Manuscript title: Activating invasion and metastasis in small cell lung cancer

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Abstract

Background: Small cell lung cancer (SCLC) harbours the most aggressive phenotype of all lung cancers to correlate with its bleak prognosis. The aggression of SCLC is partially attributable to its strong metastatic tendencies. The biological processes facilitating the metastasis in SCLC are still poorly understood and garnering a deeper understanding of these processes may enable the exploration of additional targets against this cancer hallmark in the treatment of SCLC. **Recent findings:** This narrative review will discuss the proposed molecular mechanisms by which the cancer hallmark of activating invasion and metastasis is featured in SCLC through important steps of the metastatic pathway. The review will discuss SCLC VEGF family expression and vascular mimicry as a means of vasculogenesis, and the role of tumour heterogeneity, DLL3, NFIB, selectin, and B1 integrin in enabling epithelial to mesenchymal transition and subsequent invasion, and the molecular markers expressed by SCLC to assist organ-specific homing during metastasis. The review will also discuss a recent article observing mir-1 mrna upregulation as a potential therapeutic option in targeting the metastatic activity of SCLC. **Conclusion:** Treatment of SCLC remains a clinical challenge due to its recalcitrant and aggressive nature. Amongst the many hallmarks used by SCLC to enable its aggressive behaviour, that of its ability to invade surrounding tissue and metastasise is particularly notable and understanding the molecular mechanisms in SCLC metastasis can identify therapeutic targets to attenuate SCLC aggression and improve mortality.

Introduction

Lung cancer is the foremost contributor to cancer mortality worldwide.[1] Lung cancer is broadly classified as small cell lung cancer (SCLC) and non-small cell lung cancer (NSCLC),[2] with SCLC accounting for 15% of all lung cancers.[3]

SCLC, a neuroendocrine tumour of the lung,[4] is the most aggressive lung cancer subtype,[5] characterised by rapid proliferation and metastasis, and frequent chemoresistance.[6] SCLC carries a poor prognosis, with a 5-year overall survival rate under 7%.[5] There has been minimal therapeutic advancement in SCLC for several years, and etoposide combined with platinum-based chemotherapy remains as standard treatment in all patients.[7] Unfortunately, chemoresistance to this regimen often occurs within 1 year of treatment, resulting in disease recurrence.[7] SCLC carries a high somatic mutational burden, and the mutations are highly associated with tobacco-related carcinogens[5]. SCLC almost universally demonstrates loss of function mutations in the Rb and p53 tumour suppressor genes, amongst many other mutations, which collectively contribute to cancer hallmarks including that of sustaining proliferative signalling, avoiding immune destruction, enabling replicative immortality, angiogenesis, and induction of invasion and metastasis.[5]

Notably, SCLC harbours a strong propensity for metastasic activity,[8] commonly to the liver, bones, brain and lymph nodes.[9] Metastasis accounts for 90% of all cancer related deaths,[10] and in SCLC, 70% of patients exhibit metastatic disease at the time of diagnosis,[8]with metastatic propensity contributing strongly to poor survivability in SCLC. It is therefore important to understand the biology of metastasis that underpins

SCLC to identify potential therapeutic targets against SCLC metastasis. The following sections will address important factors noted to facilitate the metastatic mechanisms of SCLC.

Drivers of SCLC metastasis

The vasculogenic properties of SCLC

Angiogenesis and lymphangiogenesis feature in SCLC, enabling metastasis through the creation of haematogenous and lymphatic routes of dissemination.[11,12]

Expression of the VEGF family

Human SCLC cell lines express VEGF-A and VEGF-C, in addition to their respective receptors VEGFR-2 and VEGFR-3[13] to promote vasculogenesis. The VEGF subclasses can act on their receptors, also expressed by the tumours, in an autocrine fashion.[13-14] VEGF-A binds VEGFR-2 to induce angiogenesis and vascular permeability,[15,16] and VEGF-C acts on VEGFR-3 to facilitate lymphangiogenesis.[16,17]

