Original Research Article

Identification of Potential Biomarkers for Cerebral Palsy Using Bioinformatics Approaches

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Abstract
Cerebral palsy has affected millions of people worldwide. There are many causes that contributed to this condition, and it might happen during maternal, prenatal, perinatal, or postnatal life. Epidemiological studies have shown that most of the causes of cerebral palsy are prior to labour. However, there is no cure for this condition, and the diagnosis is still lacking in many aspects. Early assessments and interventions are crucial because they can improve patients' health and quality of life. The purpose of this study is to predict the potential biomarkers for cerebral palsy by using bioinformatics approaches. Two datasets, GSE31243 and GSE11686, were downloaded from Gene Expression Omnibus (GEO) and GEO2R was performed to obtain the differentially expressed genes (DEGs). Enrichr, a gene set search engine was used to perform GO functional, enrichment analysis, functional annotations and KEGG pathway analysis for the DEGs. Protein-protein interaction (PPI) networks was constructed through STRING database and visualised by Cytoscape software. In total, 450 DEGs and ten hub genes were identified including LPL, LIPE, ACSL1, MT1E, MT1G, MT1X, MT1H, FABP3, PLIN2 and MT2A. In conclusion, by using bioinformatics approaches, several DEGs related to cerebral palsy were screened and the hub genes identified are crucial in differentiating cerebral palsy from other neurodevelopmental disorders.

Keywords: cerebral palsy, biomarkers, bioinformatics approaches

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Received 19 July 2022; accepted 14 Sept 2022
Available online: 1 April 2023

1.0 Introduction

Cerebral palsy (CP) is a neurological disorder that can affect individual's ability to move, balance and coordinate their body systems. Cerebral palsy can be defined as a non-progressive and permanent disorder that affects movement and posture and is attributed to the brain disturbances in brain development (1,2).

People with CP will experience spasticity, dystonia, muscle contractures, weakness and difficulty in coordination due to the disturbances (3). It has become a major problem as it affects the quality of life of the affected individuals and leads to subsequent long-term disability. As the disease developed early in life, children are the most affected with 40% of those diagnosed will lose the ability to walk independently (4). Adding to the problem, the burden of the annual cost for the treatment of children with CP is 10-26 times higher, and this problem persists into adulthood, where it is further worsened by poor access to care, disability, lower employment and absenteeism, all of which ultimately diminish the quality of life of its victims (5). All of these problems may be the major contributing factors to stress and depression among patients with CP (6).

CP can be classified into four main types which include spastic, dyskinetic, ataxic and mixed CP. These are classified according to symptoms and parts of the body. The classification of CP is important because it helps determine the appropriate intervention for the patients' management. However, classifying CP in infants is complicated and hard to do due to the lack of characteristics being expressed, which results in further misclassification.

There are many causative pathways that could be associated with CP aetiology. Sadly, in most cases, the cause of CP remains unknown. Researchers believe that genetics or epigenetic factors may contribute to the cases with unknown causes (7). In fact, it is estimated that 30% of the cases are genetic in nature. There are several situations and circumstances that could put a child at greater risk for CP, including maternal health problems, infant diseases, and pregnancy complications. Preterm infants are commonly associated with CP. The pathophysiology of CP can include prematurity, perinatal hypoxia, infection and inflammation, congenital anomalies and genetic contributors. With vast contributing factors, therefore, bioinformatic approaches are crucial for a better understanding of the disorder and a less invasive approach.

The clinician only depends on clinical or imaging biomarkers such as general movement assessment, neurological examination, and neuroimaging to diagnose CP. These are the only accessible, acceptable and traditional biomarkers that have been used by clinicians in the clinical setting. Currently, the combination of neurologic assessment, neuroimaging findings, and recognition of clinical risk factors is used to diagnose individuals with CP (8). In recent years, albeit the possibility of early diagnosis at the age of below 6 months corrected age (9), this situation may lead to false-positive screening resulting in unnecessary parental stress (8). Nonetheless, studies have shown that parents prefer to be informed sooner rather than later if their children are at high risk or have CP (10). Moreover, the diagnosis itself is a complex procedure that requires skilled and professional physicians to assure a highly precise diagnosis. Three diagnostic tools that provide high accuracy and sensitivity include neonatal magnetic resonance imaging (MRI), general movement assessment (GMA) and the Hammersmith infant neurological examination (HINE) (9).

