

World Journal of *Stem Cells*

World J Stem Cells 2020 June 26; 12(6): 406-526



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Monthly Volume 12 Number 6 June 26, 2020

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INDEXING/ABSTRACTING

The *WJSC* is now indexed in PubMed, PubMed Central, Science Citation Index Expanded (also known as SciSearch®), Journal Citation Reports/Science Edition, Biological Abstracts, and BIOSIS Previews. The 2019 Edition of Journal Citation Reports cites the 2018 impact factor for *WJSC* as 3.534 (5-year impact factor: N/A), ranking *WJSC* as 16 among 26 journals in Cell and Tissue Engineering (quartile in category Q3), and 94 among 193 journals in Cell Biology (quartile in category Q2).

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 Proofing Production Department Director: *Yun-Xiaojuan Wu*
 Responsible Editorial Office Director: *Jin-Lei Wang*

NAME OF JOURNAL
World Journal of Stem Cells

ISSN
ISSN 1948-0210 (online)

LAUNCH DATE
December 31, 2009

FREQUENCY
Monthly

EDITORS-IN-CHIEF
Carlo Ventura

EDITORIAL BOARD MEMBERS
<https://www.wjgnet.com/1948-0210/editorialboard.htm>

PUBLICATION DATE
June 26, 2020

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INSTRUCTIONS TO AUTHORS
<https://www.wjgnet.com/bpg/gerinfo/204>

GUIDELINES FOR ETHICS DOCUMENTS
<https://www.wjgnet.com/bpg/GerInfo/287>

GUIDELINES FOR NON-NATIVE SPEAKERS OF ENGLISH
<https://www.wjgnet.com/bpg/gerinfo/240>

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<https://www.wjgnet.com/bpg/GerInfo/288>

PUBLICATION MISCONDUCT
<https://www.wjgnet.com/bpg/gerinfo/208>

ARTICLE PROCESSING CHARGE
<https://www.wjgnet.com/bpg/gerinfo/242>

STEPS FOR SUBMITTING MANUSCRIPTS
<https://www.wjgnet.com/bpg/GerInfo/239>

ONLINE SUBMISSION
<https://www.f6publishing.com>

Defining lung cancer stem cells exosomal payload of miRNAs in clinical perspective

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Author contributions: All authors equally contributed to this paper with conception and design of the study, literature review and analysis, drafting and critical revision and editing, and final approval of the final version.

Conflict-of-interest statement: No potential conflicts of interest.

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Manuscript source: Invited manuscript

Received: January 16, 2020

Peer-review started: January 16, 2020

First decision: April 7, 2020

Revised: April 29, 2020

Accepted: May 27, 2020

Article in press: May 27, 2020

Published online: June 26, 2020

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Abstract

Since the first publication regarding the existence of stem cells in cancer [cancer stem cells (CSCs)] in 1994, many studies have been published providing in-depth information about their biology and function. This research has paved the way in terms of appreciating the role of CSCs in tumour aggressiveness, progression, recurrence and resistance to cancer therapy. Targeting CSCs for cancer therapy has still not progressed to a sufficient degree, particularly in terms of exploring the mechanism of dynamic interconversion between CSCs and non-CSCs. Besides the CSC scenario, the problem of cancer dissemination has been analyzed in-depth with the identification and isolation of microRNAs (miRs), which are now considered to be compelling molecular markers in the diagnosis and prognosis of tumours in general and specifically in patients with non-small cell lung cancer. Paracrine release of miRs *via* "exosomes" (small membrane vesicles (30-100 nm), the derivation of which lies in the luminal membranes of multi-vesicular bodies) released by fusion with the cell membrane is gaining popularity. Whether exosomes play a significant role in maintaining a dynamic equilibrium state between CSCs and non-CSCs and their mechanism of activity is as yet unknown. Future studies on CSC-related exosomes will provide new perspectives for precision-targeted treatment strategies.

Key words: Cancer; Cancer stem cells; Exosomes; Lungs; miRNA; Microvesicles; Non-small cell lung cancer

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Core tip: The role of cancer stem cells (CSCs) in tumour aggressiveness, progression, recurrence and resistance to cancer therapy is well appreciated. However, therapeutic strategies to target CSCs for cancer therapy has still not progressed sufficiently, particularly in terms of exploring the mechanism of dynamic interconversion between

P-Reviewer: Gazouli M, Tanabe S**S-Editor:** Dou Y**L-Editor:** A**E-Editor:** Xing YX

CSCs and non-CSCs. Similar to other cells, CSCs also release exosomes loaded with microRNAs (miRs) as part of their paracrine activity. Our review focusses on the exosomal payload of miRs released by cancer cells and their role in the diagnosis as well as prognosis of lung cancer patients.

