Integrated microRNA Analysis Identifies miR-512-3p as a Potential Biomarker of Poor Outcome in Pediatric Medulloblastoma

Carolina Alves Pereira Correia

Universidade de Sao Paulo Faculdade de Medicina de Ribeirao Preto

Pablo Ferreira Chagas

Universidade de Sao Paulo Faculdade de Medicina de Ribeirao Preto

Mirella Baroni

Universidade de Sao Paulo Faculdade de Medicina de Ribeirao Preto

Augusto Faria Andrade

McGill University

Rosane Gomes de Paula Queiróz

Universidade de Sao Paulo Faculdade de Medicina de Ribeirao Preto

Veridiana Kill Suazo

Universidade de Sao Paulo Faculdade de Medicina de Ribeirao Preto

Gustavo Alencastro Veiga Cruzeiro

Harvard Medical School

Paola Fernanda Fedatto

Universidade de Sao Paulo Faculdade de Medicina de Ribeirao Preto

David Santos Marco Antonio

Seven Bridges Biotech Consulting, São Paulo

Silvia Regina Brandalise

Boldrini Children's Center, Campinas

Jose Andres Yunes

Boldrini Children's Center, Campinas

Rodrigo Alexandre Panepucci

Fundação Hemocentro Ribeirão Preto

Carlos Gilberto Carlotti Jr

Universidade de Sao Paulo Faculdade de Medicina de Ribeirao Preto

Elvis Terci Valera

Universidade de Sao Paulo Faculdade de Medicina de Ribeirao Preto

Luiz Gonzaga Tone

Universidade de Sao Paulo Faculdade de Medicina de Ribeirao Preto

Carlos Alberto Scrideli (✉ scrideli@fmrp.usp.br)

Research Article

Keywords: Medulloblastoma, microRNA profile, miR-512-3p

Posted Date: November 22nd, 2021

DOI: <https://doi.org/10.21203/rs.3.rs-1068266/v1>

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Abstract

Background: Medulloblastoma, a genetically heterogeneous tumor, is the most frequent malignant brain tumor in children. Although several studies have been carried out, the molecular mechanism underlying medulloblastoma tumorigenesis is not completely known. microRNA (miRNA) expression profiles have been associated with development, progression, and prognosis of human cancers, including medulloblastoma. However, the role of miRNAs in pediatric medulloblastoma has been poorly explored.

Methods: Global miRNA expression in 24 microdissected medulloblastoma specimens (19 pediatric and 5 adult specimens) was evaluated by microarray assay. miR-512-3p, the most differentially expressed miRNA in these two groups, was analyzed by qRT-PCR in a cohort of 51 consecutive pediatric medulloblastoma samples and 7 pediatric non-neoplastic cerebellum control samples, and its clinical significance was assessed. Further *in silico* miRNA prediction of target genes was performed with bioinformatics tools.

Results: Compared to the controls, miR-512-3p was significantly downregulated in the pediatric medulloblastoma samples. Higher miR-512-3p was associated with incomplete degree of resection, high risk group classification, and poor overall survival. *In silico* analysis in an independent cohort of medulloblastoma identified that some of the miR-512-3p target genes (*SMAD9*, *SSX2IP*, *MAPK10*, *PTCH1*, *CCDC6*, and *BMPR2*) were statistically correlated with overall survival, metastasis, and death.

Conclusions: For the first time, our results have shown that miR-512-3p is significantly associated with poor clinical outcome in pediatric medulloblastoma, suggesting that miR-512-3p is a potential biomarker of prognosis.