Canonical tumorigenic pathways such as MAPK or PI3K are often involved in mediating the effector functions of the VEGF family.[18] VEGF-A enables angiogenesis to create routes for tumour cell dissemination, and assist circulating tumour cell survival, extravasation, deposition, and colonisation into secondary sites.[19] The lymphangiogenic capacity of VEGF-C enables lymphatic metastasis,[19] and the expression of VEGF-C in SCLC cell lines[13] may explain the frequently noted pattern of lymphatic metastasis in SCLC. Exogenous VEGF has been shown to incite SCLC cell proliferation and migration in vitro,[13] reinforcing the understanding of VEGF as a facilitator of SCLC metastasis. Unfortunately, VEGF inhibitors and angiogenesis inhibitors have conferred variable therapeutic effect in SCLC and are not yet mainstays of SCLC treatment.[20]

Vascular mimicry

SCLC also exhibits vascular mimicry potential, with vascular mimicry positively associated with invasion and metastasis.[21]Williamson et al. first uncovered the expression of VE-Cadherin in a subpopulation of SCLC circulating tumour cells, noting VE-Cadherin as an important marker of vascular mimicry. They further demonstrated the formation of vascular mimicry networks by SCLCs on matrigel in a VE-Cadherin dependent manner, thereby inferring vascular mimicry capabilities in SCLC. Vascular mimicry is a means of de novo tumour vascularisation[21] where the tumour cells demonstrate plasticity to adopt an endothelial phenotype.[22] In vascular mimicry, the VE-Cadherin transmembrane protein, which is normally present in adherens junctions between endothelial cells, binds tumour cells as they arrange into vessel- like structures.[23] Vascularisation by vascular mimicry consequently provides additional routes for invasion and metastasis.[22]

SCLC invasion and migration

SCLC can enact epithelial-mesenchymal transition (EMT), and express various ligands and receptors to facilitate invasion and circulatory migration.

Tumour heterogeneity in Epithelial-mesenchymal transition

EMT is an important step in metastasis, whereby cancer cells adopt a mesenchymal trait to enable circulatory invasion.[24]The biology of EMT in SCLC is poorly understood,[9,25,26] however it is possible that tumour heterogeneity contributes to its mechanisms of EMT.[25]Krohn et al. observed differing phenotypes of the 'floating' cell or 'adhering' cell within a single SCLC cell line, where the 'adhering' cells are characterised by an EMT phenotype and greatly express EMT markers such as vimentin and Matrix metalloproteinase (MMP)-2 and 9, while the floating cells exhibited an epithelial phenotype and highly expressed E-cadherin.[25] As such, the metastatic behaviour of SCLCs may be partially attributed to tumour heterogeneity, whereby the more malignant cellular subtype drives metastatic behaviour including that of EMT.

DLL3 is a key orchestrator of SCLC invasion and migration through the expression of Snail

Recently, SCLC has been shown to undergo migration and invasion by DLL3- mediated upregulation of Snail proteins. DLL3 is a ligand of the Notch signalling pathway, and is expressed in over 80% of SCLC

surfaces, while being minimally expressed on healthy lung tissues.[27] Nuclear expression of Snail occurs in 31% of SCLC, which represents the highest of all lung cancer subtypes.[28] Furuta et al. demonstrated DLL3 to be an in vitro inducer of Snail expression in SCLC.[29] Snail is widely implicated in EMT by suppressing E-cadherin, a mediator of cell-cell adhesion.[30] Snail also upregulates mesenchymal markers such as MMP-9 and fibronectin and downregulates epithelial markers in claudins and occludins.[31]Through migration and invasion assays, Furuta et al. demonstrated significant reduction of SCLC migration and invasion upon Snail downregulation, while DLL3 overexpression increased migration.[29] Snail as an inducer of EMT likely confers invasive properties to SCLC, however It has been shown in breast cancer that Snail can facilitate migration in an EMT- independent manner,[32] and the potential role for Snail in EMT- independent migration in SCLC[29] requires further study.

