



Extracellular vesicle-associated epidermal growth factor receptor as a potential liquid biopsy biomarker in lung adenocarcinoma: a case-control study

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Background: Extracellular vesicles (EVs) have recently emerged as potential noninvasive biomarkers for liquid biopsy because of the limitations of tissue biopsies in lung cancer. This study investigated the presence of EV-associated epidermal growth factor receptor (EGFR) in lung adenocarcinoma.

Methods: EVs were collected from the serum samples of 32 patients with lung adenocarcinoma, 32 healthy controls, and conditioned culture media from A549 and BEAS-2B cell lines. EVs were isolated using ultracentrifugation and size-exclusion chromatography. Their characteristic features were confirmed by transmission electron microscopy, nanoparticle tracking analysis (NTA), and western blotting.

Results: NTA revealed a two-fold increase in EV concentration in the serum of patients with lung cancer compared to healthy controls. Similarly, A549 cells secrete significantly more EVs than BEAS-2B cells. Western blotting validated the detection of canonical EV markers, such as TSG101, CD81, and flotillin-1, as well as the absence of calnexin. Notably, EGFR was highly packaged in the EVs isolated from both A549 cells and patient serum, whereas it was minimally present or absent in the EVs isolated from healthy controls and BEAS-2B cells.

Conclusion: Our findings indicated that EGFR was selectively packaged into EVs derived from lung adenocarcinoma and was absent in non-cancerous controls. EV-associated EGFR could be a noninvasive indicator for the early detection of lung adenocarcinoma through liquid biopsy.

Keywords: ErbB receptors; Extracellular vesicles; Liquid biopsy; Lung adenocarcinoma

Introduction

Lung adenocarcinoma is the most predominant subtype of non-small cell lung cancer (NSCLC) [1] and a major cause of cancer-related mortality globally [2]. Although tyrosine kinase inhibitors (TKIs) targeting the epidermal growth factor receptor

(EGFR) offer therapeutic benefits to subsets of patients harboring EGFR mutations, clinical challenges such as late-stage diagnosis and acquired drug resistance persist [3]. In recent decades, the urgent need for sensitive, noninvasive biomarkers to enable early detection and monitoring of disease progression and therapeutic responses has shifted attention to extracellular vesicles (EVs) as po-

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tential candidates for liquid biopsies [4].

EVs are lipid bilayer nanoparticles secreted by almost every cell type and are increasingly recognized for their role in cancer biology [5]. They are categorized into three main types according to their biogenesis pathways and size: exosomes (30–180 nm) [6], microvesicles (50–1,000 nm) [7], and apoptotic bodies (500–5,000 nm) [8]. These membrane-bound vesicles contain proteins, lipids, and nucleic acids, and promote intercellular communication by transporting these biomolecules to distant cells [9], thereby affecting the tumor microenvironment [10] and modulating immune responses [10]. Importantly, the cargo composition of EVs represents the physiological and pathological status of the secreted cells, making them highly attractive as potential diagnostic and prognostic biomarkers [11]. Among the diverse cargoes carried by EVs, the presence of oncogenic proteins, such as EGFR, has been recognized as a critical factor influencing tumor behavior and therapeutic resistance in lung adenocarcinoma.

EGFR is a transmembrane receptor that controls cell proliferation, differentiation, migration, and survival [12]. Aberrations in EGFR, including gene amplification and activating mutations in exons 19 to 21 [13], are common oncogenic drivers in lung adenocarcinoma, found in 20% to 50% [14] of cases, and are closely associated with disease progression and poor prognosis. These molecular alterations have been extensively exploited as therapeutic targets, with TKIs, such as gefitinib [15], erlotinib [16], and osimertinib [17] forming the cornerstone treatment strategies for patients with EGFR mutations.

Recent studies have indicated that EGFR is not solely confined to the plasma membrane or intracellular compartments but is also selectively packaged into EVs secreted by cancer cells. Kharmate et al. [18] investigated the presence of EGFR within EVs derived from prostate cancer cells, LNCaP xenograft models, and serum from patients with prostate cancer, highlighting the application of EV EGFR as a biomarker for monitoring disease diagnosis and progression. Similarly, Zhang et al. [19] detected EGFR in serum-derived EVs from patients with stomach cancer, suggesting that circulating exosomal EGFR plays a pivotal role in modulating the liver microenvironment and facilitating metastasis. Purcell et al. [20] identified EGFR mutations in EVs released from EGFR-mutated lung cancer cell lines.

Although EGFR has been shown to be upregulated in lung tumor tissues, there is limited research on circulatory EGFR transported by EVs. While our previous study focused on EVs from lung adenocarcinoma cell lines [5] and other studies, such as that by Yamashita et al. [21], identified EGFR within plasma-derived EVs from patients with lung cancer, these investigations are often limited by small sample sizes and a lack of longitudinal or function-

al analyses. These findings led us to hypothesize that EGFR is specifically secreted by EVs and may contribute to the progression of lung adenocarcinoma. However, few studies have directly compared EV-associated EGFR expression in clinical samples and corresponding cell line models to explore consistency across systems. Additionally, the relationship between EV EGFR and EGFR mutation status, particularly the presence of phosphorylated (activated) EGFR, remains largely unknown. This study addresses these critical gaps by examining EGFR expression in EVs from both patient serum and cultured lung cancer cells, while laying the foundation for future work on mutation-specific EV profiling. The presence of EGFR in EVs in the serum of patients with lung cancer and in the culture medium of NSCLC cell lines is crucial for evaluating its viability as a biomarker and therapeutic strategy.

