RETRACTED: REGULATION OF CELL GROWTH AND KI-67 EXPRESSION BY GHRELIN: ANALYSIS OF PRIMARY BRAIN TUMOR CELL LINES [KJMS April – June 2025, Volume 18, No. 2 DOI: https://doi.org/10.70520/kjms.v18i2.645]

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This article has been retracted.

The article has been retracted at the request of the corresponding author due to concerns regarding the authenticity and integrity of the data. These concerns include 'data fabrication or manipulation''. Following COPE (Committee on Publication Ethics) guidelines, the journal has retracted the article to maintain the integrity of the scientific record.

ABSTRACT

Objective: The current study aimed to determine the proliferative effect of ghrelin on primary brain tumor cell lines.

Methodology: A laboratory-based experimental study was designed and performed in the Department of Anatomy, Khyber Medical University, Peshawar. Three Primary brain tumor cells i.e., glioma, glioblastoma, and meningioma, samples were obtained from patients after craniotomy. Cell lines (IK148, IK155, and IK169) were established and treated with ghrelin (0-400nM). Cell migration and proliferation were assessed through wound closure assays while Ki-67 expression was analyzed using immunofluorescence.

Results: Treatment with 20 nM ghrelin significantly enhanced tumor cell proliferation, with complete wound closure observed within 72 hours in the scratch assay. Higher ghrelin concentrations (>20nM) showed reduced migration, with incomplete wound closure even after 6 days. Immunofluorescence analysis demonstrated that cells treated with 20nM ghrelin exhibited markedly elevated Ki-67 expression, indicating enhanced proliferation, while those exposed to 50nM ghrelin showed minimal Ki-67 expression."

Conclusion: Ghrelin hormone has a proliferative effect on brain tumor cells in low concentration (20 nM). Higher concentrations of ghrelin up to 50nM have anti-proliferative effects.

Keywords: Primary brain tumor, Glioma, meningioma, ghrelin, Ki-67.

INTRODUCTION

A brain tumor is a type of solid tumor in which abnormal cells or mass accumulate in brain tissues. The primary malignant type of brain tumor is more prevalent in men than women. The annual global prevalence of brain tumors is 3.7 per 100,000 for men and 2.6 per 100,000 for women. The incidence is more frequent in Western countries than developing nations (1). Brain tumors in all forms represent a group of feared tumors that lead to the death of a patient within 2 years after its diagnosis.

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Glioblastomas are the most severe and aggressive type of brain tumor. Approximately 5% per 100,000 population around the globe is affected by gliomas, and no exact cause has been defined to date while ionizing radiation exposure is the only well-understood risk factor. About 5% of glioma patients have a family history of primary brain tumors (2). Glioblastomas are the most proliferative type of primary brain tumors. The invasion of tumor cells to nearby healthy brain tissues involves poorly understood complex neurobiological

mechanisms (3). According to the Pakistan Brain Tumor Epidemiology Study (PBTES), a total of 2,750 patients in Pakistan were diagnosed with various types of brain tumors, with gliomas being the most common, accounting for 28.29% of cases (4). The treatment and management of tumors usually involve three types of interventions, i.e., surgery, radiotherapy, and chemotherapy. The exact cause and onset of these tumors are still unknown, but research studies have shown the role of different environmental and genetic factors in the formation of brain tumors. Permanent mutations in DNA lead to genomic instability, which results in more devastating events such as changes in chromosome numbers in new cells, clonal evolution, and cancer genomic diversity (5). These genomic instabilities can range from single nucleotide polymorphism to whole chromosome transformation. Similarly, the effect of hormones, especially sex hormones, on brain tumors has been extensively studied (6).

Ghrelin is a hormone discovered in 1999, consisting of 28 amino acids, secreted from the stomach, intestine, and pancreas, and a small amount is released from brain tissues in the hypothalamus and pituitary gland. Ghrelin, with the leptin hormone, acts as a powerful appetite stimulant. The concentration of ghrelin is at its peak before taking a meal and at night, which suddenly falls after taking a meal. This suggests the role of ghrelin in food ingestion. The release of ghrelin stimulates the release of growth hormone from the pituitary gland (7). It is the first known stimulatory effect of ghrelin on releasing growth hormone. Ghrelin stimulates the neurons in the arcuate nucleus in the brain, from where growth hormone-releasing hormones (GHRH) are released. Thus, the concentration of growth hormone in serum is increased. Ghrelin is involved in the reduction of blood pressure without reducing heart rate. This physiological action is achieved by a direct vasodilator effect on blood vessels. Ghrelin receptors are also found in kidneys, where these receptors cause sodium and water excretion, i.e., diuresis. This further causes a reduction in blood pressure (8). Ghrelin receptors are expressed in bones, especially in osteoblast cells, which increase osteoblast cell proliferation and bone mass density. According to studies, ghrelin suppresses the sympathetic nervous system, which has deleterious effects on bone mass, and it is independent of food intake. The effects of ghrelin on insulin release are controversial, i.e., previous studies have shown that ghrelin promotes insulin secretion from the pancreas (9). Previously, the proliferative effect of ghrelin on some cancer cell lines, such as pituitary, cardiac, liver, prostate, adrenal, and adipose cells, has been established. Its association with cell proliferation and the stimulatory influence on growth hormone secretion indicate that ghrelin is involved in the growth of tumors. Ghrelin expression is also observed in cancer metastasis, which suggests the role of ghrelin in cancer progression in different body organs and cancer metastasis (10).

