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Whole transcriptome sequencing analyses of DHA treated glioblastoma cells

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ABSTRACT

Glioblastoma (GBM) is a typical malignant tumor, and there are no effective drugs capable of improving patient survival. Docosahexaenoic acid (DHA), a nutrient essential to animal health and neurodevelopment, exerts an anticancer effect in several types of cancer. However, the function of DHA in GBM is still unclear. Here, we showed that DHA could repress the migration and invasion of GBM U251 cells and promote their apoptosis in a dose- and time-dependent manner, indicating that DHA has an anticancer effect on GBM cells. Whole-transcriptome analysis indicated that DHA treatment mainly regulates the genes associated with receptor binding, oxidoreductase activity, organic acid transmembrane transporter activity, and carboxylic acid transmembrane transporter activity. Long non-coding RNAs (LncRNAs) involved in the regulation network of DHA were also identified, and their targets were assigned to the Gene Ontology (GO) categories. In silico analysis was conducted to predict the pathways related to the differentially expressed genes by DHA treatment. Our findings suggest that DHA acts as an antitumor agent in GBM, which may provide a suitable means of improving the efficacy of GBM treatment in the future.

1. Introduction

Glioblastoma (GBM) is the most common mainly malignancy tumor in Central Nervous System, while radio-/chemotherapy and surgery are the most common forms of treatment of GBM. However, it is inefficient to cure GBM tumors just by using radiation-combined therapies. GBM has a tendency to migrate for long distances, and that can render both surgical and drug treatment difficult. Many drugs have difficulty crossing the blood-brain barrier [1], and radiotherapy often causes radio-resistance [2,3]. There have been no drugs that could improve the overall survival of GBM to date, with the median patient survival time under 18 months [4–7]. Therefore, it is an urgent need for efficacious therapies for GBM.

Currently, molecules from natural materials for GBM treatment are attracting researchers' attention. Docosahexaenoic acid (DHA), omega-3 polyunsaturated fatty acid (C22:6n-3), has a crucial role in the growth and physiology of the central nervous system [8]. DHA could be incorporated into the membrane and to regulate the physicochemical properties of cancer cells, allowing DHA to cross the blood-brain barrier more efficiently [9]. DHA can exert an anti-inflammatory effect by adjusting oxidative stress reactions. In pancreatic cancer, DHA is a promising therapeutic agent for this cancer by inducing apoptosis and down-regulating Wnt/β-catenin signaling [10]. DHA has an anticancer effect in breast cancer cells by attenuating cancer cell migration to the bone through inhibition of the expression of CD44 [11]. Recent studies have reported autophagy is involved in DHA-treated SiHa cells, and DHA induces p53-mediated AMPK/mTOR signaling [12]. DHA is toxic to glioma cells, and its content is lower than that in normal brain cells [13]. However, the molecular mechanism by which DHA repressed neuroglioma U251 cell growth is not yet clear.

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LncRNAs are functional RNA molecules with > 200 nucleotides [14]. In cancer cells, lncRNAs can regulate cell apoptosis [15], autophagy [16], and malignant transformation [17]. Recently, lncRNAs have been reported to show significant anti-cancer function and therapeutic potential [18]. As in other cancer cells, lncRNAs also participate in GBM regulation. LncRNA X-inactive specific transcript (XIST) acts as a miR-429 molecular sponge to promote glioma tumorigenicity [19]. CRNDE/miR-136-5p/mRNA interactions inhibit the Bcl-2 and Wnt2 degradation that inhibits GBM proliferation, migration, and invasion [20]. In addition, PACT-1, MEG-3, and HOTAIR have been shown to play anti-cancer roles in human GBM [21]. However, whether lncRNAs are involved in the process of DHA function in GBM cells is still unclear.

In the present study, we evaluated the effects of DHA on proliferation, migration, and invasion of the GBM cell line U251. We monitored the differentially expressed mRNAs and lncRNAs regulated by DHA administration by whole-transcriptome sequencing, which could be helpful for determining the molecular mechanism by which DHA restrains GBM.

