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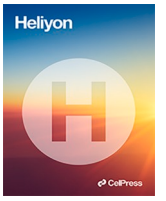

Research article

Differential gene expression analysis supports dysregulation of mitochondrial activity as a new perspective for glioblastoma's aggressiveness

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ABSTRACT

Brain cancer is considered one of the most aggressive and lethal types of cancer, including primary tumors, being subdivided into milder forms such as low-grade gliomas and glioblastoma, considered the most aggressive form with higher invasion. Among the hallmarks of glioblastoma, the deregulation of mitochondrial metabolism has not yet been fully elucidated. Therefore, the search for mitochondrial biomarkers that can be used as indicators of the progression of this type of cancer is necessary. The aim of this study was to investigate the difference in gene expression between astrocytoma-type gliomas and glioblastomas, and how genes involved in mitochondrial metabolism can influence the proliferative cascade and be associated with tumor invasion. From the differential analysis of glioblastoma expression when compared to the milder form, 11 differentially expressed genes (DEGs) were found in our study, six of which were upregulated (*ATP5MGL, C15orf48, MCUB, TERT, AGXT* and *CYP27B1*) and four downregulated (*SLC2A4, GK2, SLC25A48, ETNPPL* and *HMGCS2*). To validate the findings, we used other independent bulk RNA-seq datasets and evaluated the number of normalized counts of the DEGs founded. Among these genes, we highlight that none of them had been reported in glioblastoma until this research, and we suggest these genes as possible biomarkers to be further explored, since they are associated with essential pathways for the tumor, such as glucose metabolization, gluconeogenesis, calcium and vitamin D metabolism, tumor progression and activation of the invasion cascade.

1. Introduction

Brain cancer is a tumor that starts and remains in the central nervous system (CNS), and it includes some of the most aggressive cancers in children and adults [1,2]. Overall, brain tumors can be divided into two categories: primary brain tumors derived from cells intrinsic to the CNS and metastases derived from extracranial sites [3]. In addition, brain tumors can be classified as: (i) benign, when

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they rarely spread and invade surrounding tissues, exhibit distinct borders and have very slow-growing cells; and (ii) malignant, when they easily invade other parts of the brain and spinal cord, lack distinct borders and have fast-growing cells [2].

Among the most common primary brain tumors, there is glioma, which arises from glial cells in the CNS [4,5]. In general, gliomas can be classified according to their histological characteristics into astrocytomas, oligodendrogliomas and ependymomas, which are grouped as low-grade gliomas (LGGs) [6]. Considering that cancer is a genetically based disease, a growing body of evidence has suggested the association of mutations in specific genes with the development of various types of gliomas, including mutations in the genes *TERT* [7] and *IDH* [8].

On the other hand, glioblastoma (GBM) is characterized as a grade IV astrocytoma-type glioma (ATG) and represents the most common and aggressive primary malignant brain tumor, with great genetic heterogeneity [9], being the subtype of glioma with the worst prognosis, due to their invasive and aggressive nature [10]. As most malignant tumors, glioblastomas exhibit altered metabolism to support a variety of bioenergetic and biosynthetic demands for tumor growth, invasion, and drug resistance. Changes in glycolytic flux, oxidative phosphorylation (OXPHOS), pentose phosphate pathway (PPP), fatty acid biosynthesis and oxidation, and nucleic acid biosynthesis, are observed in GBM to help drive tumorigenesis [11].

In this sense, when the cell becomes neoplastic, it is common for it to show various alterations in its metabolism [12]. Among these, the reprogramming of energy metabolism stands out, as it is essential to meet the demand for cell proliferation, since it promotes the generation of energy and reallocation of essential substrates for the biosynthesis of macromolecules necessary for the growth [13]. Despite the well-established Warburg Effect [14], evidence shows that some cancers may prioritize the OXPHOS pathway over glycolysis as the primary source of ATP production [15,16]. However, given the vast heterogeneity of different cancer types, the preference for the glycolytic or OXPHOS pathways remains unclear and under investigation.

Therefore, since mitochondria control OXPHOS and play an active role in the biosynthesis of macromolecules [17], their role in the pathophysiology of tumors is undoubtful, as the relevance of mitochondrial function in various types of cancer has been increasingly demonstrated. These include breast cancer [18], hepatocellular carcinoma [19], gastric cancer [20] and LGGs and GBMs [19]. Especially in brain tumors such as LGGs and GBMs, mitochondria-mediated abnormalities are observed, such as a preference for ATP generation by glycolysis, high production of reactive oxygen species (ROS) and dysfunctions of the intrinsic and mitochondria-dependent apoptotic pathway, as well as mutations in mtDNA and alterations in mitochondrial structure and energy metabolism [15].

Given that the proliferative processes of cancer are associated with mitochondrial mechanisms and knowing that these include essential cellular functions (such as oxidative metabolism, apoptosis, cellular inflammation, mitochondrial and nuclear transduction, invasion and other pathways), there is a need for research into mitochondrial details in GBM [21]. In this study, we investigated the difference in gene expression between ATGs and GBMs, thus verifying how genes involved in mitochondrial metabolism may be differentially expressed between less severe and more aggressive glioma subtypes, which may influence the proliferative cascade and be associated with tumor invasion.

2. Materials and methods

2.1. Sampling and data extraction

To carry out this research, differential gene expression was performed using ATG and GBM samples available in The Cancer Genome Atlas (TCGA) genomic database. A total of 349 samples were used: 194 ATGs samples from the TCGA-LGG dataset (dbGaP Study Accession: phs000178; accessed and downloaded on October 17, 2023, and currently available at https://portal.gdc.cancer.gov/projects/TCGA-LGG) and 155 GBM samples from the TCGA-GBM dataset (dbGaP Study Accession: phs000178; accessed and downloaded on October 17, 2023, and currently available at https://portal.gdc.cancer.gov/projects/TCGA-GBM) were included. No adjacent tissue or blood samples were used, only patients who donated primary tumor tissue were included.

High-throughput RNA-Seq counts data from both ATG and GBM were downloaded for differential gene expression analysis; unstranded counts were used as indicated by the GDC (https://gdc.cancer.gov/subject-tag/general-gdc); repeated individuals were filtered out, only one sample per person was considered. As the study was conducted with transcriptome from public databases without individual identification and did not include new biological samples from patients, there was no need for submission or approval of this research to the Research Ethics Committee.

Regarding data extraction, to create the expression count matrix for the primary ATG and GBM samples, individual count files were downloaded by selecting a new cohort from the TCGA-LGG and TCGA-GBM datasets (available at https://portal.gdc.cancer.gov/) and labeled according to tumor type (ATG or GBM). This cohort was moved to the data repository, where RNA-seq data in STAR count format (tab-separated values, TSV) was selected using the "Experimental Strategy" filter. The pipeline for generating STAR count files (available at https://docs.gdc.cancer.gov/Data/Bioinformatics_Pipelines/Expression_mRNA_Pipeline/) was not executed in this work; instead, we used the open access available files.

In addition to the RNA-seq data, we also downloaded metadata such as clinical data, sample data, and the sample sheet, which contains file names and their corresponding samples. Using Microsoft Excel [22], we then formatted a text file, retaining the gene_id, gene_name, and gene_type columns and selected gene counts for each sample from the unstranded column. The RNA-seq count matrices were then constructed, and subsequent importing, filtering, indexing, and functional analyses were performed using R [23] within the RStudio IDE environment [24], utilizing the Tidyverse [25] and dplyr [26] packages. Our script, available at https://github.com/ricardoolveira/GBM_ATG_Analysis/, provides all software and code used, as present in our Table S1 of Supplementary Material 1.

