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Role of tumour derived exosomes in manuring the metastatic niche in patients with lung cancer liver metastasis: Beyond seed soil hypothesis

Kanisha A. Shah [a], Shanaya S. Patel [b], Kinjal P. Bhadresha [a], Kaid Johar SR [c], Rakesh M. Rawal [a,*]

[a] Department of Life Science, Gujarat University, Ahmedabad, Gujarat, India
[b] Division of Biological and Life Science, Ahmedabad University, Ahmedabad, Gujarat, India
[c] Department of Zoology, Gujarat University, Ahmedabad, Gujarat, India

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ABSTRACT

Metastatic lung cancer is often diagnosed at a late stage and is widely known to metastasize to the liver in the Asian population, but the underlying mechanism still remains unclear. Tumour derived exosomes (TDEs) play an important role in metastasis and its contributions to the development of the pre-metastatic niche formation is of utmost importance. In this study serum, derived tumour exosomes form lung cancer liver metastatic patients showed active incorporation by A549 cells in a concentration and time-dependent manner and induced migratory/invasive properties. Moreover, it was observed that cellular uptake of exosomes was increased during G2/M phase stimulating the cells to enter cell cycle phases leading to cell proliferation. Further, we observed that E-cadherin, beta catenin, VEGFA, CDKN2A and TGFBR2 were differentially expressed in treated A549 cells demonstrating an important role of these TDEs in altering the tumour microenvironment. *In vivo* model demonstrated an increase in serum SGPT and SGOT levels whereas the histopathological examination showed patches of pneumonitis in lungs and advanced inflammation in the liver. Conclusively, our results depict an undisputable role of these exosomes as key modulators in the formation of the pre-metastatic niche required for the colonization of circulating tumour cells (CTCs) ultimately leading to distant metastasis.

1. Introduction

Lung cancer is one of the most common tumours leading to cancer-related death worldwide [1]. The 5-year overall survival rate is nearly 80% in patients with stage I non-small cell lung carcinoma. As a matter of fact, approximately 75% of lung carcinomas are diagnosed as locally advanced or metastatic disease (stage III or IV), are associated with poor disease survival (37 and 6%, respectively) and is a leading cause of death [2,3]. It has become well known that lung cancer metastasis is not a random process. Moreover, it has been observed that in the Asian population, lung cancer metastasizes most commonly to the liver. However, patients with untreated liver metastases have a median survival of nearly 5–7 months and surgical resection remains the only known effective intervention for treating liver metastasis. Nevertheless, the 5-year survival rates of patients after surgeries are only 20–30%. Therefore, it is of utmost importance to understand the mechanisms of lung cancer liver metastasis, so that it can be treated effectively and thus be prevented and/or significantly delayed [3]. Moreover, it will aid us in developing new diagnostic molecular markers and effective therapeutic targets.

In the past decade, the study of NSCLC metastasis has immensely focused on the tumour microenvironment (TME). TME contains many components such as immune cells, inflammatory mediators, and substances secreted by tumour cells, which together affect the growth, invasion, and metastasis of primary tumour cells. Intercellular communication in the TME is mainly accomplished by extracellular vesicles particularly known as exosomes. Previous studies have shown that chemokines, cytokines and other factors are secreted as a result of the communication between tumour cells and bone marrow-derived hematopoietic progenitor cells causing a major role in lung cancer metastasis. These signals initiate a microenvironment making it suitable for the homing and proliferation of tumour cells [4]. Recently, it has been observed that exosomes (small membrane bound vesicles) are actively involved in pre-metastatic niche formation and metastasis [5,6]. Exosomes, are small membrane vesicles ranging from 30 to 200 nm in diameter and participate in cell communication by transferring proteins and genetic materials (e.g., mRNAs and microRNAs) between cells [7]. In the past decade, numerous studies have shown that exosomes

* Corresponding author. Department of Life Science School of Sciences Gujarat University, Navrangpura, Ahmedabad, 380009, Gujarat, India.
E-mail addresses: rakeshmrawal@gmail.com, rakeshrawal.gu@gmail.com, rakeshrawal@gujaratuniversity.ac.in (R.M. Rawal).

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functionally modulate the growth and metastasis in colorectal cancer, melanoma, breast cancer, glioblastoma and pancreatic adenocarcinoma [8]. Exosome are released under normal physiological conditions but their number abnormally increases during diseased condition such as cancer. The magnitude of exosome release has been directly associated with tumour invasiveness both *in vitro* and *in vivo*. Exosomes have been known to affect different stages of tumour progression, including angiogenesis, escape from immune surveillance, extracellular matrix degradation & metastasis and also promotes increased migration and proliferation of the tumours [9]. Moreover, tumour-derived exosomes upon release into the circulation act as complex signalling messengers and are taken up by the host cells to induce a pro metastatic cellular response ultimately leading to the formation of a pre-metastatic fertile niche [10–12]. Epithelial-mesenchymal transition (EMT), a major hallmark of tumour progression and invasion, can only be accomplished through intercellular communication and it has recently been reported that tumour exosomes play a vital role in this process [13].

Studies have reported that exosomes are key metastatic regulators and are also released into the metastatic microenvironment ultimately developing metastasis by transferring miRNAs to target cells [6,8,14]. The underlying metastatic mechanisms differ between varied tumours, and how exosomes regulate the metastasis of different carcinomas is most likely to vary. Transcriptome profiling studies have reported that lung cancer derived exosomes contain many microRNAs and proteins that are associated with oncogenes or metastasis-related genes [15] suggesting that exosomes might play an important role in lung cancer liver metastasis. However, a large number of studies have confirmed that lung-cancer-derived EVs are involved in lung cancer metastasis through distinct pathways, and they are expected to become therapeutic targets with predictive value in diagnosis, prognosis, and anti-tumour drug resistance. Hence, the present study aimed to explore the potential role of exosomes in promoting lung cancer liver metastasis.

In the present study, we isolated exosomes from the serum of patients with lung cancer liver metastasis and treated A549 cells with these micro vesicles. Furthermore, we explored the functional role of these isolated exosomes on invasion, cell cycle functionality, migration and various downstream signalling pathways on A549 cell line. We further developed an *in vivo* rat model for substantiating the results of the *in vitro* model. Our results support the hypothesis positive signals for cell motility and progression are carried by exosomes and these signals are stronger in exosomes derived from cells with higher metastatic potentials. Thus, our findings suggest a critical role for exosomes in driving tumour progression and identifying new biomarkers that could be used as therapeutic targets or predictors of metastasis.

2. Materials and methods

2.1. Patient sample characteristics

This study was approved by Gujarat University & The Gujarat Cancer & Research Institute (GCRI) Institutional Review Board (IRB). All procedures performed in the study involving human participants were in accordance with the ethical standards of the institutional ethics committee of Gujarat University & The Gujarat Cancer and Research Institute and/or national research committee and with the 1964 Helsinki Declaration and its later amendments or comparable ethical standards. All the patients recruited in the study provided written consents for their sample procurement and use of them for research purpose. From December 2016 through July 2017, a total of 15 specimens from patients that passed the inclusion/exclusion criteria were recruited in the study and these samples can be considered as a representation of a larger population. Peripheral blood (5 ml) was collected in serum vacutainers from patients with primary NSCLC with liver metastasis and healthy individuals (n = 15). Patients with only liver metastasis from primary lung cancer were included in the metastatic cohort and for the primary cohort only those patients with NSCLC subtype were included. Patients with multiple metastasis, HIV/HBsAg positive were excluded from the present study. The samples were processed within 30 min to avoid genomic DNA contamination for serum isolation and stored at −80 °C until further analysis.

