Identification of *ITPR1* as a hub gene of Group 3 Medulloblastoma and coregulated genes with potential prognostic values

Pablo Ferreira das Chagas (✉ pablochagas@usp.br)

University of Sao Paulo Faculty of Medicine of Ribeirao Preto: Universidade de Sao Paulo Faculdade de Medicina de Ribeirao Preto <https://orcid.org/0000-0002-0652-728X>

Graziella Ribeiro de Sousa

University of Sao Paulo Faculty of Medicine of Ribeirao Preto: Universidade de Sao Paulo Faculdade de Medicina de Ribeirao Preto

Luciana Chain Veronez

University of Sao Paulo Faculty of Medicine of Ribeirao Preto: Universidade de Sao Paulo Faculdade de Medicina de Ribeirao Preto

Andrea Martins-da-Silva

University of Sao Paulo Faculty of Medicine of Ribeirao Preto: Universidade de Sao Paulo Faculdade de Medicina de Ribeirao Preto

Carolina Alves Pereira Corrêa

University of Sao Paulo Faculty of Medicine of Ribeirao Preto: Universidade de Sao Paulo Faculdade de Medicina de Ribeirao Preto

Gustavo Alencastro Veiga Cruzeiro

Harvard Medical School

Luis Fernando Peinado Nagano

University of Sao Paulo Faculty of Medicine of Ribeirao Preto: Universidade de Sao Paulo Faculdade de Medicina de Ribeirao Preto

Rosane Gomes de Paula Queiroz

University of Sao Paulo Faculty of Medicine of Ribeirao Preto: Universidade de Sao Paulo Faculdade de Medicina de Ribeirao Preto

Suely Kazue Nagahashi Marie

Sao Paulo University Faculty of Medicine: Universidade de Sao Paulo Faculdade de Medicina

Silvia Regina Brandalise

Boldrini Children's Center

Carlos Alberto Scrideli

University of Sao Paulo Faculty of Medicine of Ribeirao Preto: Universidade de Sao Paulo Faculdade de Medicina de Ribeirao Preto

Luiz Gonzaga Tone

University of Sao Paulo Faculty of Medicine of Ribeirao Preto: Universidade de Sao Paulo Faculdade de Medicina de Ribeirao Preto

Elvis Terci Valera

University of Sao Paulo Faculty of Medicine of Ribeirao Preto: Universidade de Sao Paulo Faculdade de Medicina de Ribeirao Preto

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Abstract

The Group 3 Medulloblastoma (Grp3-MB) is an aggressive molecular subtype with a high incidence of metastasis and deaths. In this study, we used an RNA sequencing data (RNA-Seq) from a Brazilian cohort of MBs to identify hub genes associated with the metastatic risk. Data validation was performed by using multiple large datasets from MBs (GSE85217, GSE37418, EGAS00001001953). DESeq2 package in R software was used to identify the differentially expressed genes (DEGs) in our RNA-Seq data. The DEGs data were accessed to construct the modules/graphs of coexpression and to identify hub genes through Cytoscape platform. The co-regulated genes were enriched by the Kyoto Encyclopedia of Genes and Genomes (KEGG) pathway and the Protein-protein interaction (PPI) network was visualized by Cytoscape. The Kaplan–Meier plotter and ROC curves were used to validate the diagnostic and prognostic values of specific biomarkers identified through this model. We identified that Inositol 1,4,5-triphosphate receptor type 1 (ITPR1) as a downregulated hub gene, with a high diagnostic accuracy to Grp3-MBs and associated with tumor metastasis. In addition, we identified genes significantly correlated with ITPR1 that were associated with metastasis in Grp3-MB (ATP1A2 , MTTL7A and RGL1) , and worst overall survival in MBs (ANTXR1 and RGL1). Our findings suggest that the ITPR1 hub gene is potentially involved in the metastatic process for Grp3-MB. Our data also provide evidence of targets that may serve as prognostic predictors and/or regulators for the metastatic process that maybe explored for further research of individualized therapy to Grp3-MBs.

