ROLE OF EXOSOMES IN MALIGNANT TRANSFORMATION OF ASTROCYTES

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ABSTRACT

ROLE OF EXOSOMES IN MALIGNANT TRANSFORMATION OF ASTROCYTES

Glioblastoma multiforme (GBM) is the mainly common type of primary brain tumors with dismal prognosis. Every year, nearly 3 people out of 100.000 become affected by the disease. Glioblastomas belong to the group of astrocytomas which originate purely from astrocytic cells in the brain parenchyma and usually do not affect neurons. The World Health Organization (WHO) classifies the gliomas into four grades according to their morphologies and the severity of their malignant behavior. Exosomes are the small nanovesicles which are known to be released by all cell types and are present in all biological fluids. Proteins, lipids, mRNA and miRNA molecules can be transported with exosomes. They thereby have specialized functions in a variety of key biological processes like intercellular genetic and metabolic communication as well as modulation of immune responses. Recently, their role in cancer and other pathological conditions has gained more attention. Their content has been studied in GBM as well as other cancer cells and they are considered as potential diagnostic biomarkers. However, their role in diffuse glioblastoma invasion has been overlooked. In this study human astrocyte cells (NHA) cells were treated with exosomes harvested from commercially available cell lines of GBM which are U87-MG and A172. Phenotypic and behavioral changes of NHAs were observed and analyzed. These changes were determined by cell proliferation assays (like MTS and EDU), cell cycle analysis, real-time PCR, TG2 Elisa, Western Blotting and as well as direct monitoring via live cell imaging. The obtained results were shown that GBM-derived exosomes play important role in invasion and metastasis. Only a few studies have been published about the possible mechanism of exosomes in diffuse glioblastoma invasion. We think that understanding the intercellular communication of exosomes will open a new approach in the desperate search of therapeutic options for glioblastomas. The next step should include *in vivo* studies to shed light on the role of exosomes in GBM invasion in CNS.

ÖZET

ASTROSİTLERİN MALİGN DÖNÜŞÜMLERİNDE EKSOZOMLARIN ROLÜ

Glioblastoma Multiforme (GBM) beyin tümörleri içinde en çok rastlanan ve prognozu en kötü olan tümör tipidir. 100.000 kişide 2 veya 3 kişi bu hastalığa yakalanmaktadır. Gliomlar beyin parankiminde oluşan, astrosit kökenli tümörlerdir ve genelde nöronları etkilemezler. Dünya Sağlık Örgütü (DSÖ) hastalığın adını, gliomları morfolojilerine ve habis davranışlarının şiddetine göre dört evreye ayırarak belirlemiştir ve GBMleri IV. evre olarak sınıflandırmıştır. Eksozomlar vücuttaki bütün sıvılardan elde edilebilirler ayrıca hücreler arası iletişim, sinyal iletimi, genetik materyal transferi ve immünolojik tepkinin düzenlenmesi gibi çoğu biyolojik süreçte mühim görevler alırlar. Bu sebepten dolayı hastalık patogenezindeki etkileri üzerinde durulmaktadır. Eksozomlar, protein ve lipitlere ek olarak haberci RNA (mRNA) ve mikroRNA (miRNA) de taşıyabilirler. Glioblastoma da dahil olmak üzere kanser hücrelerinden salınan eksozomların içerikleri çeşitli çalışmalarda araştırılmış ve de hem fizyolojik görevleri hem de tanısal biyobelirteç olma potansiyelleri üzerinde durulmuştur. Fakat, gliomların komşu dokuya invazyonunu açıklamada, eksozomlar üzerine de detaylı bir çalışma yapılmamıştır. Bu çalışmada, glioblastoma hücre hatlarından elde edilerek karakterize edilmiş eksozomlar sağlıklı astrosit hücre kültürlerine uygulandı. Astrosit hücrelerindeki fenotipik ve davranışsal değişiklikler gözlemlendi ve analiz edildi. Bu değişiklikler hücre canlılığı ve çoğalması deneyleri (MTS, EDU), gerçek zamanlı Polimeraz Zincir Reaksiyonu (PZR), TGM2 Elisa, Western Blottting ve canlı görüntüleme sistemi ile belirlendi. Elde edilen sonuçlar GBM türevli eksozomların invazyon ve metastazda önemli bir rol oynadığı göstermiştir. GBMlerin diffüz özelliğini açıklama konusunda eksozomlar üzerinde yeterince durulmamıştır. Eksozomların GBM invazyonundaki rolünün anlaşılması, hastalığa karşı verimli terapi yöntemleri geliştirmede gelecek çalışmaların önünü açacaktır. Bir sonraki adım eksozomların GBM invazyonundaki rolünün anlaşılmasına ışık tutmak için *in vivo* çalışmaları olacaktır.

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LIST OF SYMBOLS/ABBREVIATIONS

°C	Degree centigrade
µg/ml	Microgram per milliliter
µM	Micromolar
ABM	Astrocyte Basal Medium
ANOVA	Analysis of Variance
BAX	Bcl-2-Associated X Protein
BBB	Blood brain barrier
BCL2	B-cell lymphoma 2
CNS	Central nervous system
DAPI	4′,6-diamidino-2-phenylindole
DEX	Dextran
DMEM	Dulbecco's Modified Eagle's Medium
DMSO	Dimethyl sulfoxide
EBRT	External beam radiation therapy
ECM	Extracellular matrix
EdU	5-ethynyl-2'-deoxyuridine
EGFR	Epidermal growth factor receptor
FBS	Fetal bovine serum
GAPDH	Glyceraldehyde 3-Phosphate Dehydrogenase
GBM	Glioblastoma multiforme
GLI-1	Glioma-associated oncogene homolog 1
MDM2	Mouse double minute 2 homolog
MTS	3-(4,5-di-methyl-thiazol-2-yl)-5-(3-carboxy-methoxy-phenyl)-2-(4 sulfo-phenyl)-2H-tetrazolium
NHA	Normal human astrocytes
NSCs	Neuronal stem cells
PBS	Phosphate buffered saline
PEG	Polyethylene glycol
PFA	Paraformaldehyde

PI	Propidium iodide
PMSF	Phenylmethylsulfonyl fluoride
PSA	Penicillin-Streptomycin-Amphotericin B
PTEN	Phosphatase and tensin homolog
RT-PCR	Real time polymerase chain reaction
SEM	Scanning electron microscopy
TGM2	Transglutaminase 2
TMZ	Temozolomide
WHO	World Health Organization

1. INTRODUCTION

1.1. GLIOBLASTOMA MULTIFORME

Glioblastoma multiforme (GBM) is one of the main primary central nervous system (CNS) tumor type which reveal from astrocytes. Glioblastomas are group of astrocytomas which originate purely from astrocytic cells in the brain parenchyma and usually do not affect neurons [1]. The World Health Organization is classified gliomas into grade I to IV according to their malignancy level. While grade I and II tumors are called pilocytic and fibrillary astrocytomas, respectively and exhibit a rather benign behavior, grade III and IV tumors are called anaplastic astrocytomas and Glioblastoma multiforme (GBM). In 2016, WHO developed a new classification system incorporating the molecular alterations that corresponded better with the chemotherapeutic response and prognosis. With the new classification, glioblastomas are categorized according to whether they display the wild type or mutant version of isocytrate dehydrogenase (IDH) with the IDH-mutant tumors responding better to chemotherapy [2, 3].

GBM is the mainly malignant, invasive and aggressive type of brain cancer [1-3]. Every year 3 in 100,000 people diagnosed with this cancer, accounting for >51 percent of gliomas (Figure 1.1.). The survival rate of patients is low, ranging from 15 to 17 months. The mean age of the disease onset is about 64 but it can occur at any age [4]. GBM frequency is lower in women than in men (M:F ratio = 3:1) and also increases with age [2, 5].

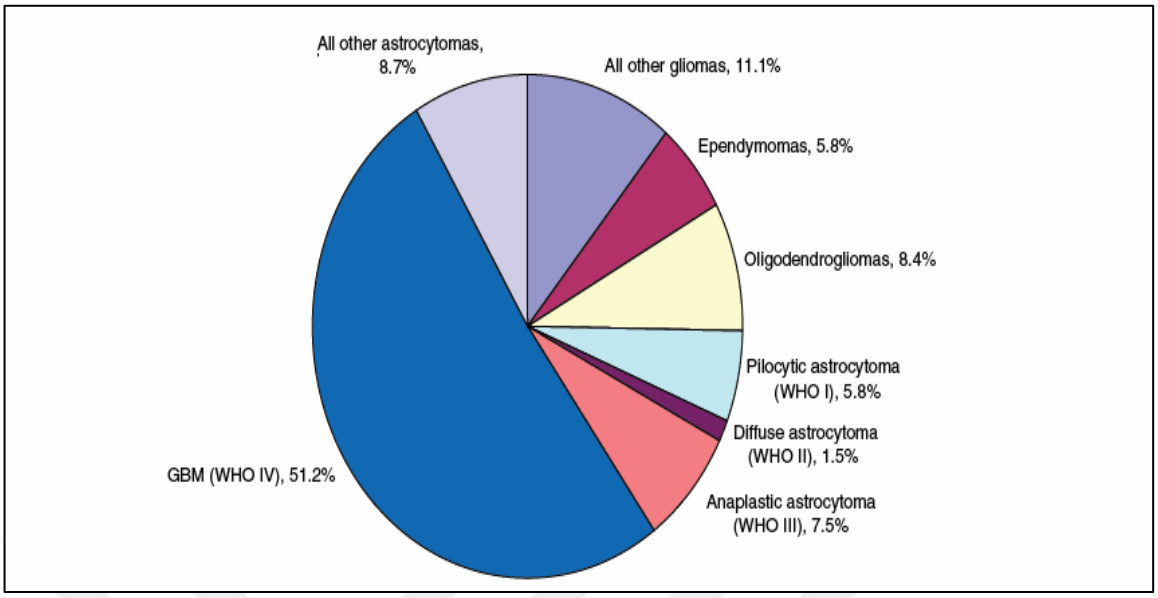


Figure 1.1. Distribution of all Central Nervous System gliomas.

The risk factor or factors leading to GBM development have not been fully established except for radiation. Apart from this, in general cancer risk factors like cigarette, electromagnetic waves, allergy, viral infection and genetic predisposition can be also listed for GBM. A number of familial diseases such as Li-Fraumeni's syndrome, neurofibramatosis, tuberous sclerosis, Turcot syndrome were reported to be associated with GBM [5, 6].

GBMs are divided into two groups as primary (de novo) and secondary. As it is understood by the name, primary comes from wild-type tissue and secondary occurs with malignant hazards in lower grade GBMs; Grade I and II.Secondary GBMs mainly arise in younger people, the average of age 45 whereas primary GBM typically consists de novo and present itself with a mean age of 62 year [1]. Primary GBs account for 90 percent of all GBs, and are tumors that develop directly from the glial precursor cells and diagnosed as GBM in the initial histopathological examination. The clinical symptoms occur due to the rapid development of the tumor within the first 3 months. Secondary GBMs, which constitute 10 percent of the remaining group, develop secondary to low grade gliomas. The average time for low grade astrocytomas to be transformed into GBM is 4 to 5 years. Primary and secondary GBMs cannot be morphologically differentiated and when the patient age is not considered both have a poor prognosis [7, 8].

Primary GBMs show distinct features from secondary one; it has specific genetic abnormalities that give each class the different characteristics (Figure 1.2.) [9]. Primary GBMs have dominant characteristic features such as Epidermal growth factor receptor (EGFR), EGFR variant III deletion (EGFRvIII), p16INK4A deletion and phosphatase and tensin homolog (PTEN) mutations but they can also be seen in secondary GBM. Similarly, mutations in tumor suppressor p53 seen in secondary GBM are also observed in primary GBM. Activation of the PI3K / AKT pathway resulted in the loss of chromosome 10, EGFRvIII amplification, O-6-methylguanine-DNA methyltransferase (MGMT), CD133, and isocitrate dehydrogenase 1 (IDH1) activation and various prognostic markers in GBM [10]. Recent investigations have also described the molecular properties of grade II and grade III gliomas, including the mutations in most of the aforementioned markers in GBM, for example p53, IDH1 /2 and 1p/19q [11–14].

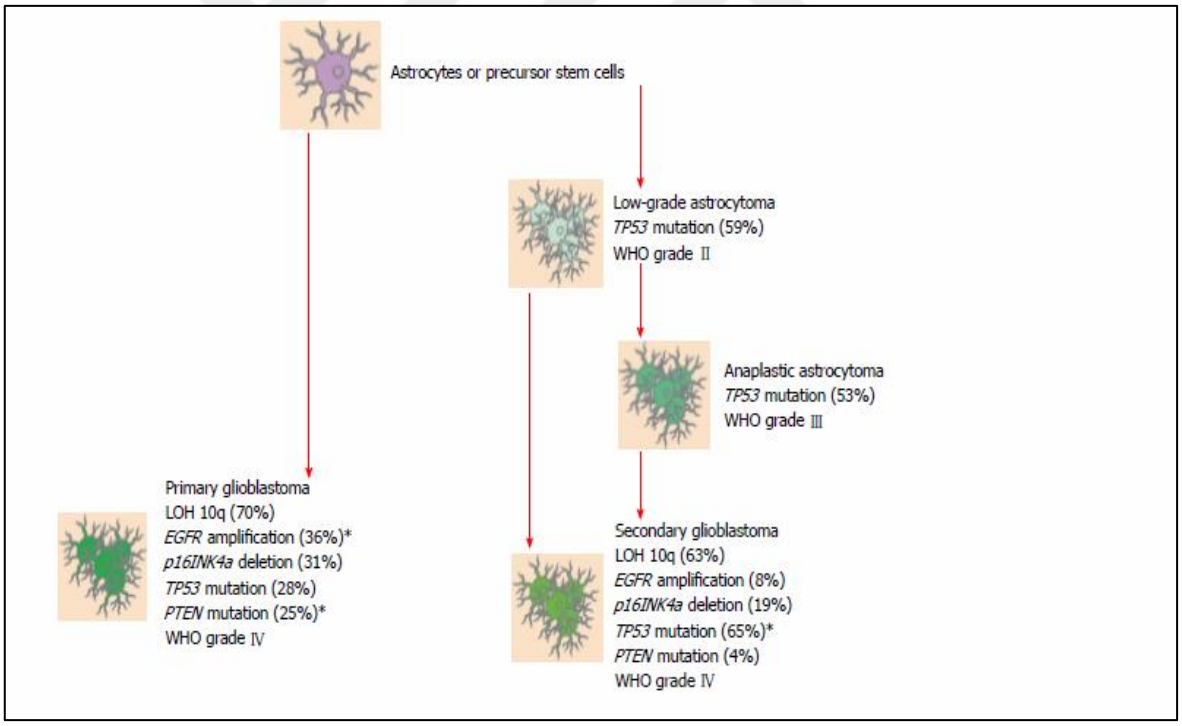


Figure 1.2. Genetic mutation least common one affected in the development of malignant gliomas [9].