2. Materials and methods

2.1. Chemical treatment

DHA (D2534, Sigma-Aldrich, Shanghai, China) was dissolved in 100% DMSO (D2652, Sigma-Aldrich, Shanghai, China) and stocked at −20 °C.

2.2. Cells and cell culture

Human GBM cells (U251) gifted by Dr. Allan Zhao (Guangdong University of Technology, Guangdong, China) were cultured in Dulbecco's modified Eagle's medium (DMEM: 31600–034, GIBCO, USA) supplemented with 10% fetal bovine serum (FBS: P30–2602, Pansera ES, Germany), and 1% penicillin and streptomycin (15140–122, GIBCO, USA) in a humidified incubator with the atmosphere of 5% CO2 at 37 °C.

2.3. Cell viability assay

Cell viability was evaluated by CCK8 cell proliferation kit (CK04, DOJINDO, Japan). Briefly, 1×10^4 cells seeded in a 96-well plate and were cultured overnight. Various concentrations of DHA (0, 50, 100, 150, 200 μM) were added to each well and then incubated for 0 h, 12 h, 36 h, and 48 h. 10 μL of CCK-8 solution was added to each well and then were incubated for another 1–2 h and then was measured using a microplate reader (Bio-Tek, CA) at 450 nm.

2.4. Apoptosis assays

Cell apoptosis assays were measured evaluated using an Annexin V-FITC/PI apoptosis detection kit (KGA106, KeyGEN, Nanjing, China) according to the manufacturer's introduction. Briefly, cells were seeded in 6 cm and digested with 0.25% EDTA-free trypsin (Gibco, USA), rinsed with ice-cold PBS twice. Then, the cells stained with FITC labeled Annexin V and propidium iodide for 5 min. Flow cytometry (BD FACSV, USA) performed to detect cell apoptosis rate. All experiments were done in triplicate.

2.5. Wound-healing assay

The cells were grown to 80–90% confluence in six-well plates. Artificial wounds generated by scraping a pipette tip across the cell surface. After the removal of the detached cells by gentle washing with PBS, the cells fed with DMEM with 5% FBS and incubated over time to allow the cells to migrate into the open area. Cell movement during wound closure was measured by phase-contrast photography at 37 °C for incubations of 0 h, 12 h, and 24 h, and three randomly selected wound areas were analyzed.

2.6. Cell migration and invasion assay

Cell invasion assay requires advancement of matrigel to incubate the upper surface of the transwell chamber. For the migration assay and invasion assays 5×10^4 cells resuspended in serum-free medium and placed in the upper chambers (356,234, Corning, USA). Next, 100 μL DMEM with 5% FBS and 150 μM of the DHA was added into the upper chamber, while 500 μL DMEM with 15% FBS was added into the lower chamber. After 36 h and 48 h of incubation, the non-migrated cells on the upper surface of the membrane were removed, and the cells on the lower surface were fixed and stained with 0.1% crystal violet. The cell was washed with 10% acetic acid, and the absorbance was measured OD595 with the microplate reader.

2.7. RNA-seq

Total RNA was isolated and ribosomal RNA was depleted. Strand-specific adapters were added to fragmented RNA (average fragment length 200 nt) before reverse transcription followed the manufacturer's instructions. The quality of cDNA libraries was quality evaluated and sequenced by RIBOBIO (Guangzhou, China). The sequences files were comprehensively assessed using TopHat2. The threshold value of differentially expressed mRNA and lncRNAs were set by$|\log2FoldChange| > 1$ and q-value < 0.001.

2.8. Bioinformatics analysis

Principal component analysis (PCA) used to assess the correlation between replicates and differential gene expression. Differential expression was analyzed by Audics and the differentially expressed genes after DHA treatment were assigned to the GO terms (http://www.geneontology.org/). The biological pathway was analyzed by searching the Kyoto encyclopedia of genes (KEGG) database (http://www.genome.ad.jp/kegg/).