Methods

Ethics statement: This study was conducted in accordance with the amended Declaration of Helsinki and was approved by the Institutional Ethics Committee of the University of Foggia, Italy (No: DDG N. 651 OF 27.08.2024). Written informed consent was obtained from all participants before their inclusion in the study.

1. Patient samples

Whole blood samples were obtained from 32 individuals diagnosed with lung adenocarcinoma and 32 healthy participants with no signs of cancer or other illnesses. These samples were collected prior to treatment at Foggia Hospital in southern Italy. Venous blood (3 mL) was drawn into coagulation-promoting tubes (Becton, Dickinson and Company, Franklin Lakes, NJ, USA) and left undisturbed in an upright position for 30 minutes to allow clotting. The samples were centrifuged at $2,500 \times g$ at 20°C for 15 minutes. After being carefully moved to a 15-mL reaction tube, the resultant serum layer was centrifuged at $3,200 \times g$ for 25 minutes at 20°C . Finally, the serum was distributed into fresh 1.5-mL reaction tubes or cryotubes, ensuring that the pellet remained undisturbed. A $0.45\text{-}\mu\text{M}$ filter was used to filter the serum, which was then stored at -80°C for later use.

2. Cell culture

Two lung cell lines were used in this study: a lung adenocarcinoma cell line (A549) and a normal human bronchial epithelial cell line (BEAS-2B). The cells exhibited an epithelial morphology and were maintained under specific culture conditions. A549 cells were grown in Dulbecco's Modified Eagle Medium (Sigma-Aldrich, St.

Louis, MO, USA) enriched with 10% fetal calf serum (FCS), 100 µg/mL streptomycin and 100 U/mL penicillin. BEAS-2B cells were seeded in Roswell Park Memorial Institute-1640 medium (Thermo Fisher, Waltham, MA, USA) containing the same components. The cells were incubated in a humidified environment with 5% CO₂ at 37°C. To collect EVs, five T-175 flasks were seeded with 4×10^6 cells each. When the cells reached 80% confluence, they were rinsed in cold phosphate-buffered saline (PBS) and re-incubated for 24 hours in a medium containing 2% EV-depleted FCS.

3. Isolation of extracellular vesicles

The process of isolating cell line-derived EVs began with the collection of conditioned culture media (CCM). Initially, approximately 20 mL of CCM from each flask underwent centrifugation at $300 \times g$ for 4 minutes (low speed), $2,000 \times g$ for 10 minutes, and ultimately, $10,000 \times g$ for 50 minutes (high speed) at 4°C.

The final product was filtered through a 0.22-µm polyethersulfone filter (Nalgene, Rochester, NY, USA) to remove particles greater than 220 nm. To obtain smaller EVs, the supernatant was placed into ultracentrifuge tubes and subjected to ultracentrifugation at $100,000 \times g$ for 110 minutes at 4°C utilizing an Optima ultracentrifuge XPN-100 fitted with a SW32 Ti Swinging Bucket rotor (Beckman Coulter, Brea, CA, USA). The pellet was subsequently resuspended in 35-mL PBS and subjected to a second ultracentrifugation step under identical conditions to improve purity. Following the final centrifugation, the pellet was resuspended in 100-µL PBS and stored at -80°C until future experiments.

Size-exclusion chromatography was performed using quick EV (qEV) columns (iZON Science, Christchurch, New Zealand) to isolate EVs from human serum samples. The columns were equilibrated at room temperature by flushing out the 20% ethanol storage solution with PBS. Serum samples, previously thawed on ice, underwent initial centrifugation at $1,500 \times g$ for 10 minutes at 10°C to remove large particles and a second centrifugation at $10,000 \times g$ for 10 minutes at the same temperature. The processed serum was either used immediately or preserved at -80°C. To isolate EVs, 500 µL of serum was added to the qEV column, discarding the first eluate. Next, the column was washed three times with 900-µL Hank's Balanced Salt Solution (HBSS) per wash and three times with 500-µL HBSS per wash, and the collected fractions were supplemented with a protease inhibitor. The purified EV fraction (1.5 mL) was concentrated further by ultracentrifugation at $130,000 \times g$ for 2.5 hours at 4°C. After carefully removing the supernatant, 115 µL of liquid remained, which was used to gently resuspend the EV pellet without creating bubbles. Finally, the EV suspensions were stored at -80°C until needed.

4. Transmission electron microscopy

Transmission electron microscopy (TEM) was used to determine the size of EV particles. For this purpose, 3 µL of the EV sample was placed for 5 minutes on special copper grids covered with pioloform carbon. Subsequently, two drops of deionized water were used to rinse the grids, followed by the addition of 4-µL drops of 0.5% aqueous uranyl acetate. Prior to TEM examination, the grids were allowed to air dry following the removal of excess stain with filter paper. A JEOL JEM 1400 TEM (JEOL Ltd., Tokyo, Japan) was employed to examine the samples at an accelerating voltage of 80 kV. Photographs were captured using MegaView III digital cameras and iTEM software (EMSIS GmbH, Münster, Germany).

