Chapter

Targeting Leader Cells in Ovarian Cancer as an Effective Therapeutic Option

Nazanin Karimnia, Gwo-Yaw Ho, Andrew N. Stephens and Maree Bilandzic

Abstract

Majority of ovarian cancers are diagnosed at advanced stages with intra-peritoneal spread as the most common mode of disease metastasis. The formation of cancer spheroids is essential for the collective migration process, where shed tumour cells from the primary tumour form aggregates rather than disseminating as individual cells and seed within the peritoneal cavity. These cancer spheroids consist of leader cells (LC) and follower cells (FC), with the LC subset as key drivers of cellular movement and invasion. LCs have stem cell-like properties and are highly chemo-resistant with a specific survival addiction to several cell signalling pathways, such as the PI3K/AKT/mTOR pathway. We explore in this book chapter, the evidence supporting the role of LC in OC metastasis and the suppression of LC as an attractive therapeutic option for the treatment of advanced OC.

Keywords: Ovarian cancer, Leader Cells, KRT14, PI3K/AKT/mTOR, Collective migration

1. Introduction

1.1 The majority of ovarian cancers disseminate passively within the intraperitoneal space via ascitic fluid

The majority of ovarian cancers (OC), up to 70%, are diagnosed at advanced stages (stage III-IV) with intra-peritoneal spread as the most common mode of metastasis [1]. OC dissemination is often accompanied by the formation of ascitic fluid within the peritoneal cavity [2–4]. Under normal conditions, a small amount of fluid is secreted by the peritoneal capillaries into the cavity to lubricate the movement of abdominal organs which is normally re-absorbed by the lymphatic channels as a result of intrathoracic pressure [5]. However, in the presence of malignant cells, fluid can accumulate in large volumes in the peritoneum and facilitate passive cancer cell dissemination [6]. Whilst haematogenous spread may account for some ovarian tumour metastasis [7], it is largely the passive peritoneal dissemination of spheroids that results in ovarian cancer spread [8].

Prior to detachment from the primary tumour, OC cells are believed to exhibit a unique gene expression profile. This includes co-expression of both epithelial and mesenchymal markers and the acquisition of an epithelial-mesenchymal transition

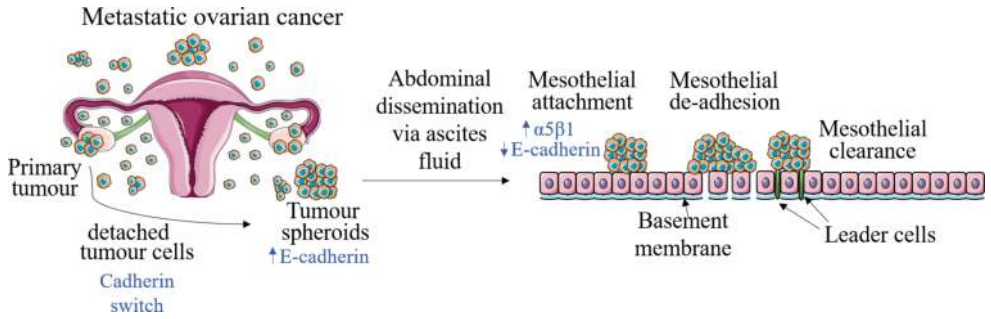

Figure 1.
Ovarian cancer passive mode of metastasis. Ovarian cancer cells from the primary tumour are exfoliated into the peritoneal cavity. Exfoliated cancer cells aggregate to form compact multicellular spheroids and disseminate within the peritoneal cavity, where single cells are subject to anoikis. Spheroids further attach to and invade the perineal lining by displacing the mesothelial cell layer in a process mediated by ovarian cancer leader cells.

(EMT)-like phenotype [9, 10]. The detached OC cells are then shed into the peritoneal cavity and simultaneously, E-cadherin expression is replaced by P-cadherin and N-cadherin, an event known as the global cadherin switch [11]. A fluctuation in E-cadherin levels is once again observed when detached cells form multicellular spheroids and E-cadherin levels are elevated [12], collectively demonstrating OC phenotypic plasticity is crucial for each step of the metastatic process [13].

1.2 OC spheroids play a key role in intra-peritoneal spread of malignant cells

Detached tumour cells from the primary tumour aggregate as spheroids in the ascites to overcome anoikis [2]. We believe that these cancer cell spheroids "floating" in the ascites are a key component in OC passive dissemination and play a pivotal role in both invasion and metastasis [6]. Furthermore, OC spheroids exhibit remarkable chemoresistance and progenitor-like properties [14, 15].