The role of NFIB in SCLC migration

The transcription factor Nuclear factor I/B (NFIB) was first recognised by Dooley et al. to possess oncogenic properties in SCLC,[33] and it has since been acknowledged as a driver of SCLC metastasis[34,35]. In murine models, NFIB is a physiological regulator of lung maturation and brain development.[36] NFIB is highly expressed in human SCLC,[33] especially in metastatic disease, and is correlated with shorter progression-free survival.[34]In human SCLC cell lines, NFIB binds to DNA and induces chromosome instability by creating a hyper accessible chromatin state, permitting increased expression of specific distal gene segments.[34] Interestingly, many genes upregulated by NFIB overexpression and NFIB-induced transcriptional accessibility are implicated in axon guidance and synaptic arrangement, in addition to cell-cell adhesion and motility.[34,37,38] While the neuronal gene expression pattern by NFIB is consistent with its physiological role in brain development, the expression of genes pertaining to developmental neuronal migration are likely also utilised to assist SCLC cells in their migration, a phenomenon that is also seen in the metastatic variants of other cancers.[34]Furthermore, NFIB downregulates E-cadherin,[35] and loss of E-cadherin is often associated with EMT, further implicating its role in invasion and migration.[39]

Selectin driven migration

SCLC expresses ligands for selectin, a cell adhesion molecule, and SCLC also contains selectin binding sites to interact with the endothelium, by which they undergo selectin-mediated migratory patterns similar to leukocyte migration during inflammation.[40-41] These selectin ligands include CEA, PSGL-1 and CD44, while the binding sites include sites for E-selectin and P-selectin.[40]Selectins including E- and P-selectin are key regulators of leukocyte adhesion and diapedesis.[42-43] In particular, SCLC cells adhere strongly to E-selectin, and were recorded by Richter et al. to roll along E-selectin coated microslides resembling the rolling pattern of leukocytes along endothelial linings expressing E-selectin.[41] In patient derived xenograft (PDX) models of SCLC, metastatic capacity is greatly reduced with knockout of E-/P-selectin.[40] It is thereby postulated that selectins can mediate SCLC metastasis by facilitating circulatory transport along the endothelium, mimicking leukocyte rolling patterns.

B1 integrin-mediated cancer cell migration

Integrins are another set of cell adhesion molecules implicated in solid tumour metastasis,[44] including SCLC.[45] Integrins are comprised of paired a and B subunits, which heterodimerise to form receptors for extracellular matrix (ECM) ligands,[45-47] and result in the assembly of the adhesome, a molecular network of scaffolding and signalling proteins.[46,48] In SCLC, the B1 integrin is highly expressed[49] and acts as a pro-metastatic oncoprotein.[45] The B1 integrin functions to recruit and activate kinases including the FAK and SRC kinases,[46] which are strongly complexed adhesome components.[50] SRC activates FAK,[51]causing disassembly of focal adhesions between the ECM and the cancer cells, triggering cell migration pathways.[52] The role of the B1 integrin in SCLC metastasis was confirmed on studies of human SCLC samples by Zhao et al., where late-stage disease was correlated with high B1 integrin expression and low expression of the CUL5 and SCOCS3 proteins, whereby CUL5 and SOCS3 are components of the E3 ubiquitin ligase that normally targets integrin B1 for degradation.[45]

Homing of metastatic SCLC

Secondary site colonisation serves as the final step of the metastatic cascade,[53] and while little is known about the mechanisms by which SCLC metastases preferentially home to their secondary organs, the molecules produced by SCLC can provide explanatory cues about the seeding patterns.[9]

The brain is a common site of SCLC metastasis, with up to 80% of patients experiencing brain metastasis through the course of disease despite treatment.[54] SCLC brain metastases highly express PLGF. PLGF secreted by SCLC facilitates activation of the Rho kinase, which activates ERK 1/2, leading to disassembly of tight junctions in the blood brain barrier (BBB), enabling seamless transendothelial migration of metastases into the brain. The expression of PLGF may explain the proclivity for SCLC to metastasise into the brain.[55]

The bone is another common site for SCLC metastasis, and the expression of the CXCR4 chemokine receptor by SCLC has been correlated with bone- specific metastasis in preclinical studies.

CXCR4 is highly expressed by SCLC, amongst other cancers, and binds to the CXCL12 ligand[56] which is secreted by osteoblasts of the bone endosteum.[57] The CXCL12-CXCR4 axis has been demonstrated to facilitate SCLC migration in vitro,[58] and Ma et al. demonstrated that CXCR4 knockout significantly reduced bone metastasis of an injected human SCLC cell line in murine models.[58] As such, the CXCL12-CXC4 axis potentially mediates a chemotactic migratory pattern of SCLC metastases towards the CXCL12 secreting bone, however this postulation pertaining to bone- specific metastasis still requires further in vivo study.

