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OSMR Has Potential Functional Significance in Mitochondrial Dynamics that affects Tumor Progression in Glioblastoma Multiforme

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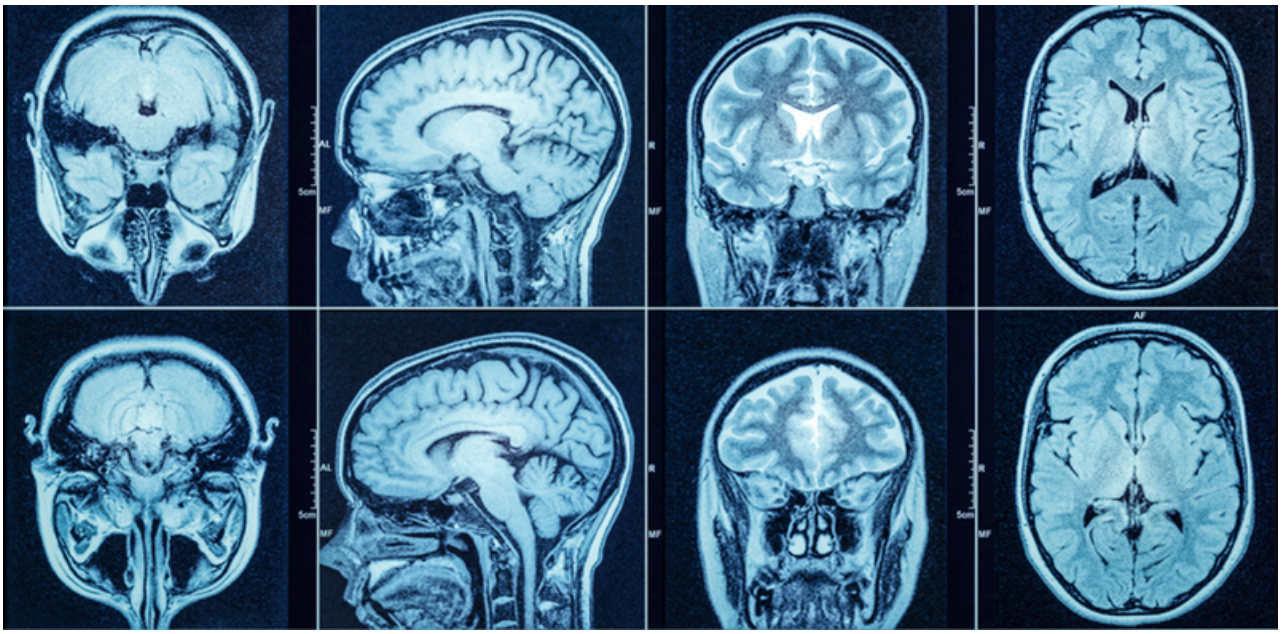


Abstract

Background: Glioblastoma Multiforme (GBM) is a type of glioma that has the characteristic of rapid growth. While the treatment for GBM is aggressive, the tumor carries a dismal prognosis. One difficult aspect of treating GBM is the blood brain barrier, often rendering therapeutic agent delivery ineffective. Conversely, gene therapy portends promise. Previously, it was discovered that high expression of the EGFRvIII gene, often co-expressed with EGFR in glioma cells, correlates to low survival probability. Moreover, the OSMR gene forms a coreceptor complex with EGFRvIII but has been found to function independently of EGFRvIII in maintaining

mitochondrial respiration. Finally, unbalanced mitochondrial dynamics has been linked to tumor growth in glioblastoma. The role of OSMR in affecting the mitochondrial respiration process and tumor progression in GBM is not extensively discussed and requires investigation.

Methods: The prognostic value of OSMR for GBM was evaluated with a KM plotter from the UALCAN dataset and confirmed by the TCGA samples. Cell cultures GAMG, U-138 MG, U-251 MG, U-87 MG and human BTSC lines 112, 145, and 172 harvested were used. Localization of OSMR to the mitochondria was validated by cell fractionation and fluorescent microscopy. Finally, correlation in expression specific to GBM between OSMR and BHLHE40 as suggested by Pearson-CC graph would be confirmed by Co-IP assay and WGCNA.