The mesothelial monolayer covering all of the abdominal organs is the initial point of contact for the disseminating spheroids during the metastatic process [16]. This layer lies on top of basement membrane, which is composed of collagen I, IV, laminin and fibronectin and contains a milieu of macrophages and fibroblasts populating the extracellular matrix (ECM) space [17–19]. It was observed that transcriptional reprogramming occurred within the floating spheroids which transformed tumour cells from a proliferative to an invasive phenotype to facilitate invasion through the mesothelium via the ECM [6]. Studies have shown that α5β1-integrin expression by spheroids binds fibronectin expressed by mesothelial cells and is critical for spheroid adhesion to the mesothelial lining [20–24]. However, multiple preclinical studies targeting individual integrin complexes failed to prevent the adhesion of spheroids to the peritoneum, hence the role of non-integrin-based adhesion molecules, such as CD44 and L1CAM, may be crucial to the spheroid adhesion process [25]. The attachment of OC spheroids to the peritoneum initiates the process of infiltration and invasion. The process of passive dissemination is illustrated in **Figure 1**.

2. Collective migration and leader cells

2.1 Collective migration occurs during epithelial cancer metastasis

During embryonic development, tissue homeostasis and also cancer invasion, cells migrate as multicellular clusters with a directed and coordinated movement – this

process is called collective migration [26]. Collective migration is characteristic of metastatic tumours in transit, particularly cancers with epithelial origin [27, 28] including pancreatic cancer [29], colon cancer [30], sebaceous cancer [31], melanoma [32], breast cancer [33–35], lung cancer [36] and OC [37, 38]. There are three key features that define the collective phenomenon; (i) the preservation of the physical connections and cell–cell junctions to orchestrate collective movement; (ii) the shared cytoskeletal dynamics within the cell clusters, allowing groups of cells to proceed as a single unit and maintain multicellular polarity; and (iii) the interactions with other cells and ECM along the migration path [26, 39, 40]. Interestingly, not all cells within the collective invading cell cluster are invasion competent [26] and it is now understood that the complex cohesive movement of collective invasion is orchestrated by a subset of cells called "leader cells" (LCs) [37, 41–44].

2.2 Cancer leader cells are the key drivers in cancer cell migration

The LCs have been well characterised in the context of collective migration in normal physiological events such as wound healing [41], nephric ducts growth [45], angiogenesis [46], and mammary branching [47]. More recently, cancer LCs have been identified in bladder [48–50], breast [34, 35, 51], prostate [50], pancreatic [52], small cell lung cancer (SCLC) [53], and now in metastatic OC [37]. These cells have a distinct front-rear polarity and membrane protrusions to sense environmental cues in order to direct the invading cluster [28, 54]. Studies have shown that within a collectively migrating cancer cluster the cancer LCs will be situated at the invasive front, followed by follower cells (FCs) in a packed morphology [28, 54, 55]. It has been shown that the removal of the LCs from an invading cluster of kidney epithelial cells results in the loss of orientation and speed in movement of the FCs - this highlighted the importance of LCs in the organisation of collective movement [44]. However, the dynamic interaction between the LCs and FCs is required to ensure the success of collective movement. Therefore, the FCs play a critical role in LCs polarisation, gradient sensing, and chemotaxis [54, 56, 57], and thus in return actively influence LC function.

2.3 Leader cells exhibit remarkable ability to alter their surrounding tissue micro-environment, which is crucial in their role as cell migration drivers

Within the collective migration process, LCs are able to lose or rearrange their baso-apical polarity during cellular elongation, while maintaining attachment to FCs by retaining molecular plasticity through the expression of epithelial markers such as *CDH1,* which encodes for E-cadherin [34, 55, 58]. LCs can mediate cytoskeletal organisation by displaying front-to-rear polarisation [28, 59]. Activation of phosphoinositide 3-kinase (PI3K) [60], GTPase proteins, cell division cycle 42 (Cdc42) and Ras-related C3 botulinum toxin substrate (Rac) [54] at the front of the spheroid induces actin polymerisation and integrin-based interactions with ECM components [61], while the expression of matrix metalloproteinases (MMPs) by LCs generates a track within the ECM and the basement membrane allowing for cell invasion into these spaces [62].

In the absence of a known LC marker, earlier studies have focused on the physical positioning of LCs within a collectively invading cluster to investigate the LCs profile. Carey et al., shed light on heterogeneous tumour subpopulations within 3D spheroids and showed different invasion and ECM remodelling capacities with LCs driving malignant protrusions [63]. Later, Yamaguchi et al., used the same approach and showed that by removing the LCs from a collectively invading cluster of epithelial kidney cells, the follower population movement lost direction [44].