2.1.1. Selection of genes

Considering that the focus of this research was the investigation of nuclear genes involved in mitochondrial functions, we used the MitoXplorer 2.0 platform [27] to screen genes present in nuclear DNA that are involved in functions such as OXPHOS, glycolysis, apoptosis, mitochondrial dynamics and translation. At first, the platform indicated 1229 genes transcribed from the mitochondrial (mtDNA) and nuclear genomes (nDNA). In this research we focused on using nuclear protein-coding genes (mRNA) indicated by MitoXplorer 2.0. Thus, no mtDNA genes were included, nor genes that encode transporter or ribosomal RNA. The screening revealed 1192 genes in the high-throughput RNA-Seq overview, hereinafter referred to as "mitochondrial genes" because of their association with mitochondrial function. For this analysis, we used gene names from the HUGO Symbol database (available at: https://www.genenames.org/tools/search/#!/genes) as gene identifiers. RNA-seq datasets with other identifiers were converted using the annotate [28], AnnotationDbi [29] and org.Hs.eg.db [30] packages.

2.1.2. Differential expression analysis

Differential gene expression was analyzed with R language [31] in Rstudio [23] using the DESEq2 package [32]. Initially, redundant genes and patient entries were eliminated, retaining a singular representation for each. Subsequently, cluster filtering, standardized by DESeq2, was employed. This step was followed by the application of a criterion where only counts exceeding 10 were included, alongside a requirement for presence in more than 10 samples. This final filtering process revealed information on 1151 nuclear genes with mitochondrial function.

Standardization was based on the methods applied by DESeq2. The trimmed mean of M values (TMM) normalization method was used to standardize and normalize, and Cook's distance was applied to remove outliers. Batch correction was also performed. Finally, the p-value of the data was corrected using the Benjamini-Hochberg (BH) method. Principal component analysis (PCA) analysis was performed to verify sample homogeneity and differences in expression variation between the groups, using DESeq2's own plotPCA function. In this study, differentially expressed genes (DEGs) were those with an adjusted p-value of less than 0.05. The cutoff of Log2FoldChange = 2 (upregulated >2, downregulated < −2) was also applied. This method was adapted from a previous work [33].

We then performed gene set enrichment analysis (GSEA) using the Gene Ontology (GO) [34,35], Kyoto Encyclopedia of Genes and Genomes (KEGG) [36], and Reactome Pathways [37] databases. This analysis focused on nuclear genes with mitochondrial function, with terms considered significant at an adjusted p-value <0.05 using the Benjamini-Hochberg (BH) method. The packages used to perform the GSEA were clusterProfiler [38] and ReactomePA [39]. For more information, our script, the software, applications, tools, the versions and all the databases used in this research are available in Table S1 of our Supplementary Material 1.

2.1.3. DEGs in normal tissues

This step was carried out using GTEx Portal (available at: http://gtexportal.org) [40], which enables the visualization of gene expression in normal, non-cancerous tissues, and, for this research, it helped to understand the brain tissues in which the DEGs are normally expressed, thus contributing to the knowledge of how the pathological processes may be possibly influenced. All available tissues from the brain were included and other tissues with higher expression were used to demonstrate non-cancerous profiles here.

2.1.4. Clinical and statistical analyses

The demographic and clinical information of the patients and samples was taken to the bioinformatics analysis, where the following variables were considered: sex, age, clinical follow-up, type of treatment and histological subtype. The R language in Rstudio was used for the statistical analysis, with the ggplot2 [41], EnhancedVolcano [42], org.Hs.eg.db [30] and cowplot [43] packages that were used to plot the differential expression data. The statistical tests used were the following: descriptive (mean, minimum - min, maximum - max, standard deviation), Shapiro-Wilk normality test (T-test used for analyses with normality and Wilcoxon-Mann-Whitney test, or *U* test, used for samples without normality) and Bayesian frequency test.

2.1.5. In silico validation

The genes considered to be differentially expressed in this research were validated using three additional databases. Two of these databases were sourced from the Gene Expression Omnibus (GEO): GSE113474 (currently available at https://www.ncbi.nlm.nih.gov/geo/query/acc.cgi?acc=GSE113474) [44] and GSE196694 (currently available at https://www.ncbi.nlm.nih.gov/geo/query/acc.cgi?acc=GSE196694), [45]. The third additional database analyzed was the Chinese Glioma Genome Atlas (CGGA), identified as mrnaseq_693 in a cohort of Chinese samples (currently available at http://www.cgga.org.cn/download.jsp) [46–49]. All three databases were accessed and downloaded on October 19, 2024. It is important to note that the version of GSE113474 used was last updated on May 17, 2019, and the version of GSE196694 was updated on October 28, 2022. The CGGA database was last updated on January 4, 2023. Regarding the gene identification used, any other identifiers were converted to gene names: identifiers from GSE196694 in Ensembl format were converted to gene names, while those from GSE113474, already in gene names, were retained. CGGA identifiers were also in gene names and therefore remained unchanged. All RNA-seq count data was downloaded from their respective websites in.txt or.csv format, including CGGA and GEO.

For this validation, the RNA-seq count data was imported into RStudio IDE, filtered out of the count matrix only the genes that were considered to be differentially expressed, and then grouped for each of the sample types (ATG or GBM), finally the data was normalized using the Log2 transformation. Subsequently, the groups were analyzed for comparison using the Wilcoxon test with the ggpubr [50], rstatix [51] and svglite [52] packages in the R IDE environment. Genes with a p-value <0.05 were considered to be significantly validated and their means were used to compare whether the gene is upregulated or downregulated in GBM. The two databases extracted from GEO were compared because GSE196694 contains ATG data (12 samples), while GSE113474 contains GBM data (24

samples). In contrast, the CGGA database includes both ATG (300 samples) and GBM data (249 samples); therefore, we identified the molecular subtype before conducting comparisons within the same database. The script used for validation can be found in Table S1 of Supplementary Material 1. Therefore, Fig. 1 presents all the steps carried out during the study analyses (sampling, filtering mitochondrial genes, DEGs in glioblastoma, astrocytoma, and non-cancerous tissues, as well as statistical analyses, including the *in silico* validation).

3. Results

3.1. Sample characterization

In the study cohort, it is possible to observe that both ATG and GBM groups had a higher proportion of male individuals than female individuals (Table 1), which is in accordance with the global literature for this type of cancer [8,53]. A single individual with GBM did not have sex information and was therefore not included in Table 1. In addition, while ATG is a type of brain cancer that mainly affects younger people, GBM is a type that significantly affects older people, based on age visualization in our analyses (p-value <2.2e-16), which is consistent with the literature where there is conformity in the ATG for adolescents and young adults, and the GBM for older people [54,55]. Considering that GBM is an evolutionary form of ATG progression, it is understandable that it can affect older people who have had a worsening of their ATG prognosis [56].

When viewing the vital status of these individuals, it is possible to confirm a significant prognostic worsening (p-value <2.2e-16) between the GBM compared to the ATG, since the GBM group is mostly composed of deceased patients. This is one of the limitations of this study, since not all the samples used are from before the death of the patients, which is a common factor in studies of neurological tissues [57,58]. Although treatment for people with GBM is on the increase, it is worth noting that 95 of the 122 patients who have undergone some kind of treatment are already deceased (approximately 78 %), according to the data available. After performing a subsequent Fisher's exact test analysis to verify significant differences, we observed a p-value = 0.009953, indicating a significant difference in vital status between individuals undergoing treatment. This highlights the difficulty in treating people with this cancer since there is currently no known treatment that eliminates GBM cells and, in short, treatment for this type of cancer aims to reduce symptoms with palliative characteristics and low survival among this group [59,60].