Introduction

Medulloblastoma (MB) is the most common malignant embryonal brain tumor in the pediatric population (Northcott et al. 2019). The current consensus established by the World Health Organization (WHO) recognizes four main molecular and clinico-pathological distinct MB subtypes: Wingless-activated (WNT-MB), Sonic Hedgehog-activated (SHH-MB), and non-WNT/non-SHH MBs: Group 3 (Grp3-MB), and Group 4 (Grp4-MB) (Louis et al. 2021). Grp3-MBs represent approximately 25% of all MB cases, being the deadliest across molecular subgroups and frequently (15-20%) showing metastasis at diagnosis in infants and older children (Cavalli et al. 2017; Juraschka and Taylor 2019).

Next Generation Sequencing (NGS) techniques such as RNA Sequencing (RNA-Seq) and robust bioinformatics analysis have been used over the years to uncover potential biomarkers in MBs (Guo et al. 2020b). Although integrative genomic studies have clarified several mechanisms on MBs tumorigenesis, (Juraschka and Taylor 2019; Menyhárt and Györfy 2019) it is of note the paucity of genes with clinical relevance that ultimately could be used as metastatic biomarkers and predictors for prognostication, improving therapeutic strategies for Grp3-MB.

In this study, we identified the Inositol 1,4,5-trisphosphate receptor 1 (*ITPR1*) gene, a regulator of calcium signaling (Ca^{2+}), homeostasis and autophagy in cancer, (Gambardella et al. 2020; Sneyers et al. 2020) as a hub gene for Grp3-MBs. In addition, we showed that *ITPR1* is specifically downregulated with a high diagnostic accuracy to Grp3-MB in our and multiple independent cohorts of MB. Interestingly, the low

expression of *ITPR1* was associated with the presence of metastasis in MBs. In addition, we identified 17 genes significantly coregulated with *ITPR1*, highlighting the *ATP1A2*, *MTTL7A* and *RGL1* which demonstrated association with metastasis in Grp3-MB, and *ANTXR1* and *RGL1* correlated to worse overall survival (OS) in MBs. Our study provides for the first-time insights about the association of *ITPR1* as a prognostic biomarker and a potential therapeutic target to Grp3-MBs.

Materials And Methods

RNA-Sequencing from a data generated in-house cohort

In a previous study of our group, 17 pediatric MB samples from Brazilian patients were sequenced and deposited in the GEO database under there Cord number GSE181293. The RNA-Seq data of our in-house cohort was used to identify the differentially expressed genes (DEGs) and also to gain insights on hub genes for Grp3-MBs as follows.

Co-expression network and Hub Gene selection

DESeq2 package in R software (version 3.4.3) was used to identify the DEGs into our RNA-Seq data (Love et al. 2014). The counts expression data (for Grp3-MB) were normalized and used as input for network construction in CEMiTool (followed script standard), (Russo et al. 2018) which DEGs with similar expression patterns were grouped into modules via the average linkage hierarchical clustering. Within the networks of coexpression were identified the genes that have the highest number of connections and/or interactions, using the connectivity module measured by the absolute value of the Pearson correlation coefficient > 0.5 and the graphs were generated to represent interacting genes (hub genes) through the extension called GeneMania (Montejo et al. 2010) in the Cytoscape 3.7.1 platform (<https://cytoscape.org/>). We screened genes closely linked in the modules and assessed the correlation of these genes' expression value with presence of metastasis using the clinical data of the pediatric MBs GSE85217 (n=643) (Cavalli et al. 2017), that were downloaded from R2 Platform (Genomics Analysis and Visualization Platform - <http://r2.amc.nl>). We used the Student's t-test (non-parametric Mann Whitney test) and a p-value ≤ 0.05 was considered as statistically significant. Thus, the *ITPR1* hub gene was retained for further analysis.