This study further showed that LCs express high level of proteins involved in cell migration and polarisation, such as Rac, integrin β1 and PI3K [44]. Konen and colleagues established a novel image-guided manipulation technique to isolate the LCs from collectively invading lung cancer spheroids [64]. The spatiotemporal genomic and cellular analysis (SaGA) technique involved labelling cells within the spheroid with a green-to-red photoconvertible fluorescent protein. Invasive cells at the front were tagged with a laser beam which converted the fluorescence to red allowing the isolation of the invasive LCs by fluorescent activated cell sorting (FACS) [64, 65]. Using SaGA, transcriptomic analysis of lung cancer LCs identified 788 differentially expressed genes comparing LCs and FCs. Among them, genes involved in VEGF signalling, focal adhesion and RNA polymerase II transcription were significantly over-expressed in the LCs population [64]. The authors further demonstrated that although LC function was not dependent on VEGF signalling, it was necessary to drive the collective movement of FCs [64]. In SCLC, a distinctive mutation profile between LCs and FCs showed that mutations in the actin related protein-3 (*ARP3*) gene enhanced LCs function [53]. Further, introducing this mutation into the non-invasive follower population promoted invasion and collective movement [53].

2.4 Cancer LCs have stem cell-like phenotype

Cancer LCs play a critical role in early-stage invasion and tumour micrometastatic seeding [34, 35, 42, 66, 67]. Multiple studies investigating cancer micrometastasis in patient-derived-xenograft (PDX) models further characterised cancer LCs at a single cell level. A study by Lawson et al. analysing breast cancer PDX micrometastases by single cell sequencing demonstrated a distinct basal/stem-cell signature in early-stage metastatic cells [68]. This study demonstrated a distinctive molecular signature for low and high- burden metastatic tumours with elevated stem cell signatures and dormancy in low burden tumours and high proliferation and differentiation signatures in high-burden tumours [68]. Another study with the same approach for the analysis of breast cancer micrometastasis identified 330 differentially expressed genes. Among the genes significantly upregulated in the micrometastatic lesions were those encoding heat shock proteins HSPB1, HSPA8 and HSPE1 as well as cytokeratins KRT14, KRT16, KRT7 and KRT17 [69]. HSPB1 is involved in protein folding, apoptosis evasion and actin remodelling [70, 71], whereas KRT14 is a marker of invasion driving LCs in breast and ovarian cancer [34, 37]. This study also showed that mitochondrial oxidative phosphorylation (OXPHOS) was significantly up-regulated in metastatic cell seedings, suggesting a potential alternative metabolic pathway is utilised by the LCs to fuel the metastatic process [72–74].

2.5 KRT14 is a reliable dynamic cancer LC marker

KRT14 is a member of the intermediate filaments (IFs) and is generally expressed within the basal layer of epithelium to provide structural support [75]. In cancer cells, the direction of collective migration cell cluster movement and formation of protrusive structures are mediated via the interplay between the keratin IFs and cadherin [76]. Elevated expression of KRT14 has been identified in invasive LCs of breast [34], ovarian [37], bladder [49], and salivary adenoid cystic carcinoma (SACC) [77]. *In vitro* studies on KRT14 expressing LCs in OC demonstrated that spheroids generated from KRT14 depleted cells failed to maintain stable attachment with the mesothelial layer and to generate invasive protrusions [37]. RNA-sequencing revealed that the KRT14^{+} breast cancer LCs show a significantly higher level of DSG3, encoding a major desmosomal protein, as well as gene

expression signatures associated with cell and matrix adhesion [34]. Desmosomes play a critical role in maintaining cell–cell adhesion throughout the collective movement via intracellular connection of keratin filaments in neighbouring cells [78, 79]. However, the exact mechanisms of KRT14 involvement in driving collective invasion remains unknown. It was hypothesised that keratin IFs may regulate focal adhesions via intertwined interactions with the AKT and integrin/focal adhesion kinase (FAK) pathways [80–83]. More specifically, KRT14 has been shown to stabilise hemidesmosomes by regulating the levels of integrin β4 on the surface of keratinocytes [80]. Furthermore, KRT14 can mediate the phosphorylation of desmosomal cell junctions via PKCα, which is important in regulating epithelial cell adhesion [81, 82]. These results suggest that the KRT14 expression in LCs can be a determining factor to maintain the integrity of the collective movement via cell–cell and cell-matrix adhesion [54, 83].