3.1.1. Differentially expressed genes

As shown in Fig. 2, no individual had a large variation between the GBM and ATG groupings from the PCA analysis. The grouping between vital statuses is recurrent between the groups, since the ATG group is on the left and the GBM group is on the right. This analysis indicates that the groups are homogeneous in terms of vital status and that there is a difference in expression between both.

The analysis of the differential expression of the 1151 screened mitochondrial genes revealed 11 DEGs (downregulated - *SLC2A4, SLC25A48, ETNPPL, GK2* and *HMGCS2*; upregulated - *ATP5MGL, C15orf48, MCUB, TERT, AGXT* and *CYP27B1*) in GBM when compared to ATG, as demonstrated in Fig. 3 and Table 2.

In the more specific count analysis for the DEGs, the *HMGCS2, GK2, ETNPPL, SLC2A4,* and *SLC25A48* genes were downregulated, while the *TERT, MCUB, CYP27B1, ATP5MGL, AGXT, and C15orf48* genes were upregulated in GBM when compared to ATG (Fig. 4).

3.1.2. Expression profile in non-cancerous tissues

When examining the expression profile of these genes in normal (non-cancerous) tissues (Fig. 5), a different profile stands out

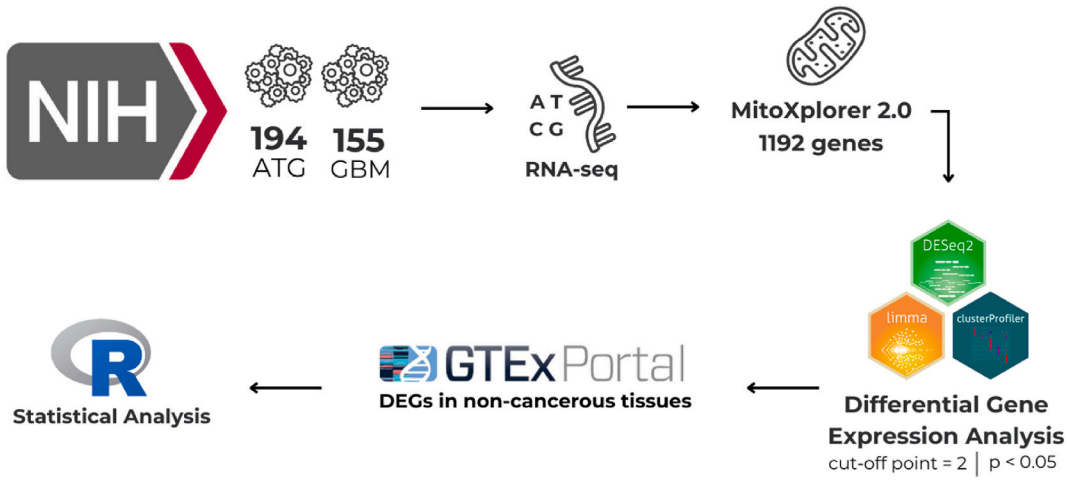

Fig. 1. Flowchart showing the workflow of filtering and differential analysis of mitochondrial genes in glioma, glioblastoma and non-cancerous tissues. The "Cancer cell", "Mitochondria'' and "RNA" icons in the flowchart were obtained from nuengrutai, dDara and Imron Sadewo, respectively, from thenounproject.com.

Table 1
Clinical data of the investigated ATG and GBM sample groups.

Groups	Sex		Age		P-value (U test)
	Male (%)	Female (%)	Min ~ Max	Mean	
GBM	100 (65.22 %)	54 (34.78 %)	21–89	59.71 ± 13.55	<2.2e-16
ATG	108 (55.34 %)	86 (44.66)	20–74	41.81 ± 12.64	

Groups	Vital Status			
	Alive (%)	Deceased (%)	Not Reported (%)	p-value (Fisher)
GBM	30 (18.63 %)	122 (80.12 %)	2 (1.24 %)	<2.2e-16
ATG	136 (75.34 %)	58 (24.47)	0 (0.0 %)	

Groups	Treatment			
	Yes (%)	No (%)	Not Reported (%)	p-value (Fisher)
GBM	122 (80.12 %)	22 (13.66 %)	10 (6.21 %)	0.002611
ATG	121 (57.28 %)	53 (35.34 %)	20 (7.38 %)	

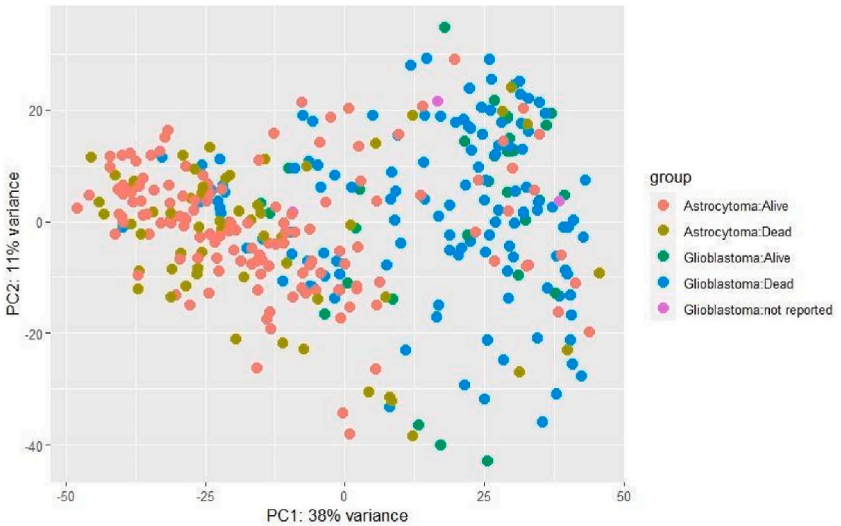

Fig. 2. Principal component analysis (PCA) between GBM and ATG individuals considering vital statuses based on the individuals' gene expression.

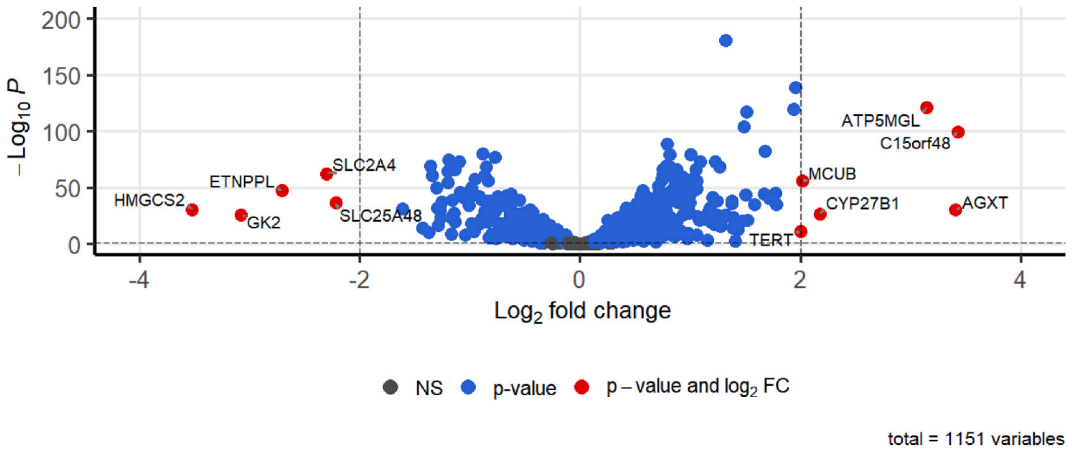

Fig. 3. Differentially expressed genes (FCcutoff = 2 and P-value <0.05) among the investigated genes from mitochondrial processes.