These examples ascribe to the 'seed and soil' theory, where organ- specific metastasis is strongly influenced by the compatibility between features of the cancer (seed) and the secondary organ (soil).[59]

New understandings of miRNA as potential therapeutic target in SCLC metastasis

While loss of function of P53 and RB are common to most SCLCs,[5] these major tumour suppressors are not classically amenable to therapeutic intervention, and there remains a constant need to identify more therapeutic targets.[60]microRNA (miRNA) targeting has recently been suggested as a possible therapeutic avenue in SCLC.[61] miRNA are short noncoding RNAs bearing translational control over mRNA, and they may be oncogenic or tumour suppressing depending on the genes they downregulate.[62] Specific miRNA involved in SCLC proliferation and metastasis is poorly understood. However, Khan et al. have recently identified the miR-1 miRNA as a tumour suppressor in SCLC, acting on the CXCR4/FOXM1/RRM2 axis to reduce proliferation and metastasis.[61]

Through RNA sequencing and a biosensing, Khan et al. identified significantly lower miR-1 in the tumour and serum samples of SCLC patients relative to healthy controls. In their studies, the authors used two main human SCLC lines: SBC3 (an miR-1 expressing cell line) and SBC5 (a cell line devoid of miR-1). Variations of the cell lines were also created whereby SBC3 was infected with lentivirus containing the miR-1Zip (an anti-miR-1), to induce SBC3-miR-1Zip: a miR-1-knockout version of SBC3. Furthermore, SBC5 was placed under the control of a Doxycycline inducible system, whereby Doxycycline could induce the expression of miR-1 to create an miR-1 overexpression version of SBC5 (SBC5-DOX-On-miR-1).

In vitro studies of these cell line variations suggested miR-1 expression to be strongly associated with decreased tumour growth and decreased migratory ability. These findings were translated in vivo, where SBC3-miR-1Zip and SBC5 cells injected into PDX mice models demonstrated significantly greater tumorigenesis and metastasis than SBC3 and SBC5-DOX-On-miR-1respectively, with metastasis sites commonly mimicking that of human SCLC metastasis patterns into the liver, brain, bone, and lungs. Overexpression of miR-1 on the other hand, appeared to diminish metastatic activity.

Subsequent RNA sequencing of each cell line variation noted CXCR4 to be the most upregulated gene in cells with low miR-1. RNA sequencing and a chromatin immunoprecipitation assay also identified the CXCR4, FOXM1 and RRM2 genes to be strongly co-expressed with downregulation of miR-1. As previously noted, CXCR4 is commonly upregulated in SCLC, and it is often involved in the metastatic behaviour of various cancers.[63] FOXM1 is a known transcription factor of RRM2, and RRM2 is often implicated in cancer aggression.[64] Through cell surface expression analysis, target scanning, and chromatin immunoprecipitation assays, Khan et al. deduced that miR-1 inhibits CXCR4 expression, which subsequently decreases FOXM1-

mediated RRM2 expression in SCLC. miR-1 was shown to inhibit expression of metastatic markers such as AKT, ERK and Snail. Expression analyses showed CXCR4, FOXM1 and RRM2 to be highly expressed in the liver metastases resected from SCLC murine xenografts. Altogether, they reasoned that miR-1 expression confers an inhibitory effect on the CXCR4/FOXM1/RRM2 axis, where the axis is otherwise heavily implicated in SCLC metastasis (Figure 1).

Due to its high plasticity and mutational burden, it has been difficult to establish therapeutic targets for SCLC.[65] CXCR4 antagonists are under study as promising means of attenuating SCLC metastasis,[58] and Khan et al. have provided new understanding of the miRNA miR-1 as a direct inhibitor of CXCR4 expression, with downstream inhibition of FOXM1/RRM2, leading to reduced SCLC metastasis. As such, there appears to be potential in creating therapies with activating or inducing effects on miR-1 to limit the metastatic capabilities in SCLC by targeting CXCR4 at the gene expression level.