GBMs are infiltrating tumors mainly located in cerebral hemispheres. The most common localization is frontal lobe and the least neighbouring is occipital lobe. Macroscopically, although GBMs are distinguished from the surrounding healthy tissue with good margins,

when examined microscopically, it shows infiltration into healthy tissue away from the mass. Normal brain tissue around the tumor is originated by a high degree of invasive tumor, which causes edema and expansion [15].

Most of the symptoms caused by GBMs depend on the rapidly growing tumor, the tumor around it, obstruction and increased intracranial pressure. Headache, nausea, vomiting, epileptic seizures, loss of neuromotor function and mental changes are the most common complaints. Focal findings may also be seen due to retained tumor localization. GBMs are non-curative type of cancer and have high morbidity and mortality characteristics. The average survival time of the patients is 14 months in spite of the most advanced treatment practices. It is known that 10 percent of the patients can reach the 2-year survival period [16].

Surgery, radiotherapy and chemotherapy are the standard forms of treatment that are currently applied. All three treatments alone are not enough. Therefore, respective implementation of these three treatment approaches are preferred. The current method of treatment of GBM includes adjuvant radiotherapy following the largest possible surgical resection and application of chemotherapy in suitable patients [16].

1.1.1. Genetic Properties of GBM

As with all cancers, GBM cells also have angiogenesis, increased activation of life signals, tissue invasion, uncontrolled proliferation and resistance to apoptosis. The genetic heterogeneity is common in GBM. It has been reported that the genetic profile shows changes within the same tumor tissue and between the tumor samples of different patients and therefore the disease is called ‘multiform’ [3]. Clinical and cytogenetic differences are found between primary and secondary GBM tissues [11].

EGFR, cyclin dependent kinase inhibitor 2 (CDKN2A) genes and loss of heterozygosity in chromosome 10q23 are the most common genetic alterations in primary GBMs [17]. A tumor suppressor gene, the phosphate and tensin homolog (PTEN) gene is placed in the chromosome 10q23 site, and the loss of heterozygosity (LOH) in that region occurs in 60-80 percent of the cases [18].

In secondary GBM, various genetic changes have been found at suppressor protein 53 (TP53) genes, platelet-derived growth factor receptor A (PDGFRA) and PDGFRA ligand. Some of the genetic changes identified in secondary GBM some are p16INK4A, mutations in retinoblastoma susceptibility locus protein 1 (pRB1) genes, amplification of cyclin dependent kinase 4/6 (CDK) and human double minute 2 (HDM2) genes and chromosome 10 deletions [11]. In addition, in recent years, it has been reported that there are mutations in the isocitrate dehydrogenase 1 (IDH1) gene involved in the protection against oxidative stress [18].

TP53 mutation for secondary GBM and EGFR amplification for primary GBM are characteristic genetic changes [11]. Genomic analysis of the genes most frequently seen in GBM cases were reported as TP53 (40 percent), EGFR (37 percent) and PTEN (30 percent) [13]. However, it is known that these genetic changes have no importance for the treatment of GBM in the prognosis. Although there are no precise limits for this classification, suppressive PTEN gene deletion, loss of chromosome 10 are common genetic changes detected in primary and secondary GBM. Therefore, genetic changes such as CDK4 amplification and TP53 mutation are found in both types of GBM [11, 12].

Phosphatidylinositol 3 kinase (PI3K) pathway is the most important pathway detected in GBM cells and triggers viability. PI3K / protein kinase B (PCB, AKT) pathway is activated by virtue of activation of various tyrosine kinase receptors, like EGFR, PDGFR, with growth factors and genes that regulate of life and proliferation activation is provided through nuclear factor κB (NFκB). The PTEN mutation detected in GBM does not inactivate this pathway, but PTEN can block this pathway. The mitogen-activating protein kinase (MAPK) pathway via Ras oncogen is another way that is caused by the activation of tyrosine kinases. It stimulates the activation of transcription factors that enable expression of genes that control life and growth in the glial cell together with the activation of this pathway.
The mutations in tyrosine kinase 19 receptors in GBM cause activation of these signals and uncontrolled activation of signaling pathways. The use of inhibitors and monoclonal antibodies to inhibit the kinases at the beginning of this pathway is an alternative treatment method in GBM [18].

GBMs are quite complex in terms of cytogenetic structure. Common chromosomal anomalies are the translocations t (15; 19) and t (10; 19), the losses in the 9p, 10p, 10q, 13q,

17p and 19q regions. The chromosomal losses rather than chromosomal gains are reported in GBMs [19].

Many of the mutations associated with gliomas occur in the cell cycle pathway and apoptotic pathways [20]. These mutations trigger gliogenesis by causing abnormal rearrangement of cell growth, survival and migration. Genomic and chromosomal variations in genes, mutations, abnormal changes in gene expression levels caused by mutations or hypo / hypermethylations along with other components in these pathways cause tumor growth and differentiation in the process from low-grade astrocytomas to GBM [21].

1.1.2. GBM Treatment

The identified optimal treatment modality in glioblastoma multiforme; adjuvant radiotherapy followed by the largest possible surgical resection and chemotherapy in selected cases. Temozolomide (TMZ) chemotherapy and external-beam radiation therapy (EBRT) have been currently used as methods to treat GBM [22].

The aim of surgery in brain tumors is to provide cure by removing the tumor completely without causing neurological damage. If surgical curing is not possible in infiltrating glioblastoma multiforme, the aim is to reduce tumor mass and decompression should be ensured [11]. The width of the surgery performed is an important prognostic factor that improves the survival and influences the adjuvant treatment results [23]. However, it has been reported that the effect of surgical resection on survival may be related to other prognostic factors [24].

Glioblastoma multiforme; due to its infiltrative structure, can replicate within normal brain tissue from residual neoplastic cells at microscopic level even after the largest surgical resection. In order to prevent the proliferation of residual cells or macroscopic tumor remaining after subtotal resections radiotherapy should be added to the treatment [24].

Chemotherapy is another treatment modality in which postoperative application of glioblastoma multiforme treatment. In a meta-analysis of 16 randomized trials evaluating the importance of chemoradiotherapy in high-grade brain tumors, a 10 percent average survival increase (9.4 months versus 12 months) was found with chemoradiotherapy compared to radiotherapy alone [11]. In a meta-analysis of 300 randomized studies including

3004 patients published in 2002, it was found that chemotherapy added to postoperative radiotherapy in high-grade brain tumors showed a statistically significant survival advantage [25].

Blood brain barrier (BBB) is a prominent problem in chemotherapy. Because the diffusion of chemotherapy agents is a slow process, even if the blood brain barrier is impaired, most of the drugs used in chemotherapy still do not achieve adequate tissue concentrations in the central nervous system [25]. High-grade brain tumors with good activity and frequently used some of the agents are procarbazine, nitrosoureas and vincristine. Other agents having some activity are hydroxyurea, cyclophosphamide, platinum analogs, 6-thioguanine and 5-fluorouracil. Usually procarbazine or carmustine as adjuvant (BCNU) monotherapy or combination therapy with PCV (procarbazine, lomustine [CCNU], vincristine) is applied [25]. Today, the efficacy of various cytotoxic agents for the simultaneous use of radiotherapy is being investigated. One of these investigated treatment modalities is temozolomide [26].

Temozolomide (TMZ) is frequently used in the chemotherapy treatment of glioblastoma multiforme. TMZ, an alkylating agent, acts by attaching methyl group to the O6 position of guanine in the most critical region of DNA [24, 27]. These damages lead to apoptosis by stopping cancer cells in G2/M cell cycle. However, this TMZ chemotherapy attempts in patients with GBM have been found to differ in their efficacies [28]. Besides, patients were found to be resistant to temozolomide during the treatment period and some patients did not respond to TMZ treatment. The repair of DNA damage in GBM cells with TMZ effect provides O6-methyl guanine transferase (MGMT) enzyme. This enzyme catalyzes a covalent bond between the cystine group in the structure and the methyl group attached to O6-guanine and removes this methyl group added by TMZ from the DNA. Some GBM patients did not respond to TMZ treatment. The reason for this is that DNA damage in cancer cells due to high expression of O6-methyl guanine transferase (MGMT) in GBM cells is not repaired and killed with TMZ rapidly [29].

Studies, have shown that MGMT in patients not responding to TMZ therapy showed that DNA damage caused by overexpression of the enzyme can be repaired quickly and prevent the death of cancer cells. MGMT inhibitor is aimed to inhibit the MGMT enzyme in order to make GBM treatment more effective in these patients and therefore MGMT inhibitors are being developed [30].

GBM tumor cells are heterogeneous and have the ability to renew themselves. Studies have shown these cells express stem cell markers for instance CD133, Nestin, SOX2 and Olig2. Thus, these cells are identified as cancer cells or cancer stem cells with stem cell features. Cancer stem cells within the GBM cell population are thought to cause tumor re-formation. However, they are thought to be drug and radiotherapy resistant [29, 30].

Another problem frequently encountered in the treatment of GBM patients with TMZ is the resistance of some patients to the cytotoxicity of TMZ. Therefore, it is essential to identify the TMZ resistance mechanism and to test potential therapeutic agents. It has been found that many GBM cell lines gain TMZ resistance. However, the characteristics of true and adaptive TMZ resistant GBM cell populations were not fully studied [29, 30].

In summary, although it is seen frequently, treatment options in GBM which have a negative effect on life quality and expectancy of patients have not changed much in many years. The optimal treatment modality is adjuvant radiotherapy (RT) and chemotherapy (CT) in selected cases after the widest possible surgery. According to the studies, radiotherapy performed in addition to the chemotherapy applied after the surgical treatment prolongs the median survival time by 2 months [31].

1.1.3. Malignancy Potential and Invasion of Glioblastoma

In primary brain tumors, lesion localization is more important than any histopathological feature. Systemic findings typical of systemic cancers,cachexia, the cause of death rather than metastatic spread is usually local recurrence [32]. GBM is truly malignant brain tumor also lacking of systemic cancer findings due to these reasons [33].

Contrary to other solid cancer types, GBM seldom metastasize outside of brain and GBM cells do not use lymphatic or intravascular courses to migrate; in default of they prefer pass by use of the extracellular space of brain tissue. This invasion progression is distinct from the migration process because cell motions need glioma cells to overcome tissue barriers by gluing and disintegrating the extracellular matrix (ECM) and by altering the cell cytoskeleton and cell volumes (Figure 1.3.) [33].

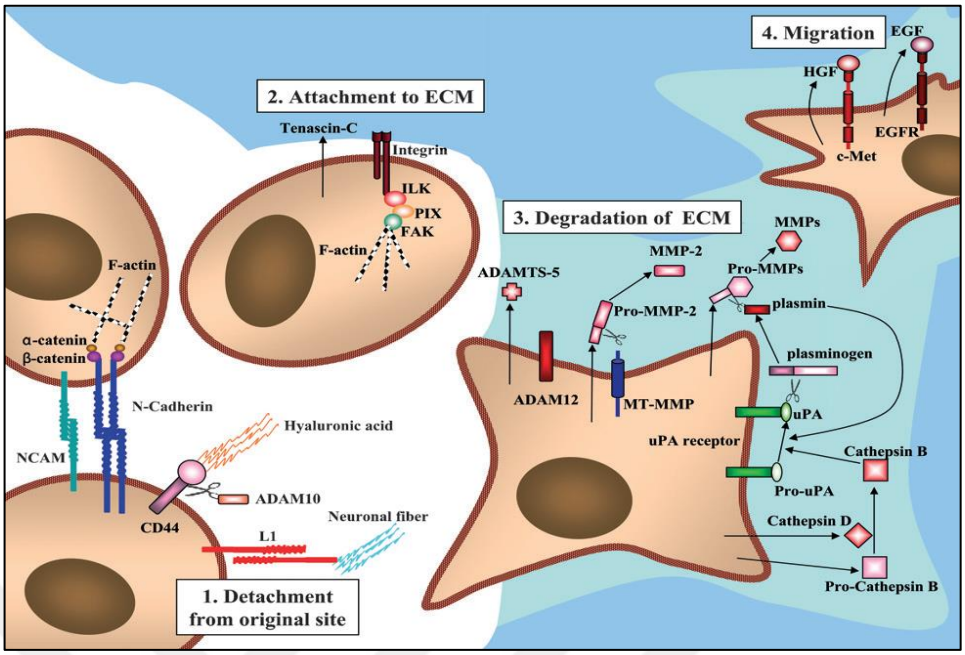


Figure 1.3. Putative mechanism of glioma invasion [33].

The use of known cancer cells can pervade cells using mesenchymal or amoeba like movement, similar to individual cells, or spread from the original tumor mass arranged in clusters or sheets. [34]. GBM cells mostly use the former kind of immigration, one of the most effective way these cancer cells embrace to migrate distant from the basic tumour bulk. That kind of incursion is evocative of that neural stem cells (NSCs) along the embryonic improvement either throughout the maintaining durations in brain tissues injured [35].

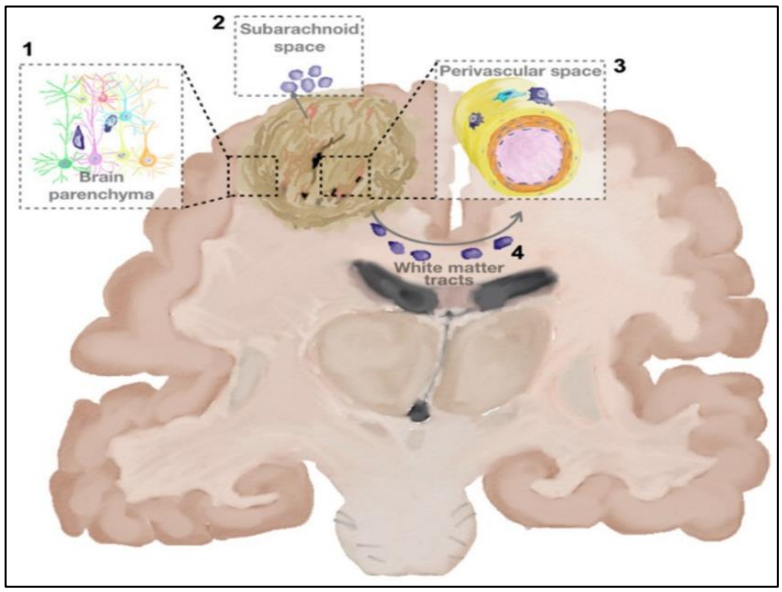


Figure 1.4. Migration routes of glioblastoma cells [34].

