Integrative Bioinformatics and Experimental Investigation Introduces *OBI1-AS1* as a Key LncRNA in the Progression of Low-grade Glioma to Glioblastoma

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Research

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Abstract

Background:

Long non-coding RNAs (LncRNAs) are widely known for their multiple functions in the context of cancer from tumor initiation to tumor progression and metastasis. Gliomas are the most prevalent primary forms of brain tumor, classed from grades I to IV according to their malignant histological features with grade IV also known as Glioblastoma Multiforme (GBM) displaying the highest level of malignancy. This fact intensifies the importance of searching for Differentially Expressed LncRNAs (DELncRNAs) between GBM and Low-Grade Glioma in the hope of finding new targets for diagnostic and therapeutic measures.

Methods:

In the current study, we performed bioinformatics analysis to obtain a list of DELncRNAs and further chose the unprecedentedly studied *OBI1-AS1* for further investigations. We also carried out Real-Time PCR to validate our bioinformatics findings.

Results:

Both analyses were in concordance and pinpointed downregulated expression of *OBI1-AS1* in GBM compared to LGG samples. Additional supplementary bioinformatics studies exhibited *OBI1-AS1* role in synaptic signal transduction and neural differentiation in addition to its role in pluripotency and maintenance of stemness.

Conclusion:

All the aforementioned findings introduce *OBI1-AS1* as a new attractive lncRNA to be further studied for a better clarification of its roles in glioma progression and a deeper understanding of malignant transformation of LGG to GBM.

1. Backgound

Glioma is the most prevalent primary brain tumor. It is classified into grades I to IV by the World Health Organization (WHO) based on its malignant features with grades I, II and III classified as Low-Grade Glioma (LGG) and grade IV as High-Grade Glioma (HGG) also known as Glioblastoma Multiforme (GBM) [1]. GBM has the highest mortality rate and lowest 5-year survival rate in less than 10% of patients when compared with LGG with 26 % in patients aged 55–64, 73% in patients aged 20–44 and 46% for those in the 45–54 age group [2]. GBMs can either be primary (pGBM), arising *de novo*, or can occur as a consequence of LGG recurrence with higher grades, known as secondary GBM (sGBM). Primary and secondary GBM can differ in regards to their genetic and epigenetic landscape [3] with sGBM displaying more aggressive features and poorer prognosis than pGBM [4]. It is also noteworthy that the progression of LGG to GBM increases the mortality rate [5], which makes this an important area of research to find key

genes implicated in the malignant progression of LGG to GBM for potential future therapeutic and interventional measures in order to enhance survival of patients.

Recently, a massive group of genes coding for non-coding RNAs (ncRNAs) rather than proteins have been in the spotlight. Non-coding RNAs exert their functions either at the RNA level by inhibiting the transcription of RNAs or at the protein level by impeding translation [6]. They can be grouped according to their number of nucleotides into small (Small non-coding RNA < 200 nucleotides) or long (Long non-coding RNA > 200 nucleotides). They have been proven to play significant roles in cancer. They play roles in tumorigenesis and tumor progression through DNA damage response, immune escape of cancer cells and metabolic dysregulation of cancer cells that help provide them with the energy required for aberrant cell growth. Additionally, they are implicated in tumor metastasis by modulating epithelial mesenchymal transition (EMT), a complex process through which cancer cells lose their epithelial features and gain mesenchymal features, enhancing their migration and invasive abilities [7]. ncRNAs are involved in regulation of gene expression through modulation of epigenetic, transcriptional and post-transcriptional mechanisms [8]. HOTAIR is just one of a plethora of LncRNAs involved in glioma by promoting cell cycle progression and is tightly linked with poor prognosis [9]. Linc-POU3F3 increased expression has been revealed to induce proliferation and viability of glial cells [10]. Moreover, *CASC2* has been documented to perform malignancy inhibitory functions through negative regulation of *miR-21* resulting in reduction of migratory and invasive abilities [11]. With all the existing evidence, some of which have been stated above, it can be clearly concluded that non-coding RNAs can be key regulators in glioma progression.

