21, miR-128, and miR-342-3p were observed to be significantly altered in GBM patients compared to non-tumor controls (9). Diagnostic potential of tissue miRNAs for brain metastases have been investigated recently (10). Nevertheless, there is a need for further research to identify accurate and reliable biomarkers for the early detection of brain metastases. Comparing CSF and blood plasma, the former is considered to be a more suitable and cleaner option, as it is in direct contact with the brain and neural system and should reflect the actual tumor microenvironment. However, due to its invasiveness, its collection represents an additional burden for patients. On the other hand, circulating miRNAs in blood plasma tend to be more affected by various factors associated with preanalytical phase including hemolysis associated with highly abundant miR-16, or presence of erythroid-specific miRNAs, such as miR-486 or miR-451 (11, 12), which can lead to the waste of sequencing capacity and biased data generation, and consequently can affect the accuracy of the diagnosis.

Our aim was to identify miRNA profiles in tumor tissue, blood plasma, and CSF from BM patients and compare them. For this purpose, we used a unique set of biological specimens from BM patients and investigated their suitability for diagnosis of the 5 most frequent types of BMs. Our results contribute to the research in the field of new diagnostic tools that would pose less burden than the currently used biopsy, and at the same time help to refine and accelerate the diagnosis of these patients in the future.

2 Materials & Methods

2.1 Patient samples

Native BM tissue and peripheral blood samples from each patient were collected by cooperating neurosurgical departments of University Hospital Brno and St. Anne's University Hospital Brno (both Brno, Czech Republic). CSF samples were collected by the neurosurgical department of University Hospital Brno. Native BM tissue samples were collected during surgery as a part of the standard treatment protocol. Peripheral blood and CSF samples were collected for the purposes of diagnostics, and the aliquots were used for the study. The study and the informed consent form were approved by the research ethics committee of University Hospital Brno under the code EKFNB-17-06-28-01. A signed informed consent form was obtained from each patient prior to the beginning of all procedures and the collection of patient tissue, peripheral blood, and CSF samples. The study methodologies obeyed the standards set by the Declaration of Helsinki. In total, 30 fresh tissue samples were collected for the study and immediately stored in RNAlater Stabilization Solution (Thermo Fisher Scientific, Waltham, MA, USA) at 4˚C for 24 h after the collection and then frozen at − 80˚C until further use. All tissue samples were histologically diagnosed according to the WHO 2021 classification scheme independently by two histopathologists. Peripheral blood samples collected for the study were centrifuged (2000 × g, 10 min, 4˚C) immediately after collection to obtain blood plasma, which was separated, transferred to clean tubes, and then frozen at − 80˚C until further use. CSF samples collected for the study were immediately centrifuged (500 × g, 10 min, 4˚C) after the collection to separate higher-density particles and contaminants from the supernatant, which was then transferred to new tubes and frozen at − 80˚C until further use.

2.2 RNA isolation and purification

Total RNA enriched for small RNA species from fresh-frozen tissue samples was isolated using mirVana miRNA Isolation Kit (Thermo Fisher Scientific) according to the manufacturer's protocol as described in detail earlier (10). After thawing plasma samples on ice, 250 µl of the sample were transferred to a clean tube and centrifuged (1000 × g, 5 min, 4˚C). Subsequently, 200 µl of supernatant were used for isolation of total RNA enriched for small RNA species using the miRNeasy Serum/Plasma Kit (Qiagen, Hilden, Germany) according to the manufacturer's instructions. After thawing CSF on ice, 1 ml of sample was used for isolation and purification of total RNA enriched for small RNA species using the Urine microRNA Purification Kit (Norgen Biotek Corp., ON, Canada) according to the manufacturer's instructions. Purified RNA was then frozen at − 80˚C until further use.

2.3 Nucleic acid quantity and quality control

Nucleic acid quantity and quality control were conducted as described in detail earlier (10). Low concentrations of RNA isolated from liquid biopsies could not be measured by standard spectrophotometry or fluorometry, therefore, maximum volume was used as the input for the cDNA library preparation as recommended in the manufacturer's protocol.

2.4 Library preparation, pooling, and sequencing

Small RNA libraries were constructed from 30 total RNA samples from tissue, 30 from plasma and 27 from CSF using QIAseq miRNA Library Kit (Qiagen) according to the manufacturer's instructions. The RNA input was 100 ng for tissue and 5 µl for plasma and CSF. The preparation and cDNA libraries quantity and quality control were described in detail earlier (10). Subsequently, libraries were normalized and pooled in equimolar ratio using online weight to molar quantity converter. Library pools (24 libraries in each pool) were then processed according to the NextSeq System Denature and Dilute Libraries Guide (13). Denatured and diluted PhiX Control v3 was added at 1% to all pools as an internal standard and single-read sequencing with 75 bp read length was performed using NextSeq 500 Sequencing System and NextSeq 500/550 High Output v2 kit (75 cycles) (all Illumina, San Diego, CA, USA). Approximately 16.7 M sequencing reads per library were expected.

2.5 Processing of small RNA sequencing data

The pre-alignment quality control (QC) of the sequencing data was done using FastQC (version 0.11.9) (14). Adaptors present within sequenced reads were trimmed off with cutadapt (version 3.3) (15). Adapter-trimmed small RNA sequencing reads were collapsed exploiting unique molecular identifiers (UMIs) with FASTX-Toolkit (version 0.0.14) (16). Subsequently, reads were quality trimmed using cutadapt and reads shorter than 15 bp were removed from the dataset. The remaining reads were mapped against the database miRBase (version 21) (17) using the miraligner tool (version 3.2) (18). All generated numerical and graphical output from QC was gathered in cohesive reports via MultiQC (version 1.7) (19). All statistical analyses were performed in the R environment (version 4.0.4). Differential expression analysis was carried out using the Bioconductor (version 3.11) package DESeq2 (version 1.30.1) (20). When performing multiple comparisons, we performed adjustment using the Benjamini-Hochberg procedure. The complete linkage (farthest neighbor clustering) method with Manhattan distance measure was used for the unsupervised clustering. Results were summarized in heatmaps with dendrogram, column graphs, and PCA plots.

3 Results

3.1 A subset of microRNAs is highly expressed in tissues and present in high levels in plasma and cerebrospinal fluid samples of patients with brain metastasis

We successfully performed small RNA sequencing on 30 tissue, 30 plasma, and 27 CSF samples of BM patients and generated ~ 1.7 billion reads (16.9 ± 2.5 million per tissue sample, 21 ± 3.0 million per CSF sample, and 21 ± 4.2 million per plasma sample). Using miraligner, we identified 34.2 ± 9.2% of tissue sample reads, 6.8 ± 8.3% of plasma sample reads, and 1.9 ± 2.2% of CSF sample reads as miRNAs (Supplement 1). Bioinformatics analysis indicated fewer mapped miRNA reads in liquid biopsies. Moreover, we compared the highly abundant miRNAs in all samples (Fig. 1a), as well as in tissue (Fig. 1b), CSF (Fig. 1c), and plasma (Fig. 1d) samples separately. Highly expressed miRNAs/high miRNA levels were similar across all types of biopsies, with hsa-miR-16-5p and hsa-miR-21-5p being the most

frequently observed. Plasma samples showed significant contamination of erythropoietic miR-486-5p, which was also present in CSF and tissue samples but at lower levels. In addition to lower miR-486-5p contamination, tissue samples had lower overall percentage of highly expressed miRNAs (~ 22.4% for 2 most expressed miRNAs) compared to plasma (~ 38.9%) and CSF samples (~ 34%).