Furthermore, the microenvironment of glioma consists of tumour cells, endothelial cells, astrocytes, microglia and also extracellular matrix (ECM). The existence of necrotic regions can induce oxygen, nutrient and pH gradients. Moreover, tumor areas are characterized by raised hardness respect to enclosing brain parenchyma [36].

As a summary, glioblastoma multiforme is a deadly primary CNS cancer. Despite many risk factors have not been identified for the development of GBM, risk factors for example ionizing radiation exposure have proven to be harmful for disease development in some cases. Another risk factors, such as using a cell phone, exposure to pesticides and head trauma have not yet been demonstrated to be an increased risk for gliomagenesis [35].

Despite our increased knowledge of the molecular genetics of high-grade glial tumors, our knowledge is still limited about the cells originating from these tumors, molecular mechanisms of the formation of these cells, and the molecular players that cause the aggression of the tumor [34–36].

1.2. EXOSOMES

1.2.1. Biogenesis, Release and Uptake of Exosomes into Target Cells

Extracellular vesicles are involved in biological events for example intracellular and intercellular signaling, coagulation. They are divided into four classes according to their size and formation; microvesicles which are secreted directly out of the cell membrane, apoptosomes as a result of apoptosis, retrovirus-like vesicles and exosomes that consisted indirectly from cell membrane [37]. Exosomes are small nanovesicles released by almost all cell types in cell culture and in vivo, found in all body fluids such as blood, urine, oral secretion, amniotic fluid, cerebrospinal fluid, joint fluid, nasal secretions, breast milk, serum and plasma [38]. Their size is maximum 150 nm [39]. They secrete diverse biomolecules such as proteins, mRNA, microRNA and membrane receptors. The important features of exosomes distinguishing them from other extracellular vesicles (EVs) are: their specific biogenesis pathways, lipid compositions and cargo contents [39, 40].

Exosomes have important functions for instance; obliteration of obsolete molecules, facilitation of the immune response, antigen presentation, programmed cell death

(apoptosis), angiogenesis, clotting, inflammation, spreading of oncogenes from tumor cells, airing of pathogens for example prions and viruses from one cell to another. Also exosomes are delivering macromolecular messages (RNA and protein)-supplying signaling and cell-to-cell communication [41, 42].

Exosomes are generated indirectly from the plasma membrane, to be recognized by the target cells membranes of serine membrane lipids, cholesterol, such as sphingolipid, glycerophospholipid, sifingomyelin, ceramide they also carry membrane lipids. Also, they carry specific proteins found in the plasma membrane of the cell they originate from, this makes it easy for them to be recognized by other cells [43].

One of the significant feature of exosomes is that they transport cargo contents. Cargoes of exosomes differ according to the purpose of secretion and the contents of the from their originated cells. As protein cargo, they contain integrins, immunoglobulins, many adhesion proteins (adhesion molecule-1, CD146, CD9, milk-fat-globule EGF-factor VIII (MFG-E8), CD18, CD11a, CD11b, CD11c, CD166 and LFA-3 / CD58), cytoskeleton proteins (tubulin, actin), ESCRT (required for transport endosomal separation complex proteins involved in exogenous biogenesis, and Alix, Tgs101, ceramide and tetraspannin family, heat-shock proteins (hsp70, hsp90), endosomal vesicle traffic in CD9, CD81 and CD63, Lord of the GTPase subgroup, fusion events and the Annexin family (Annexin I, II,V and VI) and flotillin proteins [39, 42, 44, 45].

Protein cargoes are packed into vesicles via three mechanisms to form intraluminal vesicles. These three mechanisms are ESCRT- or lipid- or tetraspanin-dependent. Like protein, lipid cargoes also vary depending on the cells they originate from. As lipid cargo; cholesterol, lysophosphatidylcholine, sphingomyelin, phosphatidylcholine, phosphatidylserine, phosphatidylethanolamine and diglyceridesfunctional lipids are transported [46, 47]. RNA cargoes (mRNA,miRNA) transported by exosomes make them more important than other EVs. Exosomes act a role in cell-to-cell interaction through their RNA contents. When exosomes are transferred to the receiving cell they are capable of changing the physiology of the cell with their RNA cargos [42].

Exosomes occur by differentiation of early endosomes created through budding from the plasma membrane. Early endosomes create a large number of intraluminal vesicles (ILV) by budge into themselves. Tetraspanins like CD9 and CD63 play crucial roles in the composition of ILVs [48]. The early endosomes containing ILV are called multivesicular

endosome (MVE) / multivesicular body (MVC) / late endosome. MVCs turn into different end products according to their contents and targets. After transition of the early endosome into MVC (late endosome), MVCs over the trans-golgi network can be eliminated by going to lysosome or by going to the plasma membrane, to be named exosome and secreted out of the cell with exocytosis. ESCRT proteins on the surface of ILV, which form exosomes, consist of four subunits. ESCRT-0 is the primary protein responsible with the identification and collection of ubiquitin proteins. ESCRT-1 and ESCRT-2 cause membrane budding, while ESCRT-3 initiates the eventual separation of the budding membrane. This mechanism provides by the formation of ESCRT-1, Tsg101, Alix and ESCRT-3 complex [46, 49].

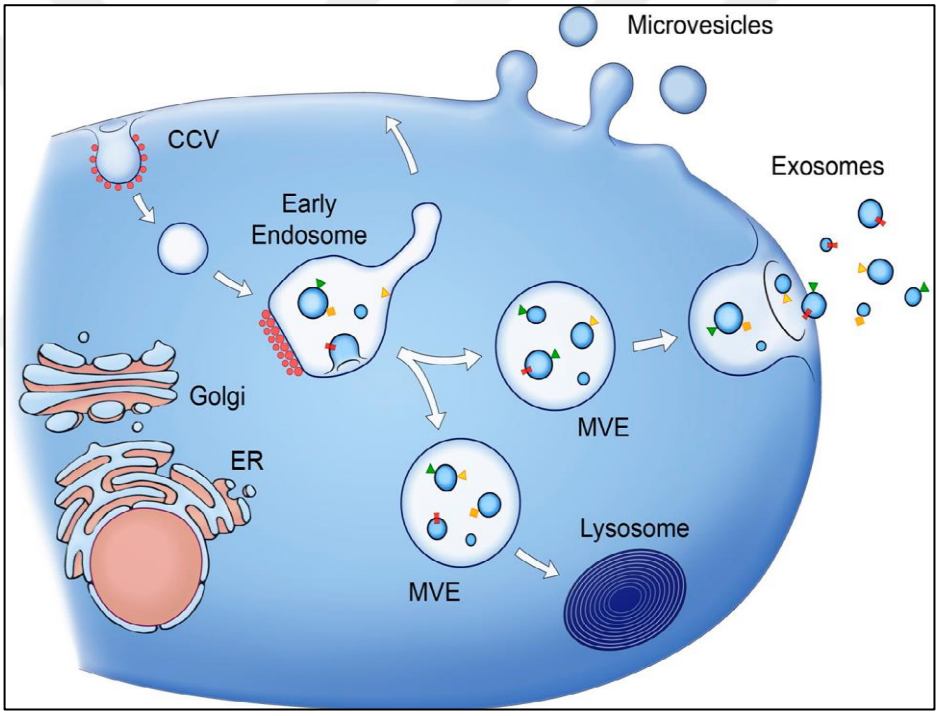


Figure 1.5. Release of exosomes [46].

The target cell recognition of exosomes and target cell entry mechanisms are still not fully elucidated, but there are three known methods for uptake of exosome content into the cell. The proteases in the plasma membrane of the target cell, combined with receptor interaction, combine with exosome membrane proteins. Soluble ligands in the regions where the target receptors are bound on the cell surface are released. The cargo in the exosome is taken into the lumen with selectively permeable intracellular signals [44].

Although there are no specific receptors that mediate uptake of exosomes into the cell, there are many proteins on the surface with potential receptors, for the APCs (Antigen Presenting Cells), an example of which is ICAM-1 and for B cells such as Tim1/4. Heparan sulfate proteoglycan (HSPGs) acts as receptors that are effective in taking of cancer cell origin exosomes into the cell. Enzymatic degradation of HSPG on the cell surface has been shown to significantly reduce the exosomal cell uptake [50, 51].

Exosomes carry out the transmission of the cargo by interacting with the target cell membrane through the cytoskeleton proteins (actin, tubulin, profile, kofilin), metabolic enzymes they carry (GAPDH and pyruvatcinase) and membrane lipids (cholesterol, ceramide, sphingolipid) in the cell membrane they originate [50].

In direct fusion, exosomal cargo is released to the cytoplasmic space via the fusion of the exosome membrane with the membrane of the recipient cell. Exosomes of dendritic origin transfer directly to the target cell's cytoplasm by fusion with the target cell membrane via CD9 tetraspanins 5 on their surface [52].

In phagocytosis (internalization), the exosome is taken into the whole cell by actin-cytoskeleton and phosphatidylinositol -3-kinase-dependent phagocytosis. The transport of the exosome by means of mechanisms in the cell, disperses into the cytoplasm [42].

1.2.2. Exosomes and Cancer

Molecular events that leading to cancer cells are not limited with mutations, defects of DNA damage repair, cell aging and epigenetic factors. In recent year studies have been shown the function of exosomes in the formation of cancer [53, 54].

Exosomes are secreted by healthy cells, cancer cells and tumor-associated stromal cells under physiological conditions and cellular compositions to their particular cell types. Exosomes allow the between cancer cell interactions of autocrine, paracrine and endocrine to create the intense a network and this network and a large number of actors extending from endothelial cells to immune system cells within this network. Cancer cells can escape from immune system, inhibit immune cells, increase angiogenesis around the tumor microenvironment, gain drug resistance and obtain the ability to invade and metastasize by means of exosomes [55]. Exosomes allow for progression and metastasis of tumor through

oncogenic signal proteins on their surface or in vesicles, ligands, enzymes and miRNAs [56]. Exosomes have functions in many points of the metastasis process. For instance, exosomes derived from glioblastoma cells increase cell mobility by activating extracellular matrix via 'hsp90alpha' [52–54, 57–59].

Exosomes play a significant role in the pathology of the disease due to the molecular markers they carry to the cells they originate. Therefore, exosomes have active functions in the formation and spread of cancer [60, 61]. Exosomes secreted from cancer cells show their effects by fusion with cell membranes. Exosomes have been shown to contribute to the formation of microenvironment, triggering angiogenesis, altering the adhesion, mobility and invasion of the target cells, enhancing metastasis and developing drug resistance [62].

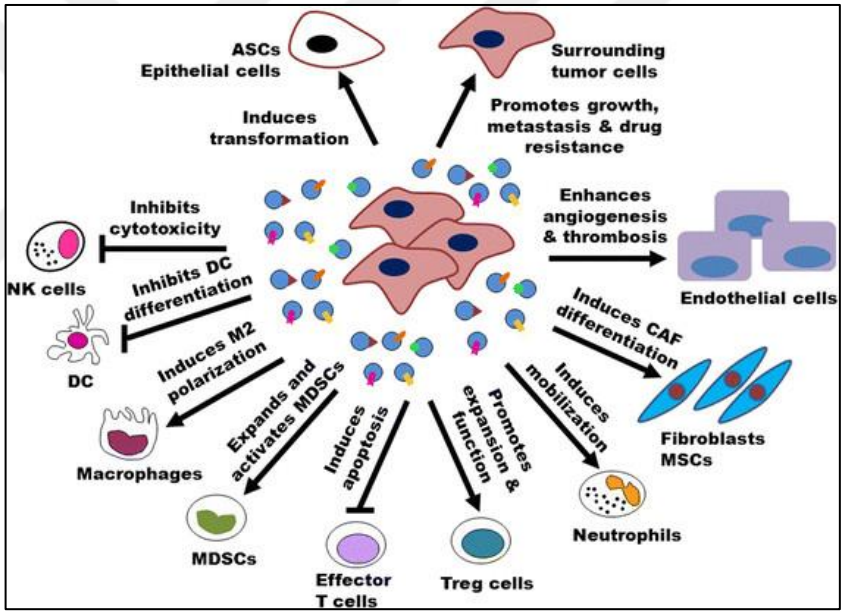


Figure 1.6. Roles of exosomes in cancer [63].

Micro-environmental changes that contribute to increased proliferation, invasion, migration, epithelial-mesenchymal transformation and tumor progression in cancer cells, are closely related to ligand and signaling pathways for example MAPK / ERK, PI3K / AKT, Wnt / b-catenin, receptor tyrosine kinases and TGF-beta. Exosomes directly or indirectly affect these signaling pathways that contribute to tumor progression at both cancer and tumor related stromal cells. For example, PTEN (Phosphatase and Tensin homolog) located in the upper steps of the PI3K / AKT pathway is transported through exosomes between the cells. In malign gliomas, EGFRvIII is transported via exosomes. EGFR-mediated receptor tyrosine

kinase activation was shown to activate proliferation, angiogenesis, and enhanced migration of glioblastoma cells [64].

Exosomes take place at many points of the metastasis process [65]. Metastasis supporting biomolecules have been identified in exosomes which are released from metastatic tumor cells. For example, Wnt-Beta catenin pathway components that are effective in epithelial-mesenchymal transition, or Matrix Metalloproteinase enzymes (MMP) that alter the extracellular matrix. Another group of proteins are integrins that are carried in exosomes and involved in the metastatic process [66]. These proteins play crucial roles in the targeting of metastasis destinations. Studies have displayed that exosomes secreted from metastatic tumor cells make these cells metastatic by transferring various oncogene and oncogenic proteins to non-metastatic tumor cells [64].

In one study, CD39 + / CD73 + enzymes were determined on the surface of exosomes released by metastatic tumor cells. CD39 is an ectoATPase which induces adenosine formation from ATP. Also, CD73 is a 5-ectonucleotidase and allows the formation of adenosine from AMP. These enzymes carried by exosomes are found to stimulate adenosine formation around the tumor microenvironment where ATP is abundant. Adenosine inhibits the antitumor function of the cells and stimulates the metastasis by facilitating access to the lymph node [67].

Consequently, exosomes are crucial players in the formation and progression of cancer due to their biological and morphological properties. The fact that they differ according to the types of cells they originate and can be obtained from all fluids in the body is a significant advantage and potential marker for cancer-related studies. However, the exosome biology has not yet been fully elucidated, it constitutes a potential barrier to future studies [68].

1.2.3. Exosomes and GBM

Exosomes released from cells into the external environment do not only carry mRNA and miRNA molecules, but can also alter the transcriptome of the recipient cell by these molecules. The best example to this function is that exosomes released by glioblastoma cells are transformed into protein in brain endothelial cells from which RNA cargos are taken and they have been shown to take part in the arrangement of tumor microenvironment [69]. Similarly, glioblast derived exosomes trigger the growth of glioblast cells [70].