Evaluation of *ITPR1* expression and identification of co-regulated genes in multiple pediatric MBs retrospective cohorts

Different datasets were downloaded from R2 Platform (Genomics Analysis and Visualization Platform - <http://r2.amc.nl>) to evaluate *ITPR1* expression levels. Two out of three datasets were derived of microarray data: GSE85217 (n=643) (Cavalli et al. 2017); GSE37418 (n=73) (Robinson et al. 2012) and one of RNA-Seq data: EGAS00001001953 (n=186) (Northcott et al. 2017). *ITPR1*-coregulated genes were identified using common elements of all four MB datasets with a p-value < 0.05 and a Pearson's correlation coefficient > 0.5 . Further, a Venn diagram was performed to overlap coregulated genes with *ITPR1* in all cohorts (data not shown). To better visualize the profile expression of *ITPR1*-coregulated

genes we generated a heatmap using the Complex Heatmap package (Gu et al. 2016) with pre-set metrics as Pearson's correlation distance parameter and the algorithm WardD2.

Protein–protein interaction (PPI) network

The Protein-protein interactions (PPI) among the common *ITPR1*-coregulated genes were evaluated using the Search Tool for the Retrieval of Interacting Genes (STRING) database (<http://www.stringdb.org/>) (Szklarczyk et al. 2015). Validated interactions with a combined score >0.9 were selected as significant (Bozhilova et al. 2019). Then, integration of PPI networks was constructed using the Cytoscape software (<https://cytoscape.org>) (Shannon et al. 2003) with GeneMANIA plugin to identify biological network integration for gene prioritization (Warde-Farley et al. 2010).

KEGG pathways enrichment analysis

We conducted Kyoto Encyclopedia of Genes and Genomes (KEGG) pathway enrichment analysis to better understand the putative functions of *ITPR1*-coregulated genes using the Enrichr database (<https://maayanlab.cloud/Enrichr/>) (Kanehisa and Goto, 2000). A p-value of < 0.05 was considered to represent a statistically significant enrichment of DEGs in pathway.

Survival and metastasis analysis

Kaplan-Meier curves and the long-rank tests were performed to evaluate overall survival (OS) according to the expression of the *ITPR1*-coregulated genes using the GSE85217 dataset (Cavalli et al. 2017). This dataset was preferred to this analysis due to the higher number of MB cases available to be analysed, along with better clinical data associated to genetic dataset. Patients were divided into two groups (low vs. high) according to the median expression of each gene. First, we considered the data from all subtypes of MBs samples primary (n=343) and metastasis (n=167). Then, we used only data of Grp3-MBs primary samples (n=62) and metastasis (n=43) information.

Statistical analysis

We performed statistical analysis comparing expressions among two different groups by Student's t-test (non-parametric Mann Whitney test). Analyzes between more the two groups were used One-way-ANOVA non-parametric followed Tukey post test. Receiver operating characteristic (ROC) curves were used to evaluate the discrimination of *ITPR1* expression levels into molecular MBs subtypes. The accuracy was determined by the area under the curve (AUC). Graphs were generated with GraphPad Prism 8.0 (GraphPad Software, San Diego, CA, USA), considering p-value <0.05 as statistically significant.

Results

RNA-Seq analysis reveals the *ITPR1* as a hub gene in our pediatric cohort of MB

In order to identify key genes in Grp3-MB tumor we first identified DEGs in Grp3-MB compared to other molecular subtypes (WNT-MB, SHH-MB and Grp4-MB) in our cohort. Next, *co-expression* network analysis identified a total of five specific key *modules* related to Grp3-MB (Supplementary Fig. 1). In one out of these modules, we identified *ITPR1* as a hub gene (Fig. 1). Previously, the *ITPR1* was found downregulated in Grp3-MBs highlighting a novel and promising therapeutic target (Beckmann et al. 2019; Castillo-Rodríguez et al. 2018). Thus, we selected the *ITPR1* as an interesting gene related with tumor biology for subsequent analysis given that *ITPR1* into Grp3-MBs carcinogenesis are poorly understood to date.

