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Novel lncRNA-miRNA-mRNA competing endogenous RNA regulatory networks in glioma

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Abstract

Gliomas the most frequent type among primary brain tumors are highly heterogeneous. Developing successful therapies to overcome glioma requires a comprehensive knowledge of its molecular mechanisms. Several altered signaling pathways and cross-linked relationships of ncRNAs and coding RNAs remain to be investigated. Evidence demonstrates that ceRNA networks play a critical role in cellular processes, and dysregulation of any component of these networks could result in pathogenesis. Thus, identifying unknown interconnections between these genes may provide valuable clues for developing strategies for cancer therapy. In the present study, we aimed to identify potential regulatory networks involved in tumorigenesis of glioma, based on the ceRNA hypothesis. We used integrated bioinformatics analysis to construct a regulatory network associated with glioma tumorigenesis we acquired five axes, "CRNDE/has-mir-223/STAB1", "CRNDE1/has-mir-150/TOP2", "NEAT1/has-mir-150/TOP2", "GRM3-AS1/has-mir-128/TOP2" and "GRM3-AS1/has-mir-128/STAB1 "as potential ceRNA regulatory networks in glioma patients.

Key words: LncRNAs, microRNAs, mRNAs, Bioinformatics

Introduction

Gliomas are the most frequent type of primary brain tumors [1, 2] Each year more than 250 000 cases are diagnosed as primary malignant brain tumors worldwide and 77% of these cases are gliomas [3]. Despite their low incidence, they have a high mortality rate because of their delicate location [4]. The World Health Organization (WHO) classifies gliomas into 4 grades, grades I and II as low-grade gliomas (LGGs) and grades

III and IV as high-grade gliomas (HGGs) [5]. Despite recent advances in surgical, radiotherapy, and chemotherapy treatments, the effectiveness of current therapies is not desirable and leads to a short survival period for most patients with HGGs. Gliomas are characterized by poor prognosis, heterogeneity, rapid cell proliferation, high diffusion and invasion capacity, recurrence rate, and resistance to treatment [6] [7].

More than 75 percent of the genome is transcribed into RNA, but only approximately 2 percent encodes proteins, and the rest are classified as noncoding RNAs (ncRNAs) [8]. Regulatory ncRNAs with lengths smaller than 200nt are sorted as small non-coding RNA, and some well-known classes are small interfering RNAs (siRNAs), microRNAs (miRNAs), piwi-interacting RNAs (piRNAs) and small nucleolar RNAs (snoRNAs) [9]. Those with lengths larger than 200nt are sorted as long non-coding RNA, such as long intergenic non-coding RNAs (lincRNAs) and natural antisense transcript (NAT). Non-coding RNAs (ncRNAs) participate in multiplex networks of interactions with RNAs and proteins. They perform structural, catalytic, and regulatory roles, and participate in proliferation, differentiation, development, and apoptosis in a wide variety of biological processes. Studies proved that their altered expression levels can lead to carcinogenesis [10, 11].

Increasing evidence suggests that Competitive endogenous RNA (ceRNA) networks are critical regulators of gene expression. The ceRNA hypothesis proposes a new approach in which the functions of coding and noncoding RNAs are linked, and a regulatory lncRNA-miRNA-mRNA network is constructed. Existing data show that lncRNAs can indirectly modulate mRNA expression levels by interacting with miRNAs: therefore, they can affect multiple target genes and biological functions. LncRNAs can act as ceRNAs because they contain miRNA response elements (MREs). LncRNAs compete to isolate miRNAs from their target genes. Subsequently by sponging miRNAs, they regulate gene expression through these complex networks [12, 13].