3.2 MiRNA expression profiles are origin-specific for tissue samples of patients with brain metastasis

We compared miRNA expression profiles in tissue samples of patients with BM and identified 451 miRNAs (Supplement 2) with adjusted p-value < 0.05, fold change (FC) ≥ 1.5, baseMean > 1, this level of significance was also used for all other comparisons in tissue samples. We performed 10 comparative analyses among 5 studied groups, from which we distinguished top 5 miRNAs and identified 28 unique miRNAs that displayed potential to be diagnostic biomarkers of BM with high sensitivity and specificity (Table 1 and Fig. 2a).

We then performed a differential analysis of global miRNA expression in tissue samples from BM patients and identified significantly differentially expressed miRNAs that could distinguish metastasis of a specific origin from all other BMs. For example, we compared miRNA expression levels in breast carcinoma BMs (BMBs) (Fig. 2b) with levels in the rest of BMs and identified 86 significantly differentially expressed miRNAs. Among these, 46 were downregulated and 40 were upregulated in BMB. Similarly, we compared miRNA expression levels in colorectal carcinoma BMs (BMCs), non-small cell lung carcinoma BMs (BMLs), melanoma BMs (BMMs), and renal clear cell carcinoma BMs (BMRs) with miRNA expression levels in the rest of BMs and found significantly differentially expressed miRNAs in each case. Specifically, we identified 166 significantly differentially expressed miRNAs in BMC (Fig. 2c), of which 93 were lowly expressed and 73 were highly expressed in BMC. We also found 82 significantly differentially expressed miRNAs in BML (Fig. 2d), of which 53 miRNAs had low expression and 29 miRNAs had high expression. In BMM, 80 significantly differentially expressed miRNAs were identified (Fig. 2e), with 45 being downregulated and 35 being upregulated. Finally, in BMR (Fig. 2f), we identified 64 significantly differentially expressed miRNAs, with 35 being lowly expressed and 29 being highly expressed. All miRNAs that showed significant differential expression can be found in Supplement 2.

Table 1
A diagnostic table based on the top 5 significantly differentially expressed miRNAs from all comparisons (28 unique miRNAs identified in total), with sensitivity and specificity values calculated for every type of BM. The correctly classified cases are highlighted in green.

Origin of the primary tumor	BMB	BMC	BML	BMM	BMR
BMB	5	0	1	0	1
BMC	1	6	1	0	0
BML	0	0	3	0	0
BMM	0	0	1	6	1
BMR	0	0	0	0	4
Sensitivity (%)	83.3	100.0	50.0	100.0	66.7
Specificity (%)	71.4	75.0	100.0	75.0	100.0

3.3 MiRNA level profiles do not show significant specificity in CSF samples of patients with brain metastases

Next, we compared miRNA levels in CSF samples from 27 patients with BMs originating in the 5 most frequent primary tumor types (Fig. 3a) and found that we could distinguish one type of BM from other BMs based on origin-specific miRNAs only to a limited extent. We observed significant differences in the levels of 153 miRNAs (Supplement 2) in the 5 BM types with p-value < 0.05, FC ≥ 1.5, baseMean ≥ 1. We also refer to this cut-off value of significance level in all subsequent comparisons in CSF samples.

After comparing miRNA levels in CSF samples of BMB patients with those in CSF of patients with other BMs, we identified 17 significantly differentially expressed miRNAs, with 7 showing decreased levels and 10 showing increased levels in CSF of BMB patients (Fig. 3b). Similarly, when we compared miRNA levels in CSF samples of BMC patients with CSF of patients with other BMs, we found 34 miRNAs with significantly different levels, with 11 present in high levels and 23 in low levels in CSF of BMC patients (Fig. 3c). In the case of BML patients, we identified 20 miRNAs with significantly different levels, with 14 showing decreased amounts and 6 showing increased amounts in CSF of BML patients compared to CSF of patients with other BMs (Fig. 3d). We also observed significantly different levels of 61 miRNAs in CSF samples of BMM patients compared to CSF of patients with other BMs, with 43 downregulated and 18 upregulated in CSF of BMM patients (Fig. 3e). Finally, when we compared CSF samples of BMR patients with CSF samples of patients with other BMs, we found 19 miRNAs with significantly changed levels, with 15 showing reduced levels and 4 showing increased levels in CSF of BMR patients (Fig. 3f). All miRNAs with significantly different expression levels can be found in Supplement 2.

3.4 MiRNA level profiles do not show significant specificity in blood plasma samples of patients with brain metastases

MiRNA levels were compared in plasma samples of 30 patients with BM originating in the 5 most frequent types of primary tumors. However, it was only possible to distinguish BM of a specific origin from other BMs to a very limited extent (Fig. 4a). We identified 155 miRNAs (Supplement 2) with significantly different levels in the 5 most frequent BM types with p-value < 0.05, FC ≥ 1.5, baseMean ≥ 1. This cut-off of significance level was used for all other comparisons of plasma samples.

Firstly, plasma samples of BMB patients were compared with plasma samples from patients with all other types of BM (Fig. 4b), identifying 20 miRNAs, including 12 miRNAs with reduced expression and 8 miRNAs with increased levels in plasma from BMB patients. Plasma samples of BMC patients had 44 miRNAs with significantly different expression levels compared to plasma samples from patients with other types of BMs; specifically, 25 miRNAs with decreased and 19 miRNAs with increased expression levels in plasma from BMC patients (Fig. 4c). Similarly, plasma samples of BML patients were compared with plasma samples from patients with other BMs, identifying 24 miRNAs present in significantly different levels, with 14 miRNAs found in reduced levels and 10 miRNAs having increased level in plasma from BML patients (Fig. 4d). After comparing plasma samples of BMM patients with plasma samples from patients with other BMs, 15 miRNAs showed significantly different levels, including 10 miRNAs with reduced and 5 miRNAs with elevated levels in plasma from BMM patients (Fig. 4e). Lastly, plasma samples of BMR patients were compared with plasma samples from patients with other BMs, leading to identification of 56 miRNAs with significantly different plasma levels. Specifically, 26 miRNAs were found in decreased levels and 30 miRNAs in increased levels in plasma from BMR patients (Fig. 4f). All miRNAs with significantly different levels can be found in Supplement 2.

3.5 Analysis of blood plasma samples from patients with brain metastasis, colorectal carcinoma, and healthy controls reveals similarities between primary tumor and metastasis

To determine if the miRNA levels in plasma of BM patients are influenced by the primary tumor, we performed a differential analysis of miRNAs in plasma samples from BMC patients, CRC patients, and healthy controls. Although the principal component analysis (PCA) did not separate groups when comparing all metastatic plasma samples with primary CRC and healthy control samples, similarities in miRNA levels were found in plasma samples from primary CRC and BM patients compared to healthy controls (Fig. 5). Similar plasma levels of 32% of all miRNAs with significantly different expression (p.adj < 0.05, FC ≥ 1.5, baseMean ≥ 1) were found in comparisons of primary CRC samples vs. healthy control samples and BMC samples vs. healthy control samples (Supplement 3).

4 Discussion

BMs are a common occurrence in advanced stages of primary solid cancers and often have a poor prognosis. The incidence of BMs has risen, possibly due to improved diagnostics and therapy methods that extend patient survival but also provide more opportunities for cancer cells to metastasize to the brain. Despite recent progress in BM treatment and imaging, the outlook for patients remains poor. Accurate and early diagnosis of the BM origin is crucial for tailoring adequate therapy to improve

patients' prognosis. Particularly for those with BMs that are poorly accessible to biopsy, diagnosis to differentiate primary tumor from BM and determine the origin can be arduous (21, 22). Therefore, it is crucial to identify new molecular biomarkers for precise diagnosis of BMs using less invasive liquid biopsies.