2.2. Cell lines and cell culture

Human lung adenocarcinoma alveolar basal epitelial (A549) cell line was obtained from National Center for Cell Science (NCCS), Pune, Maharashtra, India. A549 cells were cultured in DMEM medium (Gibco, Life technologies, USA). The culture media were supplemented with 10% fetal bovine serum (FBS) and PSN antibiotic solution (Gibco, Life technologies, USA). The cells were kept in a humidified atmosphere of 5% CO2 at 37 °C. Exponentially growing cells were used for throughout study [9].

2.3. Exosome purification and characterization

Exosomes were isolated from 1 ml of serum from lung cancer liver metastasis patients (n = 15) using a Total Exosome Isolation Kit (Invitogen, Thermo Fisher Scientific Inc., Carlsbad, CA, USA), according to the manufacturers protocol. Exosomal pellets were split and resuspended (i) in 100 µl PBS for scanning electron microscopy, (ii) in 100 µl PBS for flow cytometry analysis (iii) in 100 µl PBS for Nanoparticle tracking analysis and the remaining exosomes were stored at −80 °C.

2.4. Nanoparticle tracking analysis

The size distribution and concentration of exosomes were determined by Nanoparticle Tracking Analysis (NTA) according to the manufacturer's instruction (Nanosight NS300 NanoSight Ltd., Amesbury, UK). Exosomes were resuspended in 1 ml PBS and mixed, then the diluted exosomes were injected into the instrument (Nanosight NS300, UK). Particles were tracked and size of particles was measured based on Brownian motion and the diffusion coefficient. Filtered PBS was used as controls. All samples were measured with parameters of 405 nm laser and 0.64 V voltage using NP100 membranes. Samples were calibrated by CPC100 standard particles diluted 1000-fold under identical settings. Three 60 s videos were captured with a frame rate of 30 frames/sec. Data was analysed using the in-built NTA software (version 2.3; Nano-Sight Ltd.) which was optimized to identify and track each particle on a frame-by-frame basis [9].

2.5. Flow cytometry

Exosomes isolated from the patient serum were incubated for 60 min in 10 µl of mouse anti-CD63-PE, CD-9, CD-81 antibodies. Before data acquisition, the samples were diluted with 480 µl of 0.22 µm pre-filtered PBS (final dilution 1:25). BD FACSCalibur flow cytometer was used for data acquisition and BD FACStation was used for calculation. Data were obtained using 2 individual apogee A50/Micro flow FCs equipped with 50 mW 405 nm (violet), 488 nm (blue), and 638 nm (red) lasers [8,16].

2.6. Scanning electron microscopy

The purified exosomes were fixed with 2% (w/v) para-formaldehyde (PFA) in PBS (pH 7.4); dropped onto a carbon-coated grid and left to dry at room temperature. Further they were fixed with 1% (w/v) glutaraldehyde and stained with saturated aqueous uranyl oxalate. Exosomes were then re-suspended in a solution of 0.4% (w/v) uranyl acetate & 1.8% (w/v) methylcellulose, incubated on ice, excess liquid was drained off and the grid was air dried. Exosomes were viewed using a Field Emission Scanning Electron microscope (JSM-7600F; JEOL, Tokyo, Japan) [8].

2.7. Cell co-culture

A549 cells in culture medium containing 10% exosomes-free FBS were seeded into a 24 well plate at a density of 2×10^3 cells/well. At 50–60% confluence, cells were co-incubated with serum-derived exosomes (50 μg/ml/well & 100 μg/ml/well) for 24 h & 48 h. These, cells were then collected for further functional assays [8,12].

2.8. Uptake assay

1×10^6 A549 cells were seeded on a 12 well plate (Corning, NY, USA) a day prior to the treatment. 5 μl of DiL DYE was added to 170 μl of each exosome sample and was then incubated for 30 min at 4 °C with intermittent gentle mixing. Further, exosomes were washed twice with 1xPBS. 1 mL of serum free media was added to 20 μl of DiL Labelled exosome and these were incubated at 37 °C & 5% CO_2 for 6 h, 12 h, 18 h and 24 h. The labelled exosomes were further washed twice with 1 x PBS. A549 cells were fixed using 4% PFA and incubated at 37 °C 5% CO_2 for 20 min. Further the cells were washed thrice with 1 x PBS. Coverslip were placed on confocal slides and mounted using Moviol + DAPI (5 μl/Coverslip). Hoescht was used for nuclei staining. Dil DYE labelled exosomes were captured with a Leica-TCS- SP8 microscope (Leica Microsystems Inc., Buffalo Grove, IL, USA) equipped with a 577–656 nm laser. Images were processed with Fiji ImageJ Software (U. S. National Institutes of Health, Bethesda, MD, USA). Exosome uptake was assessed by measuring the fluorescent signals in regions of interest (ROIs) in the perinuclear region of the cell [9,30].

2.9. MTT assay

A549 cells were plated into a 96-well plate at the density of 5×10^3 cells/well and cultured in an incubator at 37 °C supplemented with 5% CO_2. After culturing for 0, 24, 48 and 72 h, 10 μL MTT solution (Beyotime) was added into each well, incubating for another 2 h. Then, DMSO was added to remove formazan. The value of optical density (OD) in each well at 570 nm was measured using a microplate reader (Thermo Fisher Scientific; Waltham, MA, USA) [27].

2.10. Cell migration assay

Cell motility was evaluated using the *in-vitro* wound-healing assay. Cells in exponential growth phase were grown in 6-well plates until they reached confluency. Exosomes were then added at a concentration of 50 & 100 μg/ml to the cells. Using a 20 μl plastic pipette tip, we scraped vertical line across the entire diameter at the bottom of each well inducing the 'wound'. Cell media were removed and the wound area was photo-graphed at 0 h after scraping. To compare cell motility of control and exosome supplemented cells, we measured the gap distance and determined the wound-closing rate at 24 and 48 h. At the indicated time points, the wound closure was photographed by a camera (Model DXM1200, Nikon, Tokyo, Japan) attached to an inverted microscope (Eclipse TE300, Nikon) [8].

2.11. Transwell invasion assay

The migration assay was performed in 6-well Transwell® plates (Corning, New York, NY, USA; cat. no. 3422) using inserts with 8 μm pore size membranes. To investigate the role of exosomes in cell migration, A549 cells were co-cultured with exosomes derived from the serum of metastatic patients. A549 cells were suspended at 1×10^6 cells/ml and mixed with 50 & 100 μg/ml exosomes in Dulbecco's modified Eagle's medium (DMEM) without FBS, then added to the upper chambers, and the same medium containing 20% FBS was placed in the lower chamber. Blank controls without exosomes were seeded at the same time. After 24 & 48 h, cells on the lower membrane were stained with crystal violet for 15 min and counted under a light microscope in at least five different fields (original magnification, ×200). Each clone was plated in triplicate in each experiment [8,9].

