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Cigarette smoking induces lung cancer tumorigenesis via upregulation of the WNT/ß-catenin signaling pathway

--Manuscript Draft--

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Abstract:	Lung cancer has the highest mortality rate compared to any other cancer worldwide, and cigarette smoking is one of the major etiological factors. How cigarette smoke (CS) induces tumorigenesis in healthy cells is still not completely understood. In this study, we treated healthy human bronchial epithelial cells (16HBE14o) with 1% cigarette smoke extract (CSE) for one week. The CSE exposed cells showed upregulation of WNT/ß-catenin pathway genes like WNT3, DLV3, AXIN and ß-catenin, 30 oncology proteins were found to be upregulated after CSE treatment. Further, we explored whether the role of extracellular vesicles (EVs) obtained from CSE exposed cells can induce tumorigenesis. We observed that CSE EVs induced migration of healthy 16HBE14o cells by upregulation of various oncology proteins in recipient cells like AXL, EGFR, DKK1, ENG, FGF2, ICAM1, HMOX1, HIF1a, SERPINE1, SNAIL, HGFR, PLAU which are related to WNT signaling, epithelial mesenchymal transition (EMT) and Inflammation, whereas inflammatory marker, GAL-3 and EMT marker, VIM were downregulated. Moreover, ß-catenin RNA was found in CSE EVs, upon treatment of these EVs to healthy cells, the ß-catenin gene level was decreased in recipient cells compared to healthy 16HBE14o cells, indicating the utilisation of ß-catenin RNA in healthy cells. Overall, our study suggests that CS treatment can induce tumorigenesis of healthy cells by upregulating WNT/ß-catenin signaling in vitro and human lung cancer patients. Therefore targeting the WNT/ß-catenin signaling pathway is involved in tumorigenesis inhibition of this pathway could be a potential therapeutic approach for cigarette smoke induced lung cancer.
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Declaration of interests

☐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.

☒The authors declare the following financial interests/personal relationships which may be considered as potential competing interests:

Vamshikrishna Malyla reports financial support and equipment, drugs, or supplies were provided by University of Technology Sydney. Student reports a relationship with University of Technology Sydney - City Campus that includes: funding grants.

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Dr. Loren E. Wold
The Editor-in-Chief
Life sciences

Dear Dr. Wold

We are excited to submit our research article entitled, **"Cigarette smoking induces lung cancer via upregulation of WNT/ß-catenin signaling pathway"** for consideration for publication in the *Life Sciences*.

Lung cancer is the leading cause of cancer related deaths globally. The key reason is a lack of effective diagnostic markers, poor understanding of the mechanisms and lack of effective therapeutic interventions. In the submitted manuscript, we have explored for the first time how cigarette smoke treated healthy airway epithelial cells derived extracellular vesicles (EVs) induce tumorigenesis in healthy epithelial cells by upregulation of WNT/ß-catenin pathway. We have highlighted the relevance that the detection of EVs can be an effective diagnostic tool and inhibition of WNT/ß-catenin pathway can be a potential therapy in managing Cigarette smoke-induced lung cancer. This would be of broad interest to discovery, translational researchers, respiratory scientists, clinicians and oncologists and the readership of the journal.

It was written by a PhD student (**Malyla**) and a fellow funded by the IASLC (**Paudel**), and expert in lung diseases (**Hansbro**), and a formulation specialist (**Dua**). This manuscript is specifically written for Life sciences. Our work has not been submitted for publication elsewhere. We hope that you find our manuscript acceptable for publication.

Kind regards,

Kamal Dua

The authors are very thankful to the Editor, Editorial team and Reviewers for consideration of our manuscript and providing their valuable suggestions.

We have now addressed all the comments and incorporated changes into a revised version (highlighted in red font in track changes) as described point by point response below. This has improved the manuscript, which we now hope is acceptable for publication in the journal.

Reviewer #1: The manuscript has been improved significantly. However, there are still some issues that were not clarified/corrected.

Response: Thank you for the feedback.

Abstract
- 16HBE14o are ''human bronchial epithelial cells''
- The abbreviation EMT was not described in the Abstract section.

Response: Thank you for pointing this out. This has been modified in the abstract section.

Materials and Methods
- In the 3.5. section some sentences were deleted and now part of the sentence is missing: ''the final pellet containing CSE EVs was resuspended in 1ml PBS...''

Response: Thank you, I have included this in the section 2.5.

Results
- There is a change made in 3.1.: ''1% CSE was treated just over a period of one days''. From the Fig. 1A it is obvious that the treatment was not just one day. This should be clearly written.

Response: Thank you for noticing this. Yes, it's over eight days and not one day. This has been modified in the manuscript.

- 3.3. ''We found that AXL, EGFR, DKK1, CD105, Progranulin, VIM, HIF1a, FGF2, ICAM1, HMOX1, HGFR, GAL-3, SERPINE1, SNAIL and PLAU oncology proteins were upregulated in healthy cells indicating that CSE EVs can induce tumorigenesis (fig. 3A&B). Also, vimentin and Gal-3, which are usually upregulated in cancer, are downregulated compared to the healthy cells, which need further investigation [61, 62].''

Again it is stated ''in healthy cells'' but should be changed into CSE EVs treated healthy 16HBE14o cells, as authors agreed.

Response: This is modified as suggested. Thanks.

Secondly, some of these proteins were not upregulated (VIM and GAL-3 were downregulated), they should be deleted and commented separately!

Response: We have deleted the VIM and GAL-3 from upregulated proteins and commented on them separately. Thanks.

The same is also mentioned in the Discussion section:
'' The cancer related inflammatory proteins like HMOX1, HIFA (is this HIF1a???), ENG, FGF2, GAL3 were upregulated in recipient 16HBE14o cells along with cell adhesion molecule ICAM and EMT inducer Snail and vimentin, WNT/ß-catenin pathway endogenous inhibitor DKK1, and other markers like AXL, EGFR, HGFR, SERPINE1, PLAU were expressed in the recipient cells''.

Also needs to be changed in the Abstract!

Response: We have deleted the VIM and GAL-3 from upregulated proteins and commented on them separately throughout the manuscript. Also, HIFA is modified to HIF1a throughout the manuscript. Many thanks.

- 3.3. There are still repetitions at the end of this paragraph (not the last sentence!):
''We observed that the recipient cells have utilised the RNA from CSE EVs indicating that ß-catenin could have translated to protein in the recipient cells (fig. 3C). As EVs are known to carry RNA in them [57], we also performed RT-qPCR on healthy cells, CSE treated cells and EVs and recipient cells. Interestingly, we found that the main component of the WNT/ß-catenin pathway, ß-catenin itself, had its RNA packaged into the CSE EVs. The treated cells had utilised the RNA from CSE EVs indicating that ß-catenin could have been translated into protein in the recipient cells (fig. 3B).''
(and it is not fig 3B, but 3C as stated above!)

Response: Thank you so much for pointing this out. We have now adjusted the repeated sentence.

Discussion
- Correction still needed: ''To further understand the role of cigarette smoke-induced lung cancer progression, we have treated CSE at 1% CSE treatment (???) for one week, significantly increasing EV production.'' (You did not test nor show a significant increase in EV production in your results, so you should paraphrase the last part of the sentence).

Response: Thank you, I agree with you. I have paraphrased this sentence.

- Correction still needed: '' this study provides evidence that studying CS induced EVs

that contain inflammatory and WNT signaling molecules (in EVs) can be a potential diagnostic marker for the early detection of CS induced lung cancer''.

Response: Thank you. We have modified this as per your suggestion.

Cigarette smoking induces lung cancer tumorigenesis *via* upregulation of the WNT/ß-catenin signaling pathway

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Abstract: Lung cancer has the highest mortality rate compared to any other cancer worldwide, and cigarette smoking is one of the major etiological factors. How cigarette smoke (CS) induces tumorigenesis in healthy cells is still not completely understood. In this study, we treated healthy human bronchial~~o~~ epithelial cells (16HBE14o) with 1% cigarette smoke extract (CSE) for one week. The CSE exposed cells showed upregulation of WNT/ß-catenin pathway genes like *WNT3, DLV3, AXIN* and *ß-catenin*, 30 oncology proteins were found to be upregulated after CSE treatment. Further, we explored whether the role of extracellular vesicles (EVs) obtained from CSE exposed cells can induce tumorigenesis. We observed that CSE EVs induced migration of healthy 16HBE14o cells by upregulation of various oncology proteins in recipient cells like AXL, EGFR, DKK1, ENG, FGF2, ICAM1, HMOX1, HIF1a~~A, GAL-3~~, SERPINE1, SNAIL~~, VMN~~, HGFR, PLAU which are related to WNT signaling, epithelial mesenchymal transition (EMT) and Inflammation, whereas inflammatory marker, GAL-3 and EMT marker, VIM were downregulated. Moreover, ß-catenin RNA was found in CSE EVs, upon treatment of these EVs to healthy cells, the ß-catenin gene level was decreased in recipient cells compared to healthy 16HBE14o cells, indicating the utilisation of ß-catenin RNA in healthy cells. Overall, our study suggests that CS treatment can induce tumorigenesis of healthy cells by upregulating WNT/ß-catenin signaling *in vitro* and human lung cancer patients. Therefore targeting the WNT/ß-catenin signaling pathway is involved in tumorigenesis

inhibition of this pathway could be a potential therapeutic approach for cigarette smoke induced lung cancer.

Keywords: Extracellular vesicles, cigarette smoke, tumorigenesis, lung cancer. WNT/ß-catenin signaling.

ABBREVIATIONS

LC: lung cancer, CS: cigarette smoke, 16HBE14o: healthy human broncho epithelial cells, CSE: cigarette smoke extract, COPD: chronic obstructive pulmonary disease, PAH: polyaromatic hydrocarbons, NNK: 4-(methylnitrosamino)-1-(3-pyridyl)-1-butanone, KRAS: Kirsten rat sarcoma viral oncogene homolog, TP53: tumor protein, DNA: Deoxyribonucleic acid, miRNA: micro Ribonucleic acid, nAChRs: Nicotinic acetylcholine receptors, β-ARs: β-Adrenergic receptors, cAMP: adenylyl cyclase and cyclic, NF-kB: nuclear factor-κB, NSCLC: non-small cell lung cancer, WNT: Wingless, FBS: foetal bovine serum, DMEM: Dulbecco's modified eagle medium, IL: interleukin, COX2: cyclooxygenase2, PBS: Phosphate buffered saline, DLS: Dynamic light scattering, RIPA: Radioimmunoprecipitation assay buffer, TEM: Transmission electron microscopy, GAPDH: Glyceraldehyde-3-Phosphate Dehydrogenase, AXL: AXL receptor tyrosine kinase, DKK1: Dickkopf, CTSD: Cathepsin D ,CTSB: Cathepsin D, CAPG: Capping Actin Protein, Gelsolin Like, ENG: Endoglin, ENO2: Enolase, ERBB5: erythroblastic oncogene B, FGF2: Fibroblast Growth Factor 2 , ICAM1: Intercellular Adhesion Molecule 1, HMOX1: Heme Oxygenase 1, HIF1a~~A~~: Hypoxia-inducible factor, MET: MET Proto-Oncogene, Receptor Tyrosine Kinase, CAG: Cytosine, Adenine, Guanine, GAL-3: Galectin-3, FOXC1: Forkhead Box C1 , FOXC2: Forkhead Box C1, MSLN: Mesothelin, PGDFA: Platelet Derived Growth Factor Subunit A, SPP1: Secreted Phosphoprotein 1, GRN: Granulin Precursor, SERPINB5 serine protease inhibitor B5, SERPINE1: serine protease inhibitor E1, SNAIL: Zinc finger protein SNAI1, VIM: Vimentin.

1. Introduction

Cigarette smoke (CS) exposure accounts for 90% of lung cancer in men and 70-80% in females (LC) cases [1, 2]. For the remaining % of people with LC, the cause is likely due to other factors such as pre-existing lung diseases such as chronic obstructive pulmonary disease (COPD), genetic, environmental, viral, and hormonal factors [3, 4]. In 2020, 2.2 million new cases and 1.7 million mortalities were attributed to lung cancer [5]. LC is the most complicated lifestyle-related cancer with a poor diagnosis and prognosis [6]. Moreover, there is a lack of promising early diagnostic markers and the current therapeutic approach cannot significantly

improve the overall survival rate [7-9]. It is widely accepted that cigarette smoking is the primary risk factor, but how cigarette carcinogens contribute to LC is not elucidated [10, 11].