The goal of our study was to uncover specific miRNA patterns in tissue, CSF or blood plasma that can distinguish BMs in patients, compare these biological materials with respect to their suitability for use in the early diagnosis and find a diagnostic approach to accurately diagnose BMs with less invasive procedures than tissue biopsy. By doing so, treatment plans could be made in advance, improving patients' quality of life. Based on the fact that tissue miRNAs were previously found to be useful in classifying BMs, we hypothesized that CSF, as a biological fluid unique to the central nervous system (CNS), would be an even better option for miRNA detection since miRNAs have been proven to be stable in this body fluid (10). Additionally, CSF washes only the CNS, and, unlike blood plasma, is in direct contact with the tumor microenvironment. It should, therefore, contain fewer non-specific miRNAs compared to blood plasma or serum, making it less vulnerable to contamination and more tissue specific (8).

Based on our results, tissue miRNAs in patients with BMs could discriminate the individual analyzed BMs with a sensitivity of 50.0–100.0% and specificity of 71.4–100.0% (Table 1). The lowest sensitivity was observed in BML, which corresponds with high heterogeneity of histological types (23). These findings and identified miRNA profiles are consistent with the results of Roskova *et al.* (10). However, biopsy and subsequent histopathological examination is not always indicated due to the fragility of cancer patients and the high invasiveness of the surgical procedure or the BM localization. For these reasons, using small RNA sequencing, we analyzed and compared 2 types of liquid biopsies from BM patients, which are less invasive than needle or conventional biopsy. MiRNAs possess favorable biochemical properties that make them ideal candidates as they are easily accessible indicators from a technical perspective. These small transcripts exhibit high stability and have a prolonged half-life in biological samples, thereby eliminating the need for specialized handling. Moreover, miRNA analysis can be applied to readily available samples and quantified using standard techniques that are already employed in clinical laboratories, such as quantitative PCR, at a relatively low cost with high sensitivity and specificity. Nevertheless, the identification of new circulating miRNA biomarkers is challenging due to multiple factors.

The profiling of circulating miRNAs in peripheral blood has attracted significant attention as potential diagnostic and prognostic biomarkers for various diseases. However, it is important to note that the presence of non-specific miRNAs in peripheral blood can lead to a bias in data interpretation and other undesirable consequences. The two most prevalent miRNAs in human peripheral blood are miR-16-5p and miR-486-5p, both of which belong to the erythroid-specific group. Comparison of the potential use of serum instead of plasma for translational studies was performed by Dufourd *et al.*, however, non-specific miRNAs were present at similar levels in both materials, and, in addition, plasma performed better with respect to overall sequencing data yield (24). Regarding the purity of the different types of biopsies

analyzed in our present study with respect to contaminating miRNAs, the plasma samples were highly contaminated compared to CSF and tissue (Fi g. 1). Their high abundance in small RNA sequencing libraries can have negative impact, such as reducing the diversity and complexity of the sequencing libraries, leading to difficulty in detecting and accurately measuring less abundant miRNAs (25, 26). Furthermore, we observed more similar expression profiles among highly expressed miRNAs in tissue and miRNA levels in CSF samples compared to plasma samples. This result supports our hypothesis of the possible use of patients' CSF for the diagnosis of BMs. Overall, based on these data, we can consider CSF as a biological material less contaminated by non-specific miRNAs. This could be presumably caused by the fact that CSF surrounds only CNS, whereas peripheral blood circulates throughout the body and thus presumably contains more non-specific molecules. On the other hand, we observed overall higher levels of possible contaminants in plasma and CSF samples compared to tissue samples and neither miRNA level profiles in CSF nor plasma did not show significant specificity in samples from patients with brain metastases.

In CSF of BM patients, we found miRNAs with different levels characterized by a low diagnostic potential (Fig. 3a). In plasma, identified miRNAs showed no potential to serve as diagnostic biomarkers (Fig. 4a). In previous study, we demonstrated that miRNA profiles in CSF can distinguish between different brain tumors (8), however, we could not confirm this finding within the BMs. Nevertheless, we were able to determine the specificity and sensitivity with which the identified miRNAs can classify BM patients into diagnostic groups, whereas this could not be determined for plasma indicating further complications for potential use of this body fluid in clinical practice. To conclude, our data show that although we are able to discriminate individual BM types with limited specificity and sensitivity within a larger number of miRNAs based on their CSF level profile, this would probably not be possible within a potential diagnostic panel with a limited number of miRNAs. The use of plasma based on our data is not an option at all, as miRNA profiles showed no diagnostic potential even when using a large number of molecules. Nonetheless, these findings would need further confirmation or refutation in a larger cohort of patients.

We also investigated how the miRNA levels in plasma of BM patients originating in colorectal cancer may be influenced by the primary tumor or whether they are specifically indicative of the BM. It is evident from Fig. 5 that much more similarity was observed between the miRNA profiles corresponding to colorectal cancer and the BMs derived from this cancer compared to healthy controls. These observations would preclude the use of small RNA sequencing of plasma from patients with suspected BM as a diagnostic tool since it could not distinguish between primary tumor and the metastasis, nevertheless, these data may be limited to a certain extent by a possible batch effect between sequencing runs. Although it was not possible to make the same comparison for CSF in this study, as we did not have available samples adequate for this analysis, we suggest this to be explored in future research.

Recent advances in next-generation sequencing (NGS) technology have revolutionized the field of miRNA research, allowing for the identification of thousands of miRNAs simultaneously. NGS can provide high-throughput and comprehensive profiling of miRNAs in biological samples, including plasma and CSF. Moreover, the use of bioinformatics tools for data analysis and interpretation can aid in the identification

of miRNA biomarkers with high sensitivity and specificity for BMs. Furthermore, the identification of circulating miRNAs with prognostic and predictive value could improve personalized treatment strategies for BM patients. However, as far as the miRNA profiling workflow is concerned, there is no standardized protocol so far, which complicates the achievement of final outputs and causes variability in the findings. This is particularly a problem for circulating miRNAs from liquid biopsies, which are present generally in low levels in body fluids. Low RNA input can lead to an increased proportion of adapter dimer and non-miRNA reads, while decreasing the number of reads that are mapped to miRNAs. This outcome requires a higher raw sequencing depth to compensate for the loss of miRNA reads. Moreover, a greater amount of contaminating RNA molecules from exogenous RNAs is frequently observed in low input samples, resulting in the detection of numerous non-target reads. In a recent study, Wong *et al.* demonstrated significant differences in the detection of individual miRNAs and their representation in sequencing data depending on the kit used for RNA extraction and subsequent preparation of cDNA libraries. Based on their data, it can be concluded that a consensus of the scientific community and the establishment of a standard protocol for miRNA profiling and subsequent bioinformatic evaluation of the obtained data is needed before routine use of miRNAs as biomarkers in clinical practice (27). Factors that affect their secretion into body fluids may also be problematic for the use of miRNAs. These include dynamic changes in their levels within a day or disease phase (28), but also factors related to the patients themselves such as gender, age, diet, smoking/non-smoking or physical activity (29–31). In the context of research aimed at analyzing and profiling miRNAs, patients should then correspond not only by diagnosis but also by the aforementioned factors, which is not always easy to do, especially in less common diseases such as BMs, which are associated with a very limited number of rare samples overall. The advantage is the relatively high level of miRNA stability even with long-term freezing, evidenced by the study of Balzano *et al.*, who observed that miRNAs were detectable in the sample even within 14 years. However, their levels decreased with time, so it is advisable to use only fresh samples frozen for a maximum of 1 year for biomarker studies, which is often not achievable in practice and, again, may cause bias within the available data (32).