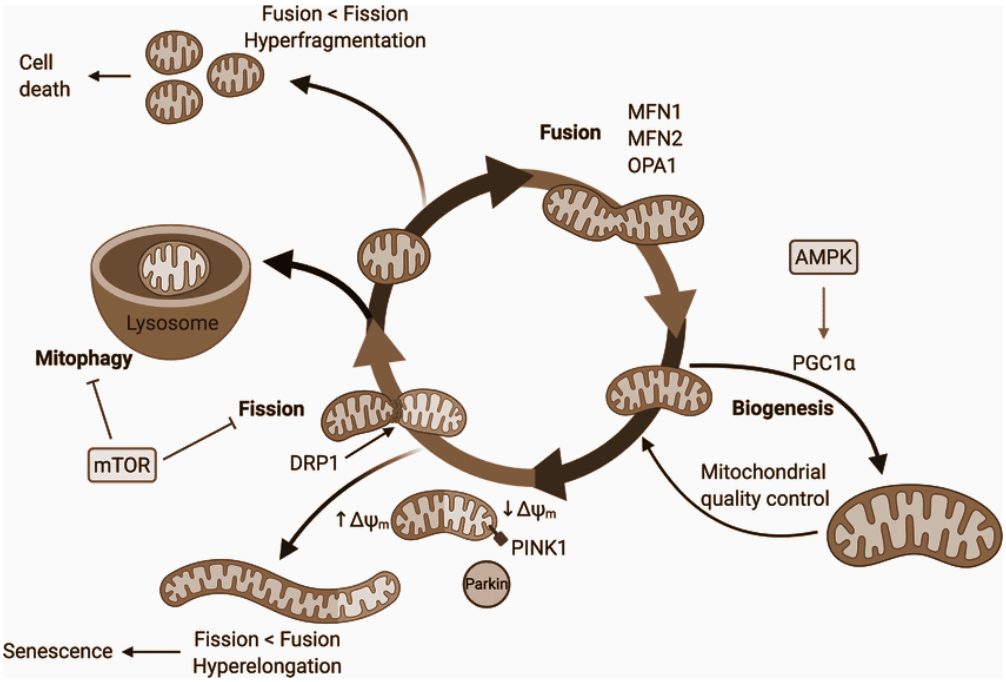
Results: The OSMR gene is strongly expressed in GBM, and there are distinct associations between OSMR expression and prognosis in GBM patients. OSMR is localized to the mitochondria in BTSCs and developed GBM cells. Finally, OSMR would interact independently within the signaling pathway of BHLHE40 in controlling the mitochondrial dynamics process, fueling tumor growth in glioblastoma multiforme through an upregulation process.

Introduction

Glioblastoma Multiforme (GBM) is a fast-growing and aggressive brain tumor. It is often invasive to nearby brain tissue but does not metastasize to distant organs (*Glioblastoma Multiforme – Symptoms, Diagnosis and Treatment Options*, n.d.). GBM is the most malignant primary tumor in adults and its many characteristics, including the presence of BBB, an immunosuppressive tumor microenvironment, and drug resistance deter effective treatment (*Current Approaches for Glioma Gene Therapy and Virotherapy*, 2021). More than 13,000 Americans are expected to receive a GBM diagnosis in 2022 and the cancer accounts for 49.1 percent of all primary malignant brain tumors (*About GBM - GBM Awareness Day*, n.d.). Gene Therapy is a novel solution to treat GBM, and understanding the mechanism by which genes are attributed to cancer hallmarks could improve therapeutic approaches.

In GBM, rare brain tumor cancer stem cells (BTSCs) are responsible for therapeutic resistance and tumor growth (*Gene Targeting Helps Overcome the Resistance of Brain Cancer to Therapy*, 2020). BTSCs can undergo self-renewal to replenish themselves and in glioblastoma tumors, destroy the functional heterogeneity of cells. Metabolic reprogramming is a characteristic hallmark of cancer growth, and such activity is present in BTSCs in the form of an upregulation in oxidative phosphorylation and low glycolytic rate.

The OSMR gene provides instructions for making a protein called oncostatin M receptor beta subunit (OSMRβ). The receptors formed, embed in the cell membrane of many types of cells in the body and function in cell signaling. Some of its role is not completely understood. Previously, it has been determined that the OSMR gene plays a role in fortifying brain tumor stem cells' resistance to therapy by translocating to and strengthening the mitochondria. However, from OSMR's close connection to the EGFRvIII gene, there is significance to investigate the OSMR gene's role in affecting the mitochondrial dynamics process, potentially via interacting with proteins involved at the site of the mitochondria in GBM.



Reference Figure | Mitochondrial Dynamics

Mitochondrial dynamics involve the process of fission and fusion. mtFusion occurs when two adjacent mitochondria join, while fission separates one mitochondrion into two. Under normal conditions, these two events counterbalance each other (*Protocol For Immunoprecipitation*, 2017). The fission and fusion processes are connected to cell cycle regulation, quality control, and transmission of energy status. In human cancers, mitochondrial morphology and ROS levels are closely related and parallel changes are found in patient primary fibroblasts. ROS participates in signaling pathways linked to cancer cell proliferation, apoptosis resistance, and cancer stem cell maintenance. Research on mitochondrial dynamics in GBM indicates that Drp1 activation is found to relate to poor prognosis in glioblastoma where the promotion of Drp1-dependent fission by the NF-κB-inducing kinase leads to tumor growth **(Table 1)**. Current understanding of mitochondrial

dynamics demonstrates that developing inhibitors against mitochondrial fusion and fission proteins is a promising strategy to overcome the drug resistance and metastasis of cancers such as GBM.

