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Research Article

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The Exosome Isolation and Characterization From Dld-1 Cell Line Media

DLD-1 Hücre Besiyerinden Elde Edilen Eksozomların İzolasyonu ve Karakterizasyonu

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ABSTRACT

Exosomes, as extracellular organelles, are the most well-known vesicles among microvesicles. They are released from almost all body fluids as well as cells. Exosomes, which have gained popularity in biology studies with their nano-level dimensions (30-200nm) and membrane structures, are primarily used in drug encapsulation, regeneration, and cell-free cellular therapy. The fact that exosomes, which also function as biomarkers, have an ever-expanding area of use has led researchers to search for the isolation and characterization of this vesicle. In this study, the isolation of exosomes of the DLD-1 colorectal cancer line was carried out by ultracentrifugation method and characterized biophysically and biochemically by SEM, NTA, and flow cytometry, and the proliferative effect of these exosomes on HT29 cells, one of the other colorectal cancer lines, was investigated. As a result, it was proved that the isolated vesicles were morphological and size-appropriate to the literature and expressed CD63 and CD81 proteins. It was observed that DLD-1 exosomes increased proliferation on the HT29 cell line in a dose-dependent manner and 3.134µg exosomes were the statistically most effective dose. Within the framework of the findings, this study could be an excellent reference for exosome isolation and characterization.

Key Words

Exosome, isolation, characterization, proliferation effect.

ÖZ

Hücre dışı organel olarak kabul edilen eksozomlar, mikroveziküller içinde en çok bilinen veziküldür. Hücrelerin yanı sıra hemen bütün vücut sıvılarından salınır. Nano düzeydeki boyutları (30-200nm), membranlı yapılarıyla biyoloji çalışmalarında popülerlik kazanan eksozomlardan başta ilaç enkapsülasyonu, rejenerasyon, hücresiz hücresel terapi gibi alanlarda yararlanılmaktadır. Biyobelirteç işlevi de gören eksozomların günden güne genişleyen bir kullanım alanına sahip olması, araştırmacıları bu vezikülün optimum izolasyon ve karakterizasyonuna yönelik arayışlara yönlendirmiştir. Bu araştırmada DLD-1 kolorektal kanser hattına ait eksozomların izolasyonu ultrasantrifüj yöntemiyle, karakterizasyonu ise SEM, NTA ve akım sitometrisi kullanılarak gerçekleştirilmiş, bu eksozomların diğer kolorektal kanser hatlarından biri olan HT29 hücreleri üzerindeki proliferatif etkisi incelenmiştir. Sonuç olarak izole edilen veziküllerin morfolojik ve boyut olarak literatüre uygun olduğu ve CD63 ve CD81 proteinlerini ifade ettiği kanıtlanmıştır. DLD-1 eksozomlarının HT29 hücre hattı üzerinde doza bağlı olarak proliferasyonu arttırdığı görülmüştür ve 3.124µg eksozom istatistiksel olarak en etkili doz olarak belirlenmiştir. Ulaşılan bulgular çerçevesinde, bu araştırma DLD-1 eksozomlarının izolasyon ve karakterizasyonu için referans bir çalışma olabilir.

Anahtar Kelimeler

Eksozom, izolasyon, karakterizasyon, proliferatif etki.

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INTRODUCTION

Exosomes are plasma membrane-derived vesicles with phospholipid bilayer membranes as mini parental cells and extracellular organelles. As early as the 1980s, exosomes were identified from harvesting sheep reticulocytes using ultracentrifuged [1]. They are the most known and studied extracellular vesicles and all body fluids secreted from cells. Their primary role is cell-to-cell communication and cargo vesicles containing DNA, RNAs, lipids, proteins, and bioactive materials and are accepted as a growth factor cocktail. Exosomes are enriched in proteins such as heat shock proteins (HSP70, HSP90), tetraspanins (CD9, CD63, CD81, CD82), multivesicular body formation proteins (Alix, TSG101), and membrane transport proteins (Annexins, Rab) [2, 3]. With their nano size of 30-200 nm, they were recently used in nano-biotechnology. Exosomes have been more critical in drug encapsulation studies, cancer biomarkers, oncobiology, and cell-free cellular therapy.