5. Nanoparticle tracking analysis

Nanoparticle tracking analysis (NTA) was used to quantify the size distribution and concentration of EVs following previously published methods [22]. The dilution ratios of the samples ranged from 1:50 to 1:2,000 in HBSS, resulting in a total volume of 1 mL. To ensure optimal measurement conditions, the particle count per frame was adjusted to fall between 140 particles and 200 particles. Each measurement consisted of two cycles, during which 11 different cell positions were scanned and 30 frames were captured for each position. The analysis was done by using predefined settings: autofocus for focus adjustment, a camera sensitivity of 79 for all samples, a shutter speed of 70, automatic detection of scattering intensity, and a cell temperature of 24°C. After recording, the videos were analyzed using ZetaView software (ver. 8.05.11 SP1; Particle Metrix GmbH, Inning am Ammersee, Germany) with the following defined parameters: a minimum area of five, minimum brightness threshold of 30, and maximum area of 1,000. The hardware setup included a complementary metal oxide semiconductor camera and an embedded 40-mW laser operating at a wavelength of 488 nm.

6. Western blotting

Protein lysates from A549 and BEAS-2B cells were extracted in $1 \times$ radioimmunoprecipitation assay buffer containing a mixture of protease and phosphatase inhibitors. The lysates were centrifuged at $10,000 \times g$ for 10 minutes at 4°C to eliminate debris. A BCA protein assay kit was used to quantify the concentration of protein (Thermo Fisher). Isolated EVs were lysed in M-PER 1X lysis buffer (Thermo Scientific) and subsequently stored at -80°C until further analysis. For protein separation, lysates containing 10 µg of protein (either cell lysates or EV samples) were resolved on 10% sodium dodecyl sulfate-polyacrylamide gel electrophoresis and then transferred onto 0.45-µm nitrocellulose membranes (Sigma-Aldrich). Following transfer, the membranes were rinsed with

Tris-buffered saline (TBS). Ponceau S staining was used to verify protein transfer, followed by a 1-hour blocking with 5% bovine serum albumin in TBS with Tween 20 (TTBS) at room temperature to reduce non-specific binding. The membranes were washed with TBS and incubated overnight at 4°C with specific primary antibodies. The following day, the membranes were washed with TTBS and incubated for 1 hour at ambient temperature with horseradish peroxidase-conjugated secondary antibodies. A series of washes was conducted, and the bands were identified using an enhanced chemiluminescence substrate (GE Healthcare Life Sciences, Marlborough, MA, USA). The resulting signals were visualized and examined using an Odyssey Fc Imager (LI-COR Biosciences, Lincoln, NE, USA). The antibodies and their dilutions are listed in Table 1.

7. Statistical analysis

Statistical analyses were performed using IBM SPSS ver. 22.0 (IBM Corp., Armonk, NY, USA), and GraphPad Prism ver. 10.1.2 (Dotmatics, Boston, MA, USA) was used to generate the charts and graphs. The chi-square test was used to assess differences in categorical variables, such as age, sex, and smoking status, between patients with lung cancer and healthy controls. Statistical significance was set at $p < 0.05$.

Results

1. Characteristics of participants

From January 2023 to December 2023, serum samples were obtained from 32 patients with lung cancer as well as from 32 healthy control donors who visited the Department of Thoracic Surgery, Azienda Ospedaliero-Universitaria, Policlinico in Foggia, Italy. All samples from patients with lung cancer were obtained prior to treatment initiation. The study included both male and female participants, with a breakdown of smoking history, tumor staging, and EGFR mutation status. Most of the patients with lung cancer had stage I and II disease, and EGFR mutation testing was performed in a subset of patients. Further details regarding the clinicopatho-

logical characteristics of the patients are provided in Table 2.

2. Characteristics of extracellular vesicles extracted from serum and culture medium

The characterization of EVs is necessary before performing a specific analysis. In agreement with the guidelines of the International Society of Extracellular Vesicles (ISEV), minimal criteria must be met to confirm the proper isolation of EVs. To evaluate the heterogeneity of EV populations, ISEV recommends combining electron or atomic force microscopy with at least one particle-tracking method. Additionally, the presence of key EV markers should be verified using western blot analysis. To rule out cellular contamination, these markers include transmembrane proteins, such as CD9; intracellular proteins linked to membrane structures, such as Alix and heat shock protein 70 (Hsp70); and intracellular proteins not associated with the plasma membrane, such as calnexin. Thus, we

Table 2. Characteristics of the participants

Characteristic	Patient	Healthy control	p-value
No. of group	32 (100)	32 (100)	
Age (yr)			
< 60	7 (21.9)	10 (31.2)	0.57
≥ 60	25 (78.1)	22 (68.8)	
Sex			
Male	19 (59.4)	21 (65.6)	0.79
Female	13 (40.6)	11 (34.4)	
Smoking			
Non-smoker	16 (50.0)	15 (46.9)	0.21
Former smoker	13 (40.6)	9 (28.1)	
Current smoker	3 (9.4)	8 (25.0)	
TNM stage			
I	7 (21.9)		
II	12 (37.5)		
III	9 (28.1)		
IV	4 (12.5)		
EGFR status			
EGFR wild-type	27 (84.4)		
EGFR mutant	5 (15.6)		

Values are presented as number (%).
EGFR, epidermal growth factor receptor.