The aim of managing CP is not to cure or achieve normalcy, but to increase

function, improve skills and maintain health based on cognitive development, locomotion, independence and social interaction (11). The best clinical outcomes are usually a result of early, intensive management. Currently, there is still no cure for CP and the treatments done are mainly to treat the symptoms and improve the patient's quality of life. The treatments for CP might be too complicated as they focus on only a specific disability experienced by the patients, while CP might also be associated with some other complications such as spasticity, osteopenia, and gastrointestinal abnormalities. The clinical management of CP should be unique and personalised for each patient, depending on the type and severity of their condition. Thus, a team approach is required to obtain optimal treatment and management for CP patients.

Biomarkers, also known as biological markers, are indicators that can be found in the blood or other body fluids or tissues and show the presence of biological activities and processes (12). Molecular biomarkers may be categorised according to their biophysical properties, including nucleic acids, proteins, peptides, metabolites, lipids and other small molecules (12). Biomarkers have a major influence on the management of patients with suspected disease, or those who have or do not have an apparent disease from a clinical perspective (13). Innovation in omics-profiling technologies enables the systemic analysis and characterization of alterations in RNA, genes, metabolites and proteins, providing the chance for the discovery of novel biomarkers and pathways activated in disease or associated with diseases pathogenesis (13).

Currently, there is no molecular biomarker platform promptly available to pinpoint individuals suffering from CP. However, screening assays that would measure blood biomarkers, ideally collected during infancy, may enable earlier assessment, intervention, and the development of novel therapeutics (14). Bioinformatics approaches, including microarray data analysis and an integrated systems pharmacology approach, were used in this study to predict the potential biomarkers for CP. The utilisation of a bioinformatics approach to determine the biomarkers for CP can help with early detection of the disease resulting in early intervention. In alignment with the lack of possible CP biomarkers, the objective of this study is to identify the potential biomarkers for CP by using bioinformatics approaches. This computational study highlights the potential generation of molecular biomarkers that can be useful in the diagnosis and prognosis of cerebral palsy treatments and management.

2.0 Materials and methods

2.1 Microarray data

The Gene Expression Omnibus (GEO) database (https://www.ncbi.nlm.nih.gov/geo/) is a public functional genomics data repository including array- and sequence-based data of gene profiles and next-generation sequencing (15) was used to obtain the datasets with the keywords of 'cerebral palsy' and 'homo sapiens'. As a result, two datasets which include GSE31243 and GSE11686 were obtained. The GSE31243 dataset is an Affymetrix human genome U133A 2.0 array of 40 microarrays which are categorised into four groups for analysing the effect of cerebral palsy and differences between muscles. The GSE11686 dataset was based on GPL96 platforms ([HG-U133A] Affymetrix Human Genome U133A Array), which comprised samples from both cerebral palsy patients and control patients.

2.2 Identification of DEGs in CP

These two datasets were processed by the online tool GEO2R (http://www.ncbi.nlm.nih.gov/geo/geo2r) (16). GEO2R is a web tool linked to the GEO database that can be used to compare multiple groups in the GEO series. Therefore, the differentially expressed genes between normal individuals and cerebral palsy patients were determined. Thereafter, the overlapping differentially expressed genes (DEGs) between GSE31243 and GSE11686 were identified based on Venn diagram analysis in Funrich (17).

2.3 Functional and KEGG pathway analyses of the DEGs

The functional annotations of DEGs were measured through the analysis of the Gene Ontology (GO) enrichment (18) and Kyoto Encyclopedia of Genes and Genomes (KEGG) was applied to categorise the pathways in which such genes function (19). DEG expression matrix was investigated through GO and KEGG enrichment to verify whether there was a statistical difference between different results and a P value < 0.05 had statistical significance. The online tool Enrichr (https://maayanlab.cloud/Enrichr/) was used to identify three ontologies in the GO analysis: biological process (BF), cellular component (CC), and molecular function (MF) (20).

2.4 Construction of Protein-Protein Interaction Network and Hub Gene Identification

The protein-protein interaction network for the DEGs was constructed using the Search Tool for the Retrieval of Interacting Genes (STRING-https://string-db.org/)(21). The connectivity network of the proteins is important for a full understanding of the biological phenomena. The organism was set as Homo sapiens, and the minimum required interaction score was set to medium confidence (>0.400) as the cut-off point. Next, the Cytohubba tool in Cytoscape software version 3.9.1 was utilised to determine the hub gene. Cytoscape is software for the visualisation and analysis of biological networks (22). Protein-protein interaction data from STRING was imported into the Cytoscape software for the identification of hub genes. The top ten hub genes were ranked and selected based on their degree.