Since high-throughput sequencing technologies have become ubiquitous, the predicted number of miRNAs has skyrocketed. However, the validation of the results from exploratory phase of biomarker studies using qPCR poses many pitfalls as far as miRNAs are concerned. The lack of a validated intrinsic miRNA control in CSF/plasma remains a point of contention in the analysis of circulating miRNAs. Although 5S, U6, and other snoRNAs are commonly used as intrinsic controls for miRNA analysis in tissues or cells, their instability in serum/plasma from nuclear or cytosolic compartments renders them unsuitable for this purpose (33, 34). This absence of a validated intrinsic control has impeded research into the use of circulating miRNAs as biomarkers. Nevertheless, certain endogenous miRNAs, such as miR-15b, miR-16, and miR-24, have been proposed as suitable intrinsic controls for circulating miRNA analysis (35). Finally, miRNA isoforms (isomiRs) must also be considered, as they may not always be distinguishable from each other by conventional methods and may cause inconsistencies both in the evaluation of sequencing data and especially in the validation by qPCR (36). Various qPCR-based miRNA

profiling platforms are being developed to address some of the associated bottlenecks (37), but to date there is no standardized procedure for validating circulating miRNAs in CSF or plasma.

Our findings indicate that the diagnostic value of miRNAs in liquid biopsies is low and remains to be further studied. Although aberrant levels of some circulating miRNAs are clearly associated with different BMs, given the relatively small patient cohort size and the limited number of patient groups included in our study, we suggest that further research is necessary to validate or refute these results. In particular, the study should be extended to a larger cohort with a higher number of patients and, possibly, more diverse groups of BMs. Such an investigation would provide a more comprehensive assessment of the diagnostic potential of circulating miRNAs in CSF and plasma for BMs and may lead to the identification of novel biomarkers that could improve diagnosis and, ultimately, patient outcomes. Although the use of liquid biopsies, especially CSF, would provide many advantages for the diagnosis of patients not only with BMs, such as lower invasiveness, the possibility of serial sampling and monitoring the dynamics of the disease course or earlier and faster diagnosis, it is necessary to standardize the methodology and solve some technical pitfalls associated with the analysis of circulating miRNAs. The potential of combining small RNA analysis of liquid biopsies with other diagnostic tools, such as imaging and clinical assessments, should also be investigated to improve the accuracy of BM diagnosis.

5 Conclusion

We performed a high-throughput analysis of global tissue miRNA expression and miRNA levels in CSF and blood plasma of BM patients using small RNA sequencing to reveal the potential of circulating miRNAs to serve as diagnostic biomarkers and thereby improve the management of these patients. The uniqueness of this study lies primarily in the patient sample sets, with nearly every BM patient having all three types of biological material analyzed – 6 patients from each of the 5 BM origins (non-small cell lung carcinoma, breast carcinoma, colorectal carcinoma, melanoma, and renal clear cell carcinoma). We described significant differences in tissue miRNA expression and CSF and plasma miRNA levels in patients with all analyzed BM types. We compared these three biological materials, using evidence to describe the advantages of using CSF as a possible diagnostic material for BM patients compared to plasma. However, based on our results, we found that miRNAs circulating in CSF may have low diagnostic potential, and for future research, we proposed to extend the study to a larger number of patient samples to confirm or refute these findings.

Abbreviations

BM
Brain Metastasis
BMB
Breast Carcinoma Brain Metastasis
BMC
Colorectal Carcinoma Brain Metastasis

BML
Non–small Cell Lung Carcinoma Brain Metastasis
BMM
Melanoma Brain Metastasis
BMR
Renal Clear Cell Carcinoma Brain Metastasis
cDNA
Complementary Deoxyribonucleic Acid
CSF
Cerebrospinal Fluid
FC
Fold Change
GBM
Glioblastoma
miRNA
MicroRNA
PCA
Principal Component Analysis
QC
Quality Control
qPCR
Quantitative Polymerase Chain Reaction
RNA
Ribonucleic Acid
SD
Standard Deviation
UMI
Unique Molecular Identifier
WHO
World Health Organization

Declarations

Ethics approval and consent to participate

Native BM tissue and peripheral blood samples from each patient were collected by cooperating neurosurgical departments of University Hospital Brno and St. Anne's University Hospital Brno (both Brno, Czech Republic). CSF samples were collected by the neurosurgical department of University Hospital Brno. Native BM tissue samples were collected during surgery as a part of the standard treatment protocol. Peripheral blood and CSF samples were collected for the purposes of diagnostics, and the aliquots were used for the study. The study and the informed consent form were approved by the

research ethics committee of University Hospital Brno under the code EKFNB-17-06-28-01. A signed informed consent form was obtained from each patient prior to the beginning of all procedures and the collection of patient tissue, peripheral blood, and CSF samples. The study methodologies obeyed the standards set by the Declaration of Helsinki.

Consent for publication

Not applicable

Competing interests

The authors declare that they have no conflict of interest.

Availability of data and materials

The data that support the findings of this study are available from the corresponding author upon reasonable request.

Funding

This work was financially supported by the Ministry of Health of the Czech Republic (grant project nr. NV18-03-00398), by the Ministry of Education, Youth, and Sports of the Czech Republic (MEYS CR, program EXCELES; grant projects nr. LX22NPO5102 and LX22NPO5107; financed by the European Union – Next Generation EU), and conceptual development of research organizations (Masaryk Memorial Cancer Institute, 00209805; University Hospital Brno, 65269705). The funders had no role in the design of the study, the collection, analysis, and interpretation of data, and in writing of the manuscript. We gratefully acknowledge the Core Facility Genomics, supported by the NCMG research infrastructure (project LM2018132 funded by MEYS CR), and Core Facility Bioinformatics of CEITEC Masaryk University for their support with obtaining of the scientific data presented in this paper.

Authors' Contributions

JS, MS, and RJ conceptualized the study and acquired funding. RJ, MHer, MS, and OS supervised the study. JS took care of the project administration. MV, JS, and OS devised the methodology used in the study. RJ, HV, MHer, MHen, VV, PF and MS provided the patient samples. MR, DA, MV, and TD performed experimental investigations, including RNA isolation, quality and quantity control, library preparation, small RNA sequencing, bioinformatics processing of measured data, and statistical analyses. MHen, VV, LK, and HV curated all clinicopathological and experimental data. DA performed the data visualization. MR and DA wrote the original draft. JS, OS, TK, and MS revised the original draft. All Authors read and approved the manuscript.