Table 1. Primary and secondary antibodies

Antibody	Dilution	Catalog No.	Source
CD9	1:1,000	13118	Santa Cruz Biotechnology, Dallas, TX, USA
CD81	1:1,000	166029	Santa Cruz Biotechnology, Dallas, TX, USA
TSG101	1:5,000	7964	Santa Cruz Biotechnology, Dallas, TX, USA
β-Action	1:5,000	47778	Santa Cruz Biotechnology, Dallas, TX, USA
EGFR	1:1,000	4267	Cell Signaling, Danvers, MA, USA
Calnexin	1:1,000	2433	Cell Signaling, Danvers, MA, USA
Anti-mouse immunoglobulin G (IgG)	1:5,000	7076	Cell Signaling, Danvers, MA, USA
Anti-rabbit IgG	1:5,000	7074	Cell Signaling, Danvers, MA, USA

evaluated the EVs obtained from the serum of patients with lung adenocarcinoma, healthy controls, and the CCM of A549 and BEAS-2B cell lines by conducting comprehensive characterization using TEM, NTA, and western blotting.

TEM imaging validated the existence of EVs, demonstrating a predominant group of vesicles within the 100-nm size range, consistent with small EVs. These vesicles exhibited a characteristic cup-shaped morphology typically associated with exosomes (Fig. 1).

Further validation was performed using NTA. As shown in Fig. 2, the NTA profiles exhibited a single peak corresponding to a mode size of 85 to 150 nm, which is aligned with the widely accepted exosome size range reported in the literature. A difference in the concentration of EVs was observed between patient and control samples. The EV count in healthy control serum was 9.94×10^{10} particles/mL (mode size of 123 ± 9.5 nm), whereas serum from patients with lung cancer exhibited a twofold increase, reaching 2.08×10^{11} particles/mL (mode size of 128 ± 6.6 nm) (Fig. 2A–2C). This suggests that the number of circulating EVs is higher in patients with lung cancer than in healthy donors ($p < 0.0001$). To confirm these results, EVs were isolated from A549 and BEAS-2B cells. NTA analysis showed that the concentration of EVs derived from the CCM of BEAS-2B was 6.01×10^8 particles/mL (mode size of 161 ± 1.5 nm), while in A549 cells, it was 1.20×10^9 particles/mL (mode size of 151 ± 3.3 nm) ($p < 0.0001$). The data are shown in Fig. 2D–2F.

3. Identification of markers confirming extracellular vesicle isolation

Next, we used western blotting to detect EV-specific biomarkers (Fig. 3). The presence of CD81, a member of the tetraspanin family, which is a family of integral membrane proteins enriched in EVs that plays a potential role in the biogenesis of EVs and cargo sorting, has been consistently observed in the serum-derived EVs of patients and controls, as well as in cancer and normal lung cell lines. Flotillin-1, a scaffolding protein associated with lipid rafts, was also detected in the EVs from all sample types. This protein is commonly found in EVs and is involved in membrane trafficking and signaling, providing further confirmation of successful EV isolation. Additionally, a key component of the endosomal sorting complex required for transport machinery, TSG101, was observed, confirming the endosomal origin of the isolated EVs across all groups. The absence of calnexin, a marker protein of the endoplasmic reticulum, indicated the high purity of the EVs isolated from patients with lung cancer, lung cancer cells, healthy controls, and normal lung cells, ensuring minimal contamination from cellular compartments.

4. Epidermal growth factor receptor packaged into lung cancer-derived extracellular vesicles

In addition to classical EV markers, we assessed whether lung cancer cells and serum-derived EVs possess EGFR, a significant cell regulator linked to advanced lung cancer progression. The data