2.9. RT-PCR and quantitative real-time PCR

Total RNA was isolated with RNeasy Mini Kit (74,104, QIAGEN, Germany), and reverse-transcribed into cDNA with RT-PCR assays (R223–01, Vazyme, Nanjing China). Real-time PCR was performed in triplicate with SYBR Green master mix (4,913,850,001, Roche, Switzerland) and using the 7000 Applied Biosystems Sequence Detection System (ABI, Waltham, USA). GAPDH was used as endogenous control. Primers used to amplify were listed in Supplementary Table S1. The $2^{-\Delta\Delta Ct}$ method was used to calculate the mRNA fold changes.

2.10. Construction of pathway act network and co-expression network

Correlations between lncRNAs and mRNA were constructed into co-expression network according to the normalized signal intensities of differentially expressed lncRNAs and mRNAs. Pearson's correlation coefficients > 0.87 were calculated, and Cytoscape (Institute of Systems Biology, USA) was used to construct a pathway act network for graphical representations of central pathways.

2.11. Statistical analyses

All experiments were repeated at least three times. Data were represented as the mean ± S.E. Statistical analyses performed using the two-tailed Student's *t*-test by GraphPad Prism. A *P* value of < 0.05 was considered statistically significant. Data were represented as the mean ± S.E.

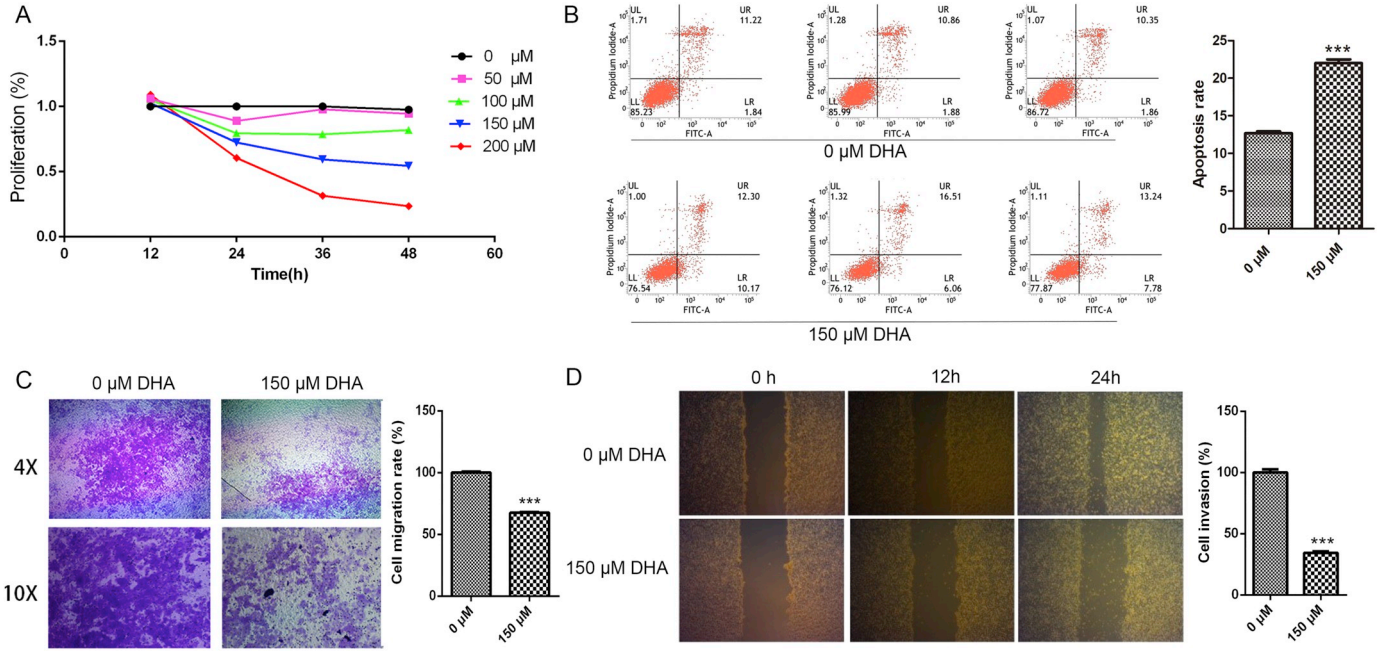

Fig. 1. A: U251 cell viability test performed by the CCK-8 assay as a function of time (0, 12 h, 24 h, 36 h, and 48 h) and concentration of DHA (0, 50, 100, 150, and 200 μM). B: U251 cell apoptosis monitored by Annexin V-FITC/PI staining after treatment with DHA. ***$p < .001$. U251 cell migration and invasion response to DHA. *trans*-Well assay for migration (C) and invasion (D) were used to assess the inhibitory effect of DHA on U251 cell. ***$P < .001$.