3.0 Results and Discussion

3.1 Microarray data

In this study, two gene datasets (GSE31243 and GSE11686) were selected and downloaded from the GEO database. The annotation information of the datasets is shown in Table 1.

Table 1 General information of the datasets

Reference	PMID	Sample	GEO	Platform	Normal	CP
Smith LR et al. (2012) (23)	22956992	Muscle	GSE31243	GPL571	20	20
Smith LR et al. (2009) (24)	19602279	Muscle	GSE11686	GPL96	4	12

3.2 Identification of DEGs for CP

From the datasets, GSE31243 contained 20 normal samples and 20 cerebral palsy samples, whereas GSE11686 contained 4 normal samples and 12 cerebral palsy samples. Based on GEO2R analysis, a total of 308 DEGs were identified from GSE31243193, of which 257 were upregulated and 56 were downregulated. Another dataset which is GSE11686 has a total of 193 DEGs, including 137 upregulated genes and 56 downregulated genes. All the DEGs met the selection criteria which are $P<0.05$ and $|logFC|\geq1.0$. Next, Venn analysis in Funrich software was used to find the correlation between the DEGs of the two datasets. Based on the Venn diagram, 58 DEGs have been highly expressed, of which 2 were upregulated genes and 56 were downregulated genes (Figure 1). The lists of upregulated and downregulated DEGs are listed in Table 2.

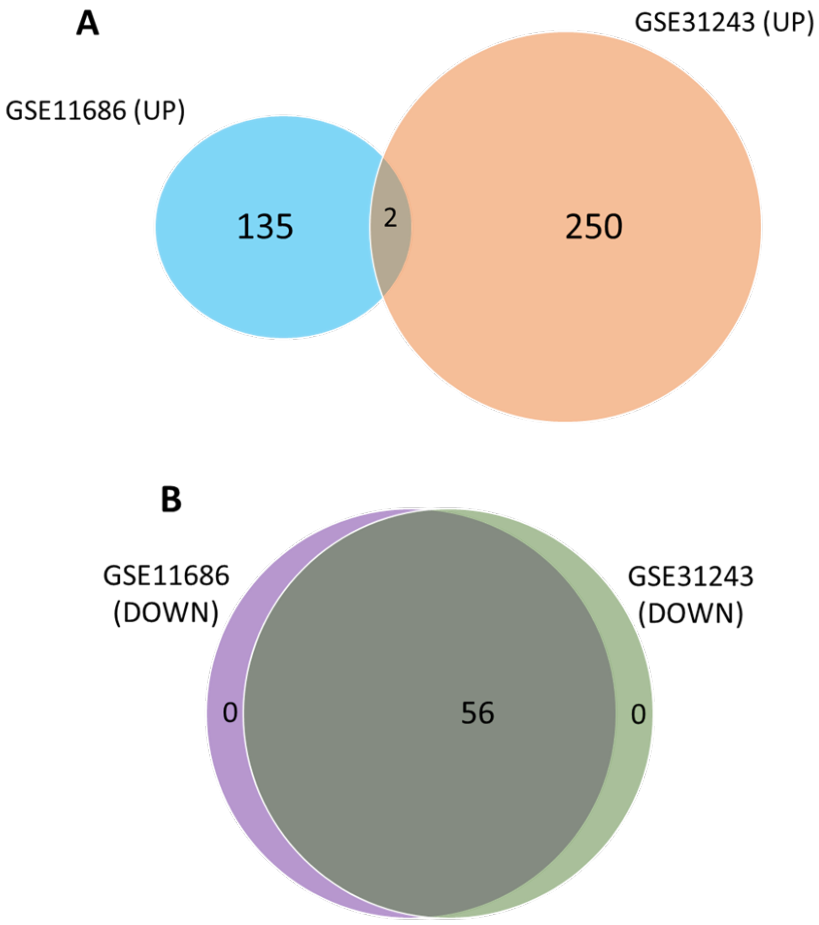

Figure 1 The Venn analysis shows the correlation between the two datasets. Venn diagram (A) shows the correlation of upregulated DEGs and Venn diagram (B) shows the correlation between downregulated DEGs