In the present study, we have conducted a systematic bioinformatics study utilizing The Cancer Genome Atlas (TCGA) database for analysis of differentially expressed LncRNAs (DELncRNAs) in LGG and GBM samples in the hope of finding potential LncRNAs that might play a role in the transformation progress of LGG to GBM. This analysis showed that *OBI1-AS1* is one of the most significant DELncRNAs downregulated in GBM. Real-Time PCR confirmed this finding experimentally. Using the GEPIA web server, we also investigated the existing correlation between *OBI1-AS1* expression and overall survival of patients. ChIP-Seq analysis was performed to elucidate the transcription factors in close contact with the *OBI1-AS1* promoter to clarify the interacting proteins contributing to regulation of *OBI1-AS1* expression and therefore playing their part in glioma malignant progression. Finally, Gene Ontology and pathway enrichment analyses were performed to underpin the molecular function, biological process, cellular component and the signaling pathway *OBI1-AS1* is involved in. Our bioinformatics analysis shows that *OBI1-AS1* has an important role in regulation of pluripotency and oligodendrocyte differentiation.

2. Methods

2.1. Data download and analysis

GBM and LGG RNA-seq datasets and their associated clinical information were obtained from the TCGA database [12]. Forty GBM and 80 LGG samples were selected for Differential Expression Analysis (DEA). Transcriptome data were analyzed using the edgeR package[13]. The p-values were adjusted using the

Benjamini-Houchberg method[14]. Subsequently, differentially expressed LncRNAs were chosen for further investigations and volcano plot was created to visualize the DELncRNAs utilizing R Enhanced Volcano package[15] on the basis of False Discovery Rate (FDR) and Log 2 Fold Change.

2.2. Survival Analysis

GEPIA web server was used for analysis of any correlation between *OBI1-AS1* gene expression level and patient overall survival[16]. Patients were grouped into low expression and high expression categories according to whether their *OBI1-AS1* gene expression level was below the first quartile or above the third quartile, respectively. Consequently, Kaplan-Meier survival analysis was performed for the survival data as presented in Fig.1c.

2.3. ChIP-Seq

The ChIP-Atlas-Enrichment Analysis online tool[17] was used to find the transcription factors (TF) binding to -3000 < TSS < 3000 of *OBI1-AS1* transcription start site (TSS) in neural cells. The significance threshold was selected as greater than 100 based on peak caller MACS2 score (-10*Log10 [MACS2 Q-value]) which means that peaks with MACS2 Q-value (FDR) lower than 10E-10 are considered. Moreover, we downloaded Histone marks which were enriched in proximity of *OBI1-AS1* TSS using the determined cut-off from the National Bioscience Database Center (NBDC)[18].

2.4. Gene Ontology (GO)

We used *OBI1-AS1* co-expressed genes to perform functional annotation. We downloaded FPKM count for TCGA-GBM and TCGA-LGG project from the GDC data portal. Subsequently, we used the Pearson method to compute the correlation coefficient and p-value. Genes with R > 0.5 were selected for functional annotation. Enrichment analysis for Biological Process, Molecular Function, Cellular Component and Pathways involved were conducted by the R TCGAbiolinks package[19].

2.5 Patient samples and IHC diagnosis

Twenty-six GBM and 26 LGG samples were collected from the Shariati Hospital affiliated with Tehran University of Medical Sciences (TUMS). Pathological diagnosis and immunohistochemical analysis of the tumor type was carried out by an expert neuropathologist based on the World Health Organization classification of tumors (grade I to IV). The histopathological diagnosis of the obtained tissue samples was conducted based on immunohistochemical detection of the following proteins: GFAP, OLIG2, p53, KI67, IDH1, ATRX, EGFR. All samples were included in the immunohistochemical and Real-Time PCR analyses. Tumor samples were all collected in RNA Later immediately after surgical resection and stored at -80° centigrade until RNA extraction. Written informed consent was obtained from all patients enrolled in this study. This study fully conforms to the ethical standards of Tehran University of Medical Sciences and the 1975 Helsinki Declaration.

2.6. RNA extraction, cDNA synthesis and quantitative Real-Time PCR

RiboEx™ (GeneAll) was used for RNA extraction and RNA extraction was carried out based on manufacturer's protocol. Presence of genomic contamination was checked by agarose gel electrophoresis before cDNA synthesis. RNA concentration and presence of contaminants were determined using the NanoDrop 2000 spectrophotometer (Thermo Scientific). cDNA synthesis was performed using the PrimeScript RT reagent (TakaraBio Inc, Shiga, Japan) and qRT-PCR was carried out using the AMPLIQON Real Q Plus 2× Master Mix Green low ROX in the Light Cycler® 96 System (Roche Life Science, Germany) based on the manufacturer's instructions. Real-time PCR was conducted in duplicates. Primers were designed using the Oligo software and were blasted to check their specificity afterwards. Primer sequences are presented in Table.1 below. Standard curves were created for the setup of the primers and the primers were set up with efficiency equal with 2. Relative quantification of target gene expression was performed using the $2^{-\Delta\Delta Ct}$ method with *B2M* as the normalizer gene. The Real-time procedure for each sample was as follows:

Incubation for 10 min at 95°C followed by 40 cycles of elongation including 10 s at 95°C and 30 s at 60 °C. To exclude presence of any primer dimers or by-products, dissociation curves were carefully analyzed to check the specificity of the product melting peak. The PCR products were ultimately confirmed by 2% agarose gel electrophoresis.

Table 1.

primers sequences

Gene	Forward primer	Reverse primer
OBI1-AS1	GCCCTGAAGCATACCAAAATGT	CACAGAAAGTGCCCAAGAGGT
B2M	AGATGAGTATGCCTGCCGTG	GCGGCATCTTCAAACCTCCA

2.7. Statistical analysis

The Q-Q plot was used to assess normal distribution of the data. The Mann-Whitney test was carried out for comparison of groups. The ROC curve and the Area Under the Curve (AUC) were used to evaluate the sensitivity and specificity of the LncRNA in distinguishing GBM from LGG. A p-value less than 0.05 was deemed statistically significant for a confidence interval of 95%. Statistical analysis was performed with GraphPad Prism8.

3. Results

3.1. OBI1-AS1 is downregulated in GBM

In this study, 1,109 LncRNAs exhibited differential expression between LGG and GBM among which 728 had FDR < 0.05. The 728 LncRNAs were further filtered for the |Log2 Fold change|>2 and this reduced the number of validated DELncRNAs to only 84 (file S1). Subsequently, the top 10 DELncRNAs with the

lowest FDRs were prioritized and selected for further investigations as presented in the volcano plot in Fig. 1a below. *OBI1-AS1* was chosen because it displayed the highest level of |Log2 Fold change|=3.67 (Fig. 1b). Furthermore, high expression level of this gene showed tight association with prolonged patient survival period as is illustrated in Fig. 1c. Interestingly, thus far, no previous studies or investigations have been performed on this gene. To further validate the results obtained from our bioinformatics analyses, 26 GBM and 26 LGG tumor samples were collected and checked utilizing Real-Time PCR technique for confirmation of RNA-Seq findings.

3.2. Real-Time PCR revealed lower expression of OBI1-AS1 in GBM compared to LGG, confirming RNA sequencing findings

Real-Time PCR revealed downregulation of *OBI1-AS1* in samples of GBM compared with LGG. The relative expression level of this gene is illustrated in Fig. 1d. Real-Time PCR showed that Log2FC was −3.54 which was in complete compatibility with our DEA results (Log2FC = -3.67). To analyze the sensitivity and specificity of *OBI1-AS1* in distinguishing GBM from LGG, ROC curve was created and the Area Under the Curve (AUC) was measured (Fig. 1e). These experimental findings add weight to the bioinformatics findings obtained from RNA-Seq which revealed *OBI1-AS1* as a DELncRNA in GBM samples compared to non-GBM.

3.3. Potential Role of OBI1-AS1 in Synaptic Signal Transduction and Neural Differentiation

Gene Ontology results for *OBI1-AS1* are presented in Fig. 2. The top 4 significant GO terms in Biological Process (BP) (orange boxes) were pertinent to cell adhesion. This was compatible with other GO terms in Molecular Function (MF) and Cellular Component (CC). GO: CCs (blue boxes in Fig. 2) showed that most of the genes are located in the plasma membrane of neurons especially in neural projections, dendrites, synapses, and postsynaptic density. Interestingly, the most significant Molecular Function GO terms were related to calcium ion binding proteins which have an important role in formation of cell-cell adhesion and postsynaptic density complexes. Other GO: MF terms were linked to ion trafficking across the neural membrane or ionotropic glutamate receptors, which meant that most of the *OBI1-AS1* co-expressed proteins have a binding domain for calcium. Intriguingly, when we conducted pathway enrichment analysis, glutamate receptor signaling was found to be the most significant pathway. This signaling pathway has a crucial role in response to glutamate in the post synaptic neurons at the postsynaptic density. Some other signaling pathways which were significantly enriched were related to neural communication especially in post synaptic neurons.