Accurate identification of the different stages of GBM can greatly improve treatment options for patients suffering from the disease. For this reason, new biomarkers such as exosome dependent diagnostics could be vital as a platform for early diagnosis, and improved treatment of GBM patients [71][64].

1.2.4. The Importance of Exosomes in Diagnosis and Treatment

Exosomes are potential markers in the treatment and diagnosis of many diseases including cancer. It is thought that the cargo contents of exosomes (especially mRNA and miRNA content) will be an important advantage in the diagnosis of diseases. Exosomes carry membrane and cytoplasmic properties from secreted cells as a characteristics [72]. Exosomes can be obtained from all fluids in the body with different isolation methods, has led to the introduction of marker-based examinations that facilitate the diagnosis stage for many diseases [73].

Many studies have been done diagnosis of cancer with exosomes. The most significant data for diagnostic use were obtained from studies on prostate cancer cell line (PC3) and primary tissue cultures. 36 of the miRNAs used as markers of prostate cancer were determined in PC3 cell line exosomes. In addition, exosomes have been shown to be a potential tool for the diagnosis of cancer as a result of studies on different cancer types such as colorectal cancers, hepatocellular carcinoma, lung flat cell carcinoma, ovarian cancer and glioblastoma. These properties of exosomes, as a biomarker in clinical research strengthens the possibility of being used [74, 75].

Exosomes are also important in treatment-oriented researchs due to they have natural liposomal properties. Exosomes can circulate in the blood as well as capable to cross the blood brain barrier because their size and protein and lipid content in their membranes. Therefore, exosomes can be easily use in brain cancers especially Glioblastoma Multiforme (GBM) [76, 77].

Researches on diagnosis and treatment are continuing because of the interesting and unusual properties of exosomes [77].

1.3. AIM OF THE STUDY

Exosomes are the intercellular cargo and communication systems that are secreted from cells (normal and cancer) and have been discovered in recent years. Cancer cells derived exosomes show their effects by fusion with cell membranes. They have been shown to contribute to the formation of microenvironment, triggering angiogenesis, altering the adhesion, mobility and invasion of the target cells, enhancing metastasis and developing drug resistance.

The aim of this thesis is to investigate the interactions of exosomes in malignant transformation of astrocytes and progression of GBM. For this purpose, in this study normal human astrocyte cells (NHA) were treated with exosomes harvested from glioblastoma cell lines which are U87-MG and A172.

In current study, we aimed to evaluate the role of exosomes in the formation and progression of cancer and their potency for diagnosis and treatment in the light of current literature.

2. MATERIALS AND METHODS

2.1. MATERIALS

2.1.1. Consumables

Table 2.1. Consumables used for cell culture experiments

#	Brand	Item	Catalog No
1	TPP	Cell culture Flask T150,T75, T25	90150,90075,90025
2	CAPP	Serological Pipette 5,10,25,50 mL	SP-5-C,SP-10-C SP-25-C,SP-50-C
3	Isolab	Microcentrifuge tubes 1.5,2 mL	078.03.002, 078.03.003
4	Axygen	Micropipette tips 10,200,1000 uL	301-03-051
5	Isolab	Falcon tubes 15,50 mL	LB.IS.078.02.003
6	Isolab	Cryotube (2 mL)	091.11.102
7	Eppendorf	Micropipette (2.5,10,20,200,1000 uL)	2231300002
8	Thermo Fisher	Countess Cell Counting Chamber Slides	C102288
9	Integra	Pipette Pump	156403
10	Merck	Millicell EZ Slide,4 well glass	PEZGS0416

2.1.2. Chemicals

Table 2.2. Chemicals used for cell culture experiments

#	Brand	Item	Catalog No
1	ThermoFisher	DMEM,high glucose	11965092
2	ThermoFisher	FBS	A3840002
3	ThermoFisher	PBS	14190144
4	ThermoFisher	PSA	15240062
5	Gibco	Trypsin	25200056
6	Lonza	Astrocyte Basal Medium (ABM)	CC-3186
7	Sigma-Aldrich	DMSO	D8418
8	Roche	DAPI	10236276001
9	Sigma-Aldrich	Heparin	H3393
10	Sigma-Aldrich	PKH26	PKH26GL
11	Sigma-Aldrich	PKH67	PKH67GL
12	GeneCopoeia	iClick™ EdU Andy Fluor™ 488 Imaging Kit	A003
13	Novus Biologicals	Transglutaminase 2/TGM2 Assay Kit	NBP1-37008
14	ThermoFisher	RNase A	EN0531
15	ThermoFisher	Propidium Iodide	P1304MP
16	ThermoFisher, Invitrogen	Nonyl phenoxypolyethoxyl ethanol	FNN0021
17	ThermoFisher	Pierce BCA Protein Assay Kit	23227
18	Sigma-Aldrich	Polyethylene glycol	81310

19	Sigma-Aldrich	Dextran	9004-54-0
20	ThermoFisher Scientific	TRIzol™ Reagent	15596-018
21	Abcam	Anti-CD9 antibody	ab2215
22	Abcam	Anti-CD63 antibody	ab8219
23	Abcam	Anti-Hsp70 antibody	ab61907
24	ThermoFisher Scientific	Aldehyde/Sulfate Latex Beads,4% w/v, 4 μm	A37304
25	SantaCruz	RIPA Buffer	sc-24948
26	Biorad	Mini-PROTEAN Precast Gel	4561023
27	ThermoFisher Scientific	Page Ruler Protein Ladder	26619
28	Biorad	PVDF membrane	1620177
29	Biorad	Blotting Grade Blocker	17006404
30	Invitrogen	TGM2 Antibody	PA5-14966
31	SantaCruz	Anti-rabbit secondary Antibody	sc-2004
32	ThermoFisher Scientific	SuperSignal™ West Femto Maximum Sensitivity Substrate	34096
33	Qiagen	QuantiTect SYBR Green PCR Kit	204145
34	Roche	Transcriptor First Strand cDNA Synthesis Kit	04897030001
35	Promega	CellTiter96 AqueousOne Solution	G3582

2.1.3. Instruments

Table 2.3. Instruments used in experiments

#	Brand	Item	Catalog No
1	Heat Force	Cell Culture Hood	HFsafe-1200
2	Sigma-Aldrich	Centrifuge	Sigma 3-18KS
3	ThermoFisher	Forma™ 310 Direct-Heat CO_2 Incubators	310TS
4	Zeiss	Inverted Microscope	Axio Vert.A1
5	Zeiss	Fluorescent Microscope	Axio Vert.A1
6	Olympus	Live Cell Imaging System	Olympus IX81
7	BD Bioscience	Flow Cytometry	BD FACS Calibur
8	ThermoFisher	Mr. Frosty™ Freezing Container	5100-0001
9	BIO-RAD	Real Time PCR Detection System	CFX96
10	ThermoFisher	Varioskan LUX Multimode Microplate Reader	VL0000D0
11	Biotek, Winooski, VT	ELISA plate reader	10320053
12	Malvern Panalytical	NanoSight NS300	NS300
13	Eppendorf	Vacuum Centrifuge	5301
14	ThermoFisher	NanoDrop	2000

2.1.4. Cell Lines

- Normal Human Astrocytes (NHA)- Lonza, CC-2565
- U87-MG and A172 Glioblastoma Cell Lines-ATCC, HTB-14 and CRL-162

2.2. METHODS

2.2.1. Cell Culture

U87-MG and A172 cell lines provided by American Type Culture Collection. NHA (normal human astrocyte cell line) cell line provided by Lonza. U87-MG and A172 cells were incubated in Dulbecco's Modified Eagle's Medium. NHA celss were incubated with Astrocyte Basal Medium. Media are supplemented with 10 percent exosome depleted fetal bovine serum and 1 percent Penicillin/Streptomycin/Amphotericin. Condition of humidified incubator's were 37 ℃ and 5% CO2. 0.25% trypsin-EDTA was used to detached of cells from surface in seeding and passage.

2.2.2. Exosome Isolation

Isolation of exosomes was done with a modified protocol of one described by U87-MG and A172 cell culture mediums were collected and centrifuged at 10,000 g for 10 minutes. Resulting supernatant was passed through 0.22 μm filters to remove contaminating particles and to reduce larger extracellular vesicles. Filtered supernatants were mixed at a 1:1 volume ratio with PEG/DEX exosome isolation solution, and were then centrifuged at 1,000g for 10 minutes for phase separation. Twice, 80 percent of the volume was removed from the PEG-rich upper phase to remove contaminating proteins and replaced with a PEG-rich washing solution [78]. In order to remove DEX from the exosomes, samples were mixed at a 1:1 volume ratio with methanol and centrifuged at 22.000g for 20 minutes for precipitation of DEX. After centrifugation, supernatants were collected in new eppendorf tubes. Concentrator was used to suspended the methanol from mixture. The concentrations of exosomes were determined by BCA assay.

2.2.3. Flow Cytometry

Three commonly used exosome markers, CD9, CD63 and Hsp70 were used to identify of exosomes. The purified exosomes (50 μg/mL) were incubated at room temperature for 15 minutes with 5 μL of 4-μm diameter aldehyde/sulfate latex beads. This was followed by the

dilution with 2 percent BSA containing PBS. The binding reaction was continued for 2 hours with shake gently. 100 mM glycine was used to stop the reaction and incubated for 30 minutes. Then beads were washed with PBS and centrifuged at 2700xg for 3 minutes. Exosome coated beads were stained with exosome specific 1µl of antibodies (CD9, CD63 and Hsp70) and incubated at 4°C overnight. Lastly, samples were centrifuged at 2700 g for 3 minutes and PBS added to final volume of 500 µl [79]. BD-Cell Quest Pro software was used for data analysis and BD FACS Calibur was used to flow cytometry.

2.2.4. Nanoparticle Tracking Analysis (NTA)

Measurements of the exosomes were performed by the nanoparticle tracking analysis. The isolated exosomes were diluted to 1:10 with ddH_20 and loaded in syringe for analyze the size (nm) and concentration (particles/mL) of exosomes using NanoSight NS300. Analysis of exosomes was calculated with NTA software v.3.30. Capture and analysis settings were shown in Figure 2.1.

Capture Settings

Camera Type:	sCMOS
Laser Type:	Blue488
Camera Level:	16
Slider Shutter:	1300
Slider Gain:	512
FPS	25.0
Number of Frames:	1498
Temperature:	15.0 - 15.0 °C
Viscosity:	(Water) 1.134 - 1.135 cP
Dilution factor:	1 x 10e1

Analysis Settings

Detect Threshold:	10
Blur Size:	Auto
Max Jump Distance:	Auto: 9.9 - 11.0 pix

Figure 2.1. Capture and analysis settings for NTA.

2.2.5. Scanning Electron Microscopy (SEM)

Circles of 1 to 2 cm in diameter were drawn on glass slide with histology pencil, and were marked with arrows. 20 µL of isolated exosome was dropped the center of the drawn circle and sample was dried in concentrator at 30°C. Lastly, prepared sample screened in scanning electron microscope (SEM).

2.2.6. Exosome Uptake Analysis

Firstly, NHA cells were trypsinized and centrifuged at 1300 rpm for 5 minutes. After that, pellet of $1x10^6$ cells was resuspended with 4 µL of PKH26 which is mixed with 1 mL Diluent C and incubated for 10 min at room temperature. When incubation was finished, cells were washed two times with PBS then seeded on 6 well plate. On the other hand, exosomes were labelled with PKH67 green cell membrane dye. Exosomes were mixed with 4 µL of PKH67 and 1 mL Diluent C then they incubated for 5 minutes at RT. After incubation, mixture was centrifuged at 3000 rpm for 10 minutes and exosomes (labelled with PKH67) were added on NHA cells (labelled with PKH26). After 48 hours, images were taken by using fluorescent microscopy. Secondly, heparin was used with aim of control the exosome uptake which is endocytosis inhibitor. Briefly, NHA cells and exosomes were labelled with PKH26 (red) and 20 µg/mL heparin was added on cells then incubated for 30 minutes at 37 °C. Exosomes were labelled with PKH67 (green). After incubation, heparin containing medium was removed and labelled exosomes were added on cells [80].

2.2.7. Cell Viability Assay

Effect of glioblastoma cells exosomes (U87-MG and A172) on NHA cells were measured with MTS assay. Shortly, cells were seeded on 96 well plates at a cell density of $5x10^3$ cells/well and incubated in 37 °C and 5% CO_2 humidified incubator for 24 hours. After that cells were treated different (1µg/mL, 5µg/mL, 10µg/mL and 20µg/mL) concentrations of exosomes. 24, 48 and 72 hours later, exosome containing medium was removed and replaced with PBS containing 4.5 g/L glucose and 10% MTS as described elsewhere [81]. Cells were incubated at growing conditions to let them metabolize MTS reagent. One hour later,

viability changes were measured with the absorbance rates at 490 nm by using an ELISA plate reader.

2.2.8. Edu Cell Proliferation Assay

Effect of U87-MG and A172 exosomes on cell viability of NHA cells was determined by using iClick™ EdU Andy Fluor™ 488 Imaging kit. Briefly, cells were seeded on 4 well glass slides and incubated for overnight. On the other hand, cells were seeded on 96 well tissue culture black with clear bottom plate at a cell density of $5x10^3$ cells/well and incubated in 37°C and 5% CO_2 humidified incubator for 24 hours. Next day cells were treated with determined exosome concentration (20 μg/mL) and labelled via EdU. After 48 hours cells were fixed with 3.7% formaldehyde in PBS and incubated for 15 minutes at room temperature. Then fixative was removed and washed the cells in each well with 3% BSA in PBS. For permeabilization, 0.5% Triton® X-100 in PBS was added to each well, then incubate at room temperature for 20 minutes. iClick reaction additive cocktail was prepared according to Table 2.4. Permeabilization buffer was removed and each wells were washed twice with 3% BSA in PBS. Subsequently, iClick reaction cocktail table 2.4 was added on each well and incubate the plate for 30 minutes. After incubation, reaction cocktail was removed and washed once via %3 BSA in PBS. Washing solution was removed and each wells were washed again with only PBS. Hoechst 33342 was diluted to 1:1000 in PBS for nuclear staining (the final concentration was 5 μg/mL) and it was added on wells and plate was incubated at RT. After incubation wells were washed via PBS [82, 83]. Finally, cell proliferation changes were measured with absorbance rates at 495 nm for Andy Fluor 488 and 350 nm for Hoechst 33342 by using Varioskan LUX Multimode Microplate Reader and confocal microscopy.

