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Lung Cancer Stem Cells

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Lung Cancer Stem Cells: Relevance for Disease and Phytotherapeutic Opportunities

Heng Win Sen

Heng, W.S.

Lung Cancer Stem Cells: Relevance for Disease and Phytotherapeutic Opportunities

Thesis, University of Groningen, Groningen, The Netherlands

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Chapter 1

General Introduction and thesis outline

A brief history of lung cancer: from unknown to well-known

Lung cancer is a collective term to describe malignancies comprising of tumors arising in the trachea, bronchi, bronchioles, and alveoli. The first clinical report in 1761 associating cancer with the use of tobacco products, highlighted the importance of chemical agents as carcinogens in the development of cancer¹. This was recognized in lung cancer etiology after realizing sharp increases in incidence in parallel with increasing smoking activity². Almost 200 years ago, lung cancer was not very common with only 1% of incidence recorded out of all cancer². In the early 20th century, lung cancer was still so rare that it took 17 years to witness two successive cases³. However, the third case and subsequent seven cases were seen in only the next six months likely related to the increased prevalence of smoking activity. In the last decades, the disease has worsened to the extent that this disease recorded a nearly two million worldwide deaths annually⁴. Today, lung cancer is one of the most fearful diseases being one of the most common causes of death, together with cardiovascular, respiratory, and neurodegenerative diseases⁵.

Risk factors of lung cancer

As mentioned above, the occurrence of lung cancer was presumed to be associated with carcinogens that were found in cigarettes⁶. Besides smoking behavior, Witschi discussed that lung cancer was also associated with miners and should be considered as an occupational disease due to the high incidence of the disease among the miners². The mining cave was filled with a high concentration of radon gas, a known lung cancer risk factor today. Similarly, inadequate ventilation in uranium mines also led to lung cancer-related deaths. These occupational threats along with other industrial pollutants such as asbestos, chromium (VI), and silica that are released to the environment that lacks adequate ventilation constitute the environmental risk factors for lung cancer⁷. Behavioral patterns that prefer consumption of processed meat or red meat especially those being prepared fried, might increase the risk of lung cancer^{8,9}. In addition, a family history of lung cancer and certain genetic polymorphisms were found to genetically predispose individuals to develop lung cancer⁷. These polymorphisms occur in coding genes including tumor protein p63 (TP63), telomerase reverse transcriptase (TERT), and DNA repair protein RAD52 among others⁷.

Statistics and prevalence of lung cancer

Lung cancer's incidence and mortality are continuing on the rise as depicted in Figure 1. In 2008, the worldwide estimated numbers of incidence and death were 1.6 million and 1.4 million, respectively¹⁰. In 2012, the number rose to 1.8 million and 1.6 million, respectively¹¹. The 2015's report estimated an annual figure of 2 million incidences and 1.7 million deaths¹². The report in 2018 predicted a total of 2.1 million new cases and 1.8 million deaths in that year¹³. The most recent report estimated a figure of 2.2 million new diagnoses and 1.8 million deaths in 2020⁴. With this number, new lung cancer diagnosis represents 11.4% of total cancer cases and 18% of all cancer-related deaths, and thus far, it remains to be the second most commonly diagnosed cancer after female breast cancer and the leading cause of cancer-related death worldwide.

Figure 1. Lung cancer incidence and mortality of the last decade based on recent five reports^{4,10–13}. The increasing trend of both incidence and mortality is still at an alarming rate, therefore the effort to address management of disease and prevention needs to be improved.

The basis of diagnosis of lung cancer: histology, cytology, and molecular characteristics