Citation: Aramini B, Masciale V, Haider KH. Defining lung cancer stem cells exosomal payload of miRNAs in clinical perspective. *World J Stem Cells* 2020; 12(6): 406-421

URL: <https://www.wjgnet.com/1948-0210/full/v12/i6/406.htm>

DOI: <https://dx.doi.org/10.4252/wjsc.v12.i6.406>

INTRODUCTION

Stem cells related to cancer [cancer stem cells (CSCs)] were first reported in 1994 during a study on human acute myeloid leukaemia (AML)^[1]. The authors of the study identified a rare population of AML-initiating cells isolated from AML patients post-engraftment in severe immunocompromised SCID mice. These cells expressed CD34+/CD38- surface markers and were less mature than the colony-forming cells besides possessing a higher proliferative capacity^[1]. Subsequently, in 2003, human CSCs were identified in solid tumours in various organs, including the breast and brain^[2-4]. Hence, a small CSC population (approximately 100 cells) developed into a tumour in an immunodeficient mice model^[2]. A consensus regarding the criteria for classifying CSCs has not been established as yet. However, they are defined as a sub-population in a given tumour with the ability to self-renew and produce cells that can differentiate^[4]. There is growing evidence that CSCs are highly resistant to different types of stresses, including anti-cancer therapy^[5], and hence, they are generally associated with an increased risk of cancer relapse, metastasis and an overall low survival rate^[5]. CSCs have also been linked with tumours identified in various cell lines but not so frequently in all human tissues (*e.g.*, in human lung cancer).

EPIDEMIOLOGY OF LUNG CANCER AND TREATMENT PERSPECTIVES

According to recently published reports, in 2018, there were 121680 new lung cancer cases for men and 112680 for women in the United States, totaling 234030 cases^[6], which is equivalent to an average of 641 lung cancer cases diagnosed per day. These epidemiologic data rank lung carcinoma as the 2nd most prevalent in men behind prostate cancer and for women after breast cancer^[6]. On a positive note, Siegel *et al*^[7] stated that there was an overall 27% decline in cancer-related death cases between 1991 and 2016, translating into more than 2 million fewer deaths than expected if the rate had stayed at the range of the standard values^[7-9]. Conventional therapeutic strategies including surgery, radiotherapy, and chemotherapy are used for lung cancer treatment either singly or in combination at different cancer pathological stages. However, issues associated with chemotherapy and radiotherapy resistance are well known, and recurrence is still a challenge in advanced lung cancer patients. This inability to be 100% curative has been attributed to the sub-populations of stem cells that are capable of self-renewal, undergoing differentiation and producing multi-lineage progenies that may be tumourigenic or non-tumourigenic. This sub-population of cells contributes to the establishment and maintenance of tumours. Although the underlying molecular mechanisms behind these CSCs' properties are less well-defined, the intrinsic resistance of CSCs to therapy is now generally ascribed to a lack of the capacity to induce the apoptotic signalling, increasing telomere length as well as interfering with the cell membrane's ability to act as a transporter, favoring cell migration, and metastasis^[10-12]. Owing to the multifactorial mechanistic nature of resistance, the current treatment methods are inadequate for cancer treatment, and hence, warrant in-depth molecular studies in the future to improve the contemporary therapeutic regimens^[13].

TARGET THERAPY FOR LUNG CSCS

Given their significant role in poor prognosis, relapse and drug resistance in cancer patients^[14,15], the current treatment modalities are being focussed to target CSCs. Some of these emerging treatment modalities include immunotherapy directed against the CSCs' specific surface antigens, interference with signalling pathways (*i.e.*, Notch signalling) and epigenetic approaches^[16,17]. For example, a recent pre-clinical study targeted the self-renewal regulator BMI1 to attenuate CSCs' self-renewal and tumour-initiating potential in oral cavity squamous cell carcinoma^[18]. Based on the promising results during pre-clinical studies, these therapeutic strategies are now entering into the clinical phase of assessment. Another interesting area of research targeting CSCs involves analyzing the underlying mechanisms regulating the dynamic interconversion between CSCs and non-CSCs (Figure 1). An understanding of the molecular mechanism underlying such a bidirectional interconversion of cells would have significant implications on the future development of therapeutic strategies^[19,20].

Besides the CSCs paradigm, the issue of cancer dissemination has been analyzed in-depth with the identification and isolation of microRNAs (miRs). miRs are short (20-24 nucleotides), non-coding RNAs, which are now suggested to be the most promising molecular markers for the diagnosis and prognosis of tumours^[21]. Their regulatory role in various cellular processes is now well-established, although some of the known miRs are dispensable for the normal functioning of cells but have instrumental participation in the initiation as well as the progression of diseases. The main function of miRs is to play an epigenetic role in regulating gene expression at the post-transcription stage^[22]. In the context of their role in cancers, they are grouped as oncomirs that either function as oncogenes or as tumour suppressors. Given the pivotal role of miRs in the physiological functioning of a cell, their dysregulation has been associated with various pathologies, including the initiation and progression of cancer^[23]. Their prognostic value as qualitative and quantitative biomarkers in plasma, either in free form or encapsulated in the microvesicles, has been reported in various cancers^[24]. There have been attempts to classify human cancers based on miR expression profiles^[25].