2.12. Cell cycle analysis

A549 cells (1×10^6 cells per ml) were incubated with 100 μg/ml exosomes for 24 & 48 h. Cells were washed with PBS and harvested by re-suspending in fresh media. Cells were fixed with 90% ethanol overnight at 4 °C for flow cytometry. For cell cycle analysis, fixed cells were washed in PBS and treated with RNase for 20 min at 37 °C. 5 μg/mL of Propidium Iodide (PI) were added to each sample and analysed by flow cytometry. Flow cytometry with was conducted using a BD Aria II (BD Biosystems, USA) [27,29].

2.13. Real time PCR (qRT- PCR)

Total RNA from exosomes was isolated from serum of metastatic patients as well as healthy individuals using TRIzol reagent (Invitrogen, Carlsbad, CA, USA) following the manufature's protocol. RNA integrity was further checked on 1% formaldehyde gels on Mini-Sub-Cell GT (BioRad) and gels were then visualized on Gel Documentation System (BioRad; Gel Doc XR+). The extracted RNA was stored at −80 °C until further analysis. 1 μg total RNA was reverse transcribed to cDNA using the cDNA archive kit (Applied Biosystems – ABI; Cat no: 4368813) in 50 μl reaction volume following manufacturer's instructions and the resulting cDNA was used as a template for Real Time PCR analysis. qRT-PCR was performed using AriaMX (Agilent Technologies, USA) with a SYBR Green PCR Master Mix. Primer sequences are provided in Table 1. Amplification was performed at the following cycling conditions: 1 cycle of 3 min at 95 °C for the initial denaturation step and 40 cycles of 5 s at 95 °C for the denaturation step, 20 s at 60 °C for the annealing and extension step. Melting curve analysis was performed following the amplification in order to distinguish the accumulation of specific reaction products from non-specific products or primer dimmers. The generation of a specific PCR product was tested using the ΔΔCT method with β-actin as the endogenous control. All experiments were performed in triplicates independently and average C_T value was calculated for the quantification fold change analysis [8].

2.14. Network analysis and string protein-protein interaction

Network analysis was performed for the 5 most variable genes present obtained from the real time PCR data. The network analysis was conducted using online software known as NetworkAnalyst. The analysis was used to find clusters (network networks) of high-correlation genes. The network clusters formed and the protein-protein correlation obtained was based from two different databases: (i) IMEx Interactome (ii) STRING Interactome. Further, to identify the possible pathways involved protein-protein interactions from both the database were subjected to String protein-protein database.

2.15. Animals

Animal care and all animal experiments were performed according to the national guidelines and approved by the local government authorities of the state (Gujarat University – GU/IEC/07/2018). A total of 20 adult female Wistar rats 8–10 weeks old were purchased and used, ranging in weight from 150 to 200 g. Animals were randomly divided into four groups with 5 rats in each group including the control cohort. Rats were housed in specific pathogen-free conditions and experiments were conducted in accordance with the guidelines of the Animals Act and approved by the Institutional Animal Care and Use Committee.

2.16. Metastasis and liver colonization studies

To analyse the role of exosomes in tumour metastasis, 8-10-week-old

Table 1
List of primers used for Real Time PCR analysis.

Gene	Forward Primers	Reverse Primers
CXCL12	5' -AAGCCCGTCAGCCTGAGCTA-3'	5' -TTAGCTTCGGGTCAATGCACAC-3'
CXCR4	5'-AATAAAATCTTCCTGCCCACC-3'	5'-CTGTACTTGTCCGTCATGCTTC-3'
CK7	5'-GACATCGAGATCGCCACCTAC-3'	5'-ATTGCTGCCCATGGTTCCC-3'
CDH1	5′-GACTCGTAACGACGTTGCAC-3′	5′ -GGTCAGTATCAGCCGCTTTC-3′
CTNNB1	5′-TGGATACCTCCCAAGTCCTG-3′	5′-CAGGGAACATAGCAGCTCGT-3′
CLDN4	5′-AGATGGGTGCCTCGCTCTAC-3′	5′-CCAGGGAAGAACAAAGCAGA-3′
HIF-1α	5' –ACAGCCTCACCAAACAGAGCAG-3'	5'–CGCTTTCTCTGAGCATTCTGCAAAGC-3'
VEGFA	5'-CTTGCCTTGCTGCTCTACC-3'	5'-CACACAGGATGGCTTGAAG-3'
MMP9	5'-GAGTGGCAGGGGGAAGATGC-3',	5'-CCTCAGGGCACTGCAGGATG-3'
p53	5′-CCGTGTTGGTTCATCCCTGTA-3′	5′-TTTTGGATTTTTAAGACAGAGTCTTTGTA-3′.
OPN	5′- ACTCGTCTCAGGC CAGTTG-3′	5′-CGTTGGACTTGGAAGG- 3′
CDKN2A	5'-CCCAACGCACCGAATAGT-3'	5'-GGGGATGTCTGAGGGACCTT-3'
TGFβR2	5'-GTAGCTCTGATGAGTGCAATGAC-3'	5'-CAGATATGGCAACTCCCAGTG-3'
MUC16	5'-CTGAGACCCCAACATCCTTG-3'	5'-GGTCACTAGCGTTCCATCAG-3'
CD44v6	5'- CCAGGCAACTCCTAGTAGTACAACG-3'	5'- CGAATGGGAGTCTTCTTTGGGT-3'
β- actin	5'-TGACGTGGACATCCGCAAAG-3'	5'-CTGGAAGGTGGACAGCGAGG-3'

Wistar female rats were injected intra-abdominally with the isolated exosomes from serum of patients with liver metastasis. The exosome treatment was administered in 3 doses at different time intervals. The first dose of exosomes was administered at a concentration equivalent to 100 μg protein whereas the second and third doses were at a concentration equivalent to 50 μg protein. The doses were given at an interval of 15 days and control group rats were injected with normal saline. Each group contained 3–5 rats. Blood from the eyes of these rats was collected after every 4 weeks for serum albumin and the Liver Function Tests (LFT). Moreover, the autopsies were performed after 4th, 8th and 12th weeks and the lung, liver and other vital organs were fixed in 4% (w/v) PFA for histologic analysis.

2.17. Hematoxylin and eosin staining

Tumours and tissues used to evaluate metastasis in the liver and the metastatic microenvironment were fixed in 10% neutral buffered formalin at least one day before paraffin embedding. Serial 4 μm sections were cut and stained with H&E for histopathological examination. The sections were stained with hematoxylin and eosin or immunohistochemically stained and two pathologists independently observed the tissue sections in 10 high power fields using an inverted microscope (IX71; Olympus, Tokyo, Japan).