Drp1's regulation of brain tumor-initiating cells along the downstream metabolic stress sensor AMPK has been well studied, but the cell signaling upstream is to be investigated. This study investigates the potential for the OSMR gene and the BHLHE40 gene to participate in the upregulation of said fission process upstream of the Drp1 protein. Such findings could contribute to more design opportunities for therapeutic approaches to suppress GBM development. The implication would be that inhibition of OSMR diminishes the metabolic properties of the tumor stem cells, and in turn leading to favorable prognosis.

Materials and Methods

Datasets and Data Processing

TCGA data Glioblastoma Multiforme expression profile, mutation, and clinical data. Data of protein interaction, localization, pathology on EGFR and OSMR gene from the Human Protein Atlas. Prognostic value analyzed, controlling for patient age, tumor grade, STAT3 expression, and IDH1 status.

Cell Culture

GAMG, U-138 MG, U-251 MG, U-87 MG are human glioblastoma cancer cell lines from Uppsala University, Sweden (*Brain Related Cell Lines*, n.d.). The human BTSC lines 112, 145, and 172 were provided by Harvard Medical school. The BTSC cell lines were characterized for mutations in EGFRvIII, p53, PTEN, and IDH1. Prior to use, all cell cultures were recovered from cryopreservation in 10% dimethyl sulfoxide and cultured in Nunc ultra-low attachment flasks as neurospheres in NeuroCult NS-A medium supplemented with 100 U/mL penicillin, 100 μg/mL strep- tomycin, heparin, human EGF, and Human FGF.

Survival Analysis

To explore whether expression level of the OSMR gene is related to prognosis in GBM, the cancer sample was divided into two groups of high and low expression. The KM curve plotted by the prognostic value of OSMR in GBM was evaluated. TCGA samples were analyzed from UALCAN, and expression level compared based on sample size.

GO Enrichment Analysis

Pan cancer was analyzed by TIMER2 (tumor immune estimation resource, version 2) web (http://timer.cistrome.org/) and observed the expression difference of OSMR between tumor and adjacent normal tissues for different tumors.

Cell Fractionation

BTSCs were washed with 0.9% sodium chloride solution and resuspended in a lysis buffer. Tissues were homogenized using the TissueRuptor rotor-stator homogenizer. Tissues and cells were incubated at 4 °C for 10 min on a shaker and were subjected to centrifugation (1000 g, 10 min, 4 °C). The supernatants containing cytosolic proteins were carefully removed. The pellets were resuspended in a disruption buffer and were subjected to centrifugation (1000 g, 10 min, 4 °C). The pellets containing nuclei were resuspended in RIPA lysis buffer and the supernatants were centrifuged at 6000 g (10 min, 4 °C) to obtain the mitochondrial fraction. Protein concentration of mitochondrial, cytosolic, and nuclei lysates was determined using the Bradford assay (*Protocol For Immunoprecipitation*, 2017).

Fluorescence Microscopy

Following protocol from ThermoFisher Scientific, DAPI was prepared to stain the nuclei region of the GBM human cultures GAMG and U-138 MG. Cell lines were washed 3 times in PBS. 300 nM DAPI stain solution added to cover the cells. Incubated for 1-5 minutes, protected from light. Stain solution was then removed, and cells washed with PBS. Mitochondrial matrix protein ATPIF1 stain and antigen to OSMR applied to cell culture with corresponding protocol (*DAPI Protocol for Fluorescence Imaging*, n.d.).

Coimmunoprecipitation Assay

To identify potential unknown protein complexes in the mitochondrial fission process, coimmunoprecipitation assay is used on OSMR, BHLHE40, and Drp1. GBM cultured cells were pe-chilled PBS for 2 times and added in a cold RIPA lysis buffer. The tissues were then centrifuged at 14,000 g 4 °C for 15min, 50% protein A/G agarose with ratio of 100µl for 1 ml were added to the sample solution. Protein A/G agarose discarded. Total protein quantified with BCA assay, diluted to 1 µg/µl with PBS. Appropriate amount of primary antibody added. Antigen-antibody

complex shaken on rotating shaker at 4 °C overnight. Prior to supernatant collected, it was washed with a pre-chilled washing buffer for 3 times.