Exosomes are extracellular vesicles with a small size of approximately 30 to 100 nm. They are formed by the fusion of intracellular components surrounded by the plasma membrane and released from cells^[26]. It is now becoming well-established that exosomes, besides other components, also transfer their nucleic acid payload including miRs, from the cell of their origin to the recipient cells. The importance of exosomes is mainly due to their capacity to transport miRs into the body, and this process forms an important focus for providing a deep understanding of the possible genetic implications between cancer and non-cancer cells^[27]. The miRs thus transferred significantly affect the gene expression and cellular signalling pathways in the recipient cells, including maintenance of a dynamic equilibrium between CSCs and non-CSCs by delivering their miR-payload^[28,29].

Mature miRs that are about 70 nucleotides long are derived from pre-miRs composed of 100 nucleotides and then transcribed^[30]. Exosomes can transport small intracellular components, such as proteins and lipids, which are included by an endocytosis process from pre- to late-mature exosomes^[31]. Hence, exosomes are currently the smallest cellular components carrying miRs from the cell to the human organs. They are detected in many fluids, such as urine^[32,33], blood and saliva, and this peculiarity makes them a unique mediator against tumour development and progression. Each miR has various targets, although different miRs may have a single target^[34], and this characteristic highlights their significant involvement in many genetic and cellular processes, such as in particular the preservation of cellular differentiation. The most interesting characteristic is that many human genes depend on miRs; this aspect reflects the roles of these small molecules in the genome^[35,36].

In recent studies, it has been found that operable non-small cell lung cancer (NSCLC) patients showed a significant association between recurrence and survival^[37-40]. In adenocarcinoma patients, a miR score has been defined for distinguishing between patients in stage I developing recurrence within two years from surgery, and those patients that were disease-free after three years^[41,42]. We believe that determining the roles of exosomes and their miR-payload in the prevention and cure of cancer is the future of personalized cancer medicine. However, targeting exosomes and monitoring miRs in biological fluids will be the pillar for the setting of new approaches in terms of follow-up for cancer patients, with the focus being to determine more details in the history of each person affected from this disease^[43-45].

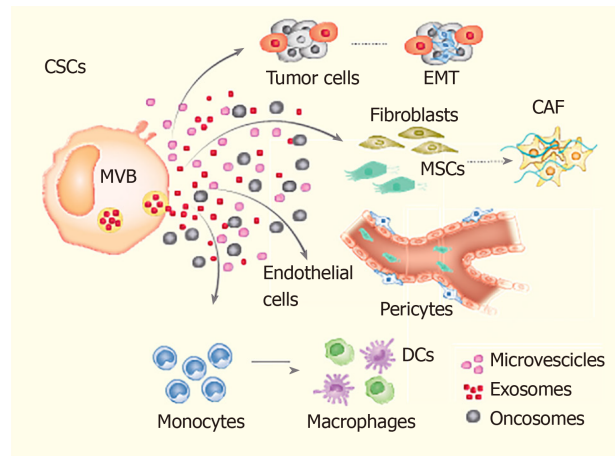


Figure 1 The mechanism of the dynamic interconversion between cancer stem cells and non-cancer stem cells. The cancer stem cells interact with various cell populations of the tumour microenvironment, *i.e.*, tumour epithelial cells, mesenchymal stem cells, endothelial cells, epithelial mesenchymal transitioning cells, fibroblasts, immune cells (monocytes, macrophages *etc.*). This interaction can cause cancer cell stemness phenotype, promotion of tumour cell invasion, angiogenesis and metastasis. EMT: Epithelial to mesenchymal transition; CAF: Cancer-associated fibroblasts; MSCs: Mesenchymal stem cells; CSCs: Cancer stem cells; DCs: Dendritic cells.

CANCER CELL GROWTH, METASTASIS AND ROLE OF EXOSOMAL MIRS

The amount of miRs in exosomes is influenced by the intracellular components as well as by the physiological context^[46-50]. In particular, exosomes can maintain the stability of miRs by preventing their degradation^[48]. The consequence of this exosome-driven role is to drive them in cancer progression as miR-142-3p, miR-150, and miR-451, all of which have been found in exosomes in gastric cancer cells^[46]. Similarly, microsomal miR-21, let-7f, miR-20b, and miR-30e-3p are differentially expressed in patients with solid tumours as compared with healthy people^[46,49]. The role of exosomal miRs is undoubted in terms of cancer growth, development and recurrence. It is apparent that miRs also important players in angiogenesis and metastasis as they can influence host immunity-inducing chemoresistance and tumour microenvironment (TME) re-adaptation^[51].