2.18. Statistical analysis

All statistical analyses were performed using the SPSS 18.0 software, and the graphs were generated using GraphPad Prism 6.0 (GraphPad Software, San Diego, CA, USA). The comparisons of means among groups were analysed by one way ANOVA, and the Dunn Multiple Comparison Test was further used to determine significant difference between groups. Quantitative data are presented as means ± standard error of the mean (s.e.m.). *P* value less than 0.05 was regarded as statistically significant. All experiments were repeated at least in triplicate [8,9,30].

3. Results

3.1. Characterization of the exosomes derived from serum of lung cancer liver metastatic patients

To investigate the role of exosomes in lung cancer liver metastasis, exosomes were isolated from the serum of patients with lung cancer liver metastasis, and their identity was confirmed by electron microscopy, particle size distribution and flow cytometry. The vesicles isolated from the serum contained a heterogeneous population of vesicles comprising both round-shaped 30–70 nm diameter vesicles, as well as few larger vesicles ~110–150 nm in diameter consistent with exosomes. We further confirmed whether these vesicles were exosomes by performing Nanoparticle tracking analysis using Nanosight based on the principle of particle size distribution [Fig. 1 a]. The vesicles isolated were approximately 40 nm in size which confirmed that these vesicles are exosomes [Fig. 1 b]. Results of flow cytometry also showed CD63 and CD81 to be present in exosomes from the serum of lung cancer liver metastatic patients [Fig. 1 c].

3.2. Tumour derived exosomes are internalised by A549 cells

To study the effects of tumour derived serum exosome uptake and internalization by lung cancer cells, exosomes were labelled with fluorescent dye Dil and added to fresh A549 monolayers for various times periods (6hr, 12hr, 18hr, 24hr). Cells were analysed for the presence of Dil granules for intracellular distribution by confocal laser microscopy [Fig. 1 d]. The results indicated that exosomes derived from lung cancer liver metastatic serum were taken by A549 cells, localized around the nucleus and were time dependant.

3.3. Effect of tumour-derived exosomes on the cell proliferation, migration and invasion ability of A549 cells in vitro

To study the effects of tumour serum-derived exosomes on biological functions of A549 cells *in vitro*, we exposed A549 to lung cancer liver metastatic serum-derived exosomes. MTT assay was performed to determine cell proliferation, and the data showed that serum exosomes reinforced cell proliferation [Fig. 2 a & 2 b]. We investigated whether liver metastatic-derived exosomes could affect the migration and invasion of A549 cells. *In vitro* cell migration and matrigel transwell invasion assays were performed by treating A549 cells with liver metastatic-derived exosomes. A549 cells without treatment with exosomes served as the experimental control. At both 50 and 100 μg/ml concentrations, treatment with tumour derived liver metastatic exosomes resulted in a clear and significant increase in migration and closure of the wound of A549, in a seemingly dose-dependent fashion as compared to the control group ($p < 0.001$) [(Fig. 2 c & d]. Similarly, these liver metastatic derived exosomes resulted in a significant increase in fibroblast invasion through matrigel at both the concentrations, and the effects were more prominent with increased exosome concentration [Fig. 2 e & f] after 24 as well as 48 h ($p < 0.05$). The invasion and migration rate was significantly low in the control cells as compared to that in the treated A549 cells at both concentrations and at different time intervals.

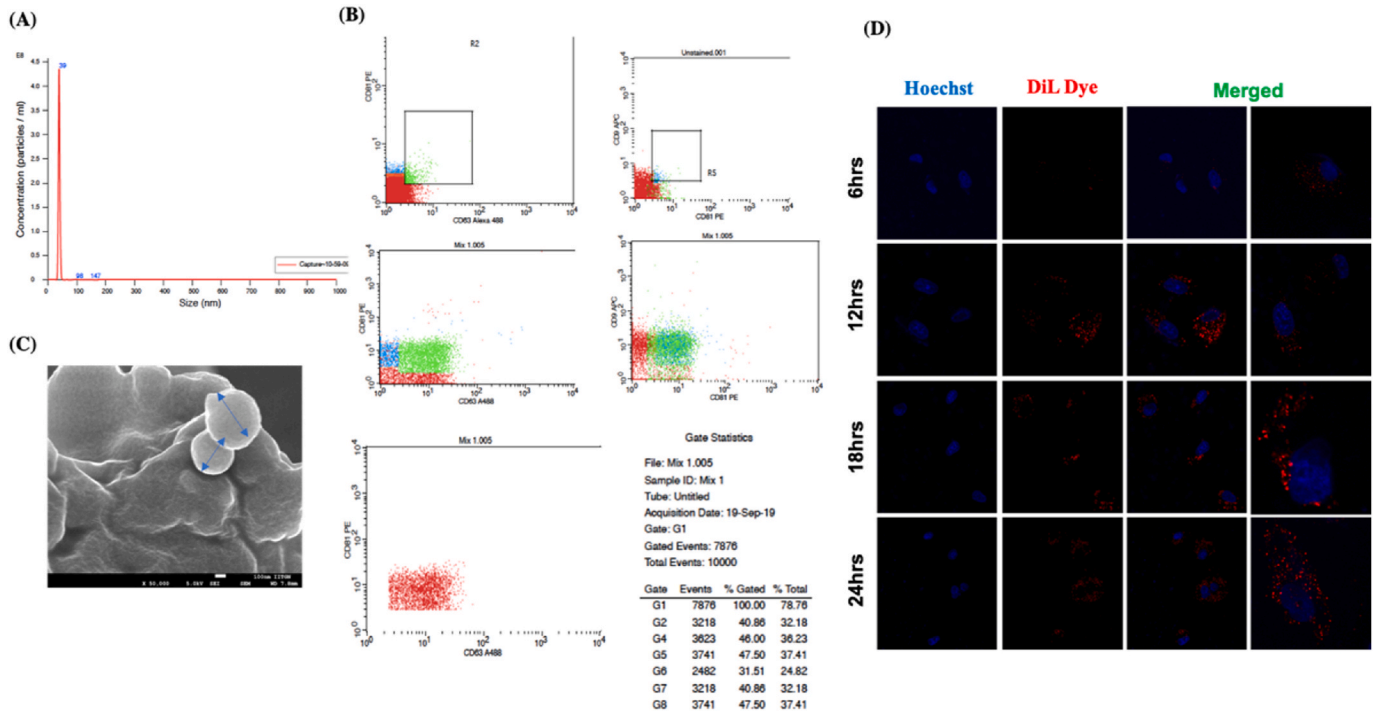


Fig. 1. Characterization and uptake of exosomes derived from serum of lung cancer liver metastatic patients by A549 cells. (a) Electron microscopic images of exosome isolated from metastatic patients which revealed that isolated exosomes comprised round-shaped 30–100 nm diameter vesicles. (b) CD63 and CD81 expression of exosomes was detected by flow cytometry in the tumour derived exosomes. (c) Size distribution of purified tumour derived exosomes by Nanosight NS300. (d) Fluorescently labelled exosomes entered into A549 cells. Representative images were filmed after cells were fixed and stained (scale bar, 20 μm; magnification, 400 ×) (Blue, nuclei; red, exosomes). (For interpretation of the references to color in this figure legend, the reader is referred to the Web version of this article.)