The CREB signaling pathway was revealed to be the second significant pathway in our analysis. There are many studies suggesting a role for this pathway in brain tumor proliferation, calorie restriction in brain, synaptic plasticity and neural cell differentiation[20]–[22]. Taking all these findings into account in addition to the fact that CREB is known as a key regulator in neural differentiation and glioma progression, the CREB signaling pathway can be considered as a potential target for therapeutic applications[23], [24]. This plausibly proposes that *OBI1-AS1* has a role in regulation of neural

differentiation in this pathway. Additionally, the second significant groups of GO: BPs were related to neural differentiation and neurogenesis which strengthened our hypothesis.

3.4. Transcription factor enrichment analysis unravels OBI1-AS1 potential involvement in regulation of glioma stemness

A high proportion of transcription factors that bind to *OBI1-AS1* promoter contribute to regulation of pluripotency and cell cycle. The OCT4 pluripotency signaling pathway was the most significant pathway in our analysis (Fig. 3a). Oct4 is known as a major mediator to maintain stemness in cancer cells and Embryonic Stem Cells (ESC). Most genes downstream of this pathway are regulated by methylation and histone modifications[25]. Moreover, DNA Methylation and Transcriptional Repression Signaling were presented as the third significant pathway which can suggest a possibility for contributory roles of these TFs in regulation of stemness by epigenetic modifications in this locus. We assessed histone modifications in this region to find significant histone modifications around the *OBI1-AS1* TSS (file S2). H3K27ac and H3K4me3 were the two major histone modifications in this site (Fig. 3b). Both modifications have been attributed to active cell-specific enhancers (H3K27ac) and promoters (H3K4me3)[26]–[28].

4. Discussion

In this study, we showed that *OBI1-AS1* is downregulated in GBM in comparison with LGG by bioinformatics analysis and confirmed this finding experimentally. In addition, we demonstrated presence of poor prognosis in patients with low expression level of *OBI1-AS1*, in stark contrast with patients with high expression level. These findings suggest the possible fundamental implication of *OBI-AS1* in transformation of LGG to GBM. Hence, *OBI1-AS1* is expected to act as a tumor suppressor and its functions are, therefore, contributory to reducing tumor invasion.

Pathway enrichment analysis has revealed the plausible role of *OBI1-AS1* in synaptic response especially glutamate receptor signaling. Ionotropic glutamate receptor signaling was the prioritized enriched pathway in the current study. Glutamate receptors are transmembrane proteins highly expressed in the CNS. Not only are glutamate receptors expressed in synapses, but they are also, more interestingly, expressed in non-synaptic regions and non-neuronal cells including glial cells and glioma cells. In recent years, this has attracted a lot of attention in glioma. Michael C. Oh *et al*. has proven overexpression of AMPA-type glutamate receptors in cancer stem cells derived from GBM [29]. The rise in intracellular calcium concentration level mediated by glutamatergic receptors is a well-established fact [30]. Promoted intracellular calcium levels may help trigger glutamate secretion by glioma cells to their surrounding cells [31]–[33]. The resumption of this feedback loop, therefore, results in more extracellular glutamate levels and intracellular calcium concentrations ultimately leading to excitotoxicity of the neurons while contributing to progression of glioma cells, putting forth the possibility that GBM expansion and LGG malignant changes are mediated by excessive glutamatergic signaling [34], [35]. The active engagement of the glutamatergic system has rendered it as one of the most substantial oncogenic pathways in glioma with roles in migration, differentiation and survival mechanisms [36]–[38]. These notions are

further supported and strengthened by observing the additional impact of this pathway in the tumor microenvironment conducive to glioma progression as has been reported in a study by Venkatesh [39] shedding light on neuron-to-glioma synapses.

The CREB signaling pathway and neuron differentiation was the second most prioritized pathway our LncRNA, *OBI1-AS1*, is seemingly implicated in. CREB is a nuclear-localized super family transcription factor with three major isoforms. They are activated by serine-threonine tyrosine kinases and ultimately bind cAMP-Response elements (CREs) in the promoters of their target genes [40], [41]. CREB has previously been recognized to be an important contributor to neurogenesis by orchestrating multiple signaling cascades highly demanded for stem cell survival and growth. CREB elevated expression level has been linked to diverse types of cancer and, above all, brain tumors in terms of cell growth and differentiation [42]–[45]. One of the major routes through which CREB has implemented its role in glioma is through PTEN loss-of-function mutations, which leads to CREB loss of deactivation giving rise to cell survival and growth in Glioma Stem Cells (GSCs), contributing to gliomagenesis .