Weighted Gene Co-expression Network Analysis

Weighted gene co-expression network analysis (WGCNA) was used to construct a scale-free network using OSMR and BHLHE40 gene expression data. Experiments were conducted in accordance with the criteria of outlier samples detection and appropriate soft threshold power selection. Module identification procedures included topological overlap matrix (TOM) formation representing adjacency, hierarchical gene clustering with a deep-split value of 2, a minimum size cutoff of 30, and similar modules merging with a height cutoff of 0.4. All procedures were computed with the module preservation function implemented in the WGCNA package (*Systematic Transcriptome*, 2020).

Results

OSMR Has Prognostic Value in GBM and There is Clinical Correlation

To determine the functional role of OSMR in carcinogenesis and tumor progression in GBM, we must first analyze the expression level and prognostic value of the gene. The TIMER database shows the expression of OSMR in 37 cancer types **(Figure 1a)**. Notably, OSMR is overexpressed in 9 cancers compared to normal samples including CESC, CHOL, ESCA, GBM, HNSC, KIRC, KIRP, LUAD, STAD. Analysis of the KM curve from the UALCAN dataset showed higher expression level of OSMR proportional to decreased probability of survival compared to low/medium expression level (tumor immune estimation resource, version 2) web (http://timer.cistrome.org/) **(Figure 1b)**. Finally, in comparing the expression of OSMR in normal and primary GBM tumor tissues from the box plot of **Figure 1c**, OSMR is found to be significantly overexpressed in the cancerous tissues. There is an indication of correlation between the expression of this gene and the prognostics of patients (UALCAN, 2022).