Introduction

Medulloblastoma (MB) is a malignant embryonic tumor that affects the cerebellar region of the central nervous system. According to the World Health Organization, it is classified as grade IV[1]. MB accounts for 20–25% of all the tumors of the central nervous system in children. It is highly aggressive and is responsible for over 10% of children's death due to cancer [2]. Genetically, MB is a heterogeneous disease classified into four molecularly distinct subgroups: WNT, SHH, Group 3 (Grp3), and Group 4 (Grp4). Molecularly, the two former subgroups are the best understood and characterized [3, 4]. In these subgroups, there are molecular gaps with distinct transcriptional profiles, leading to worse outcomes. This reinforces the need for a global effort to search for biomarkers and to develop new therapeutic interventions for these specific subgroups [5].

microRNAs (miRNAs) are small non-coding RNA molecules that act in post-transcriptional regulation. These molecules are involved in neurological disorders, neurodevelopment, and cancer pathological processes such as proliferation, migration, invasion, and metastasis. They also play key roles in chemosensitivity and radiosensitivity [6]. In addition, in recent years, genomic analyses like analysis of microarray data have been widely used to identify miRNAs that drive worse prognosis. This may be

useful in future therapeutic interventions for cancer treatment because miRNAs are differentially expressed in pediatric central nervous system brain tumors such as gliomas [7] and neuroblastomas [8], and differential miRNA expression has been detected in in vitro and in vivo studies of MBs [9, 10]. However, the role and the underlying mechanisms of miRNAs in the regulatory network of tumorigenesis in specific MB subgroups remain poorly understood.

To fill this gap, we have carried out microarray data analysis of pediatric and adult MB samples of a Brazilian cohort to identify differential expression patterns of miRNAs. Additionally, we have selected the most differentially expressed miRNA in these two groups and performed pathway enrichment and Gene Ontology analysis to evaluate how miRNAs are correlated with several potential target genes and to establish a prognostic correlation. For the first time, we have found that miR-512-3p is downregulated in human MB pediatric samples, and that its high expression is significantly associated with overall survival. We have also found a link between miR-512-3p and crucial targets predict and validated with overall survival, metastasis, recurrence, and death.

Material And Methods

Patients

For the microarray assay, 19 microdissected pediatric MB specimens and five adult MB specimens were analyzed. For validation by RT-qPCR, 51 samples from consecutive pediatric and adolescent patients with MB admitted and treated at the two participating institutions (University Hospital of the Ribeirao Preto Medical School – University of Sao Paulo and the Boldrini Children's Center - Campinas/SP) were used. Seven pediatric non-neoplastic cerebellum samples were employed as controls. Supplemental Table 1 shows the pediatric patients' data. Molecular classification was done as previously described [11]. Patients aged less than three years, tumor residue $>1.5\text{ cm}^2$ after surgery, or disseminated disease at diagnosis were classified as high risk. Patients were submitted to three different treatment protocols that included radical surgical resection, craniospinal radiotherapy (for children older than three years), and chemotherapy, at the discretion of each center. The five-year overall survival for high-risk patients was $26.8\% \pm 8.7\%$; for the low-risk group, this value increased to $72.4 \pm 8.8\%$.

All the procedures performed in this study were in accordance with the ethical standards of the institutional and/or national research committee and with the 1964 Helsinki declaration and its later amendments. The Ethics Committee approved this study (protocol number: 8380/2010), and each patient or their Legal Guardians provided a signed statement of informed assent and consent.

Microarray analysis and validation by qRT-PCR of differentially expressed miRNAs

Total RNA was extracted from samples by using the reagent TRIzol™ (Thermo Fisher Scientific, USA); the manufacturer's instructions were followed. cDNA was synthesized from total RNA by using the High-

Capacity cDNA Reverse Transcription Kit (Thermo Fisher Scientific, USA).