Cancer is one of the health problems that cause uncontrolled cell growth. It is known that cancer cells need metastases to develop. Some theories about cancer metastases are not fully understood [4]. These days, cancer-derived exosomes play a critical role in cancer biogenesis and metastases because the cancer-derived exosomes can connect with tumor cells, epithelial cells, and host immune cells. Cancer-derived exosomes manipulate the other cells to form tumor bodies and cancer metastases and present a new mode of intercellular communication, cellular process, and cellular cargo [5, 6]. So, their isolation and surface marker characterization are crucial in exosome research.

Colorectal cell carcinoma, the most common type of cancer that affects both genders, is seen in one to every ten cancer patients. Approximately 600,000 people die annually from metastatic colon cancer [6, 7].

In this study, we isolated and characterized DLD-1 colorectal cancer cell exosomes biophysically and biochemically. Then, we analyze the DLD-1 cell-derived exosomes on the HT29 colorectal cell line using MTT.

MATERIALS and METHODS

Cell Culture and Exosome Isolation

DLD-1 colorectal adenocarcinoma cell line was used as a cell source. Cells cultured in 10% FBS, 1% L-Glutamine, and 1% penicillin-streptomycin contained high glucose DMEM (4500 mg/ml) as a growth medium for cell growth supporting at 75cm^2 flasks in humidified 37°C CO_2 incubator. When cells reached 85-90% confluency, the medium was aspired, and cells were washed two times with dPBS to avoid FBS contamination. Then, the FBS-free growth medium was added to cells and incubated in the same conditions for exosome enrichment. After 24 hours, cell media was collected in a conical bottom centrifuged tube and centrifuged to remove the cells and cell debris at 300x*g* for 30 minutes at 4°C. Then, the cell supernatant was filtered through a 0,22µm syringe filter to remove contaminating apoptotic bodies, microvesicles, and cell debris. Clarified cell media was transferred into 5 ml tubes (HIMAC, #332245a) and centrifuged at 120.000xg_{avg} for 90 minutes at ultracentrifuge (HIMAC, CP100NX) with swinging bucket rotor (P55STN2) to pellet exosomes. Then, the supernatant was carefully aspired, and the pellet was resuspended with 1 mL cold dPBS. The pellet was centrifuged again in the same conditions to remove residue proteins [8]. After the last centrifuge, the exosome pellet was resuspended with dPBS in a sterile tube, and the sample was kept at -80°C for further exosome analysis.

Exosome Characterization

Samples were thawed on ice for the characterization test. First, scanning electron microscopy (SEM) analysis was performed to image the exosome morphology. For this, 30 µl exosome samples were dropped onto a clean microscopic slide and dried in a sterile laminar air flow hood. The dry exome samples were fixed with 2.5% glutaraldehyde, washed with dPBS, then dried again and coated with Au-Pa for SEM. Then, samples were visualized and imaged.

Nanoparticle analysis (NTA) was performed for exosome amount and dimensional distribution. Samples diluted 1:1000 in ddH2O. The samples were put in a chamber device NTA (Malvern, USA), and a video was recorded at 25 frames. The video was analyzed with NTA software version 3.4.4.

The last and most crucial characterization of exosomes is the surface marker as biochemically analysis by flow cytometry. The exosome's protein concentration was measured using BCA protein analysis (BCA kit, Thermo Fisher) as the direction's manufacturer for this test. Furthermore, a 50-µg exosome sample was used for analysis. For bead binding, 10 µl 4% aldehyde/sulfate

latex beads were added to the 90 µl exosome sample and incubated overnight at +4°C. Then 2M, 90µl glycine was added and incubated at +4°C for 30 minutes. Then, samples were centrifuged at 20.000x*g* for 10 minutes. The pellet was resuspended with dPBS, and anti-CD63 and anti-CD81 antibodies were added to the samples and incubated for one hour at room temperature in the dark. Then, samples were washed and centrifuged to remove unbinding antibodies. Exosomes were analyzed with flow cytometry (FacsAria III, BD). FlowJo performed flow cytometric data analyses and generation of flow plots.