Study		**Model**	**LC-specific signatures**
Yamaguchi et al. [43]	LCs isolated from invasive strands of a spheroid embedded in collagen matrix using a micromanipulator	Kidney epithelial cells	Rac Integrin β1 PI3K
Lawson et al. [66]	FACS-based isolation of single metastatic cells followed by Fluidigm dynamic array experiments identified signatures of micrometastases.	Breast cancer PDX model	Differentiation Proliferation Dormancy exit
Cheung et al. [33]	RNA-sequencing identified 239 DEGs comparing $KRT14^+$ LCs and $KRT14^-$ FCs	Breast cancer cells/ PDX model	ECM proteins Immune system regulators Cell–cell and cell-matrix adhesion Regulators of the metastatic niche
Konen et al. [62]	SAGA identification of 788 DEGs in the LCs isolated from a collectively invading spheroid model	Lung cancer	VEGF signalling Focal adhesion molecules RNA polymerase II transcription
Sonzogni et al. [50]	RNA sequencing and secretome analysis of $KRT14^+$ LCs and $KRT14^-$ FCs	Breast cancer	Pro-metastatic genes Matrix adhesion
Zoeller et al. [52]	SAGA identification of genomic and transcriptomic signatures for LCs via parallel mutation and RNA-seq analysis	NSCLC	collective movement Actin filament proteins Mitochondrial enzymes
Davis et al. [67]	Single-cell RNA sequencing of micrometastases compared to the primary tumour.	Breast cancer / PDX model	Heat shock proteins Cytokeratins OXPHOS Mitochondrial electron transport Mitochondrial ribosomal genes

Table 1.
Summary of studies investigating LCs profile.

2.6 KRT14 positive cells are linked to LC with distinct gene expression profile

Transcriptome analysis of the KRT14 expressing LCs in breast cancer by RNA sequencing identified 239 differentially expressed signatures between the $KRT14^+$ LCs and the $KRT14^-$ FC population. Gene ontology (GO) analyses, revealed that the expression of genes encoding ECM proteins, intermediate filaments, cytoskeleton organisation, and cell adhesion were significantly elevated in LCs compared to the FC population [34]. Interestingly, this study demonstrated that the LC subset is not a fixed lineage, however, the mechanisms regulating the interconversion of LCs and FCs remains unclear [34]. Recent studies suggest that the behaviour of breast cancer LCs can be mediated by CD44 expression levels where a high level of expression induces a shift towards an invasive LC phenotype [84]. Sonzogni et al. showed that KRT14 expressing LCs have a significantly higher expression of genes involved in metastasis progression including metallothionein-2 (*Mt2*), glycoprotein non-metastatic B (*Gpnmb*), and adhesion molecule Amigo2, and secrete significantly higher levels of the collagen VI subunit A (*Col6a1*) [51]. In bladder cancer, stem-like $KRT14^+$ cells gave rise to differentiated cells and were shown to be necessary for epithelial layer establishment following tissue damage [49]. A summary of studies and pathways involved in LC function is provided in **Table 1**.

2.7 LCs are implicated in OC metastasis and invasion

We have recently identified the OC LCs [37]. A study using spheroid-mesothelium co-culture model was utilised to identify molecules that were specifically expressed at the early stages of invasion via matrix-assisted laser desorption/ionisation (MALDI) tissue imaging. Among the identified proteins, KRT14 was shown to mark the invading cells universally across the different subtypes of EOC, while KRT14 expression was absent from the normal ovarian and fallopian tube tissue [37]. This study confirmed that cells lacking KRT14 proliferate at the same rate as the WT cells, however, demonstrate significantly impaired migration and matrix-adhesion [37]. These results suggest the explicit role of LCs in invasion and metastasis in OC.

3. Novel OC therapeutic approach by targeting the collectively migrating cell population

3.1 Collectively migrating cell clusters may be targeted to reduce cancer spread

Current cancer therapies are mainly evaluated by cytotoxicity and their effect on tumour shrinkage; however, bulk tumour regression is not the only factor in effective cancer therapies [85]. In OC, the majority of patients are diagnosed with metastatic disease which is associated with a significantly poorer prognosis, hence strategies to interrupt metastasis through the disruption of cell motility, collective movement, directed cell migration and invasion have gained interest [86]. Targeting the cytoskeletal stability through actin is one such approach that has shown inhibitory effects on invadopodia formation and outgrowth in lung [87, 88], melanoma [88] and prostate [89] cancers. Unfortunately, these drugs are usually associated with significant toxicities due to the lack of discriminative drug effects between the malignant and healthy cells [88, 89]. Targeting other processes involved in actin polymerisation such as Rho GTPases and RhoA/Rho-associated kinase (ROCK) signalling pathway is potentially beneficial since the cytoskeletal dynamics play an important role during invasion and metastasis of a collectively

invading cluster [90, 91]. However, cancer cells generally are able to establish alternative mechanisms to bypass these targets leading to early drug resistance [92].