It is estimated that nearly 7000 chemicals are generated from CS, out of which more than 60 are reported to be carcinogens, like polyaromatic hydrocarbons (PAH), benzo[a]pyrene, and nitrosamine derived compound 4-(methylnitrosamino)-1-(3-pyridyl)-1-butanone (NNK) [12-14]. Generally, these carcinogens are metabolised by phase 1 detoxifying enzymes such as cytochrome p420, which adds an oxygen atom to make them water soluble to be excreted from the body [15]. PAH are highly active compounds that are metabolised by cytochrome p450 enzymes, but regular smoking can lead to accumulation in the body; these potent PAH carcinogens can bind to DNA and form DNA adducts leading to a process called metabolic activation, which may lead to mutations in the tumor protein (TP53) gene [16, 17] as well as mutation mainly in 12 codons of Kirsten rat sarcoma viral oncogene homolog (KRAS) and Epidermal growth factor receptor (EGFR) [18, 19]. Thus, balancing the deactivation and formation of DNA adducts determine the progression of LC. Therefore, the excretion of these carcinogens is critical in preventing tumorigenesis.

Besides the DNA mutations, CS dysregulates various carcinogenic pathways like Nicotinic acetylcholine receptors (nAChRs) signaling as nicotine binds to various α and β subunits of nAChRs and interrupts ion influxes. It is already known that this pathway is upregulated in LC [20, 21]. NNK and nicotine binding to α7 subunit of nAChRs can activate phosphoinositide 3-kinases/protein kinase B signaling pathway [22, 23]. Along with nAChRs, nicotine and other carcinogens are reported to bind to β-Adrenergic receptors (β-ARs), leading to β-ARs mediated signaling, causing increased cell proliferation of epithelial cells. This can lead to adenylyl cyclase and cyclic (cAMP) signaling leading to LC [24-26]. Besides the cancer pathways, inflammatory pathways are crucial for the LC progression [27]. For example, the nuclear factor-κB (NF-kB) pathway has a role in inducing the production of pro-inflammatory markers like interleukin (IL)-6, IL-8, and cyclooxygenase (COX2) and influence cancer progression by recruiting neutrophils and macrophages [28-31]. Other pathways dysregulated by cigarette smoking are Notch signaling and embryogenesis pathways like Hedgehog and WNT/ß-catenin [32-34]. WNT/ß-catenin signaling is an evolutionarily conserved signal transduction pathway that helps in cell-to-cell communication and aids in maintaining cell proliferation, cell polarity, cell homeostasis, embryogenesis, and cancer [35-37]. Mutations or over-activation of these

pathways leads to various human diseases, including cancer [38]. WNT/ß-catenin signaling is widely studied in colon cancer. However, its role in LC is not fully elucidated [39, 40].

Extracellular vesicles (EVs) are tiny particles released from all cells in the human body and don't have the capability to divide. Based on the size, EVs are categorised into medium EVs (> 200nm) and small EVs (< 200nm) [41]. These EVs carry a lot of functional molecules like proteins, RNAs, DNA, and lipids [42-44]. The packages of different cargo into EVs depend on the cell of EVs origin and diseased condition [45, 46]. The EVs are mediators in cell-to-cell communication and help maintain homeostasis in the body [42]. Researchers have recently tried to validate if the tumors derived EVs stimulate tumorigenesis in healthy recipient cells by transferring oncogenic material like miRNA and oncogenic proteins to healthy cells [34, 47, 48]. Therefore, further understanding the role of cancer-derived EVs helps understand tumorigenesis pathways and find effective diagnostic and prognostic tools, as these EVs are primarily released into the biological fluids [49, 50].

It is recently known that cigarette smoke can induce changes in EV composition and release, but their role is not entirely understood [51]. However, few reports highlight that smoking enhances EV release and plays a crucial role in the pathogenesis of non-small cell lung cancer (NSCLC) [34, 52, 53]. This study investigated how CSE treatment to healthy bronchial epithelial cells can impact WNT/ß-catenin signaling pathways and how the CSE treatment derived EVs can induce tumorigenesis in healthy 16HBE14o cells.

2. Materials and Methods

2.1. EVs depletion from foetal bovine serum

As commercially available foetal bovine serum (FBS) contains small EVs, removing the small EVs from FBS is essential while performing an *in vitro* experiment with the EVs [49]. In our study, to deplete the exosomes from FBS, the heat inactivated FBS was initially centrifuged at 18,000g for 90mins to remove any medium size EVs present in the FBS. Soon after, the supernatant was centrifuged for 12 hours at 100,000g, maintaining $4^{o}C$ to remove small EVs pellets at the bottom. The supernatant passed through a 0.22 micron filter. These small EVs depleted FBS were used for culturing the human normal bronchial epithelial cells (16HBE14o) [54].

2.2. Cell culture

Healthy human bronchial epithelial cells (16HBE14o) were cultured as previously mentioned [34].

2.3. Cigarette smoke extract(CSE) preparation

CSE was generated by a custom built smoking device where one research cigarette (Reference Cigarette 3R4F, University of Kentucky) was lit and drawn manually into 10ml of PBS through silicon tubing. This 10ml extract was considered 100% cigarette smoke extract and filtered through a 0.22 micron filter to sterilise it. The CSE was then diluted in low glucose DMEM media based on the usage percentage. CSE was prepared fresh before the start of any experiment [55, 56].

2.4. Exposing cells to CSE

A density of $2x10^6$ of 16HBE14o cells were seeded into multiple T-175 flasks using small EVs depleted low glucose DMEM media(as described in section 2.1). Next day, discard the media and wash cells with PBS to remove any unattached or dead cells. Freshly prepared 1% CSE was added to the cells on Monday and left for 24 hours. The following day media was replaced with fresh media (without CSE). This cycle continued daily until Friday. On Friday, cells were treated with 1% CSE and left over the weekend and on Monday, media was collected to isolate CSE EVs (Fig 1A). Along with the CSE EVs, 1% CSE treated cells were harvested for protein and RNA extraction (n=5 biological replicates).

2.5. Isolation of EVs

1% CSE treated supernatant was collected and centrifuged as discussed previously, the final pellet containing CSE EVs was resuspended in 1ml PBS and stored at -80°C for characterisation and downstream protein or RNA extraction (Fig 1B) [34, 46].

2.6. Dynamic Light Scattering

1% CSE treated 16HBE14o EVs size and polydispersity index were measured using dynamic light scattering (DLS). A small portion of the EV fraction was diluted in PBS, equilibrated to 37°C, and DLS was measured using a 1cm path length cuvette. DLS measurements were recorded in triplicates using Zetasizer Nano ZS equipped with a He-Ne 633nm laser light source and readings measured at 25°C in a size range of 0.3–10,000nm. The Zeta average

diameter of 1% CSE treated EVs was 492.1(n=6 biological replicates and 3 technical replicates) [34].

2.7. Protein extraction and quantification

200µl of CSE EVs suspension was centrifuged as described above to isolate EVs. Radioimmunoprecipitation assay buffer (RIPA) buffer containing phosphatase and protease inhibitor cocktail was added to the CSE EVs followed by vortexing and incubation on ice for 15mins. The mix was then sonicated at 30% amplitude three times for 2 seconds and incubated again on ice for an additional 15mins. the lysate was then centrifuged for 30mins at 18,000g and 4°C, and the supernatant collected. The process for extracting proteins from cells is similar except the protein lysate was centrifuged for 12,000g at 4°C for 10mins and the protein concentration was quantified using a Pierce^{TM} BCA Protein Array Kit (Thermo Scientific) [34].

2.8. Flow cytometry

5µg of CSE 16HBE14o EVs were analysed by flow cytometry. EVs stained with V450 annexin V (BD HorizonTM catalogue No. 560506) for 30mins without light. 5 µg of unstained samples were run to identify the concentration of EVs for flow and accordingly gating strategy was made. Annexin V stained EVs were run with and without Calcium, as annexin V binds to EVs only with Calcium (n=3 biological replicates) [34].

2.9. Transmission electron microscopy (TEM)

EV samples were suspended in PBS on carbon coated copper grids (Mesh 200) (GSAU200F-50, ProSciTech) and left to attach for 1hr. The EVs were then fixed onto the grid with 1% glutaraldehyde, washed with Milli-Q water and negatively stained with 1.5% uranyl acetate and dried overnight in desiccator images were taken the following day using a Tecnai T20 is a 200kV TEM TWIN electron microscope (n=3 biological replicates) [57].

2.10. Protein array of CSE EVs and Cells

Protein from 16HBE14o cells, 1% CSE treated 16HBE14o cells, 1% CSE treated 16HBE14o EVs and CSE EVs treated healthy 16HBE14o cells were isolated and equal amounts (600µg) were used to run the oncology array as per the manufacturer's instructions (https://www.rndsystems.com). Blots are imaged using the Biorad imaging platform. Data was analysed by measuring pixel density in ImageJ software [34, 55, 58].

2.11. Migration (scratch wound) assay

16HBE14o cells were seeded in a 6-well plate at $2x10^4$ cells per well and incubated for 24 hrs. Following day media was removed and washed twice with PBS. A 200ul yellow pipette tip was used to make a vertical scratch and washed 3 times with PBS to remove unattached cells. 9 images of each well taken at 10x at zero hours. Cells were then treated with 0, 5, 10 and 20µg protein equivalent of 1% CSE 16HBE14o EVs and the migration length measured 24 and 48hrs later (n=5 biological replicates and 9 technical replicates) [34, 59, 60].

2.12. RNA extractions and assessment of gene expression level by real-time PCR.

RT-PCR was performed as previously mentioned [34] with genes related to WNT/β-catenin signaling pathway table 1.

Gene Name	Forward	Reverse	Ref Seq ID
CTNNB1	CTTGGAATGAGACTGCTG	AGAGTGAAAAGAACGATAGC	NM_001904
DVL3	ATGTCACTCAACATCATCAC	CCTTCATGATAGAGCCAATG	NM_004423
AXIN1	CCGACCTTANATGAAGATGAG	CAGGATCCATACCTGAACTC	NM_003502
WNT3	CTGTGACTCGCATCATAAG	ATGTGGTCCAGGATAGTC	NM_030753
GAPDH	ACAGTCAGCCGCATCTTCTT	GACCAAATCCGTTGACTCCGA	NM_008085

Table 1: WNT/β-catenin signaling human primer sequences

The quantitative expression of the genes was calculated through $2^{-\Delta\Delta Ct}$, corresponding to the house keeping gene GAPDH. The relative abundance was calculated with the help of untreated control [34].

2.15. Statistical analysis

Data are presented as mean ± SEM. Statistical analyses were performed using one-way ANOVA for multiple comparisons and T-test for two group comparisons using the Graph Pad Prism software (version 9.3). Statistical significance was accepted at $p < 0.05$.

3. Results

3.1. CSE treatment and characterisation of EVs

To determine whether treating healthy 16HBE14o cells with CSE over time can induce any cancer-like phenotype, we have optimised the concentration of CSE to 1% so that there is a

gradual induction of tumorigenesis. 1% CSE was treated just over a period of eight~~one~~ days and EVs were collected as shown from day 7&8(48hrs) (Fig. 1A) [56]. The isolation of medium size EVs (200-1000nm) involves very well established multiple centrifugation steps (Fig.1B) [50]. According to the guidelines [36], the EVs are characterised by at least three different methods. Among these methods, we used DLS for size distribution. We observed that EVs were in the size range of medium EVs (>200-1000nm) with no contamination from small EVs (<200nm), indicating the purity of EVs with an average particle size of Z Average is 492.1 nm (Fig. 1C).

Transmission electron microscopy (TEM) is often used to visualise EVs and to determine EVs from non-EVs material. Traditionally, EVs resemble a cup-shape in TEM images due to a dehydration phase during sample preparation [51]. We also found cup-shaped EVs with no additional non-EV material (Fig. 1D). Another characterisation technique of EVs is identification with a maker that is important in the context of biological properties. As EVs contain lipid membranes, characterising via membrane lipids is another way to confirm EVs. We have carried out Annexin V staining of phosphatidylserine (PS) on EVs [52]. The process of membrane shedding releases EVs and therefore PS is carried with medium EVs [53]. Annexin V is known to bind to PS only in a Calcium dependent manner [49, 53]. Therefore, we have visualised PS by flow cytometry and confirmed the binding of Annexin V in the presence and absence of Calcium (Fig. 1E).