Proliferation Assay

For test DLD-1 cell exosome proliferation, the HT29 colorectal carcinoma cell line was used. HT29 cell line was seeded in 10% FBS, 1% L-Glutamine, and 1% penicillin-streptomycin contained high glucose DMEM (4500 mg/l glucose) as a growth medium in a 75cm^2 flask with the same conditions as DLD-1. When cells reached up to 80-85%, cells were washed with dPBS and harvested with 0.05% trypsin/EDTA for three minutes. Then, cells were centrifuged at 300x*g* for five minutes. The pellet was resuspended with fresh growth medium, and cell count by thoma cell counter chamber. Furthermore, 3x10^3 cells were seeded in 96 well plates in 200 µl. The day after, 100µg DLD-1 cell exosome was added to the wells and diluted serially (100µg, 50µg, 25µg, 12.5µg,6.25µg, 3.125µg) and incubated for 24 hours. Then, 20 µl MTT solution (0.5mg/ml final concentration) was added to the wells. After three hours, formazan crystals were observed, and well contained aspired carefully, and 100 µl DMSO was added to the wells to solve formazan crystals. Then, the wells' absorbance was measured at 560nm at an ELISA plate reader (Promega, Glomax). The statistical evaluation was made using a t-test.

RESULTS and DISCUSSION

Cell Culture and Exosome Isolation

DLD-1 cells were cultured, and exosomes were isolated without contamination or other problems. SEM, NTA, and Flow Cytometry analysis were performed to characterize vesicles as exosomes. SEM images show that the isolated vesicles are cup-shaped, membraned, and tented to collapse because they are hydrophobic (Figure 1). It measured the exosome size from the SEM images, and according to the SEM results, exosome vesicles were 90.162nm±17,2 nm.

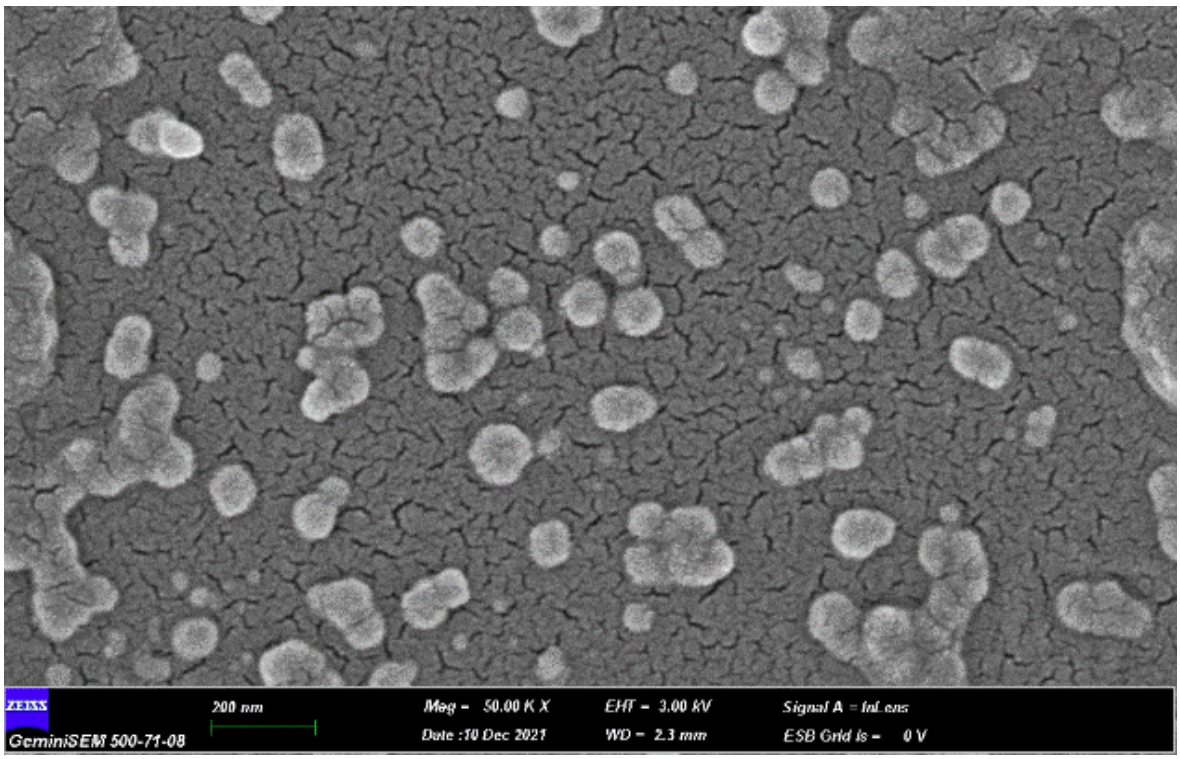


Figure 1. SEM images distribute exosome morphology.