As has been indicated in our bioinformatics results section, a high proportion of the transcription factors which bind to *OBI1-AS1* promoter are involved in OCT4 pathway to maintain pluripotency. Oct4, a stem cell marker, is a transcription factor expressed in stem cells for induction of the pluripotency state and body of evidence elucidates its role in Cancer Stem Cells, rendering them the ability to recur and escape complete treatment in addition to self-renewal and differentiation ability. It has also revealed higher expression level in the more malignant grades of astrocytoma. Together with the SOX axis, Oct4 helps maintain the tumorigenicity of tumor initiating cells in glioma [46]. Oct4 and Sox2 work in collaboration to promote transcription of genes involved in pluripotency features. Additionally, they repress genes involved in differentiation [47]. Based on ChIP-Seq data in glioma, *SOX2* has a binding site around the *OBI1-AS1* TSS (Fig, 3b) which puts forward the idea of Oct4 working in complex with Sox2 to suppress expression of *OBI1-AS1* in GBM and help maintain the state of pluripotency in glioma stem cells. In addition, eukaryotic promoter database (EPD) predicted a significant binding site (p-value = 0.00001) 206 bp upstream of *OBI1-AS1* TSS (supplementary) which lends support to this hypothesis (Fig. 3c). However, more experimental investigations are required to assess the validity of such hypothesis.

5. Conclusion

Taking all the findings mentioned above into account, *OBI1-AS1* is differentially expressed between GBM and LGG, introducing it as a potential LncRNA involved in malignant progression of LGG into GBM. What makes it more attention worthy, is its calcium ion-binding properties in the ionotropic glutamate receptor signaling pathway, unravelling it as a mediator in the neuron-glial communication and signal transduction that ultimately lead to tumor expansion. The current study also underscores its plausible roles in cancer stemness owing to it being regulated by Oct4, a stem cell marker well-established for its role in tumor recurrence and invasiveness.

Abbreviations

LncRNA: Long Non-coding RNA

DELncRNA: Differentially-Expressed LncRNA

GBM: Glioblastoma multiforme

pGBM: Primary GBM

sGBM: Secondary GBM

EMT: Epithelial-to-Mesenchymal Transition

TSS: Transcription Start site

ChIP: Chromatin Immunoprecipitation

LGG: Low-Grade Glioma

HGG: High-Grade Glioma

FDR: False Discovery Rate

EPD: Eukaryotic Promoter Database

WHO: World Health Organization

TCGA: The Cancer Genome Atlas

DEA: Differential Expression Analysis

TF: Transcription Factor

NBDC: National Bioscience Database Center

GO: Gene Ontology

AUC: Area Under the Curve

BP: Biological Process

MF: Molecular Function

CC: Cellular Component

CRE: Camp-Response Element

GSC: Glioma Stem cell

Declarations

Ethics approval and Consent to participate

All procedures followed were in accordance with the ethical standards of Tehran University of Medical Sciences and the Helsinki Declaration of 1975, as revised in 1983 and its subsequent revisions. All participants signed the written informed consent form.

Consent for publication

Not applicable

Availability of data and materials

The datasets used and/or analyzed during the current study are available from the corresponding author on reasonable request

Competing interests

The authors declare that they have no competing interests

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Authors' contributions

AM has contributed to the design of the study and bioinformatics investigations and data interpretation. ShB has contributed to sample collection, experimental investigations and drafting the manuscript. AM and SSh have contributed to experimental investigation and statistical analysis of the data. AKh and MTR have contributed to the surgical resection of the tumor samples. HS has contributed to the pathological diagnosis of the tumor type and grade. MT has supervised the whole project and has contributed to manuscript edition and submission.