A total of 24 primary MB samples (19 pediatric and 5 adult specimens) were used in the microarray assay. To determine the miRNA expression profiles, the miRNA library was constructed with the Agilent “Human microRNA Microarray Kit (v3)” (G4470C, Agilent Technologies, Santa Clara, CA, USA), as described previously [12]. Hierarchical clustering was carried out to show the distinguishable miRNA expression patterns among the samples. Subsequently, miR-512-3p expression was evaluated in 51 pediatric MB and 7 pediatric non-neoplastic cerebellum samples by qRT-PCR; Taqman microRNA assays (Applied Biosystems Inc., Foster City, CA, USA; Cat. #4427975; Assay ID: 1823) were performed according to the manufacturer’s recommendations. Relative expression was determined by the $2^{-\Delta\Delta CT}$ method by using the mean of non-neoplastic cerebellum samples as calibrator and the expression of noncoding small nuclear RNA RNU6B (Cat. #4427975; Assay ID: 1093) and RNU19 (Cat. #4427975; Assay ID: 1003) as endogenous reference [13].

Pathway enrichment of differentially expressed miRNAs and Gene Ontology analyses

Pathway enrichment was done with the DIANA-miRPath webserver [14]. First, the miWalk2.0 database [15] was applied to identify validated and predicted miR-512-3p targets. This analysis was carried out with genes bearing miRNA binding sites; at least six existing miRNA-target prediction programs were employed. To identify the biological process of these predicted target genes, Gene Ontology enrichment was accomplished by using ShinyGO [16], and the 10 most significant terms with p-values <0.05 were considered.

Clinical relevance and profile expression analysis of predicted target genes of miRNAs by MB dataset

After the validated and predicted targets of our miRNA of interest were identified, the public database GSE85217 [4], composed by 763 primary MB samples and available on the website R2: Genomics Analysis and Visualization platform (<http://r2.amc.nl>), was used to evaluate how the expression of these genes was associated with overall survival, metastasis, and death. In addition, the expression profile of these genes was evaluated in relation to the MB molecular classification.

Statistical analysis

Differences in the miRNA expression levels of the MB and control samples, as well as the clinical and biological characteristics (metastasis, gender, recurrence, degree of tumor resection after surgery, age at diagnosis, high or low risk, molecular subgroups) were determined by the Mann–Whitney test. Overall survival was calculated on the basis of Kaplan–Meier plots and the log-rank test; the median expression value was used as the cut-off point for low and high miRNA expression level. The association of the expression of the genes downloaded from GSE 85217 [4] with metastasis and death was analyzed by the unpaired t-test, and Overall survival was calculated by using Kaplan-Meier plots and the log-rank test.

Data were analyzed with the IBM® SPSS® Statistics software v.20 (SPSS Inc, IL, USA) or GraphPad Prism 8 (GraphPad Software, San Diego, CA). The level of significance was set at $p < 0.05$ in all analyses.

Results

Microarray analysis of differentially expressed miRNAs and cohort validation by qRT-PCR

The microarray assay showed that, compared to adult MB samples, nine human miRNAs had reduced expression in pediatric MB.

We normalized the miRNA expression levels as shown in the hierarchical clustering heatmaps (Supplemental Fig. 1). Moreover, to investigate the role of miR-512-3p, the most differentially expressed miRNA in the microarray assay, we validated its expression by qRT-PCR, which revealed its reduced expression in 51 pediatric MB samples as compared to seven non-neoplastic cerebellar samples ($p=0.003$; Fig. 1). This suggested that this miRNA contributes to pediatric MB carcinogenesis.

Higher miR-512-3p expression is associated with poorer prognosis in MB

Interestingly, we found that higher miR-512-3p expression was associated with poor overall survival (38.5% *versus* 66.4%; $p=0.048$) (Fig. 2a). Higher miR-512-3p expression was also significantly associated with incomplete degree of tumor resection after surgery ($p=0.008$), patients classified as high risk ($p=0.036$), and patients classified as Grp4-MBs ($p=0.008$) (Fig. 2b-d). Based on these results, miR-512-3p may play a crucial role in pediatric MB biogenesis, especially in patients with an unfavorable clinical outcome.