We know that the metastasis of cancer cells is an intricate process that implicates the colonization of cancer cells from their primary site to a secondary location^[52]. It encompasses a cascade of molecular events that facilitates cell migration, invasion, angiogenesis and epithelial to mesenchymal transition (EMT)^[53]. From amongst the oncomirs, specific miRs are also grouped as “metastamirs” due to their association with the molecular processes and signalling pathways that underlie cancer cell metastasis^[54]. For example, Coebergh *et al*^[55] have recently identified signature miRs, including let-71 and miR-10, which can serve as biomarkers to predict colon cancer patients who are at a risk of metastatic cancer spread. Both miRs’ signatures were successfully used for prediction of hepatic recurrence of cancer in stage-I and -II patients. Similarly, miR-126 suppresses EMT to influence lung cancer cell metastasis^[56]. Molecular studies have revealed the inhibition of the PI3K/Akt Snail pathway with the involvement of miR-126 that could be a potential therapeutic target for lung cancer treatment. Similar observations have also been reported with a possible role of miR-30a *via* targeted regulation of Snail^[57]. Further, the role of Wnt/ β -catenin in EMT has been reported in human colorectal carcinoma metastasis that involved *GNA13* and *PTP4A* genes’ regulation *via* β -catenin signalling and which are targeted by the miR-126 pathway *via* ERK/GSK3 β / β -catenin and Akt/GSK3 β / β -catenin signalling pathways^[58]. The role of β -catenin in EMT has also been reported in a recently published study that involved miR-1246 as a regulator of EMT in A549 cells by inhibiting E-cadherin expression *via* regulation of the Wnt/ β -catenin pathway through GSK3 β / β -catenin targeting^[59]. These data provide vivid evidence for the significant participation of miRs in supporting the metastatic spread of cancers from their primary origin.

There has been a recent interest in miR dissemination through exosomes. In this regard, an important role is played by the cancer-associated fibroblasts into the TME, a process that seems to release exosomes, inducing tumour development or control depending on the presence of some nutrients^[60]. Besides EMT, angiogenesis is

important for tumour maintenance and recurrence. In this context, exosomes released by cancer contribute to increased angiogenesis and tumour growth through the transforming growth factor β 1-dependent pathway, which induces the fibroblast evolution process^[61,62].

In lung cancer, exosomal miR-23a from hypoxic lung cancer cells and hypoxamir-210 from exosomes derived from such cells can improve permeability of the vessel membranes and increase vascularization through the STAT3 mechanism, which can transform normal bronchial cells into malignant ones^[63]. One of the mechanisms that may induce tumour progression involves tumour-derived exosomal interactions with TME. For example, it has been shown that tumour-derived exosomes in lung cancer may induce bone marrow-derived mesenchymal stem cells to change themselves into a phenotype stimulating inflammation^[64]. Hence, the immune system inside TME may be affected by the tumour-derived exosomes with the final result being tumour progression, most likely due to the reprogramming of the immune cells influenced by tumour exosomes^[64-66]. Akin to other cells, the exchange of exosomal miRs from cancer cells to endothelial cells (ECs) significantly influences their angiogenic activity. Tumour cell-released miR-221-3p facilitates lymphangiogenesis in cervical squamous cell carcinoma by its transfer to lymphatic ECs^[67]. Similarly, cancer cell-derived exosomes transfer miR-25-3p to the ECs and regulate VEGF expression by targeting KLF2 and KLF4, thus promoting angiogenesis^[68].

EXOSOMAL MIRS AS BIOMARKERS AND THEIR ROLE IN DRIVING RECURRENCE

As discussed before that the exosomes carrying miRs drive angiogenesis and cancer progression^[69]. For example, it has been shown that miR-103 enhanced angiogenesis and induces tumour metastasis in hepatocarcinoma patients. This process involves several endothelial target proteins, such as VE-cadherin, p120-catenin and zonula occludens 1 in ECs^[70]. In other blood diseases, such as leukaemia, exosomal miR210 secreted by hypoxic leukaemia cells have an important impact on angiogenesis through the receptor tyrosine kinase ligand Ephrin-A3 of ECs^[71]. In contrast, exosomes may include miRs that can harm leukaemia cells, influencing motility and their capacity to adhere. This process is induced by the loss of C-X-C motif chemokine ligand 12 and vascular cell adhesion molecule-1 proteins in ECs^[72].