Table 2 List of DEGs conducted using GEO2R and Funrich software

Differentially Expressed Genes (DEGs)	Gene Symbol
Upregulated	SFRP4, LUM
Downregulated	HMGCS2, DGKG, LIPE, CEACAM21, MT1F, MT1HL1, KCNJ8, MT1E, FABP3, MICALL1, PFKFB3, CEBPD, MT1H, GLUL, TGM2, MT2A, LPL, MT1X, SLC12A8, RTP4, BDH1, RETSAT, CNGB1, GPX3, MT1G, TEN1-CDK3, TST, CKB, AMOT, GOT1, PPIF, ACSL1, IL6R, COL4A2, PECAM1, PREB, CD93, MPC1, ADM, UCP3, SV2B, ALDH6A1, APLNR, ILVBL, HSPB6, COL4A1, ECHDC3, GIMAP6, BSG, PLIN2, MLYCD, GSTO1, MRPL12, UCP2, CLDN5, SH3TC1

3.3 Functional and KEGG pathway analyses of the DEGs

Enrichr, a gene set search engine was used to perform the GO functional and KEGG pathway analyses for all the DEGs. Figure 2 shows the functional and pathway analysis for upregulated DEGs while Figure 3 shows the functional and pathway analysis for downregulated DEGs.

The result shows that the upregulated DEGs were mainly enriched in biological processes (BP), including trivalent inorganic anion homeostasis, regulation of keratinocyte apoptotic process, positive regulation of epidermis development, regulation of the non-canonical WNT signalling pathway, phosphate ion homeostasis and keratan sulphate catabolic process. This indicates that the DEGs are mainly responsible for the normal balance of cellular function in the human body. Upregulation of the said DEGs might alter normal biological functions such as cellular structural integrity, intracellular signaling and many more, thus disturbing the homeostatic process in CP individuals. The downregulated DEGs were enriched in cellular responses to zinc ions, cellular responses to copper ions, cellular responses to cadmium ions and cellular zinc ion homeostasis.

Next, the cellular component (CC) shows that the upregulated DEGs were mainly enriched in the lysosomal lumen, Golgi lumen, vacuolar lumen and lysosome. Modifications in the biological processes will directly affect the construction of the cellular components. DEGs overexpression mainly affect lumen region which is responsible for the structural integrity of cellular components. The facilitation of materials in and out of the cells is greatly safeguarded by the stability of these components. While the downregulated DEGs are mainly expressed in the mitochondrion, perinuclear region of the cytoplasm, the mitochondrial matrix, the lipid droplet and the bicellular tight junction. Interestingly, many studies have linked the contribution of low mitochondrial numbers in CP patients, which is in parallel to our findings in downregulated DEGs. Mitochondria are the powerhouse of the human cell, and without them, energy production in the muscles and tissues will be depleted. CP individuals are shown to have low abundance and functioning of mitochondria in the skeletal muscle, resulting in significant movement decline and short endurance despite increases in movement attempts (25).

As for molecular function (MF), the downregulated DEGs were mainly enriched in zinc ion binding, metal ion binding, transition metal ion binding, monocarboxylic acid transmembrane transporter activity and olfactory receptor binding.

Based on Figure 2, the KEGG pathway for upregulated DEGs were mainly expressed for the WNT signaling pathway and proteoglycans in cancer. Since CP normally begins to develop in the womb, dysregulation of WNT signalling pathway seems to be the contributing culprit as it is

