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Εξωσώματα ο ρόλος των μικρών κυστιδίων στην ανάπτυξη, την μετάσταση και την θεραπεία του καρκίνου του πνεύμονα

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Περίληψη

Τα εξωκυτταρικά κυστίδια (ΕΚ) περιλαμβάνουν έναν μεγάλο πληθυσμό κυστιδίων που απελευθερώνονται από διάφορα είδη κυττάρων του σώματός. Με βάση το μέγεθος και τη βιογένεσή τους, έχουν κατηγοριοποιηθεί σε υποτύπους οι οποίοι περιλαμβάνουν τα εξωσωμάτα, τα μικροκυστίδια, τα εκτοσώματα, τα ογκοσώματα και τα αποπτωτικά σωμάτια. Τα ΕΚ αποτελούν ένα γενικό ορισμό που περιλαμβάνει το σύνολο των εκκρινόμενων από τα κύτταρα κυστίδια.

Τα ΕΚ είναι ένας σημαντικός φορέας διαφόρων βιολογικά ενεργών μορίων που προέρχονται από τα κύτταρα, όπως πρωτεΐνες, νουκλεϊκά οξέα, λιπίδια και μεταβολίτες. Τα κυστίδια αυτά κυκλοφορούν στον εξωκυττάριο χώρο όπως το αίμα, το ασκιτικό υγρό, τα ούρα και το σάλιο. Στη βιολογία του καρκίνου, ο ρόλος των ΕΚ αναδεικνύεται στην ογκογένεση, την μετάσταση και την αντίσταση των καρκινικών κυττάρων στην χορηγούμενη στη χημειοθεραπεία. Τα κυκλοφορούντα ΕΚ φαίνεται να έχουν επίσης σημαντικό ρόλο σαν δείκτες προγνωστικής και προβλεπτικής αξίας, καθώς φέρουν μόρια ειδικά για τον όγκο, όπως πρωτεΐνες νέο-αντιγόνα, RNA και DNA. Ταυτόχρονα, τα ΕΚ έχουν επίσης πολλά υποσχόμενες δυνητικές κλινικές εφαρμογές, καθώς μπορούν να λειτουργήσουν ως φορείς για την χορήγηση και την μεταφορά θεραπευτικών παραγόντων ενώ παράλληλα μελετάται η χρήση τους σαν βιοδείκτες προγνωστικής και προβλεπτικής αξίας. Στην παρούσα μεταπτυχιακή διατριβή παρουσιάζουμε μια λεπτομερή επισκόπηση των μοριακών καταρρακτών, μέσω των οποίων σχηματίζονται και απελευθερώνονται στην κυκλοφορία τα ΕΚ. Παράλληλα αναφέρεται ο ρόλος τους στον σχηματισμό και την εξέλιξη του καρκίνου του πνεύμονα. Τέλος αναλύεται το πιθανό θεραπευτικό πλεονέκτημα που προκύπτει από την αξιοποίηση των ΕΚ στην σύγχρονη κλινική πρακτική.

Λέξεις Κλειδιά: εξωσώματα, καρκίνος του πνεύμονα, εφαρμογές, καρκινογένεση, υγρές βιοψίες

Oncology: from Oncogenesis to Therapy

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Exosomes, the role of small vesicles in lung cancer proliferation and treatment

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Abstract

Extracellular vesicles (EV) include a diverse population of membrane-bound vesicles released by various cells of our body. Based on their size and biogenesis, they have been categorized into subtypes including exosomes, microvesicles (MVs), ectosomes, oncosomes, and apoptotic bodies; EV is understood to be a generic term that refers to secreted vesicles.

EVs are an important cargo carrier of various cell-derived bioactive molecules, including proteins, nucleic acids, lipids, and metabolites, and circulate in extracellular spaces in biofluids such as blood, ascites, urine, and saliva.

The potential for EV applications has been expanding quite rapidly, particularly in cancer-related fields. In cancer biology, the role of EVs is now recognized in oncogenesis, metastatic cascade, and resistance to chemotherapy. Circulating EVs are also considered an essential clinical target for use as disease biomarkers as they carry tumor-specific molecules such as neoantigen proteins, RNA, and DNA, leading to the realization of liquid biopsies for cancer diagnosis and management. Moreover, nano-technology-based approaches have also emerged to obtain high-quality EVs through easy and high-throughput methods. Moreover, EVs also have promising potential as therapeutics, as they can act as carriers to deliver therapeutic agents and contribute to other therapeutic approaches.

In the current thesis we present a detailed overview of the molecular cascades, through which EVs are being formed and released into the circulation, as well as their reported role in the formation and progression of lung cancer, with notable emphasis on the exosome mediated tumor metastasis and tumor alterations regarding immune surveillance. There will be reviewed the potential therapeutic advantage of exosome manipulation production in modern clinical practice, as those small vesicles can be applied as both biomarkers of predictive value and as a novel, specific targeted cancer therapy.

Keywords: exosomes; lung cancer; applications; tumor progression; liquid biopsies

Section 1: Introduction

Extracellular vesicles (EVs) are lipid bilayer-enclosed extracellular structures which can be formed by outward budding of the plasma membrane or by intracellular pathways. These events result in the extracellular release of intraluminal vesicles, generating a subtype of EVs termed exosomes. Exosomes are extracellular communication platforms that form a connective network between tumor cells and their surrounding environment that transport information bidirectionally. Those small vesicles have a diameter of 40-160 nm and can be secreted by various cell types. EV cargo is comprised of lipids, nucleic acids and proteins and can be isolated from body fluids like plasma, lavage fluids, serosal effusions and cerebrospinal fluid. Exosomes participate in multiple and important biological processes both physiological and pathological as they are essential to maintain normal cellular homeostasis but they have the potential to promote carcinogenesis as well by regulating tumor proliferation, invasion, metastasis and immunosuppression.

Different types of cancer cells can secrete exosomes with different cargos and all the factors that affect cell proliferation (hypoxia and survival pressure) can also induce exosome production. Cancer cells subsequently have the potential to secrete larger volume of exosomes than normal cells.

The study of exosomes has provided novel insight regarding the application of those vesicles both as predictive biomarkers and as new target therapies in cancer, as they come with a plethora of benefits that can be harnessed to tailor modern clinical practice.

This review will highlight the key points of exosome biogenesis, components as well as the main processes that they mediate so as to promote cancer proliferation. Additionally, there will be summarized the existent knowledge regarding the exosome role in lung carcinogenesis, and the potential implications of those small vesicles in the diagnosis and therapeutics of lung cancer.

Section 2: Main Part

2.1 Exosomes

Exosomes are small vesicles that are secreted by different types of cells. They carry various substances and are widely distributed in body fluids including urine, plasma, lavage fluid, serosal effusions and cerebrospinal fluid (1). These small vesicles come with significant roles in both physiological and pathological processes. They are important to maintain intracellular homeostasis and they mediate important tumorigenic activities as well. This duality in the nature of exosomes comes with a scientific interest as to how they can be applied in disease diagnosis and treatment.

2.1.1 Brief History

The earliest descriptions of EVs, emerged in the 1960s when Bonucci (2) and Anderson (3) observed that chondrocytes secrete small vesicles, that budded directly from the plasma membrane (3). Simultaneously, in 1967 Wolf reported that platelets also harbor the ability to secrete small extracellular vesicles, with an important clotting activity, which were originally described as platelet dust (4). In future years, those scientific discoveries were robustly confirmed and extended further, highlighting the role of small vesicles in osteogenesis (5), while platelet derived exosomes were further associated with both physiological hemostatic conditions and pathological clinical manifestations (6). Similarly, exosome related studies were also emerged in the 1980s, including the ectoenzyme work of Trams et al (7), as well as the discovery of prostate produced exosomes in seminal fluid and their potential role in modifying the composition and biological functions of human sperm (8). In 1983 studies of transferrin receptor (TfR) elimination from the plasma membrane of maturing reticulocytes through the selective TfR endocytosis and budding from endosomes membranes into the endosomes lumen and subsequent fusion of vesicle-containing endosomes with the plasma membrane demonstrated that exosome genesis is a process of plasma membrane protein control (9),(10).

Collectively those hallmark studies underlined four major principles of exosome biological functions; 1) exosomes are formed through the budding of plasma and

endosome membrane, 2) exosomes harbor macromolecules that affect the function of target cells, 3) exosome formation is a mechanism of protein quality control and 4) exosomes have a vital role in numeral biological processes in both pathological and non-pathological contexts (11).

In 2013 the Nobel Prize in physiology or medicine was awarded to James E. Rothnan, Randy W. Schekman and Thomas C Sudhof for their studies on extracellular vesicles (12) highlighting thus this evolving field of study . Nowadays, exosomes significance is mainly demonstrated in the field of cancer research (13), as the complex communication network formed between cancer and non-cancer cells through exosomes seems to be involved in every step of tumor progression, from tumor growth to metastasis (14).

2.1.2 Exosome Structure

Both prokaryote and eukaryote cells, secrete extracellular vesicles (EVs) as part of their normal biological function as well as during abnormal conditions (15). Those EVs can be widely categorized into two main categories, ectosomes and exosomes. Exosomes are single-membrane extracellular vesicles (EVs) with a size range of ~40 to 160nm (average ~100nm) (15), that have the same topology as the cell (11), while their size varies significantly even among the same cell line (11), as each isolation technique comes with its own bias in the estimation of exosomal size (16). For instance, techniques that measure exosomal hydrodynamic size are sensitive to the size of proteins and glycans that extend from the exosome membrane. Transmission electron microscopy is insensitive to adhered molecules (16) (17), although its measurements are widely affected by other variables such as swelling, flattening and shrinking which could occur during sample fixation (11). Fluorescence microscopy can detect exosomes, if the exosomes are labeled with fluorescent probes specific to the membrane bilayer or exosome proteins, nucleic acids or carbohydrates (18). Super resolution microscopy can determine both the exosome size and sub-exosome cargo distribution (19), while single-particle interferometric reflectance imaging has the potential to identify exosomes in the 50~200nm range, and if combined with fluorescence microscopy can additionally detect the presence of specific molecules (20). As noted, accuracy in exosome measurements still remain a challenge to overcome. Experiments with cryon-electron microscopy demonstrate that nearly all the EVs circulating the human body display a spheroid morphology. The presence of other more complex structures that have been observed, have been attributed to other

biological processes such as mechanical resealing and physical force fragmentation or specimen contamination with lipoprotein particles, RNA-protein particles and protein aggregates (11).

The density of exosomes can heavily vary and is mostly affected by the protein-lipid ratio, which varies among those small vesicles (11). Additionally, it has been demonstrated that the expression of the protein cargo of the exosomes can affect heavily the exosome density as well as the size and the shape of those vesicles (21).

Regarding the aforementioned, it can be concluded that the exosome size, shape and density, are highly variable parameters, which are specific to each exosome and are immediately correlated with their specific cargo (protein, lipids, and mineral contents) and structural characteristics, therefore such features are individualized to each vesicle and cannot be defining aspects of exosomes. It has been noted that the exosome properties are strongly related to the ways they are purified.