Several exosomal miRs are essential in the process of recurrence. In particular, in metastatic breast cancer, exosomal miR-210 is involved in EC transport as well as improving angiogenesis^[73]; in nasopharyngeal carcinoma (NPC) cells, miR-23a exosome enhances tumour growth and recurrence^[74], although exosomal miR-9 suppresses NPC cell migration and the consequent vascular formation by targeting midkine and modulating the phosphoinositide-dependent protein kinase/protein kinase B (Akt)-signalling pathway^[75].

Due to their already demonstrated crucial participation in metastatic processes and their presence into human fluids, exosomal miRs are the future of personalized medicine as biological biomarkers^[76]. Exosomal miRs are already in practice as reliable biomarkers for the diagnosis of lung cancer patients^[77-79]. Cazzoli *et al*^[77] performed a thorough exosomal miR-analysis of 30 plasma samples (including $n = 10$ each from lung-adenocarcinoma, lung-granuloma and healthy-smoker subjects) and all the donors were matched for age and sex. The expression level of four miRs distinguished between tumour and healthy-smoker subjects^[77]. These findings were subsequently used on a larger group of patients with 96% sensitivity and nearly 70% specificity. Several upregulated miRs-derived exosomes (such as miR-21 and miR-155) have been found in patients who developed lung cancer recurrence^[78]. In particular, Li *et al*^[63] used a qPCR-based array to analyze plasma from 10 patients affected by lung adenocarcinoma, and he found an increased level of 3 exosomal miRs (miR-23b-3p, miR-10b-5p, and miR-21-5p) which seemed to be correlated with decreased survival^[63]. The expression profile of specific exosomal miRs in lung adenocarcinoma patients vindicated the previously published results that circulating exosomal miRNAs were a useful and possible marker for further diagnostic and therapeutic purposes in lung cancer^[79]. Dejima *et al*^[80] profiled plasma exosomal miRs derived from lung cancer patients to demonstrate that miR-21 and miR-4257 were significantly upregulated as compared with those patients without recurrence and healthy individuals as controls. The microarray data also showed that exosomal miR-21 and miR-4257 exosomes were significantly associated with tumour growth and metastatic invasion in lung cancer. These data were also supported by the low percentage of disease-free survival in patients with high expression of both exosomes levels^[80,81].

Besides the recent reports regarding the pivotal role of exosomal miRs in driving

recurrence and invasion of cancer in patients, their use as biomarkers seems to represent an effective method for diagnostic and prognostic purposes. That is justified by the fact that there are currently no markers that can satisfactorily diagnose presence of tumour in the early stage as well as predict long-term survival with respect to many solid cancers^[82].

Although quantification of a panel of miRs in the plasma or serum of patients at high-risk to develop lung cancer has been proposed, the difficulty in discriminating between miRs for normal and cancerous tissue remains an obstacle in the development of an effective screening method^[83]. Nigita *et al.*^[84] analyzed data from 87 NSCLC patients, attempting for the first time to identify and differentiate miRNAs for normal and tumour tissues; in particular, miR-441-5p was the most consistently detected among the NSCLC exosomes.

One of the mechanisms by which exosomal miRs affect the functioning of CSCs is *via* regulation of the interaction between CSCs and their microenvironment^[85]. The exosomes serve as carriers of the genetic information (*i.e.*, miRs) required for regulation of the signalling involved in the transformation of cancer cells into CSCs to achieve a dynamic equilibrium between the two cell types.

EXOSOMAL MIR PAYLOAD AND TME

Reflecting their role in metastatic processes, it is apparent that exosomal miRs have an important impact on TME^[86]. It has been demonstrated that exosomes driven by specific miRs, such as miR-223 derived from macrophages, may induce drug resistance in the hypoxic TME^[87].

The interactions between cancer cells, exosomal miRs, and the TME rely on a complex network that has yet to be satisfactorily clarified. In particular, exosomes can mediate immune regulation, and several studies are attempting to understand the direct effects of exosomes in T-cell activation^[88]. However, there is currently a lack of understanding regarding the identification of the connections between the immune system, miRs, exosomes, and CSCs. Recently, a correlation between tumour-infiltrating lymphocytes (TILs) and CSCs in NSCLC patients has been demonstrated^[89]. This is important to highlight the existence of interactions and relations of these cellular components to further connect exosomes carrying miRs, CSCs and TME. Further clarification of the associations between TILs and CSCs will be helpful for the development of targeted therapies that may focus on miR exosomes, CSCs, and TME cells.

The complexity of TME leads to difficulty in understanding the mechanisms involved. TME is an amalgam of both cellular and non-cellular components that encompasses the surrounding microvascular structures, immune cells, fibroblasts, ECs, cancer cells, signalling molecules and the extracellular matrix (ECM) (Figure 2). Put together, these components offer a growth factor and cytokine-rich TME that is conducive for phenotypic plasticity, immune surveillance, survival, angiogenesis and cancer cell metastasis^[90,91]. More importantly, TME contributes significantly to the spatiotemporal dynamics of pattern formation and is one of the primary factors that substantially influence tumour heterogeneity^[92]. The link between TME and CSCs generation is represented by the EMT^[93].