Acknowledgements

Not applicable

References

1. Ostrom QT, Wright CH, Barnholtz-Sloan JS. Brain metastases: epidemiology. Handb Clin Neurol. 2018. 10.1016/B978-0-12-811161-1.00002-5.
2. Kotecha R, Gondi V, Ahluwalia MS, Brastianos PK, Mehta MP. Recent advances in managing brain metastasis. F1000Res. (2018). 10.12688/f1000research.15903.1.
3. Schouten LJ, Rutten J, Huveneers HAM, Twijnstra A. Incidence of brain metastases in a cohort of patients with carcinoma of the breast, colon, kidney, and lung and melanoma. Cancer. 2002. 10.1002/cncr.10541.
4. Polyzoidis KS, Miliaras G, Pavlidis N. Brain metastasis of unknown primary: a diagnostic and therapeutic dilemma. Cancer Treat Rev. 2005. 10.1016/j.ctrv.2005.03.006.
5. Carnevale JA, Imber BS, Winston GM, Goldberg JL, Ballangrud A, Brennan CW, et al. Risk of tract recurrence with stereotactic biopsy of brain metastases: an 18-year cancer center experience. J Neurosurg. 2022. 10.3171/2021.3.JNS204347.
6. Rehman AU, Khan P, Maurya SK, Siddiqui JA, Santamaria-Barria JA, Batra SK, et al. Liquid biopsies to occult brain metastasis. Mol Cancer. 2022. 10.1186/s12943-022-01577-x.
7. Teplyuk NM, Mollenhauer B, Gabriely G, Giese A, Kim E, Smolsky M, et al. MicroRNAs in cerebrospinal fluid identify glioblastoma and metastatic brain cancers and reflect disease activity. Neuro Oncol. 2012. 10.1093/neuonc/nos074.
8. Kopkova A, Sana J, Machackova T, Vecera M, Radova L, Trachtova K et al. Cerebrospinal Fluid MicroRNA Signatures as Diagnostic Biomarkers in Brain Tumors. Cancers (2019). 10.3390/cancers11101546.
9. Wang Q, Li P, Li A, Jiang W, Wang H, Wang J, et al. Plasma specific miRNAs as predictive biomarkers for diagnosis and prognosis of glioma. J Exp Clin Cancer Res. 2012. 10.1186/1756-9966-31-97.
10. Roskova I, Vecera M, Radova L, Trachtova K, Siegl F, Hermanova M, et al. Small RNA Sequencing Identifies a Six-MicroRNA Signature Enabling Classification of Brain Metastases According to their Origin. Cancer Genomics Proteomics. 2023. 10.21873/cgp.20361.
11. Kirschner MB, Edelman JJB, Kao SCH, Vallely MP, Van Zandwijk N, Reid G. The impact of hemolysis on cell-free microRNA biomarkers. Front Genet. 2013. 10.3389/fgene.2013.00094.
12. Juzenas S, Lindqvist CM, Ito G, Dolshanskaya Y, Halfvarson J, Franke A, et al. Depletion of erythropoietic miR-486-5p and miR-451a improves detectability of rare microRNAs in peripheral blood-derived small RNA sequencing libraries. NAR Genom Bioinform. 2020. 10.1093/nargab/lqaa008.
13. NextSeq 500. and 550 System Denature and Dilute Libraries Guide [Internet]. https://emea.support.illumina.com/downloads/nextseq-500-denaturing-diluting-libraries-15048776.html Accessed 14 August 2023.
14. Babraham Bioinformatics -. FastQC A Quality Control tool for High Throughput Sequence Data [Internet]. https://www.bioinformatics.babraham.ac.uk/projects/fastqc/ Accessed 14 August 2023.

15. Martin M. Cutadapt removes adapter sequences from high-throughput sequencing reads. EMBnet J. 2011. 10.14806/ej.17.1.200.
16. FASTX-Toolkit [Internet]. http://hannonlab.cshl.edu/fastx_toolkit/ Accessed 14 August 2023.
17. Griffiths-Jones S, Grocock RJ, van Dongen S, Bateman A, Enright AJ. miRBase: microRNA sequences, targets and gene nomenclature. Nucleic Acids Res. 2006. 10.1093/nar/gkj112.
18. Pantano L, Estivill X, Martí E. SeqBuster, a bioinformatic tool for the processing and analysis of small RNAs datasets, reveals ubiquitous miRNA modifications in human embryonic cells. Nucleic Acids Res. 2010. 10.1093/nar/gkp1127.
19. Ewels P, Magnusson M, Lundin S, Käller M. MultiQC: summarize analysis results for multiple tools and samples in a single report. Bioinformatics. 2016. 10.1093/bioinformatics/btw354.
20. Love MI, Huber W, Anders S. Moderated estimation of fold change and dispersion for RNA-seq data with DESeq2. Genome Biol. 2014. 10.1186/s13059-014-0550-8.
21. Lam TC, Sahgal A, Chang EL, Lo SS. Stereotactic radiosurgery for multiple brain metastases. Expert Rev Anticancer Ther. 2014. 10.1586/14737140.2014.940325.
22. Tong E, McCullagh KL, Iv M. Advanced Imaging of Brain Metastases: From Augmenting Visualization and Improving Diagnosis to Evaluating Treatment Response. Front Neurol. 2020. 10.3389/fneur.2020.00270.
23. de Sousa VML, Carvalho L. Heterogeneity in Lung Cancer Pathobiology. 2018. 10.1159/000487440.
24. Dufourd T, Robil N, Mallet D, Carcenac C, Boulet S, Brishoual S, et al. Plasma or serum? A qualitative study on rodents and humans using high-throughput microRNA sequencing for circulating biomarkers. Biol Methods Protoc. 2019. 10.1093/biomethods/bpz006.
25. Juzenas S, Lindqvist CM, Ito G, Dolshanskaya Y, Halfvarson J, Franke A, et al. Depletion of erythropoietic miR-486-5p and miR-451a improves detectability of rare microRNAs in peripheral blood-derived small RNA sequencing libraries. NAR Genom Bioinform. 2020. 10.1093/nargab/lqaa008.
26. Smith MD, Leemaqz SY, Jankovic-Karasoulos T, McAninch D, McCullough D, Breen J, et al. Haemolysis Detection in MicroRNA-Seq from Clinical Plasma Samples. Genes (Basel). 2022. 10.3390/genes13071288.
27. Wong RKY, MacMahon M, Woodside JV, Simpson DA. A comparison of RNA extraction and sequencing protocols for detection of small RNAs in plasma. BMC Genomics. 2019. 10.1186/s12864-019-5826-7.
28. Samandari N, Mirza AH, Kaur S, Hougaard P, Nielsen LB, Fredheim S, et al. Influence of Disease Duration on Circulating Levels of miRNAs in Children and Adolescents with New Onset Type 1 Diabetes. Noncoding RNA. 2018. 10.3390/ncrna4040035.
29. Duttagupta R, Jiang R, Gollub J, Getts RC, Jones KW. Impact of Cellular miRNAs on Circulating miRNA Biomarker Signatures. PLoS ONE. 2011. 10.1371/journal.pone.0020769.

30. MaClellan SA, Macaulay C, Lam S, Garnis C. Pre-profiling factors influencing serum microRNA levels. BMC Clin Pathol. 2014. 10.1186/1472-6890-14-27.
31. Liang H, Zhang S, Fu Z, Wang Y, Wang N, Liu Y, et al. Effective detection and quantification of dietetically absorbed plant microRNAs in human plasma. J Nutr Biochem. 2015. 10.1016/j.jnutbio.2014.12.002.
32. Balzano F, Deiana M, Giudici SD, Oggiano A, Baralla A, Pasella S, et al. miRNA Stability in Frozen Plasma Samples. Molecules. 2015. 10.3390/molecules201019030.
33. Benz F, Roderburg C, Cardenas DV, Vucur M, Gautheron J, Koch A, et al. U6 is unsuitable for normalization of serum miRNA levels in patients with sepsis or liver fibrosis. Exp Mol Med. 2013. 10.1038/emm.2013.81.
34. Xiang M, Zeng Y, Yang R, Xu H, Chen Z, Zhong J, et al. U6 is not a suitable endogenous control for the quantification of circulating microRNAs. Biochem Biophys Res Commun. 2014. 10.1016/j.bbrc.2014.10.064.
35. Mitchell PS, Parkin RK, Kroh EM, Fritz BR, Wyman SK, Pogosova-Agadjanyan EL et al. Circulating microRNAs as stable blood-based markers for cancer detection. Proc. Natl. Acad. Sci. U. S. A. (2008). 10.1073/pnas.0804549105.
36. Avendaño-Vázquez SE, Flores-Jasso CF. Stumbling on elusive cargo: how isomiRs challenge microRNA detection and quantification, the case of extracellular vesicles. J Extracell Vesicles. 2020. 10.1080/20013078.2020.1784617.
37. Blondal T, Jensby Nielsen S, Baker A, Andreasen D, Mouritzen P, Wrang Teilum M, et al. Assessing sample and miRNA profile quality in serum and plasma or other biofluids. Methods. 2013. 10.1016/j.ymeth.2012.09.015.