Exosomes and microvesicles have traditionally been defined on the basis of differential centrifugation, with microvesicles pelleting upon 1–2 10,000 × *g* spins for 30 min, whereas exosomes are pelleted by spinning the 10,000 × *g* spin supernatant at 70,000–100,000 × *g* for 90– 120 min. However, these exosome pellet fractions contain more than just exosomes. For instance, if the examined biofluid is plasma, this pellet could contain significant amounts of chylomicrons and other lipoprotein particles (11). Also, centrifugation parameters can lead to the pelleting of some exosomes in the microvesicle fraction and the pelleting of some microvesicles in the exosome fraction and while such obstacles can be surpassed by introducing immunoaffinity purification techniques, those methods come with their own categorization bias (11).

2.1.3 Exosomal cargo: proteins, nucleic acids and lipids

Depending on the cell of origin EVs can harbor several components of the cell, such as DNA, RNA, lipids, metabolites and cytosolic and cell surface proteins (15), which provide them with the ability to communicate with nearby cells so as to modulate the local or distant microenvironment (22) *[Figure 1].*

In detail, exosomes come with a wide heterogeneity, regarding their size as well as their composition as they contain a broad spectrum of transmembrane proteins, lipid anchored proteins, peripherally associated membrane proteins and soluble proteins of

the exosome lumen. It is well established that exosomes are enriched in tetraspanin proteins. Those proteins do not mediate catalytic processes, but rather contribute to the trafficking, function and stability of the plasma membrane. Such proteins are the molecules CD81, CD82, CD37 and CD63 (23), while the proteins CD81 and CD63 are molecular markers frequently used in exosome identification (23). Other proteins that are present on the exosome membrane include the major histocompatibility complex (MHC) II, immunoglobin superfamily member 8 (IGSF8), intracellular adhesion molecule- 1 (ICAM-1), syndecans (SDC1-4) and integrins and those proteins are considered to be imported in the exosomal membrane by the tetraspanins. Integrins are of highly clinical significance, as they are considered to have a role in the formation of the premetastatic niche in the process of cancer development process (23), (24), (25), (26). Additionally, other proteins of biological importance that appear to be present in the exosomal membrane are the programmed death ligand 1 (PD-L1) and CD200, molecules that bare significant immunosuppressive properties (27), (11) since the presence of those molecules is considered to provide cancer cells with the ability to immune-modify their surrounding environment, even in faraway sites, in favor of their survival. Exosomes also contain integral membrane signaling proteins, like epidermal growth factor receptor (EGFR), mast/stem cell growth factor receptor (c-Kit), vascular endothelial growth factor type 2 (VEGFR-2), insulin like growth factor I, T cell receptor, cytokine receptors, G protein-coupled proteins, Notch receptors (28), (29), (30), (31), (32). The existence of those transmembrane proteins that harbor the ability to function as surface signaling molecules, emphasize that exosomes could deliver those functional receptors and their signaling cascades to cells that do not have such biological functions.

Exosome surface is also enriched with lipid-anchored proteins (11). Those proteins are a number of C-terminal glycosylphosphatidylinositol (GPI) -anchored proteins, like CD39 and CD73 which enrich exosomes with immune modulation properties as they can suppress T cell activation (33). Additionally, other lipid enriched proteins in the exosome surface are the complement inhibitory molecules CD55 and CD59 (34), as well as the Hedgehog signaling molecules (35), with a significant role in tumor progression (35).

Peripheral surface proteins have also been reported to be involved in molecular signaling mediated by exosomes. Several wingless proteins (Wnt), transforming growth factors-β, tumor necrosis factor (TNF), TNF- related apoptosis- inducing ligand, first apoptosis signal (FAS) ligand, cytokines and several other proteinic molecules

have the potential to mediate complex signaling procedures, transforming exosomes into complex trans cellular communication platforms (36), (37), (38), (39). Also, the surface of the exosomes is enriched in extracellular matrix (ECM) proteins providing to them additional signaling capabilities (40).

The inner exosomal cortex is also rich in molecules that connect the cytoplasmic parts of membrane proteins to one another, as well as to other molecular mediators so as to form a complex network, creating thus a scaffold web. Such proteins are the ezrin–radixin-moesin (ERM) proteins which responds to phosphorylation by cross linking plasma membrane to cytoskeletal proteins, in order to achieve cortical structure and signal transduction (41) and syntenin with significant interactions with the tetraspanin CD63, as demonstrated by the observation that high syntenin expression appears to preserve CD63 on the exosomal plasma membrane (42). Also, cortex exosomal proteins contain the endosomal sorting complexes required for transport (ESCRTs). The ESCRT proteins are composed of ESCRT-0, ESCRT-I, ESCRT-II and ESCRT-III which function synergistically with the vacuolar protein sorting gene 4 (VPS4) protein AAA in complex molecular cascades such as the biogenesis of multivesicular bodies, cytokinesis, autophagy and other biological processes (43). ESCRT activities have created the speculation that those proteins could also take part in exosome budding (44), although such hypothesis has yet to be experimentally confirmed. Finally, of high biological significance remains the presence of heat shock proteins (HSPs) in exosomal cortex as demonstrated by various studies (45), (46), (47).

Exosomes, apart of being communication vesicles between cells and their surrounding environment, are also metabolically active extracellular platforms, containing enzymes that catalyze biological processes. Such molecules are RNA editing enzymes, lipases, proteases, glycosyltransferases, glycosidases, as well as metabolic enzymes, capable of altering the exosomal context (48), (49). Interestingly, cancer derived exosomes have a great enzymatic activity, carrying proteins like mutant Ras proteins and receptors (30), (50).

Consequently, it can be concluded that concerning their protein cargo, exosomes are small vesicles that appear to be highly heterogenous. The protein concentration within their cortex and on their membrane varies in a stochastic manner, since their formation is the result of diffusion, relocation of proteins, other chemical processes, as well as different gene expression within the cell, which also fluctuates in probabilistic manner.

Exosomes, contain extracellular RNAs, as was firstly described by Ratajczak et al in 2006 (51) who reported that stem cell exosomes carried RNAs like Oct-4 and could transfer this molecules to the recipient cells. Several studies since then have demonstrated that exosomes are particularly enriched in small noncoding RNA including small nuclear RNAs (snRNAs), microRNAs (miRNAs), transfer RNAs (tRNAs), Y RNAs, vault RNAs, repetitive element RNAs and fragmented RNAs (11). Exosomes have several molecular pathways through which RNAs are loaded and then transferred in recipient cells. MiRNAs are molecules with the potential to modulate immune responses, inhibit apoptosis and promote angiogenesis, biological processes important in cancer development (52). Additionally, the discovery of molecules responsible for miRNA process (Dicer and Ago2) inside exosomes, come with particular interest, as it underlines the notion that exosomes, apart from passive carriers could also be extracellular miRNA-producing factories (52).

Exosomes also contain DNA such as single-stranded DNA, double stranded DNA, genomic DNA and mitochondrial DNA (11), while it is not clear whether those molecules are contained inside the small vesicles, or are bound to their surface. Exosomal DNA secretion participates in various biological functions like regulation of inflammation and DNA quality control. Additionally it has been proposed that exosomal DNA could be a significant molecular marker in cancer identification (11). In detail, exosomes have the potential to offer a sustainable identification method of rare mutations and translocations in oncogenes, as they can be used as an alternative method to tissue biopsy and conventional circulating tumor DNA isolation (76). In this setting, Kim et al studied epidermal growth factor (EGFR) mutations on bronchoalveolar lavage (BAL) EV from advanced stage non small cell lung carcinoma (NSCLC) patients and compared it with tissue genotyping and plasma liquid biopsies. They demonstrated that there existed 97% accordance between BAL EV genotyping and standard tissue based genotyping, higher even than that of plasma cell free DNA based liquid biopsy (53).

2.1.4 Exosome Biogenesis

Exosomes are formed by vesicle budding into endosomes and subsequently they are released through the budding of multivesicular bodies and the plasma membrane. Originally this exosome biogenesis model was based on the vesicular secretion of TfR by maturing reticulocytes (9), (10). Since then electronic microscopy and genetic studies (54), (55) have heavily supported this hypothesis (11). In detail, cells capture

extracellular materials through endocytosis forming early endosomes, those endocytic bodies sprout inside to envelope specific proteins and nucleic acids to form intraluminal bodies (ILVs) known as late/advanced endosomes. Those late endosomes which contain multiple ILVs, are also known as multivesicular bodies (MVBs). Most MVBs will be digested by lysosomes and only a few of those vesicles have the potential to bud with the cell membrane and provoke exosomal release. Those MVBs contain molecules such as CD63, recombinant lysosomal-associated membrane protein (LAMP)1 and LAMP2 on their surface (56). ILVs and MVBs act as pre-exosomes. Exosomes are finally released from the cells by fusing with the cell membrane *[Figure 1].*

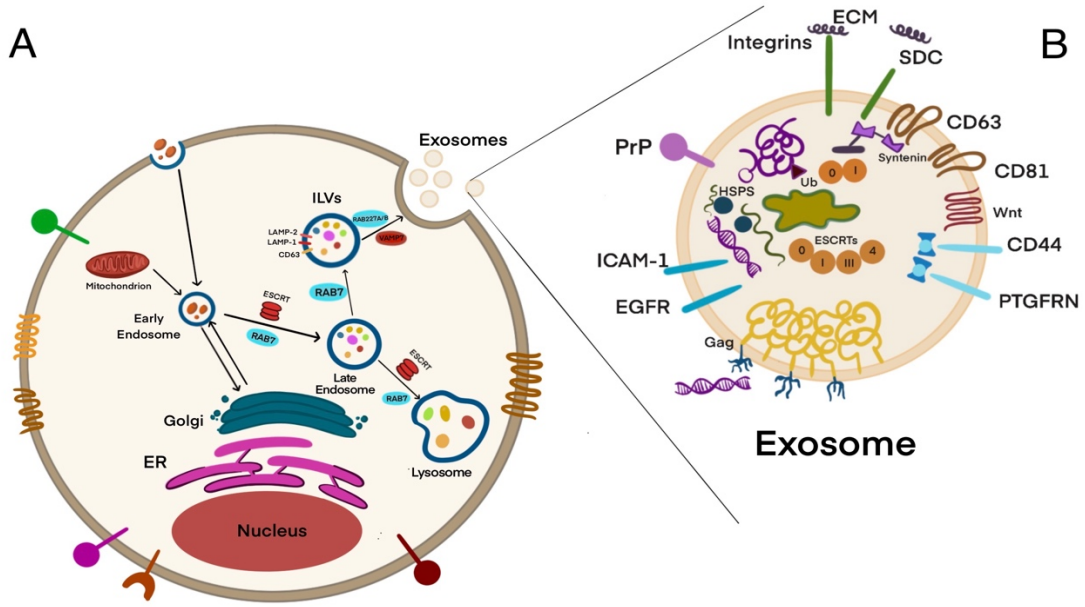

Figure 1: (A) Formation and secretion mechanism of exosomes. Early endosomes are formed through endocytosis, and then as a result of ESCRT, become late endosomes and then MVBs. ILVs are broken down to form exosomes and then released outside the cell. The whole process requires the action of Rab. **(B)** Exosomal components. Exosomes are heterogeneous in size, heterogeneous in composition, and enriched in membrane-associated, high-order oligomeric protein complexes. Although it is likely that no exosome contains all of these proteins, exosomes are rich in tetraspanins, adhesion molecules, enzymes, scaffolds, RNA-binding proteins, RNAs, DNAs, and complex glycans.