DIANA-miRPath database analysis revealed pathways significantly associated with these miRNAs

To investigate in which enriched pathways and predicted miRNA–target gene interactions miR-512-3p participates, we carried out DIANA-miRPath analysis. We focused on signaling pathways related to cancer initiation and progression, and we observed that miR-512-3p was involved in eight interesting signaling pathways (Adherens Junction, Proteoglycans in cancer, Pluripotency of stem cells, Hippo signaling pathway, Glioma, Prolactin signaling pathways, Wnt signaling pathway, and Pathways in cancer). Supplemental Table 2 lists the target genes associated with these signaling pathways.

miR-512-3p targets are associated with poorer overall survival, metastasis, and death in medulloblastoma

To explore the prognostic significance of the genes associated with miR-512-3p (Supplemental Table 2), we analyzed their association with metastasis, death, molecular subgroup classification, and overall survival in an independent cohort of patients with medulloblastomas by using the public database

GSE85217 [4]. Higher expression of *SMAD9* ($p=0.0017$) and *SSX2IP* ($p=0.0215$) and lower expression of *MAPK10* ($p<0.0001$) and *PTCH1* ($p=0.044$) were associated with metastasis (Fig. 3a-d). In addition, higher expression of *SMAD9* ($p=0.0003$), *SSX2IP* ($p=0.0007$), *CCDC6* ($p=0.0017$) and lower expression of *MAPK10* ($p=0.0035$) and *BMP2* ($p=0.0059$) were associated with death (Fig. 3e-i). Patients classified into MB Group 3 and Group 4 had greater expression of *SSX2IP*, *SMAD9*, and *BMP2* and lower expression of *PTCH1* and *MAPK10* in relation to other MB subgroups ($p<0.0001$; Fig. 4a-f). Furthermore, overexpression of *SSX2IP* ($p=0.016$), *SMAD9* ($p<0.001$), and *CCDC6* ($p=0.034$) and underexpression of *PTCH1* ($p=0.027$), *BMP2* ($p<0.001$), and *MAPK10* ($p<0.001$) were associated with poor overall survival in this cohort (Fig. 5a-f). These data could suggest that miR-512-3p is associated with deregulation of genes that are related to poorer prognosis.

MiR-512-3p is involved in regulation of cellular processes and central nervous system development

Next, we investigated in which biological processes this miRNA may be involved by using the miRWalk 2.0 database and selecting the target genes that were found in at least six different databases (miRWalk, miRDB, PITA, RNA22, miRanda, RNAhybrid, PICTAR2, and TargetScan). Then, we used these genes for enrichment through Gene Ontology, to observe that miR-512-3p may play a biological role in central nervous system development, cell development, and cell morphogenesis, among other processes (Supplemental Fig. 2).

Taken together, our data suggested that miR-512-3p is downregulated in pediatric MB, can modulate the target genes demonstrated in this study, and is associated with poor clinical outcome in children with MB, especially those classified as higher risk subgroups. To confirm these findings, further investigations must be carried out to verify whether these genes are regulated by miR-512-3p and to find out how they are involved in MB progression.

Discussion

miRNAs are predictors of prognosis in several tumors and can contribute to tumorigenesis by interacting with target genes involved in cancer development and progression [17]. The incidence of MB in pediatric patients is very high in contrast with adult patients [18] and it is already discussed that these two groups of patients differ genetically, showing different chromosomal amplifications [19]. To date, it noted the paucity of studies with focus on comparing the differential expression of miRNAs between the pediatric and adult patients. Thus, we performed the microarray analysis of miRNAs in pediatric and adult MBs tissue, and we showed that several miRNAs are differentially expressed when comparing these two groups of patients segregated by age, and we highlight miR-512-3p, which was the miRNA with the lowest expression in pediatric patients.

In agreement with our results, miR-512-3p has been found to be downregulated in breast cancer [20], lung cancer [21] and classic Hodgkin lymphoma [22]. In association with the clinical outcome, higher miR-512-3p expression was related to the worse prognosis of these pediatric MB patients, such as worse overall

survival, high risk classification, and incomplete resection level in Grp-4 MBs patients. Likewise, miR-512-3p is more expressed in patients with a worse prognosis of prostate cancer [23].