3.2 Targeting LCs within the collectively migrating cluster may be a better therapeutic option for the treatment of OC

As highlighted earlier, the molecular features of LCs are cancer-specific and this represents a challenge for developing clinically relevant therapies against LCs. Despite this, multiple targets have emerged from LCs studies (listed in **Table 1**: Summary of studies investigating LCs profile). These include targeting the LC stimulatory pathways such as the PI3K/mTOR pathway (with tyrosine kinase inhibitors and Ivermectin), metabolic/energy pathways (statins, cardiac glycosides and metformin) and inflammatory pathways (non-steroidal anti-inflammatory drugs).

3.3 Disrupting the PI3K/AKT/mTOR pathway is an attractive therapeutic strategy to inhibit LCs

There is an enrichment of LCs observed in late-stage OC associated with the up-regulation of the PI3K/AKT/mTOR pathway [37, 93]. Yamaguchi et al.'s study revealed the up-regulation of PI3K in kidney epithelial LCs [44] implicating this pathway as a potential target for LC inhibition. The PI3K/AKT/mTOR signalling pathway mediates major cellular events such as growth, motility, metabolism, and survival [94].

PI3Ks are a group of membrane-associated kinases that form heterodimeric structures comprised of regulatory and catalytic subunits classified based on their structure, regulation and substrates [95]. Class I PI3Ks are hugely implicated in cancer and are comprised of a p85 regulatory and a p110 catalytic subunit [96]. The catalytic subunit in class IA has three variants including p110α, p110β, and p110δ encoded by *PIK3CA*, *PIK3CB* and *PIK3CD* respectively, whilst the catalytic subunit of the only class IB PI3K, p110γ, is produced from *PIK3CG* gene [96]. Class IA PI3Ks are activated via ligand binding to receptor tyrosine kinases (RTKs), while activation IB PI3Ks is mediated by G-protein-coupled receptors (GCPRs) [97]. Upon ligand binding, activated PI3Ks catalyse phosphorylation of phosphatidylinositol (PtdIns) [4, 5] P2 (PIP2) to produce PtdIns [3–5] P3 (PIP3), an event that is inhibited by the tumour suppressor Phosphatase and tensin homologue (PTEN) in normal cells [94]. Following PIP2 to PIP3 conversion, proteins with a PH domain are recruited to the plasma membrane to activate downstream signalling proteins such as AKT, triggering multiple downstream pathways regulating survival, growth and invasion [94, 98]. AKT, also known as protein kinase B (PKB) is the main effector of PI3K and other than direct activation by PI3K, can be activated indirectly by mTOR and phosphoinositide-dependent kinase-1 (PDK1) that phosphorylate AKT at Ser 473 and Tyr 308 residues, respectively [99–101]. A schematic overview of the PI3K/AKT/mTOR pathway is demonstrated in (**Figure 2**).

3.4 Dual PI3K/mTOR kinase inhibitors may be required to effectively suppress OC leader cells

Activation of PI3K/AKT/mTOR pathway is frequently observed in oncogenic events contributing to tumour development, metastasis and therapy resistance [98] and irregularities in the PI3K/AKT/mTOR pathway corresponds with a poor prognosis in OC patients [99, 102, 103]. Activating mutations and genomic amplification of *PIK3CA* [104] and AKT and mTOR are more prevalent in women with