3. Results

3.1. DHA inhibits U251 glioma cell viability, migration, and invasion but promotes cell apoptosis

The inhibition effect of DHA on cancer viability has been studied in many kinds of cancer cells; we extended the survey to GBM in the present study. CCK-8 assays indicated that DHA treatment significantly reduced U251 cell viability in a dose- and time-dependent manner (Fig. 1A). About 50% cell death was observed at 48 h in 150 μM DHA-treated U251 cells; therefore, 150 μM was applied in the DHA treatments thereafter. DHA significantly increased the rate of apoptosis in U251 cells (Fig. 1B). As shown in Fig. 1C and 1D, migration and invasion of U251 cells were also significantly inhibited by DHA, indicating DHA could act as an anticancer agent against GMB.

3.2. RNA sequence analysis

RNA sequence analysis was performed to assess the gene expression profile of DHA-treated U251 cells. As shown in Fig. 2A, a total of 293 differentially expressed genes were identified. Among them, 139 genes were up-regulated, and 154 genes were down-regulated. These differentially expressed genes between DHA-treated and control groups were clustered (Fig. 2B) and further classified into Molecular Function, Biological Process and Cellular Components according to the standard Gene Ontology terms (GO; http://www.geneontology.org) (Fig. 2C-E). As for the Cellular Components, DHA mainly initiates the expression changes of genes located in the extracellular region compared with the control cells (Fig. 2C). With regard to the category of Biological Process, biological adhesion (28%) was the most highly represented category. The following categories were cell adhesion (27%), wounding (16%), response to toxic substances (8%), and neuron recognition (5%) (Fig. 2D). Under the category of Molecular Function, DHA mainly regulates genes associated with receptor binding, oxidoreductase activity, organic acid transmembrane transporter activity, and carboxylic acid transmembrane transporter activity (Fig. 2E). A search of the KEGG database indicated that DHA functioned in U251 cells mainly through the following pathways: (1) PPAR signaling pathway; (2) AMPK signaling pathway; (3) EGFR tyrosine kinase inhibitor resistance; (4) glioma; (5) glutathione metabolism; and (6) PPAR signaling pathway (Fig. 2F).

3.3. Validation of differentially expressed genes by real-time qRT-PCR

In order to confirm the differentially expressed genes revealed by RNA sequencing, seven up-regulated genes, including *SCD*, *RCAN1*, *AKR1B10*, *FTH1*, *HMOX1*, *PRDX1*, *FTL*, and five down-regulated genes including *ISG15*, *GPC1*, *INSIG1*, *GLIPR1*, and *FABP7* were selected and subjected to real-time qRT-PCR analysis. These genes are functionally associated with fatty acid metabolism, cell viability, cell proliferation, migration, invasion, and apoptosis. As shown in Fig. 3, the expression profiles of these genes are consistent with the RNA sequencing data.