The Ki-67 protein exists in all active stages of the cell cycle (G1, S, G2, and mitosis); however, it is lacking in inactive cells (G0), making it an ideal marker for cell proliferation. Cells having the highest percentage of Ki-67 were observed in one choroid plexus carcinoma, one primary melanoma of meninges, three medulloblastomas, one anaplastic astrocytoma, and six different types of glioblastomas (11). In another study on anaplastic gliomas, the Ki-67 antibody expression was higher in anaplastic mixed gliomas (14.2%) as compared to anaplastic astrocytomas (8.6%) (12). However, the exact role of Ki-67 protein in early diagnosis of brain tumors may require further research, as previous studies have shown controversial results on the association between Ki-67 protein and cancer metastasis. The primary objectives of the current study were to analyze the effect of ghrelin on primary cell lines of brain tumors and assess the proliferative effects of ghrelin using Ki-67 biomarkers.

MATERIAL AND METHODS

This experimental lab-based study was done at the Department of Anatomy, Khyber Medical University, Peshawar, from 1st January 2022 to 31st December 2022. For our study, we prepared tumor cell lines from three different nervous system tumor samples: glioma, glioblastoma, and meningioma. These samples were obtained after craniotomy through informed consent from the patient or their attendants. Ethical approval was obtained from the Ethical Committee of the Khyber Medical University (Ethical approval No: DIR/KMU/EB/PG/000822). Cell lines, i.e., IK148 (glioma), IK155 (glioblastoma) and IK169 (meningioma) from 3 patients were obtained by simple random sampling technique. The inclusion criteria were primary brain tumors with no patient's age gender limitations while the tumors other than brain tumor and metastatic tumors were excluded from the study. Tumor samples were collected from department of Neurosurgery, Hayatabad Medical Complex Peshawar after informed consent from patient's attendant. Demographics & disease details

including all pre-operative investigations and radiological Investigations reports were recorded on a separate excel sheet. A sterilize sample bottle, EDTA tube and syringe were provided for collection of tumor sample and blood respectively. Following the craniotomy, the dry tumor samples were immediately transported to the laboratory within 15 minutes in an ice box. Upon arrival, the samples were sterilized with 70% ethanol, placed in petri-dishes under a flow hood, and each sample was carefully divided into 04 portions; three portions were saved in cryovials for further studies. Remaining portion was washed first with Phosphate Buffer Saline (PBS) and then with Hank's Balanced Salt Solution (HBSS) and it was dissociated into small pieces. Collagenase enzyme was added to the sample and transferred to falcon tube and placed in a shaking water bath for complete enzymatic dissociation. The samples were then centrifuged at maximum speed for 5 minutes. The supernatant was discarded and the pellet media was transferred to labelled T25 flask for cell culture. T25 flask was placed in carbon dioxide incubator (5% CO_2, 37°C, 95% humidity) and were checked daily under microscope to assess the growth of cells.

To assess the effects of ghrelin on tumor cells lines, the samples were initially treated with various concentrations of ghrelin starting from 400nM and then reduced to 200nM, 100nM, 50nM, 25nM, 12.5nM and 6.25nM. Ghrelin untreated cells were considered as control. To assess the proliferative effect of ghrelin, scratch test was performed. Primary brain tumor cells were incubated with various concentrations of ghrelin, and in vitro migration activities were measured by using 6-well plate. The migration cells were visualized by phase-contrast imaging. The tumor cells were treated and incubated with two types of ghrelin solutions i.e. 20nM and 50nM concentration. In order to assess the expression of Ki-67 against various concentration of ghrelin on cancer, immunofluorescence assay tests were carried out. For this purpose, three cover slips were used to which tumor cells were applied. One cover slip was left as control while to other two cover slips, ghrelin concentration of 20nM and 50nM was applied and observed under flouro-microscope. Obtained data were analyzed using SPSS version 24. Mean, median, mode and standard deviation were calculated for both groups.