Table 2
Characterization of the significant DE genes among the investigated genes of mitochondrial functions.

Status	Gene	baseMean	log2FoldChange	stat	pvalue	padj
Downregulated	*HMGCS2*	4.131178e+00	−3.5375630	−11.944574	6.930415e-33	6.387180e-32
	GK2	2.005448e+00	−3.0916193	−10.859516	1.796985e-27	1.201128e-26
	ETNPPL	4.459516e+03	−2.7081317	−14.847644	7.205722e-50	1.695609e-48
	SLC2A4	1.618719e+02	−2.3006376	−17.121448	1.026893e-65	5.425836e-64
	SLC25A48	8.051872e+02	−2.1976805	−12.855795	7.980099e-38	9.811364e-37
Upregulated	*TERT*	3.263206e+01	2.0024732	6.987137	2.805525e-12	7.501578e-12
	MCUB	1.255430e+03	2.0222633	16.245346	2.409799e-59	9.476892e-58
	CYP27B1	1.982761e+02	2.1597497	11.037068	2.531599e-28	1.778417e-27
	ATP5MGL	4.597258e+00	3.1397391	23.832408	1.541421e-125	9.155507e-123
	AGXT	7.100821e+00	3.3906601	11.779347	4.987780e-32	4.367794e-31
	C15orf48	2.247924e+01	3.4092441	21.418543	8.975214e-102	2.342395e-99

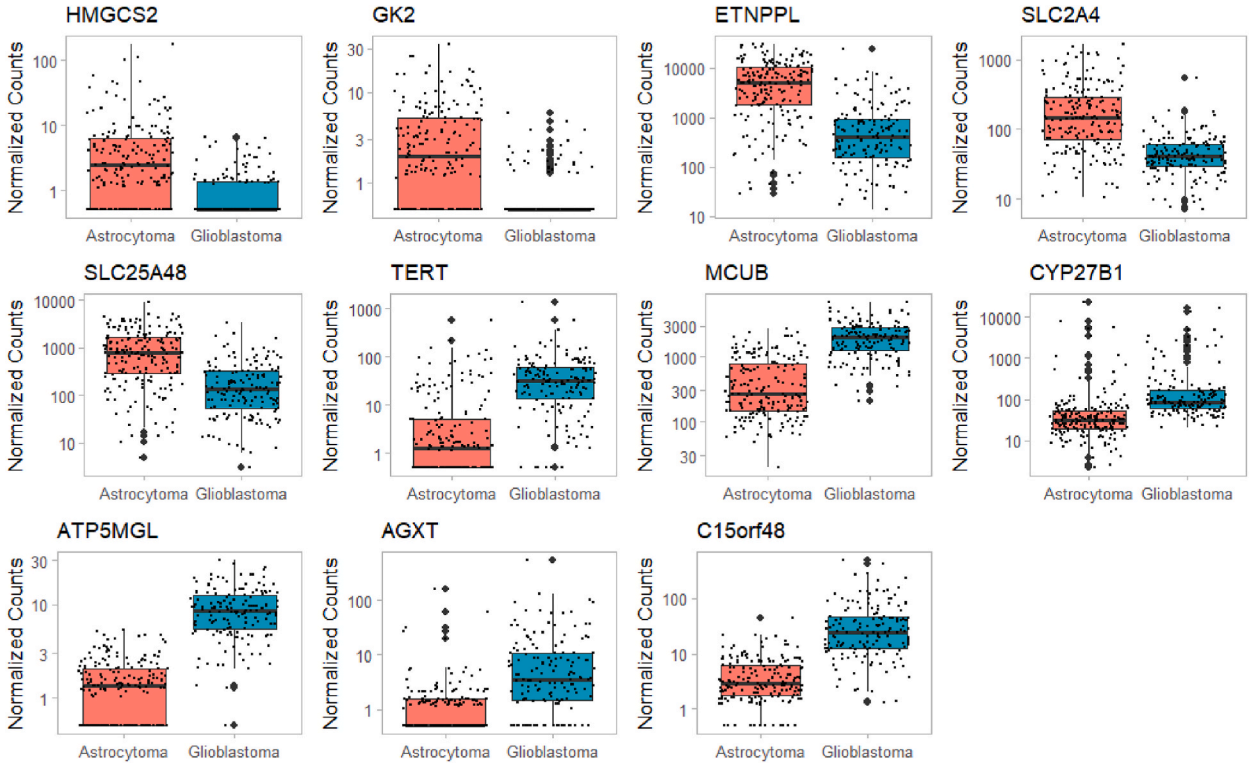

Fig. 4. Differential gene expression of upregulated (*ATP5MGL, C15orf48, MCUB, TERT, AGXT* and *CYP27B1*) and downregulated (*SLC2A4, SLC25A48, GK2, ETNPPL* and *HMGCS2*) genes in GBM when compared to ATG.

compared to that observed in GBM. For instance, *SLC25A48 and ETNPPL* show higher expression in non-cancerous brain tissues, in contrast to GBM. Moreover, *ATP5MGL* (also called *ATP5L2*), *C15orf48, MCUB, TERT, AGXT* and *CYP27B1* genes, which were upregulated in GBM, exhibit low expression in non-cancerous brain tissues.

3.1.3. Enrichment analysis

In the gene enrichment of our expression analysis, we ranked the main terms based on the adjusted p-value (Table 3). In a broader context, there is an observed upregulation of processes potentially linked to the maintenance of mitochondrial functionality, particularly mechanisms facilitating the synthesis of proteins crucial for preserving the integrity of both mitochondria and cells, specifically within GBM cells, encompassing terms such as GO:0098798 ("mitochondrial protein-containing complex"). Across the three analyzed enrichment databases (GO, KEGG and Reactome), data pertaining to the expression of mitochondrial translation is evident. Based on the enrichment scores obtained, there is suggestive evidence indicating its overexpression in GBM. Notably, within the Reactome enrichment analysis, two terms associated with neuronal function emerge, both downregulated. These findings potentially correlate with the upregulation of functions aimed at maintaining cellular integrity.

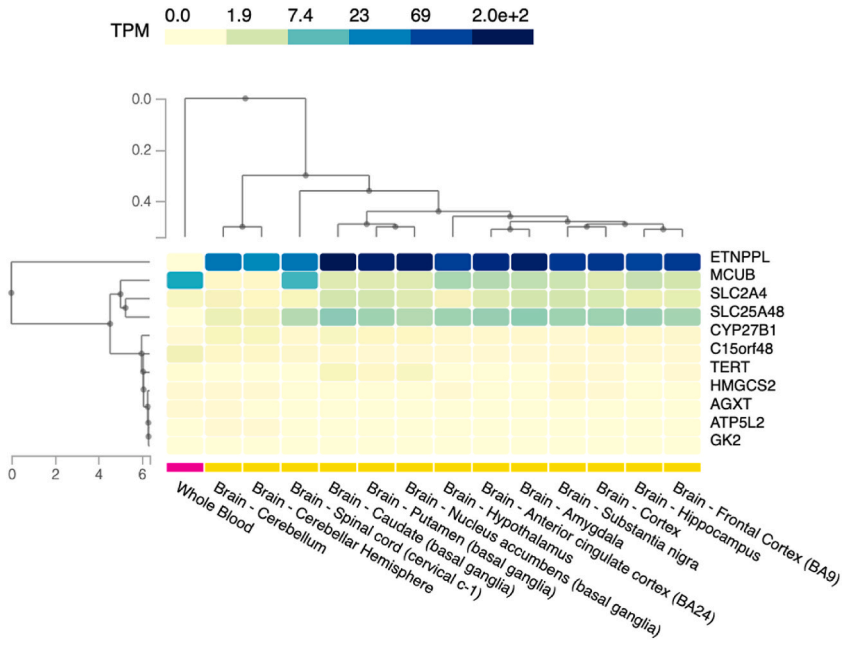

Fig. 5. Normal expression of the found DEGs in different brain tissues, as well as whole blood.