ESCRT, endosomal sorting complexes required for transport; MVB, multivesicular body; ILV, intraluminal vesicle; Rab, rabphilin; HSP, heat shock protein; TSG101, tumor susceptibility gene 101 protein; ALIX, apoptosis-linked gene 2 interacting protein X; CD, cluster of differentiation; ER, endoplasmic reticulum;

This model of exosomal biogenesis however while the most renown is not the only one, as there exist studies that support that exosomes can also bud from the plasma membrane (5), (18), (21), (32). Such hypothesis has also been robustly confirmed through atomic force microscopy experiments that support that the exosome budding at the plasma membrane is proportionate to their genesis from endosomes (57). Further, it is supported that certain cell types contain deep invaginations of the plasma membrane. These intracellular membrane-connected compartments (IPMCs) are continuous with the extracellular environment through necks that do not allow the release of vesicles, serving thus as reservoirs (58).

Consequently it can be concluded that exosome biogenesis occurs by three modes: 1) vesicle budding into discrete endosomes that mature into MVBs which release exosome into the plasma upon plasma membrane fusion; 2) immediate release of exosomes by direct budding from the plasma membrane; 3) delayed exosome release at IPMCs *[Figure 2].*

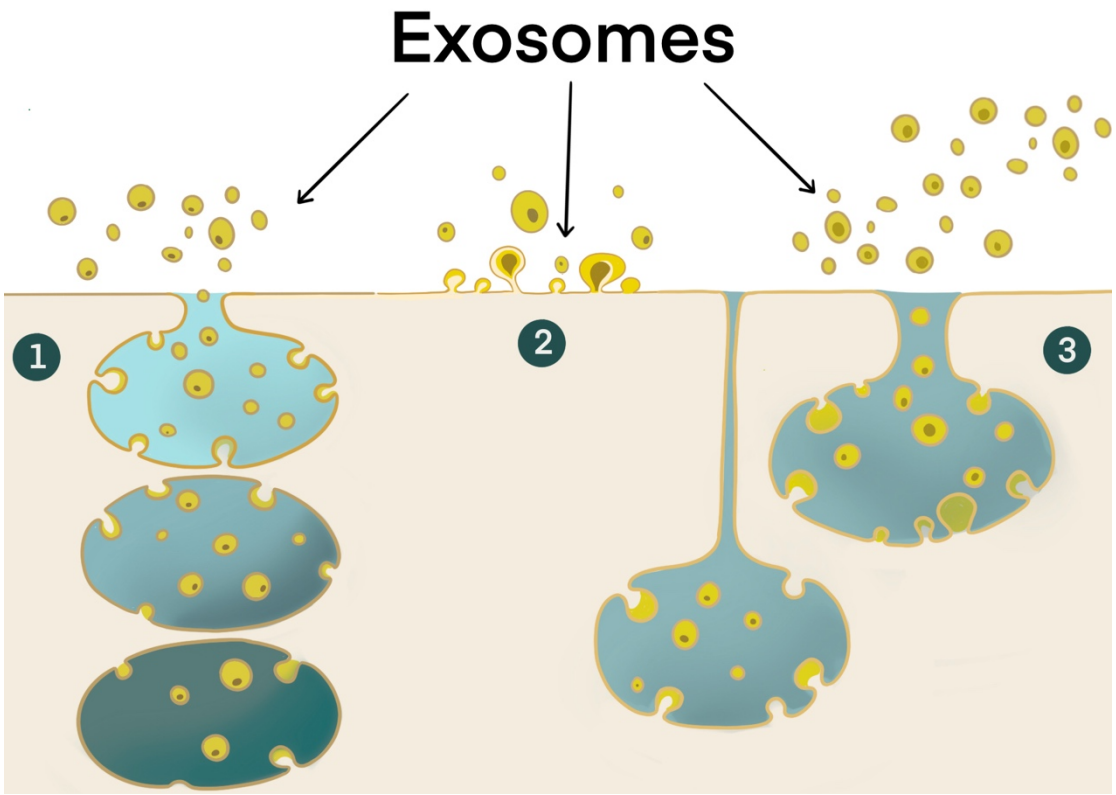

Figure 2: Exosome biogenesis occurs by three modes: 1) vesicle budding into discrete endosomes that mature into MVBs which release exosome into the plasma upon plasma membrane fusion; 2) immediate release of exosomes by direct budding from the plasma membrane; 3) delayed exosome release at IPMCs

The process of exosomal biogenesis is mediated by complex interactions between various protein molecules. The Ral/Arf6/PLD2/syntenin/Alix axis takes part in such cascades. Inhibition of the Ral family of small GTPases has been reported to accumulate MVBs near the plasma membrane and result in subsequent reduction in exosome secretion and exosomal marker proteins (59). Ral GTPases act through multiple effectors to mediate biological processes of exosome creation, two of those are the small GTPase Arf6 and the phospholipase PLD2. Those molecules contribute to the exosomal release of SDCs which is also affected by a pair of exosomal scaffolds, syntenin and Alix. This axis seems to be vital in exosomal budding of the syndecan proteins (11).

Additionally the ESCRT machinery has been reported to have an important key role in exosome formation as it contributes to membrane deformation and sealing through various biological processes (MVB formation, nuclear envelope sealing, plasma membrane repair and cytokinetic abscission) (60). However the exact role of the ESCRT machinery in exosome creation still remains a question, with the current belief being that ESCRTs have multiple functions in exosome biogenesis, such as limiting the size of highly oligomeric cargoes and scaffolding (11), while their main function is considered to be the sorting of specific substances into ILVs (56).

Exosome secretion is also created by autophagy pathways. Autophagy relates proteins participate in initiation, nucleation and elongation during autophagosome biogenesis, a process that also requires ESCRT-III components. This hypothesis is further supported by the observation that lack of autophagy protein 5 reduces the exosome production in cancer cells (11).

Rabphilin (Rab) proteins can also regulate the exosome formation, as they modulate interactions taking place on the endosomes and plasma membrane. Also those proteins are the main motion controller of exosomes on cytoskeleton. In detail, Rab27a, Rab27b, their effector Slp4, Slac2b and Munc13-4 participate in MVBs biogenesis as well as positioning. Such hypothesis has been supported by studies that

demonstrated that loss of Rab27 function results in drop in exosome secretion in certain cell lines. However the mechanism by which Rab27 mediate exosome biogenesis is not yet fully comprehended, with the current speculation being that these proteins orchestrate MVB maturation and trafficking. Additional studies by Ostrowski et al demonstrate that Rab27a is required for assembling plasma membrane microdomains, involved in vesicle- plasma membrane budding, highlighting thus that Rab27 proteins regulate exosomal bio formation in both the endosome and plasma membrane. Rab35, has been proven to regulate exosome production as well, since its loss of function also resulted in reduction of the released exosomes. Rab35 is localized on the plasma membrane and the loss of this molecules impairs the rapid cycling of plasma membrane proteins and lipids back to the cell surface (11).

Finally vesicle-associated membrane protein 7 of the soluble N-ethylmaleimide-sensitive fusion protein attachment protein receptor proteins family plays the main role in orchestrating the fusion between exosomes and cell membranes so as to excrete exosomes (56).

2.1.5 Exosomes: Mechanisms of uptake

Exosome uptake bares many similarities to the interactions occurring between cells and viruses. However exosomes are much more complex vesicles regarding their structure as well as their cargo and biological processes that they mediate and therefore it is expected that their uptake probably come with more complicated cell-binding kinetics (11).

Several studies have demonstrated the existence of specific ligand-receptor interactions that can have a strong impact on exosomal-cell biding and subsequent uptake. In detail, there has been reported that direct and indirect binding of exosomes to cells is mediated by phosphatidylserine receptors, lectins, glycans, integrins and other adhesion molecules (26), (61), (62). The mechanism of those interactions has not yet been deciphered, although exosomal surface proteins such as CD9 and CD81 are believed to be implicated in those events (11).

2.1.6 Exosome biology & cancer formation

The biological roles of exosomes is a rather extended aspect, with multifactorial effects to the recipient cells and their surrounding environment. Briefly it should be noted that

exosome biogenesis is a biological process of protein quality control that allows cells to selectively eliminate proteins from the plasma membrane. Additionally, those small vesicles are molecule carriers with the potent to transmit signals to neighboring cells and therefore set in motion changes regarding their function, while their ability to communicate and alter the structure of ECM is of high interest.

Over the last years an increasing number of studies have demonstrated that exosomes have a robust role in cancer progression, invasion, metastasis and immunomodulation, as well as in the creation of a favorable nest for tumor growth. In further detail, it is believed that exosomes take part in most of the biological processes that promote tumor creation, affecting thus many aspects of cancer hallmarks.

Firstly, exosomes are capable of modulating immune responses. Human derived Epstein-Barr Virus transformed B-lymphocytes exosomes contained the MHC class I and II at their surface, was the first evidence that those small vesicles could interfere with immune interactions and cause alterations in immune responses (63). The MHC-I and -II proteins are responsible for antigen presentation to CD4+ and CD8+ T-cells which regulate antigen specific immune responses, and since exosomes bear MHC-II molecules they have the capability of presenting antigens to CD4+ cells and thus activating them. This argument was further endorsed by the isolation of MHC-I and -II on human dendritic cells (DC) derived exosomes (64), (65). Since DCs have been correlated with antitumor immune responses, the question on whether DC derived exosomes could present antigens to T-cells was further examined and murine bone marrow-derived DCs were incubated with tumor peptides along with MHC-stimulatory production molecules to create exosomes expressing tumor antigens (65). The isolated exosomes were successively used in vivo as immune treatment that initiated an antitumor immune response in murine models (65). Those findings demonstrated that exosomes bare a significant immune modulatory effect and set the basis for the hypothesis that cancer derived exosomes could express MHC complexes as well and prime immune responses. Since multiple studies have demonstrated that tumor-derived exosomes have the potent to escape immune surveillance, by decreasing T-cell proliferation, reducing natural killer (NK) cells' cytotoxic activity and impairing DCs maturation, while the molecular cascades through which those biological processes take place have not yet been identified (13). Additionally, cancer exosomes have been observed to interact with pathways related to macrophage biology in a tumor favoring way. Tumor-associated macrophages secrete growth factors and promote fibrosis in an oncogenic and pro-inflammatory pattern (66). In detail cancer derived exosomes

have been described to induce NF-κB activation in macrophages and the successive production of cytokines like IL6, CCL2 and TNFa with a clear inflammatory effect (13). Moreover, exosomes secreted by tumor cells modulate macrophages towards an M2 phenotype, so as to promote tumor survival (56). Additionally, tumor exosomes carry PD-L1 from tumor cells, and transfer it to DCs or macrophages , thus blocking T-cell function (56). In summary cancer derived exosomes have the ability to interact with every type of immune cell, while those complex interactions could be immunosuppressive and immunoactive as well (56).