3.4. Bioinformatic analysis of differentially expressed lncRNAs

As lncRNAs have been found to play important roles in the regulation of cancer, we also analyzed the differentially expressed lncRNAs in U251 cells after administration of DHA. In total, 75 ncRNAs were identified as DHA-modulated, including 43 up-regulated and 32 down-regulated differentially expressed ncRNA (Fig. 4A). The heatmap of differentially expressed ncRNAs between the DHA-treated and control groups is shown in Fig. 4B. The possible targets of these lncRNAs were predicted in order to explore the underlying mechanism by which DHA acts against GBM. The target genes were predicted base on the effect of lncRNA with mRNA by Cis or Trans. GO analysis (Fig. 4C) indicated the target genes of lncRNA were mainly predicted to be cell membrane and intracellular proteins. In Biological Proccess, DHA mainly participate in the regulation of genes which involved in cellar process (Fig. 4D). The target genes are mainly associated with binding and catalytic activity in the Molecular Function categories (Fig. 4E). Pathways analysis (Fig. 4F) showed that 78 genes were involved in metabolic pathways, and 19 genes were involved in the lysosome system. Detailed lncRNA–mRNA network data are shown in Supplemental S2. To further investigate the correlation between the differentially expressed lncRNAs and their targets, 25 mRNAs and 20 lncRNAs regulated by DHA were selected to construct a co-expression network (Fig. 5).