The discovery of the TME implies the possibility of a novel treatment strategy that goes beyond the paradigm of cancer genetics that restricts its focus only on cancer cells^[94]. The presence of CSCs in the TME is crucial for 'tumour nutrition' due to their capacity to reproduce themselves, inducing tumour growth in NOD-SCID mice and facilitating the spread and chemoresistance of the tumour. There is mounting evidence that conservative therapeutic strategies (*i.e.*, radiotherapy and chemotherapy) largely fail to eliminate CSCs, which are now associated with minimal residual disease and cancer relapse^[95,96].

The physiological functioning of cells is affected by a multitude of physical factors that alter both genetic and epigenetic states. These molecular changes influence the intracellular regulatory circuitry that enables the body to achieve as well as sustain an appropriate response to the changing environment^[97-99]. In this regard, ECM is an elastic barrier able to change the mechanical properties of its proteins through genes' profile mutations. Studies of the TME have led to immunotherapy and other new-generation immune therapies, such as CAR-T cells^[100-102] and anti-cancer vaccines^[103-105]. The mechanism underlying the role of TME-associated transformation of normal cells into tumour cells and then converting to malignancy upon cancer initiation is less well defined^[94]. However, this mechanism is associated with the mechanical/scaffold (elasticity) effect of ECM^[106]. Li *et al.*^[94] suggested that the elasticity is an important aspect in ECM development, playing a key-role in miR exosome expression,

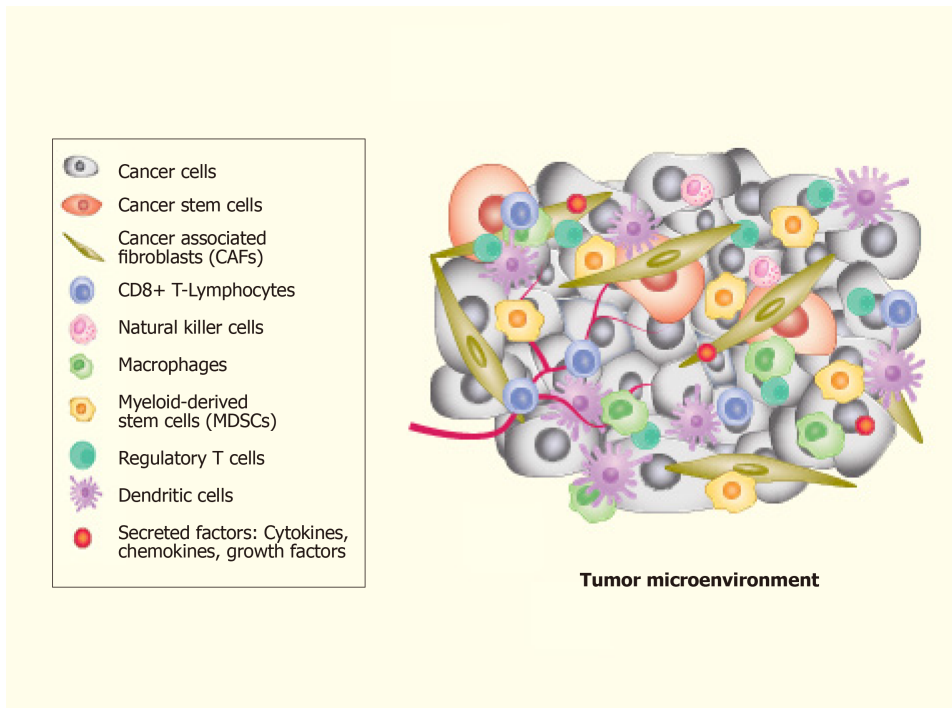


Figure 2 The components of the tumour-microenvironment. The cellular components of the tumour-microenvironment include different types of cells including such as epithelial tumour cells, cancer stem cells, and immune cells. Every type of cellular component contributes to maintain the tumour alive and to develop neo-vessels for promoting the tumour cells dissemination.

regulating tumour gene expression and tumour growth. Further understanding of the role of tumour-TME interaction will significantly help in understanding the acquired resistance of cancer cells towards contemporary cancer therapies including surgery, radiotherapy, chemotherapy and anti-angiogenic therapy^[107]. This understanding will open up a new avenue to target the CSC niche within the TME. In this context, in particular, the exosomes derived from the tumour (TEX) seem to have a strong influence on the TME for their capacity to expand themselves in other tissues and contributing to cancer dissemination to the organs^[108]. Additionally, besides TME, Javeed *et al*^[109] hypothesized the presence of a tumour “macroenvironment” (TMaE) that results from the pathological interaction between tumour cells with other organs and systems of the body to mediate immunosuppression and promote genetic and metabolic reprogramming of the cells through exosomal-miR payloads^[109]. Further understanding of the TMaE would be helpful for two reasons: first, it would help in identifying those patients who would benefit from systemic therapy and second, it would help in the future development of novel systemic therapies^[110].