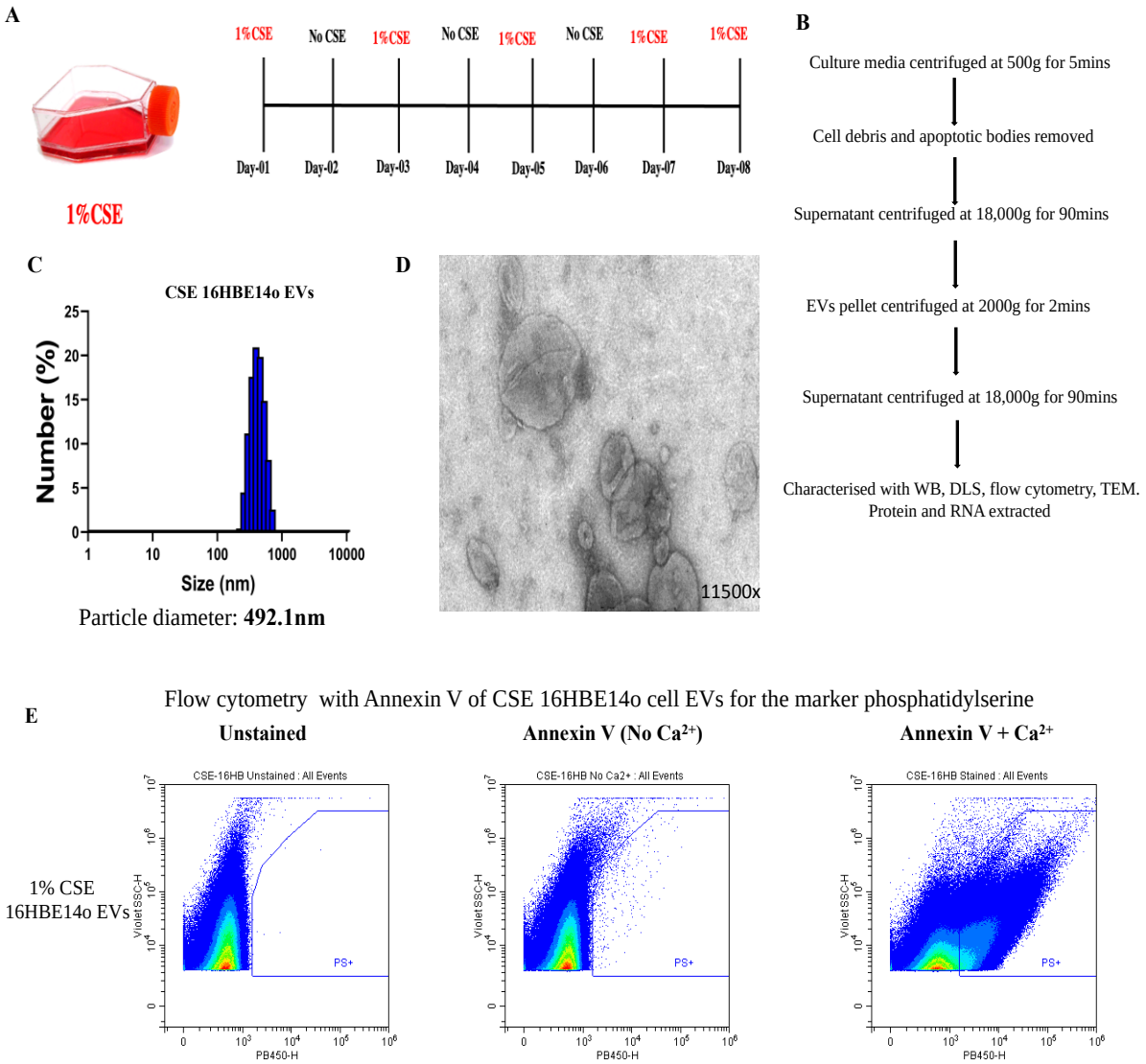
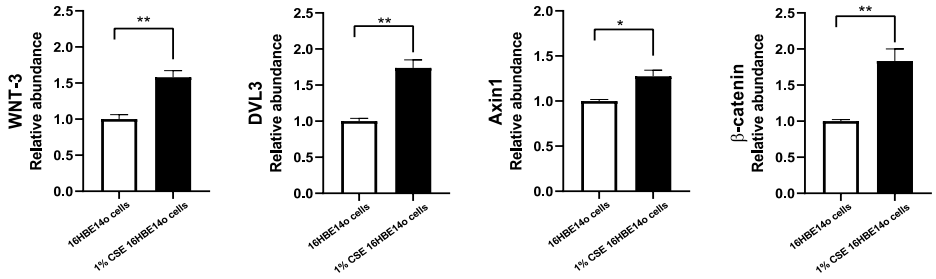


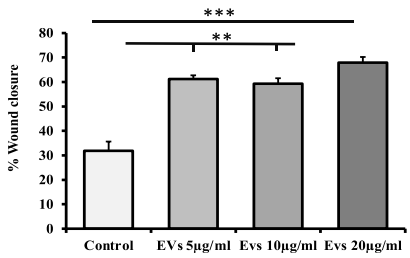
Figure 1. Isolation and characterisation of 1% CSE EVs

1A) Healthy 16HBE14o cells were treated with 1% CSE for 8 days. 1B) Flow chart of CSE EVs isolation process 1C) DLS of CSE EVs found to be in the range of medium EVs with an average particle diameter of 492.1nm. 1D) TEM image of CSE EVs, cup shaped EVs indicate the dehydration of EVs during sample preparation, 11,500x magnification. 1E) Representative dot-plots from flow cytometric analysis of phosphatidylserine exposure. Unstained CSE EV samples (left) analysed as control for autofluorescence, staining in the absence of Calcium (middle) was performed to control for specific Annexin V binding, stained with Annexin V in the presence of Calcium (right) to detect phosphatidylserine surface exposure.

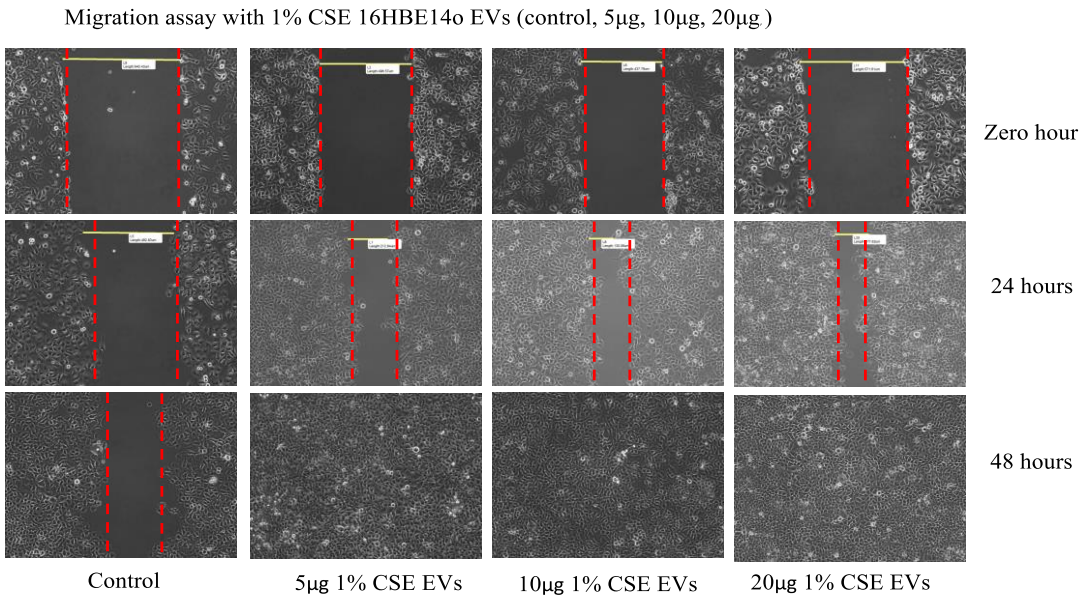
A


Upregulation of WNT/β-catenin pathway in CSE 16HBE14o cells
WNT-3
Relative abundance
DVL3
Relative abundance
Axin1
Relative abundance
β-catenin
Relative abundance
16HBE14o cells
1% CSE 16HBE14o cells

B


% Wound closure
Control
EVs 5μg/ml
Evs 10μg/ml
Evs 20μg/ml

C


Migration assay with 1% CSE 16HBE14o EVs (control, 5μg, 10μg, 20μg)
Zero hour
24 hours
48 hours
Control
5μg 1% CSE EVs
10μg 1% CSE EVs
20μg 1% CSE EVs

D

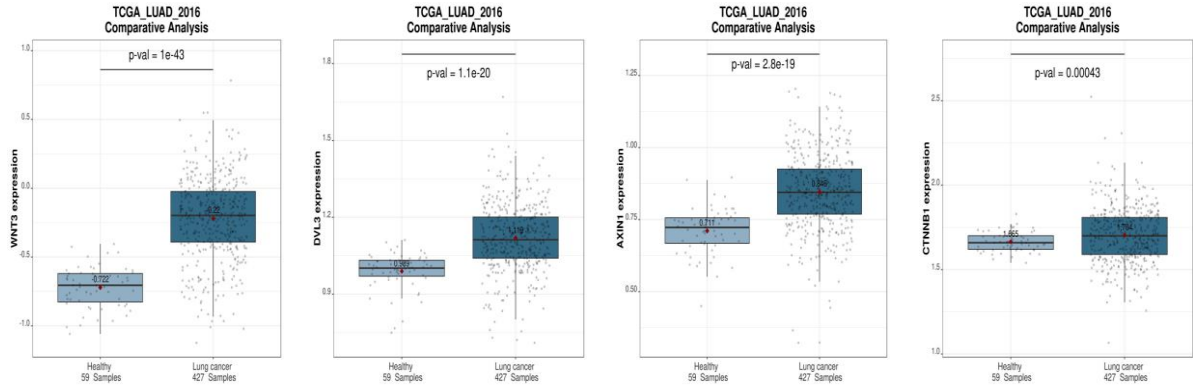

TCGA_LUAD_2016
Comparative Analysis
p-val = 1e-43
WNT3 expression
p-val = 1.1e-20
DVL3 expression
p-val = 2.8e-19
AXIN1 expression
p-val = 0.00043
CTNNB1 expression
Healthy
59 Samples
Lung cancer
427 Samples

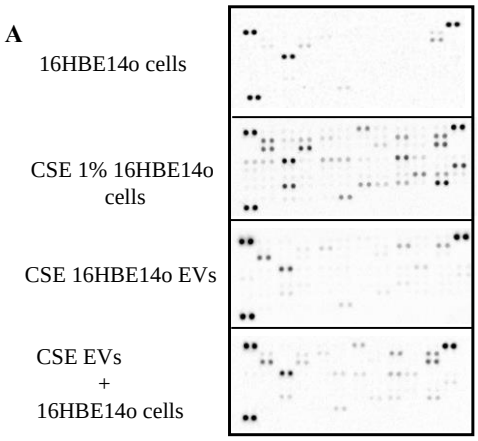
Figure 2. CSE EVs induced Migration in healthy cells

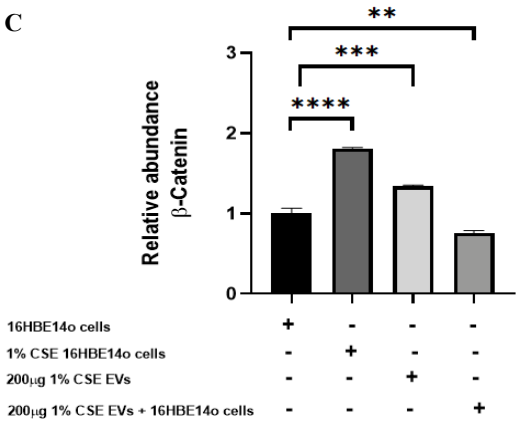
2A) 16HBE14o cells treated with CSE had expressed high levels of WNT/ß-catenin RNA levels including *WNT3, DLV3, Axin1* and *ß-catenin*. T-test was performed ** P ≤ 0.01, * P< 0.05 vs control. 2B) Healthy 16HBE14o cells treated with 1% CSE EVs showed significant Migration compared to the control. Data were analysed using an ANOVA and % wound closure was calculated. ** P ≤ 0.01, ***p<0.001. 2C) Microscopic images of wound closure induced by CSE EVs at 5 µg, 10µg, and 20µg protein equivalent of EVs compared to control. Images were taken at 0hr, 24hr, and 48hrs. 100% of wound closure was achieved by 48hrs at all concentrations. Images were taken at 10x magnification. 2D) Comparative analysis of healthy lung tissue vs lung tumors in smokers of *WNT3, DLV3, Axin1* and *ß-catenin*.