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Figures

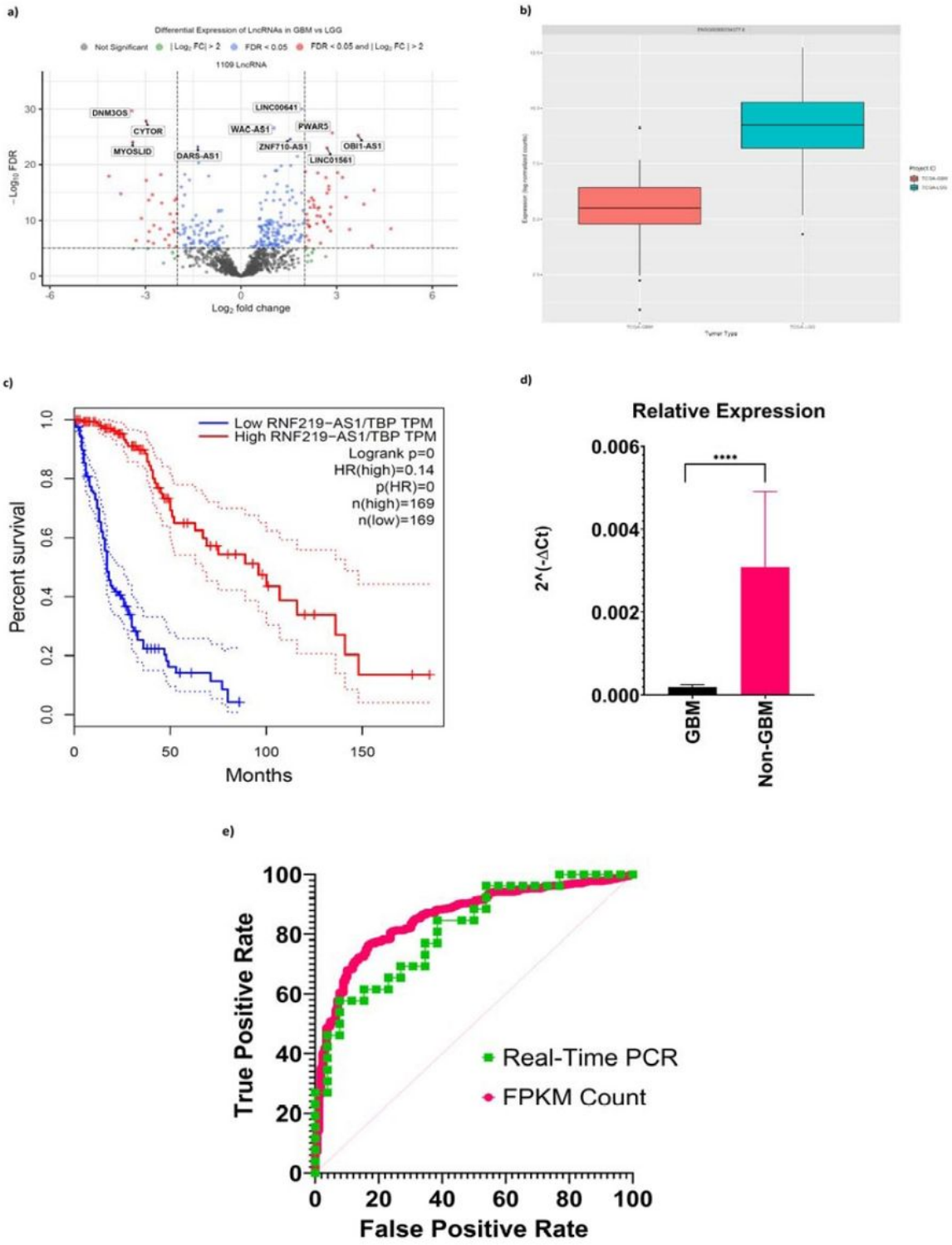

Figure 1

a) In this volcano plot, 1,109 differentially expressed LncRNAs in GBM compared to LGG are presented. The names of the top 10 with the least FDR are displayed in the figure with OBI1-AS1 having the highest |Log2FC|.b) RNA-Seq Analysis of 120 glioma samples reveals decrease of OBI1-AS1 expression in GBM compared to LGG by Log2FC= -3.67. c) Based on survival analysis of 338 glioma patients (169 in the GBM and 169 in the LGG group), those with low expression of OBI1-AS1 exhibit significantly poor

prognosis with Hazard Ratio (HR)= 0.14. d) Real-Time PCR results confirm our DEA findings. As is presented, OBI1-AS1 is overexpressed in non-GBM glioma samples by log2FC = -3.54 which is in accordance with our RNA-Seq data analysis findings. e) The ROC curve illustrates AUCs equal with 81% and 85% for Real-Time PCR and RNA seq data, respectively, with 52 samples (26 for GBM and 26 for LGG) for Real-Time PCR and 698 for RNA-seq investigation. These AUCs also highlight the agreement between RNA-seq and Real-Time PCR findings. Note that ENSG00000234377.6 and RNF219-AS1 both refer to OBI1-AS1.