One of the most interesting aspects in this context is that TEX is associated with some antigens, such as programmed death-ligand 1 (PD-L1), which induces the pre-metastatic pathway for tumour dissemination^[111]. PD-L1 expression has been reported to be enhanced by exosomes from melanoma, and this justifies the roles of exosomes in tumour growth, metastasis and immune modulation^[112,113]. There is interest in establishing an “exosome screening” approach due to their presence in many fluids of the human body, and this aspect is highly important in terms of providing potential biomarkers as well as drug carriers for targeted drug delivery^[114-116].

THE POTENTIAL ROLE OF MIRS IN TUMOUR AGGRESSIVENESS

“Tumour aggressiveness” is typically used to define a highly incurable end-stage cancer that is also able to resist the standard treatments^[117-119]. Dysregulated miRs can be oncogenes (oncomirs) if upregulated, or tumour suppressors (anti-oncomirs) if downregulated, depending on their target transcripts^[120,121]. Tumour aggressiveness is generally associated with altered miRs profile that has already been reported in various solid tumours^[121-125]. Specifically, miRs have been implicated in a substantial number of intracellular mechanisms that include tumour growth, proliferation, recurrence, tumour metabolic alterations and chemo- and radio-resistance. However,

they are also influenced by many extracellular factors, such as hypoxia, which seems to contribute to poor clinical outcomes by increasing the recurrence risk^[126-128].

The mechanism of oxygen sensing at the cellular level has remained an area of intense investigation, as evidenced by the two Nobel Prizes won by Otto Warburg in 1931 and Corneille Heymans in 1938 for their findings on the role of enzymes and the nervous system in respiratory cellular mechanisms^[126]. Moreover, the genetic component of adaptation to oxygen flux remained oblivious for the major part of this century. More recent studies have successfully addressed this issue to elucidate the molecular biology of hypoxia-inducible factor (HIF) signalling as part of the body's hypoxic response in health and disease^[127,128]. HIF-1 signalling and HIF-1-dependent miRs, hypoxamirs, are being extensively studied for their role in cell survival and angiogenesis in the context of cancer cell biology^[129-132] as well as regenerative medicine^[133]. The data thus generated has been exploited to promote stem cell survival and angiogenesis either as part of physical or genetic manipulation of cells for hypoxamir expression^[134-138]. In this context, the homeostasis of reactive oxygen species (ROS) is the key for maintaining normal biological processes^[139]. Higher-level oxidative stress produces irreversible damage to intracellular and cellular components, thus altering the stability of the genome with the induction of malignant cell transformation^[140]. For example, altered production of ROS is also associated with EMT, tumorigenesis and tumour progression^[141]. In particular, the mechanism of ROS on CSC mechanism regulation is not yet fully clarified.

The precise mechanism by which ROS regulates CSCs and EMT characteristics with the HIF-mediated pathways is unclear^[140-142]. It has been documented that normal stem cells, as well as CSCs, have a low level of ROS, potentially due to their strong defense system against DNA damage^[140-143]. Low-level ROS in CSC-like cells is associated with higher free radical scavenger production. The inhibition of ROS scavengers by drug treatment in mouse breast Thy-1 + CD24 + Lin- CSC-enriched cells markedly decreased their clonogenicity with increased radio-sensitization^[144]. Hence, low levels of ROS and enhanced ROS defense may contribute to tumour radio-resistance as compared to non-tumorigenic counterparts^[145]. These findings strongly suggest that ROS may play an important role in the pathogenesis of CSCs.

Surprisingly, there is a link between ROS and miRs. It has been demonstrated that H₂O₂ treatment can dysregulate the expression of certain miRs in vascular smooth muscle cells and macrophages. Moreover, it has also been demonstrated that miR-30e contributes to regulating oxidative stress and ROS levels through SNAI1 mRNA in human umbilical endothelial vein cells^[146]. Given the significant regulatory role of miRs in metabolic processes in the cells, many of the oncomirs, such as miR-21, are directly involved in the formation of ROS and hence, promote tumorigenesis^[147]. Both ROS and miRs control each other's expression in cancer cells to maintain a balance that is supportive of cancer cells in terms of their ability to produce the hallmarks of cancer^[148,149]. More investigations are mandatory to clarify the possible importance of ROS in CSC regulation.