Lung cancer is mainly categorized into two histological types, namely non-small cell lung carcinoma (NSCLC) and small cell lung carcinoma (SCLC) which account for 85% and 15% of total lung cancer cases, respectively. NSCLC is further categorized into three major subtypes consisting of adenocarcinoma (ADC), squamous cell carcinoma (SCC), and NSCLC-not otherwise specified (NOS)^{14,15}. Whether they are derived from pathological classifications based upon resection, small biopsies or cytological specimens, these histological stratifications help medical practitioners to prescribe appropriate therapy for patients. NSCLCs are identified from light microscopy based on their size and respective morphologies. ADC usually displays glandular differentiation with acinar, lepidic, micropapillary, papillary, or solid architectural features¹⁶. Its tumor cells usually have clear basophilic cytoplasm with nuclei located irregularly and typically having a single macronucleolus¹⁶. Squamous differentiation is distinguished by its keratinization, pearls, and intercellular bridge features¹⁶. Its tumor cells comparatively have more opaque cytoplasm with centrally located nuclei and less developed nucleoli¹⁶. Immunoreactivity to either transcription termination factor 1 (TTF1) or p40 that favors ADC or SCC, respectively can be utilized to ascertain the diagnosis decision for poorly differentiated tumor¹⁷. Alternatively, napsin-A for ADC and cytokeratin (KRT) 5/6 and p63 for SCC can be used to distinguish these histology subtypes, although their usability is at a lesser extent¹⁷. If glandular and squamous differentiation are absent together with the negative immunoreactivity of the aforementioned biomarkers, the tumor is classified as NSCLC-NOS¹⁸.

SCLC and large cell neuroendocrine carcinoma (LCNEC) are high-grade neuroendocrine (NE) tumors that are characterized by high mitotic activity and extensive necrosis¹⁸. LCNEC can be distinguished from SCLC by its prominent nucleoli and more abundant cytoplasm. When NSCLC diagnosis has immunoreactivity to NE markers such as synaptophysin (SYP), chromogranin A (CHGA), and neural cell adhesion molecule (NCAM/CD56), LCNEC should be concluded as diagnosis. SCLC is determined by light microscopy by its small cell size, scant cytoplasm, and absence of nucleoli¹⁹. SCLC can be both

NE markers positive (classic) and negative (variant) although such categorization has not yet had clinical impact²⁰.

A number of genetic alterations associated with NSCLC, are targetable with available therapies. Hence, molecular testing to identify the presence of specific genetic alterations is an important part of disease management. Mutations are often detected in specific receptor tyrosine kinases (RTKs) resulting in aberrant signal transduction in cells that induce and drive pro-tumorigenic phenotypes. For example, in ADC, mutations for epidermal growth factor receptor gene (*EGFR*), *ALK* receptor tyrosine kinase, erb-b2 receptor tyrosine kinase 2 (*ERBB2*), B-Raf proto-oncogene (*BRAF*), ROS proto-oncogene 1 (*ROS1*), and neurotrophic tropomyosin-related kinase (*NTRK*) family members, recurrently occur for which targeted therapies are available that improve patient prognoses²¹.

Lung cancer therapies, their efficacy in clinics, and their limitations

Today, there are extensive choices of therapy available for lung cancer patients. Depending on the status of the disease based on the tumor, node, and metastasis (TNM) staging, histological subtyping, and presence of treatable mutations, the appropriate treatment is chosen from the Food and Drug Administration (FDA)-approved drugs list. For early-stage disease (stage I–II) of NSCLC, surgical resection is the standard care and preferred treatment option. When surgery is not an option, conventional or stereotactic body radiotherapy (SBRT) may be employed^{22,23}. For stage II–III NSCLC, patients may opt for surgery followed by adjuvant chemotherapy²³. For an unresectable locally-advanced disease (stage III), concurrent/sequential chemo-radiotherapy is recommended, but when not feasible, it should be treated as stage IV disease²³. Adjuvant immunotherapy following concurrent chemoradiotherapy has resulted in improved survival rates for stage III NSCLC patients and is currently standard of care²⁴. NSCLC patients with advanced disease (stage IV) can receive systemic treatment such as chemo-immunotherapy (when an actionable oncogenic target is absent), targeted therapy (when actionable oncogenic target, also known as driver mutation, is present), or immune monotherapy^{22,25}. SCLC patients with limited disease-stage (LS) are usually treated with concurrent chemo-radiotherapy^{26,27}. When SCLC is detected upon surgery (as an incidental finding) then patients should be treated systemically with chemotherapy after surgery²⁷. In contrast, patients with extensive-stage (ES, stage IV) SCLC are usually treated with chemotherapy^{26,27}.