The low expression of *ITPR1* gene is associated with metastasis and represents a potential biomarker in Grp3/Grp4-MBs

In our cohort, we showed that low expression gene of *ITPR1* in Grp3-MBs when compared to other MBs subtypes (Fig 2a). Thus, the expression profile of *ITPR1* was also evaluated in three additional independent databases GSE85217 (Fig. 2b), GSE37418 (Fig. 2c), and EGAS00001001953 (Fig. 2d) of MBs. Interestingly, it was possible to validate a specific low expression profile of *ITPR1* in Grp3-MBs or Grp4-MBs compared to WNT and SHH-MBs.

Once we observed that *ITPR1* is downregulated in both Grp3 and Grp4-MBs, we evaluated its potential as a biomarker of these two specific subgroups. ROC analysis showed that the AUC was 0.846, suggesting a high diagnostic accuracy in the discrimination between Grp3/Grp4-MBs from non-Grp3/Grp4-MBs (Fig. 2e). Remarkably, the lower expression of *ITPR1* was also associated with the presence of metastasis in MB (Fig. 2f). Together, these results suggest that the low expression of *ITPR1* gene is a potential biomarker of Grp3/Grp4-MBs and is potentially related to metastasis process in this setting.

***ITPR1*-coregulated genes potentially regulate pathways related to metastasis in MBs**

To gain further insights into the putative functions of the hub gene *ITPR1*, we identified genes significantly coregulated with *ITPR1* expression exploring data of three MBs public databases (GSE85217, GSE37418, EGAS00001001953) and our RNA-Seq data. A total of 17 genes were shown to be commonly coregulated with *ITPR1* in all four datasets, which roles previously described in cancer are summarized in Supplementary Table 1. Interestingly, the expression profile of these 17 *ITPR1*-coregulated genes in four datasets converges to a low expression of these genes to Grp3-MBs in the most of samples, as shown in Fig. 3a-d. The PPI network and KEGG enrichment analysis revealed that the seventeen *ITPR1*-coregulated genes present genetic and co-expression interactions (Fig. 4a) and are significantly associated with the signaling pathways regulating pluripotency of stem cells, regulation of actin cytoskeleton, Ras signaling pathway and cell tight-junction (Fig. 4b). The genes that are involved into each correlated pathway are described in Supplementary Table 2. Altogether, our results indicate an important regulatory network between *ITPR1* and co-regulated genes that potentially are involved into a signaling net associated with metastatic processes in MBs.

***ITPR1*-coregulated genes are associated with metastasis and poor prognosis in MBs**

To evaluate the prognostic value and clinical relevance of the 17 *ITPR1*-coregulated genes we performed association and survival analysis using the GSE85217 dataset (Cavalli et al. 2017). Interestingly, the low expression of all the 17 genes was significantly associated with the occurrence of metastasis when analyzed in all MBs datasets ($p < 0.001$) Supplementary Fig. 2). In addition, we observed that the expression of three out of 17 common-genes, the *ATP1A2*, *MTTL7A* and *RGL1* were also significantly associated with presence of metastasis specifically in Grp3-MBs patients ($p < 0.001$) (Fig. 5a-c). Regarding prognostic value of the 17 *ITPR1*-coregulated genes, lower expression levels of *ANTXR1* and *RGL1* were significantly associated with worse OS in MBs ($p = 0.0037$ and $p = 0.029$, respectively- Fig. 5d-e). Collectively, our findings suggest that *ITPR1* and coregulated genes are potential biomarkers for poor prognosis and metastasis in MBs.

Discussion

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“Omics” studies (e.g., exome/proteomics/single-cell approaches) and integrative large NGS data analysis are valuable tools to identify novel biomarkers in cancer. Although many studies regarding biomarkers for MBs have been published (Huang et al. 2020), a sharper identification of hub genes and their interactions can be very helpful to unravel molecular mechanism associated with prognosis and metastasis, particularly for Grp3-MBs.