Table 3
Enrichment Analysis of nuclear genes of mitochondrial function differentially expressed.

Database	ID	Description	pdaj	enrichmentScore
GO	*GO:0098798*	mitochondrial protein-containing complex	0.00548012	0.4031142
	GO:1990904	ribonucleoprotein complex	0.01092437	0.4965351
	GO:0003735	structural constituent of ribosome	0.01476710	0.5293781
	GO:0005198	structural molecule activity	0.01476710	0.5247663
	GO:0032543	mitochondrial translation	0.01476710	0.4583425
	GO:0044248	cellular catabolic process	0.01476710	−0.3187708
KEGG	*hsa03010*	Ribosome	0.007042611	0.6038421
	hsa04920	Adipocytokine signaling pathway	0.014992144	−0.6796592
	M00002	Glycolysis, core module involving three-carbon compounds	0.01395138	0.8051921
Reactome	*R-HSA-5368287*	Mitochondrial translation	0.004644261	0.4917053
	R-HSA-72766	Translation	0.005249472	0.4573345
	R-HSA-112315	Transmission across Chemical Synapses	0.036384962	−0.7597380
	R-HSA-112316	Neuronal System	0.036384962	−0.7597380

3.1.4. Protein-protein interaction

Finally, we performed a protein-protein interaction network on the STRING platform [61] (Fig. 6), where it was possible to observe that *AGXT* and *ETNPPL* genes present co-expression interactions (score 0.101 and black lines), which is an interesting finding, considering that these two genes have opposite expressions in our study. Moreover, most genes exhibit various types of interaction, such as *SLC25A48* with *MCUB*, which are linked in textual databases (light green line) and demonstrate interactions between them (*TXNDC15*), experimentally validated (pink line) and through co-expression. It is also noteworthy that genes *C15orf48* and *ATP5MGL* possess accurately depicted interaction relationships in the literature (blue line), as well as experimentally verified interactions. *PCK1* also acts as a bridge between *AGXT* and *GK2*, genes responsible for regulating glycolysis, and is being co-expressed along with the *SLC2A4* gene. These interactions provide valuable insights into underlying biological processes, such as metabolic regulation and cellular signaling.

3.1.5. Validation analysis

Using the number of normalized counts in Log2 from three independent RNA-seq datasets, we compared the expression levels of the upregulated and downregulated DEGs found in our study, to validate our results *in silico* (Fig. 7). It is observed that, despite using distinct and independent datasets from the original study data, the DEG expression results remained consistent in the three databases for most genes. Furthermore, in the comparison results of GBM with ATG from CGGA, all genes agreed with the current study data, with statistically significant values (Fig. 7A). In the GEO datasets results, it was observed that the *ATP5MGL* gene was downregulated in GBM compared to ATG, contrary to the findings of CGGA and the current study, and the genes *ETNPPL, SLC2A4, SLC25A48,*

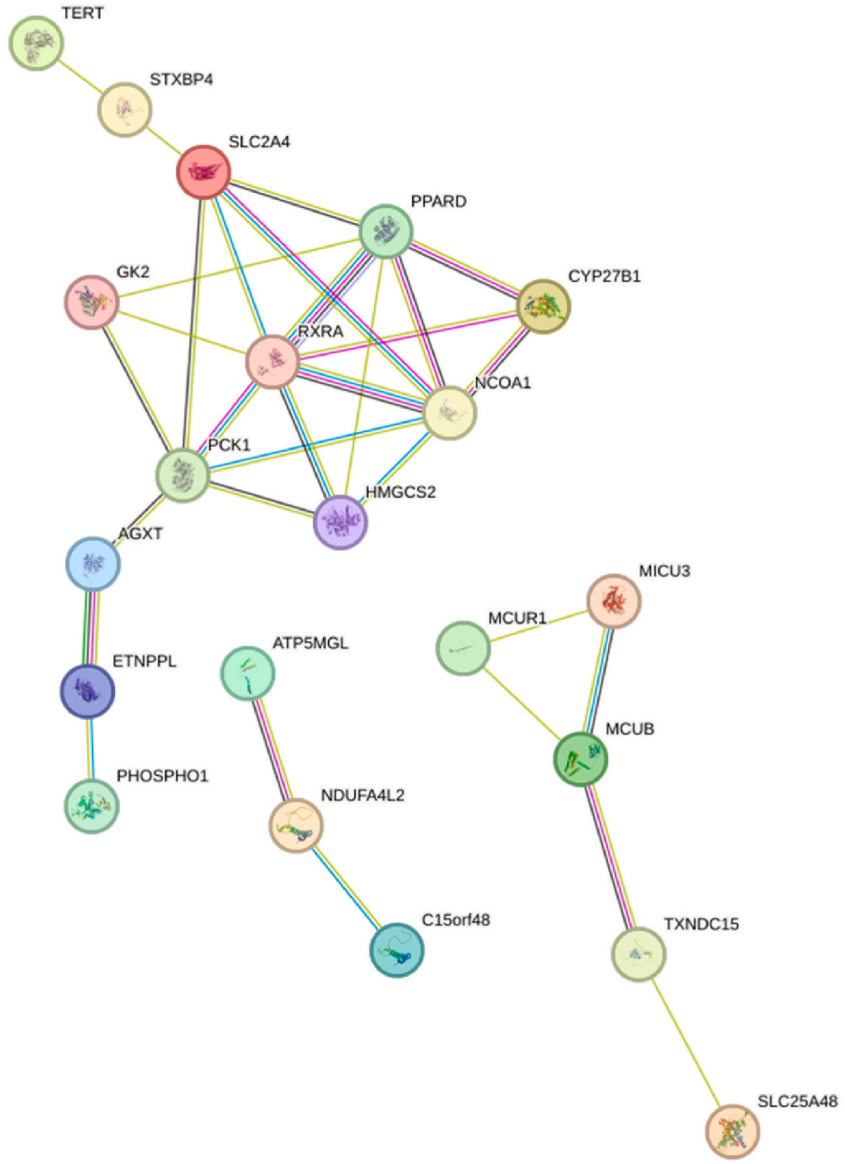

Fig. 6. Protein-protein interaction network of downregulated (*SLC2A4, GK2, SLC25A48, ETNPPL* and *HMGCS2*) and upregulated (A*TP5MGL, C15orf48, MCUB, TERT, AGXT* and *CYP27B1*) genes in GBM when compared to ATG.

HMGCS2 and *GK2* did not present statistically significant values of comparisons, the latter due to ATG not having expression information for this gene in these databases. However, the other genes agreed with the current study and CGGA and the comparisons were statistically significant (Fig. 7B).