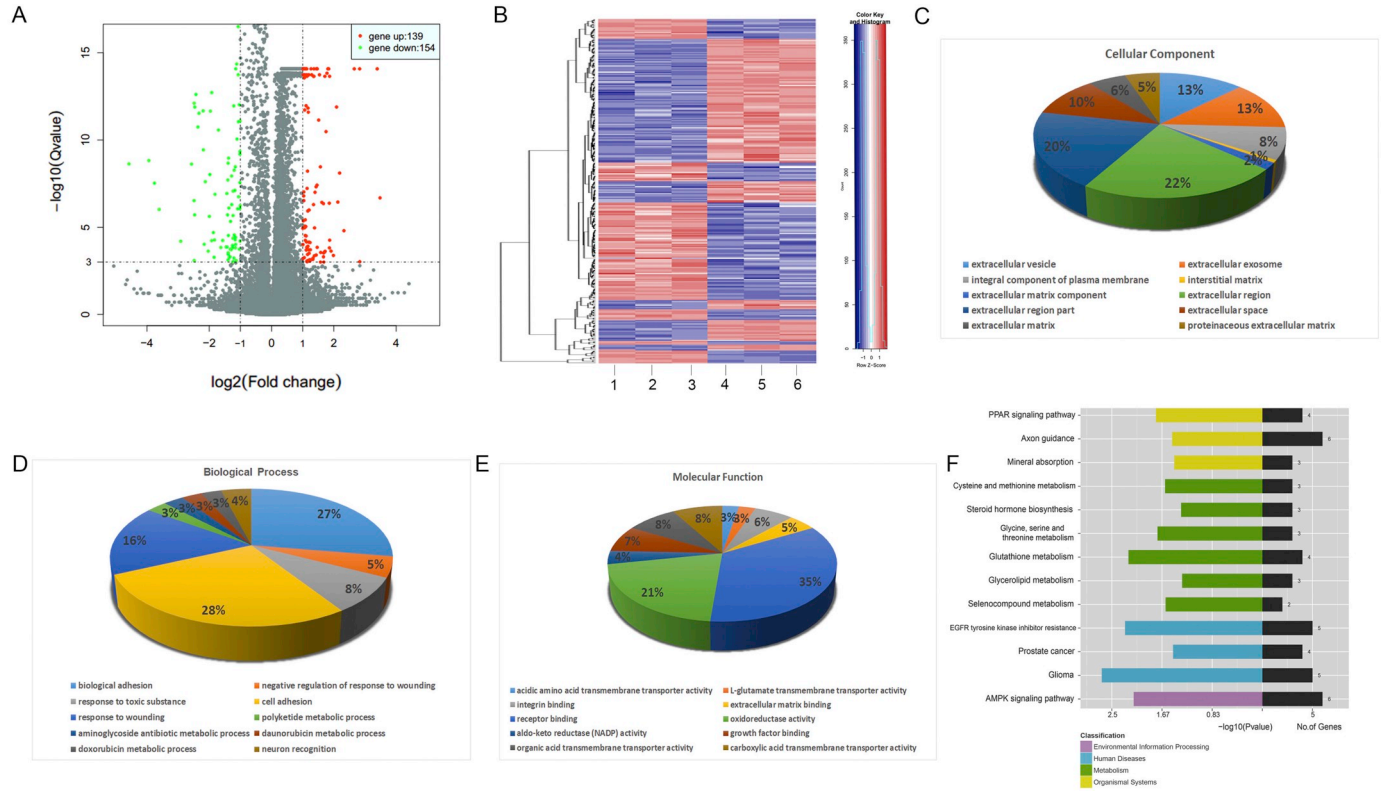

Fig. 2. The statistically significantly differentially expressed genes regulated by DHA. (A) Volcano Plot and heatmap (B) of the differentially expressed mRNAs: 1,2,3 0 μM DHA; 4,5,6150 μM DHA. (C-E) GO analysis of the differentially expressed genes in Cellular Component, Biological Process and Molecular Function. (F) KEGG pathway analysis the pathways involved in DHA regulation.

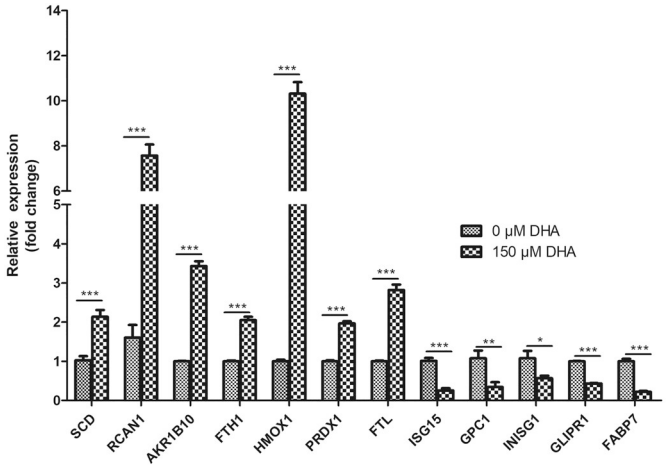

Fig. 3. qRT-PCR validation of up-regulated (A-F) and down-regulated genes (H-L) by DHA treatment. ***P < .001, **P < .01; *P < .05.

4. Discussion

GBM is a diffusely infiltrative and highly aggressive brain tumor associated with poor survival, even after resection and adjuvant chemotherapy. Surgery usually causes significant acute and chronic morbidities [22–24], and the adjuvant chemotherapy can cause peripheral neuropathy, particularly pain, motor impairments, ototoxicity and impaired cognition [25–27].

During the past decade, efforts have facilitated the molecular understanding of GBM [28]. However, until now, there have only have been two FDA-approved systemically administered chemotherapy drugs: temozolomide and bevacizumab [6]. Although there have been many molecular-targeted therapies against GBM, like the Ras pathway [29], vascular endothelial growth factor receptor [30], and mammalian target of rapamycin, they are unable to eradicate GBM completely. The overall survival of GBM patients has hardly improved over the past two decades. This may because most of the drugs have the poor BBB permeability of anticancer compounds, and they are not lipophilic. In addition, high doses of drugs can cause chemoresistance [3]. Therefore, it is necessary to search for a natural molecule for GBM treatment. Here, we found that DHA could function as an anticancer agent in GBM by inhibiting U251 cell vitality, migration, and invasion and by promoting apoptosis.

DHA, a naturally occurring molecule, has several advantages over other agents. Researchers have reported that an excess of DHA is not harmful to the brain [31], so chemoresistance is not likely to develop. Our studies indicate that DHA may contribute to GBM treatment, and DHA may act as a drug delivery system in order to improve the efficiency of therapeutic regimens. DHA is esterified into the membrane phospholipids, released, and converted to bioactive mediators during brain injury. The bioactivity of DHA could regulate the signaling pathways for synaptogenesis, cell survival neuroinflammation and neurological diseases [32]. Reports showed that DHA could alter the function of glial cells by inducing oxidative stress and inflammation [33]. Because DHA can be esterified into membrane phospholipids, it may alter the activities of transmembrane enzymes and binding of receptor proteins by changing the membrane's physical properties [34]. In many studies, DHA can affect membrane fluidity [35,36] and the activities of G-protein-coupled receptors (GPR40 and GPR120) [37]. In our research, DHA mainly causes changes in relative gene expression in extracellular regions and in components that function in the extracellular regions. The genes that are differentially expressed in response to DHA treatment are mainly involved in biological adhesion, response to wounding, response to toxic substances, and neuron recognition. Therefore, DHA may regulate the malignancy of GBM by esterifying the