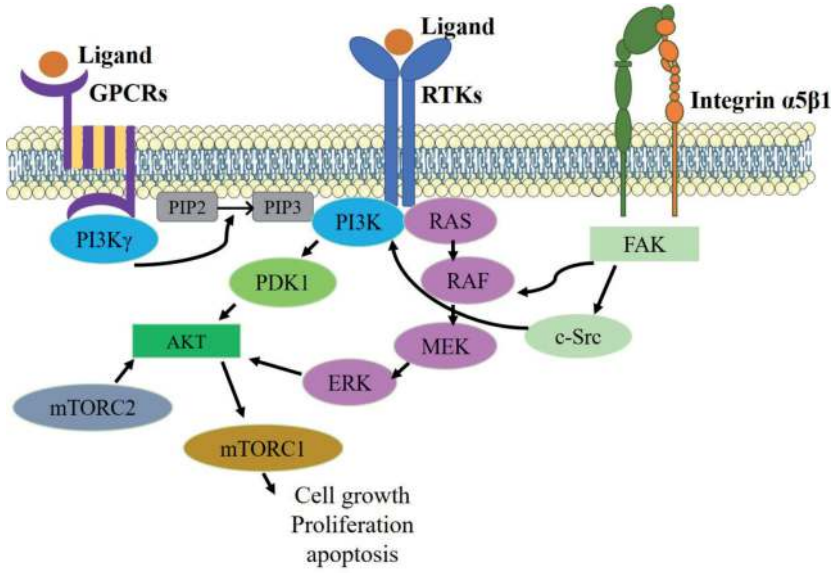

Figure 2.
Overview of the PI3K/AKT/mTOR pathway. Class IA PI3Ks are activated via ligand binding of receptor tyrosine kinases (RTKs), while class IB PI3Ks depend on G protein-coupled receptor (GPCRs) activation. Activated PI3K facilitates the conversion of PIP2 to PIP3 and in turn induces AKT phosphorylation. Activated AKT mediates the phosphorylation mTOR and a signalling cascade that drives cellular proliferation and cell death. In concert, the RAS/RAF/MEK/ERK pathway is activated by RTKs, acting as an escape mechanism for PI3K inhibition. The focal adhesion kinase (FAK) pathway also feeds into the PI3K pathway through c-Src activated by integrin-based adhesion molecules including integrin α5β1.

clear cell ovarian carcinoma and associated with drug resistance phenotype [101]. Importantly, pharmaceutical inhibition of the PI3K/AKT/mTOR pathway was shown to increase *in vitro* sensitivity of OC cell lines to multiple chemotherapy agents [105, 106]. Moreover, PI3K inhibition via LY294002 disrupted the directional movement of kidney LCs [44], further highlighting the importance of the PI3K pathway for LC function. Inhibition of PI3K/AKT/mTOR pathway can be achieved via pan or isoform specific PI3K inhibitors, AKT inhibitors or dual pan PI3K/mTOR inhibitors [107–109]. However, PI3K/AKT/mTOR inhibition as a therapeutic option can be challenging due to the potential toxicities compounded by the activation of compensatory pathways and enhanced insulin production upon inhibition of PI3K [94, 98, 100, 101, 104, 110]. Currently, the PI3K inhibitor idelalisib and the mTOR inhibitor everolimus have gained FDA approval for the treatment of lymphoma [111] and renal cancer [112], respectively. Unfortunately, the clinical use of single agent inhibitors has shown minimal efficacy and high toxicities in treatment of OC [113–115].

The PI3K/AKT/mTOR pathway is interconnected with other signalling pathways including focal adhesion kinases [116] and RAS/RAF/MEK/ERK [117]. There are multiple canonical and non-canonical crosslinked pathways that could bypass single protein inhibition resulting in therapeutic failure. Therefore, targeting the pathway cascade at multiple levels via dual PI3K/mTOR inhibitors, might circumvent the negative feedback loops that occur with single target inhibitors [118]. Pre-clinical data from the PI3K/mTOR dual inhibitors omipalisib (GSK2126458), CMG002 and BEZ235 have indicated effective inhibition of ovarian cancer tumour growth and progression *in vitro* and *in vivo* [93, 106, 119, 120]. Currently, there are no ongoing clinical trials investigating the efficacy of dual inhibitors in OC patients mainly due to toxicity and off target effects of the dual inhibitors in clinical setting [121].

3.5 Anti-helminth, Ivermectin, may be effective in sensitising OC LCs to chemotherapy by disrupting the AKT/mTOR pathway

Ivermectin belongs to a family of drugs widely used to treat parasites and pest insects [122]. The anti-cancer property of ivermectin can be related to the inhibition of the Pgp pumps and MDR protein expression [123], inhibition of AKT/mTOR pathway [124], and targeting the yes-associated protein 1 (YAP1) [125], all of which are involved in the OC tumorigenesis [100, 126–128]. *In vivo,* ivermectin treatment of a xenograft mouse model of EOC showed a significant reduction in tumour growth and a reversal in tumour growth without severe toxicity effects when the drug was combined with cisplatin [129]. Currently there is a phase II clinical trial in recruitment to study the long-term effect of ivermectin treatment (NCT02366884).