Figures

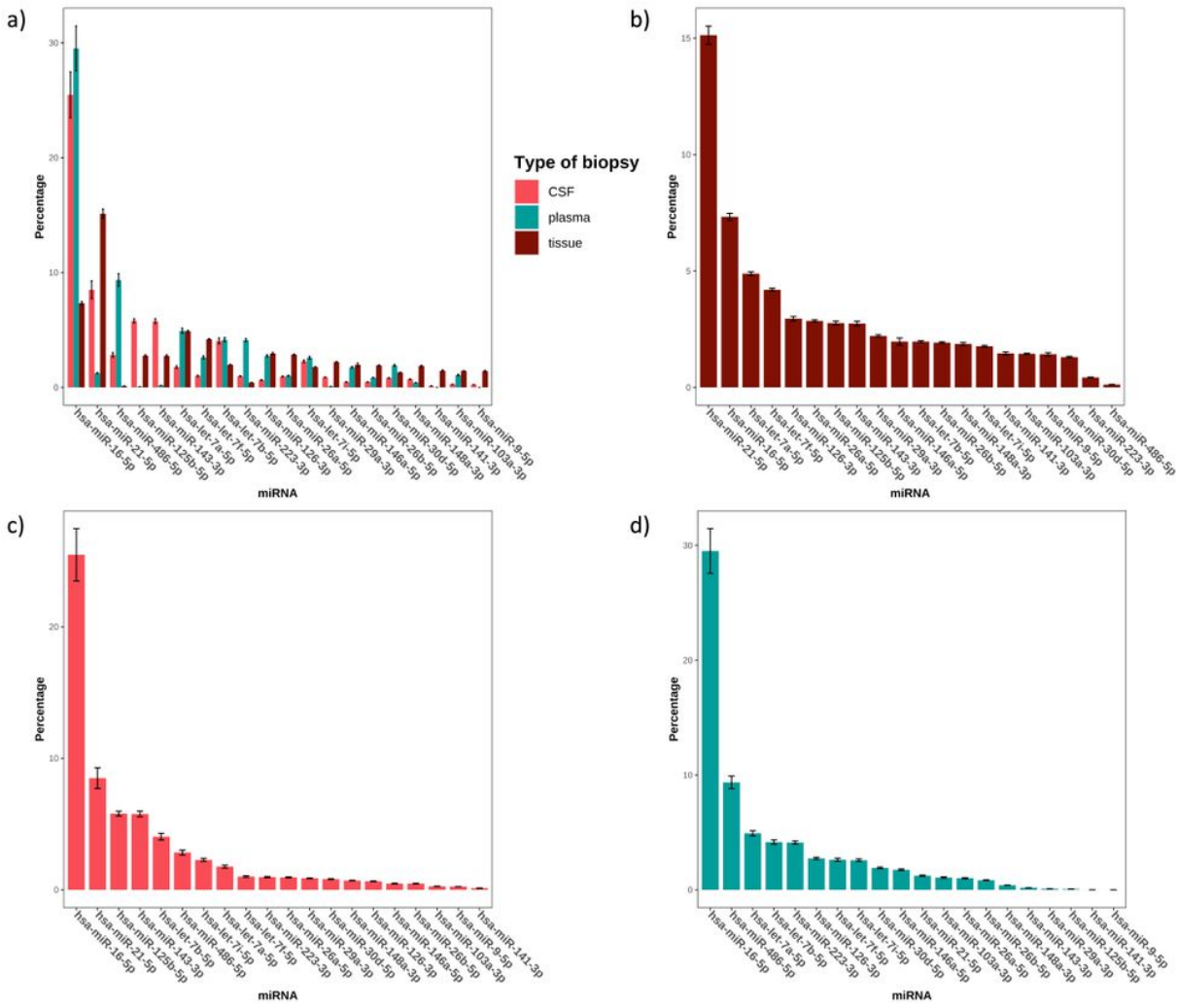

Figure 1

Column graphs representing the percentage of most abundant miRNAs in a) all analyzed samples, b) tissue samples, c) cerebrospinal fluid samples (CSF), d) blood plasma samples (mean ± SD).