The tumor microenvironment is primary consisted of mesenchymal cells, such as, fibroblasts, endothelial cells, hematopoietic cells (of myeloid and lymphoid origin) and the ECM that provides physical and biochemical support (67). Cancer cells communicate with their surrounding environment bidirectionally through the release of soluble compounds and the release of exosomes as well. Cancer exosomes have been reported to alter surrounding cell interactions in favor of tumor growth and dissemination (13). In detail, tumor exosomes have been demonstrated to promote fibroblast differentiation into activated fibroblasts and myofibroblasts in a TGFβ-dependent way (68). Those activated cells are usually found in tumor surroundings and can promote tumor progression by the secretion of growth factors, chemokines, and deposition of ECM constituents, favoring tumor growth and invasion (13). In addition, tumor growth and survival depends greatly on cancer cells' access to nutrients, oxygen and waste removal, all of which are provided by vasculature (69). Tumor cells achieve their survival by secreting angiogenic factors like VEGF to promote endothelial cells' proliferation and migration and cancer exosomes can mediate those processes by carrying mRNAs and proteins that regulate hypoxic conditions, by mediating hypoxia-related intracellular signaling (13).

The role of the exosomes in cancer metastasis has also been the subject of various studies. During cancer dissemination, one of the primal metastatic events is the capability of cell motility and the invasive capacity of the cancer cell. Exosomes released by tumors mediate such processes as they have been reported to promote the formation of invadopodia, in vitro, which are subcellular structures found in cancer cells that are composed of actin and degrade ECM. Also invadopodia were reported to be significant docking sites for CD63 and Rab27a MVBs, highlighting a tight binding between exosome secretion and cancer invasion (70). Additionally exosomes have been demonstrated to regulate cell movement and increase cancer invasion potential by orchestrating ECM degradation. The aforementioned process is mediated by

proteases in cancer exosomes that can degrade collagen (71) and exosomal HSP90a that activates extracellular proteases MMP-2 and plasmin in cancer cells (72). During the process of acquiring a metastatic phenotype, cancer cells gain the ability to cross the endothelial barrier, a procedure known as intravasation (73). miRNA-105 promotes this metastatic features of cancer cells as it is involved in the disruption of vascular endothelial tight junctions by regulating the tight junction protein 1 (74), miRNA-105 has been found in circulating exosomes in patients being in premetastatic stage. After invasion and intravasation cancer cells have the potential to colonize secondary organs (73). In this metastatic stage, there has been studied the role of the exosomes in the organotropism of metastasis (13). Hoshino et al demonstrated that metastasization could be redirected using exosomes derived from cells known to metastasize to specific sites (75), additionally it was reported that integrin expression in exosomes correlates with cancers' cell metastatic organotropism, with integrins $\alpha_6\beta_4$ and $\alpha_6\beta_1$ being associated with lung dissemination (75). Those findings could have a clinical application extension with predictive value to future metastatic formation (75), while the idea that cancer exosomes biodistribution could preclude metastatic dissemination remains of high interest, as it demonstrates that blocking cancer exosome dissemination could halt the cancer metastasis in secondary sites.

Exosomes role in the formation of pre metastatic niche (PMN) has also been the subject of various studies (13). The PMN describes the ability of hematopoietic precursors cells from the bone marrow to move to specific sites before the arrival of cancer cells, paving the way for metastatic cells through the formation of niches that favor their seeding and proliferation (76). Tumor exosomes are ideal candidates for mediating such processes, as they can deliver long distance signaling due to their biology.

The role of EVs in lung cancer can be demonstrated from their potential clinical use in the setting of liquid biopsies. Lung cancer still represents the main cause of cancer related death worldwide as the diagnosis of the disease is mostly obtained when the disease is disseminated and therefore unresectable. Over the last years targeted oncogene therapies and immune modulatory regimens have been introduced to treating those patients. Tumor identification as well as molecular and immunological characterization rely heavily upon bioptic samples. However, tumor biopsy is usually a risky invasive procedure that involves accurate radiological and clinical preliminary study. In this setting EVs and specifically those isolated in body fluids in contact with lung cancer cells (e.g. pleural fluid, bronchoalveolar lavage) might transport clinically

significant molecular cargo such as tumor features and key driver mutations of the disease, harboring thus the advantage of the identification of tumor specific markers less invasively, overcoming potential drawbacks in biopsy related processes (77).

2.1.7 Exosome Isolation Techniques

EV separation methods are continuously evolving, as the majority of existing isolation techniques often fail to simultaneously ensure both high purity and yield of EVs (140). Moreover, there is a risk of compromising vesicle integrity during the isolation process, which can negatively impact the accuracy of subsequent experiments (140). Presently, a variety of techniques have been devised to separate extracellular vesicles, primarily relying on their biophysical and/or biochemical characteristics, such as size, density, shape, and the presence of specific surface markers (140). When selecting a specific method for isolating vesicles, it is crucial to carefully consider both the examination or research needs and the intricate nature of the biological fluids in which EVs circulate (140) .

2.1.7.1 Differential ultracentrifugation

Differential ultracentrifugation is the most commonly used “gold standard” technique for EVs isolation. This process involves the fractionation and separation of substances with different densities and sizes by using different centrifugal speeds and forces. Initially, a series of straightforward steps are undertaken to eliminate dead cells, cellular debris, and large extracellular vesicles (140). The resulting pellet is then reconstituted in phosphate buffered saline, and a final ultracentrifugation step is carried out to eliminate any remaining proteins that may be contaminating the sample. Centrifugation speed is selected according to the experimental requirements, and the temperature is maintained at 4 °C throughout the process to ensure that protease, DNase, and RNase is inactive (140). Subsequently, the EVs can be subjected to characterization analysis. Ultracentrifugation demonstrated the highest isolation purity compared to other size- based methods and therefore, can be preferentially selected when separating EVs from plasma. Controlling the results achieved through ultracentrifugation can be relatively challenging as they are susceptible to the type of biological material, specific rotor type, and duration of centrifugation (140).

2.1.7.2 Isopycnic density gradient centrifugation

Isopycnic density gradient centrifugation, an advancement over differential ultracentrifugation, is a density-dependent isolation technique that utilizes a gradient tube. This method relies on the principle that objects possessing a specific density will remain suspended within a liquid layer of comparable density following centrifugation (140).

2.1.7.3 Rate zone ultracentrifugation

Compared with isodensity ultracentrifugation, rate zone ultracentrifugation is mainly based on particle diameter and can be used to separate particles with the same density but different diameters, eg. rate zone ultracentrifugation can separate platelets from EV fractions (140).

2.1.7.4 Size Exclusion Chromatography

Size Exclusion Chromatography (SEC) is a well-established technique that employs polymers to create a porous stationary phase within a chromatographic column (140). Through SEC, EVs are segregated based on variances in the path length of molecules or particles with distinct sizes (140). The EVs separated using SEC exhibit a more comprehensive physical structure and biological functionality when compared to those obtained through ultracentrifugation. SEC can also efficiently separate EVs from soluble contaminants and is suitable for various biological fluids (140).

2.1.7.5 Percipation

Precipitation is a separation method that relies on the dispersibility of the buffer containing the EVs. Hydrophilic polymers, such as polyethylene glycol (PEG), are commonly employed to create a highly hydrophilic environment that interacts with the surroundings, facilitating the precipitation of the EVs by forming a hydrophobic microenvironment (140). The specific procedures involve the elimination of sizable impurities such as cell debris and apoptotic bodies, as well as precipitation operations. Precipitation also preserves the structural integrity and biological function of EVs. Significantly, precipitation is especially valuable for small-volume samples and finds extensive application in RNA analysis of EV fractions (140).

2.1.7.6 Asymmetric flow field flow fractionation

Asymmetric flow field flow fractionation (AsFIFFF4, AF4) is a technique for separating EV subtypes (140). The flat channel in Asymmetric Flow Field-Flow Fractionation (AF4) consists of two plates. The upper wall of the AF4 channel is constructed using a water-impermeable polycarbonate glass plate, while the lower channel plate is breathable and made of porous stainless-steel frit material (140). Within the channel, there is a polyester trapezoidal separator and an ultrafiltration membrane positioned in the middle (140). The combination of the bottom channel plate and the ultrafiltration membrane serves as aggregation barriers and size-based isolation is accomplished through a transverse flow that runs perpendicular to the parabolic flow pattern (140). The particles to be analyzed flow towards the channel floor or accumulation wall under the action of the transverse flow (140). At the same time, the particles diffuse to the center of the channel under Brownian motion. This technique is characterized by its gentle isolation approach, which minimizes the application of strong shearing forces. As a result, it helps preserve the structural and biological integrity of EVs and allows the isolation of EVs subtype (140).

2.1.7.7 Ultrafiltration

Ultrafiltration (UF) is an isolation technique based on the size of EVs (140). UF uses membranes with molecular weight cut-off, those filtration membranes are usually made of cellulose, polyethersulfone or hydrogenated salts, among which cellulose film is mostly used (140). UF is a straightforward method that does not require specialized equipment. By adjusting the pore size of the filtration membrane, UF can effectively separate EVs into distinct particle sizes (140).

2.1.7.8 Immunoaffinity capture

Immunoaffinity capture technology is primarily based on EVs membrane surface protein markers such as CD9, CD63, CD81, CD82, annexins, programmed cell death 6 interacting protein, Rab5, and epithelial cell adhesion molecules (140). Various immunoaffinity capture-based techniques have been devised, employing microtiter plates, affinity columns, or magnetic beads, and those methods are preferred for isolating EV subtypes based on markers rather than isolating all EVs at one time (140). For instance when investigating specific subpopulations of EVs, immunoaffinity capture serves as an effective method to isolate the desired EVs following UC or SEC. In comparison to UC and SEC techniques, the immunoaffinity method significantly reduces the contamination of proteins and apolipoproteins from blood, thereby

enhancing purity. Furthermore, this method ensures the preservation of EV integrity (140).

2.1.7.9 Charge based separation techniques

Due to the heterogeneity of cells and their varying charges, extracellular vesicles (EVs) exhibit diverse negative charges that differ from their parent cells (140). This inherent variability in charge can have an impact on the efficiency of EV isolation. Therefore, the distinct methods should be selected by considering different cell sources. Several techniques have been devised, utilizing the negative charge characteristics of EVs, such as chromatography-based systems, magnetic bead-based ion exchange technology, and chitosan based isolation techniques (140).

2.1.7.8 Synthetic peptide (Vn96) based isolation method

There have been designed a series of peptides, and also a new class of peptides Vn96 which demonstrate affinity for typical heat shock proteins (140). Multiple experiments have provided evidence that Vn peptides can selectively and effectively capture EVs containing heat shock proteins (HSPs) from cell culture growth media and plasma (140). Those EVs were first isolated from the samples by ultracentrifugation, subsequently the isolated samples were incubated with Vn96. The characteristics of the final product were similar to those obtained from UC isolation, which proved the reliability of the technique (140).