3.6 The mevalonate pathway in LC can be potentially targeted with HMG-CoA inhibitors

Statins are among the most commonly prescribed medications to reduce cholesterol and inflammation through blocking 3-hydroxy-3-methyl-glutaryl-CoA (HMG-CoA) reductase [130]. Inhibiting the mevalonate pathway can have a protective effect against cancer progression and reduce LC activity [131, 132]. Furthermore, the mevalonate pathway has been shown to be significantly activated in *TP53* mutated cells [133]. Therapeutic effects of statins in OC are further supported by the *in vitro* studies showing anti-metastatic and anti-tumorigenic effects through the inhibition of MAPK and mTOR pathways [134]. Lovastatin significantly reduced the development of serous tubal intraepithelial carcinomas, the purported precursor ovarian cancer lesions, in mice through the inhibition of the mevalonate pathway and dysregulation of the Rho signalling pathway [135]. Currently, a phase III clinical study for evaluating the safety, tolerability and effects on tumour progression of Atorvastatin is at the recruitment stage for ovarian and pancreatic cancer patients (NCT 02201381).

3.7 Cardiac glycosides, such as digoxin, may be able to suppress LC population

Cardiac glycosidases (CGs) are a family of drugs used for the treatment of congestive heart failure and cardiac arrhythmia by regulating cardiac muscle contraction through the inhibition of the NA^+-K^+-ATPase pump [136]. The first anti-proliferative effects of CGs were reported more than five decades ago in HeLa cells [137] and since then, multiple studies have highlighted the anti-neoplastic effects of CGs by inducing cancer cell apoptosis [138], activating autophagic cell death through the Ras-dependent extracellular signal-regulated kinase (ERK1/2) pathway [139], inhibiting hypoxia-inducible factor-1 alpha (HIF-1α) protein synthesis [140] and inhibiting FA/BRCA pathway activation [141]. CGs have been shown to have a higher cytotoxicity effect when combined with chemotherapy in prostate, breast, non-small cell lung, colorectal, and pancreatic cell lines as well as advanced stage melanoma patients compared to single agents [141–144]. However, so far epidemiological studies have yielded inconsistent results. For example, while digoxin was found to inhibit tumour growth *in vitro* and was associated with a 25% lower prostate cancer risk [145], systematic review and meta-analyses indicated an increased prostate cancer risk in digoxin users [146]. Nevertheless, the number of clinical trials specifically designed for cancer patients being treated with CGs is very limited and most of these conflicting results come from re-analysing data present in the medical databases with limited numbers of patients. So far, there are no clinical

trials designed to investigate the relationship between CGs and OC. Despite this, there is a recent study retrospectively analysing the Surveillance, Epidemiology, and End Results (SEER) program, the national cancer institute (NCI), and Medicare healthcare claim record data to assess whether digoxin use enhances chemotherapeutic responses in OC treatment [147]. The study suggested that digoxin use during chemotherapy did not have any survival benefits in patients with EOC, however, the research was limited by small sample size. Furthermore, 46% of the patients had a prior history of heart disease complicating the interpretation of subject fatality rates. More importantly, only 7% of the studied population were treated with digoxin during chemotherapy which may describe the opposing results with other cancer types. Since cardiac glycosidases regulate ion transport via the NA^+/K^+-ATPase, they interact with a wide variety of the intracellular signalling pathways, including those driving cellular proliferation and apoptosis [148], therefore, future clinical trials specifically designed for OC patients is highly expected. Our laboratory drug screening pipeline used to identify therapies against LCs has identified digoxin as a potent LC inhibitor, demonstrating synergistic effects when sublethal concentrations of digoxin were combined with platinum-based chemotherapies (result not published).

3.8 Metformin is a potential LC targeting agent by suppressing the AMPK pathway

Metformin is an anti-diabetic drug reducing blood glucose and insulin levels through activation of adenosine monophosphate-activated protein kinase (AMPK) to inhibit gluconeogenesis in the liver [149]. In cancer cells, AMPK activation results in mTOR pathway inhibition and therefore inhibition of cell proliferation [150]. So far, several epidemiological studies focusing on ovarian cancer patients with type 2 diabetes who were taking metformin at the time of diagnosis showed that these patients had a significantly improved 5-year survival rate compared to those who did not take metformin [151, 152]. Currently, there are multiple clinical trials submitted in the national institute of health (NIH) clinical trial database focusing on non-diabetic ovarian cancer patients being treated with a combination of metformin and first line chemotherapy. The results from one of the completed phase II studies (NCT01579812) showed that the tumours in women treated with metformin had a significantly fewer $ALDH1^+$ cells representing OC stem cells [153], therefore, supporting the use of this drug in the next phase of clinical trials. Furthermore, investigations in our lab evaluated the effect of sitagliptin, a drug used for the treatment of type 2 diabetes, in a murine model of ovarian cancer showing that sitagliptin enhanced the immune response via T cell recruitment to the tumour and inhibited several pro-tumorigenic cytokines, therefore reducing tumour burden and improving survival [154].