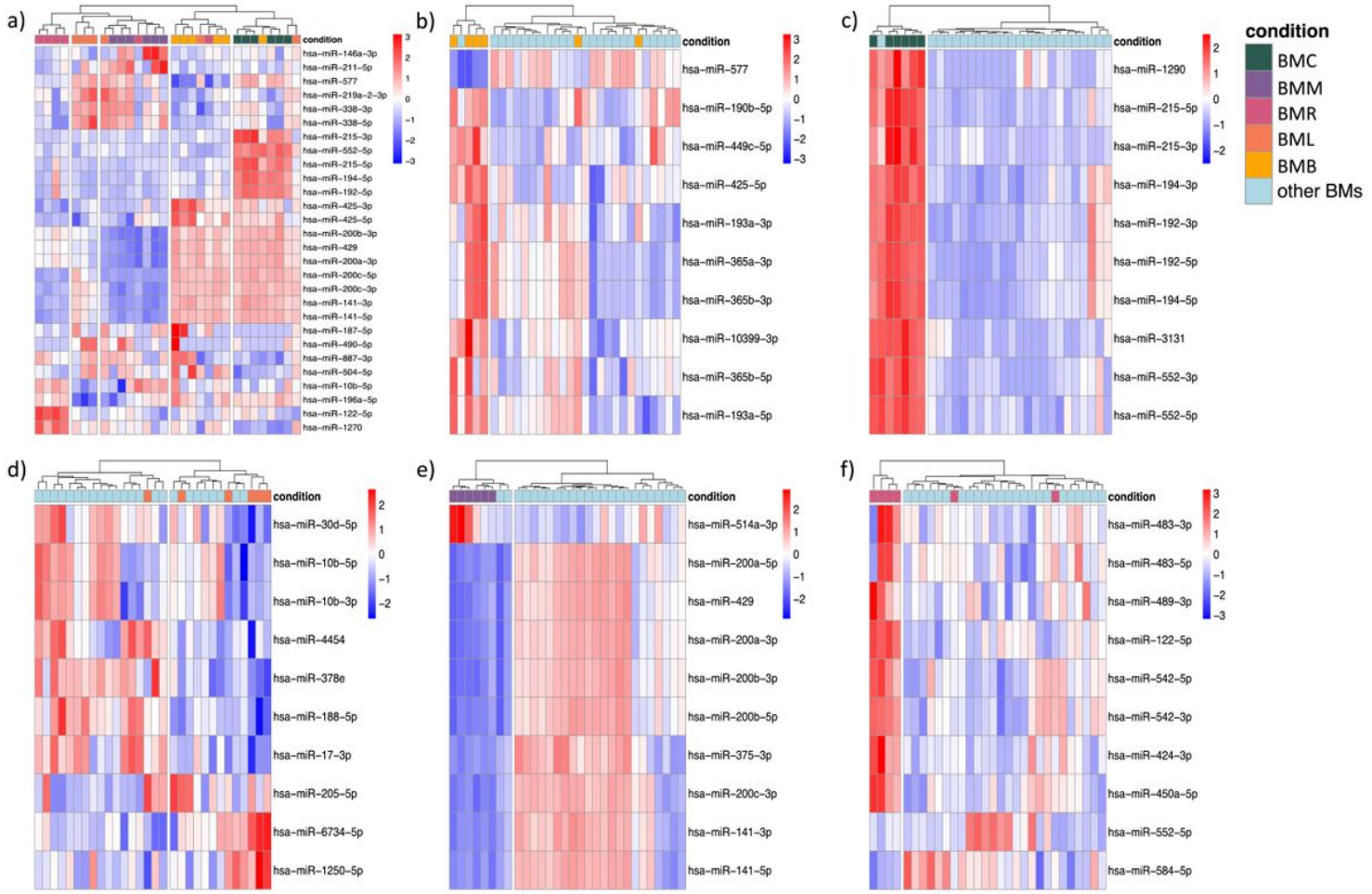

Figure 2

Heatmaps with unsupervised hierarchical clustering based on the expression of: a) 28 unique miRNAs acquired from top 5 miRNAs with adjusted p-value<0.05, FC≥1.5, and baseMean≥1 for each comparison; b-f) top 10 miRNAs with adjusted p-value<0.05, FC≥1.5, and baseMean≥1 of each metastatic origin compared to all other metastatic origins derived from tissue samples of patients with b) breast carcinoma (BMB), c) colorectal carcinoma (BMC), d) non-small cell lung carcinoma (BML), e) melanoma (BMM), and f) renal clear cell carcinoma (BMR).

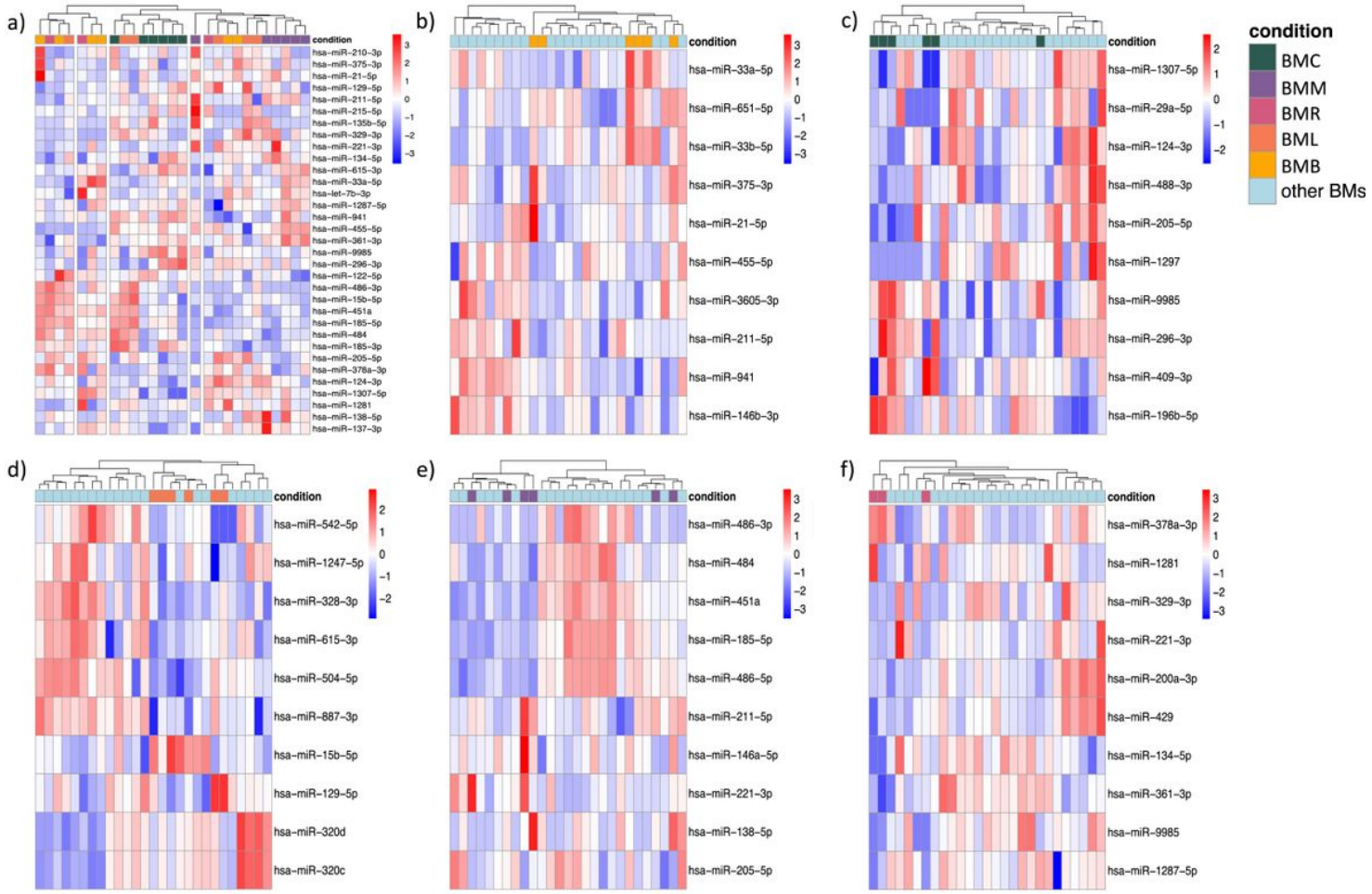

Figure 3

Heatmaps with unsupervised hierarchical clustering based on the cerebrospinal fluid (CSF) levels of: a) 33 unique miRNAs generated from top 5 miRNAs with p-value<0.05, FC≥1.5, and baseMean≥1 for each comparison; b-f) 10 miRNAs with p-value<0.05, FC≥1.5, and baseMean≥1 of each metastatic origin compared to all other metastatic origins derived from CSF of patents with b) breast carcinoma (BMB), c) colorectal carcinoma (BMC), d) non-small cell lung carcinoma (BML), e) melanoma (BMM), and f) renal clear cell carcinoma (BMR).