3.2. Upregulation of WNT/ß-catenin pathway genes and CSE induced tumorigenesis

Treatment of 16HBE14o cells with 1% CSE for 8 days (every alternative days for 6 days) induced upregulation of WNT/ß-catenin pathway genes such as *WNT3*, Dishevelled Segment Polarity Protein 3 (*DVL3*) a key molecule in binding to the cell surface and propagating actions of WNT based signals [54], and *Axin1* a vital component of the *ß-catenin* destruction complex which regulates the activity ß-catenin [55] (the critical component of tumorigenesis and EMT) (Fig. 2A). Furthermore, we validated these findings with publicly available datasets of lung cancer(Fig. 2E) and compared healthy lung tissue in smokers with lung tumor tissue of smokers, indicating similar observations in *WNT3, DVL3, Axin1* and *ß-catenin*. This further confirms that CS induces upregulation of the WNT/ß-catenin pathway [56] (https://lce.biohpc.swmed.edu/, accessed on 15 June 2022). We performed an oncology array of 84 cancer proteins from CSE treated cells and EVs and CSE EVs treated healthy 16HBE14o cells. We found 30 oncogenic proteins such as AXL, EGFR, DKK1, CTSD, CTSB, CAPG, ENG, ENO2, ERBB5, FGF2, ICAM1, HMOX1, HIF1a~~A~~, MET, CAG, GAL-3, FOXC1, FOXC2, IL-8, MSLN, PGDFA, TP53, P27, SPP1, GRN, SERPINB5, SERPINE1, SNAIL, VIM, PLAU. All of these are related to cancer induced inflammation, ß-catenin regulators and EMT (Fig. 2B). Among these proteins, a few of the oncogenic proteins are encapsulated in CSE derived EVs like AXL, EGFR, DKK1, CTSD, ENG, ICAM1, IL-8, SPP1, GRN, SERPINE (Fig. 2B). This led us to treat healthy cells with these EVs to determine whether these can induce tumorigenic features in healthy cells. To validate this, we performed scratch wound/migration assays using different concentrations of CSE EVs and found that there was a

significant amount of wound closure after 24hr and by 48hr there was 100% wound closure (Fig. 2C and D)





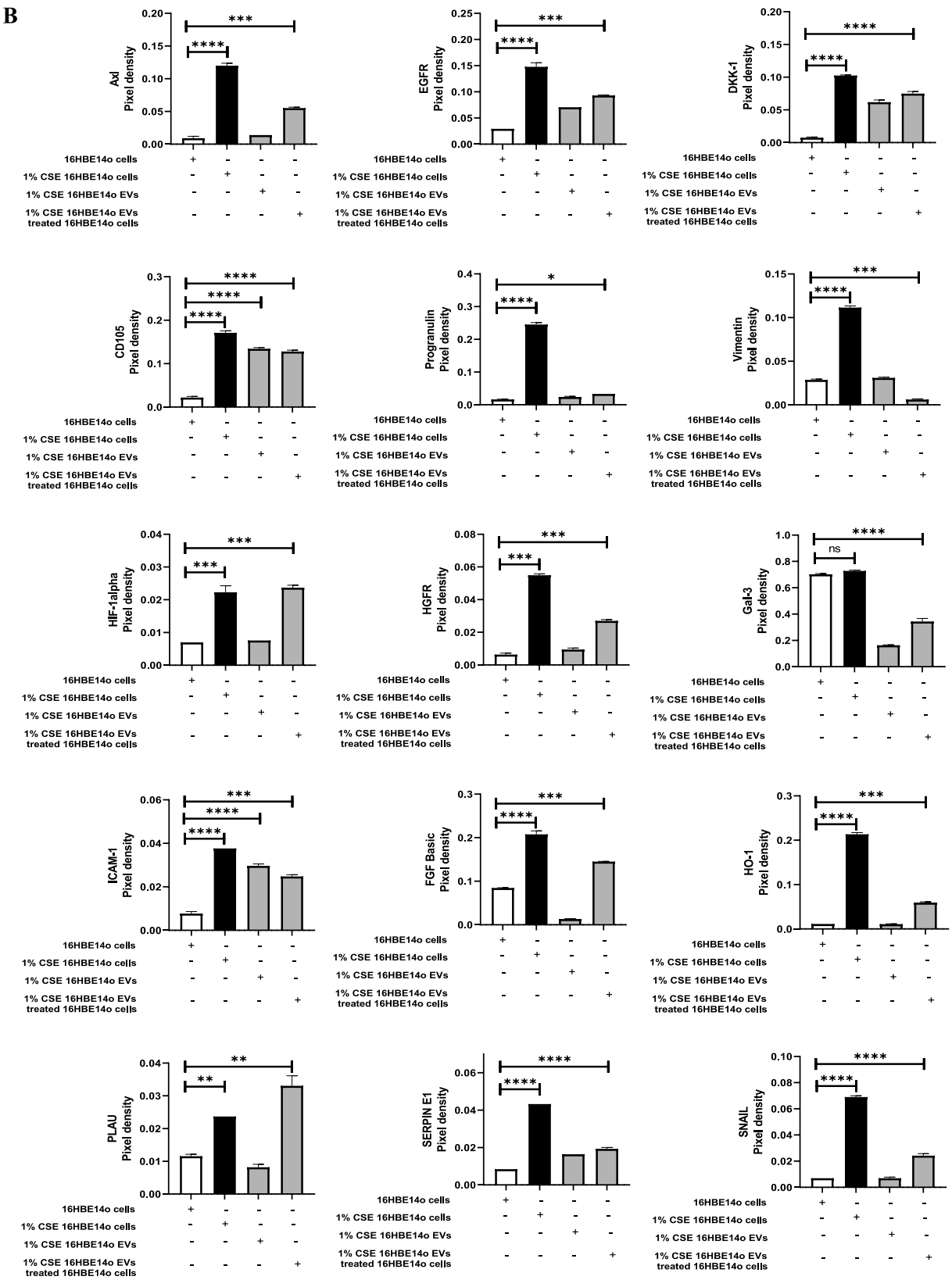


Figure 3: CSE-treated 16HBE14o cells-derived EVs induced tumorigenesis protein and gene expression in healthy bronchial epithelial cells

3A) Oncology array blots of healthy 16HBE14o cells, CSE exposed 16HBE14o cells, CSE induced 16HBE14o-derived EVs, and EVs treated to the healthy 16HBE14o cells. 3B) Pixel density graphs of oncology array showing cancer markers expressed in healthy 16HBE14o cells, CSE exposed 16HBE14o cells, CSE induced 16HBE14o-derived EVs and recipient cells, analysis was done with one-way ANOVA. ns= non-significant ** P ≤ 0.01, ***p<0.001, ****p<0.0001. 3C) Gene ex-pression of ß-catenin in healthy 16HBE140cells, CSE treated 16HBE14o cells, 200 µg of CSE in-duced 16HBE14o-derived EVs and EVs treated recipient 16HBE14o cells after 48hrs. Relative abundance was measured with respect to *GAPDH* gene. Analysis was done with one-way ANOVA. ** P ≤ 0.01, ***p<0.001, ****p<0.0001.

3.3. WNT/ß-catenin pathway in recipient cells (*in vitro*)

To further understand the tumorigenesis induced by CSE induced 16HBE14o-derived EVs, we performed an oncology array on recipient cells. We again performed an oncology array on CSE EVs treated 16HBE14o cells. We found that AXL, EGFR, DKK1, CD105, Progranulin~~, VIM~~, HIF1a~~a~~, FGF2, ICAM1, HMOX1, HGFR, ~~GAL-3,~~ SERPINE1, SNAIL and PLAU oncology proteins were upregulated in CSE EVs treated healthy 16HBE14o cells ~~healthy cells~~ indicating that CSE EVs can induce tumorigenesis (fig. 3A&B). Also, vimentin and Gal-3, which are usually upregulated in cancer, are downregulated compared to the healthy cells, which need further investigation [61, 62]. Among the cargo carried by EVs from parent/originating cells, RNA is one of them [57]. As EVs are known to carry RNA in them [57]. ~~We performed RT-qPCR to reveal the ß-catenin mRNA expression. We observed that the recipient cells have utilised the RNA from CSE EVs indicating that ß-catenin could have translated to protein in the recipient cells (fig. 3C). As EVs are known to carry RNA in them [57],~~ W~~w~~e also performed RT-qPCR on healthy cells, CSE treated cells and EVs as well as ~~and~~ recipient cells(3C). Interestingly, we found that the main component of the WNT/ß-catenin pathway, ß-catenin itself, had its RNA packaged into the CSE EVs. The treated cells utilised the RNA from CSE EVs indicating that ß-catenin could have been translated into protein in the recipient cells (fig. 3C~~B~~).

4. Discussion

Cigarette smoke significantly contribute to tumorigenesis and the mechanism underlying this response remains poorly understood [63]. A few studies indicate that planar aromatics in cigarette smoke can form DNA adducts leading to mutations and cancer [64]. To further

understand the role of cigarette smoke-induced lung cancer progression, we have treated CSE at 1% ~~CSE treatment~~ for eight days~~one week, significantly increasing EV production. W~~and isolated~~e also~~ ~~characterised the EVs by shape using TEM, by size using DLS, and by EVs marker Annexin V using flow cytometry to demonstrate the purity and quality of the EVs collected.

We found that treatment with 1% CSE for one week increased upregulation of WNT/ß-catenin signaling genes such as *WNT3, DVL3, AXIN1* and *ß-catenin* in 16HBE14o cells and similar findings were observed in human lung cancer patients. In a healthy scenario when WNT signaling is not activated (Fig. 4A) ß-catenin is degraded by the Axin complex via phosphorylation [65], However, in the presence of WNT signaling (Fig. 4B) the Axin complex moves towards the membrane leaving inhibition of axin complex mediated phosphorylation. This leads to the accumulation of free ß-catenin and results in the activation/expression of various oncogenic and WNT genes [66]. Exactly how CSE induces the expression of this pathway is still not known. We found that CSE treatment induced expression of ß-catenin, and the RNA of ß-catenin was found in the EVs. In addition, our protein oncology array demonstrated the expression of various inflammation and EMT related proteins in CSE treated cells. Interestingly, a few of them were found in the CSE EVs, that lead us to investigate whether treatment of healthy epithelial cells with CSE EVs could induce tumorigenesis by transferring oncogenic proteins and RNA to the healthy cells.

We performed a scratch wound healing assay to further understand whether the CSE induced 16HBE14o derived EVs carrying oncogenic protein and ß-catenin RNA can induce tumorigenesis. We observed that treatment of CSE induced 16HBE14o derived EVs has significantly induced cell migration (as revealed by would closure after 48hr), indicating the transfer of protein and RNA cargo to healthy cells. In terms of RNA, CSE EVs containing ß-catenin RNA have been taken by recipient cells. Whether the EVs RNA has been transcribed to protein still needs to be explored . To understand the tumorigenesis induced by CSE induced 16HBE14o derived EVs in the context of proteins, an oncology protein array was performed on recipient healthy cells. The cancer related inflammatory proteins like HMOX1, HIF1a~~A~~, ENG, FGF2,~~ GAL3~~ were upregulated in recipient 16HBE14o cells whereas GAL3 is downregulated. ~~along with~~ C~~c~~ell adhesion molecule ICAM and EMT inducer Snail were upregulated whereas EMT inducr Vimentin was downregulated~~ and vimentin.~~ ~~,~~WNT/ß-catenin pathway endogenous inhibitor DKK1, and other markers like AXL, EGFR, HGFR,

SERPINE1, PLAU were expressed in the recipient cells. Overall, our finding suggests that CSE induced 16HBE140 derived EVs induce tumorigenesis by a multitude of factors through WNT/ß-catenin signaling.