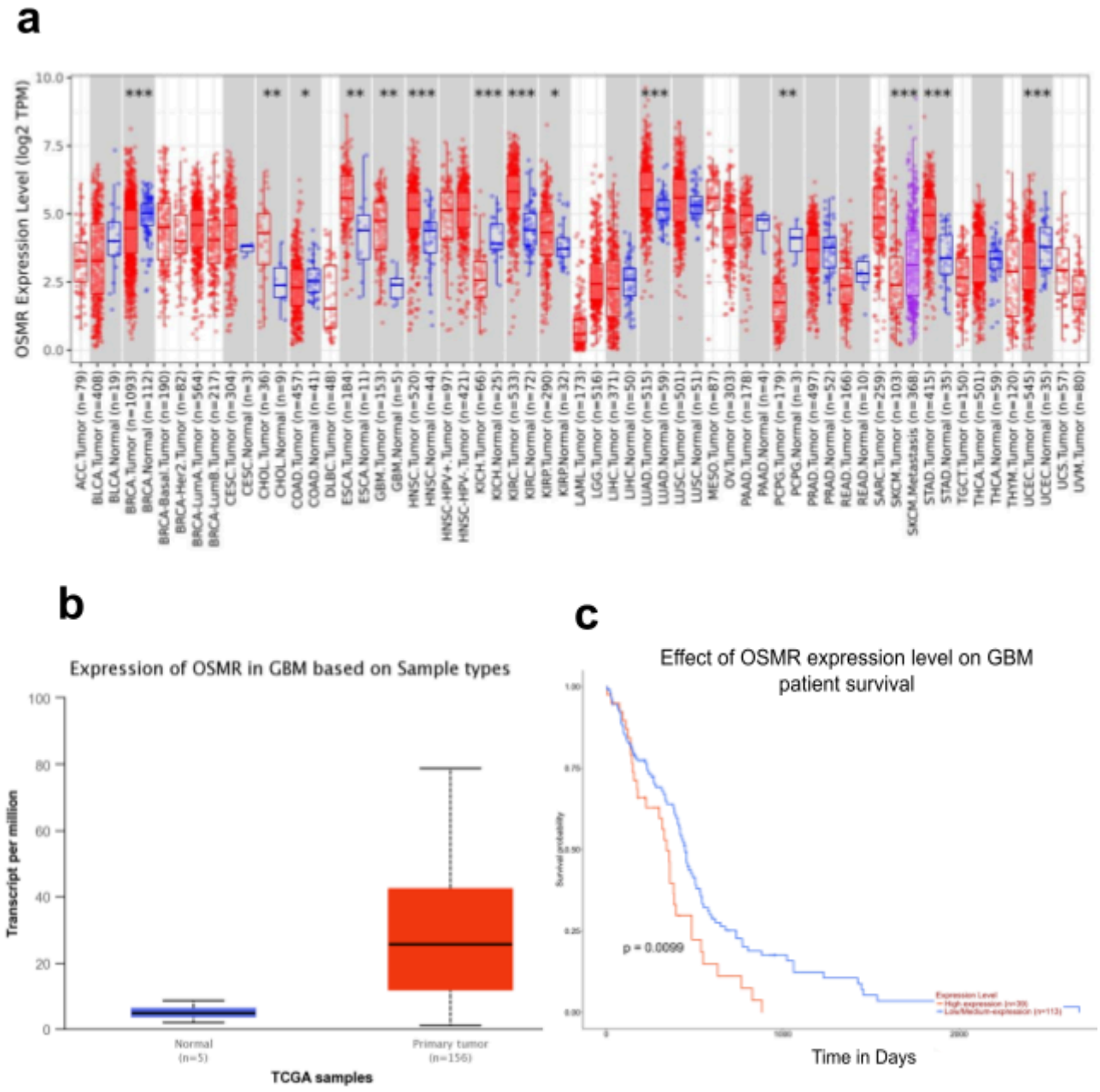


FIGURE 1 | OSMR was up-regulated in pan-cancer and has prognostic value in GBM **a.** The expression of OSMR in pan-cancer from TCGA **b.** Expression of OSMR in normal and tumor samples **c.** Survival probability over time in days for high and low/medium expression levels of OSMR *p value < 0.01.

OSMR Has Localization to the Mitochondria in GBM Tumor Stem Cells

To gain insight into the interaction of OSMR with the mitochondrial dynamics process, we characterized the role of OSMR in metabolic pathways and identified the localization of OSMR. Since it is known that cytokine receptors for OSMR regulate proliferation of a population of brain tumor stem cells (BTSCs) in GBM tumorigenesis (Sharanek et al., 2020), we presumed the involvement of OSMR in the metabolic process of glioblastoma; BTSCs have the phenotype of metabolic shift to aerobic glycolysis. To determine the functional significance of OSMR in the mitochondrial dynamics process particularly, we would first validate the presence of OSMR at the mitochondria with cell fractionation on the GAMG, U-138 MG, U-251 MG, U-87 MG cell lines **(Figure 2)**. The results are expected to demonstrate that OSMR is present at the mitochondria independent of its presence in other cell structures including the nuclei and cytoplasm. This finding could be further evidence of OSMR's independent function at the mitochondria.

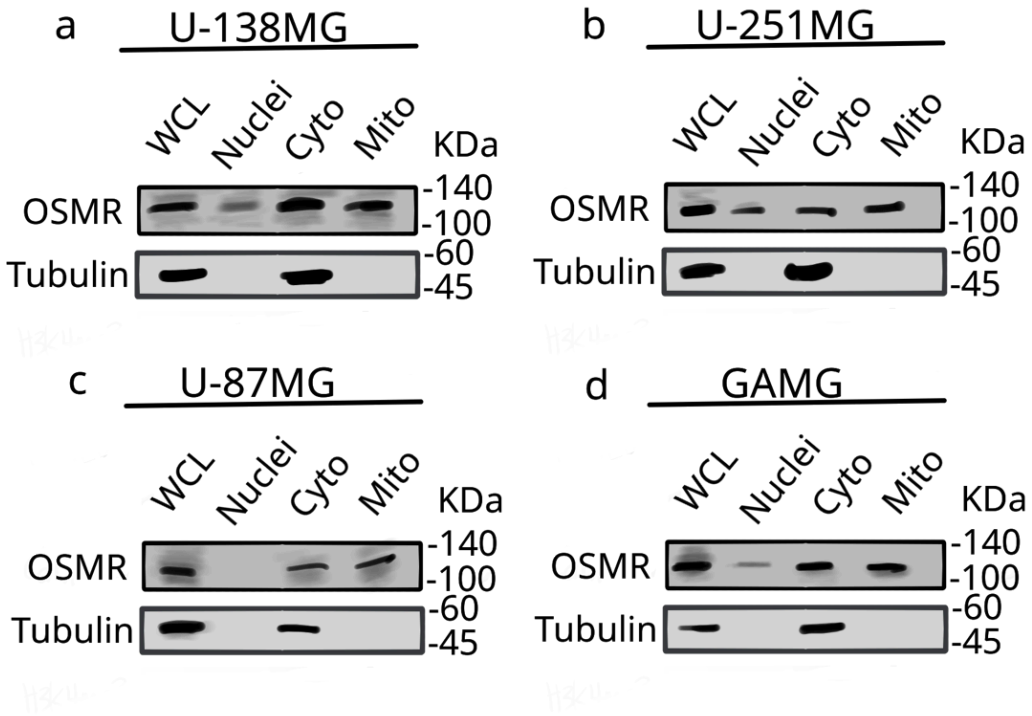


FIGURE 2 | OSMR is present at the mitochondria independent of other cellular structures. This is only a hypothetical figure of the result expected from a cell fractionation test. Cell lines used are

from glioblastoma patients. All cultures show OSMR presence in the mitochondria. The other genes presented are only for reference and do not present an accurate reflection of the results.