4. Discussion

4.1. Downregulated genes

The *HMGCS2* gene, which encodes the mitochondrial enzyme 3-hydroxy-3-methylglutaryl-CoA synthase 2, has been explored as a possible biomarker in different types of cancer due to its function in the ketogenesis process (participating as a regulator in the conversion of acetyl-CoA in ketone bodies) and mitochondrial metabolic reprogramming [62]. These essential functions for energy metabolism make *HMGCS2* fundamental in carcinogenesis and tumor progression, especially described as a tumor suppressor in renal carcinoma [62,63], hepatocellular carcinoma [64,65], intestinal carcinogenesis [66], and tumor angiogenesis of colorectal cancer [67]. Furthermore, low *HMGCS2* expression is related to lower survival in cancer patients [62,67].

Ketogenesis, a process measured by *HMGSCS2*, appears as a relevant pathway in the pathogenesis and progression of gliomas and

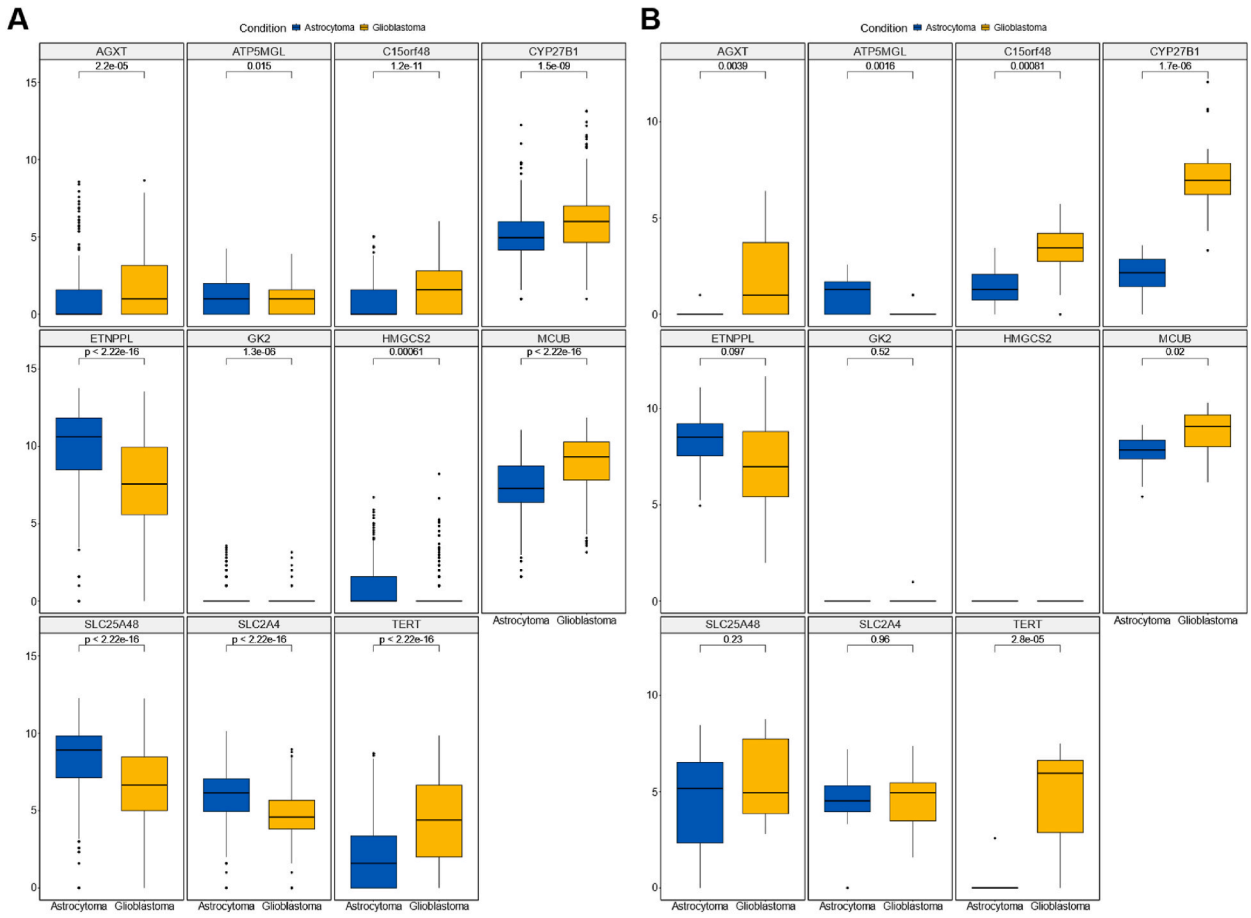

Fig. 7. *In silico* validation of the differential gene expression of upregulated (*ATP5MGL, C15orf48, MCUB, TERT, AGXT* and *CYP27B1*) and downregulated (*SLC2A4, GK2, SLC25A48, ETNPPL* and *HMGCS2*) genes in Glioblastoma when compared to Astrocytoma. A. Validation using CGGA database. B. Validation using GSE196694 (ATG) and GSE113474 (GBM) databases.

glioblastomas, which have a high metabolic rate, characterized by an increased demand for energy [68–72].

The *ETNPPL* (ethanolamine phosphate phosfoliase) was shown to be underexpressed in our analysis. This enzyme is mainly related to lipid metabolism. A previous study explored this gene as a potential astrocytic marker and its results demonstrated that *ETNPPL* has selective expression in adult astrocytes and exhibits variations in expression levels in response to different types of stimuli [73]. This finding suggests a significant role for *ETNPPL* in regulating astrocytic function in contexts of varied stimulation, indicating its possible relevance in physiological and/or pathological processes associated with astrocytic activity. Furthermore, *ETNPPL* was confirmed as a specific marker of neural stem cells in both humans and monkeys [74].

ETNPPL expression may be associated with various central nervous system pathologies, including cancer, psychiatric disorders, injuries, stroke, and inflammatory processes [73]. In gliomas, ETNPPL protein expression has been identified in both glioma cells and astrocytes in the human brain. An inverse correlation exists between ETNPPL expression and the degree of malignancy in gliomas [75]. Overexpression of ETNPPL has been shown to reduce the growth of glioma stem cells, suggesting an inhibitory effect on gliomagenesis. This gene appears to negatively regulate glioma growth, primarily by reducing phosphoethanolamine and phosphatidylethanolamine, factors associated with membrane synthesis. Consequently, low ETNPPL expression may be linked to glioma progression and poor survival [75].

Reduced expression of *GK2* (glycerol kinase 2) was seen in our examination of GBM samples, indicating a potential role for this protein in the pathophysiology of this illness. The findings pointed to a reverse correlation between GK2 expression and GBM, indicating a possible function for this gene as a tumor suppressor. Glycerol kinase is an important enzyme in glycerol metabolism, it limits the rate at which glycerol is converted to glycerol 3-phosphate, being important for the synthesis processes of glucose, glycerolipids and proteins [76–78]. Despite its significant role in energy metabolism, which makes it relevant in the investigation of different types of cancer, there are few studies that explore *GK2* in carcinogenesis and tumor progression. Furthermore, our study appears to be the first to highlight it in glioblastoma.

It has already been described in the literature that different types of tumors tend to present changes in different pathways of energy metabolism, such as glycolysis and OXPHOS [79,80]. Among the proteins that participate in the glycolytic pathway, GLUT4 is

characterized as an important insulin-dependent glucose transporter encoded by the *SLC2A4* gene [81]. Given this, a study revealed that ivermectin-mediated inhibition of GLUT4 in Glioblastoma lines *in vitro* resulted in reduced cell viability and increased apoptosis, an effect that was reversed when GLUT4 was overexpressed [82]. Another study on brain cancer, in this case medulloblastoma, revealed the elevated expression of several key glycolysis enzymes when comparing tumor, adjacent and non-tumor tissue, among them, *SLC2A4* was overexpressed [83].