Emerging studies have revealed the significant impact of dysregulated ceRNA networks on the pathogenesis of diseases. Discovering possible ceRNA networks would provide insights into the molecular mechanisms that lead to pathogenesis [14, 15]. Therefore, in the present study, we aimed to identify a potential lncRNA-miRNA-mRNA regulatory network involved in glioma tumorigenesis. By using a combination of bioinformatics tools, we acquired five axes, "CRNDE/has-mir-223/STAB1", "CRNDE1/has-mir-150/TOP2", "NEAT1/has-mir-150/TOP2", "GRM3-AS1/has-mir-128/TOP2" and "GRM3-AS1/has-mir-128/STAB1" as potential ceRNA regulatory networks in glioma patients.

Materials and Methods

Data acquisition and Differential expression analysis

Four datasets (SRP434123, SRP233221, SRP328814, SRP114556) were downloaded from the SRA database (http://www.ncbi.nlm.nih.gov/sra). These datasets contain mRNA and lncRNA expression RNA-seq from 32 glioma tissues and 32 normal ones. Also, 2 and 12 samples contain miRNA-seq of glioma and 12 normal tissues, respectively. The quality of the reads was checked by FASTqc and adapters were eliminated at the beginning of each read with Trim Galore software. The pre-processed reads were aligned by HISAT2 against the human reference genome (GRCh38/hg38). Counting of reads and their assembly were done with the htseq-count software. Eventually, the Deseq2 package was applied to calculate the differentially expressed genes (DEGs) between glioma and normal tissues. The DEGs with log2 fold change (FC) > 2 and< 0.5 and ($p < 0.05$) were selected to further analysis.

Survival and co-expression analysis of key genes

Clinical data of glioma patients were obtained from the TCGA database (HGG). We analyzed the overall survival rate of differentially expressed mRNAs, lncRNAs and miRNAs, using the survival package. Starbase database was used to analyze survival of genes in the ceRNA network and to further investigate, Correlation expression analysis of key genes was performed.

GO and KEGG enrichment analysis

We investigated the functions of DE-mRNAs and pathways in which they operate by applying GO analysis which consists of (Cellular Component (CC), Biological Function (BF), Molecular Process (MP)) and The Kyoto Encyclopedia Genes and Genomes (KEGG) analysis, using R package.

Regulatory network of lncRNA-miRNA-mRNA

To generate an interaction network based on the DEGs, the relationship of 10 up/down-regulated mRNA–miRNA, lncRNA–miRNA, and lncRNA–mRNA pairs were predicted using online tools Targetscan (www.targetscan.org/), mirDB (www.mirdb.org/), miRwalk (http://mirwalk.umm.uni-heidelberg.de/), and Starbase database (www.starbase.sysu.edu.cn/). The construction of the ceRNA networks between the commonly predicted lncRNA–mRNA, lncRNA–miRNA, and miRNA–mRNA was visualized using Cytoscape software version 4.1.

Results

Data acquisition and Differential expression analysis

RNA-seq data was analyzed to investigated the Up/down-regulated coding/non-coding RNAs in glioma. we acquired differentially expressed lncRNAs, miRNAs and mRNAs in Glioma samples compared to normal samples. Based on the expression analysis, 598 DE lncRNAs (250 upregulated and 348 downregulated), 210 DE miRNAs (110 upregulated and 100 downregulated) and 495 DE mRNAs (254 upregulated and 241 downregulated) were identified. ten of the most important genes are listed in the table 1. The dysregulated genes were significantly associated with Glioma with criteria; log Fc > 2 or < 0.5 and (P<0.05). Results are shown in (Fig.1).

Survival and co-expression analysis of key genes

The survival rate of DE-lncRNAs, DE-mRNAs and DE-miRNAs was evaluated using the survival package in R (P<0.05). Starbase was applied for survival analysis of the key genes. Results indicated that DE-mRNAs (TOP2 and STAB1), DE-lncRNAs (CRNDE and NEAT1) and were negatively correlated with overall survival of Glioma patients. DE-miRNAs including has-mir-128, hsa-miR-150, hsa-miR-223 were positively associated with overall survival in glioma. Results are shown in (Fig.2). The correlation between the expression level of key genes and their targets was explored by starbase database. According to starbase database, hsa-miR-150 negatively correlated with TOP2. hsa-miR-128 negatively correlated with TOP2. Results are shown in (Fig.3).