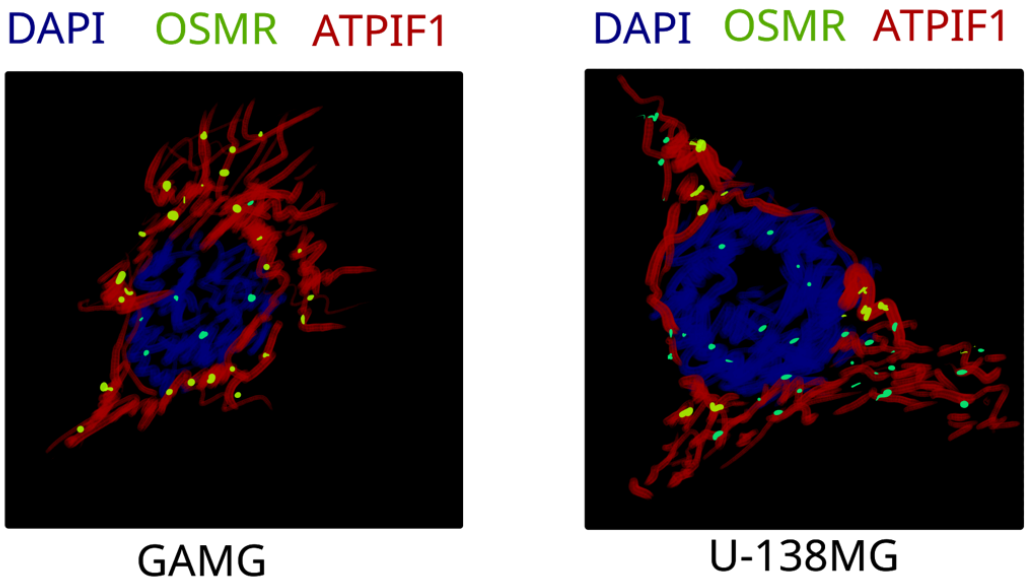


FIGURE 3 | Fluorescent microscopy confirms OSMR localization to mitochondria in GBM cell lines. Hypothesized results from the fluorescent microscopy. Antigens to OSMR (Green). Mitochondrial matrix protein ATPIF1 (Red). Nuclei stained with DAPI.

Ensuing the experiment, we would corroborate the localization of the gene with fluorescence microscopy using the patient derived GBM cell lines. The imaging is expected to indicate some presence of OMSR at the mitochondrial sites **(Figure 3).** The confirmed presence of OSMR in GBM cell lines at the mitochondria is evidence of the gene's potential functional importance in the mitochondria dynamics process. This would support the gene's role in respiration and its metabolic function in GBM propagation.

OSMR Expression is Positively Correlated to BHLHE40 in GBM

From studying the UCALN database on OSMR correlation, we noticed the BHLHE40 gene that is positively correlated to OSMR in GBM with a Pearson-CC of 0.68 **(Figure 4a).** BHLHE40 functions as a transcription factor and it could profoundly influence the biogenesis and metabolic activities of the mitochondria (Chang et al., 2018). This could be an implication of OSMR's function in regulating the metabolic pathway of GBM and co-operating with regulatory genes to

affect the proteins involved in the mitochondrial dynamics process. However, a signal transduction pathway as such is not currently elucidated and requires further study.