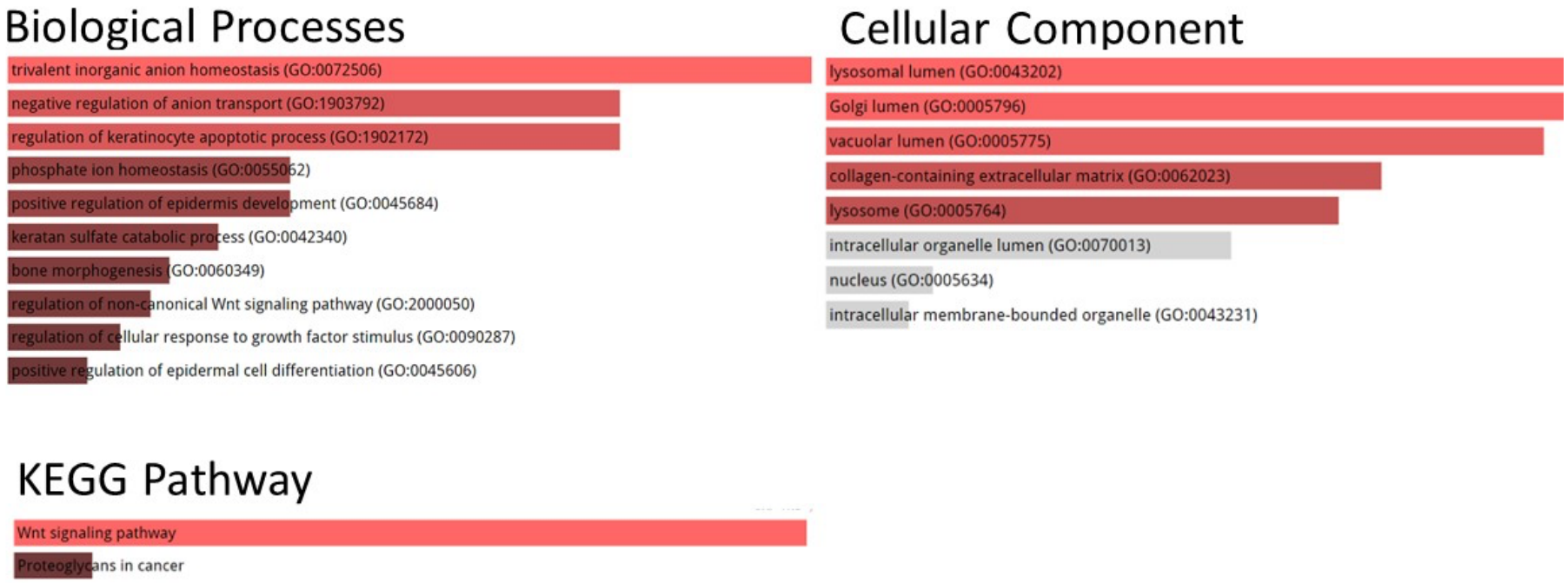

Figure 2 GO and KEGG pathway analysis results of upregulated DEGs

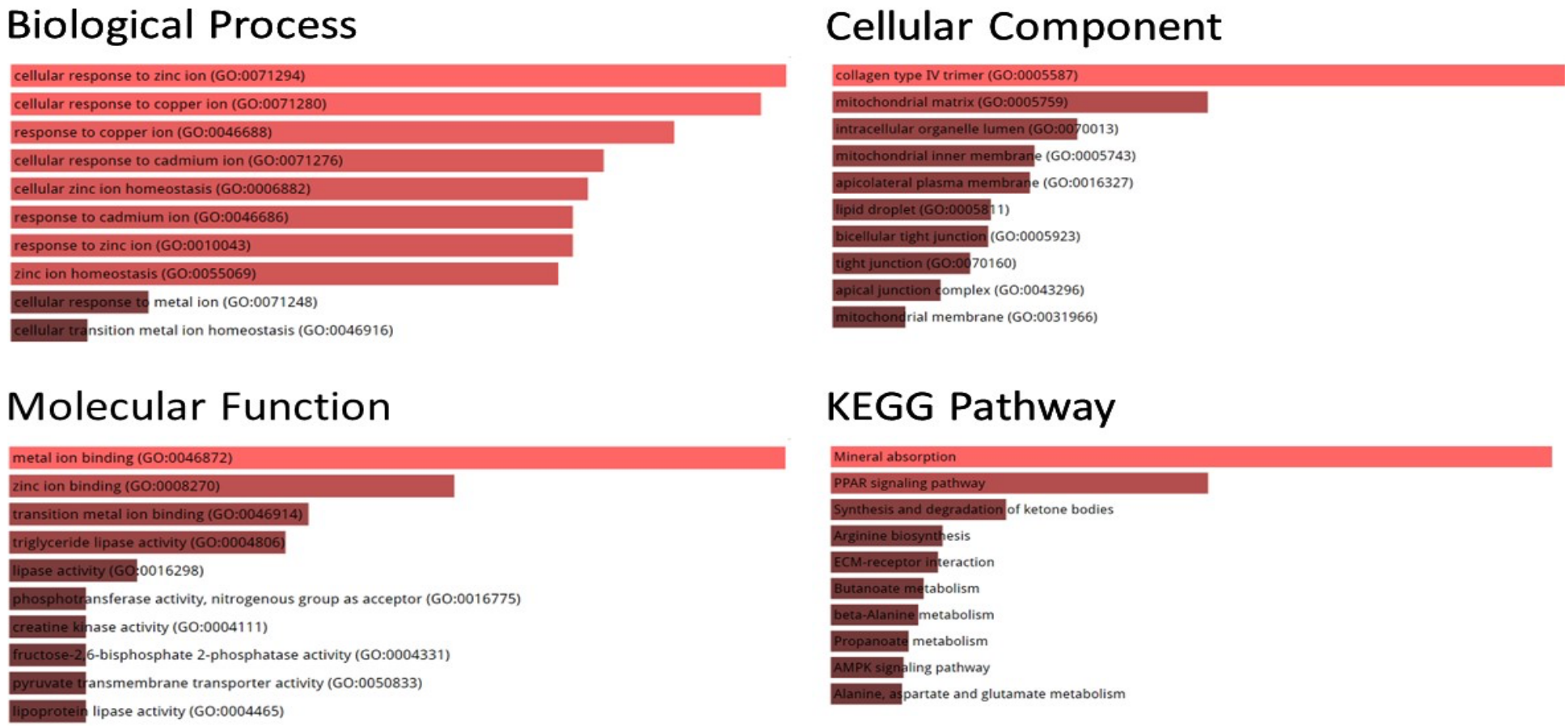

Figure 3 GO and KEGG pathway analysis results of downregulated DEGs

accountable for the normal development of the embryo and in adults, proper signaling will ensure a balance of tissue regeneration. This pathway is crucial for regulation of tissue development, differentiation, migration, renewal and repair (26). In line to its various functions, abnormal regulation will affect the ability of the embryo to develop healthily and reduce the ability of humans to respond to cellular injury and self-regenerate during normal tissue homeostasis. Lastly, based on Figure 3, the downregulated DEGs were significantly enriched in mineral absorption, the PPAR signaling pathway, the synthesis and degradation of ketone bodies, arginine biosynthesis and ECM-receptor interaction.