NTA results show that isolated vesicles' mean mode size is 155nm±76nm. According to the results, the particle diameter corresponding to 10% cumulative small size distribution (D10) was 102.6, the median diameter of the particle size distribution (D50) was 138.9nm, and the particle diameter corresponding to (D90) was 216.3 nm (Figure 2). Nanoparticle concentrations were measured as $8.96x10^7$ particles/mL. These results aligned with literature presenting exosome size between 30-200 nm [9]. The difference between the SEM and NTA could be the sample preparation. SEM was performed at dry ambient, and aqueous ambient was used for NTA.

We performed the flow cytometry analysis to determine the surface markers of vesicles. According to our results, the isolated vesicles were CD63 and CD81 positive ($C_{autoflorescence}$:2143; C_{CD63}:2557; C_{CD81}:3810) (Figure 3). This flow cytometry analysis proved that isolated vesicles were exosomes. Cells or body fluids release a lot of extracellular vesicles. Several kinds of extracellular vesicles are secreted from cells: exomeres, exosomes, ectosomes, apoptotic bodies, migrasomes, large oncosomes, prostasomes, promininosomes, telorosomes. These are generally called "communicasomes" [10-13]. Exosomes are the most studied vesicles among them, and there are several methods to isolate the exosomes, such as ultracentrifuged, density gradient centrifugation, precipitation, and ultrafiltration. However, studies show that none of the isolation methods can isolate the extracellular vesicles to homogeneity [14,15]. So, it is essential to characterize the exosomes' biochemical and biophysical techniques [16-18]. Some proteins are specific to exosomes, like CD63, CD81, CD9, HSP70, and HSP90. These proteins could help to distinguish exosomes from other membrane vesicles [19-21]. This study characterized the cell culture medium-derived exosomes using biophysical (SEM, NTA) and biochemical techniques (Surface markers CD63 and CD81).