3.4. Effects of tumour derived exosomes on upregulation on cell cycle analysis

Effect of tumour derived exosomes in the regulation of cell cycle was assessed to explore the possible mechanism of enhanced cell migration and invasion. The results of cell cycle analyses showed that post exosomal treatment A549 in the G0/G1 phases decreased significantly whereas cells in the S phase of cell cycle simultaneously increased at 100 μg/ml concentrations after 24 and 48 h [Fig. 2 g & h]. Notably, A549 cells treated with exosomes at 100 μg/ml concentration showed an increasing trend in the G2/M phase of the cell cycle as compared to its control counterparts thus revealing the plausible role of exosomes in cell proliferation and cell cycle progression ($P < 0.05$).

3.5. Tumour derived exosomal treatment in A549 cells causing altering various gene expression patterns

Quantitative gene expression patterns of CXCL12, CXCR4, CK7, CDH1, CTNNB1, CLDN4, VEGFA, HIF1A, MMP9, TP53, CDKN2A, MUC16, TGFBR2, OPN and CD44v6 were analysed from the in-vitro model generated after co-incubation with exosome exposure at 50 & 100 μg/ml concentration for 24 and 48 h. A549 cells without exosome treatment were used as control. It was observed that CXCL12, MMP9, HIF1A, CLDN4, TP53, MUC16 and CD44v6 were upregulated and showed a time and dose dependent increasing trend of gene expression as compared to the control [Fig. 3 a & b]. OPN and CDKN2A showed an upregulation in the gene expression but demonstrated a decreasing trend with increasing exosomal concentrations at different time intervals. CK7 and CTNNB1 also showed upregulation in gene expression but at 24 h they exhibited a decreasing trend with increasing exosomal concentration of 50 & 100 μg/ml. Moreover, it was observed that the expression of CK7 was same in treated as well as in control A549 cells after 48 h but CTNNB1 showed an increase in expression at 50 μg/ml concentration as compared to 100 μg/ml exosome treatment as well as control. Additionally, CDH1 showed an upregulation in the control cells whereas it exhibited a significant downregulation in the treated cells after 24 h. Contradictorily, an upregulation was seen in the control as well as treated cells with a decreasing trend with an increase in exosomal concentration after 48 h of treatment. Furthermore, it was observed that VEGFA and TGFBR2 demonstrated downregulation after 24 h of treatment. However, the control A549 cells also showed a downregulation but treated A549 cells showed a significant upregulation after 48 h of treatment [Fig. 3 a,b,c,d].

3.6. Network analysis and protein interactions

Network analysis was used to identify potential drug targets through interaction (nodes) for complex diseases primarily tumour metastasis. The differentially expressed genes namely VEGFA, TGFBR2, CXCR4, CDH1 and CTNNB1were subjected to network analysis using NetworkAnalyst software for unravelling the possible interactions amongst these genes and the pathways involved in the initiation of the metastatic cascade.

IEx Interactome and STRING Interactome both protein-protein networks were constructed for the 5 differentially expressed genes [Fig. 4 a & c] and their minimum association was also established [Fig. 4 b & d]. Furthermore, the minimum interactions from both the databases for all the genes was subjected to protein-protein interactions using String Protein-protein database and it revealed the possible pathways involved in the mechanism of metastasis such as the JAK-STAT, SMAD & TGFBR2 pathways [Fig. 4 e].