3.4 Construction of Protein-Protein Interaction Network and Hub Gene Identification

The STRING database was utilised to construct the protein-protein interaction (PPI) network for better understanding of the functional interrelationships among DEGs (Figure 4). Then, Cytoscape software was used to identify the hub gene. The PPI network is composed of 63 nodes and 79 edges. The lines represent the interaction of proteins between the genes, the circles represent the genes and the result within the circles shows the protein structure.

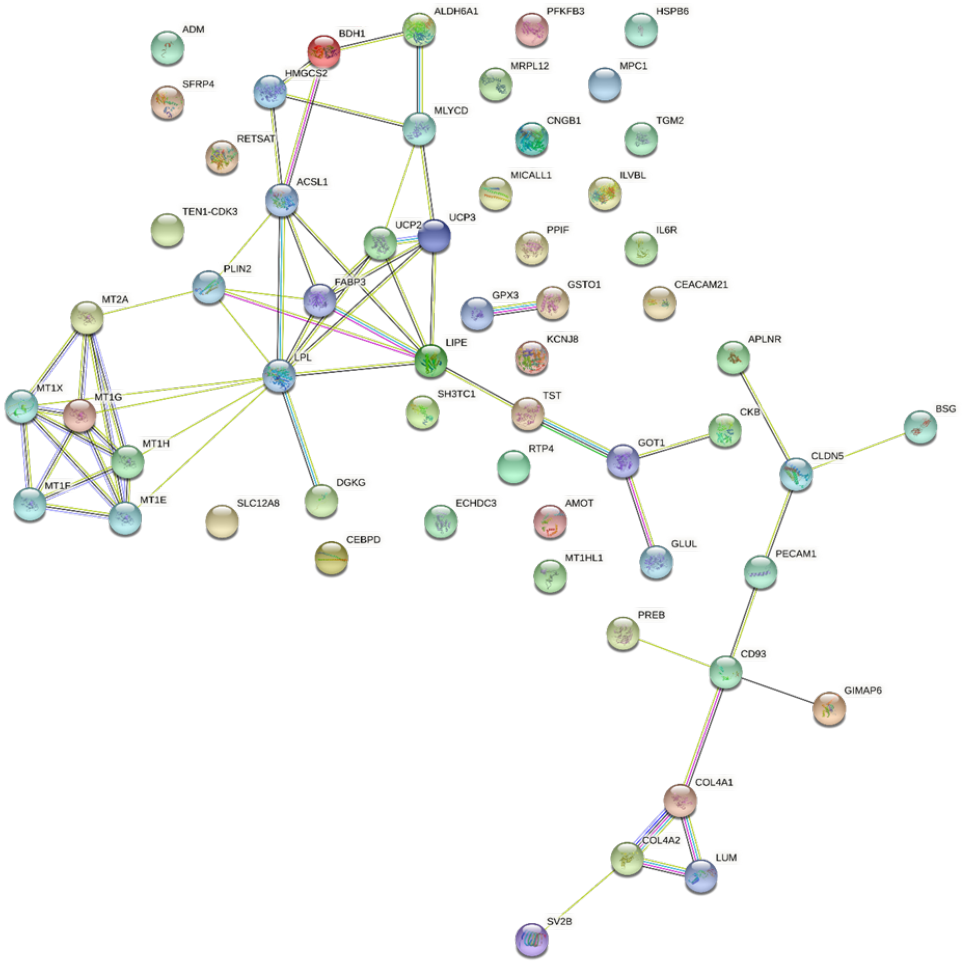

Figure 4 The STRING PPI network interactions of DEGs

Finally, the Cytohubba tool in Cytoscape software was utilised to obtain hub genes consisting of LPL, LIPE, ACSL1, MT1E, MT1G, MT1X, MT1H, FABP3, PLIN2 and MT2A, all of which are downregulated DEGs in CP patients.