CLINICAL IMPLICATIONS

Although recent studies have identified the presence of CSCs in ADENO and SQUAMO cell carcinomas of the human lung, with this aspect being important in terms of understanding that CSCs are present in all NSCLC tumours^[150-152], even in neuroendocrine tumours of the lung^[153], the possibility to target CSCs is still debated. In particular, the scientific community is focusing attention on the characterization of miR exosomes for their intrinsic characteristics as complex paracrine factors^[153]. In fact, it has been found that stem cell-derived exosomal miRs can be used to modulate the therapeutic response to stroke and may increase their therapeutic potential^[77]. This aspect may be important in future if we consider the possibility of targeting CSC-derived exosomal miRs, in consideration of CSCs' characteristics related to chemo- and radioresistance – this approach would have a key role in new cancer treatments.

Lung cancer cell-derived exosomal miRs are at the centre of interest in the present-day research for future roles as predictors of recurrence as well as biomarkers in the early stage or for their prognostic role in advanced-treated patients^[13,154-157]. The prime objective is to explore the possibility of their use as biomarkers for early diagnosis of lung cancer combined with target therapy^[158,159]. In one of the studies, immunostaining of exosomes from lung cancer tissue and chronic lung disease showed that 80% of these specimen-derived exosomes were positive for EGFR, although 2% of the inflammatory tissue was positive. This point suggested a possible role of EGFR exosomes as potential biomarkers in lung cancer^[61,160,161]. A similar result has also been identified in ALK-EML4 translocated exosomes, which are markers for first-

generation treatment ALK-TKIs^[162,163].

On the same note, exosomes from the plasma of NSCLC patients ($n = 276$) indicated that exosomes may have a prognostic role as biomarkers in NSCLC^[156]. Similar results were found in plasma exosomal miRNAs analyzed from lung adenocarcinoma patients ($n = 84$) versus healthy controls. These results showed increased levels of exosomal miR-10b-5p, miR-23b-3p, and miR-21-5p were significantly associated with poor prognosis, thus suggesting their significance as biomarkers of NSCLC prognosis^[157]. The use of exosomal miRNAs as prognostic biomarkers is the basis for new-generation target therapy against NSCLC, particularly in lung cancer for highly important proteins such as EGFR, KRAS and RAB family.

The most relevant aspect related to targetable exosomes is connected to the precision treatment of NSCLC^[164]. In this regard, exosomes can be produced as new cellular molecules of delivery for medical treatment as well as against tumours to reduce the negative collateral effects in the human body^[165,166]. Lai *et al*^[167] have established a method for loading exosomes with a drug or genetically manipulating cells with genes of interest to alter the exosomal payload derived from these cells. Mendt *et al*^[168] have reported a standard operating procedure to engineer exosomes that could target KRAS (iExosomes), with a particularly good response in terms of improved survival. The possibility to target genes such as KRAS is highly innovative because it is responsible for the highest mortality rate in NSCLC patients.

The diversity of exosomal-payload and their functions relevant to NSCLC may provide future pioneering target treatments. Yang *et al*^[169] determined that induced expression of miR-let-7 in exosomes for NSCLC treatment was specific and effective in tumour suppression^[169,170]. Similarly, exosomes isolated from H460 cells and that had been transduced for the restoration of LKB1 (liver kinase B1) had an increased ability to restore lung cancer cell migration as compared to exosomes isolated from H460 cells lacking in LKB1 activity^[171]. While elucidating the process, it was observed that H460 cells with restored LKB1 supported the emigrational activity of lung cancer cells by the suppression of exosomal secretion of migration-suppressing miRNAs, including miR-125a, miR-126a, and let7b. These data highlight the significance of LKB1 as a new target for future cancer therapy. In an interesting new development, antibody therapy with anti-CD9 or anti-CD63 to target tumour-derived exosomes effectively inhibited the progression of breast cancer in mice, suggesting a successful further treatment in lung cancer^[172].

CONCLUSION

Characterization of CSC-derived exosomes in terms of their payload and the use of exosomes for CSC targeting have emerged as potential strategies for cancer theranostics. For example, the safety and feasibility of exosome targeting were first assessed in phase I clinical trial for metastatic melanoma^[173]. The patients were treated with autologous dendritic cell-derived exosomes (DEX) loaded with the melanoma antigen gene (MAGE) by intradermal and subcutaneous^[173,174]. Although no significant outcome was reported, the data vividly demonstrated that exosome-based treatment may represent a new approach for curing cancer patients. The results from phase I trial evidenced that the immune system of treated patients was active and that exosome-treated patients showed limited disease progression^[174]. Based on these encouraging data, DEX has progressed to Phase II trials as maintenance immunotherapy after first-line chemotherapy in NSCLC patients^[175]. Besides the value and importance of exosomes as mediators against anticancer effects, it is necessary to study their clinical effects in the human body to guarantee a better standardization of the methods of processing and ensure optimal and reproducible anti-tumour immune responses after exosome-based therapy in the clinical perspective^[176,177].

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