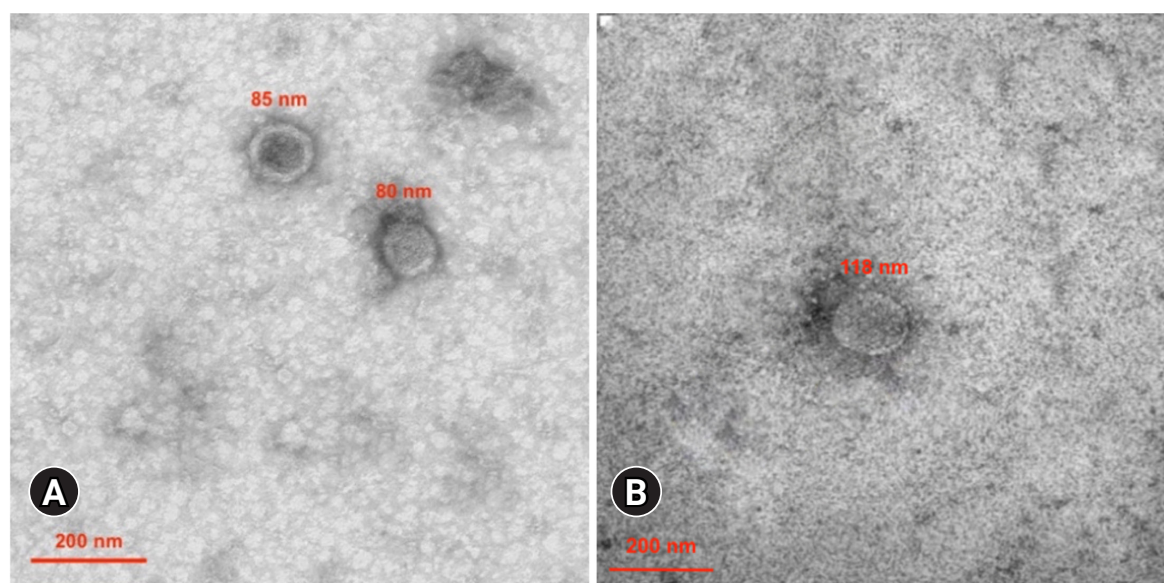


Fig. 1. Transmission electron microscopy analysis confirming the presence of extracellular vesicles (EVs). Representative transmission electron microscopy images show EVs isolated from (A) healthy controls and (B) patients with lung adenocarcinoma. The EVs have been negatively stained with 2% uranyl acetate after moisture removal. These vesicles exhibit a characteristic cup-shaped morphology and range in size from 30 to 150 nm in diameter, confirming their identification as EVs.