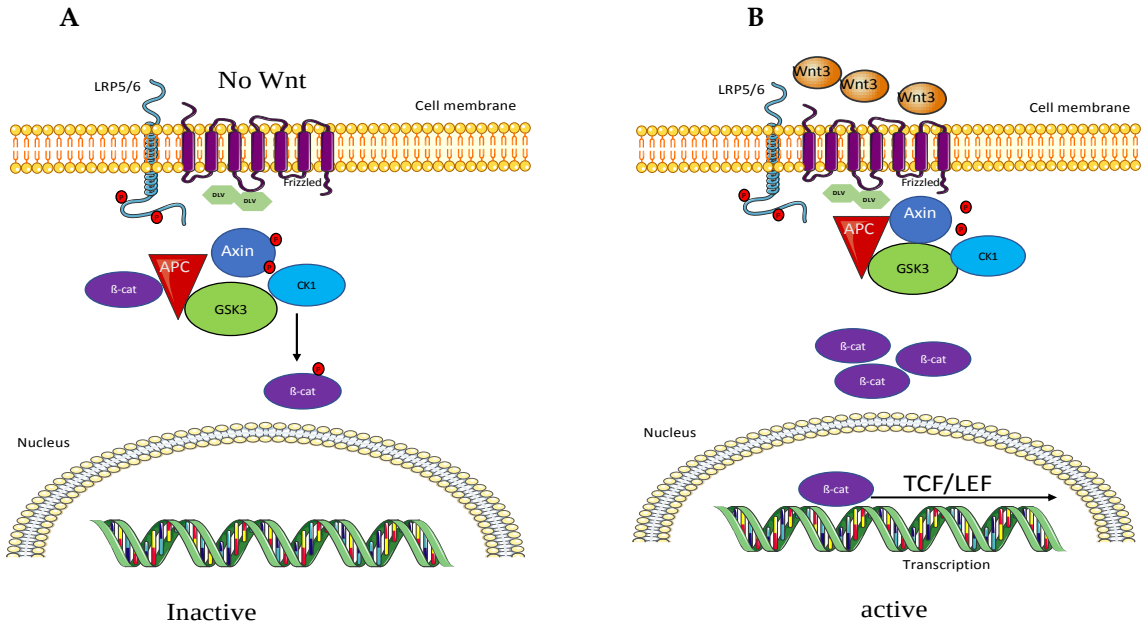


Figure 4: WNT/ß-catenin pathway in the presence and absence of WNT signal

4A) in the absence of WNT signal, ß-catenin is phosphorylated by the Axin complex leading to the degradation of ß-catenin. 4B) In the presence of WNT signal, Axin complex localises to the cell membrane and halts the degradation of ß-catenin, leading to various downstream effects. Abbreviation: LRP5/6: Low-density lipoprotein receptors, DLV; Dishevelled, Axin: Axis Inhibition, APC: adenomatous polyposis coli, GSK3: Glycogen synthase kinase 3, CK1: Casein kinase 1, ß-cat: ß-catenin, TCF/LEF T-cell factor/lymphoid enhancer factor

Despite the role of WNT and ß-catenin in lung cancer, our results further suggest that CS is a critical factor in regulating the WNT/ß-catenin pathway. Additionally, our *in-vitro* and publicly available lung cancer data confirm the high expression of EMT regulator protein ß-catenin, which is known to interact with TCF-LEF (Fig. 4B), which activates various cancer-related pathways, specially EMT molecule E-cadherin [67]. Further, ß-catenin forms ß-catenin-E-cadherin complex causing the release of tight epithelial junctions leading to metastasis, also ß-catenin induce tumorigenesis by suppression of of tumor suppressor gene adenomatous polyposis coli, inducing cell proliferation and cancer cell growth [68, 69]. Therefore, we can conclude from this study that CS induces upregulation of WNT/ß-catenin pathway, contributing to tumorigenesis. Despite the upregulation of the pathway, how carcinogens

contribute to the upregulation and whether this is inducing mutation in WNT/ß-catenin pathway is yet to be validated.

5. Conclusion

Overall, this study indicates that CSE treatment of healthy bronchial epithelial cells can result in tumorigenesis by inducing various inflammatory and WNT/ß-catenin signaling pathways in both *in-vitro* and LC patients. Further, the EVs released by these cells can carry similar markers readily taken up by healthy cells and undergo tumorigenesis. Migration assays confirmed this via regulation of WNT/ß-catenin pathway in healthy cells. Furthermore, this study provides evidence that studying CS induced EVs that contain inflammatory and WNT signaling molecules (in EVs) can be a potential therapeutic target and diagnostic marker for ~~the early~~ detection of CS induced lung cancer. Despite these findings, the mechanistic approach of how CS induced the upregulation of the pathway and the use of the inhibitors as a potential therapy in LC needs to investigated. This needs to be further investigated in pre-clinical models of lung cancer to study if CS induce LC by upregulation of the tumorogenesis via WNT/ß-catenin pathway pathway. In conclusion, the study of EVs in biofluids such as blood, and the use of WNT pathway inhibitors for smoking induced LC could be an early diagnostic and therapy, respectively for LC.

Author Contributions

Conceptualisation, VM, KD, PMH; methodology, software, validation, formal analysis, investigation, VM, GDR; writing—original draft preparation, VM; writing-review and editing, VM, NGH, KRP, PM.H, KD; supervision, KD, KRP, and PMH. All authors have read and agreed to the published version of the manuscript.

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Institutional Review Board Statement

Not applicable.

Informed Consent Statement

Not applicable.

Data Availability Statement

The data presented in this study are available on request from the corresponding author.

Conflicts of Interest

The authors declare no conflict of interest.

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Cigarette smoking induces lung cancer tumorigenesis *via* upregulation of the WNT/ß-catenin signaling pathway

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Abstract: Lung cancer has the highest mortality rate compared to any other cancer worldwide, and cigarette smoking is one of the major etiological factors. How cigarette smoke (CS) induces tumorigenesis in healthy cells is still not completely understood. In this study, we treated healthy human bronchial epithelial cells (16HBE14o) with 1% cigarette smoke extract (CSE) for one week. The CSE exposed cells showed upregulation of WNT/ß-catenin pathway genes like *WNT3, DLV3, AXIN* and *ß-catenin*, 30 oncology proteins were found to be upregulated after CSE treatment. Further, we explored whether the role of extracellular vesicles (EVs) obtained from CSE exposed cells can induce tumorigenesis. We observed that CSE EVs induced migration of healthy 16HBE14o cells by upregulation of various oncology proteins in recipient cells like AXL, EGFR, DKK1, ENG, FGF2, ICAM1, HMOX1, HIF1a, SERPINE1, SNAIL, HGFR, PLAU which are related to WNT signaling, epithelial mesenchymal transition (EMT) and Inflammation, whereas inflammatory marker, GAL-3 and EMT marker, VIM were downregulated. Moreover, ß-catenin RNA was found in CSE EVs, upon treatment of these EVs to healthy cells, the ß-catenin gene level was decreased in recipient cells compared to healthy 16HBE14o cells, indicating the utilisation of ß-catenin RNA in healthy cells. Overall, our study suggests that CS treatment can induce tumorigenesis of healthy cells by upregulating WNT/ß-catenin signaling *in vitro* and human lung cancer patients. Therefore targeting the

WNT/ß-catenin signaling pathway is involved in tumorigenesis inhibition of this pathway could be a potential therapeutic approach for cigarette smoke induced lung cancer.
Keywords: Extracellular vesicles, cigarette smoke, tumorigenesis, lung cancer. WNT/ß-catenin signaling.

ABBREVIATIONS

LC: lung cancer, CS: cigarette smoke, 16HBE14o: healthy human broncho epithelial cells, CSE: cigarette smoke extract, COPD: chronic obstructive pulmonary disease, PAH: polyaromatic hydrocarbons, NNK: 4-(methylnitrosamino)-1-(3-pyridyl)-1-butanone, KRAS: Kirsten rat sarcoma viral oncogene homolog, TP53: tumor protein, DNA: Deoxyribonucleic acid, miRNA: micro Ribonucleic acid, nAChRs: Nicotinic acetylcholine receptors, β-ARs: β-Adrenergic receptors, cAMP: adenylyl cyclase and cyclic, NF-kB: nuclear factor-κB, NSCLC: non-small cell lung cancer, WNT: Wingless, FBS: foetal bovine serum, DMEM: Dulbecco's modified eagle medium, IL: interleukin, COX2: cyclooxygenase2, PBS: Phosphate buffered saline, DLS: Dynamic light scattering, RIPA: Radioimmunoprecipitation assay buffer, TEM: Transmission electron microscopy, GAPDH: Glyceraldehyde-3-Phosphate Dehydrogenase, AXL: AXL receptor tyrosine kinase, DKK1: Dickkopf, CTSD: Cathepsin D ,CTSB: Cathepsin D, CAPG: Capping Actin Protein, Gelsolin Like, ENG: Endoglin, ENO2: Enolase, ERBB5: erythroblastic oncogene B, FGF2: Fibroblast Growth Factor 2 , ICAM1: Intercellular Adhesion Molecule 1, HMOX1: Heme Oxygenase 1, HIF1a: Hypoxia-inducible factor, MET: MET Proto-Oncogene, Receptor Tyrosine Kinase, CAG: Cytosine, Adenine, Guanine, GAL-3: Galectin-3, FOXC1: Forkhead Box C1 , FOXC2: Forkhead Box C1, MSLN: Mesothelin, PGDFA: Platelet Derived Growth Factor Subunit A, SPP1: Secreted Phosphoprotein 1, GRN: Granulin Precursor, SERPINB5 serine protease inhibitor B5, SERPINE1: serine protease inhibitor E1, SNAIL: Zinc finger protein SNAI1, VIM: Vimentin.

1. Introduction

Cigarette smoke (CS) exposure accounts for 90% of lung cancer in men and 70-80% in females (LC) cases [1, 2]. For the remaining % of people with LC, the cause is likely due to other factors such as pre-existing lung diseases such as chronic obstructive pulmonary disease (COPD), genetic, environmental, viral, and hormonal factors [3, 4]. In 2020, 2.2 million new cases and 1.7 million mortalities were attributed to lung cancer [5]. LC is the most complicated lifestyle-related cancer with a poor diagnosis and prognosis [6]. Moreover, there is a lack of promising early diagnostic markers and the current therapeutic approach cannot significantly

improve the overall survival rate [7-9]. It is widely accepted that cigarette smoking is the primary risk factor, but how cigarette carcinogens contribute to LC is not elucidated [10, 11].

It is estimated that nearly 7000 chemicals are generated from CS, out of which more than 60 are reported to be carcinogens, like polyaromatic hydrocarbons (PAH), benzo[a]pyrene, and nitrosamine derived compound 4-(methylnitrosamino)-1-(3-pyridyl)-1-butanone (NNK) [12-14]. Generally, these carcinogens are metabolised by phase 1 detoxifying enzymes such as cytochrome p420, which adds an oxygen atom to make them water soluble to be excreted from the body [15]. PAH are highly active compounds that are metabolised by cytochrome p450 enzymes, but regular smoking can lead to accumulation in the body; these potent PAH carcinogens can bind to DNA and form DNA adducts leading to a process called metabolic activation, which may lead to mutations in the tumor protein (TP53) gene [16, 17] as well as mutation mainly in 12 codons of Kirsten rat sarcoma viral oncogene homolog (KRAS) and Epidermal growth factor receptor (EGFR) [18, 19]. Thus, balancing the deactivation and formation of DNA adducts determine the progression of LC. Therefore, the excretion of these carcinogens is critical in preventing tumorigenesis.

Besides the DNA mutations, CS dysregulates various carcinogenic pathways like Nicotinic acetylcholine receptors (nAChRs) signaling as nicotine binds to various α and β subunits of nAChRs and interrupts ion influxes. It is already known that this pathway is upregulated in LC [20, 21]. NNK and nicotine binding to α7 subunit of nAChRs can activate phosphoinositide 3-kinases/protein kinase B signaling pathway [22, 23]. Along with nAChRs, nicotine and other carcinogens are reported to bind to β-Adrenergic receptors (β-ARs), leading to β-ARs mediated signaling, causing increased cell proliferation of epithelial cells. This can lead to adenylyl cyclase and cyclic (cAMP) signaling leading to LC [24-26]. Besides the cancer pathways, inflammatory pathways are crucial for the LC progression [27]. For example, the nuclear factor-κB (NF-kB) pathway has a role in inducing the production of pro-inflammatory markers like interleukin (IL)-6, IL-8, and cyclooxygenase (COX2) and influence cancer progression by recruiting neutrophils and macrophages [28-31]. Other pathways dysregulated by cigarette smoking are Notch signaling and embryogenesis pathways like Hedgehog and WNT/ß-catenin [32-34]. WNT/ß-catenin signaling is an evolutionarily conserved signal transduction pathway that helps in cell-to-cell communication and aids in maintaining cell proliferation, cell polarity, cell homeostasis, embryogenesis, and cancer [35-37]. Mutations or over-activation of these

pathways leads to various human diseases, including cancer [38]. WNT/ß-catenin signaling is widely studied in colon cancer. However, its role in LC is not fully elucidated [39, 40].

Extracellular vesicles (EVs) are tiny particles released from all cells in the human body and don't have the capability to divide. Based on the size, EVs are categorised into medium EVs (> 200nm) and small EVs (< 200nm) [41]. These EVs carry a lot of functional molecules like proteins, RNAs, DNA, and lipids [42-44]. The packages of different cargo into EVs depend on the cell of EVs origin and diseased condition [45, 46]. The EVs are mediators in cell-to-cell communication and help maintain homeostasis in the body [42]. Researchers have recently tried to validate if the tumors derived EVs stimulate tumorigenesis in healthy recipient cells by transferring oncogenic material like miRNA and oncogenic proteins to healthy cells [34, 47, 48]. Therefore, further understanding the role of cancer-derived EVs helps understand tumorigenesis pathways and find effective diagnostic and prognostic tools, as these EVs are primarily released into the biological fluids [49, 50].

It is recently known that cigarette smoke can induce changes in EV composition and release, but their role is not entirely understood [51]. However, few reports highlight that smoking enhances EV release and plays a crucial role in the pathogenesis of non-small cell lung cancer (NSCLC) [34, 52, 53]. This study investigated how CSE treatment to healthy bronchial epithelial cells can impact WNT/ß-catenin signaling pathways and how the CSE treatment derived EVs can induce tumorigenesis in healthy 16HBE14o cells.