Several miRNAs have been identified and evaluated as therapeutic targets in MB, such as miR-584-5p which has been identified as a tumor suppressor with an important role in sensitization to MB treatment with vincristine and irradiation [24]. Furthermore, studies have shown that the expression profile of miRNAs can differentiate MB tumor tissue from non-neoplastic cerebellar tissue [25], and the relationship between the differential expression of miRNAs and specific MB subgroups or important signaling pathways are also elucidated, such as decreased expression of miR-218, particularly in SHH and GRP-3MBs patients [26].

Many studies have shown that the expression profiles of miRNAs are associated with specific malignant diseases, and that their deregulation is involved in the biological process of cancer [27]. Enrichment of signaling pathways conducted herein showed that miR-512-3p may be involved in several pathways that contribute to the initiation and progression of central nervous system tumors, including MB. Examples of such pathways include adherens junctions, proteoglycans, pathways related to stem cell pluripotency, Hippo, WNT, and prolactin signaling pathway, which have been studied and elucidated in brain tumors, such as glioblastoma and MB [3–5, 28, 29]. Other studies have shown that miR-512-3p is involved in tumorigenic pathways, such as the MAPK pathway, focal adhesion, TGF- β pathway [30], proteoglycans in cancer, and signaling pathways regulating pluripotency of stem cells [31], indicating that miR-512-3p plays an important role in processes related to MB development.

Interestingly, when we used the GSE85217 [4] data for MB, we verified that predicted target genes for miR-512-3p (*SSX2IP*, *PTCH1*, *SMAD9*, *BMPR2*, *MAPK10*, and *CCDC6*) are associated with worse overall survival, presence of metastasis, death and with differential expression in Grp3-MBs and Grp4-MBs. *PTCH1* has been the most extensively studied in MB and germline mutations in this gene occur in 6% of MB cases, and these mutations are associated with increased risk for the development of malignant diseases [32]. *PTCH1* is a membrane receptor of the SHH signaling pathway, which activates *GLI1* and *GLI2* and consequently promotes activation of the mitogenic genes *CCND1* and *MYCN* [33].

BMPR2 and *SMAD9* are part of the signaling pathway of bone morphogenetic proteins (BMP), which participate in the development of the central and peripheral nervous system, including survival, proliferation, and differentiation of progenitor cells [34, 35]. Therefore, changes in the expression of these genes may be related to the development of tumors of the central nervous system. *SSX2IP*, *MAPK10*, and *CCDC6* have never been associated with MB or tumors of the central nervous system. However, high *SSX2IP* expression has been associated with worse prognosis in gastric cancer [36] and hepatocellular cancer [37], and high *CCDC6* expression has been associated with metastasis and worse survival in lung cancer [38]. Mutations in *MAPK10* have been associated with worse prognosis in pancreatic cancer [39], and this gene is underexpressed in breast cancer [40]. Thus, some of the genes associated with miR-512-3p are involved in the development of the central nervous system in general, and deregulation of these genes has already been related to the prognosis of patients with other tumors.

This is the first time that miR-512-3p deregulation has been described in pediatric MB. In conclusion, miR-512-3p may be involved in biological processes related to the development of the central nervous system, as identified by GO enrichment and by the role of miR-512-3p target genes demonstrated above. Finally, the association of this miRNA with clinical and biological findings indicates that it is a potential biomarker of poor prognosis that should be considered in future investigations into MB carcinogenesis.

Declarations

Funding Information

This work was supported by the Brazilian Public Research Agencies Fundação de Amparo à Pesquisa do Estado de São Paulo (FAPESP, Grants Numbers: 2010/07020-9 and 2014/20341-0) and Conselho Nacional de Desenvolvimento Científico e Tecnológico (CNPq, Grant Number: 471885/2013-4).

Data availability statement

The data and other items supporting the results of the study will be made available upon reasonable request.