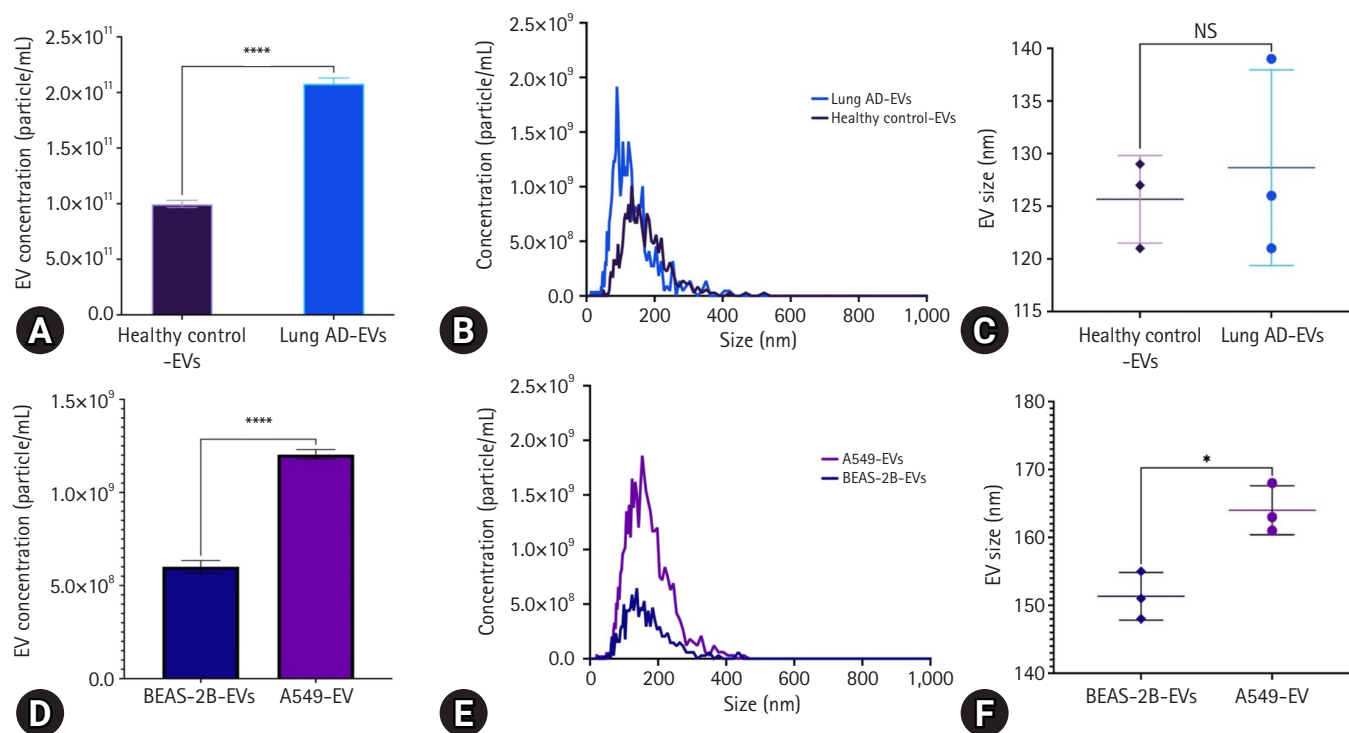


Fig. 2. Isolation and characterization of extracellular vesicles (EVs) from cell lines and patients with lung cancer. (A) Nanoparticle tracking analysis reveals a significantly higher concentration of EVs in serum from patients with lung adenocarcinoma (AD) compared with that from healthy controls ($p < 0.0001$). (B) Size distribution profiles of EVs from lung AD and healthy control serum revealed that the majority of EVs range between 30 and 200 nm, consistent with typical EV sizes. (C) Mode size distribution of EVs isolated from healthy controls and patients with lung AD. The data indicate a slight but nonsignificant (NS) increase in EV size in patients with lung AD. (D) A549-derived EVs show a significantly higher concentration than BEAS-2B-derived EVs ($p < 0.0001$). (E) The majority of EVs from BEAS-2B and A549 cells fall within the expected size range of 30 to 200 nm. (F) A549 EVs exhibit a significantly larger mode size than BEAS-2B EVs ($p < 0.05$). * $p < 0.05$ (significant), **** $p < 0.0001$ (extremely significant).

show that the BEAS-2B CCM-derived EVs exhibited very low EGFR levels, whereas the A549 CCM-derived EVs displayed variable EGFR levels. Overall, the EGFR levels were consistently higher in total cell lysates than in the corresponding EV fractions (Fig. 4A, 4B). The EGFR content in cell-derived EVs differs among cell lines, highlighting the heterogeneity in EV cargo composition. To assess the clinical relevance of EV-associated EGFR, we investigated the presence of EGFR in serum-derived EVs from controls and patients with lung cancer. Western blot analysis revealed detectable levels of full-length 170-kDa EGFR in the EV fractions from all patient serum samples. In contrast, EVs from the control serum samples showed very low or undetectable EGFR levels (Fig. 4C).

Discussion

The molecular pathogenesis of lung cancer involves a complex interplay of genetic, epigenetic, and environmental factors that lead to the conversion of normal lung cells into malignant cells, with key oncogenic drivers such as EGFR, KRAS, BRAF, and ALK

playing central roles in tumor progression and therapeutic resistance [23]. Among these, EGFR alterations are particularly common in NSCLC and are closely linked to aggressive disease behavior. Given the capacity of EVs to reflect the molecular characteristics of their original cells, the presence of EGFR within EVs highlights their potential utility as noninvasive biomarkers in liquid biopsy applications [24]. This study highlights the importance of EGFR in lung adenocarcinoma by isolating and characterizing EVs derived from both *in vitro* models and clinical samples from patients.

The presence of EVs in blood, urine, saliva, and other bodily fluids presents a promising avenue for the noninvasive diagnostic and prognostic assessment of cancer [25]. In the current study, we observed a two-fold increase in EV concentration in the serum of patients with lung adenocarcinoma and cancer cell lines compared to controls, indicating that EVs can be used to distinguish between normal and pathological conditions. This observation aligns with previous reports suggesting that, likely due to underlying physiological changes, the bloodstream of patients with cancer may con-