Table 2.4. iClick Reaction cocktail

Reaction Components	Volume of reagents
1X iClick Reaction Buffer	4.3 mL
$CuSO_4$	200 μL
Andy Fluor 488 azide	15 μL
1X Reaction buffer additive	500 μL
Total volume	5 mL

2.2.9. Cell Cycle

In order to determined to effect of GBM exosomes on NHA cells, cell cycle assay was performed. Shortly, NHA, U87-MG and A172 cells were seeded in 6 well plate then incubated for 24h. After incubation, NHA cells were treated with GBM exosomes and incubated for 48h. When incubation was finished cells were fixed with 70% ethanol and incubated for 2h. Then they were suspended in 500 µL staining solution (40 µg/mL RNase A, 33 µg/mL Propidium Iodide, 0.2% nonyl phenoxypolyethoxylethanol in PBS) and incubated for 30 min at 37 °C [84]. BD-Cell Quest Pro software was used for data analysis and BD FACS Calibur was used to flow cytometry.

2.2.10. Real Time Polymerase Chain Reaction

Protein kinase B (Akt), Glyceraldehyde 3-phosphate dehydrogenase (GAPDH), Bcl-2-associated X protein (BAX), Mouse double minute 2 homolog (MDM2), Epidermal growth factor receptor (EGFR), Tumor protein 53 (TP53), C-MYC, Phosphatase and tensin homolog (PTEN) and Glioma associated oncogene 1 (GLI-1) primers were used. Primers were produced with Primer-BLAST software and Macrogen synthesized the primers. All reagents that were used in real time PCR procedure were summarized in table 2.5 and PCR settings were given in table 2.6. Total RNAs (NHAs, exosome-treated NHAs, U87-MG, A172) isolated via Trizol reagent and their concentrations were measured with NanoDrop. mRNAs were converted to cDNAs with transcriptor first strand cDNA synthesis kit prior to polymerase chain reaction (PCR) with SYBR Green method which are used to determine mRNA gene levels. Mixture of primers, cDNAs, SYBR-mix and PCR grade distilled water were prepared in a 10µl of final volume. GAPDH was used for housekeeping gene. RT-PCR assay was performed by using CFX96 RT-PCR system.

Table 2.5. Real time PCR primer sequences

Gene	Side	Sequence
P53	Forward	5’ GCCCAACAACACCAGCTCCT 3’
	Reverse	5’ CCTGGGCATCCTTGAGTTCC 3’
MDM2	Forward	5’ GGCTCTGTGTGTAATAAGGGAGA 3’
	Reverse	5’ GGACTGCCAGGACTAGACTTTG 3’
BAX	Forward	5’ TGCAGAGGATGATTGCCGCCG 3’
	Reverse	5’ ACCCAACCACCCTGGTGTTGG 3’
EGFR	Forward	5’ AATGCAACATCCTGGAGGGG 3’
	Reverse	5’ AGGTGATGTTCATGGCCTGG 3’
PTEN	Forward	5’ TGTGGTCTGCCAGCTAAAGG 3’
	Reverse	5’ ACACACAGGTAACGGCTGAG 3’
Akt	Forward	5’ GAAGCTGCTGGGCAAGGGGCA 3’
	Reverse	5’ GTGGGCCACCTCGTCCTTGG 3’
C-MYC	Forward	5’ AATGAAAAGGCCCCCAAGGTAGTTATCC 3’
	Reverse	5’ GTCGTTTCCGCAACAAGTCCTCTTC 3’
GLI-1	Forward	5’ AGGGAGGAAAGCAGACTGAC 3’
	Reverse	5’ CCAGTCATTTCCACACCACT 3’
GAPDH	Forward	5’ AAGGTGAAGGTCGGAGTCAAC 3’
	Reverse	5’ GGGGTCATTGATGGCAACAATA 3’

Table 2.6. Reagents of Real time PCR

Reagents	Volume
Maxima™ SYBR Green qPCR Master Mix	5 µl
Forward Primer	0,5 µl
Reverse Primer	0,5 µl
Distilled water	3 µl
Template	1 µl

Table 2.7. Settings for PCR

Cycle	**Repeats**	**Step**	**Duration**	**Temperature**
Initial Denaturation	1	1	3 minutes	93 °C
Denaturation	36	1	30 seconds	93 °C
Annealing		2	40 seconds	59 °C
Extension		3	45 seconds	72 °C
Final extension	1	1	10 minutes	72 °C
Melt curve	110	1	12 seconds	-0.5 °C/cycle
Hold	1	1	-	4°C

2.2.11. Transglutaminase 2 Elisa

NHA, GBM exosomes treated NHA, U87-MG and A172 cells were seeded in 6 well plate. After 48 hour, protein was isolated with freeze/thaw method. Firstly, Tris-buffered saline, 1%Triton X-100 and Protease inhibitor mixture was prepared and cell pellets were resuspended with this mixture. Then they were freezed at -80 °C and thawed on ice, this

process was repeated to 4-5 times. Samples were centrifuged at 22.000g for 15 min. When centrifugation was finished supernatant was collected in 1.5 mL eppendorf tubes and Pierce BCA Protein Assay Kit was used to determine the protein concentrations. In order to determine the amount of TGM2 in exosome treated NHA cells, Transglutaminase 2 Assay Kit was used. Briefly, each well was washed with 1X washing solution (150 µL/well) and incubated for 15 min at 37 °C. After that, assay mixture (10 µL DTT, 0.5 m L reaction buffer) was dispensed in wells and 50 µL EDTA was added for negative control. According to Table 2.8. 50 to 60 µL of ice cold Sample/Enzyme was added in corresponding wells then incubated for 20 min at 37 °C and shaken gently. After incubation, each well was washed with respectively wash buffer, 0.1 M NAOH and again twice wash buffer. SAv-HRP solution (SAv-HRP at 1/2 000 in Wash Buffer 1X) was dispensed in wells (100 µL/well) and incubated for 15 min at 37 °C. Then wells were washed three times with 200 µL/well of Wash Buffer 1X. HRP substrate was dispensed in each well and incubated for 3 min at room temperature. Blocking reagent was added and absorbance was measured by using plate reader at 450 nm [85].

Table 2.8. Reaction scheme for TGM2 Elisa

	Sample/Enzyme	Dilution Buffer	EDTA	Assay mixture	Final volume
Blank	-	60 µL	-	50 µL	110 µL
Test	12 µL(1:5 dilution)	48 µL		50 µL	110 µL
Standard curve	50 µL	10 µL	-	50 µL	110 µL
Negative control	50 µL	-	10 µL	50 µL	110 µL

2.2.12. Western Blot

Solutions prepared for western blot analysis is given in table 2.9. In order to understand the change in synthesized protein amount, total protein was isolated from cell pellets with RIPA buffer. To sum up, cell pellet was washed twice with ice cold PBS. While washing step, RIPA buffer cocktail was prepared with the addition of PMSF, protease inhibitor and sodium orthovanadate to RIPA solution (10µl each for every milliliter of RIPA solution). RIPA

cocktail was added into washed cell pellets and mixed carefully on ice. After the 10 minutes of incubation this mixture was centrifuged at 14.000 g for 15 minutes (4°C). Supernatant includes proteins which can be stored at -80°C. BCA assay was used to determine of protein concentrations and change in TGM2 amount in total protein were determined with western blot analysis. Proteins were loaded in Mini-PROTEAN Precast Gel with the protein ladder. After running the gel at 110 V for approximately 90 minutes gel was transferred to PVDF membrane with wet transferring method. After transfer membrane was blocked with TBST containing 5% Blotting-Grade Blocker for 1 hour. TGM2 specific antibody was applied in blocking solution overnight (4°C). Primary antibody was washed in TBST for 3 times and membrane was incubated with anti-rabbit secondary antibody for 2 hours at room temperature. Finally, after the washing step of secondary antibody, membrane was incubated with SuperSignal™ West Femto Maximum Sensitivity Substrate ECL for 30 seconds. GAPDH was used to control and images were taken by using the luminometer system.

Table 2.9. Western Blot Solutions

TBS-T	20mM Tris-HCl 150mM NaCl 0.1% Tween 20, pH 7.6
Running Buffer	25mM Tris base 190mM Glycine 0.1% SDS, pH 8.3
Transfer Buffer	25mM Tris base 190mM Glycine 20% Methanol
Blocking Buffer	5% Non-fat dry milk prepared in TBS-T

2.2.13. Live Cell Imaging

Live cell imaging system was used to observe the behavior of exosome-treated NHA cells. First of all, NHA cells labelled with red fluorescent membrane dye which is PKH26. Briefly, cells were trypsinized and centrifuged. Then, 4 μL PKH26 dye was mixed with 1 mL of Diluent C and pellet was resuspended via Diluent C. After that, they were blended and incubated at room temperature for 10 minutes. When incubation was finished, 2 mL FBS was added for blocking and incubated again for 1 minute at room temperature. After

incubation, cells were washed twice with PBS and pellet was solved wit cell culture medium. 24 hours later, labelled cells were treated with exosomes which are stained via PKH67 green fluorescent dye. Both labelled cells and exosomes were placed in live cell imaging microscope system for 48 hours.

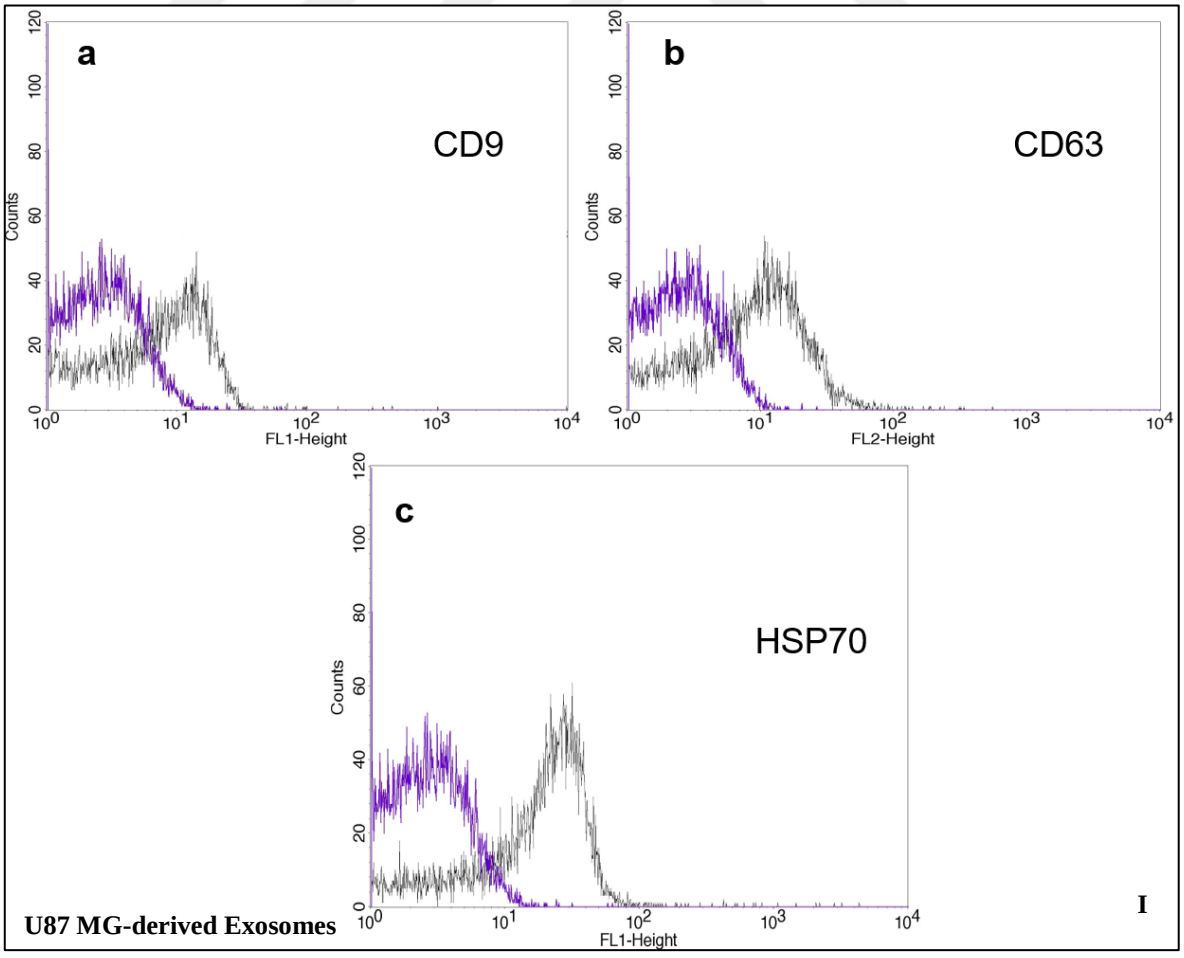
3. RESULTS

3.1. EXOSOME CHARACTERIZATION

Exosomes were characterized according to their shape, size, functionality and their markers by flow cytometry, NTA, SEM and uptake analysis.

3.1.1. Flow Cytometry

Flow cytometry was performed to identification and characterization of GBM exosomes. Three commonly exosome markers, CD9, CD63 and Hsp70, were found to be present on both U87-MG and A172 exosomes (Figure 3.1).



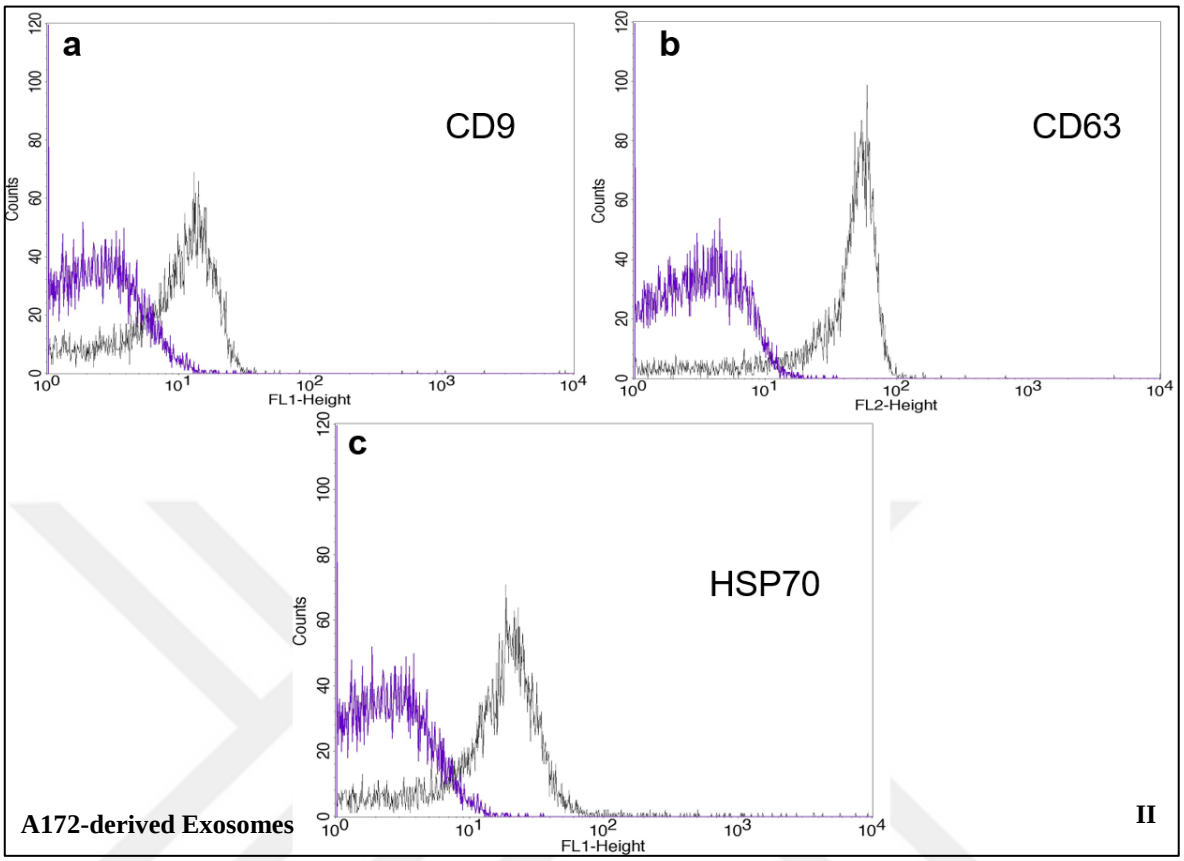


Figure 3.1. Identification and characterization of U87-MG (upper panel-I) and A172 (lower panel-II) exosomes by flow cytometry.