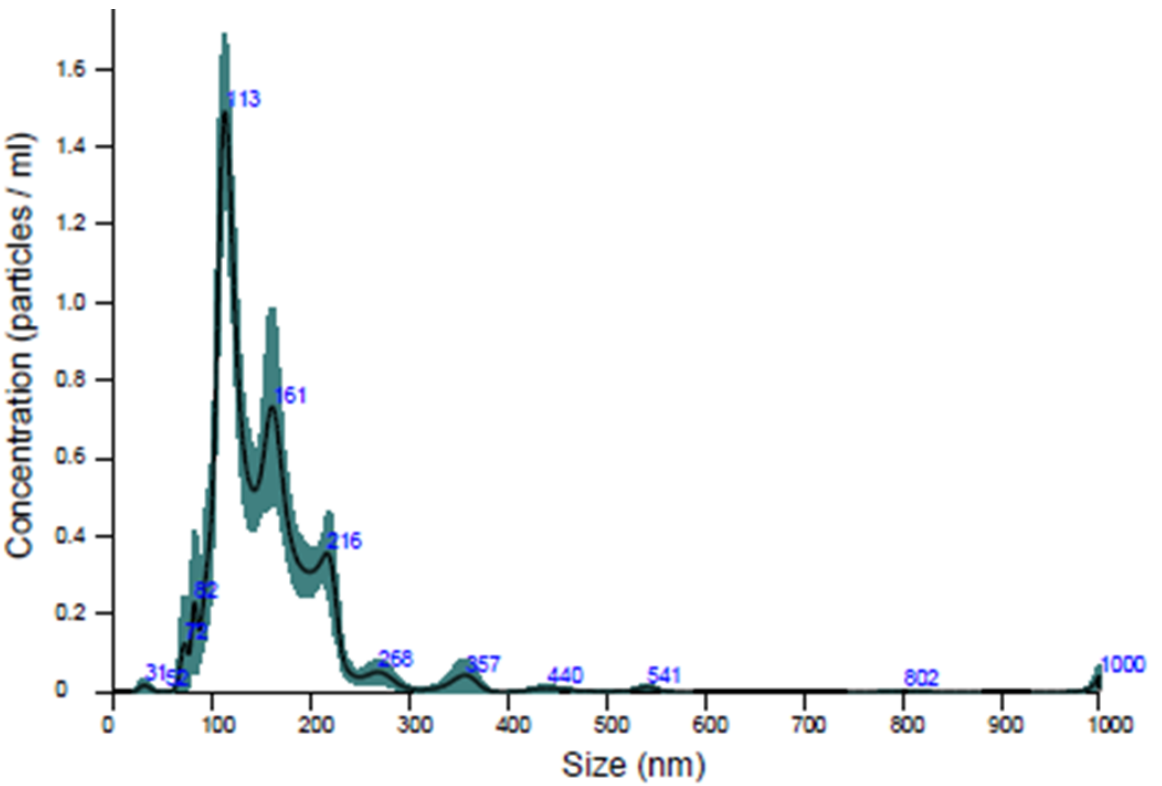
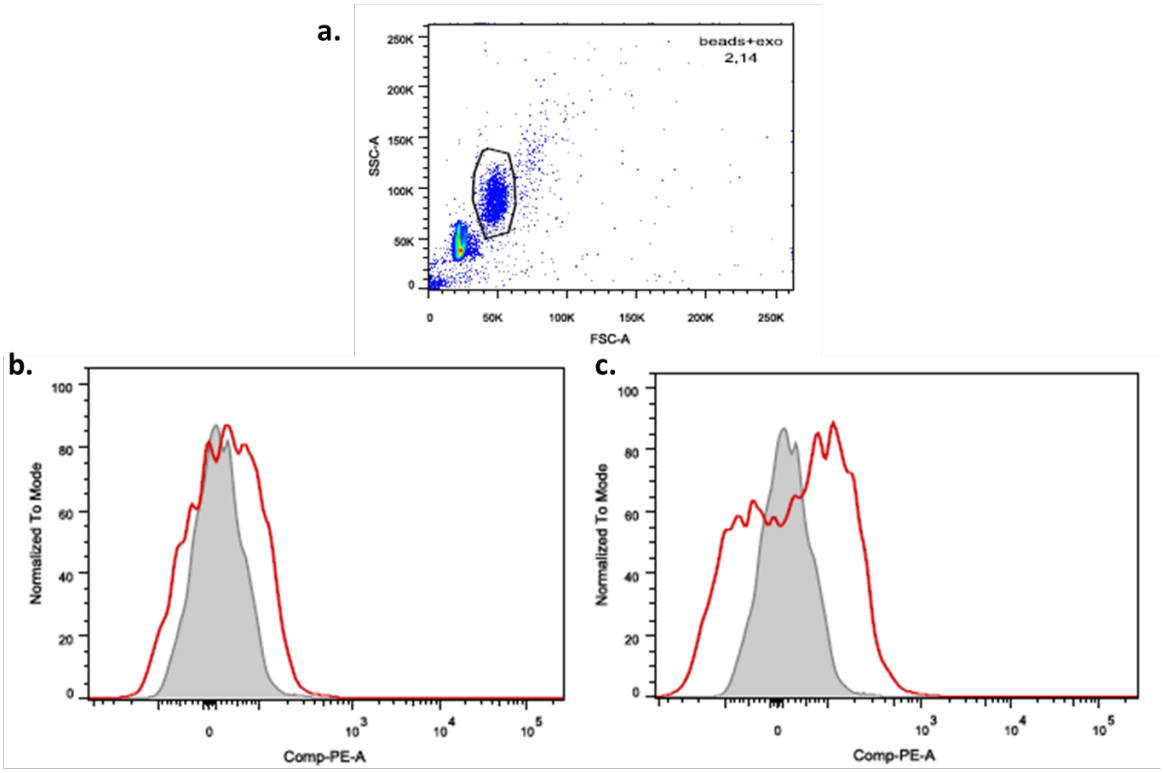


Figure 2. NTA results present concentration of exosomes.