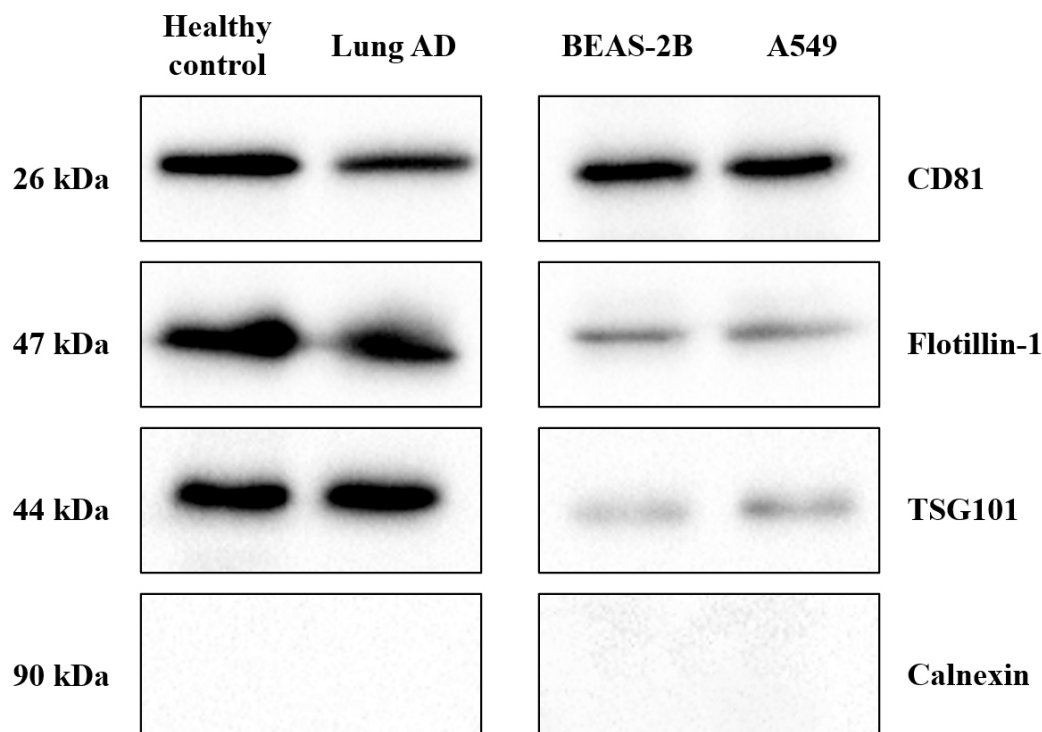


Fig. 3. Western blot analysis confirming the presence of extracellular vesicle (EV)-specific markers. Western blot analysis was performed to validate the presence of EVs by detecting established EV markers, including CD81, flotillin-1, and TSG101, in both circulating EVs (from healthy controls and patients with lung adenocarcinoma [AD]) and cell-derived EVs (from BEAS-2B and A549 cells). The expression of these markers confirms the successful isolation of EVs. Additionally, the absence of calnexin, an endoplasmic reticulum marker, indicates minimal cellular contamination, ensuring the purity of the EV preparations.

tain up to 4,000 trillion exosomes, approximately double the amount found in healthy individuals, highlighting the potential of EVs as valuable noninvasive biomarkers for various cancer detection strategies. Rodríguez-Sanz et al. [26] reported elevated levels of EVs in lung cancer plasma than in control plasma. Similarly, other studies have indicated that, compared to healthy donors, serum-derived EVs in patients with colorectal cancer were significantly higher [27], suggesting that EV release is upregulated in malignancy and may correlate with tumor burden or aggressiveness.

Another key aspect of the present study is the rigorous EV characterization performed based on ISEV guidelines, which adds robustness to our conclusions. The combination of NTA, TEM, and western blotting to assess EV morphology, size distribution, and marker expression reduces the risk of contaminating non-EV particles and ensures the reliability of our EV preparations [28].

In NSCLC, EGFR is commonly upregulated or altered by activating mutations, leading to abnormal stimulation of signaling cascades such as phosphoinositide 3-kinase (PI3K)/Akt and mitogen-activated protein kinase (MAPK)/extracellular signal-regulated kinase (ERK) [29]. These disruptions promote unchecked cellular growth, enhance cell survival, and prevent programmed cell

death. In lung adenocarcinoma, EGFR mutations play a role in tumor initiation and are strongly linked to metastatic potential and resistance to TKIs [30].

Considering the role of EVs in intercellular communication, EV-associated EGFR present in diverse tumor types, such as lung adenocarcinoma, may substantially enhance cancer progression. While previous studies have reported EGFR within EVs derived from cancer cell lines of glioblastoma [31], breast [32], gastric [33], and lung cancer [20] as well as in EVs from the serum of patients with prostate [18] and gastric cancer [19], data regarding EGFR packaging in EVs specifically associated with lung adenocarcinoma remain limited.

Our study confirmed that EVs derived from A549 lung cancer cells and patients with lung adenocarcinoma were significantly enriched in EGFR compared to those derived from controls, supporting the significant role of EV-associated EGFR as a biomarker in NSCLC. The identification of EGFR in EVs isolated from both patient serum and A549 cells is particularly significant because EGFR mutations and overexpression are well-established drivers of NSCLC pathogenesis [12].