RESULTS

The effect of ghrelin in different concentrations was assessed in tumor cell lines. Our result showed that up to 30nM concentration of ghrelin showed tumor cell proliferation and above this, ghrelin has anti-proliferative effect. The results are summarized in **Table 1** and **Fig 1** with mean percentage of cells proliferation under different volume of concentration of ghrelin.

After 24hrs of incubation, the number of cells was increased that showed that they have potential to migrate and proliferate. In control group, i.e., untreated with ghrelin, scratch completed in 6 days in case of IK148 and IK155 and IK169 . The wells treated with 20nM ghrelin, Scratch completed in 3days in IK148 and IK155 while in 5 days in IK169. Furthermore, when treated with 50nM ghrelin scratch did not complete even after 6 days in all the cancer cell lines. Ki-67 monoclonal antibody was used as biomarker for determination of tumor cells proliferation. Our results showed that Ki-67 was less expressed in cover slips where 50nM ghrelin was applied than those where 20nM ghrelin was applied while in control cover slips, there was maximum expression of Ki-67 protein. The results are summarized in **Fig 2.**

Table 1: Percentage Proliferation of Cells in Different Ghrelin Concentrations

Ghrelin Assay (%ge Expression)			
Concentration (ug)	**IK-148**	**IK-155**	**IK-169**
0	100	100	100
6.25	164.9093956	105.1774544	104.386732
12.5	223.0561123	111.0384327	113.2223239
25	168.2962477	106.2230681	140.191334
50	148.569534	93.16608625	105.2165776
100	84.81524091	98.57780421	100.1555024
200	112.8974535	96.68703301	97.45776379
400	141.0302987	99.153661	110.5856338

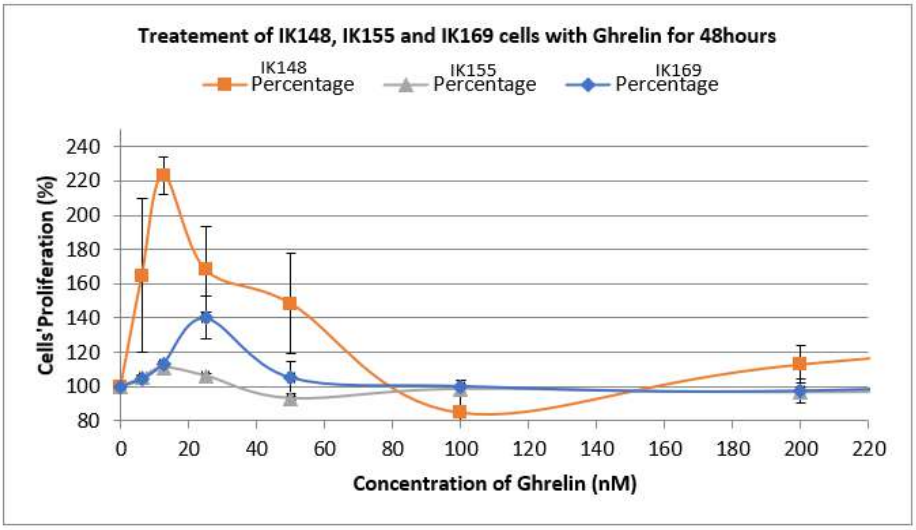

Fig 1: Effects of Ghrelin on Tumor Cell Lines

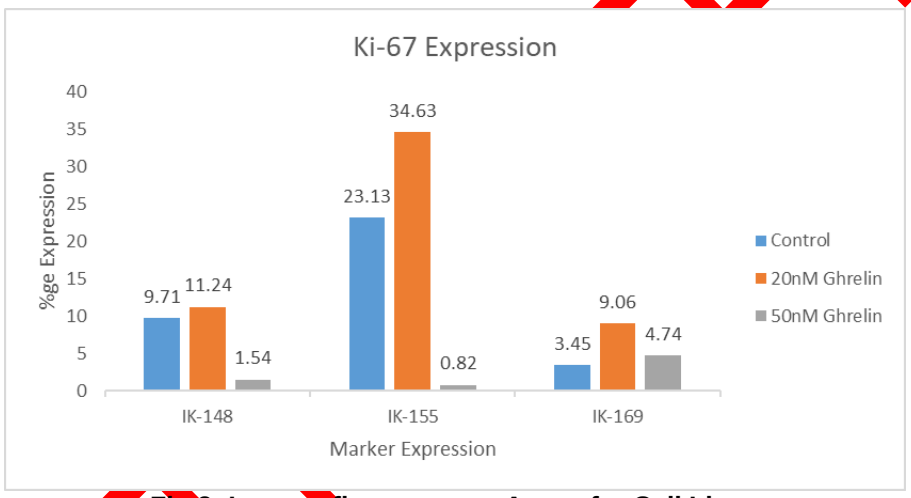

Fig 2: Immunofluorescence Assay for Cell Lines

DISCUSSION

The aim of our study was to determine the role of ghrelin in brain tumor cell proliferation.