In contrast, Azzalin et al. (2017) demonstrated that the inhibition of GLUT4 by indinavir was not harmful to GBM cell proliferation and viability when compared to the inhibition of GLUT1 and GLUT3 transporters. However, the authors point out that when GLUT4 was inhibited, there was an overexpression of GLUT1, suggesting a compensatory mechanism possibly underlying the modest impact of targeted GLUT4 inhibition [84]. In another work, a gene expression analysis of GBM biopsies identified considerably low levels of GLUT4 expression [85].

In line, our analys es revealed underexpression of *SLC2A4* in GBM samples, a finding that contrasts with most of the results described above that demonstrate glucose transporter expression as a relevant factor in tumor development. In addition to the fact that there are few studies investigating the relationship between GLUT4 and GBM, the results above highlight inconsistencies in the literature, since the decrease in its gene expression has sometimes been shown to harm tumor development, and sometimes it has not been shown to significantly influence it.

In contrast, the *TERT* gene responsible for telomere homeostasis was shown to be overexpressed in our analysis. In most healthy somatic cells, the *TERT* gene is silenced, however, reactivation of telomerase activity in several types of cancer is widely recognized to prevent senescence and promote cell proliferation [86]. It has been reported in the literature that mutations in the promoter region of this gene can result in its overexpression [87,88], up to 2.5 times, possibly due to changes in the affinity between transcription factors and their respective binding sites [89]. In view of this, the association of mutations in the promoter region of the *TERT* gene with the development of GBM has been reported with considerable frequency (70–80 %) by genomic studies [90], in addition to its association with a worse prognosis [91]. The study by Amen et al., 2021 demonstrates that inhibition of GABPB1L, a TERT transcription factor, was associated with a reduction in its expression and tumor growth, as well as a higher survival rate in trials with animal models affected by GBM [92].

Similarly, silencing *TERT* gene expression by reversing a mutation in its promoter region using the CRISPR method resulted in decreased telomere length and cell proliferation, as well as an increase in the number of anaphase bridges (a hallmark of telomere dysfunction) in GBM cell lines, and increased survival rates in animal models [93]. These findings agree with our results, in which we also found TERT to be overexpressed in GBM. Taken together, they support the premise that TERT is a markedly key factor in GBM tumor development.

SLC25A48 consists of a mitochondrial transmembrane protein responsible for transporting choline into the mitochondria [94,95], where it acts as a substrate for betaine synthesis [96]. Therefore, the knockout of SLC25A48 in HEK293 lines was shown to impair betaine synthesis, cell proliferation and O_2 consumption rates, in which all outcomes were capable of reversal when the expression of that gene was restored [95].

Recently, Zhou & Li (2023) identified a possible protective effect of the expression of the *SLC25A48* gene in GBM by building a prognostic risk model via regression analysis using the LASSO method [97]. In line with this, Tang et al. (2020) constructed a 3D cellular model of glioblastoma and observed that several transporters were underexpressed, including *SLC25A48* [98]. Both results suggest that *SLC25A48* underexpression is an important factor in the development of GBM.

Despite the lack of a variety of functional studies involving SLC25A48 and cancer, especially GBM, we outline some hypotheses about the mechanism by which underexpression of the transporter may be associated with GBM. Firstly, betaine synthesis has been shown to depend directly on the transport of its substrate, choline, to the mitochondrial matrix [99]. From this, in two studies, an inhibitory effect of betaine on cell proliferation was observed in prostate cancer cell lines and animal models with lung cancer [100, 101]. Similar results were described in two meta-analyses demonstrating that high betaine levels were associated with lower cancer incidence [102,103].

Our analys es revealed results in agreement with those mentioned above, in which SLC25A48 was underexpressed in GBM, which apparently seems to favor the development of cancer, given the possible anti-tumoral role attributed to betaine. However, due to the considerable lack of studies that directly investigate the relationship between SLC25A48, betaine synthesis and the development of cancers, mainly GBM, the importance of interpreting the hypotheses outlined with caution is reiterated, as well as the relevance of carrying out functional studies to understand the significance of the altered expression of SLC25A48 more clearly in the carcinogenic process. Other types of transporters from the same family that have also been associated with GBM include SLC9A1, SLC16A1, SLC16A3 and CA9, which are downregulated in types of GBM but may be functioning properly in other cell types from the same tumor. Whose functions include decreasing the pumping of ions and glucose into the intracellular space, and play an essential role in extrusion protons from tumor intracellular space [104].

4.1.1. Upregulated genes

Regarding *MCUB* (also known as *CCDC109B*), its main functions are in the metabolism of calcium ions (Ca^{2+}), which also involves mitochondria. MCUb in this process is an auxiliary or regulator of the MCUc (mitochondrial calcium uniporter complex) transport channel. In moments of mitochondrial stress that lead to impaired calcium transport, MCUb can incorporate itself into MCU and compensate for the function of the channel protein [105,106]. In previous studies [107–109], the *MCUB* gene was overexpressed in GBM tissue when compared to LGG, as seen in our study. Furthermore, in all three studies, it was associated with a poor prognosis in gliomas in general.

Considered a hub gene and potential oncogene, *MCUB* is associated with functions such as calcium absorption, apoptosis,

proteolysis, angiogenesis, regulation of proliferation and inflammatory response. Its expression increases according to the progression of the glioma, which was seen in our research, as well as an ov erexpression occurring in glioblastoma (severe grade) compared to astrocytoma (lower grade) tissues [108]. *MCUB* gene expression *in vitro* and *in vivo* was significantly associated with glioma progression, invasion and migration [109].

It is worth noting that, when the ATG group was divided into people with higher and lower MCUb expression, those with higher expression had lower survival rates than those with lower expression [109]. Loss of the gene in both *in vivo* and *in vitro* was associated with loss of progression, invasion and migration of tumor tissue [109]. Thus, our suggestion in relation to MCUb is that as calcium remodeling is necessary for resistance to cell death, cell invasion and metastasis, in glioblastoma, which is the metastatic form of astrocytoma, the remodeling of this ion is potentially associated with its formation, especially in neuronal tissue, where it is one of the essential roles for synapse and neuronal formation, which is present within our enrichment [110,111].

Moreover, *CYP27B1* (cytochrome P450 family 27 subfamily B member 1) is a protein associated with the inner membrane of mitochondria that participates in the synthesis and metabolism of cholesterol, steroids and other lipids, and mainly in the synthesis of the active form of vitamin D, associated with calcium metabolism [112]. Thus, individuals with GBM and many alterations in the *CYP27B1* gene have already been associated with poor survival and more severe forms of this cancer [113].

A previous study [114] compared the expression of this gene between normal and tumor human brain endothelial tissue and observed that, in addition to being expressed only in GBM, this gene is associated with tissue differentiation and proliferation. Another study [115] reported that, because *CYP27B1* is highly variable in mRNA processing, its expression can also be highly variable, as there are many splice variants that can affect this gene and it is also associated with calcium metabolism, with high expression in GBM.

Therefore, the suggestion between *MCUB* and *CYP27B1* to calcium ion metabolism in GBM is that, in addition to trying to maintain the normal function of tumor cells, their roles may activate proliferation, tumorigenesis and invasion pathways, since calcium metabolism is associated with synapses and neurocognitive functions as it is also shown in our enrichment [116]. In prostate cancer (PC), *CYP27B1* has been reported as underexpressed in relation to normal tissue, unlike GBM, and is considered a tumor suppressor in PC [117], while in Head and Neck Squamous Cell Carcinoma it is already considered a risk gene [118]. In other diseases such as Parkinson's, *CYP27B1* overexpression is associated with cell protection through the clearance of alpha-synuclein [119].