Concluding, single methods for EVs isolation demonstrate a great variability. In order to achieve high yield and purity, an amalgamation strategy employs the optimal fusion of methods by leveraging their complementary advantages to achieve the goal of high yield and high purity (140).

2.2 Lung Cancer

2.2.1 Epidemiology

Cancer is a leading cause of death worldwide, accounting for nearly ten million deaths in 2020. The most common, in term of new cancer cases, was breast cancer with 2.26 million cases, followed by lung cancer with 2.21 million cases. Regarding mortality, the most common cause of cancer related deaths in 2020 was lung cancer with 1.80 million

deaths in 2020. Among females, lung cancer was the leading cause of cancer death in developed countries, and the second leading cause of cancer deaths in less developed countries. In men, the highest lung cancer incidence rates were reported in Europe, Eastern Asia and Northern America, while the lowest rates were in sub-Saharan Asia. It is interesting to be noted, that changes in disease epidemiology among the several countries and population, reflect the practice of tobacco consumption. In accordance with that, lung cancer rates appear to be decreasing in men but increase in women in countries where the tobacco epidemic peaked later (such as Spain and Hungary). In contrast, in countries where the epidemic was established more recently and smoking habits has just peaked, cancer rates are most likely to continue to increase in the following decades, unless interventions to accelerate smoking cessation are applied (78).

2.2.2 Risk Factors

Lung cancer is a multifactorial disease which has been associated with many risk factors, most robustly of which the consumption of tobacco. The strong correlation between cigarette smoking and the appearance of lung cancer, can be demonstrated by how smoking cessation affects the epidemiology of the disease. Supporting this fact, in North America, lung cancer has become more predominant among former than current smokers, whereas in other countries such as China, a peak in lung cancer incidence is still expected, as there has been reported a significant increase in cigarette smoking the last decades (79). In addition to that, in the United States and the United Kingdom, the decline in lung cancer rates is projected to level off in 2 decades because of the slow progress in smoking cessation at present. It is well understood that lung cancer will remain among the top killers for decades to come, unless radical reductions in smoking prevalence are achieved (80). Complementary, there have been associations between the emergence of lung cancer and passive (or secondary) smoking. Supporting this fact, it is estimated that 1.6% of lung cancers can be attributed to secondhand tobacco smoking (81) and there has been published a meta-analysis, demonstrating a relative risk between 1.14 to 5.20 in people who had never smoked but who lived with a smoker (82).

Another risk factor leading to the high prevalence of lung carcinogenesis is air pollution, as lung cancer is believed to be one of the long-term adverse effects of cumulated exposure to ambient air pollution, such as emissions rich in various polycyclic aromatic hydrocarbon compounds, which through oxidative stress,

inflammation, induction of a procoagulatory state, and dysfunction of the autonomic nervous system could deregulate normal cell function (83). The proportion of lung cancers attributable to urban air pollution in Europe is estimated to be 11% (81).

2.2.3 Classification

Different types of lung cancers, are traditionally categorized based on their pathology. The histopathological features of the disease allow to discriminate two distinct categories. The first one is non-small cell lung carcinoma (NSCLC), which includes a broader spectrum of disease subtypes (adenocarcinoma, squamous cell carcinoma, large cell carcinoma, and other types), and represents the majority of lung cancers, with a percentage of 85%. The second category is small-cell carcinoma (SCC) which is accountable for 15% of lung carcinoma and is derived from neuroendocrine origins. The histopathological characteristics of each tumor accounts for a further more detailed tumor categorization, based on the main histologic pattern, the depth of invasion, the cellular mitotic activity and the presence of malignant signs (necrosis). Also NSCLC can be further subdivided regarding the molecular marks that those tumor bare, in accordance to the driving mutations that they harbor (84) *[Figure 3]*, *[Table 1].*

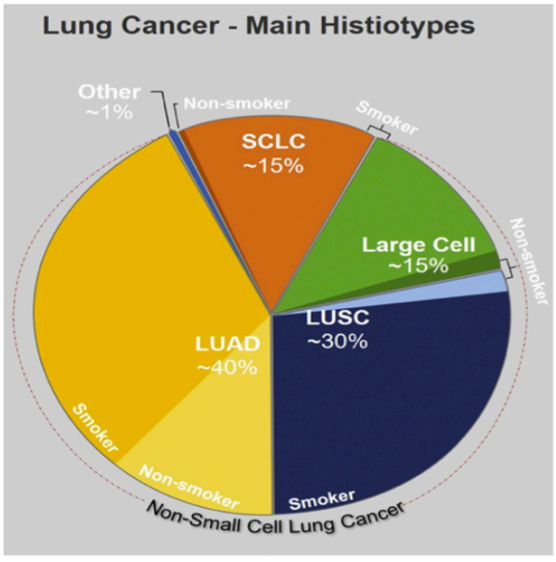

Figure 3: The main histiotypes of lung cancer and their association with tobacco smoking

TABLE 1. 2015 WHO Classification of Lung Tumors[a,b,c]

Histologic Type and Subtypes	ICDO Code
Epithelial tumors	
Adenocarcinoma	8140/3
Lepidic adenocarcinoma[e]	8250/3[d]
Acinar adenocarcinoma	8551/3[d]
Papillary adenocarcinoma	8260/3
Micropapillary adenocarcinoma[e]	8265/3
Solid adenocarcinoma	8230/3
Invasive mucinous adenocarcinoma[e]	8253/3[d]
Mixed invasive mucinous and nonmucinous adenocarcinoma	8254/3[d]
Colloid adenocarcinoma	8480/3
Fetal adenocarcinoma	8333/3
Enteric adenocarcinoma[e]	8144/3
Minimally invasive adenocarcinoma[e]	
Nonmucinous	8256/3[d]
Mucinous	8257/3[d]
Preinvasive lesions	
Atypical adenomatous hyperplasia	8250/0[d]
Adenocarcinoma in situ[e]	
Nonmucinous	8250/2[d]
Mucinous	8253/2[d]
Squamous cell carcinoma	8070/3
Keratinizing squamous cell carcinoma[e]	8071/3
Nonkeratinizing squamous cell carcinoma[e]	8072/3
Basaloid squamous cell carcinoma[e]	8083/3
Preinvasive lesion	
Squamous cell carcinoma in situ	8070/2
Neuroendocrine tumors	
Small cell carcinoma	8041/3
Combined small cell carcinoma	8045/3
Large cell neuroendocrine carcinoma	8013/3
Combined large cell neuroendocrine carcinoma	8013/3
Carcinoid tumors	
Typical carcinoid tumor	8240/3
Atypical carcinoid tumor	8249/3
Preinvasive lesion	
Diffuse idiopathic pulmonary neuroendocrine cell hyperplasia	8040/0[d]
Large cell carcinoma	8012/3
Adenosquamous carcinoma	8560/3
Sarcomatoid carcinomas	
Pleomorphic carcinoma	8022/3
Spindle cell carcinoma	8032/3
Giant cell carcinoma	8031/3
Carcinosarcoma	8980/3
Pulmonary blastoma	8972/3
Other and Unclassified carcinomas	
Lymphoepithelioma-like carcinoma	8082/3
NUT carcinoma[e]	8023/3[d]
Salivary gland-type tumors	
Mucoepidermoid carcinoma	8430/3
Adenoid cystic carcinoma	8200/3
Epithelial-myoepithelial carcinoma	8562/3
Pleomorphic adenoma	8940/0
	(*Continued*)

TABLE 1. (*Continued*)

Histologic Type and Subtypes	ICDO Code
Papillomas	
Squamous cell papilloma	8052/0
Exophytic	8052/0
Inverted	8053/0
Glandular papilloma	8260/0
Mixed squamous and glandular papilloma	8560/0
Adenomas	
Sclerosing pneumocytoma[e]	8832/0
Alveolar adenoma	8251/0
Papillary adenoma	8260/0
Mucinous cystadenoma	8470/0
Mucous gland adenoma	8480/0
Mesenchymal tumors	
Pulmonary hamartoma	8992/0[d]
Chondroma	9220/0
PEComatous tumors[e]	
Lymphangioleiomyomatosis	9174/1
PEComa, benign[e]	8714/0
Clear cell tumor	8005/0
PEComa, malignant[e]	8714/3
Congenital peribronchial myofibroblastic tumor	8827/1
Diffuse pulmonary lymphangiomatosis	
Inflammatory myofibroblastic tumor	8825/1
Epithelioid hemangioendothelioma	9133/3
Pleuropulmonary blastoma	8973/3
Synovial sarcoma	9040/3
Pulmonary artery intimal sarcoma	9137/3
Pulmonary myxoid sarcoma with *EWSR1–CREB1* translocation[e]	8842/3[d]
Myoepithelial tumors[e]	
Myoepithelioma	8982/0
Myoepithelial carcinoma	8982/3
Lymphohistiocytic tumors	
Extranodal marginal zone lymphomas of mucosa-associated Lymphoid tissue (MALT lymphoma)	9699/3
Diffuse large cell lymphoma	9680/3
Lymphomatoid granulomatosis	9766/1
Intravascular large B cell lymphoma[e]	9712/3
Pulmonary Langerhans cell histiocytosis	9751/1
Erdheim–Chester disease	9750/1
Tumors of ectopic origin	
Germ cell tumors	
Teratoma, mature	9080/0
Teratoma, immature	9080/1
Intrapulmonary thymoma	8580/3
Melanoma	8270/3
Meningioma, NOS	9530/0
Metastatic tumors	

[a]The morphology codes are from the ICDO.[2] Behavior is coded /0 for benign tumors, /1 for unspecified, borderline or uncertain behavior, /2 for carcinoma in situ and grade III intraepithelial neoplasia, and /3 for malignant tumors.
[b]The classification is modified from the previous WHO classification[3] taking into account changes in our understanding of these lesions.
[c]This table is reproduced from the 2015 WHO Classification by Travis et al.[1]
[d]These new codes were approved by the International Agency on Cancer Research/ WHO Committee for ICDO.
[e]New terms changed or entities added since 2004 WHO Classification.[3]
LCNEC, large cell neuroendocrine carcinoma, WHO, World Health Organization; ICDO International Classification of Diseases for Oncology.

Table 1: 2015 WHO classification of lung tumors

2.2.4 Staging

Each type of lung tumor, depending on its primary characteristics, its potential to infiltrate lymph nodes and metastasize to distant organs and structures, can be described by the Tumor Nodes Metastasis (TNM) staging system which evaluates the anatomic tumor extension. Clinical staging plays a crucial role in predicting survival, as well as influencing management options in lung cancer patients. The accurate staging of lung cancer is vital, as it will tailor the initial therapeutic approach and will further guide the treatment options (85).