The changes in particular gene expression in CP compared to normal individuals can lead to changes in cellular components such as proteins and RNA encoded by those genes (27). Downregulated genes or genes that have reduced expression may result in a lower amount of the cellular component being produced. Subsequently, it may interrupt the biological, chemical and molecular processes depending on where those genes may play roles.

The LPL gene provides instructions for making the lipoprotein lipase enzyme. This enzyme plays a central role in lipid metabolism. The primary function of this lipoproteins lipase is to hydrolyse triglycerides into fatty acids (FAs) and glycerol (28). This process is important for the body to utilise the fat molecules as energy or stored in adipose tissue for later use. Triglycerides are carried by lipoprotein known as chylomicrons and very low-density lipoproteins (VLDL) from various organs. It is highly expressed in adipocytes and myocytes as well as macrophages. Its protein level and activity are tightly regulated by multiple mechanisms depending on the metabolic state and energy demand of the cell (29). Activities such as fasting, feeding and exercise may change the protein level and activity of LPL to meet the energy demand of the body by increasing the transfer of FAs to specific cells.

LIPE is a gene responsible for encoding the human hormone-sensitive lipase (HSL). HSL is responsible for the breakdown of fats, triacylglycerols specifically diglycerides as well as other lipids into FAs through hydrolysis in adipose tissue, a process known as lipolysis. The rate of lipolysis is modulated by complex interactions between lipases and other proteins, such as perilipin-1 (PLIN-1) (30). Low LIPE gene expression causes inhibition of HSL activity and was previously shown to result in a lower rate of lipolysis (30). Therefore, it is proposed that individuals with CP might have problems utilising FAs as an energy source. Additionally, inhibition of HSL also results in lipogenesis stimulation (30). Furthermore, HSL were proved to be significantly important in the regulation of the mobilisation of stored lipid and are expressed predominantly in adipocytes (31). In several studies, HSL has been frequently associated with metabolic diseases such as diabetes; its actual roles in CP need to be determined further.

Acyl-coenzyme A synthetase 1 or in short ACSL1 is one of the five clonal isomers of the Acyl-CoA synthetase family (ACSL) which also plays an important part in lipid metabolism. It converts free long-chain FAs into fatty acyl-CoA esters and thereby plays a key role in lipid biosynthesis and FAs degradation. ACSL1 exists abundantly in the adipose tissue, liver and heart. In this study, ACSL1 is downregulated and not highly expressed in the CP sample as compared to the control. Zhao et al indicates that loss of ACSL1 in mouse skeletal muscle severely reduces acyl-CoA synthetase activity and FA oxidation (32) which could lead to impairment of FA utilisation. FAs are mainly utilized during both rest and prolonged exercise.

Therefore, the inability of skeletal muscle to utilise FAs for energy results in fatigue and muscle weakness (32) that may have an association with muscle spasticity experienced by CP patients. However, further possible explanation on the mechanism in which it involved with

CP could not be evaluated. This is because muscle weakness and spasticity experienced by CP patients are believed to be mainly caused by the disturbance of the voluntary movement signalling from the brain

Fatty acid-binding protein 3 (FABP3) belongs to the calycin superfamily. FABP3 is also expressed in the brain even though its function in the heart is enormously studied. FABP3 has specific developmental stages and processes in the brain (33). It is thought to play a role in the intracellular transport of long-chain FAs and their acyl-CoA esters. Additionally, it is also thought to be closely related to FAs transport and uptake of long chain polyunsaturated fatty acids (PUFAs). The cellular transportation and physiological actions of PUFAs are mainly mediated by FA binding proteins (FABPs) (33). PUFAs are critical structural components of the brain and essential for normal brain development (33). This may be the reason for retardation of brain development in individuals with CP as the gene important for encoding FABPs is barely expressed.