When a driver mutation is absent in ADC as well as in patients with SCC, the anti-programmed cell death 1 receptor (PDCD1, also known as PD1) monoclonal antibody pembrolizumab is the usual treatment when programmed cell death ligand 1 (PD-L1, also known as CD274) is detected for $\geq 50\%$ on tumor cells in diagnostic biopsies^{22,25}. If PD-L1 $< 50\%$, pembrolizumab plus pemetrexed and a platinum-based chemotherapy are used as standard therapy in ADC without drivers²⁵. For SCC with PD-L1 $< 50\%$, pemetrexed is replaced with paclitaxel or albumin-bound paclitaxel (nab-P)^{22,25}. Anti-PDL1 atezolizumab may be considered as an alternative first line immunotherapy and be used with nab-P in SCC²⁵. Atezolizumab can also be used in combination with pemetrexed and platinum-based chemotherapy or bevacizumab plus carboplatin and paclitaxel in ADC^{25,28}.

Genetic alterations in *EGFR* and *ALK* genes are by far the most successfully targeted oncogenic drivers with tyrosine kinase inhibitors (TKIs). First line TKIs standard options for *EGFR* mutations are osimertinib, erlotinib, gefitinib, and afatinib, whereas, for example, crizotinib, or alectinib can be used for ADC patients with rearranged *ALK*²⁵. When dealing with a rare or complex genomic alterations in patients, multidisciplinary molecular tumor boards (MTBs) should be consulted to yield useful recommendations for treatment. For instance, the MTB in the University Medical Center Groningen (UMCG) involves pulmonary oncologists, medical oncologists, pathologists, clinical scientists in molecular pathology, and structural biologists to generate effective treatment decision through a series of molecular assessments²⁹.

For SCLCs, the available treatment option has not improved as much as for NSCLC. Carboplatin, cisplatin, and etoposide are the only few chemotherapeutic drugs being used as standard care^{26,27}. However, a recent study of incorporating atezolizumab into first line treatment along with carboplatin and etoposide significantly improved the overall survival (OS) of patients compared to using only a combination of carboplatin and etoposide³⁰. Another study also suggested the improvement of OS by the addition of another PD-L1 inhibitor, durvalumab, to the platinum and etoposide regimen³¹. These favorable observations might lead to a change of standard care with carboplatin/etoposide of SCLC patients to also include PD-L1 inhibitors^{26,27}.

Despite the great value of standard cytotoxic therapies and the more recent successes of the available targeted therapies, treatments still face resistance problems in addition to toxicity complications. For example, widespread resistance is seen in patients treated with chemotherapeutic drugs³². Due to the mechanism of action of chemotherapeutic drugs, these drugs are associated with all sorts of side effects and toxicity symptoms^{33,34}. Evolving resistance to TKIs such as for example those targeting *EGFR* in NSCLC hamper durable treatment responses and underlying mechanisms have been identified involving the occurrence of novel mutations in *EGFR* or downstream pathways, which on their turn also provide targets for therapy to improve responses³⁵. The more recent successes in the clinic with the immune-checkpoint inhibitors are still associated with certain side-effects, such as colitis and diarrhea³⁶. Some patients with co-morbidities (especially auto-immune) are in serious consideration whether to receive such therapy³⁷. Moreover, those who qualify to receive immune-checkpoint inhibitors do not all respond to the treatment. Further stratification is still needed to optimize therapeutic outcome³⁸.

Cancer stem cells

An old theory postulating that malignancy arises from proliferative tissue-specific stem cells is re-emerging³⁹. The theory proposes that a small subpopulation termed cancer stem cell (CSCs) is responsible for tumor initiation, maintenance, and perpetuation. Such population is very similar to the normal stem cell that possesses higher potency to proliferate and differentiate as compared to a terminally differentiated functional cell in analogy to the majority cells in tumor bulk. Due to their versatility, CSCs are deemed to be the main culprit for therapeutic resistance, tumor recurrence, and metastasis.