Author Contributions

C.A.S and C.A.P.C planned and conducted data analysis and drafted the manuscript. P.F.C, M.B, A.F.A, R.G.P.Q, V.K.S, G.A.V.C wrote and organized the data, created the figures/tables, edited, and critically revised the manuscript. P.F.F, D.S.M.A, S.R.B, J.A.Y, R.A.P, C.G.C.J, E.T.V and L.G.T revised the text for important intellectual content. All authors critically read and approved the final manuscript.

Compliance with Ethical Standards

Conflict of interest

The authors declare that they have no competing interests.

Ethical approval

All the procedures performed in this study were in accordance with the ethical standards of the institutional and/or national research committee and with the 1964 Helsinki declaration and its later amendments. Ethical approval was obtained from Ribeirão Preto Medical School Ethics Committee

(protocol number: 8380/2010), and each patient or their Legal Guardians provided a signed statement of informed assent and consent.

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Figures

Figure 1

Analysis of miR-512-3p expression in MB and non-neoplastic cerebellum samples. The RT-qPCR assay showed reduced miR-512-3p expression in MB samples (n=51) compared to non-neoplastic cerebellum samples (n=7) ($p=0.003$)

Figure 2

Higher miR-512-3p expression is associated with worse prognosis for MB patients. (A) Survival analysis by the log-rank test showed that patients with miR-512-3p expression above the median (n=25) had worse overall survival than patients with miR-512-3p expression below the median (n=26) (p=0.048). (B) The Mann-Whitney test showed that patients with the highest miR-512-3p expression had incomplete degree of resection (n= 6), whereas patients with complete degree of resection had lower miR-512-3p expression (n=33) (p=0.008). (C) Likewise, patients classified as at high risk (n=27) showed greater expression of this miRNA compared to patients classified as at low risk (n=22) (p=0.036). (D) Finally, patients classified as MB Group 4 also showed greater miR-512-3p expression (n=12) (p=0.008)

Figure 3

According to the R2 online genomic analysis platform, miR-512-3p target genes are associated with metastasis and death. The unpaired t-test helped to identify some miR-512-3p target genes associated with worse prognosis. Patients who metastasized had lower expression of (A) MAPK10 ($p < 0.0001$) and (C) PTCH1 ($p = 0.044$) and higher expression of (B) SMAD9 ($p = 0.0017$) and (D) SSX2IP ($p = 0.0215$). Patients who died also had higher expression of (E) SSX2IP ($p = 0.0007$), (F) SMAD9 ($p = 0.0003$), and (I) CCDC6 ($p = 0.0017$) and lower expression of (G) BMPR2 ($p = 0.0059$) and (H) MAPK10 ($p = 0.0035$).

Figure 4

According to the R2 online genomic analysis platform, miR-512-3p target genes are associated with the subgroups with the worst prognosis in MB, groups 3 and 4. The unpaired t-test showed that the patients classified as MB groups 3 and 4 had higher expression of (A) SSX2IP, (C) SMAD9 and (D) BMPR2 and lower expression of (B) PTCH1 and (E) MAPK10 ($p < 0.05$). (F) The CCD6 gene did not show differential expression between subgroups

Figure 5

According to the R2 online genomic analysis platform, miR-512-3p target genes are associated with survival. The log-rank test and the Kaplan-Meier curves showed that higher expression of (A) SSX2IP ($p=0.016$), (C) SMAD9 ($p<0.001$), and (F) CCDC6 ($p=0.034$) is associated with worse overall survival of MB patients. On the other hand, MB patients with lower expression of (B) PTCH1 ($p=0.027$), (D) BMPR2 ($p<0.001$), and (E) MAPK10 ($p<0.001$) had worse overall survival

Supplementary Files

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

- [SUPPLEMENTALFIG.1.pdf](#)
- [SUPPLEMENTALFIG.2.pdf](#)
- [SupplementalTable1.pdf](#)
- [SupplementalTable2.pdf](#)