(a) Negative control (purple trace) and CD9 antibody labelled exosome (black trace), (b) Negative control (purple trace) and CD63 antibody labelled exosome (black trace), (c) Negative control (purple trace) and Hsp70 antibody labelled exosome (black trace)

3.1.2. Nanoparticle Tracking Analysis

NTA was used to measure the sizes, size determination and concentrations of exosomes. The mean size of U87-MG exosomes was 144.9 nm and the mean size of A712 exosomes was 147 nm (Figure 3.2).

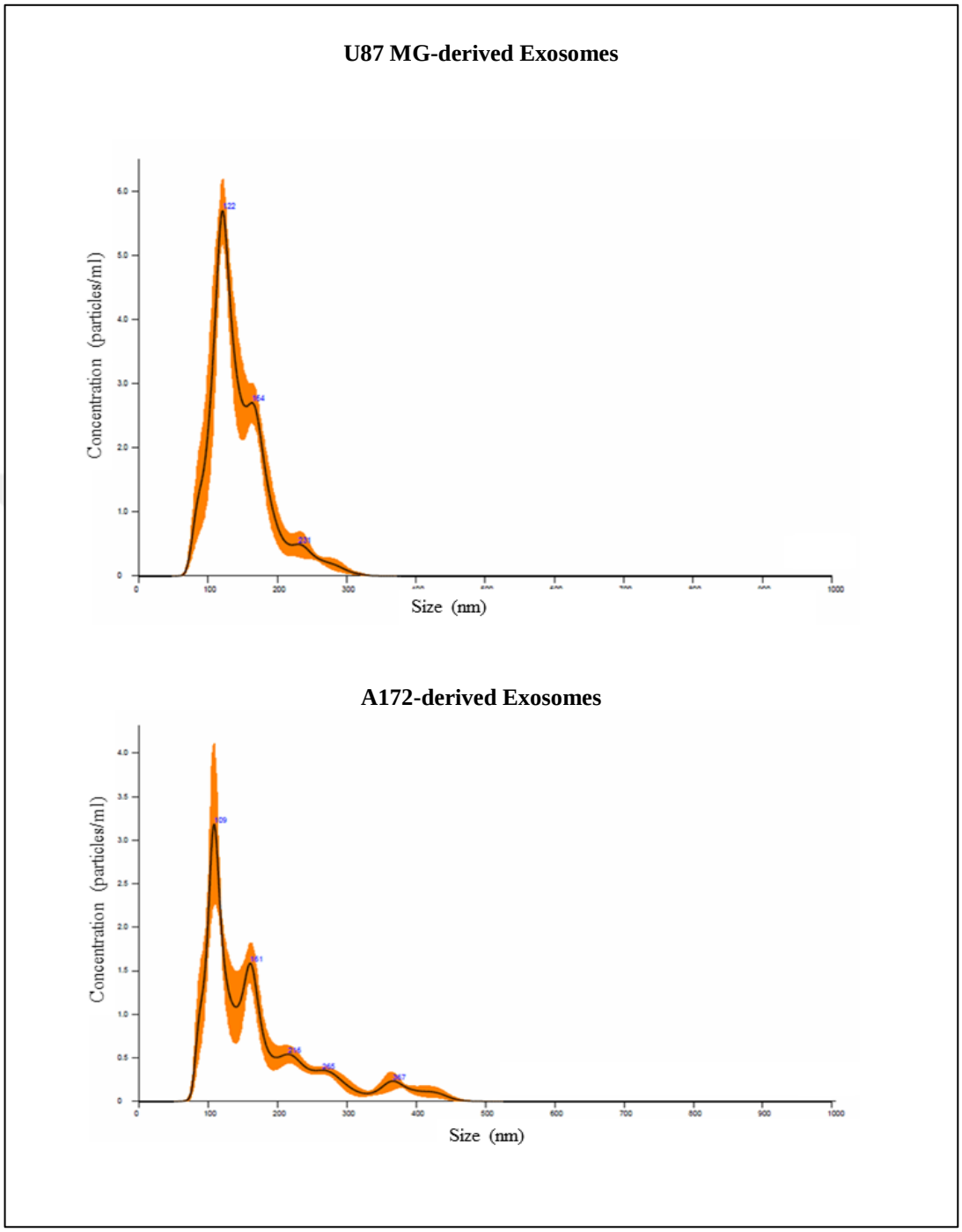


Figure 3.2. Analysis of sizes, size distributions and concetration of U87-MG (upper panel) and A172 (lower panel) exosomes as analyzed by NTA.

3.1.3. Scanning Electron Microscopy

In order to further characterize the exosomes, their morphology and size were determined by SEM. Scanning electron microscopy results were similar to NTA. Furthermore, the shape

of exosomes was observed as round with a mean size of ≈150 nm in both U87-MG and A172 exosomes (Figure 3.3).

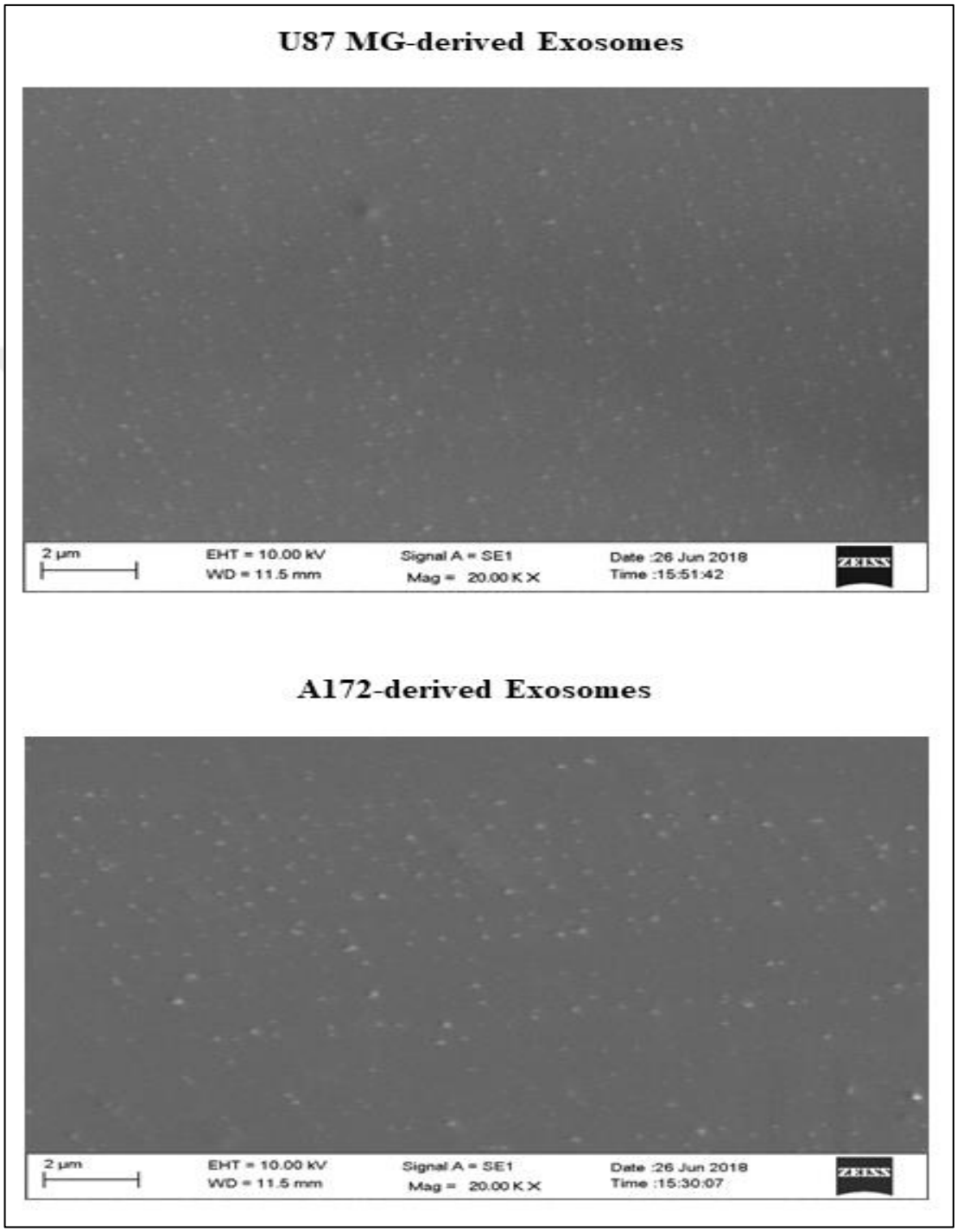


Figure 3.3. Scanning electron microscopy images of exosomes isolated from U87-MG (upper panel) and A172 (lower panel).

Upper panel: U87-MG cell line culture-derived exosomes, Lower panel: A172 cell line culture-derived exosomes. Size bar: 2 µm

3.1.4. Exosome Uptake Analysis

The exosome uptake analysis was performed to detect whether exosomes were functional. The results from fluorescence microscopy showed that NHA cells treated with the GBM exosomes demonstrated diffuse fluorescence. Normally, GBM exosomes were uptaken by NHA cells when heparin was not added. But when heparin was added, most of the GBM exosomes were not uptaken in by NHA cells. Heparin efficiently blocked uptake of GBM-derived exosomes (Figure 3.4).

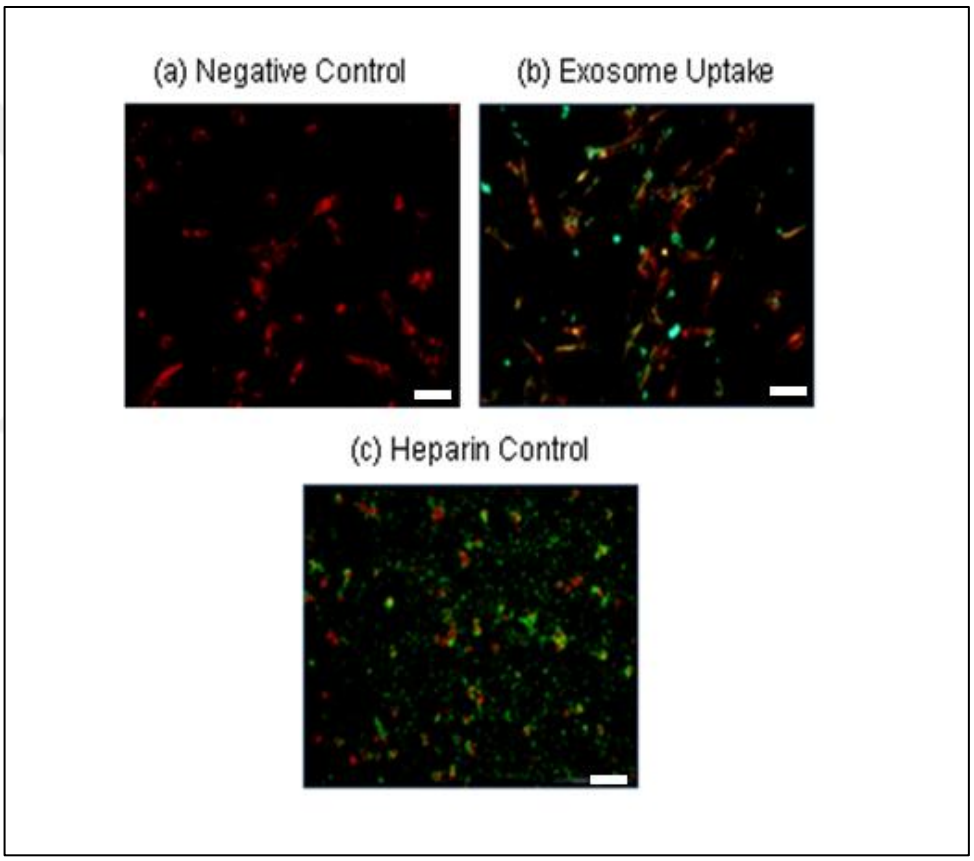


Figure 3.4. Flourescent micrographs of exosome uptake analysis (20X magnification,scale bar of 20 μm) (a)Negative Control, (b) Exosome Uptake, (c) Heparin Control

3.2. CELL VIABILITY

MTS assay was used to understand the effect of GBM exosomes on NHA cells for three days and different doses were tested. Results have been show that proliferation of GBM exosome

(20 µg/mL) treated cells were increased by approximately 30 percent in NHA cell lines after 2 days, respectively (Figure 3.5).