3.7. Liver metastasis - derived exosomes preferentially enhance the pre-metastatic niche formation and metastasis

To determine if tumour-derived exosomes play a role in liver

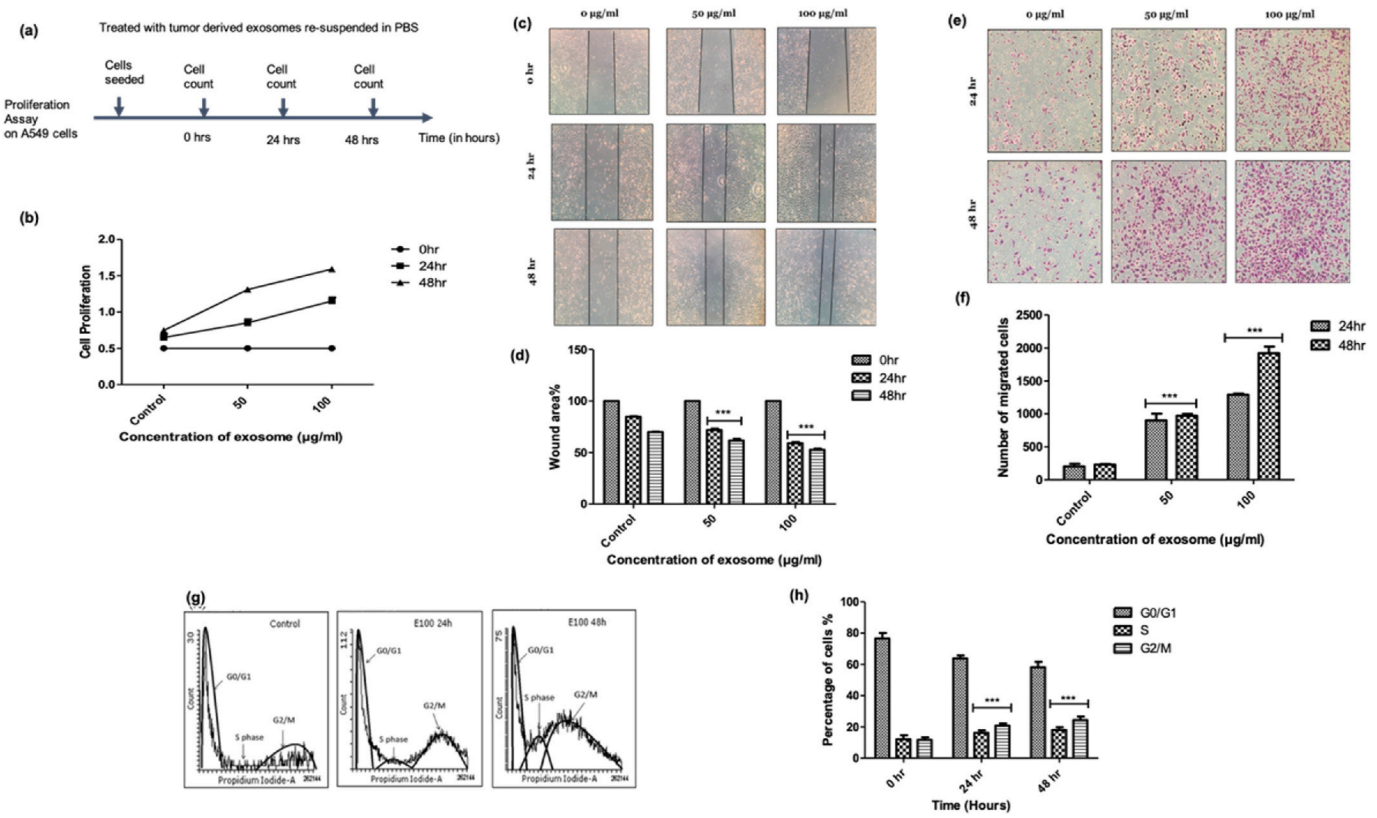


Fig. 2. Result of cell proliferation, wound healing assay, cell invasion assay and cell cycle progression on A549 cell line treated with exosome *in vitro*. (a) Representative flow chart and time course for cell proliferation assay. (b) Cell proliferation assay was assessed for different concentration (50 & 100 µg/ml) for 24hr & 48hr using MTT assay. (c) Representative image depicts the significant increase in the wound healing potential of the treated cells at different exosomal concentration (50 & 100 µg/ml) compared to control cell lines of A549 after 24 and 48 h. (d) The graph demonstrates the remarkable difference in distance migrated by treated cells compared to control. (e) Representative image depicts the significant increase in the cell invasion of the treated cells at different exosomal concentration (50 & 100 µg/ml) compared to control cell lines of A549 after 24 and 48 h. (f) The graph demonstrates the remarkable difference in cell invasion by treated cells compared to control. Error bars represent mean ± SEM of three independent experiments and significant differences were indicated as ***$p \leq 0.001$. (g) The effect of exosome on cell cycle was examined by flow cytometry. G0/G1 phase cells were significantly decreased and S phase was relatively increased in treated groups as compared to control (**$P < 0.05$). (h) Graphical representation of the cell cycle analysis after exosomal treatment.

metastasis, we used an experimental rat model for intra-abdominal injection of the isolated exosomes. We collected blood of all the animals at the 4th, 8th and 12th week after injection to check for the anatomical/physiological abnormalities if any in the liver post exosomal treatment. It was observed that after 4 weeks of treatment the serum levels of total protein & bilirubin increased as compared to the controls. Also, the levels of aspartate transaminase (AST)/serum glutamic oxaloacetic transaminase (SGOT), alanine transaminase (ALT)/serum glutamic-pyruvic transaminase (SGPT) were elevated [Fig. 5b and 5c] indicating acute liver damage and despair. However, alkaline phosphatase (ALP) levels showed a decrease in the serum levels in treated rats [Fig. 5b and 5c] as compared to the control [Fig. 5 a]. Similar results were obtained in the serum of rats that were treated for 8weeks and 12 weeks [Fig. 5 d and 5e]. Nevertheless, it was observed that when we checked for the presence of CEA – a tumour marker there was no expression seen [Table 2].

Further, the hematoxylin eosin staining of the lung and liver tissue of both the control and the treated groups are depicted in [Fig. 6 a & b]. The hepatic tissue in the exosomal treatment group exhibited inflammation whereas in the pulmonary tissue pneumonitis was observed after 4th, 8th and 12th week of treatment as compared to the control normal tissue. The other vital organs that were screened did not show any changes. Collectively, these results suggest that exosomes are capable of changing the microenvironment and suggesting their role in the formation of tumour metastasis.

4. Discussion

Liver is the major organ which is conducive to metastasis of colorectal, gastric and pancreatic tumours. However, liver metastasis arising from other types of tumours, such as breast cancer, lung cancer and renal cancer are not fully understood [17]. Moreover, liver metastasis is one of the main pathological developments that cause the death of lung cancer patients in the Asian population but the underlying mechanism still needs to be elucidated [8]. Accumulating evidence have been focused on identifying cell-intrinsic determinants of organotrophic metastasis owing to the seed soil hypothesis proposed by Stephen Paget [18,19]. Recently, exosomes have demonstrated to have a crucial role in determining organ-specific metastasis [20–22]. This study explicated for the first time the role of exosomes derived from highly liver metastatic patients, in promotion of liver metastasis by the primary lung cancer A549 cell line.

Recent studies have shed light on exosomes having a role in the early detection of malignant tumours, pathway activation and signal transduction amongst cells. These extracellular vehicles are also used as a vehicle for the delivery of targeted drugs, as well as miRNAs and siRNAs [23,24]. Moreover, exosomes are known to mediate the immune escape, drug resistance and angiogenesis of tumours but its role in creating a pre metastatic niche still remains less explored [25–27].

Cancer metastasis is a complex cascade involving multiple steps such as invasion, angiogenesis, dissemination of cancer cells through blood vessels, extravasation and organ-specific homing & growth. Tumour cell

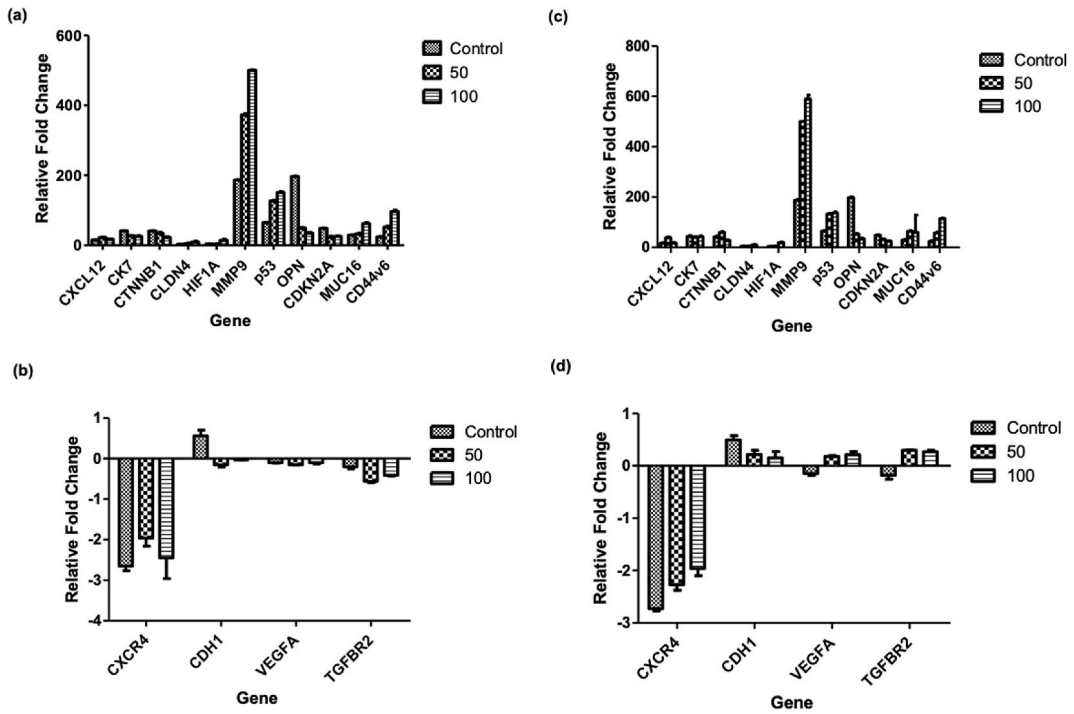


Fig. 3. RT-PCR expression of potential markers in exosome treated A549 cells Relative gene expression profile of 15 marker panel (CXCL12, CXCR4, CK7, CDH1, CTNNB1, CLDN4, HIF1A, VEGFA, MMP9, p53, OPN, CDKN2A, TGFBR2, MUC16, CD446) in treated and control cells (a) upregulated genes after 24 h, (b) down-regulated genes after 24 h, (c) upregulated genes after 48 h (d) downregulated genes after 48 h. Data shown as mean ± SD with statistical significance at $p < 0.05$.