In the current study, we demonstrated that *ITPR1* is a hub gene downregulated in Grp3-MBs and associated to metastasis. The *ITPR1* gene is highly expressed in the Purkinje cells in the central nervous system (SNC) and the modulation of intracellular calcium homeostasis is the main biological function of this gene (Foskett et al. 2007; Nakanishi et al. 1991). Recently, the *ITPR1* has been reported to present a complex role in cancer dynamics by controlling cell survival and cell death via regulation of apoptosis and autophagy (Parys et al. 2021). This is especially relevant, given that programmed cell death through autophagy plays crucial role in progression and metastasis in cancer (Su et al. 2015) and the low expression of autophagy-related genes is associated with poor prognosis and tumor progression (Wang et al. 2015a; Xu et al. 2019). Based on these reports and in our results, we suggest that the downregulation of *ITPR1* is involved in the crosstalk between reduced autophagy and increased metastatic mechanisms and may play an important role towards a malignant phenotype in Grp3-MBs. Yet, further studies are needed to test and to explore this hypothesis.

Previously, the *ITPR1* was found downregulated in Grp3/Grp4-MBs highlighting a novel and promising therapeutic target. Meanwhile, this data was not explored the underlying molecular mechanisms of the *ITPR1* into Grp3/Grp4-MBs carcinogenesis (Beckmann et al. 2019; Castillo-Rodríguez et al. 2018). We screened the gene expression profile of *ITPR1* among our and others three pediatric MBs dataset: GSE85217 (Cavalli et al. 2017); GSE37418 (Robinson et al. 2012) and EGAS00001001953 (Northcott et al. 2017), and the low expression of *ITPR1* gene was also found on Grp3/Grp4-MBs when compared to WNT/SHH-MBs. Of note, our ROC analysis showed that low expression of *ITPR1* gene is a potential predictive biomarker for segregating Grp3/Grp4-MBs subtypes.

Furthermore, by screening the degree of nodes in the PPI network and KEGG enrichment analysis, we showed that overlapping of these *ITPR1*-coregulated genes present genetic and co-expression interactions and were significantly associated with the signaling pathways regulating pluripotency of stem cells, regulation of actin cytoskeleton, Ras signaling pathway and tight junction. The Ras Signaling was reported as regulator key element of several aspects of normal cell growth and malignant transformation. Further, it was also associated with metastatic dissemination and poor survival in breast cancer (Wright et al. 2015). Surprisingly, we showed that *ITPR1*-coregulated genes are also involved in relevant hallmarks of cancer as metastasis, cell invasion, migration, differentiation and proliferation, as described in Table 1. Intriguingly, we also observed that in most of the samples of pediatric MBs tissues from all four cohorts, 17 *ITPR1*-coregulated genes were also found downregulated in the majority of samples of Grp3-MBs. Together, these *ITPR1*-coregulated genes are involved in pathways associated to the metastatic markers of Grp3-MBs.

Finally, we also found that low gene expression of *ATP1A2*, *MTTL7A* and *RGL 1* were significantly associated with the occurrence of metastasis, particularly for Grp3-MBs. In addition, we demonstrated that low gene expression of *RGL 1* was associated with worse OS in MBs. Studies addressing the role of these genes in tumorigenic processes also are very scarce. Interestingly, the low gene expression of *ATP1A2* was also found in breast cancer as a predictive and a prognostic factor (Bogdanov et al. 2017). Yet, the association between the downregulation of *RGL 1*, metastasis and worse OS on MBs were neither described, nor explored to date.

Conclusion

In summary, this study highlights *ITPR1* as a hub gene for Grp3-MB. Using an integrative bioinformatic approach, *ITPR1* is potentially involved in the metastatic process of MBs. Nevertheless, further investigations are needed to fully verify and validate the function of this gene in MB setting, and to balance their role in the underlying mechanisms of Grp3-MB metastization.

Ethical Statement

Ethics approval and consent to participate: This study was approved by the HC/FMRP-USP Research Ethics Committee (Approval number: CAAE: 62604816.4.000.5440) and informed consent was obtained from all patients included.

Consent for publication: Not applicable.

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

Competing interests: The authors declare that they have no competing interests.