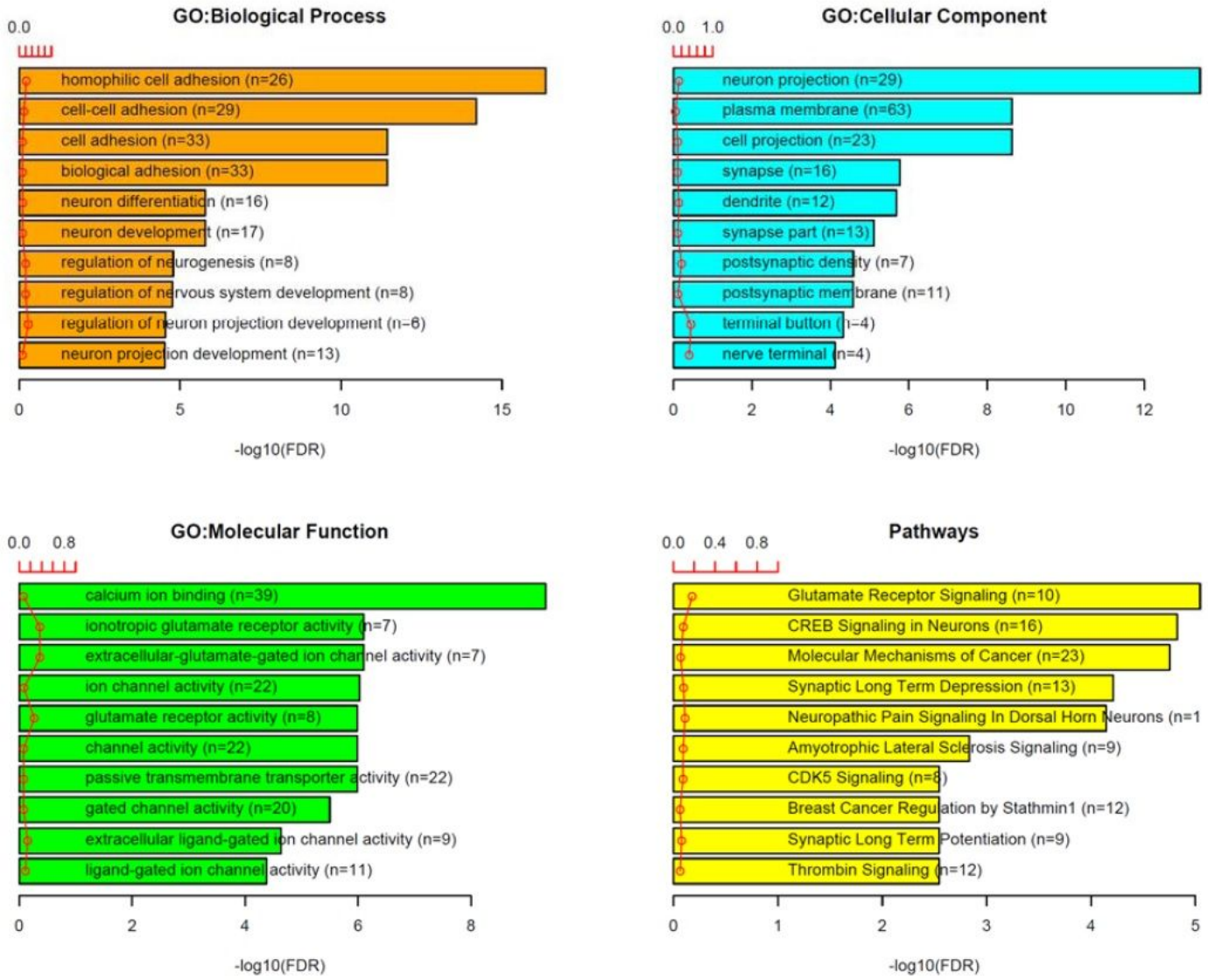

Figure 2

Gene Ontology and pathway enrichment analysis of OBI1-AS1 co-expressed genes. Most of these genes have been attributed to cell adhesion and neuron differentiation. Their locations are shown to mainly include plasma membrane in synaptic parts. GO: Molecular Function shows that the bulk of the genes have a binding site for calcium or are implicated in regulation of ion channel activity and response to glutamate. In addition, pathway enrichment analysis reveals that the majority of these genes are involved

in glutamate and CREB signaling which are the foremost pathways in neural differentiation and glioma progression. Other significant pathways are shown to be pertinent to cancer and synaptic signaling which is in reasonable compliance with our GO terms.