2. Materials and Methods

2.1. EVs depletion from foetal bovine serum

As commercially available foetal bovine serum (FBS) contains small EVs, removing the small EVs from FBS is essential while performing an *in vitro* experiment with the EVs [49]. In our study, to deplete the exosomes from FBS, the heat inactivated FBS was initially centrifuged at 18,000g for 90mins to remove any medium size EVs present in the FBS. Soon after, the supernatant was centrifuged for 12 hours at 100,000g, maintaining $4^{o}C$ to remove small EVs pellets at the bottom. The supernatant passed through a 0.22 micron filter. These small EVs depleted FBS were used for culturing the human normal bronchial epithelial cells (16HBE14o) [54].

2.2. Cell culture

Healthy human bronchial epithelial cells (16HBE14o) were cultured as previously mentioned [34].

2.3. Cigarette smoke extract(CSE) preparation

CSE was generated by a custom built smoking device where one research cigarette (Reference Cigarette 3R4F, University of Kentucky) was lit and drawn manually into 10ml of PBS through silicon tubing. This 10ml extract was considered 100% cigarette smoke extract and filtered through a 0.22 micron filter to sterilise it. The CSE was then diluted in low glucose DMEM media based on the usage percentage. CSE was prepared fresh before the start of any experiment [55, 56].

2.4. Exposing cells to CSE

A density of $2x10^6$ of 16HBE14o cells were seeded into multiple T-175 flasks using small EVs depleted low glucose DMEM media(as described in section 2.1). Next day, discard the media and wash cells with PBS to remove any unattached or dead cells. Freshly prepared 1% CSE was added to the cells on Monday and left for 24 hours. The following day media was replaced with fresh media (without CSE). This cycle continued daily until Friday. On Friday, cells were treated with 1% CSE and left over the weekend and on Monday, media was collected to isolate CSE EVs (Fig 1A). Along with the CSE EVs, 1% CSE treated cells were harvested for protein and RNA extraction (n=5 biological replicates).

2.5. Isolation of EVs

1% CSE treated supernatant was collected and centrifuged as discussed previously, the final pellet containing CSE EVs was resuspended in 1ml PBS and stored at -80°C for characterisation and downstream protein or RNA extraction (Fig 1B) [34, 46].

2.6. Dynamic Light Scattering

1% CSE treated 16HBE14o EVs size and polydispersity index were measured using dynamic light scattering (DLS). A small portion of the EV fraction was diluted in PBS, equilibrated to 37°C, and DLS was measured using a 1cm path length cuvette. DLS measurements were recorded in triplicates using Zetasizer Nano ZS equipped with a He-Ne 633nm laser light source and readings measured at 25°C in a size range of 0.3–10,000nm. The Zeta average

diameter of 1% CSE treated EVs was 492.1(n=6 biological replicates and 3 technical replicates) [34].

2.7. Protein extraction and quantification

200µl of CSE EVs suspension was centrifuged as described above to isolate EVs. Radioimmunoprecipitation assay buffer (RIPA) buffer containing phosphatase and protease inhibitor cocktail was added to the CSE EVs followed by vortexing and incubation on ice for 15mins. The mix was then sonicated at 30% amplitude three times for 2 seconds and incubated again on ice for an additional 15mins. the lysate was then centrifuged for 30mins at 18,000g and 4°C, and the supernatant collected. The process for extracting proteins from cells is similar except the protein lysate was centrifuged for 12,000g at 4°C for 10mins and the protein concentration was quantified using a Pierce^{TM} BCA Protein Array Kit (Thermo Scientific) [34].

2.8. Flow cytometry

5µg of CSE 16HBE14o EVs were analysed by flow cytometry. EVs stained with V450 annexin V (BD HorizonTM catalogue No. 560506) for 30mins without light. 5 µg of unstained samples were run to identify the concentration of EVs for flow and accordingly gating strategy was made. Annexin V stained EVs were run with and without Calcium, as annexin V binds to EVs only with Calcium (n=3 biological replicates) [34].

2.9. Transmission electron microscopy (TEM)

EV samples were suspended in PBS on carbon coated copper grids (Mesh 200) (GSAU200F-50, ProSciTech) and left to attach for 1hr. The EVs were then fixed onto the grid with 1% glutaraldehyde, washed with Milli-Q water and negatively stained with 1.5% uranyl acetate and dried overnight in desiccator images were taken the following day using a Tecnai T20 is a 200kV TEM TWIN electron microscope (n=3 biological replicates) [57].

2.10. Protein array of CSE EVs and Cells

Protein from 16HBE14o cells, 1% CSE treated 16HBE14o cells, 1% CSE treated 16HBE14o EVs and CSE EVs treated healthy 16HBE14o cells were isolated and equal amounts (600µg) were used to run the oncology array as per the manufacturer's instructions (https://www.rndsystems.com). Blots are imaged using the Biorad imaging platform. Data was analysed by measuring pixel density in ImageJ software [34, 55, 58].

2.11. Migration (scratch wound) assay

16HBE14o cells were seeded in a 6-well plate at $2x10^4$ cells per well and incubated for 24 hrs. Following day media was removed and washed twice with PBS. A 200ul yellow pipette tip was used to make a vertical scratch and washed 3 times with PBS to remove unattached cells. 9 images of each well taken at 10x at zero hours. Cells were then treated with 0, 5, 10 and 20µg protein equivalent of 1% CSE 16HBE14o EVs and the migration length measured 24 and 48hrs later (n=5 biological replicates and 9 technical replicates) [34, 59, 60].

2.12. RNA extractions and assessment of gene expression level by real-time PCR.

RT-PCR was performed as previously mentioned [34] with genes related to WNT/β-catenin signaling pathway table 1.

Gene Name	**Forward**	**Reverse**	**Ref Seq ID**
CTNNB1	CTTGGAATGAGACTGCTG	AGAGTGAAAAGAACGATAGC	NM_001904
DVL3	ATGTCACTCAACATCATCAC	CCTTCATGATAGAGCCAATG	NM_004423
AXIN1	CCGACCTTANATGAAGATGAG	CAGGATCCATACCTGAACTC	NM_003502
WNT3	CTGTGACTCGCATCATAAG	ATGTGGTCCAGGATAGTC	NM_030753
GAPDH	ACAGTCAGCCGCATCTTCTT	GACCAAATCCGTTGACTCCGA	NM_008085

Table 1: WNT/β-catenin signaling human primer sequences

The quantitative expression of the genes was calculated through $2^{-\Delta\Delta Ct}$, corresponding to the house keeping gene GAPDH. The relative abundance was calculated with the help of untreated control [34].

2.15. Statistical analysis

Data are presented as mean ± SEM. Statistical analyses were performed using one-way ANOVA for multiple comparisons and T-test for two group comparisons using the Graph Pad Prism software (version 9.3). Statistical significance was accepted at $p < 0.05$.

3. Results

3.1. CSE treatment and characterisation of EVs

To determine whether treating healthy 16HBE14o cells with CSE over time can induce any cancer-like phenotype, we have optimised the concentration of CSE to 1% so that there is a

gradual induction of tumorigenesis. 1% CSE was treated just over a period of eight days and EVs were collected as shown from day 7&8(48hrs) (Fig. 1A) [56]. The isolation of medium size EVs (200-1000nm) involves very well established multiple centrifugation steps (Fig.1B) [50]. According to the guidelines [36], the EVs are characterised by at least three different methods. Among these methods, we used DLS for size distribution. We observed that EVs were in the size range of medium EVs (>200-1000nm) with no contamination from small EVs (<200nm), indicating the purity of EVs with an average particle size of Z Average is 492.1 nm (Fig. 1C).

Transmission electron microscopy (TEM) is often used to visualise EVs and to determine EVs from non-EVs material. Traditionally, EVs resemble a cup-shape in TEM images due to a dehydration phase during sample preparation [51]. We also found cup-shaped EVs with no additional non-EV material (Fig. 1D). Another characterisation technique of EVs is identification with a maker that is important in the context of biological properties. As EVs contain lipid membranes, characterising via membrane lipids is another way to confirm EVs. We have carried out Annexin V staining of phosphatidylserine (PS) on EVs [52]. The process of membrane shedding releases EVs and therefore PS is carried with medium EVs [53]. Annexin V is known to bind to PS only in a Calcium dependent manner [49, 53]. Therefore, we have visualised PS by flow cytometry and confirmed the binding of Annexin V in the presence and absence of Calcium (Fig. 1E).

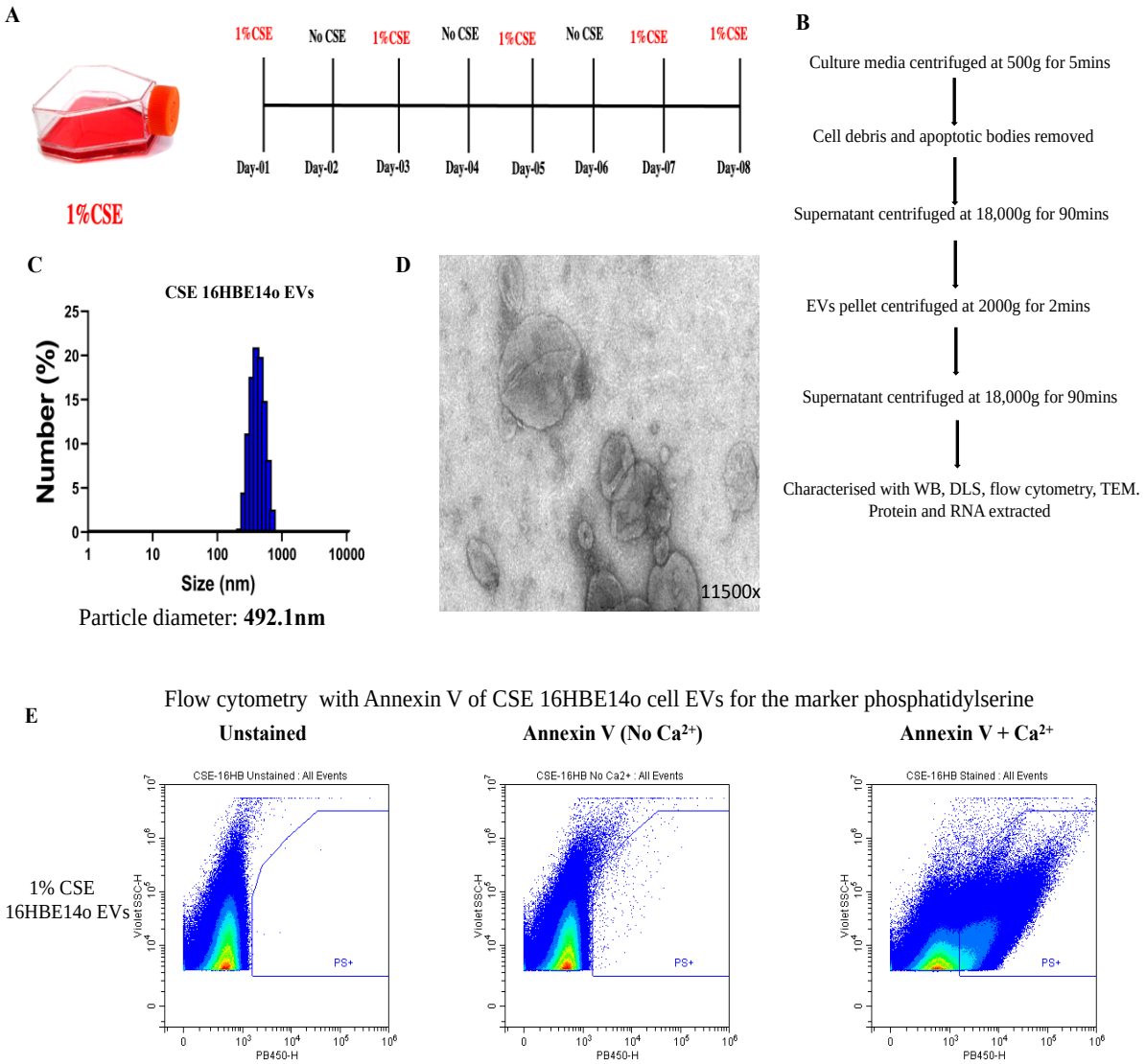
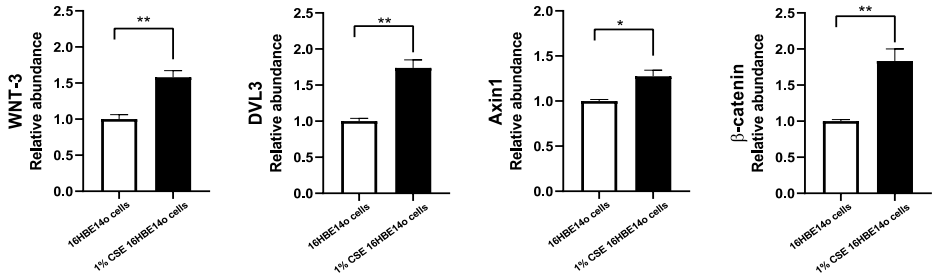


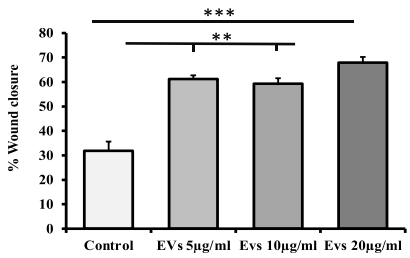
Figure 1. Isolation and characterisation of 1% CSE EVs

1A) Healthy 16HBE14o cells were treated with 1% CSE for 8 days. 1B) Flow chart of CSE EVs isolation process 1C) DLS of CSE EVs found to be in the range of medium EVs with an average particle diameter of 492.1nm. 1D) TEM image of CSE EVs, cup shaped EVs indicate the dehydration of EVs during sample preparation, 11,500x magnification. 1E) Representative dot-plots from flow cytometric analysis of phosphatidylserine exposure. Unstained CSE EV samples (left) analysed as control for autofluorescence, staining in the absence of Calcium (middle) was performed to control for specific Annexin V binding, stained with Annexin V in the presence of Calcium (right) to detect phosphatidylserine surface exposure.