As mentioned above, CSCs share similarities with normal stem cells in their characteristics. There is evidence for the existence of CSCs in lung cancer based on enriching

cell populations expressing stem cell markers and demonstrating their relevance in therapeutic resistance as the root for disease relapsing and progression. For example, Herreros-Pomares *et al.* found that tumorspheres from resected NSCLC patients were enriched with cells exhibiting stem cell properties, including self-renewal ability, resistance to chemotherapies, invasion and differentiation capacities *in vitro*, higher *in vivo* tumorigenicity, and higher expression of independent prognostic biomarkers such as cyclin-dependent kinase inhibitor 1 (CDKN1A), integrin alpha-6 (ITGA6), and zinc finger protein SNAI1⁴⁰. Another study provided evidence by enriching the putative CSC population with a triple-positive expression of epithelial cell adhesion molecule (EPCAM), CD166, and CD44 from NSCLC cell line A549. When compared to the triple-negative population, these cells exhibited self-renewal capability, migration ability, aldehyde dehydrogenase (ALDH) activity, higher proliferative activity, higher clonogenicity, higher resistance to chemotherapies, and higher expression of stem cell markers such as reduced expression protein 1 (REX1) and stage-specific embryonic antigen 4 (SSEA4)⁴¹. In another study, cisplatin-pretreated NSCLC cell lines demonstrated CSC-like behavior such as increased colony formation and sphere-forming abilities, increased expression of pluripotency transcription factors such as homeobox protein NANOG, octamer-binding protein 4 (OCT4), and sex determining region Y-box 2 (SOX2), increased expression and ALDH enzymatic activity, and increased resistant phenotype to chemotherapies⁴². In SCLC, side population (SP) fraction that exhibits Hoechst dye efflux behavior was characterized as CSC-like cells that display higher proliferation, higher self-renewal ability, lower neuronal differentiation marker expression for CD56 and CD90 but higher expression of stem cell markers such as ATP-binding cassette sub-family G member 2 (*ABCG2*), fibroblast growth factor 1 (*FGF1*), insulin-like growth factor 1 (*IGF1*), *MYC* proto-oncogene protein, *SOX1/2*, and proto-oncogene *WNT1* when compared to non-SP cells⁴³. Despite their identification, direct evidence that demonstrates CSC's involvement in the progressive worsening of lung cancer disease is still largely missing from the literature. For an extensive overview of the current proof for the existence of CSC in lung cancer and their potential clinical relevance see also chapter 2 of this thesis.

Aim of the thesis

SCLC and NSCLC have a huge difference in the way they can be managed by medical practitioners. As summarized above, NSCLC has had tremendous improvement over the years, including how the disease has been characterized and the availability of treatment modalities. However, some problems related to suitability, toxicity, and drug resistance remain to be addressed to improve current treatment options. As for SCLC, progress has been lacking in both the disease characterization aspect and the vastness of treatment choice. Hence the aims of this thesis were:

1. To more extensively review the available literature regarding the progress of research in NSCLC and SCLC concerning CSC theory
2. To provide evidence of the involvement of CSC in the disease progression of SCLC
3. To find potential therapeutics that can inhibit the activity of CSC from phytochemical source

Outline of the thesis

In **chapter 2**, a review of literature on the current views for cancer stem cells (CSCs) in lung carcinomas is provided. First, a brief overview is presented describing the activity of normal lung tissue in response to injury and how it is linked to cancer initiation and development. Various aspects of CSCs in lung cancer are discussed, including signaling pathways, tumor microenvironment, plasticity, and cell-intrinsic resistance mechanisms. Most importantly, some updates on preclinical and clinical studies assessing potential pharmacological therapies in targeting various oncogenic targets were highlighted. In the effort to provide more decisive evidence on the association between CSCs and therapeutic resistance during disease progression, the relevance of CSC populations and their properties in SCLC were examined in **chapter 3**. Here, three GLC cell lines were used, isolated from one patient representing three different progressive stages of SCLC. Using RT-qPCR and western blotting, the expression of a panel of stem cell-related genes was examined. Then, immunofluorescence and flow cytometry were subsequently used to map the expression heterogeneity of progressively enriched proteins following the direction of disease progression. The results were correlated with some functional observations, including colony formation and irradiation sensitivity. Finally, a therapeutic opportunity to inhibit one of the highest expressing proteins in the advanced tumor model, CD44, was explored. In **chapter 4**, the focus is shifted towards NSCLC in which treatment options are abundantly available, but they are still associated with some toxicity and resistance problems as highlighted in the overview above. Hence, in this chapter, a new drug candidate called chelerythrine chloride was sought from the natural source and assessed for its bioactivities *in vitro*. Its inhibitory activity was evaluated against Wnt/ β -catenin activity assessing β -catenin nuclear localization and CSC properties, including colony formation, spheroid formation, migration, and invasion. In **chapter 5**, more phytochemical compounds consisting of representatives of alkaloids, chalcones, and isothiocyanates were evaluated in similar assessments as those performed in chapter 5. In **chapter 6**, the literature is reviewed for more in-depth on the normal homeostasis and injury response of lung tissue. An overview is provided on the mechanisms involved in the initiation and maintenance of lung CSC with the assumption that cancer works the same way as normal tissue but with looser control of proliferation and differentiation. Intracellular deviations that induce cancerous traits are explored with some discussions on the role of extracellular signals from the microenvironment. Pharmacologically active phytochemical compounds were selectively picked for their frequency of research in the last decade, and they are discussed based on their pathway targeting and activity to dampen oxidation and inflammation for both preventive and therapeutic options. Finally, in **chapter 7**, the overall findings throughout the chapters are summarized and a discussion on potential future outlooks and studies is given.