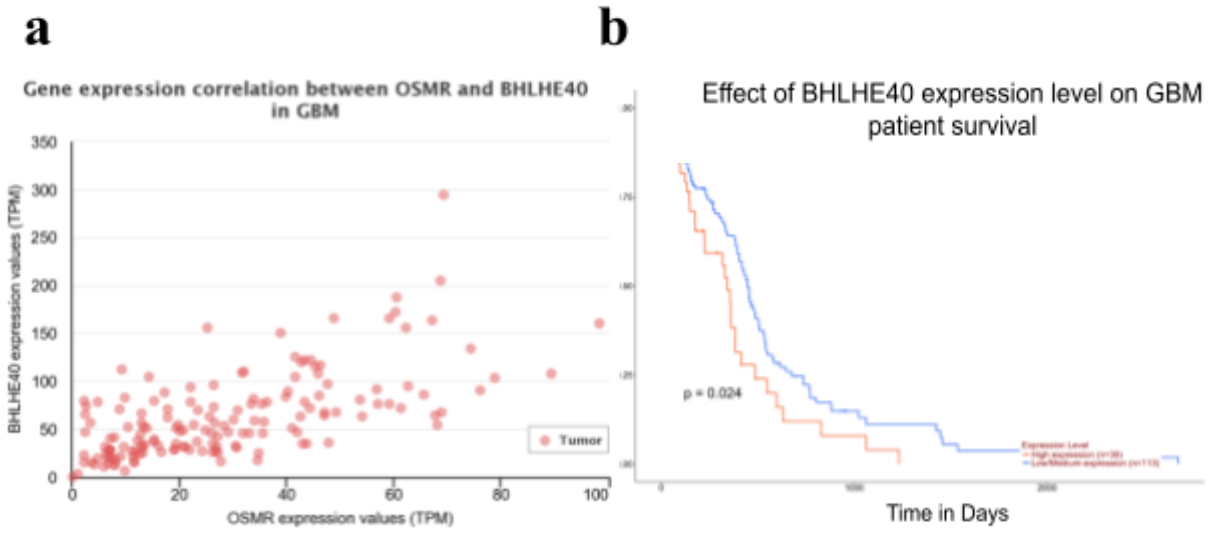
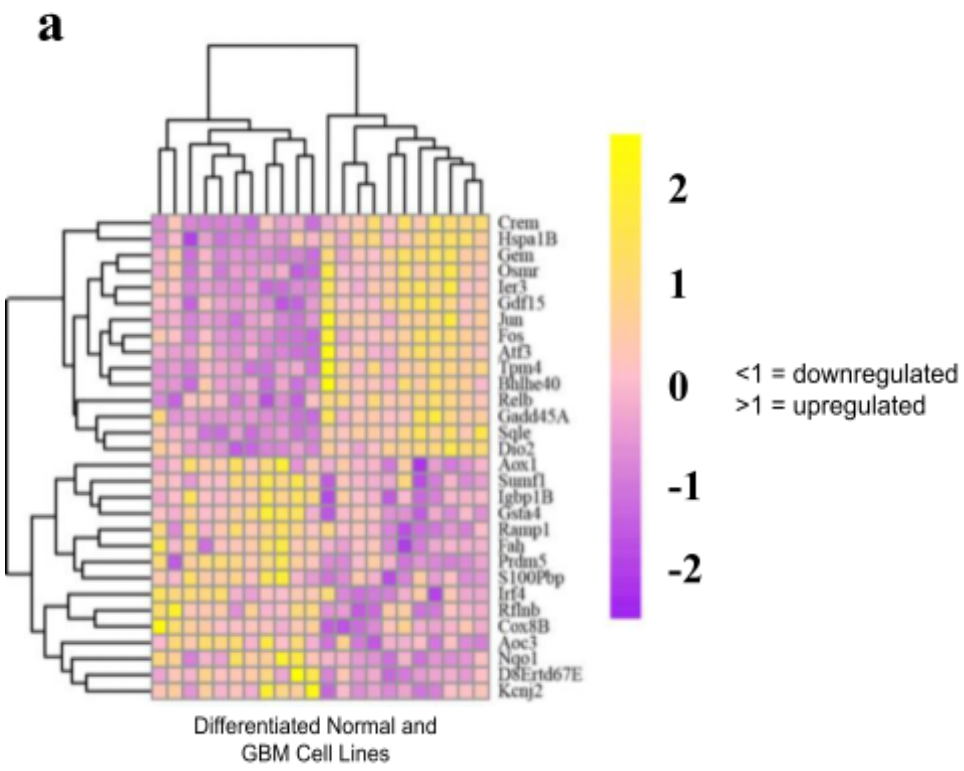


FIGURE 4 | OSMR and BHLHE40 expression correlate in GBM **a.** Plot of Pearson-CC graph shows BHLHE40 as a gene positively correlated to OSMR. **b.** Survival probability over time in days for high and low/medium expression levels of BHLHE40.

OSMR and BHLHE40 Function in The Same Signaling Pathway that Affects Metabolism in GBM

Finally, to investigate the mechanism by which OSMR and BHLHE40 potentially interact to disrupt the mitochondrial dynamics process in GBM, we must test the protein-nucleic acid interactions of the two genes with coimmunoprecipitation assay. We expect OSMR to be precipitated by BHLHE40 **(Figure 5b)**, indicating a potential for the two genes to affect the mitochondrial dynamics mechanism within the same signaling pathway. To further investigate the correlation in expression between OSMR and BHLHE40 in BTSCs, we would perform a weighted gene co-expression network analysis of OSMR and BHLHE40 from an available database. Results expected from the WGCNA would reveal similarities in upregulation and downregulation patterns among the two genes of interest **(Figure 5a)**. Such correlation could be the basis for the dual role of OSMR and BHLHE40 in controlling the metabolic changes to GBM tumor stem cells.