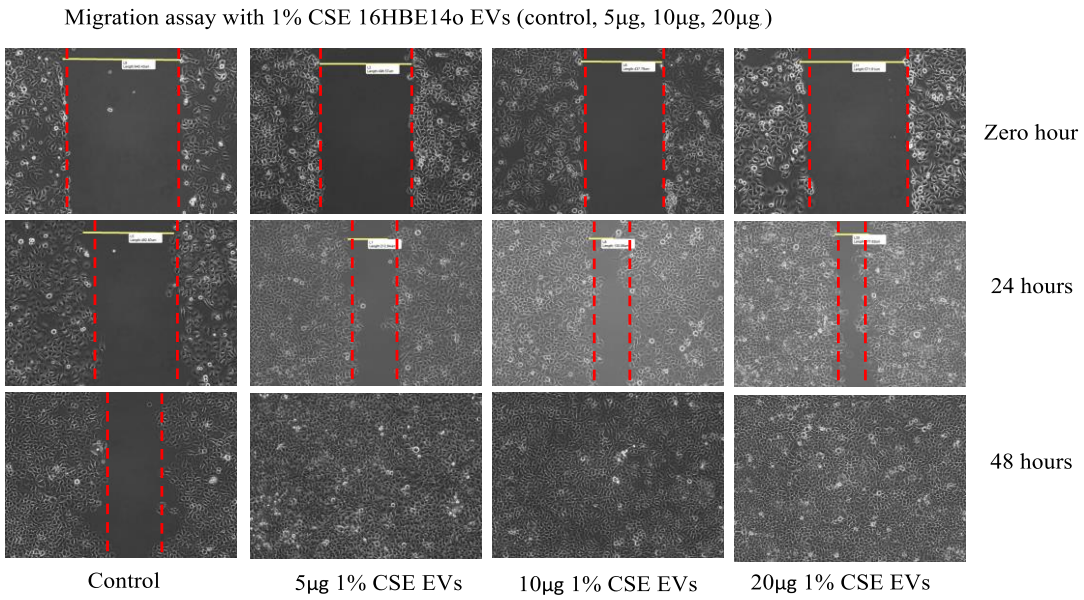
A


Upregulation of WNT/β-catenin pathway in CSE 16HBE14o cells
WNT-3
Relative abundance
DVL3
Relative abundance
Axin1
Relative abundance
β-catenin
Relative abundance
16HBE14o cells
1% CSE 16HBE14o cells

B


% Wound closure
Control
EVs 5μg/ml
Evs 10μg/ml
Evs 20μg/ml

C


Migration assay with 1% CSE 16HBE14o EVs (control, 5μg, 10μg, 20μg)
Zero hour
24 hours
48 hours
Control
5μg 1% CSE EVs
10μg 1% CSE EVs
20μg 1% CSE EVs

D

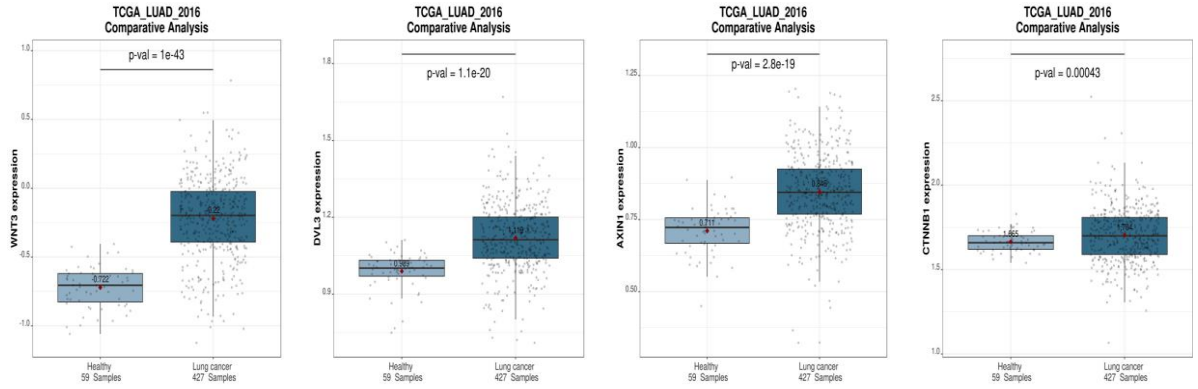

TCGA_LUAD_2016
Comparative Analysis
p-val = 1e-43
WNT3 expression
p-val = 1.1e-20
DVL3 expression
p-val = 2.8e-19
AXIN1 expression
p-val = 0.00043
CTNNB1 expression
Healthy
59 Samples
Lung cancer
427 Samples

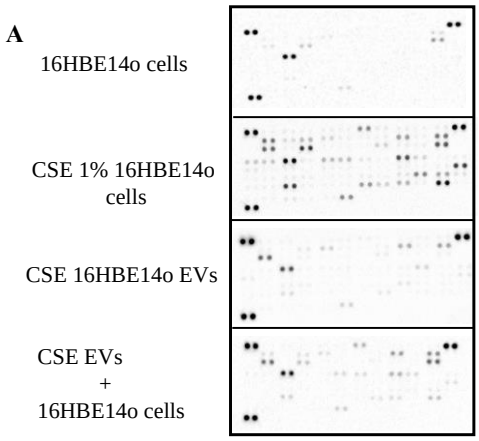
Figure 2. CSE EVs induced Migration in healthy cells

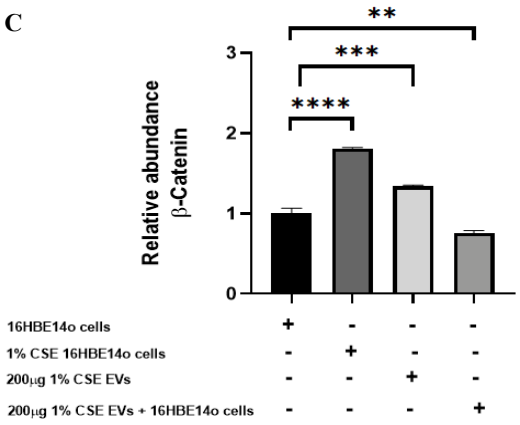
2A) 16HBE14o cells treated with CSE had expressed high levels of WNT/ß-catenin RNA levels including *WNT3, DLV3, Axin1* and *ß-catenin*. T-test was performed ** P ≤ 0.01, * P< 0.05 vs control. 2B) Healthy 16HBE14o cells treated with 1% CSE EVs showed significant Migration compared to the control. Data were analysed using an ANOVA and % wound closure was calculated. ** P ≤ 0.01, ***p<0.001. 2C) Microscopic images of wound closure induced by CSE EVs at 5 µg, 10µg, and 20µg protein equivalent of EVs compared to control. Images were taken at 0hr, 24hr, and 48hrs. 100% of wound closure was achieved by 48hrs at all concentrations. Images were taken at 10x magnification. 2D) Comparative analysis of healthy lung tissue vs lung tumors in smokers of *WNT3, DLV3, Axin1* and *ß-catenin*.

3.2. Upregulation of WNT/ß-catenin pathway genes and CSE induced tumorigenesis

Treatment of 16HBE14o cells with 1% CSE for 8 days (every alternative days for 6 days) induced upregulation of WNT/ß-catenin pathway genes such as *WNT3*, Dishevelled Segment Polarity Protein 3 (*DVL3*) a key molecule in binding to the cell surface and propagating actions of WNT based signals [54], and *Axin1* a vital component of the *ß-catenin* destruction complex which regulates the activity ß-catenin [55] (the critical component of tumorigenesis and EMT) (Fig. 2A). Furthermore, we validated these findings with publicly available datasets of lung cancer(Fig. 2E) and compared healthy lung tissue in smokers with lung tumor tissue of smokers, indicating similar observations in *WNT3, DVL3, Axin1* and *ß-catenin*. This further confirms that CS induces upregulation of the WNT/ß-catenin pathway [56] (https://lce.biohpc.swmed.edu/, accessed on 15 June 2022). We performed an oncology array of 84 cancer proteins from CSE treated cells and EVs and CSE EVs treated healthy 16HBE14o cells. We found 30 oncogenic proteins such as AXL, EGFR, DKK1, CTSD, CTSB, CAPG, ENG, ENO2, ERBB5, FGF2, ICAM1, HMOX1, HIF1a, MET, CAG, GAL-3, FOXC1, FOXC2, IL-8, MSLN, PGDFA, TP53, P27, SPP1, GRN, SERPINB5, SERPINE1, SNAIL, VIM, PLAU. All of these are related to cancer induced inflammation, ß-catenin regulators and EMT (Fig. 2B). Among these proteins, a few of the oncogenic proteins are encapsulated in CSE derived EVs like AXL, EGFR, DKK1, CTSD, ENG, ICAM1, IL-8, SPP1, GRN, SERPINE (Fig. 2B). This led us to treat healthy cells with these EVs to determine whether these can induce tumorigenic features in healthy cells. To validate this, we performed scratch wound/migration assays using different concentrations of CSE EVs and found that there was a

significant amount of wound closure after 24hr and by 48hr there was 100% wound closure (Fig. 2C and D)





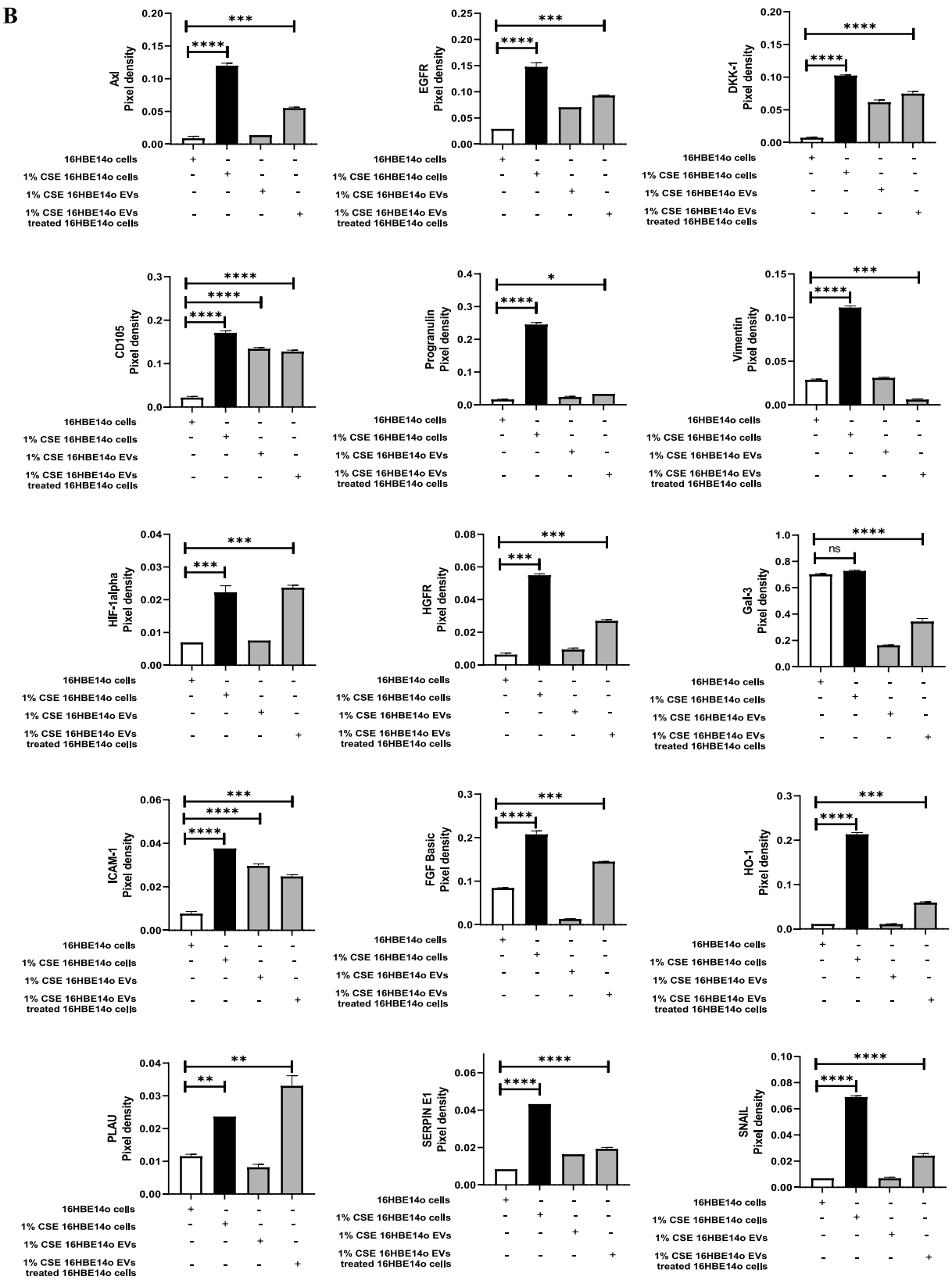


Figure 3: CSE-treated 16HBE14o cells-derived EVs induced tumorigenesis protein and gene expression in healthy bronchial epithelial cells