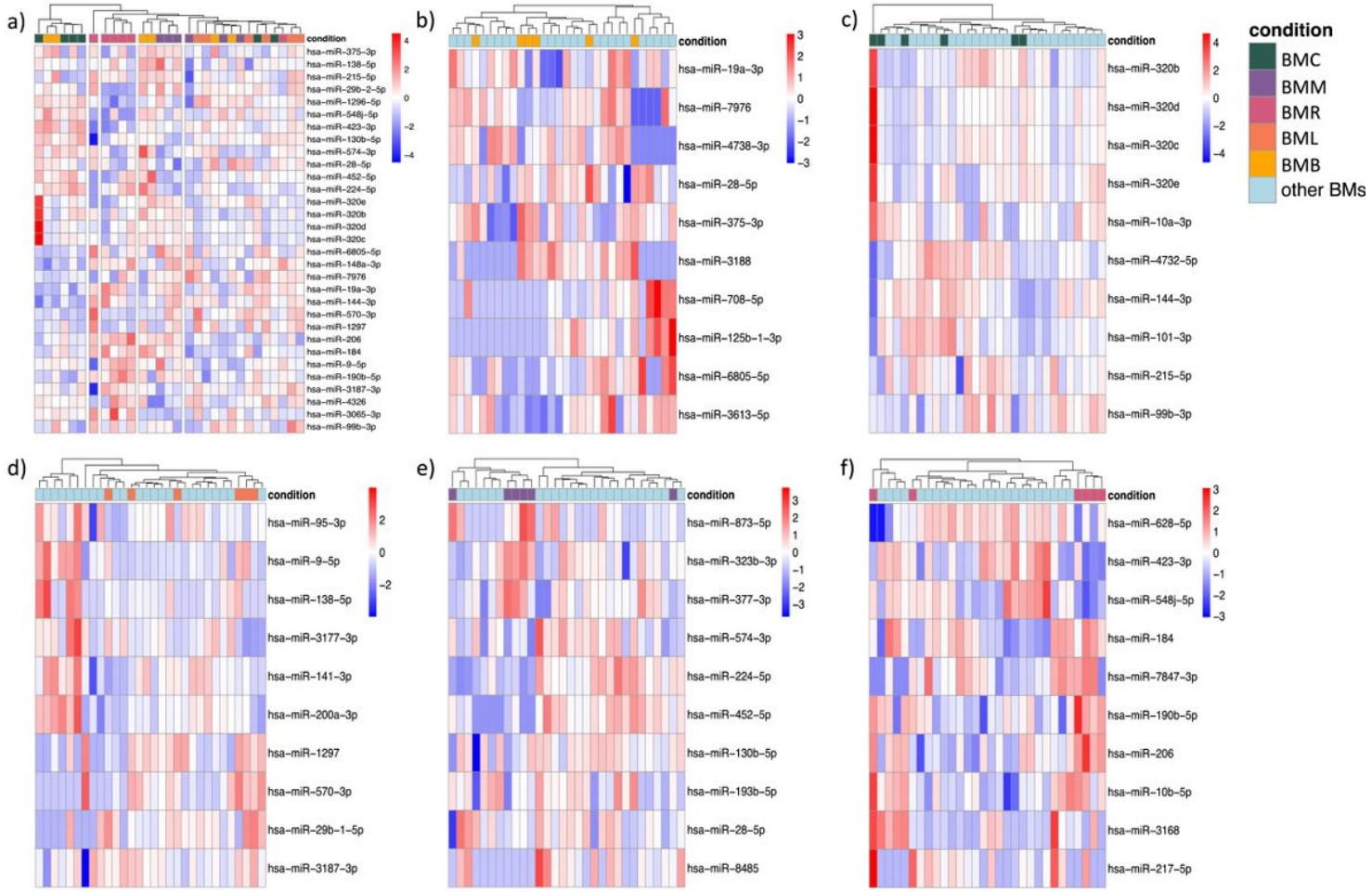

Figure 4

Heatmaps with unsupervised hierarchical clustering based on the blood plasma levels of: a) 31 unique miRNAs obtained by combining top 5 miRNAs with p-value<0.05, FC≥1.5 and baseMean≥1 for each comparison; b)-f) miRNAs with p-value<0.05, FC≥1.5 and baseMean≥1 of each metastatic origin compared to all other metastatic origins derived from plasma of patients with b) breast carcinoma (BMB), c) colorectal carcinoma (BMC), d) non-small cell lung carcinoma (BML), e) melanoma (BMM), and f) renal clear cell carcinoma (BMR).

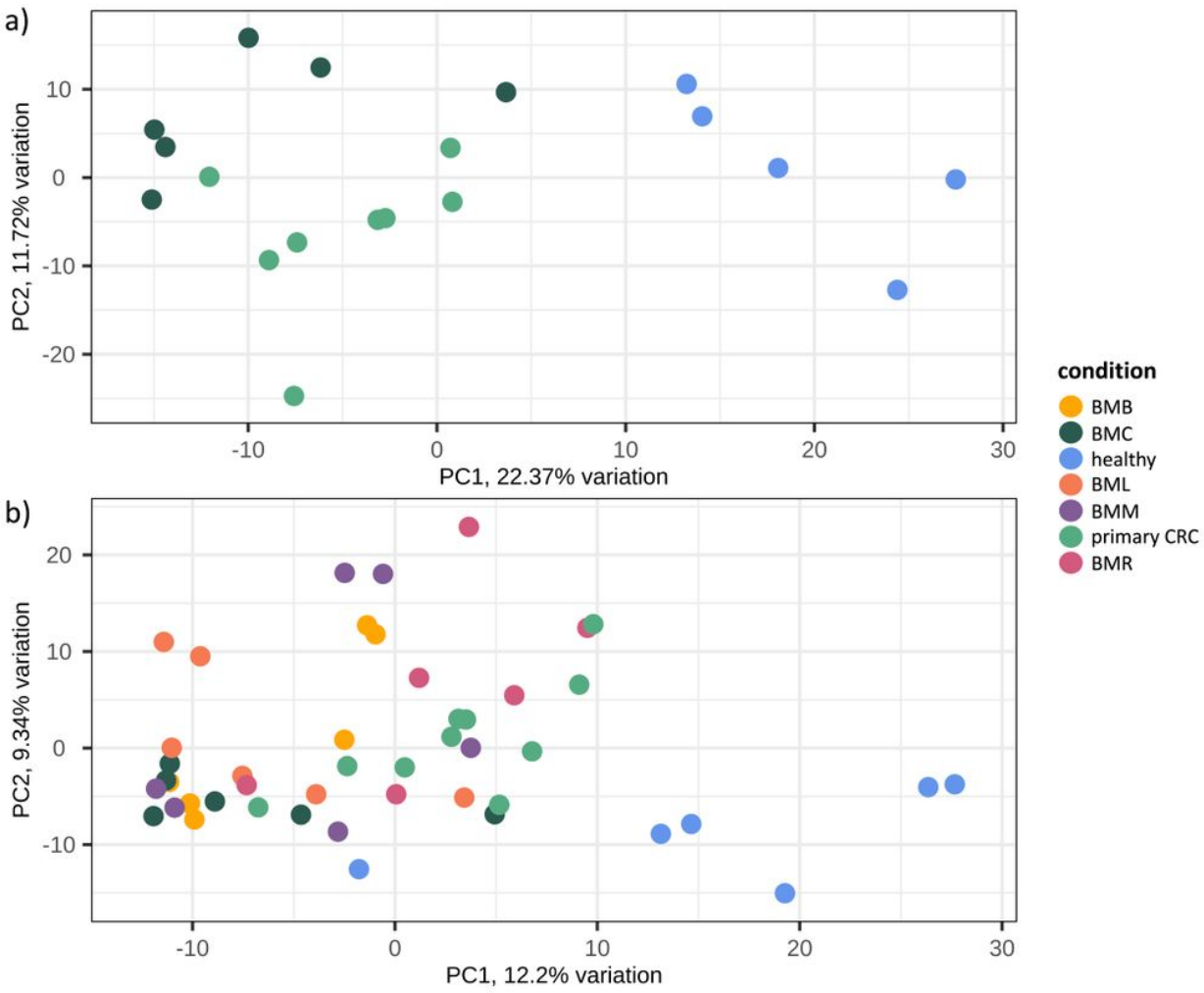

Figure 5

PCA plot showing a: a) comparison of plasma samples from healthy controls, patients with primary colorectal carcinoma (CRC) and patients with brain metastasis derived from colorectal carcinoma (BMC); b) comparison of plasma samples from healthy controls and patients with primary CRC, BMC, BM from breast carcinoma (BMB), BM from non-small cell lung carcinoma (BML), BM from melanoma (BMM), and BM from renal clear cell carcinoma (BMR).

Supplementary Files

This is a list of supplementary files associated with this preprint. Click to download.

- supplement1.xlsx
- supplement2.xlsx
- supplement3.xlsx