The role of ghrelin in brain tumors was not studied yet in our study populations, which needed to be analyzed. Ghrelin has been shown to be responsible for regulation of different physiological functions in the body including metabolism, energy balance and cellular proliferation (13). As per recent studies, ghrelin has proliferative effect in pancreatic, prostate and adrenal cancers while it shows antiproliferative effect on breast and lungs cancer cells (14). Similarly, the positive role of ghrelin in different gastric malignancies such as esophageal cancer and colon cancer have been reported earlier in different studies (15). Previously, Yousuke et al showed that ghrelin promotes the tumor cells proliferation in astrocytomas and glioblastomas (16). As per previous studies, the normal serum ghrelin concentration is usually less than 1nM. The concentration of ghrelin is increased during fasting state (17). In a clinical study performed by Vestergaard et al, a continuous infusion of ghrelin at a dose of 5pmol/kg for 3hrs does not produce significant change in cardiac function as compared to placebo (18). In our study, ghrelin shows stimulatory effects on tumor cells proliferation which is concentration dependent. Our study revealed that ghrelin in concentration up to 30nM shows proliferative effect while above this concentration, it has antiproliferative effect on primary brain tumor cell lines. The mechanism proposed by which ghrelin assists the tumor cells proliferation in brain is through the expression of Matrix Metalloproteinases (MMPs). Excess formation and expression of MMPs inside the brain promotes the infiltration of tumor cells in normal parenchymal tissues through splitting from the primary tumor mass. The production of MMPs is also mediated by

different other types of proteins such as adiponectin and leptin which have shown involvement in proliferation of glioblastoma cells in brain (19). Our results contradicts with the findings of Strasser et al which showed that ghrelin in low and high concentrations are safe in advance stages of cancer (20). For in-vitro analysis of tumor cells proliferation, different experimental models are proposed including culture dish assay, in which the trans-membranous proteins such as integrins and extracellular proteins are cultured to elaborate their role in tumor cells proliferation. Other assays include spheroid models, trans-well migration assay, microdevices, Dunn's chamber analysis and scratch test (21). In our study, scratch assay was performed for in-vitro analysis of tumor cells proliferation and concentration dependent activity of ghrelin on primary brain tumor cell lines. The results showed that the scratch was filled in few days when treated with 20nM of ghrelin while the 50nM ghrelin solution does not resulted in scratch fill even after 6 days.

Furthermore, in our designed study, the proliferative action of ghrelin on primary brain tumor cell lines was verified by immuno-analysis. Ki-67 monoclonal antibody was used to determine the activity of KI-67 protein as a potential biomarker for early detection of malignant brain tumors. The Ki-67 monoclonal antibody was added to the cultured tumor cell lines and immunofluorescence results showed that up to 20nM concentration of ghrelin has proliferative effect while above this, ghrelin shows antiproliferative effect on cells. Franca et al showed that immunohistochemical assay of ki-67 and p53 proteins are good predictors of meningiomas in early stages (22). Ki-67 protein expression was identified to be involved in cancer metastasis in brain due to primary non-small cell cancer of lungs (23). Mrouj et al demonstrated that there is high expression of ki-67 and proliferating cell nuclear antigen (PCNA) in major primary brain tumors including malignant meningioma and medulloblastoma (24). The subtypes of primary brain tumors and its grading is a complex process that depends upon the tumor size, shape and levels of biomarkers in serum. Pevelin et al found that the tumor grading and types are correlated with the levels of ki-67 (p-value = 0.009) (25). According to a study performed by Christian et al and found a significant correlation between the Ki-67 proliferation index with overall survival and increasing grades of tumor and can be used as diagnostic tool to distinguish between low grade and high-grade tumors. Similarly, the results were replicated by Ahmed et al on Turkish population, KI-67 expression in astrocytes glioma was increased as the tumor proliferated in nearby healthy brain cells and can be corelated with poor prognosis and survival rate (26).

CONCLUSION

The ghrelin hormone has proliferative effect on tumor cells in low concentration (20nM), while higher concentrations (up to 50 nM) exhibit anti-proliferative properties. The results underscore the critical importance of precise dosage selection in potential ghrelin-based therapeutic interventions, highlighting the hormone's complex and concentration-dependent impact on tumor cell dynamics.

Authors Contribution

Sumaira Javed (SJ): Laboratory work, Manuscript writing and data compilation
Muhammad Kabir khan Afridi (KA): Manuscript editing and writing
Nadira Hameed (NH): Manuscript writing
Ayesh Sadaf (AS): Laboratory work and Manuscript writing
Khabeer Ahmad Khattak (KK): Results compilation and statistical analysis
Sidra Humayun (GF): manuscript compilation.

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Conflict of Interest

The author declares no conflict of interest.

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