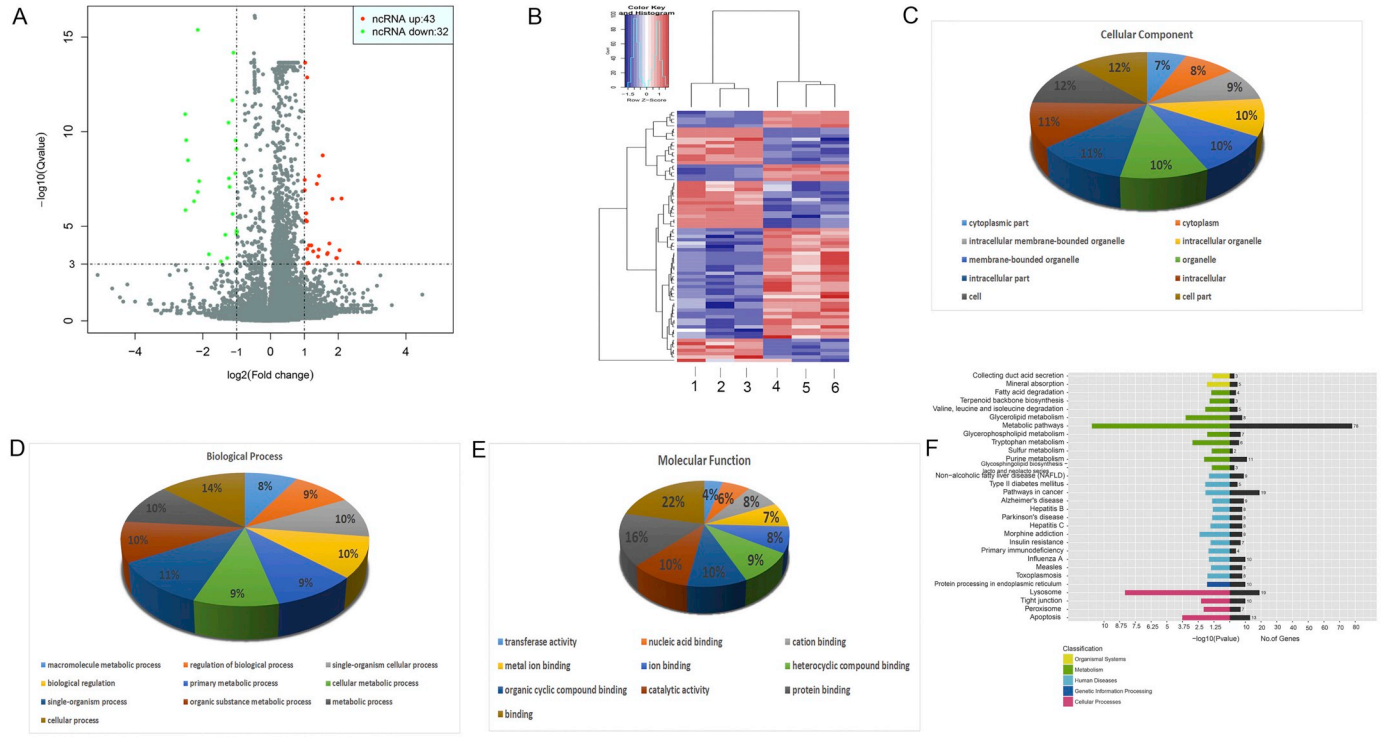

Fig. 4. The statistically of significantly differentially expressed lncRNAs and their target genes regulated by DHA. (A) Volcanoplot and heatmap (B) of the differentially expressed lncRNAs: 1,2,3 represents 0 μM DHA; 4,5,6 represents 150 μM DHA. (C-E) GO analysis of the target genes of differentially lncRNAs in Cellular Component, Biological Process and Molecular Function. (F) KEGG pathway analysis the pathways about the target genes of differentially lncRNAs involved in DHA regulation.

phospholipids of membranes and changing membrane permeability and mobility.