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Authors' contributions: E.T.V. and P.F.C planned and conducted data analysis and drafted the manuscript. G.R.S, L.C.V, A.M.S, C.A.P.C; G.A.V.C and L.F.P.N wrote and organized the data, created the figures/tables, edited and critically revised the manuscript. R.G.P.Q, S.K.N.M, S.R.B, C.A.S and L.G.T revised the text for important intellectual content. All authors critically read and approved the final manuscript

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Figures

Figure 1

Gene networks of co-expression module displaying the most connected genes (hub genes). Analysis of co-expression reveal ITPR1 as hub gene in Group 3-MBs. The size of the node is proportional to its degree. The genes with co-expression are represented in blue and the genes with interactions are written in red. The module was constructed by CEMiTool package

Figure 2

Overview of expression ITPR1 gene in MBs subtypes and clinical relevance. Violin plots showing the levels of ITPR1 gene expression among our RNA-seq data and three datasets of pediatric MBs samples obtained of R2 Genomic Analysis online platform. All analyzes were performed by separating the samples into molecular subgroups of MBs: (a) Validation of low levels of ITPR1 gene expression in our cohort of pediatric MB patients by RNA-Seq represented through the violin plots among the molecular subgroups of MBs (WNT n=4; SHH n=4; Group 3 n=4 and Group 4 n=5); (b) GSE85217(n=643), (c) GSE37418 (n=73) and (d) EGAS00001001953 (n=186). The profile of ITPR1 gene expression was significantly observed downregulated in Grp3/Grp4-MBs when compared with others molecular subgroups of MB. ANOVA were conducted using the Tukey multiple comparisons post test to assess the statistical significance of the differences. * indicates ($p < 0.001$). (e) ROC curve analysis for prediction of G3 and G4 MBs among pediatric patients by ITPR1 level. (f) Low levels of ITPR1 are associated with metastasis in MBs samples primary (n=343) and metastasis (n=167), ($p < 0.05$)

Figure 3

Hierarchical unsupervised clustering of primary MB datasets. Samples were clustered according molecular subgroups of MBs: SHH (blue), WNT (purple), Group 3 (pink) and Group 4 (green). Heatmaps summarizing a downregulated profile of expression genes positively coregulated with ITPR1 (FGL2, ALDH1A, ATP1A2, FGFR2, CEND1, GR1A4, METTL7A, NAV2, RGL1, JAM2, PCGF5, ZCCH24, ANTXR1, WWTR1, ZFP36L1, RILPL2, MSN) in (a) our cohort (n=17); (b) GSE85217(n=643), (c) GSE37418(n=73) and (d) EGAS00001001953 (n=186). Red indicates that the expression of genes is relatively upregulated, blue indicates that expression of genes is relatively downregulated. The heatmap was generated using the ComplexHeatmap package and Pearson distance as Metric. Clustering using the Ward.D2 algorithm

Figure 4

(a) PPI analysis reveals interactions potential of ITPR1 and its coregulated genes. Circles represent genes and the results within the circle represent the structure of proteins. The ITPR1-RGL1-ANTXR1 axis-regulated indicated interaction of genes associated with worse prognosis. ITPR1-ATP1A2-MTTL7A-RGLI suggests a putative gene interaction coregulated with metastatic risk potential in Group 3-MBs. Lines colors represent evidence of the interaction of proteins between genes: genetic interactions (line in blue) and co-expression (line in red). (b) KEGG pathway Enrichment analysis in MBs: Abbreviations: KEGG, Kyoto Encyclopedia of Genes and Genomes. The significant enriched KEGG terms in MBs were based on their functions. Enrichment was done by Enrichr, using KEGG databases. The grayscale bars represent the p-value, increased from the bottom up

Figure 5

Analysis of ITPR1-gene expression with biological and clinical importance in MBs samples by GSE85217: The expression levels level of (a) ATP1A2, (b) MTTL7A and (c) RGL1 in Group 3-MBs primary samples (n=62) compared to metastasis patients (n=43) ($p < 0.05$). Kplan-Meier survival plots showing that low-expression of the (d) ANTXR1 and (e) RGL1 reveals statistical significance associated with poor survival in MBs patients $p = 0.0037$ and 0.029 respectively. Red, low-risk group, blue high-risk group. X-axis, time (months); Y-axis, overall survival probability.

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