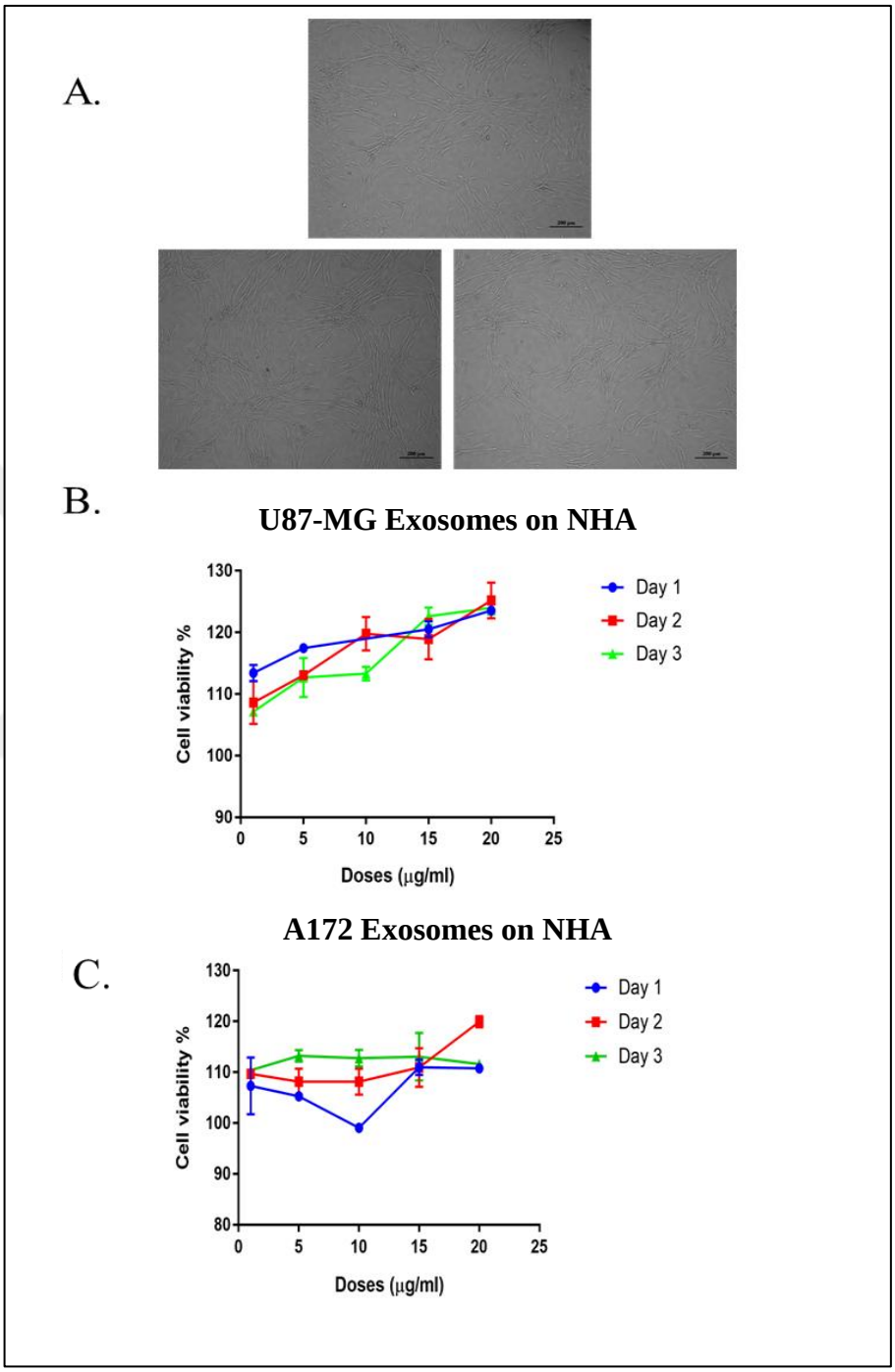


Figure 3.5. Effect of U87-MG and A172 exosomes on NHA.

(A) 5X magnified (scale bar of 200 µm) light microscopy image, (B) U87-MG exosomes effect on NHA, (C) A172 exosomes effect on NHA cells.

Y axis shows cell viability percentage and X axis shows doses.

Blue line: 24h, Red line: 48h and Green line: 72h

3.3. EDU CELL PROLIFERATION ASSAY

EdU assay was performed in order to support the MTS assay and similar results were obtained with MTS. The results show were by two way, first images were taken by 5X magnified confocal microscopy. Secondly, cell proliferation changes were measured with absorbance rates at 495 nm for Andy Fluor 488 and 350 nm for Hoechst 33342 by using Varioskan LUX Multimode Microplate Reader. DAPI positive cells were proportioned to EdU positive cells and the changes were calculated in cell proliferation. Results were analyzed by t test by using Graphpad Prism. According to EdU assay, cell proliferation was increased by approximately 30 percent (Figure 3.6).

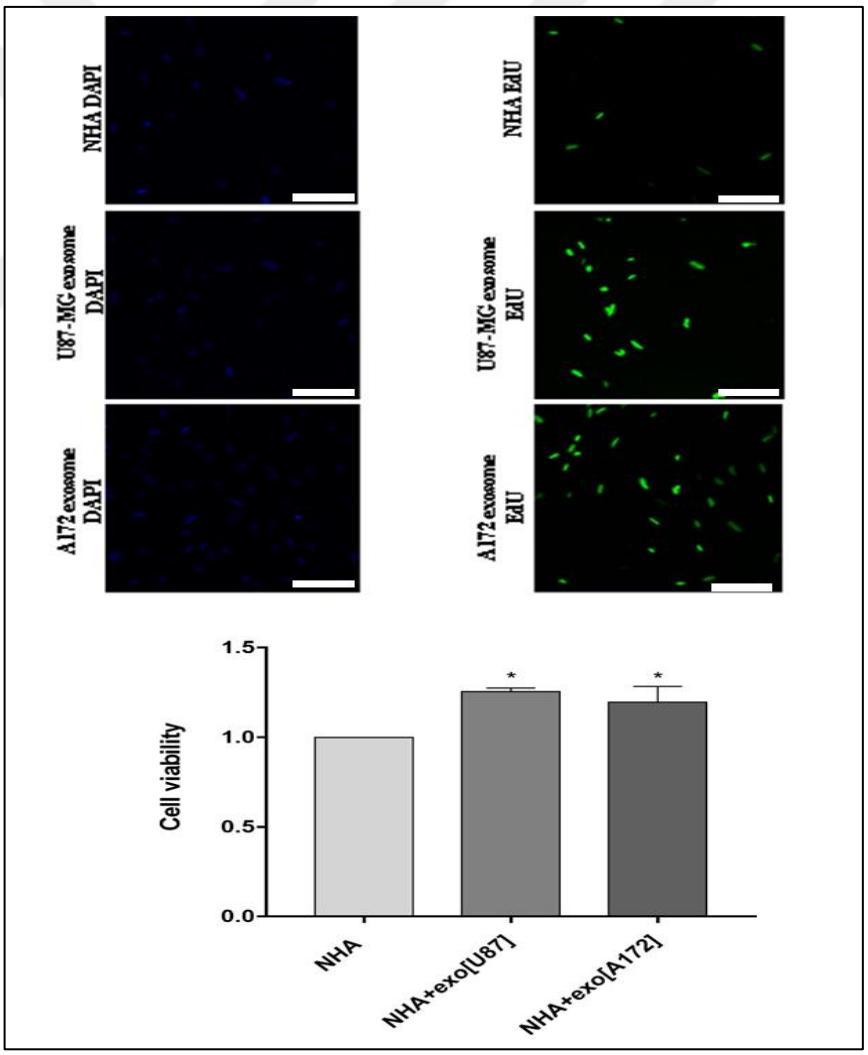


Figure 3.6. Effect of GBM exosomes (U87-MG and A712) on NHA cells proliferation. *P<0.003. Upper panel shows confocal microscopy images. Lower panel demonstrates selected GBM-derived exosomes dose's effect on NHA cells.

3.4. CELL CYCLE

Cell cycle assay was performed to understand the effect of GBM exosomes on the cell cycle and all changes of cell cycle profile were observed after U87-MG and A172 exosomes treatment. According to results, U87-MG exosomes were significantly increased the division of cell division at M phase 3.07 percent for control (NHA cells), 9.19 percent for U87-MG exosome treated cells. Also, A172 exosomes were increased the cells at M phase when compared to control NHA cells (3.07 percent for control, 6.19 percent for exosome-treated cells) (Figure 3.7).

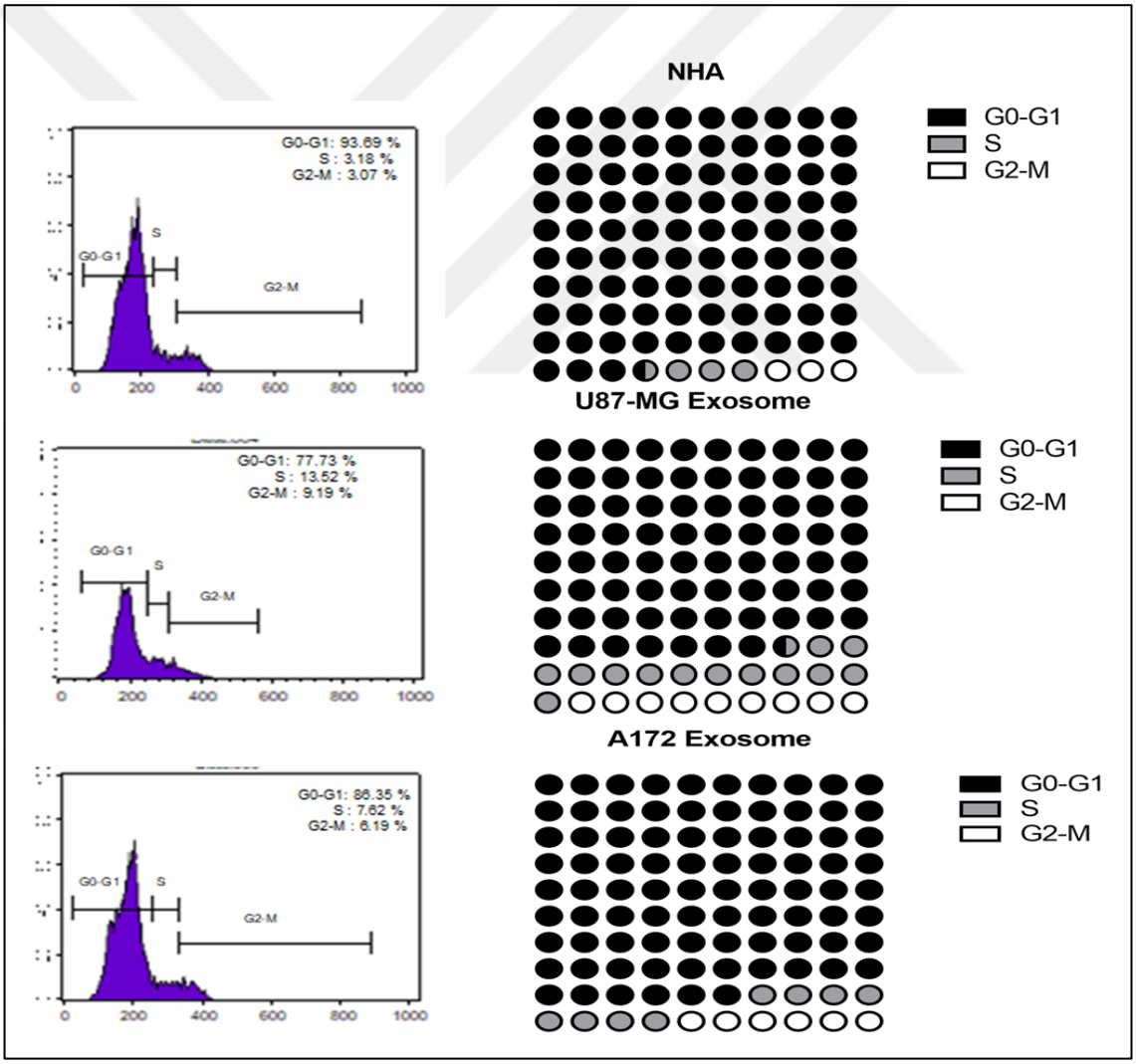


Figure 3.7. Graph of the GBM exosome's effect on cell cycle profile of NHA cells.First graph shows non-treated NHA cells, Second graph shows U87-MG exosomes-treated NHA cells, Third graph shows A172 exosomes-treated NHA cells.

3.5. REAL TIME PCR ANALYSIS

Gene expression levels of GBM exosomes treated NHA cells were determined by RT-PCR. All changes in gene expression levels were statistically analyzed by one-way ANOVA and Tukey's tests.

3.5.1. Gene Expression Effect of U87-MG Exosome on NHA cells

RT-PCR results have shown that U87-MG exosomes in NHA cells, have no significant effect on EGFR gene level as well as BAX. But, GLI-1 known as glioma-associated gene expression was increased significantly (≈5 fold).

Also, TP53 (≈2.7 fold), PTEN (≈2.8 fold), Akt (≈2.5 fold), C-MYC (≈2.9 fold), MDM-2 (≈1.5 fold) expression levels were increased (Figure 3.8).

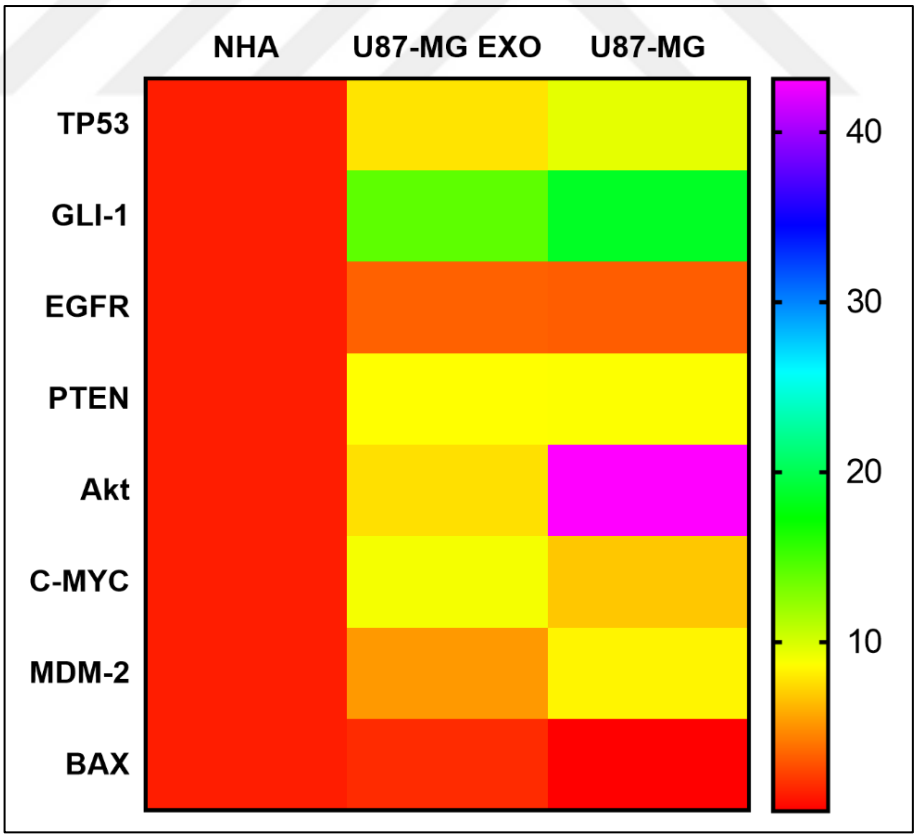


Figure 3.8. Heat map analysis of U87-MG Exosome effect on gene expression profile of NHA.