Gene Ontology(GO) and KEGG enrichment analysis

To explore the functional significance of DEGs in the ceRNA networks, we performed GO enrichment analysis and KEGG pathway analysis. Results of the GO functional annotation and KEGG pathway analysis demonstrated that, DEGs were significantly enriched in “systemic lupus erythematosus (SLA)”, “alcoholism”, and “neutrophil extracellular traps” which reported to be related to glioma. Top GO functional annotation and KEGG pathway analysis are shown in (Fig.4).

Prediction of lncRNA-miRNA-mRNA relationship and construction of ceRNA network

We constructed ceRNA networks to study the regulatory relationships between DE-miRNAs and their target genes to get a better understanding of the underlying molecular mechanism of Glioma. We identified target gene interactions by using mRNAs to predict related miRNAs and using miRNAs to predict related lncRNAs. lncRNA-miRNA–mRNA networks were constructed using the combination of lncRNA–miRNA and miRNA–mRNA pairs. Online tools such as Targetscan, mirDB, miRwalk, and Starbase were utilized. The results of interactions were visualized by Cytoscape. We predicted that hsa –mir- 223 could bind to the 3UTR of STAB1 and hsa –mir-128 could bind to 3UTR of STAB1 and TOP2. And hsa-mir-223 could be sponged by CRNDE, hsa –mir- 128 could be sponged by NEAT1 and hsa –mir- 150 could be sponged by NEAT1 and GRM3. In this study the following 5 axis, "CRNDE/has-mir-223/STAB1", "CRNDE1/has-mir-150/TOP2,” "NEAT1/has-mir-

150/TOP2", "GRM3-AS1/has-mir-128/TOP2 "and "GRM3-AS1/has-mir-128/STAB1 "were introduced as potential ceRNA regulatory networks in Glioma. Results are shown in (Fig.5).

Discussion

Gliomas are highly heterogeneous tumors [16]. Developing successful therapies to overcome glioma requires a comprehensive knowledge of its molecular mechanisms. Several altered signaling pathways and cross-linked relationships of molecules remain to be investigated. Evidence demonstrates that ceRNA networks play a critical role in cellular processes, and dysregulation of any component of these networks could result in pathogenesis. In the present study, we aimed to identify potential regulatory networks, based on the ceRNA hypothesis. We used integrated bioinformatics analysis to construct a regulatory network associated with glioma tumorigenesis. First, we obtained 598 DE-lncRNAs, 210 DE-miRNAs, and 495 DE-mRNAs in Glioma samples compared to normal samples from TCGA database. Furthermore, Survival and correlation analyses were performed and significantly associated genes were selected. Bioinformatics analysis was then performed to predict target gene interactions. Ultimately, lncRNA- miRNA–mRNA networks were constructed. Based on the results, we identified five axes, "CRNDE/has-mir-223/STAB1", "CRNDE/has-mir-150/TOP2", "NEAT1/has-mir-150/TOP2", "GRM3-AS1/has-mir-128/TOP2" and "GRM3-AS1/has-mir-128/STAB1" as potential ceRNA regulatory networks in glioma patients. There are no previous studies on the impact of miR-128, miR-150 and miR-223 on TOP2 and STAB1 in gliomas.