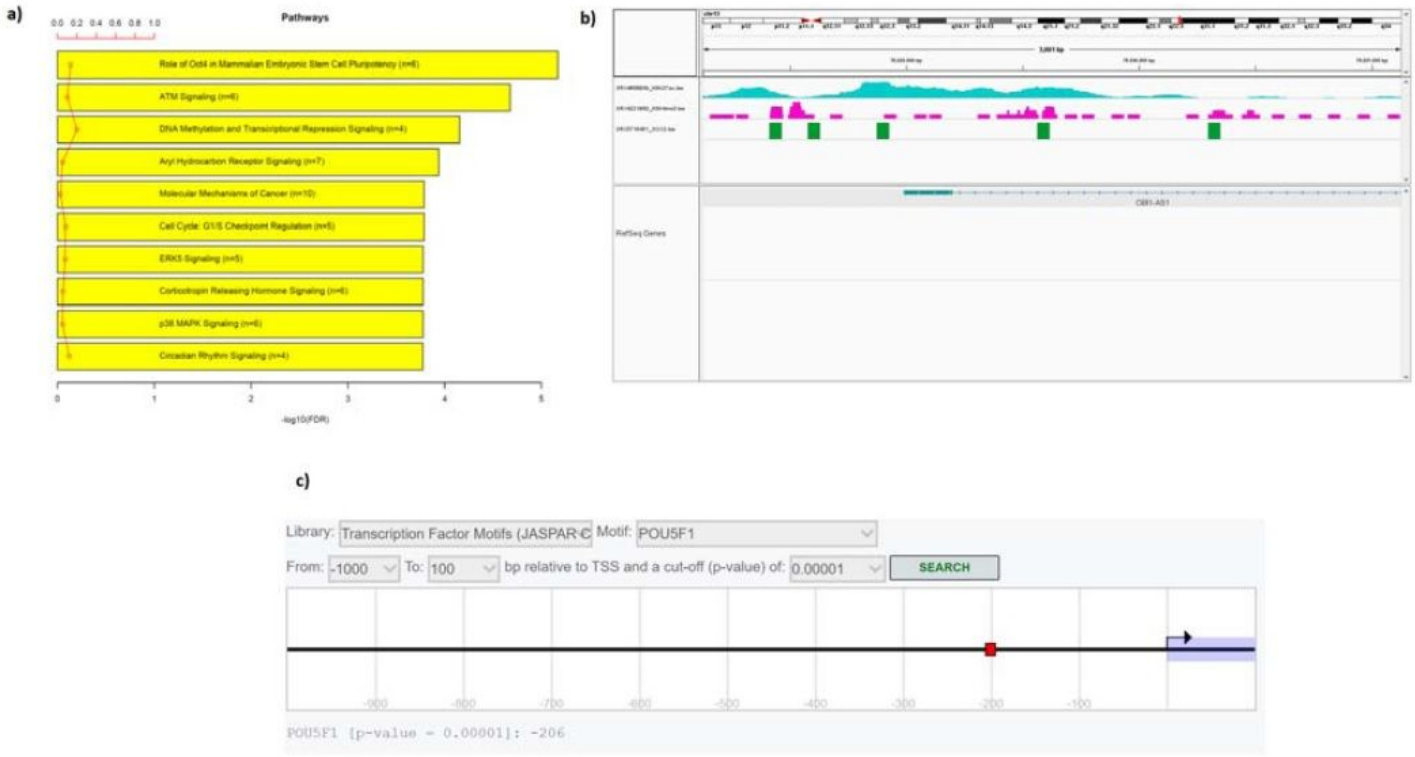

Figure 3

a) Transcription factor enrichment analysis pinpoints engagement of the preponderance of the proteins binding to OBI1-AS1 transcription start site in the OCT4 pathway to regulate pluripotency in stem cells. b) H3K27ac and H3K4me3 are the two principal histone modifications notably observed in the proximal region of OBI1-AS1 TSS. Sox2, chief partner of Oct4, binds to this region strongly. c) in-silico analysis has predicted the existence of a strong binding site for Oct4 206 nucleotides upstream OBI1-AS1.

Supplementary Files

This is a list of supplementary files associated with this preprint. Click to download.

- FileS1.csv
- FileS2.csv