It should be noted however, that a single miRNA can regulate several mRNAs,[62] and it is unknown whether therapeutic overexpression of miRNA can result in unintended effects. miR-1 has a known role in cardiomyocyte development,[66] and its overexpression has been shown to induce cardiac arrhythmias in murine models.[67] Therefore, cardiac safety profiling of potential miR-1-inducing therapy for SCLC would be required should miR-1 activating drugs be explored in clinical trials.

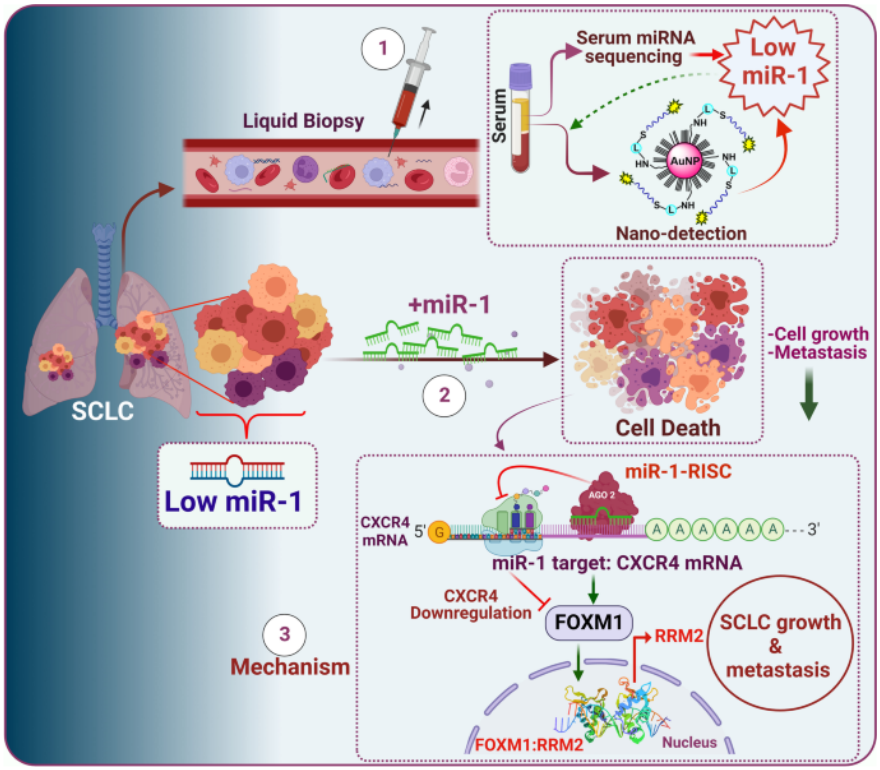

Figure 1: miR-1 downregulates CXCR4 transcription, resulting in decreased FOXM1-dependent RRM2 expression, thus limiting SCLC metastatic activity. Source: Khan et al.[61]

Open questions

Currently, in vivo studies of SCLC metastasis are largely confined to PDX models.[9] These mice models are immunodeficient, and do not replicate the tumour immune microenvironment.[68] Preclinical studies have identified tumour-infiltrating macrophages as proponents of SCLC liver metastasis[69] and natural killer cells as inhibitors of SCLC liver metastasis.[70] The immunodeficient PDX models do not capture the complex

interplay between the immune system and the metastatic activities of SCLC, and there is a great need to develop models that can overcome this experimental challenge.

As previously noted, the understanding of EMT in SCLC is still lacking. EMT is an evolutionarily conversed step in developmental biology,[71] and is often pivotal to tumour invasiveness and consequent metastasis.[72] While Notch signalling is a mediator of EMT in various cancers including NSCLC,[73] Notch appears to exert anti-EMT properties in SCLC[74] and a deeper understanding of SCLC- specific Notch signalling is required, should Notch be pursued as a therapeutic target.

Conclusion

The hallmark of Invasion and metastasis features greatly in SCLC. SCLC can drive metastasis aided by its vasculogenic properties, EMT capabilities, and its expression of migration-related factors. Furthermore, metastatic SCLC can migrate with organ-specific tropism. The prognosis of SCLC has seen little improvement over recent decades due to its recalcitrant and metastatic nature, presenting great challenge to the identification of new therapeutic targets.

Ethical statement

Not applicable

Conflicts of interest

The author declares no conflicts of interest.

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