Topoisomerase 2 (TOP2) are ATP-dependent enzyme that are found to be in nucleus and mitochondria [17]. They are involved in DNA functions such as chromatin organization, replication process and transcription [18][17]. TOP2 resolve DNA topological problems encountered in the above-mentioned functions by generating transient double-stranded breaks (DSBs) and unwinding the DNA, thus relieving the torsional stress [19]. Unrestrained TOP2-associated DSBs can cause mutations, such as deletions and translocations, and can trigger malignancies [20]. Vertebrates have two structurally similar isoforms of Topoisomerase 2; TOP2A [21] and TOP2B [22] are located on chromosomes 17 and 3 respectively. These isoforms differ in terms of expression and physiological functions [23]. TOP2A is considered a marker of cell proliferation, while TOP2B is mainly involved in other DNA metabolism processes [24]. TOP2A expression levels increase in proliferating cells and maximizes in G 2 /M phase, which represents its significant activity in replication and chromosome segregation. Knockout of TOP2A impedes development at the four or eight cell stage in mice [25]. Wang et al reported TOP2A Activates PI3K/AKT Signaling pathway and Elevates metastasis in Cervical Cancer [26]. Meng, Jiali, et al. suggested that high expression of TOP2A in hepatocellular carcinoma could be associated with tumor progression and bad prognosis [27]. Findings showed that TOP2A could play an oncogenic role in gliomas and is correlated with patients survival [32]. Owing the contribution of TOP2 in tumorigenesis, chemotherapeutic drugs targeting them are among the most effective drugs [23]. The mechanisms involved in the TOP2 regulatory networks remain to be elucidated. STAB1 encodes a multidomain type 1 transmembrane receptor, known as Stabilin-1, FEEL-1, CLEVER-1, KIAA0246. Its expression has been identified in macrophages, immunosuppressive cells,

monocytes, sinusoidal endothelial cells and lymphatic endothelial cells [34]. Stabilin-1 functions in tissue homeostasis, intracellular trafficking, scavenging, and tolerance [35]. Stabilin-1 is associated with endocytosis of acetylated low-density lipoprotein (acLDL), secreted protein acidic and rich in cysteine (SPARC), and transcytosis of the growth hormone family member placental lactogen (PL). Stabilin-1 is a known receptor for SPARC, a matricellular protein involved in cell migration, which is upregulated in glioma tissues and promotes metastasis [36]. The role of STAB1 in cancer progression remains challenging, Due to its context-dependent nature and the multiple functions of ligands in the tumor microenvironment [37]. Stabilin-1 is expressed by tumor-associated macrophages (TAM) in the tumor microenvironment in cancers such as melanoma, lymphoma, glioblastoma, and pancreatic insulinoma [38]. A study showed that STAB1 knockout mice develop smaller primary tumors and metastases, suggesting that it may play a role in tumor development [39]. Clément et al. showed that stabilin-1 is expressed in early gliomas and is downregulated during tumor progression [36]. Studies on STAB1 in Glioma are limited, and further investigations on their potential roles and regulators are urgently needed. miR-128 is reported to be a brain-enriched miRNA [41].

miR-128-3p acts as a tumor suppressor in various cancers, including esophageal squamous cell carcinoma [42], breast [43], bladder [44] cancer and gliomas [45]. Accumulating evidence suggests that it as a potential target for cancer therapy [46]. Decreased expression of miR-128 associates with aggressive glioma grades [47]. Studies indicated that miR-223 could act either as an oncogene or as a tumor suppressor in carcinogenesis [48]. miR-223-3p inhibited proliferation of glioma cells by regulating NFIA expression [49] and inflammation-associated cytokines [50]. A study reported that miR-150 inhibits the tumorigenesis of Leukemia Stem Cells by regulating the Nanog Signaling Pathway [51]. Another study reported that miR-150-3p Inhibits the proliferation of glioma cells through targeting SP1 and its decreased expression is associated with metastasis in patients with glioma. [52]. Taken together, our miRNA candidates were reported to be promising regulators in various cancers thus, investigating their novel targets including TOP2 and STAB1 could bring new insights into unexplored mechanisms in glioma.