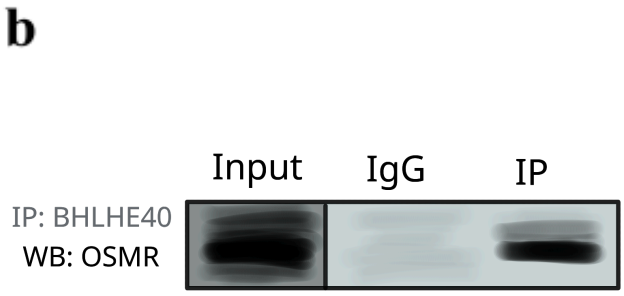


FIGURE 5| BHLHE40 and OSMR function in the same signaling pathway **a.** Computer generated heat map from WGCNA results expected to show OSMR and BHLHE40 with similar expression patterns in Normal and GBM human cell lines. **b.** Co-IP results expected indicate OSMR to precipitate with BHLHE40.

Discussion

In this study, we found that OSMR is overexpressed in glioblastoma multiforme and a correlation between an upregulation in OSMR and poor GBM prognostics. This finding ascertains the clinical value of OSMR and justifies further investigation into the cellular mechanism controlled by the gene. To further analyze the potential functional role of OSMR, we investigated the gene localization in both available GBM cell lines and their composition of brain tumor stem cell lines in which OSMR has been found to have a role in metabolic process maintenance. Our study would reveal that OSMR is localized to the mitochondria independent of its presence at other locations. In studying the GBM-specific data on OSMR, we found an apparent positive expression correlation between OSMR and BHLHE40, a gene previously found to have functional significance in the mitochondrial dynamics process. To corroborate this finding, we expect a WGCNA test on co-expression patterns and a co-immunoprecipitation assay on the two genes to indicate interaction.

In context of the discussion around the study of mitochondrial dynamic dysregulation in GBM, our study offers potential candidate genes of interest in the upstream signaling for Drp1 activation. Since mitochondrial fission, facilitated by Drp1, has been a primary effector in GBM tumorigenesis, influencing the malignant growth characteristic of cancer by maintaining metabolic capacity, our study has potential significance for future gene therapy targets.

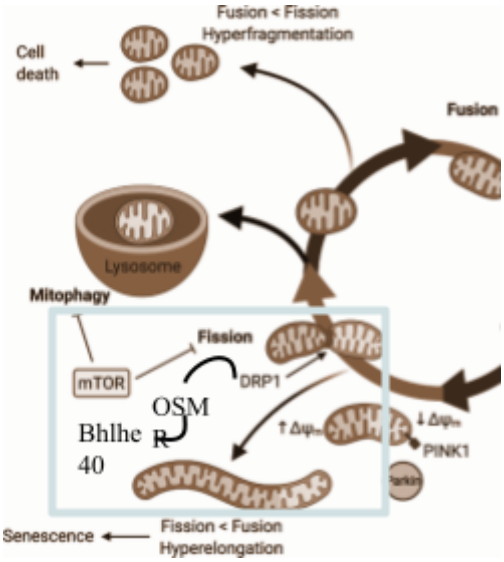


Figure 5 | Suggested diagram incorporating OSMR and BHLHE40 to the current understanding of mitochondrial dynamics within GBM

The potential of OSMR to modify the metabolic pathway in GBM has been corroborated by other evidence and previous studies, for instance, the EGFRvIII gene. The EGFRvIII gene has been studied to alter glioblastoma metabolism through interacting with the mitochondria (*Gene Targeting Helps Overcome the Resistance of Brain Cancer to Therapy*, 2020). Moreover, EGFRvIII and OSMR commonly form a coreceptor complex. Conversely, it is known that OSMR has an independent function to EGFRvIII. Based on OSMR's significant prognosis data regarding GBM, its localization at the mitochondria, and the functional role of EGFRvIII in the metabolic process of GBM progression, we propose the potential for OSMR to function in the mitochondrial dynamics of GBM.

From the current studies into the mitochondrial dynamics, results show that human cancers are often connected to the dysregulation of said process. In GBM for instance, tumor growth in glioblastoma has been attributed to the promotion of Drp1, which is involved in the mitochondrial fission process (Liu et al., 2020). Changes to the mitochondria affects the metabolic activity in GBM, potentially initiating the shift to aerobic glycolysis, affecting metastasis, drug resistance, and stem cell survival. The signal transduction pathways upstream of Drp1 is not extensively researched and we hypothesize that OSMR is involved in disrupting the mitochondrial dynamics with an upregulation in fission. This then affects tumorigenesis in GBM. One way we could test this hypothesis is through co immunoprecipitation assays on OSMR and Drp1, BHLHE40 and Drp1, and OSMR and BHLHE40. The results, if positive in any way, could further elucidate the potential functional significance of OSMR.

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