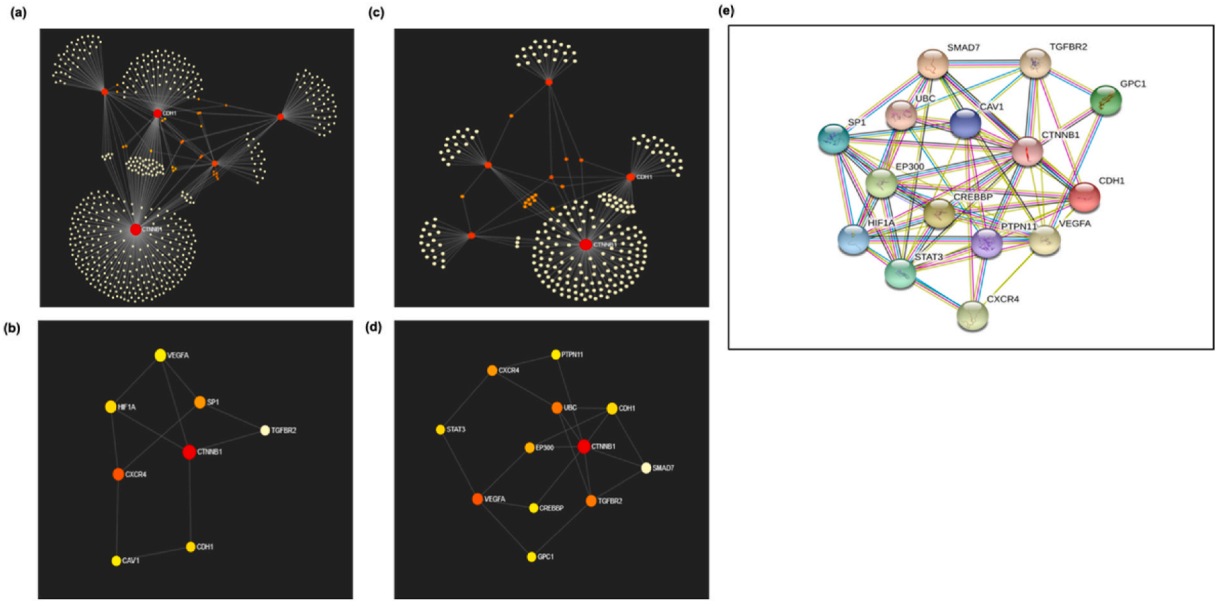


Fig. 4. **Network analysis output of the differentially expressed genes (CXCR4, CDH1, CTNNB1, VEGFA and TGFBR2).** (a) Network cluster obtained when these genes were analysed using IMEx Interactome database. (b) Minimum interactions of the genes using IMEx Interactome database. (c) Network cluster obtained when these genes were analysed using STRING Interactome database. (d) Minimum interactions of the genes using STRING Interactome database. (e) String protein – protein interactions of all the minimum interactions obtained from the IMEx and STRING Interactome.

migration, invasion and cell proliferation are one of the most important hallmarks of tumour metastasis [27,28]. Whether liver metastatic-derived exosomes can affect the cell proliferation and migration/invasion of A549 cells was examined by transwell cell migration assay *in vitro*. Our results showed that exosomes from the serum of metastatic tumours that were incorporated by the lung cancer cells promoted the migration of A549 cells, at both concentrations (50 & 100 μg/ml) thus indicating that exosomes have a greater impact on A549 cell migration than the non-treated cells. Moreover, it was also observed that at both the concentrations of exosome treatment (50 & 100 μg/ml) and at 24 & 48 h A549 cells had greater capability of wound closure as compared to the non-treated controls. Thus, exosomes might promote metastasis by increasing the migration and invasion of A549 cells.

Cell cycle mechanism of the treated A549 cells at 24 & 48 h were analysed, in order to decipher its significance in terms of functional

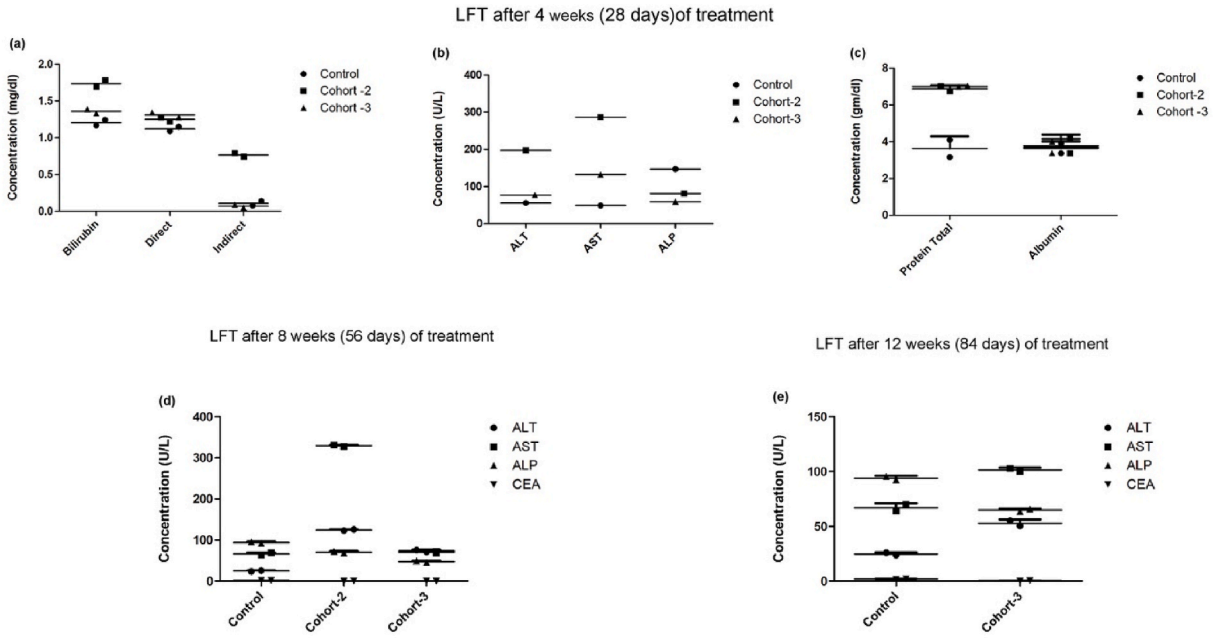


Fig. 5. Graphical representation of the Liver Function Test (LFT) performed from the serum of treated as well as control rats after 4th, 8th and 12th weeks of treatment.

Table 2
Determination of Liver Function Test (LFT) in experimental rats and control.