Changes in the clinical staging of NSCLC were revised in 2018 through the 8th lung cancer TNM classification. In detail, those revisions included changes to the T component included subclassification of T1 and T2 by size in 1-cm increments and reclassification of tumors larger than 5 cm as T3 and tumors larger than 7 cm as T4. Diaphragm invasion became a T4 descriptor. Lung atelectasis, whether partial or total, and all cases of main bronchus invasion regardless of the distance from the carina were classified as T2. Tumors with extra- thoracic metastases were subdivided into

M1b for a single distant metastasis and M1c for multiple distant metastatic lesions. No changes were made to the N component for the eighth edition (86) *[Table 2]*.

Descriptor	Definition
T descriptor	
TX	Primary tumor cannot be assessed or tumor proven by the presence of malignant cells in sputum or bronchial washings but not visualized with imaging or bronchoscopy
T0	No evidence of primary tumor
Tis	Carcinoma in situ
T1	Tumor ≤ 3 cm in greatest dimension, surrounded by lung or visceral pleura, without bronchoscopic evidence of invasion more proximal than the lobar bronchus
T1a	Tumor ≤ 1 cm in greatest dimension
T1b	Tumor > 1 cm but ≤ 2 cm in greatest dimension
T1c	Tumor > 2 cm but ≤ 3 cm in greatest dimension
T2 descriptor	
T2	Tumor > 3 cm but ≤ 5 cm or tumor with any of the following features: involvement of a main bronchus regardless of the distance from the carina; invasion of the visceral pleura; associated with partial or complete lung atelectasis or pneumonitis
T2a	Tumor > 3 cm but ≤ 4 cm in greatest dimension
T2b	Tumor > 4 cm but ≤ 5 cm in greatest dimension
T3	Tumor > 5 cm but ≤ 7 cm in greatest dimension or one that directly invades any of the following structures: parietal pleura, chest wall (including superior sulcus tumors), phrenic nerve, parietal pericardium; or separate tumor nodule or nodules in the same lobe
T4	Tumor measuring >7 cm in greatest dimension that invades any of the following structures: mediastinum, diaphragm, heart, great vessels, trachea, recurrent laryngeal nerve, esophagus, vertebral body, carina; or separate tumor nodule or nodules in a different lobe of the same lung
N descriptor	
NX	Regional lymph nodes cannot be assessed
N0	No regional lymph node metastasis
N1	Metastasis in ipsilateral peribronchial and/or ipsilateral hilar lymph nodes and intrapulmonary nodes, including involvement by direct extension
N2	Metastasis in ipsilateral mediastinal and/or subcarinal lymph nodes
N3	Metastasis in contralateral mediastinal, contralateral hilar, ipsilateral or contralateral scalene, or supraclavicular lymph nodes
M descriptor	
M0	No distant metastasis
M1	Distant metastasis
M1a	Separate tumor nodule or nodules in contralateral lung; malignant pleural effusion or pleural thickening or nodules or masses; malignant pericardial effusion or pericardial thickening or nodules or masses
M1b	Single distant (extrathoracic) metastasis in a single organ
M1c	Multiple distant (extrathoracic) metastases in a single organ or multiple organs

Stage	Tumor	Node	Metastasis
Occult carcinoma	TX	N0	M0
Stage 0	Tis	N0	M0
Stage IA1	T1a(mi)*	N0	M0
	T1a	N0	M0
Stage IA2	T1b	N0	M0
Stage IA3	T1c	N0	M0
Stage IB	T2a	N0	M0
Stage IIA	T2b	N0	M0
Stage IIB	T1a–c	N1	M0
	T2a	N1	M0
	T2b	N1	M0
	T3	N0	M0
Stage IIIA	T1a–c	N2	M0
	T2a–b	N2	M0
	T3	N1	M0
	T4	N0	M0
	T4	N1	M0
Stage IIIB	T1a–c	N3	M0
	T2a–b	N3	M0
	T3	N2	M0
	T4	N2	M0
Stage IIIC	T3	N3	M0
	T4	N3	M0
Stage IVA	Any T	Any N	M1a
	Any T	Any N	M1b
Stage IVB	Any T	Any N	M1c

Table 2: TNM descriptions for TNM-8 and stage groups for TNM-8

The primary purpose of the TNM classification is to provide a nomenclature for the anatomic extent of disease in a way that discriminates distinct patient groups. The actual outcomes of groups (calibration [e.g., 5-year survival]) in a data set may differ, reflecting the influence of the many factors besides disease extent that affect prognosis, such as the differences in the health care system, time period, treatment received, patient characteristics of the cohort (age, comorbidities), and so forth, that characterize the particular data set. The median overall survival (OS) in the NCDB clinically staged subset ranged from 37.5 months in the stage IA1 category to 4.8 months in the stage IV category. In the clinically staged cases of the IASLC data set, the median survival was not reached in any stage I category and was 8.8 months in the stage IV category (87) *[Figure 4]*.

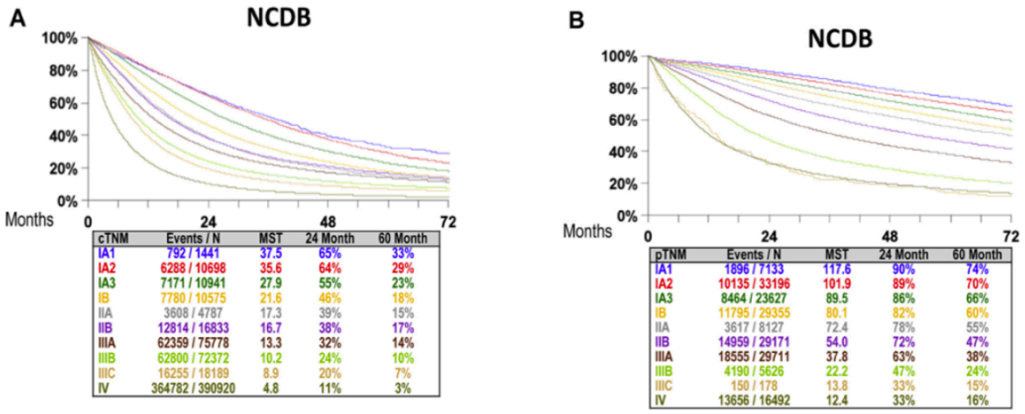

Figure 4: Stage groups for NSCLC. Overall survival in patients with NSCLC according to the eighth edition stage groups in the National Cancer Data Base (NCDB). **(A)** Clinically staged (cTNM) tumors **(B)** Pathologically staged (pTNM) tumors. MST, median survival time (months)

2.3 Exosomes and lung cancer

As already described, exosomes are extracellular vesicles excreted by all cell types, and participate in a plethora of biological processes, by mediating intracellular communications. In this section of this review, there will be summarized the existent knowledge regarding the components of those small vesicles derived from lung cancer, as well as their function in lung carcinogenesis.

2.3.1 Lung cancer exosomes components

The secretion of exosomes is affected by numerous factors, such as physical, chemical and biological stimuli. Marc Ruiz-Martinez et al demonstrated that in NSCLC cell line, miR-134 and miR-135b could regulate YKT6, a protein with important role in regulation of exosomal release (88).

Exosomal cargo, differs according to the originating cell line. Huang et al demonstrated that 80% of the exosomes isolated from NSCLC biopsies were EGFR positive when compared to chronic inflammatory lung tissue (89), providing those vesicles with immune modulation properties, like suppression of lung cancer specific CD8+ cells (89). Additionally, lung cancer exosomes can transfer EGFR to nearby endothelial cells and activate molecular cascades (MAPK and Akt pathways) which promote the overexpression of VEGF and result in the creation of tumor vascularity (28). The

proteinic studies of lung cancer exosomes have demonstrated that when compared to normal bronchial cells, NSCLC exosomes contained more cell adhesion proteins, ECM proteins, proteases and cell signaling molecules. Additionally, the ALK-EML4 translocation has been spotted inside the exosomes as well (90), (91).

As aforementioned, nucleic acids such as mRNAs, lncRNAs, tRNAs and miRNAs can all been found inside exosomes, with the latter being firstly identified in exosomes. Overexpression of 12 miRNAs has been demonstrated between lung cancer samples and normal lung tissue, with this finding bearing the potential of using exosomal miRNA as a diagnostic marker in lung cancer (92), (93).

Additionally, more recent studies by Giallombardo et al have analyzed 8 miRNAs that are known to be deregulated in NSCLC and demonstrated that these exosomal miRNA from NSCLC patients are significantly decreased when compared to healthy donors, implying thus the potential clinical uses of exosomal miRNA detection when tumor tissue is not feasible to acquire (94).

2.3.2 Functions of exosomes in lung carcinogenesis and metastasis

Exosomes harbor various molecules such as proteins and nucleic acid that take part in cascades that facilitate intracellular communications. In lung carcinogenesis, those small vesicles participate in processes such as epithelial mesenchymal transition (EMT), oncogenic cell transformation, angiogenesis simulation, PMN manipulation as well as remodeling of the immune responses *[Figure 5].*

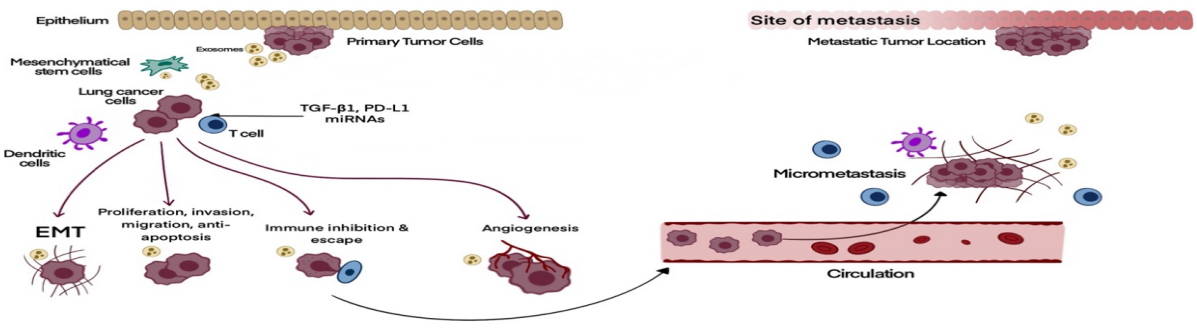

Figure 5: Tumor-derived exosomes promote lung cancer metastasis. Tumor-derived exosomes, especially lung cancer exosomes participate in lung cancer metastasis by promoting cellular epithelial–mesenchymal transformation (EMT); by regulating cell proliferation, apoptosis, and migration; by regulating immune function and angiogenesis; by activating inflammation-related pathways or inducing tumor metastasis signaling pathways; and by interacting with neighboring cells to promote lung cancer progression

2.3.2.1 Exosomes drive epithelial mesenchymal transition

EMT has been described as the loss of epithelial characteristics and the gain of mesenchymal phenotype, such process is considered to be linked to malignant transformation and remains an important step in tumor development (95). There are two key points in the EMT process, the first one is the reduction of expression of adhesion molecules, cell matrix components and molecules that participate in polarity, while the other is the increased expression of cytoskeleton remodeling proteins and proteases (96), (97).