ATP5MGL (ATP Synthase Membrane Subunit G Like) has not been reported or linked to any type of cancer thus far. There is little information about its functions and links in the literature, but part of it is highlighted by its higher expression in mammary glands during pregnancy and lactation, compared to women who are not at this stage, and it is worth mentioning that this is a time of high cell proliferation [120]. Since it was upregulated in GBM in our study, its pathway needs further investigation to verify at what step it is associated with GBM.

C15orf48 (also known as NMES1, Coxfa4l3, MISTRV or MOCCI) is a mitochondrial protein homologous to the *NDUFA4* subunit of cytochrome C oxidase (complex IV or CIV), interacting with multiple subunits in complexes I and IV [121,122]. *C15orf48* expression is induced by inflammatory stimuli, such as interleukin−1β, interferon-γ, toll-like receptor ligands and viral infection, replacing *NDUFA4* in complex IV. In this way, *C15orf48* reduces CIV activity, mitochondrial membrane potential and production of reactive oxygen species (ROS) and protects against cell death due to viral infection [121,122].

Regarding its expression in human tissues, studies report that *C15orf48* is expressed throughout the healthy gastrointestinal tract, having a negative regulation in squamous cell carcinomas of the esophagus and lung, which suggests that this gene could be a potential tumor suppressor, although its functions are still poorly understood [123–126].

In a prognostic analysis of pan-cancers, it was observed that C15orf48 was significantly associated with the prognosis of multiple types of cancer, in particular, gliomas [121]. Regarding gene expressivity, it was reported that high expression of this gene was significantly associated with shorter overall survival, progression-free survival, disease-specific survival and disease-free interval in LGGs [121]. Furthermore, *C15orf48* was significantly enriched in malignant gliomas, suggesting its role in promoting the malignant development of gliomas [121]. Further univariate and multivariate analyses of this study revealed that *C15orf48* may serve as an independent prognostic factor for glioma [121]. Interestingly, in a study analyzing changes related to ferroptosis in glioblastoma, 23 genes with high tumor risk scores were identified, and four of these genes, including *C15orf48*, had no previous reports in the literature related to ferroptosis nor in studies related to glioblastoma [127].

Lastly, the *AGXT* gene encodes the peroxisomal enzyme alanine-glyoxylate aminotransferase, responsible for glyoxylate detoxification and catalyzing L-alanine and glyoxylate to pyruvate and glycine to glycine, expressed mainly in liver cells [128–131]. Mutations in the *AGXT* gene can cause disturbances in glyoxylate metabolism, leading to the accumulation of oxalate in the body and, eventually, influencing the expression of clinical phenotypes, such as type I primary hyperoxaluria [131,132]. Furthermore, studies have shown that polymorphisms in this gene were associated with poor prognosis in patients with metastatic colorectal cancer and non-small cell lung cancer, which may indicate that the AGXT gene may be involved in the metabolic reprogramming of cancer cells [128,131]. It is noteworthy that the AGXT gene has no reports in the literature related to its overexpression in brain cancers nor studies related to glioma and glioblastoma.

4.1.2. Additional databases

The *in silico* validation demonstrated that most of the DEGs formed compatible patterns between the original study data and the additional databases analyzed. Thus, the differential expression levels between GBM and ATG in the CGGA and GEO databases, together with the statistical significance values, confirm the validity of the observed conclusions and underscore the potential impact of these genes for further understanding of GBM [133].

Nevertheless, some discrepancies were observed, particularly in the comparison with the GEO database. The *ATP5MGL* gene was found to be downregulated in GBM compared to ATG, contrary to observations from the CGGA and original study dataset, highlighting

the need to address the scarcity of studies focused on this gene. Additionally, some genes did not show statistically significant values, and *GK2* lacked data in the mentioned database. These discrepancies among the results obtained from database analyses may be attributed to methodological, ethnic, or biological differences between samples, further underscoring the need for cross-referencing these databases to corroborate the understanding of the observed expression patterns [133].

However, the other genes that were differentially expressed in our analysis (*C15orf48, TERT, AGXT, CYP27B1, SLC2A4, GK2, SLC25A48, ETNPPL,* and *HMGCS2*) maintained the same expression pattern and statistical significance when using CGGA data. Considering that GBM shows poor survival in Caucasians [134], and noting the similarity in most of genes across three databases of different ethnicities, we suggest that these DEGs could serve as potential biomarkers across diverse populations. This highlights the potential role of these genes in the pathology of GBM, validating the consistency of the results and reinforcing their replicability even in the face of the ethnic and methodological heterogeneity inherent to the databases used (TCGA: European, CGGA: Asian, and GEO: Mexico and United States) [133].

5. Conclusion

Understanding mitochondrial processes in malignant brain tumors, especially in ATG and GBM, are essential factors for the identification of new biomarkers for the diagnosis and treatment of these cancers. In our study, we identified 11 DEGs in GBM when compared to ATG (*SLC2A4, SLC25A48, ETNPPL*, *HMGCS2*, *GK2, ATP5MGL, C15orf48, MCUB, TERT, AGXT* and *CYP27B1*), which are involved in processes such as gluconeogenesis, calcium and vitamin D metabolism, mitochondrial metabolism through fatty acid oxidation, as well as transamination and transport of glucose. To the best of our knowledge, this is the first work to associate such genes with glioblastoma, being a new approach to the analysis of glycolysis and gluconeogenesis in the metabolism of malignant brain tumors. Therefore, future studies with larger and more heterogeneous cohorts, as well as further functional studies, are recommended to fully elucidate the role of these genes in brain cancer.

CRediT authorship contribution statement

Ricardo Cunha de Oliveira: Writing – original draft, Formal analysis, Data curation. **Felipe Gouvea de Souza:** Writing – original draft, Methodology, Conceptualization. **Ana Gabrielle Bispo:** Writing – original draft, Methodology, Conceptualization. **Matheus Caetano Epifane-de-Assunção:** Writing – original draft, Methodology, Conceptualization. **Giovanna C. Cavalcante:** Writing – review & editing, Supervision, Conceptualization.

Ethical approval

Not applicable. This research did not involve any studies with humans or animals; therefore, submission to or approval from an ethics committee was not required because the data in this paper is from free public databases.

Data availability statement

This work used open access high-throughput RNA-sequencing data available in public databases. The results shown here are in whole or part based upon data generated by the TCGA Research Network: https://www.cancer.gov/tcga. For the primary analysis we used the Low-Grade Glioma data to filter only Astrocytoma (available at: https://portal.gdc.cancer.gov/projects/TCGA-LGG) and Glioblastoma (available at: https://portal.gdc.cancer.gov/projects/TCGA-GBM). For validation, we gathered two datasets from GEO: GSE196694 (available at: https://www.ncbi.nlm.nih.gov/geo/query/acc.cgi?acc=GSE196694) and GSE113474 (available at: https://www.ncbi.nlm.nih.gov/geo/query/acc.cgi?acc=GSE113474). Our script is available at https://github.com/ricardoolveira/GBM_ATG_Analysis/, and includes the filtering used for the samples, the parameters for the differential expression analysis and the step-by-step validation.

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Declaration of competing interest

The authors declare there are no conflicts of interest.

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Appendix B. Supplementary data

Supplementary data to this article can be found online at https://doi.org/10.1016/j.heliyon.2024.e40414.

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