3A) Oncology array blots of healthy 16HBE14o cells, CSE exposed 16HBE14o cells, CSE induced 16HBE14o-derived EVs, and EVs treated to the healthy 16HBE14o cells. 3B) Pixel density graphs of oncology array showing cancer markers expressed in healthy 16HBE14o cells, CSE exposed 16HBE14o cells, CSE induced 16HBE14o-derived EVs and recipient cells, analysis was done with one-way ANOVA. ns= non-significant ** P ≤ 0.01, ***p<0.001, ****p<0.0001. 3C) Gene ex-pression of ß-catenin in healthy 16HBE140cells, CSE treated 16HBE14o cells, 200 µg of CSE in-duced 16HBE14o-derived EVs and EVs treated recipient 16HBE14o cells after 48hrs. Relative abundance was measured with respect to *GAPDH* gene. Analysis was done with one-way ANOVA. ** P ≤ 0.01, ***p<0.001, ****p<0.0001.

3.3. WNT/ß-catenin pathway in recipient cells (*in vitro*)

To further understand the tumorigenesis induced by CSE induced 16HBE14o-derived EVs, we performed an oncology array on recipient cells. We again performed an oncology array on CSE EVs treated 16HBE14o cells. We found that AXL, EGFR, DKK1, CD105, Progranulin, HIF1a, FGF2, ICAM1, HMOX1, HGFR, SERPINE1, SNAIL and PLAU oncology proteins were upregulated in CSE EVs treated healthy 16HBE14o cells indicating that CSE EVs can induce tumorigenesis (fig. 3A&B). Also, vimentin and Gal-3, which are usually upregulated in cancer, are downregulated compared to the healthy cells, which need further investigation [61, 62]. Among the cargo carried by EVs from parent/originating cells, RNA is one of them [57]. As EVs are known to carry RNA in them [57]. We also performed RT-qPCR on healthy cells, CSE treated cells and EVs as well as recipient cells(3C). Interestingly, we found that the main component of the WNT/ß-catenin pathway, ß-catenin itself, had its RNA packaged into the CSE EVs. The treated cells utilised the RNA from CSE EVs indicating that ß-catenin could have been translated into protein in the recipient cells (fig. 3C).

4. Discussion

Cigarette smoke significantly contribute to tumorigenesis and the mechanism underlying this response remains poorly understood [63]. A few studies indicate that planar aromatics in cigarette smoke can form DNA adducts leading to mutations and cancer [64]. To further understand the role of cigarette smoke-induced lung cancer progression, we have treated CSE at 1% for eight days and isolated characterised the EVs by shape using TEM, by size using DLS, and by EVs marker Annexin V using flow cytometry to demonstrate the purity and quality of the EVs collected.

We found that treatment with 1% CSE for one week increased upregulation of WNT/ß-catenin signaling genes such as *WNT3, DVL3, AXIN1* and *ß-catenin* in 16HBE14o cells and similar findings were observed in human lung cancer patients. In a healthy scenario when WNT signaling is not activated (Fig. 4A) ß-catenin is degraded by the Axin complex via phosphorylation [65], However, in the presence of WNT signaling (Fig. 4B) the Axin complex moves towards the membrane leaving inhibition of axin complex mediated phosphorylation. This leads to the accumulation of free ß-catenin and results in the activation/expression of various oncogenic and WNT genes [66]. Exactly how CSE induces the expression of this pathway is still not known. We found that CSE treatment induced expression of ß-catenin, and the RNA of ß-catenin was found in the EVs. In addition, our protein oncology array demonstrated the expression of various inflammation and EMT related proteins in CSE treated cells. Interestingly, a few of them were found in the CSE EVs, that lead us to investigate whether treatment of healthy epithelial cells with CSE EVs could induce tumorigenesis by transferring oncogenic proteins and RNA to the healthy cells.

We performed a scratch wound healing assay to further understand whether the CSE induced 16HBE14o derived EVs carrying oncogenic protein and ß-catenin RNA can induce tumorigenesis. We observed that treatment of CSE induced 16HBE14o derived EVs has significantly induced cell migration (as revealed by would closure after 48hr), indicating the transfer of protein and RNA cargo to healthy cells. In terms of RNA, CSE EVs containing ß-catenin RNA have been taken by recipient cells. Whether the EVs RNA has been transcribed to protein still needs to be explored . To understand the tumorigenesis induced by CSE induced 16HBE14o derived EVs in the context of proteins, an oncology protein array was performed on recipient healthy cells. The cancer related inflammatory proteins like HMOX1, HIF1a, ENG, FGF2, were upregulated in recipient 16HBE14o cells whereas GAL3 is downregulated. Cell adhesion molecule ICAM and EMT inducer Snail were upregulated whereas EMT inducr Vimentin was downregulated. WNT/ß-catenin pathway endogenous inhibitor DKK1, and other markers like AXL, EGFR, HGFR, SERPINE1, PLAU were expressed in the recipient cells. Overall, our finding suggests that CSE induced 16HBE140 derived EVs induce tumorigenesis by a multitude of factors through WNT/ß-catenin signaling.

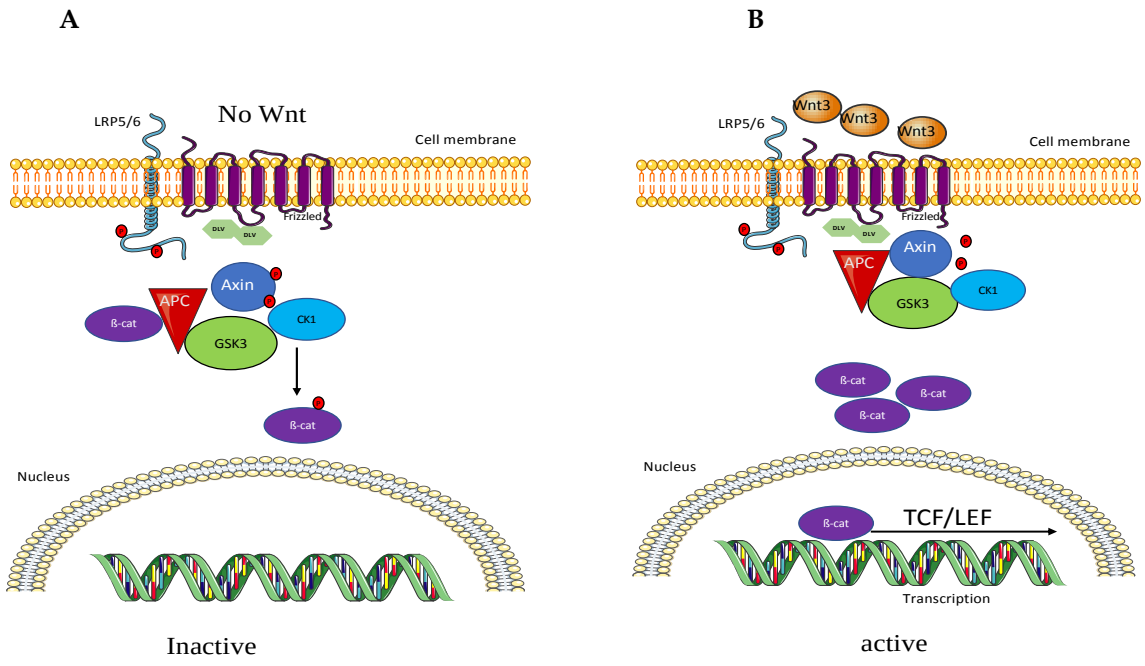


Figure 4: WNT/ß-catenin pathway in the presence and absence of WNT signal

4A) in the absence of WNT signal, ß-catenin is phosphorylated by the Axin complex leading to the degradation of ß-catenin. 4B) In the presence of WNT signal, Axin complex localises to the cell membrane and halts the degradation of ß-catenin, leading to various downstream effects. Abbreviation: LRP5/6: Low-density lipoprotein receptors, DLV; Dishevelled, Axin: Axis Inhibition, APC: adenomatous polyposis coli, GSK3: Glycogen synthase kinase 3, CK1: Casein kinase 1, ß-cat: ß-catenin, TCF/LEF T-cell factor/lymphoid enhancer factor

Despite the role of WNT and ß-catenin in lung cancer, our results further suggest that CS is a critical factor in regulating the WNT/ß-catenin pathway. Additionally, our *in-vitro* and publicly available lung cancer data confirm the high expression of EMT regulator protein ß-catenin, which is known to interact with TCF-LEF (Fig. 4B), which activates various cancer-related pathways, specially EMT molecule E-cadherin [67]. Further, ß-catenin forms ß-catenin-E-cadherin complex causing the release of tight epithelial junctions leading to metastasis, also ß-catenin induce tumorigenesis by suppression of of tumor suppressor gene adenomatous polyposis coli, inducing cell proliferation and cancer cell growth [68, 69]. Therefore, we can conclude from this study that CS induces upregulation of WNT/ß-catenin pathway, contributing to tumorigenesis. Despite the upregulation of the pathway, how carcinogens contribute to the upregulation and whether this is inducing mutation in WNT/ß-catenin pathway is yet to be validated.

5. Conclusion

Overall, this study indicates that CSE treatment of healthy bronchial epithelial cells can result in tumorigenesis by inducing various inflammatory and WNT/ß-catenin signaling pathways in both *in-vitro* and LC patients. Further, the EVs released by these cells can carry similar markers readily taken up by healthy cells and undergo tumorigenesis. Migration assays confirmed this via regulation of WNT/ß-catenin pathway in healthy cells. Furthermore, this study provides evidence that studying CS induced EVs that contain inflammatory and WNT signaling molecules (in EVs) can be a potential therapeutic target and diagnostic marker for detection of CS induced lung cancer. Despite these findings, the mechanistic approach of how CS induced the upregulation of the pathway and the use of the inhibitors as a potential therapy in LC needs to investigated. This needs to be further investigated in preclinical models of lung cancer to study if CS induce LC by upregulation of the tumorogenesis via WNT/ß-catenin pathway pathway. In conclusion, the study of EVs in biofluids such as blood, and the use of WNT pathway inhibitors for smoking induced LC could be an early diagnostic and therapy, respectively for LC.

Author Contributions

Conceptualisation, VM, KD, PMH; methodology, software, validation, formal analysis, investigation, VM, GDR; writing—original draft preparation, VM; writing-review and editing, VM, NGH, KRP, PM.H, KD; supervision, KD, KRP, and PMH. All authors have read and agreed to the published version of the manuscript.

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Data Availability Statement

The data presented in this study are available on request from the corresponding author.

Conflicts of Interest

The authors declare no conflict of interest.

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Credit Author Statement

Author Contributions

Conceptualisation, V.M., K.D., P.M.H; methodology, software, validation, formal analysis, investigation, V.M, G.D.R; writing—original draft preparation, V.M.; writing-review and editing, V.M, N.G.H, K.R.P, PM.H, K.D; supervision, K.D., K.R.P., and P.M.H. All authors have read and agreed to the published version of the manuscript.

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