Groups	Total bilirubin (mg/ml)	Total Protein (gm/ml)	AST (U/L)	ALT (U/L)	ALP (U/L)	CEA (U/L)
Control	1.17 ± 0.02	3.16 ± 0.01	49 ± 1.1	56 ± 0.9	147 ± 1.8	1.5 ± 0.02
After 4 weeks	1.51 ± 0.01	6.86 ± 0.04	209 ± 2.0	137 ± 2.7	70 ± 1.2	–
After 8 weeks	–	–	197 ± 2.5	97 ± 1.5	56 ± 0.5	0.2 ± 0.01
After 12 weeks	–	–	100 ± 1.0	50 ± 0.98	63 ± 0.5	0.2 ± 0.01

Results were expressed as Mean ± S.E.M.

relevance. After exosome treatment, G0/G1 phase were significantly decreased with a considerable increase in the S phase and marginal upregulation in G2/M phases of the cell cycle. These findings signify that exosomal treatment has a significant impact on G0/G1 phase arrest of cell cycle which might be responsible for the cell proliferation of primary tumour cells. Thus, these results speculated that enhanced proliferation of cells after treatment with exosomes could be due to cell cycle dysregulation possibly leading to metastasis.

Moreover, our real time PCR results demonstrated that exosome treatment at 50 & 100 μg/ml concentration at 24 & 48 h led to the differential expression of 5 genes such as CXCR4, CDH1, CTNNB1, VEGFA and TGFBR2. Furthermore on performing network analysis and string protein - protein interactions it was observed that these genes govern common signalling pathways such as the JAK-STAT, TGFBR2 and the SMAD signalling leading to activation of transcriptional factors and alteration of the secondary organ microenvironment. Additionally, it has been observed that Wnt/β-catenin, p53, COX, TGF-β/Smad, NF-κB, Notch, VEGF and JAKs/STAT3 signalling pathways are involved in the pathogenesis of colorectal liver metastasis by activating factors for the pre-metastatic niche development [28].

To verify the direct role of exosomes in lung cancer metastasis, we

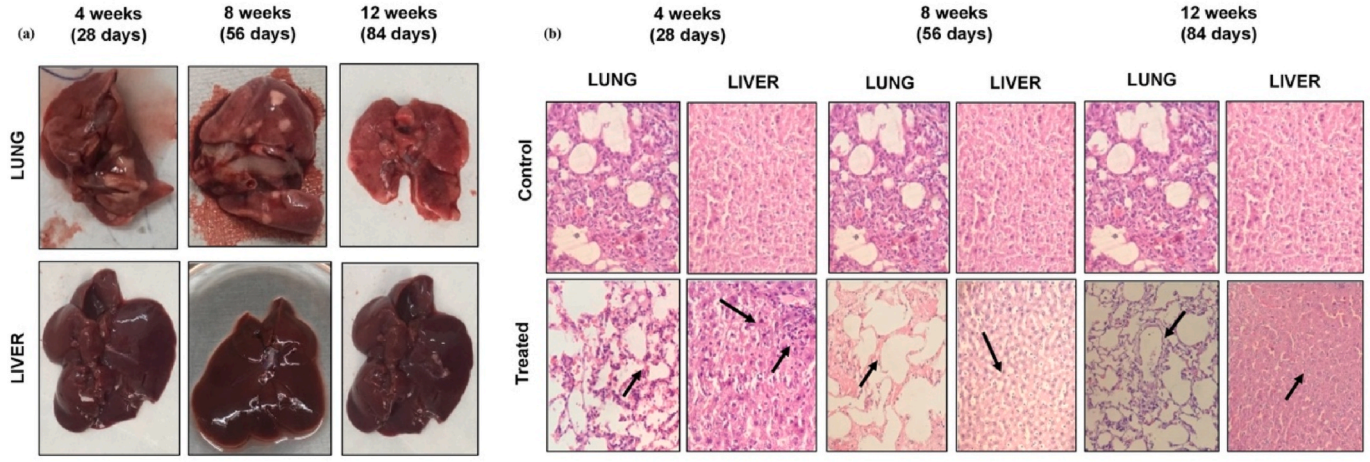


Fig. 6. *In – vivo* effect of exosomes on the Wistar rats. Gross anatomical and microscopic views of liver and lung from rats injected with exosomes at (a) 4 weeks (b) 8 weeks and (c) 12 weeks as compared to normal tissue. Scale bar: 10 mm.

established an *in-vivo* rat model using serum derived liver metastatic exosomes and intra-abdominally injecting them to examine the characteristics and functional relevance. Subsequently, tumour - derived exosomes were extracted the serum of liver metastatic patients and characterized by electron microscopy, flow cytometry and nanoparticle tracking analysis before administration in the rats. Treatment with tumour derived exosomes induced a pronounced enhancement of the liver enzymes such as the AST, ALT, total bilirubin and total protein. Nevertheless, we also observed that the lung and the liver of these treated rats has development pneumonitis and inflammation respectively post exosomal treatment signifying the ability of the exosomes to alter the organ microenvironment. These results showed that exosomes play a key role in pre-metastatic niche formation [27,29–31].

5. Conclusion

In the present study, we demonstrated a pivotal role for exosomes in the liver metastasis of lung cancer, which may function by affecting cellular migration, invasion and cell cycle. Exosomes possibly have an effect on cell cycle by increasing the cells in the S & G2/M phase leading to greater cell proliferation and progression. We also showed that the effect of tumour derived – exosomes causes the activation of various signalling pathways like the JAK/STAT, TGFB2, VEGF and SMAD. This further leads to the alteration of the secondary organ microenvironment causing the formation of a fertile pre-metastatic niche that might harbour the circulating tumour cells that have disseminated from the primary tumour and lead to the development of the complex metastatic cascade. Our research leads to the further understanding of how exosomes affect the liver metastasis of lung cancer and provide perspective regarding the therapeutic window for therapy of lung cancer by targeting the various biomarkers. To the best of our knowledge, this is the first study that has been carried out to find the pivotal role of exosomes in the development of lung cancer liver metastasis and the current approach may have considerable clinical & therapeutic implications.

Availability of data and material

All data generated or analysed during this study are included in this published article.

Ethics approval

Compliance with Ethical Standards has been taken care of for this study. Research involving Human Participants and/or Animals.

This study was approved by Gujarat University (GU/IEC/07/2018) & The Gujarat Cancer & Research Institute (GCRI – EC-O-92-2014) Institutional Review Board (IRB). All procedures/methods performed in the study involving human participants were in accordance with the ethical standards, guidelines and regulations of the institutional ethics committee of Gujarat University & The Gujarat Cancer and Research Institute and/or national research committee and with the 1964 Helsinki Declaration and its later amendments or comparable ethical standards. All the patients recruited in the study provided written informed consents for their sample procurement and use of them for research purpose.

The animal study had been approved by Gujarat University (GU/IEC/07/2018) and all the methods are reported in accordance with ARRIVE guidelines for the reporting of animal experiments.

Consent to participate

Informed consent was obtained from all individual participants included in the study.

Consent to publish

NA.

Author contribution statement

K.S. and R.R conceptualized the study, K.S., S.P. and K.B. performed the experiments, K.J. and R.R supervised the study, K.S., S.P., K.J. and R. R analysed the data, K.S. S.P. wrote the main manuscript text, K.S. and K. B. prepared the images and All authors reviewed the manuscript.

Declaration of competing interest

The authors declare that they have no known competing financial interests or personal relationships that could have appeared to influence the work reported in this paper.

Data availability

No data was used for the research described in the article.

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