	Sample Name	Subset Name	Count
☐	Specimen_001_L U 63_014.fcs	beads+exo	2557
■	Specimen_001_L U OTO_002_018.fcs	beads+exo	2143

	Sample Name	Subset Name	Count
☐	Specimen_001_L U 81_02_009.fcs	beads+exo	3610
■	Specimen_001_L U OTO_002_018.fcs	beads+exo	2143

Figure 3. Representative flow charts of autofluorescence, CD63+, CD81+ exosomes. a. Cytogram represent exosome vesicles with beads and black rounded exosomes gated as florescence labelled exosomes on beads. b. Cytogram represent CD63 positive exosomes. The gray scale is autofluorescence and red one is the positive for CD63. c. Cytogram represent CD81 positive exosomes. The gray scale is autofluorescence and red one is the positive for CD81.

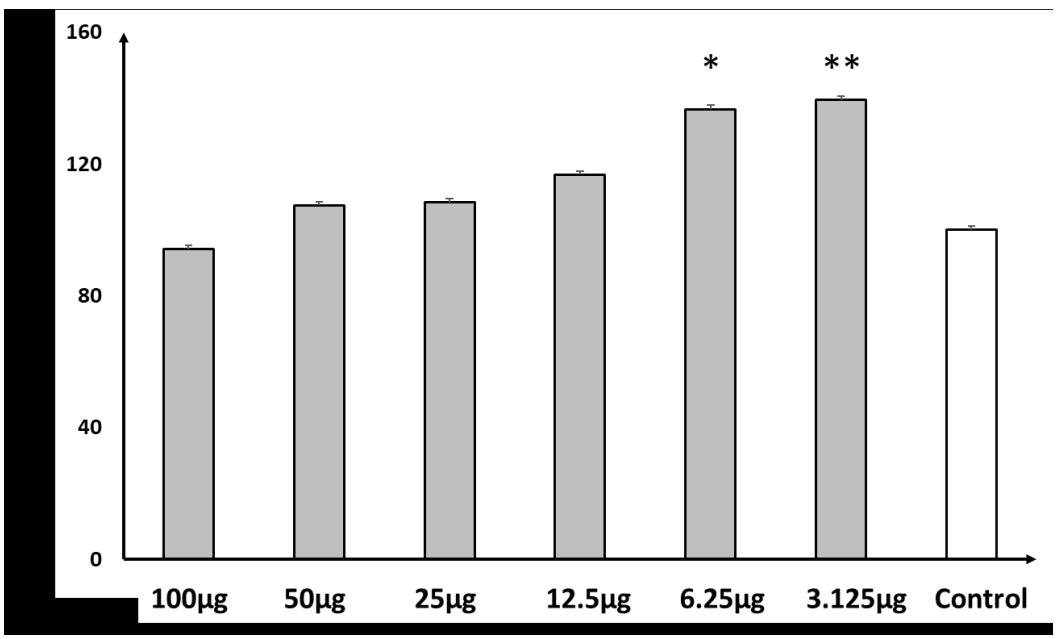


Figure 4. MTT results on HT29 cell line with DLD-1 exosome incubation for 24 hours.

The MTT test checked the effect of DLD-1 exosome on the HT29 cell proliferation. The results showed that DLD-1 exosomes induced HT29 cell proliferation dependently (Figure 4). The increase in effect as the dose decreases may be due to exosome agglomeration. Because it is known that agglomerated nanoparticles negatively affect the interfacial properties of nanoparticles [22]. There was a statistical difference between the control and 100µg exosome loaded group (P<0,05); 6.25 µg and 3.124 µg loaded cells' proliferation statistically induced ($P_{6.25\mu g}$<0,05; $P_{3.125\mu g}$<0,01). The MTT results demonstrated that the isolated exosomes were biologically active and could affect the other cells. It is concluded that the cancer-derived exosomes induced other cancer cell proliferation, which may cause secondary cancer cases by metastases.

CONCLUSION

Recently, extracellular vesicles have been considered a new cell communication player. With their nanosized exosomes, they are accepted as the next small, big thing. Exosomes are widely used for cell therapy, drug carrier systems, and biomarkers. This study isolated the DLD-1 exosomes and characterized them biophysically and biochemically. Moreover, it proved that cancer cell-derived exosomes stimulate other cancer cells in vitro. Our limitations are to find out the oncosome biomarkers. Cancer metabolism and metastases can be found with further studies on exosomes.

Acknowledgments

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