Kim et al studied changes in exosomal cargo regarding EMT, and demonstrated that exosomes isolated from lung cancer cell lines presented with alterations regarding their proteins and miRNAs towards a mesenchymal phenotype. Those results also report that the exosomal cargo can reflect the condition of the derived cancer cell. Additionally, it was demonstrated that cancer derived exosomes could carry distinct proteinic cargo that could activate specific molecular pathways, causing EMT in recipient cells (98).

Mohammad et al demonstrated that exosomes derived from highly metastatic lung cancer cells and human late-stage lung cancer serum, had the potential to induce EMT to recipient human bronchial epithelial cells, suggesting thus that cancer derived exosomes could be potential EMT mediators in lung cancer cells (99).

Furthermore, cancer associated fibroblasts (CAFs) contain SNAIl1, which is delivered to recipient cancer cells via exosomes. The level of SNAIl1 in exosomes is important for EMT initiation in lung cancer cells (100). Additionally, exosomes derived from lung cancer mesenchymal cells, contain ZEB1 mRNA which increases the expression of ZEB1 in recipient cells, promoting thus their mesenchymal phenotype since ZEB1 is an EMT major transcription factor (101).

Mesenchymal stem cells (MSCs) derived from human umbilical cord can also promote EMT, invasion and dissemination of lung cancer. TGF-β1 expression in MSCs, amplifies the role of MSCs to promote EMT and enhances proapoptotic effects on cancer cells through MSC-derived exosomes (102).

Finally, specific exosomal miRNAs, such as miR193A-3p, miR-210-3p and miR-5100 have also been identified in participating in EMT by exosomes. The miRNA profile of exosomes can change after cellular alterations and those miRNAs could potentially promote EMT (103). Accordingly, exosomes derived from lung cancer stem cells (CSCs) promote EMT, migration and invasion of lung cancer cells through the upregulation of the expression of N-cadherin, Vimentin, MMP-9 and MMP-1 and downregulation of E-cadherin (104).

Therefore the exosomal miRNA expression is correlated with EMT and dissemination and could serve as a new biomarker for the EMT process in lung cancer (1).

Inhibition of the EMT process in recipient cells could have the potential of an effective treatment for lung cancer metastatic disease. Targeting specific genes in cancer exosomes could block the exosome pathway responsible for lung cancer metastasis. Such an effort could provide clinical practice with a new method of dealing with metastatic lung disease (1).

2.3.2.2 Exosomes mediate oncogenic cell transformation

Exosomes have the potent to act as carrier platforms and transduce molecular signals to recipient cells, provoking thus a plethora of biological processes, such as direct cell transformation. Supporting this argument, Xiao et al, demonstrated that exosomes of mast cell origin, have the potential to transfer KIT to A549 cells, activate KIT-SCF pathway which leads to the increase of the expression of cyclin D1 and proliferation of human lung adenocarcinoma cells (105).

2.3.2.3 Exosomes stimulate angiogenesis

Exosomes participate in tumor angiogenesis, which can be induced by various cytokines and growth factors. In the lung cancer setting, Liu et al demonstrated that exosomal miRNA miR-21 derived from smoke cigarette smoke extract, leads to STAT3

activation, which increases the VEGF levels in recipient cells. This process is involved in angiogenesis as well as the malignant transformation of recipient human bronchial epithelial cells (106).

Additionally, several other studies have also demonstrated the exosomal input in lung cancer angiogenesis. In detail, Zhuang et al demonstrated that exosomal miRNA, miR-9 could induce strong morphological changes on endothelial cells, through the activation of JAK-STAT signaling pathway and thus promote angiogenesis (107). Also it has been reported that exosomal miR-210 derived from lung adenocarcinoma cells could stimulate angiogenesis in stromal cells through the regulation of the protein ephrin A3 (108).

Lung cancer derived exosomes contain miR-23a which blocks the propyl hydroxylases PHD1 and PHD2, leading to the accumulation of hypoxia-inducible factor-1 (HIF-1) in endothelial cells to promote angiogenesis. Also, miR-23a inhibits the junction protein ZO-1, increasing thus vascular permeability (109). Patients with NSCLC release exosomes that contain miR126 which can also induce angiogenesis and malignant transformation to human bronchial epithelial cells (110).

Furthermore, Grange et al studied renal carcinoma derived exosomes and demonstrated that such exosomes could promote lung metastasis by upregulating the VEGF-A/MMP2/MMP9 pathways resulting thus in enhanced tumor vascularization (111).

2.3.2.4 Exosomes modulate immune responses

As aforementioned, exosomes have the potent to interact with every type of immune cells and thus mediate various biological processes such as immune suppression, immune activation, immune surveillance, antigen presentation as well as intracellular communication. It has been reported that the complexity of those interactions show that exosomes can both favor the immune response against the tumor and act as immunosuppressive agents as well (13).

Fabri et al, studied the immune changes that exosomes promote in lung cancer, as they demonstrated that exosomal miR-21/29a derived from lung cancer cells have the potential to react with Toll-like receptors (TLR) of human immune cells and

successively trigger a TLR-mediated pro-metastatic immune response that leads to tumor growth and tumor metastasis (112).

Furthermore, exosomes expressing Fas ligand (FasL), released from activated CD8+ T cells have the potential to activate the ERK and NF-kB molecular cascades. The activation of those pathways subsequently increased MMP9 expression as demonstrated by Cai et al. Those effects were associated with increased invasive potential and immune evading in lung cancer cell lines (113).

Additionally, exosomes from lung cancer cells express PD-L1 and contribute to tumor growth by reducing T cell activity, evading thus immunosurveillance. Exosomes have also the potential to promote cancer growth by reducing cytokine production and by inducing apoptosis of CD8+ T cells (114).

Epidermal growth factor (EGFR) is closely correlated to the development of lung cancer. Lung cancer derived exosomes with the presence of EGFR have the potential to induce tolerant DCs. Tolerant DCs and Th0 cells were cultured to produce tumor antigen specific regulatory T cells (Tregs), which inhibit tumor antigen specific CD8+ T cells and cause immune tolerance (89).

2.3.2.5 Exosomes facilitate pre-metastatic niche

Information transmission between cancer cells and other cells on their surrounding environment plays a key role in tumor metastasis. Exosomes have a significant role in circulating information and achieving molecular and structural alterations on tumor microenvironment, as they function as communication platforms between cells, forming thus a complex intracellular network.

Exosomes have been suggested to promote ECM remodeling and recruitment of pro-tumorigenic factors at secondary metastatic sites. In detail, it has been demonstrated that tumor derived exosomes have the potential to transfer miRNAs to pre metastatic organs and modulate its stroma for tumor hosting (115). Lung epithelial cells have also a significant role in formation of lung metastatic niche and neutrophil recruitment by sensing tumor exosomal RNA via TLR3 (116). Other studies have investigated the role of tumor derived exosomes in the formation of primary tumors and pre-metastases. Exosomes appear to trigger tissue remodeling and micro anatomic niche preparations that can facilitate lymphatic metastasis (116). Additionally, exosomes can trigger

vascular leakiness at pre-metastatic sites to reprogram bone marrow progenitor cells toward a pro-vasculogenic phenotype (116).

2.3.2.6 Exosomes regulate cell proliferation, apoptosis and migration

Tumor exosomes can influence tumor metastasis by regulating cell proliferation, apoptosis and migration. Tumor derived exosomes, contain molecules such as Wnt3a/β-cantenin, circSATB2, HIF-1a/COX-2,KLF9 and LMO7 which regulate genes and signaling pathways to promote proliferation and migration of lung cancer cells. Additionally, specific mRNAs in cancer derived exosomes like miR-326, miR-135b, miR-210, miR660-5p and miR-96 have also an important stimulating role in lung cancer proliferation (117), (118), (119).

Additionally, mRNA transcripts of GGT-1, LTC4, FECR1, FECR2, miR106b, ALDOA, ALDH3A1 in exosomes derived from lung cancer have a regulatory role in promoting lung cancer invasion and migration. Tumor derived exosomes from lung cancer cells promote lung cancer growth by regulating the expression level of specific genes that encode matrix metalloproteinases like MMP-2, MMP-9, PTEN, E-cadherin (1).

Furthermore, lung cancer derived exosomes have the potential to inhibit apoptosis. Those small vesicles, can deliver the transcripts pf ASMA, S100A16 and the long non-coding RNA (lncRNA) MALAT-1, all of which prevent apoptosis, achieving thus tumor growth (120), (22).

Regarding lung cancer migration, studies have demonstrated that the presence of the transcripts of TGF-β, lnc-MMP2-2, IL-10 and other genes in exosome derived from lung cancer cells have a regulatory role in cell migration, as they affect the expression of migration related genes, promoting thus lung cancer metastasis (121), (122).

These studies collectively report that cancer derived exosomes are heavily implicated in the biological process of lung cancer metastasis, by regulating cell apoptosis, cell proliferation and cell migration. Inhibition of those genes in tumor derived exosomes could present with a novel therapeutic approach to lung cancer metastatic disease. However additional in depth studies need to be executed so as to guide those findings into clinical practice.

2.3.2.7 Other mechanisms

Lung cancer derived exosomes influence tumor growth through other mechanisms as well. Such mechanisms are inflammation pathways interactions with adjacent cells and induction of the EGFR cascade in proteoclasts.

Lung tumor derived exosomes have been demonstrated to secrete TRIM59 and affect macrophage phenotype by transforming them into tumor promoting macrophages by regulating ABHD5 proteasome degradation, which successively activates the NLRP3 inflammasome signaling pathway and produces IL-1, leading to lung cancer progression (123). Also, Kim et al reported that celecoxib, induces COX-2 expression in lung cancer cells and high expressions of COX-2 in exosomes can be transferred to other cells , participating thus in interactions with neighboring cells (98).

Additionally, NSCLC derived exosomes containing amphiregulin (AREG) have the potential to induce EGFR signaling in proteoclast cells which leads to increased RANKL expression and induces expression of proteolytic enzymes. Such molecular changes eventually leads to osteolytic bone metastasis (124).

2.3.3 Clinical potential of exosomes as biomarkers and therapeutics in lung cancer

Lung cancer is the leading cause of cancer associated deaths (116). Despite novel treatment strategies and advanced diagnostics, the prognosis of lung cancer still remains poor (89), mainly due to late diagnosis. Exosomes have been proposed as potential tools with both therapeutic and diagnostic applications, as they are tumor specific, easily available in the blood stream and relatively stable when isolated.

2.3.3.1 Exosomes as biomarkers in lung cancer

Exosomes as aforementioned, contain a plethora of proteinic molecules on their surface like CD91 and CD317 which could serve as exosomal markers for NSCLC (125). In detail, Paulsen et al studied an extracellular vesicle array with 49 antibodies that could evaluate protein profiling of exosomes from different lung cancer stage and histology and potentially diagnose lung cancer as well (126). Of those proteinic markers the presence of CD51 was correlated with increased tumor aggressiveness (126). Additionally, Park et al studied exosomes derived from malignant pleural effusions in NSCLC patients and demonstrated several potential diagnostic markers

including EGFR, K-ras, basigin, carcinoembryonic antigen-related cell adhesion molecule 6, claudin1, claudin3 and RAB family proteins. Such studies demonstrate that multimarker models derived from exosomes could make a fine stratification of patients, highlighting thus the perspectives of exosomes protein profiling as a valid biomarker (126). Furthermore exosomal proteins have also been demonstrated to have a potential role in predicting overall survival (OS) in NSCLC as multiple markers like NY-ESO-1, EGFR, PLAP, EpCam and Alix were correlated with inferior OS (126).