LncRNA Colorectal neoplasia differentially expressed (CRNDE) has been suggested to be a key player in carcinogenesis in a variety of cancer types, including glioma [53]. Accumulating studies verifies CRNDE exerts multiple carcinogenic functions, such as inhibiting cell apoptosis and inducing proliferation, invasion, migration, and chemoresistance in gliomas through multiple mechanisms. [54] [55]. Wang et al. reported up-regulated CRNDE promotes malignancy in glioma via mTOR signaling pathway [56]. Li et al. claimed that CRNDE accelerates tumor progression through regulating the miR-136-5P/Bcl-2/Wnt2 signaling axis in GBM [57]. LncRNA Nuclear paraspeckle assembly transcript 1 (NEAT1) is another oncogenic factor that regulates multiple signaling pathways in various cancers [58]. For instance, Zhao, Lun, et al. indicated lncRNA NEAT1 regulates non-small cell lung cancer (NSCLC) tumorigenesis by sponging miR-153-3p [59]. A study has shown that NEAT1 promotes breast cancer progression via modulating miR-448 and ZEB1 [60]. Zhang, Jiale, et al. found that NEAT1 sponges miR-324-5p thus positively regulates KCTD20 expression and increases the proliferation

of glioma cells [61]. Increased expression of CRNDE levels, indicates a poor prognosis and overall survival in glioma patients [61] [62]. A study showed that NEAT1 promotes proliferation in glioma cells by modulating the miR-185-5p/DNMT1/mTOR signaling pathway [63]. Reduced expression of NEAT1 inhibits tumor progression in GSCs via restoring the microRNA let-7e [64].

The aberrant expression of our candidates in a variety of tumor cells indicates their significance in tumorigenesis. Thus, identifying unknown interconnections between these genes may provide valuable clues for developing strategies for cancer therapy. Since no previous studies have investigated the impacts of miR-128, miR-150 and miR-223 on TOP2 and STAB1 in Glioma, we aimed to uncover the potential underlying regulatory network. Results of Functional Enrichment Analysis demonstrated that change in expression level of mentioned genes, directly affects systemic lupus erythematosus (SLA), alcoholism and neutrophil extracellular traps. Central nervous system is among the organs that can get involved in SLE [65]. Studies revealed there is a link between SLE and increased cancer incidence, including brain tumors such as glioma [66]. Case studies have proposed an association between high-grade glioma and SLE [67, 68]. There is inconsistent evidence concerning alcoholism and glioma therefore, further research is required to understand the exact association and mechanisms [69]. Activated Tumor Associated Neutrophils release networks composed of DNA-histone complexes and proteins known as Neutrophil Extracellular Traps in tumor microenvironment, which promotes tumor progression and metastasis [70]. It has been reported that NETs could induce the glioma cell proliferation, migration, and invasion [71]. However, involvement of these mechanisms in glioma progression remains largely unknown, we suggest experimental investigations.

In conclusion, our results provide a potential regulatory network underlying glioma genesis and displayed that CRNDE can competitively bind to miR-223, miR-150 also NEAT1 can competitively bind to miR-150 and miR-128. And GRM3-AS1acts as a ceRNA and binds to miR-128. Therefore, modulate STAB1 and TOP2 expression levels in glioma and base on Functional Enrichment Analysis affect systemic lupus erythematosus (SLA), alcoholism and Neutrophil extracellular traps pathways in glioma.

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Legends

Fig1. Identification of DE-lncRNAs, DE-mRNAs, DE-miRNAs related to glioma

(A). Volcano plot for DE-lncRNAs

(B). Volcano plot for DE-mRNAs

(C) Volcano plot for DE-miRNAs

Fig2. Survival analysis results of differentially expressed genes in the ceRNA networks Based on starbase database.

Fig3. Correlation expression analysis of TOP2A and STAB1 in starbase database.

(A) TOP2A and hsa-miR-128 and hsa-miR-150.

(B) STAB1 and has-mir-128 and has-miR-223.

Fig4. Analysis results of DE-mRNAs in the ceRNA regulatory network in the GO and KEGG pathways.

(A) Results of GO enrichment analysis of the DEGs

(B) Results of KEGG pathway analysis of the DEGs

Fig5. The result of lncRNA-miRNA mRNA ceRNA network construction by cytoscape.