3.5.2. Gene Expression Effect of A172 Exosome on NHA cells

A172 exosomes were more effective on gene expression levels than U87-MG exosomes. Especially, TP53 (≈7.38 fold), GLI-1 (≈3.8 fold), EGFR (≈5.2 fold) and PTEN (≈5.1 fold) gene expressions were significantly increased. However, A172 exosomes have no significant effect on Akt (≈1.78 fold), C-MYC (≈1.98 fold) and MDM-2 (≈1.19 fold). Also, BAX (≈0.82 fold) gene expression level was decreased (Figure 3.9).

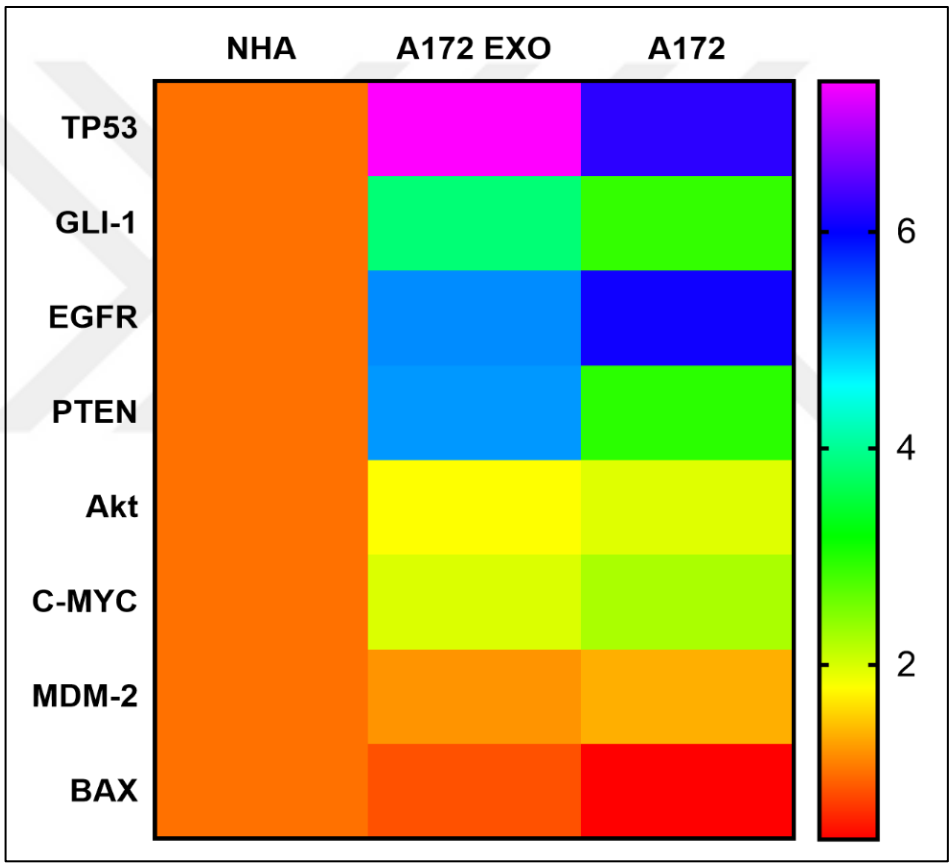


Figure 3.9. Heat map representation of A172 Exosome effect on gene expression profile of NHA.

3.6. TRANSGLUTAMINASE 2 (TGM2) ELISA ASSAY

Transglutaminase 2 elisa assay was implemented to measure the amount of TG2 in glioblastoma exosomes-treated NHA cells. Results revealed that when NHA cells treated with GBM exosomes, TGM2 expression was increased. The amount of TG2 was

approximately doubled in exosomes treated cells when compared with normal astrocyte cells. (Figure 3.10).

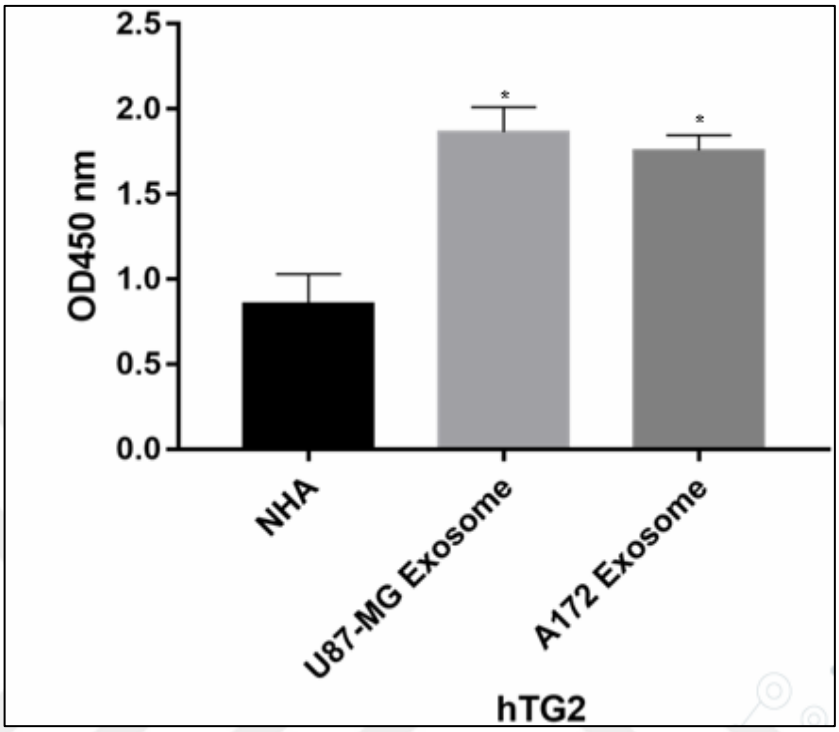


Figure 3.10. hTG2 amount in NHA cells and GBM exosomes treated NHA cells. *P<0.05

3.7. WESTERN BLOT

The changes in TGM2 protein level after exosome treatment was demonstrated with western blot. According to the results, total TGM2 protein level was increased in exosome-treated cells (Figure 3.11).

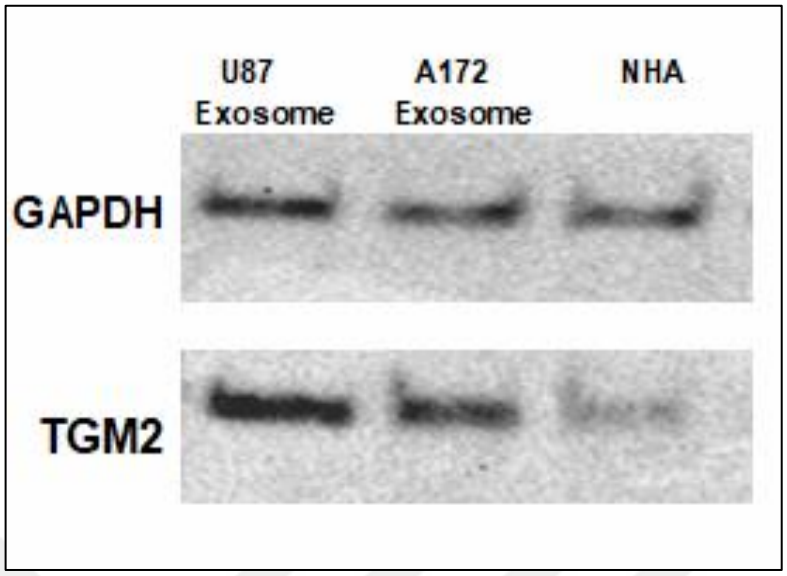


Figure 3.11. Total TGM2 protein levels of NHA and GBM exosome treated NHA cells. GAPDH was used as a control.

3.8. LIVE CELL IMAGING

To demonstrate the effect of GBM-derived exosomes on NHA cells' behaviour, cells were monitored for 48 hours following the exosome treatment by using live cell imaging microscopy. According to the results, exosomes obtained from tumor cells were uptaken by astrocyte cells. Upon the uptake of the exosomes, an increase in mobility and division of the astrocytes was observed. The effect of U87 MG-derived exosomes (first video) and A172-derived exosomes (second video) on astrocyte cell was shown in videos. In videos NHA cells were stained with red fluorescent dye (PKH26) and exosomes were labelled with green fluorescent dye (PKH67). Video files can be found attached to the CD of the thesis.

4. DISCUSSION

GBM is mainly common type of brain cancer and every year, nearly 3 people out of 100.000 become affected by the disease. However, there is not enough study about diagnosis and treatment of glioblastoma according to other tumor types. Because, there are features that distinguish glioblastoma from others. First of all, GBM is a type of cancer that can keep the brain diffuse. GBM cells can be seen even in far away from the diseased lesion site, even against the hemisphere in normal brain parenchyma. Another feature is that they make rare metastasis out of the brain. In summary, GBM is limited to the brain, but it is a diffuse disease [2, 86].

Exosomes, which are the nanovesicles released by all known cells in our body, have not been sufficiently focused on explaining the diffuse properties of GBMs. In recent studies, these vesicles have been shown to have significant physiological functions beyond being only cell debris responsible for removing unwanted molecules out of the cell [87]. Exosomes, which can be obtained from whole body fluids, have crucial roles in many biological processes such as intercellular communication, signal transduction, transfer of genetic material and regulation of immunological response [88]. For this reason, the effects of exosomes on disease pathogenesis are discussed. Furthermore, exosomes may also carry mRNA and miRNA in addition to proteins and lipids. The contents of exosomes secreted from cancer cells, including glioblastoma, have been investigated in several studies and their potential to be both physiological and diagnostic biomarkers have been emphasized [89]. However, there is no detailed study on exosomes in explaining the invasion of the gliomas to the adjacent tissue [45, 54, 63].

The purpose of this study was to investigating role of the exosomes in glioblastoma multiforme invasion. They have important roles in invasion and metastasis of cancer. In this study, effect of exosomes from glioblastoma cell lines (U87-MG and A172) was investigated on normal human astrocyte (NHA) cells.

Exosomes are small nanovesicles and they have characteristic features such as round shape, maximum of 150 nm size and the presence of specific markers for example CD9, CD63 and Hsp70. Our results were consistent with these properties, because we obtained the

characteristic round shape and size for exosomes derived from the U87-MG (≈144.9 nm) and A172 culture medium (≈147 nm) by SEM and NTA. Besides, we determined the expression of the exosome markers CD9, CD63 and Hsp70 in our both exosomes. These findings support that the vesicles isolated from U87-MG and A172 cells correspond to exosomes [90].

Previous studies have shown that heparin blocks the uptake of exosomes into the cell and also they demonstrated that heparin could be used to prevent uptake of exosomes with the potential that this could be used as a tool to study exosome function. Also heparin can be used to develop a therapy for diseases in which exosomes play a role in pathogenesis such as glioma. Our results are parallel to these studies, when heparin was added, exosomes could not enter the cells [91].

As is known, cancer is a disease caused by unbounded proliferation of cells. Previous studies have supported that tumor-derived exosomes were mediated to cell proliferation and metastasis. In this study, GBM-derived exosomes were tested on NHA cells to investigate the effect of cell division and proliferation. The obtained results supported the literature. The proliferation and division of NHA cell line were enhanced when they were treated with U87-MG exosomes. Similarly, exosomes from A172 cells were caused an increased in NHA cells, compared to non-treated cells [92].

One of the most important features of cancer is uncontrolled cell division. The mistakes in the adjustment of cell cycle cause disruption of the control of the cell division. Changes in cell cycle control points can induce cancer development. Cell division occurs in the M-phase therefore GBM exosome-treated cells were expected to increase in this phase. Obtained results demonstrated that exosome treatment was increased percentage of M phase [93, 94].

There are aberrations in the expression of different genes in glioblastoma such as TP53, PTEN, EGFR, Akt, C-MYC, MDM2 and GLI-1 [95]. Dysregulation of these genes have also been shown to increase tumor growth, invasion, migration, proliferation and evasion of apoptosis [20]. The most main mutations in gioblastoma are TP53 (40 percent), EGFR (37 percent) and PTEN (30 percent). According to obtained results, TP53 and PTEN expressions were enhanced in U87-MG exosome-treated NHA cells but there was no significant change in EGFR gene level. The exosomes from A172 were markedly increased of TP53, PTEN and EGFR gene levels. Therefore, A172-derived exosomes were more effective than U87-

MG. Furthermore, deregulation of GLI-1, C-MYC, MDM2 and Akt genes were caused of tumorigenesis. GBM-derived exosomes were enhanced the levels of these genes, especially GLI-1 expression was significantly risen in both U87-MG and A172 exosome-treated NHA cells [96–101]. Apart from these there are apoptotic genes involved in cancer, Bax is one of them. The expression of Bax gene in both GBM exosomes was reduced and this was an expected result. Because, our aim was to increase the proliferation of cancer exosomes-treated NHA cells [102].

In recent years, one of the proteins that act a significant role in many types of cancer is tissue transglutaminase (TG2 / TGM2). Cancer progression is associated with inflammatory response, tissue damage and wound healing. Inflammatory diseases have common properties such as increased TG2 expression and transamidation activity. Therefore, various cytokines and growth factors released during tissue injury and wound healing are the most important inducers of TG2 expression. Inflammatory responses play an important role in tumor onset, progression, invasion and metastasis. Immune cells infiltrate the tumor, communicates with cancer cells and allows its growth, survival and development. As a result, transglutaminase (TG2) is high in cancer which is expressed is a protein associated with drug resistance, metastasis and low survival. Additionally, studies have played an important role in the proliferative process of glioblastoma of transglutaminase. In light of this information, the results demonstrated that the amount of TG2 protein was remarkably increased in GBM exosome-treated astrocyte cells. The amount TG2 in untreated cells was about half of those treated with exosomes [103].

In this study, we found out cancer-derived exosomes play important role in malignant transformation of astrocytes and progression of GBM. However, exosomes will open a new approach in the desperate search of therapeutic options for glioblastomas.

In summary, GBM-derived exosomes effect of normal astrocyte cells, but mechanisms that causing changes in astrocytes have not been exactly elucidated. *In vivo* experiments could be necessary to better understand the role of exosomes on glioblastoma multiforme invasion.

5. CONCLUSION

Exosomes are important players in the formation and progression of cancer due to their biological and morphological properties. The fact that they differ according to the types of cells they originate and can be obtained from all fluids in the body is a significant advantage and potential marker for cancer-related studies.

Our findings support that GBM-derived exosomes are capable of molecular changes in NHA cells, in this way they were caused similarities with cancer. By understanding the role of exosomes in progression of GBM, it will pave the way for future studies in developing efficient therapy methods against the disease.

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