LncRNAs play important roles in gene expression at the transcriptional, post-transcriptional, and epigenetic levels and are involved in a series of biological processes. Moreover, lncRNAs are frequently associated with tumorigenesis, cancer initiation, and malignant progression [38,39]. TFPI2AS1, an antisense transcript of the tumor suppressor TFPI2 (tissue factor pathway inhibitor 2), is markedly upregulated in NSCLC patient tumors. TFPI2 overexpression decreases NSCLC cell proliferation and migration by upregulating TFP12 [40].

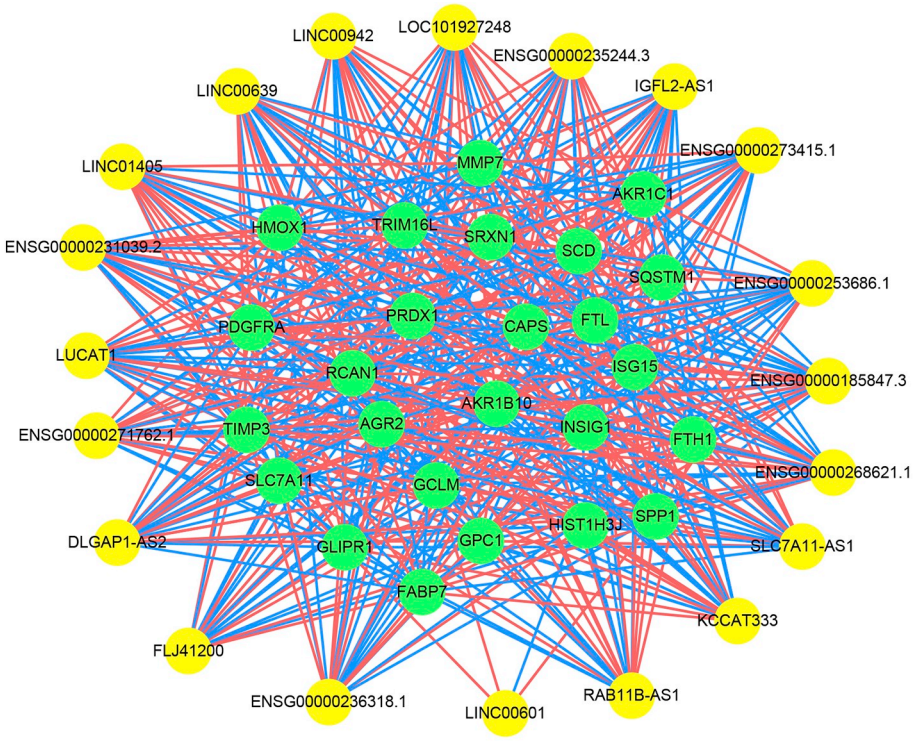

Fig. 5. The co-expression of lncRNA-mRNA network. Yellow dots indicate differentially lncRNAs and the green dots indicate mRNA. The red line represents a positive correlation, and the blue line represents a negative correlation. (For interpretation of the references to colour in this figure legend, the reader is referred to the web version of this article.)

Helicos single-molecule sequencing showed lncRNAs differentially expressed in GBM and found that Bromodomain and extracellular domain proteins can directly regulate lncRNA expression [41]. Results indicated that PCAT-1, MEG3, and HOTAIR served as tumor suppressor genes on GBM [19]. In the present study, 75 lncRNAs were significantly dysregulated in DHA-treated U251 cells compared to the control cells, which may help to elucidate the mechanism by which DHA exerts an anticancer effect against GBM.

5. Conclusions

We here showed that DHA exerts an antitumor effect on GBM by esterification into the membrane phospholipids and changing the membrane permeability, mobility, apoptosis, and expression of lncRNAs, highlighting the therapeutic potential of DHA for GBM treatment.

Conflicts of interest

The authors declare no conflict of interest.

Acknowledgments

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Appendix A. Supplementary data

Supplementary data to this article can be found online at https://doi.org/10.1016/j.jns.2018.11.027.

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