The protein encoded by the PLIN2 gene belongs to the perilipin family, members of which coat intracellular lipid storage droplets. This protein is associated with the lipid globule surface membrane material and may be involved in the development and maintenance of adipose tissue. However, it is not restricted to adipocytes as previously thought, but is found in a wide range of cultured cell lines, including fibroblasts, endothelial and epithelial cells, and tissues (34).

All the potential candidate biomarkers discovered are interconnected with lipid metabolism. This elucidates that the pathogenesis of CP may be linked to the deficits in lipid metabolism and inability to utilise FAs. Therefore, to understand further the importance of lipids in CP development and progression, a thorough and extensive study must be done to understand the association of lipid metabolism in CP.

Lipid metabolism within the brain is tightly regulated, as it is essential for normal physiological function of the neurons and structural development of the brain (33-34). They are also involved in the formation of lipid bilayers that form the membrane structure and provide the necessary channel for protein function. Furthermore, it also functions as an energy reservoir and serves as a precursor for various secondary messengers, such as 1,2-diacylglycerol (DAG) and ceramide. The normal functions of the brain are governed by these lipids. Therefore, any atypical deviation from this function, which can be caused by physical or pathological changes in the brain, may lead to different types of neurodevelopmental diseases (35).

Apart from that, the CNS is a major player in the regulation of systemic metabolism and lipid balance (34). Indeed, the content of lipid in the brain is the second highest after adipose tissue, and lipids in the brain make up half of the brain's dry weight (36). This is believed to be due to the brain's use of fat as fuel and as a source of energy (36). Although some FAs can be synthesised de novo, essential FAs must be transferred into the brain from the systemic circulation (36). Since the mechanism of CP is highly associated with injury to the brain, this may be the consequence of energy or ATP depletion as it may result in an elevated level of oxidative stress towards the brain (37). An increase in oxidative stress may induce cellular damage and cell death which contribute to brain injury (38).

MT1E, MT1G, MT1X, MT1H and MT2A genes are closely related families in which all of them encode for a protein known as metallothionein (MT). They also belong to the metallothionein type I family. MTs have a high affinity for heavy metal ions such as zinc, copper, cadmium

and mercury. Thus, they can function as regulators of the cellular metabolism of essential metals as they have the capability to bind to various metals intracellularly (39). One of the examples is modulating the activities of zinc-dependent regulatory proteins such as enzymes and zinc-finger transcription factors by the removal or transfer of zinc. Besides, MTs is also a potent antioxidant and thus provides neuroprotection for the brain from damaging substances such as reactive oxygen species (ROS) and free radicals. CP is also associated with brain injury and therefore, a decrease in the expression of genes encoding MTs may suggest an association between them. In individuals with CP, there might be a low number of MTs proteins to protect the brain from damaging factors that may occur during the maternal, prenatal, perinatal and postnatal periods.

The potential biomarkers listed in this study suggested that these genes are insignificantly expressed or dysfunctional in CP patients and are interlinked with the disturbance in lipid metabolism and FAs utilisation impairment as well as the loss of the vital protein for brain protection. The possible reasons may be thought to be related to one another in the sense that when there is an increase in oxidative stress due to impairment of lipid metabolism and FAs utilisation which comes together with dysfunction of substances responsible for brain protection, these may result in severe damage to the components of the brain.

4.0 Conclusion

As a conclusion, through comprehensive bioinformatics approaches, ten hub genes have been identified as potential diagnostic biomarkers for cerebral palsy which include LPL, LIPE, ACSL1, MT1E, MT1G, MT1X, MT1H, FABP3, PLIN2 and MT2A. All these genes are shown to be downregulated in cerebral palsy patients. Based on the study conducted, several hub genes obtained are associated with lipid metabolism, while others are linked to the antioxidant function of the specific protein being encoded by the gene as well as their neuroprotective ability towards the brain. However, further studies should be conducted to verify the roles of the genes in cerebral palsy. Additionally, this study will provide comprehensive ideas about the pathway mechanisms involved in cerebral palsy. It is important to increase our understanding of the gene's specific role in cerebral palsy which may help to develop more specific and personalised therapies for cerebral palsy patients in the future. If further experimental studies were done on the listed potential candidate biomarkers, they may successfully become validated biomarkers for cerebral palsy in the future. Subsequently, the diagnosis can be done more quickly, and we will be able to provide precise intervention and management for cerebral palsy patients.

Acknowledgement

The authors would like to thank UiTM for the facilities provided in Bioinformatics Unit and Brain Research Laboratory, Faculty of Pharmacy, UiTM Selangor Branch, Puncak Alam Campus, Selangor, Malaysia.

Conflict of interest

Authors declare no conflict of interest in the present work.

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