Circulating miRNAs from lung cancers have also been studied for their potential role as diagnostic biomarkers. Rabinowits et al demonstrated that the increased expression of 12 specific miRNAs in NSCLC also increased in circulating exosomes, suggesting thus that exosomal miRNAs can be used to accurately predict tumor profile without using tumor tissue (93). Also, Cazzoli et al studied 742 exosomal miRNAs and confirmed that 4 miRNAs (miRNA378a/379/139-5p/200b-5p) could differentiate lung cancer patients from healthy former smokers. Additionally, they demonstrated that six exosomal miRNAs (miR-151a-5p/30a-3p/200b-5p/629/100/154-3p) had the potential to segregate lung adenocarcinoma patients from lung granuloma patients (127). Furthermore, Giallombardo et al revealed that decreased expression of exosomal miR-30b and miR-30c could relate to squamous cell carcinoma histology (94). Lai et al developed a mathematical model for early stage for NSCLC using the expression of three exosomal miRNAs (miR-21, miR-205, miR155) and demonstrated that the study of the expression of those biomarkers could potentially be applied in the early NSCLC detection (128). The expression of exosomal miRNA appears to have a prognostic role as well regarding lung cancer. Liu et al reported that the increased expression of exosomal miRNAs (miR-23b-3p, miR-10b-5p and miR-21-5p) was associated with poor OS and therefore those molecules appear to be promising non-invasive prognostic biomarkers of NSCLC (129). Moreover, according to a recent meta-analysis, exosome-derived lncRNA MALAT1 shows promise as a potential biomarker for NSCLC screening, however, further validation is required due to its limited specificity (141). Additionally, exosome lncRNAs have also been associated with drug resistance. For instance, the resistance to gefitinib has been attributed to the presence of lncRNA H19, while the resistaatence to erlotinib in NSCLC has been connected to lncRNA (141).

Additionally, clinical trials and observational studies are conducted in order to demonstrate whether EV particles can be applied in the modern clinical practice as liquid biopsy biomarkers. Currently there exist an observational clinical trial which aims

to discover whether bronchial washing samples obtained during routine surgery for the resection of NSCLC tumors contain sufficient vesicles for conducting tests that could aid researchers in acquiring further knowledge about NSCLC (142). There also exists a single arm, open label, Phase 2 clinical trial which aims to evaluate the efficacy of Olmutinib administered to patients with T790M-positive NSCLC confirmed using DNA extracted from extracellular vesicles in BAL as measured by objective response rate (ORR) (143). In the same principle, there is also pending a Phase II, single-center, single-arm, prospective study, which aims to assess the effectiveness of neoadjuvant Lazertinib in treating resectable NSCLC with EGFR mutations, while simultaneously it seeks to explore the clinical utility of liquid biopsy using EVs from BAL fluid as a non-invasive method for identifying EGFR mutations, thus avoiding the need for invasive tissue biopsies (144).

2.3.3.2 Exosomes in lung cancer therapy

Considering the role played by exosomes in lung cancer metastasis promotion, it can be assumed that those vesicles could have a possible therapeutic role in cancer treatment strategy. In this notion exosomal components that suppress tumor growth could be targeted to increase their up-regulation, while tumor promoting cargoes could be inhibited, additionally exosomes could be harnessed as delivery platforms of therapeutic regimens to cancer cells.

2.3.3.2.1 Exosomes as new mode of drug delivery system

Exosomes are small vesicles circulating intracellularly in order to mediate complex biological processes and transfer molecular signals from cell to cell. Those small vesicles come with rather interest as they are natural transporting platforms harboring thus the ability to act as natural nanoparticles with the potential to surpass various barriers regarding the efficient drug transport (130). There have been reported attempts to deliver chemotherapeutic and other natural agents through cell-derived exosomes (130), in detail Kim et al developed paclitaxel's exosomal formulation (exoPTX) in order to treat MDR cancer cells. It was demonstrated that exoPTX could reduce drug elimination and have an increased therapeutic potential in drug resistant tumors. Also exosomes nearly completely colocalized with cancer cells of lung metastases in murine model, concluding thus that exosomes might have a potential in paclitaxel delivery so as to treat drug resistant lung cancer (130).

Complementary, Wu et al demonstrated that exosomes derived from curcumin pretreated H1299 cells exhibited antitumor activity in lung cancer, as curcumin has the potential to downregulate the methylating transferase DNMT1 which leads to the upregulation of transcription factor 21 (TCF21). It was also reported that encapsulation of curcumin by cancer derived exosomes had the potential to increase local drug concentrations in recipient cells and also increase its anti-cancer effect (131).

Furthermore, Aqil et al studied the anticancer activities of celastrol (CEL), a plant based derived titrepenoid, which is a known inhibitor of Hsp90 and NF-κB activation pathways. The authors encapsulated CEL in exosomes and demonstrated that CEL's exosomal formulation exhibited increased efficacy in inhibiting lung cancer cells' proliferation, while the toxicity of the regimen was significantly reduced when tested in murine models (132).

Collectively it can be concluded that exosomes have the potential to distribute medications in blood circulation and protect them from biodegradation, improving thus tissue bioavailability. Additionally, those small vesicles have the advantage to reduce treatment toxicity as well as to enhance drug stability.

2.3.3.2.2 Exosomes as new drug targets

Cisplatin (DDP) is a primary regimen for lung cancer therapy with many clinical trials having failed to predict the clinical outcome of its use due to lack of suitable biomarkers (133). In this setting, Xiao et al demonstrated that the addition of DDP to A549 cells, led to an increase in exosome secretion. Subsequently, when those secreted exosomes were added to other A549 cells, resistance to DDP was increased accordingly. The precise mechanism of such effect still has not yet been deciphered, but it can be speculated that intracellular communications mediated by exosomes and miRNA and mRNA exchange could be responsible (105). The authors also concluded that inhibition of exosome formation and release, could have a potential future role in lung cancer treatment.

Moreover, NSCLC therapeutic approaches heavily rely on molecular target therapies. Gefitinib and erlotinib are both medications widely administered in EGFR positive NSCLC patients, however the use of those regimens with platinum-based regimens does not seem to improve OS (91), (134). In the process of elucidating the antagonistic

effect between EGFR-TKIs and chemotherapeutic agents, Li et al investigated the role of exosomes (134). This study demonstrated that exosomes derived from gefitinib treated PC9 cells (Exo-GF) decreased the antitumor effect of cisplatin, while exosomes derived from cisplatin treated PC9 cells (Exo-DDP) did not affect the antitumor affect of gefitinib (134). Moreover, the authors demonstrated that adding an inhibitor of exosome secretion (GW4869), a synergistic effect of cisplatin and gefitinib was observed (134). These results conclude that inhibiting the secretion of exosomes could potentially overcome the antagonism between EGFR-TKIs and chemotherapy and thus it is proposed that before administering chemotherapy, introducing a washout period to completely eliminate TKI-related exosomes, may be a better procedure for administering chemotherapy and TKIs.

Furthermore, it is well established that cancer growth is also depended on interactions between cancer cells and their surrounding environment, particularly the immune system. There exist several evidence supporting that exosomes are immunological active platforms with potential immunotherapeutic activities. In this setting DC-derived exosomes (Dex) have been used as cell free vaccines in clinical trials as they present with multiple advantages. For instance, Dex can present tumor-associated antigens (TAAs) and provoke specific immune responses such as NK cell activation, while a wide variety of immunogenic molecules are expressed on their outer membrane. Concerning lung cancer there exist two major clinical trials one phase I trial and one phase II trial. In Phase I trial, the first generation (IFN-γ-free Dex) of Dex had the potential to provoke NK cell responses in patients, but demonstrated limited T cell reactivity. This trial validated the safety of IFN-γ-free Dex in NSCLC patients (135). Later studies reported that the second generation of Dex (IFN-γ-Dex) could improve T cell and NK cells responses (136). In Phase II trial, after 4 months platin-based chemotherapy, IFN-γ-Dex could enhance NK-cells functions in patients with unresectable NSCLC and prolong progression-free survival (PFS) in patients with defective NKp30 expression (137). Despite those results, the Phase II trial did not meet the primary endpoint and multiple reasons could account for this result. First, molecules expressed on the outer membrane of IFN-γ-Dex were not capable enough to induce an effective T cell response in the population studied, also IFN-γ-Dex could have overexpression of PD-L1 ligands and finally immunotherapy with vaccines tends to function in a less immunosuppressive environment. Overall it is possible that IFN-γ-Dex might be an effective immunotherapy in stage IIIB/IV NSCLC patients with defective NKp30 expression.

Moreover, several studies have reported the tumor suppressive effect of exosomal cargo mediated by oncogenic and tumor suppressor miRNAs. Huang et al studied pigment epithelial-derived factor (PEDF), which is a protein with anti-tumor effect that regulates cancer progression. PEDF inhibits the metastatic potential of lung cancer cells by increasing thrombloplastin 1 release in cancer derived exosomes leading thus to suppressed cytoskeletal remodeling, cell motility migration and invasion (138). The application of exosomal pretreatment with PEDF could come with antitumor effects (116) and therefore a potential promising therapeutic strategy.

Finally, NSCLC cells derived PD-L1 promotes cancer growth and immune evasion. Hong et al studied the role of circular RNA circ-CPA4, let-7 miRNA and PD-L1 axis in the regulation of NSCLC progression and drug resistance (139). This study demonstrated that circ-CPA4 and PD-L1 were highly expressed in NSCLC compared to human bronchial epithelial, while let-7 miRNA was not. Moreover, the knock-down of circ-CPA4 was demonstrated to inhibit cell growth as well as EMT and favor cell death of NSCLC cells by downregulating PD-L1. Additionally, the NSCLC derived exosomes contained PD-L1was proven to increase resistance of NSCLC to cisplatin.

Section 3: Discussion and Conclusions

Exosomes in lung cancer is an evolving field of study which has gained particular interest over the past few years. Those small vesicles have the potent to transfer molecules from one cell to another and therefore influence recipient cells behavior as well as provoke alterations on the surrounding microenvironmental conditions so as to host lung cancer cells and promote their proliferation and metastasis through the mediation of multiple biological processes. Current studies demonstrate the possibilities of the clinical use of exosomal cargo in diagnosing and treating lung cancer, while further analysis need to focus on the finding of key exosomal molecules that could be applied in the early identification of lung cancer, as well as the proper tailoring of medical treatment in the least evasive way. Those studies, have offered an insight in the practices of liquid biopsies and also come with a new direction regarding research in lung cancer, however, there still remains a large amount of work to elucidate the promising role of exosomes on clinical practice.

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