Furthermore, our findings offer a deeper understanding of the

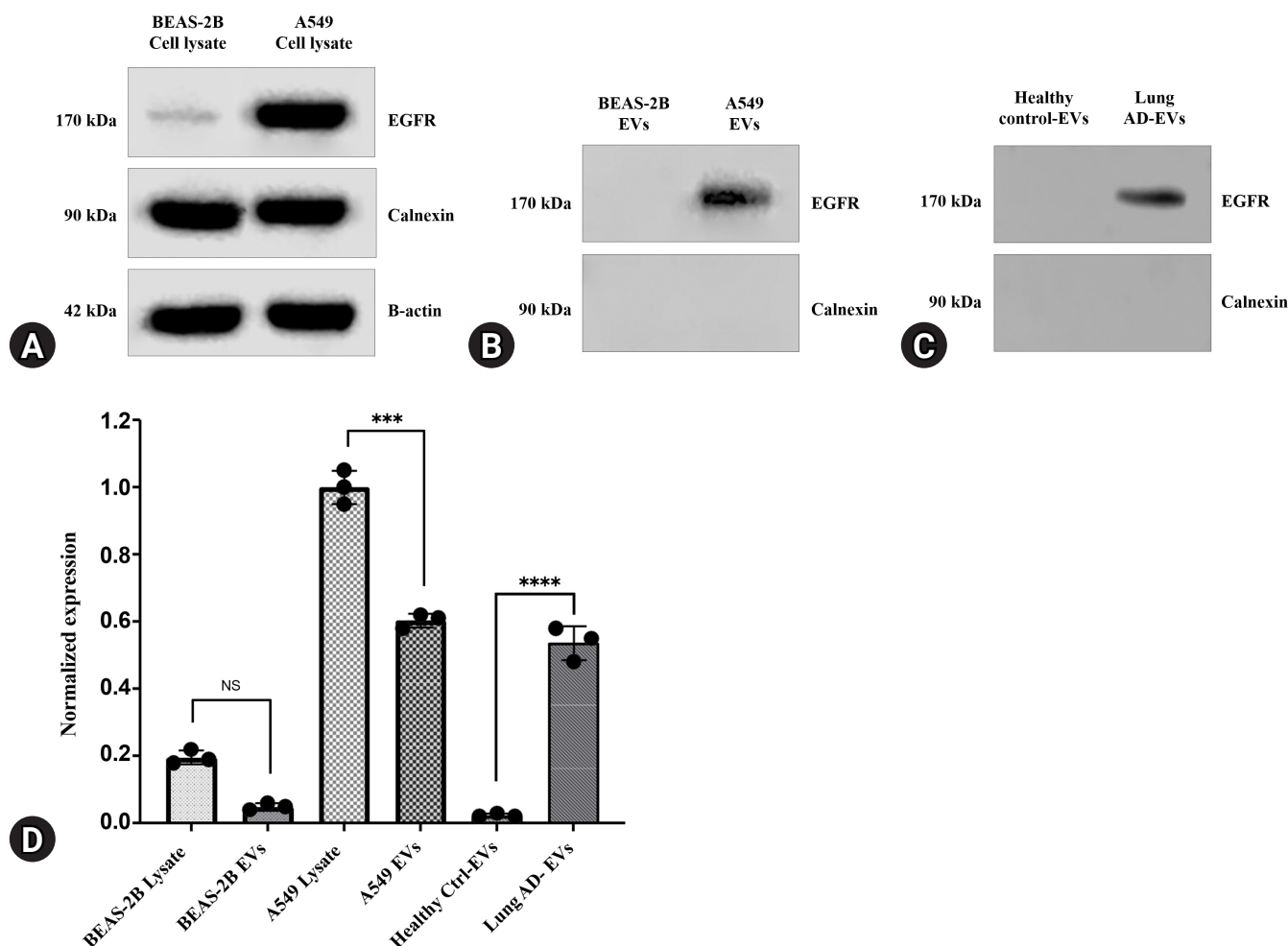


Fig. 4. Epidermal growth factor receptor (EGFR) packaging in lung cancer-derived extracellular vesicles (EVs). Western blot analysis shows EGFR expression in (A) cell lysates and (B) their derived EVs and (C) circulating EVs. BEAS-2B cells and their derived EVs exhibit minimal EGFR levels, while A549 cells and their derived EVs display variable expression. (D) Normalized expression. EGFR is consistently more abundant in total cell lysates than in EV fractions. Serum-derived EVs from patients with lung cancer contain detectable full-length 170-kDa EGFR, whereas control serum EVs show little to no EGFR levels, highlighting the clinical relevance of EV-associated EGFR. AD, adenocarcinoma. NS, not significant. *** $p < 0.001$ (highly significant), **** $p < 0.0001$ (extremely significant).

heterogeneity of EV cargo between cancerous and healthy cells. The absence of EGFR in BEAS-2B-derived EVs, despite the expression of EV markers, such as CD81 and TSG101, suggests cancer-specific enrichment of EGFR in EVs. This finding mirrors earlier reports emphasizing that the cargo composition of EVs is highly reflective of the state of their originating cells [34].

Importantly, our results suggest that EV-associated EGFR may play a role in EGFR activation in lung cancer. Given that EGFR is a transmembrane receptor that, upon activation, triggers signaling pathways, including PI3K/Akt and MAPK/ERK [13], its presence in EVs may have functional consequences for receiving cells. Our previous study further supported this hypothesis, as we found that phosphorylated EGFR (p-EGFR) was present in EVs isolated

from PC9 cells, which are lung cancer cells harboring an EGFR exon 19 deletion mutation but was undetectable in EVs from A549 cells with wild-type EGFR [35]. The selective presence of p-EGFR suggests a mutation-specific packaging mechanism and highlights the potential of p-EGFR as a precise biomarker of EGFR-driven lung adenocarcinoma. Prior research has demonstrated that cancer-derived EVs containing EGFR can modify the behavior of recipient cells in both the tumor microenvironment and distant metastatic locations. Zhang et al. [19] reported that EV-delivered EGFR from gastric cancer cells primed the liver microenvironment to facilitate metastasis. Similarly, Jouida et al. [36] demonstrated that circulating EVs in the serum of patients with EGFR-mutated NSCLC promote hybrid epithelial-mesenchymal

transition and enhance invasion of A549 cells by upregulating matrix metalloproteinase-9.

Despite these encouraging results, the present study has some limitations. The comparatively small sample size constrains statistical power, and more studies with larger patient cohorts are required to validate the clinical use of EV-associated EGFR as a biomarker. Moreover, although this study established a correlation between lung cancer and the presence of EGFR in EVs, further mechanistic studies are needed to elucidate whether EV-bound EGFR actively contributes to NSCLC progression and therapeutic resistance.

In conclusion, this study supports the exciting notion that EV-associated EGFR can act as a biomarker for lung adenocarcinoma. Its detection in serum-derived EVs provides a noninvasive approach to disease monitoring and can potentially guide EGFR-targeted therapies. Further longitudinal clinical studies are needed to investigate the prognostic significance and predictive value of EV-associated EGFR and its role in shaping the tumor microenvironment and mediating resistance to TKIs.

Article information

Conflicts of interest

No potential conflict of interest relevant to this article was reported.

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