3.9 Non-steroidal anti-inflammatory drugs are potent cytotoxic LC inhibitors

Non-steroidal anti-inflammatory drugs (NSAIDs), such as aspirin, diclofenac and celecoxib are mainly prescribed to reduce pain, fever and inflammation [155]. Inflammation has a key role in cancer development and progression, therefore, NSAIDs have been shown to exhibit protective roles against this disease [156]. This effect is mediated through the inhibition of cyclooxygenase-1 and 2 (COX-1,2) enzymes inhibiting prostaglandin (PG) synthesis [157]. While constitutive expression of COX-1 regulates tissue homeostasis through PG synthesis, COX-2 is not expressed in normal epithelial tissues and is only induced during inflammation.

In addition, this marker is found to be overexpressed in epithelial tumours [158]. COX-2 inhibition eventually leads to the induction of apoptosis and the inhibition of tumour invasion [159]. The action of NSAIDs has been further linked to PI3K signalling pathway [160, 161] and the inhibition of NF_KB that leads to dysregulation of the genes involved in cancer progression and apoptosis [162]. The benefit of NSAIDs in cancer prevention and treatment remains controversial and tumour type dependant [156]. Re-assessing case–control and cohort studies from 1950 to 2011, that reported associations between aspirin uptake and cancer, showed that cancer prevention becomes significant only when the aspirin usage proceeds 5 years [162] and in this case, the overall benefit from the long-term use of NSAIDs was compromised by side-effects, such as gastrointestinal bleeding [163–165]. *In vitro* investigation of a panel of NSAIDs in ovarian cancer, showed significant apoptosis induction and reduced tumour growth in four cell lines treated with diclofenac [166]. Moreover, *in vivo* evaluation of diclofenac in mice implanted with ovarian cancer cells, showed significantly smaller tumours formed in diclofenac-treated animals compared to the control group [166, 167]. In line with this data, the drug screening platform established in our laboratory also identified diclofenac as a potent cytotoxic LC inhibitor. However, despite the growing body of evidence regarding the anti-neoplastic effects of diclofenac in OC, currently there are no clinical trials evaluating the effectiveness of this drug in patients. A phase II clinical trial to examine the effect of celecoxib treatment in combination with carboplatin in recurrent resistant ovarian cancer patients has shown promising results with a 28% RR and PFS [168], however this study did not provide any evidence of COX-2 inhibition in patients after treatment. Likewise, a phase II investigation of celecoxib plus carboplatin and docetaxel as a first-line treatment for ovarian cancer failed to demonstrate COX-2 inhibition with 82% of patients expressing COX-2 and no improvement in PFS or OS observed [169]. Furthermore, two systematic analyses on the effect of NSAID use and OC risk on big cohorts of patients failed to show such an association [170, 171]. However, both studies have indeed critical limitations with regards to the cancer subtypes, type of NSAIDs used, drug doses and the duration of treatments.

4. Conclusion

Despite the introduction of several novel therapeutics that include targeting DNA repair pathways with Poly (ADP-ribose) polymerase (PARP) inhibitors (PARPi), and vascular endothelial growth factor (VEGF) pathways with bevacizumab, the overall survival outcome for women with platinum-resistant OC remains poor. Unfortunately, women with advanced metastatic OC will eventually succumb to their disease due to the emergence of drug resistance. Understanding the mechanisms of OC migration and metastasis is crucial for the development of an effective therapeutic approach. Targeting the OC LC population serves as an attractive strategy given LCs are instrumental in orchestrating OC spread within the intra-peritoneal cavity. LCs are often highly chemo-resistant due to their stem cell-like nature and their survival post cytotoxic chemotherapy treatment may lead to therapy resistance and tumour recurrence. Multiple potential targets have been identified based on the understanding of LC biology, some of which may be targeted by re-proposing established drugs, such as dual PI3K/mTOR inhibitors, anti-helminths, statins, NSAIDs and metformin. Suppressing and eliminating LCs may be an effective therapeutic option for management of this lethal disease and is worth further exploration.

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