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An illustration of human lungs, rendered in a semi-transparent blue style, set against a teal gradient background. A red, irregular mass representing a tumor is visible in the lower-left lung. The trachea and bronchial tree are also depicted in blue.

Chapter 2

Lung cancer stem cells: origin, features, maintenance mechanisms and therapeutic targeting

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Abstract

Lung cancer remains the leading cause of cancer-related deaths despite recent breakthroughs in immunotherapy. The widely embraced cancer stem cell (CSC) theory has also been applied for lung cancer, postulating that an often small proportion of tumor cells with stem cell properties are responsible for tumor growth, therapeutic resistance and metastasis. The identification of these CSCs and underlying molecular maintenance mechanisms is considered to be absolutely necessary for developing therapies for their riddance, hence achieving remission. In this review, we will critically address the CSC concept in lung cancer and its advancement thus far. We will describe both normal lung stem cells and their malignant counterparts in order to identify common aspects with respect to their emergence and regulation. Subsequently, the importance of CSCs and their molecular features in lung cancers will be discussed in a preclinical and clinical context. We will highlight some examples on how lung CSCs attain stemness through different molecular modifications and cellular assistance from the tumor microenvironment. The exploitation of these mechanistic features for the development of pharmacological therapy will also be discussed. In summary, the validity of the CSC concept has been evidenced by various studies. Ongoing research to identify molecular mechanisms driving lung CSC have revealed potential new cell intrinsic as well as tumor microenvironment-derived therapeutic targets. Although successfully demonstrated in preclinical models, the clinical benefit of lung CSC targeted therapies has thus far not been

demonstrated. Therefore, further research to validate the therapeutic value of CSC concept is required.

Keywords: NSCLC, SCLC, stemness, molecular features, tumor microenvironment, therapy

Introduction

Lung cancer is the most common cancer type worldwide with the highest mortality rate. In 2018, global estimates of incidence and death were at 2.1 million and 1.8 million respectively¹. Lung cancer is histologically categorized into small cell (SCLC) and non-small cell lung carcinoma (NSCLC), constituting about 15% and 85% of the disease, respectively. Lung cancer is often diagnosed at late stages of disease, limiting the applicability of surgery and radiotherapy, making chemotherapeutic regimens the preferred treatment. Significant advancement has been achieved with NSCLC treatment, particularly in adenocarcinoma (ADC) and squamous cell carcinoma (SCC), indicated by the number of treatments approved by the FDA that include epidermal growth factor receptor (EGFR) and ALK targeted therapy and, more recently, biological therapy using immune checkpoint inhibitors². On the other hand, for SCLC, which is already metastatic at early stages of disease, no effective targeted therapy has been identified as yet. First line treatment chemotherapy in SCLC has not changed since the last decades, although recently promising results were obtained with immune checkpoint inhibitors that may lead to the long looked for improvement in survival of SCLC patients³. Despite these encouraging developments in subgroups of patients, therapeutic improvement for lung cancer patients as a whole are still needed.

In the last two decades, the cancer stem cell (CSC) hypothesis has attracted immense attention from both tumor conceptual as well as therapeutic perspective⁴. CSCs, representing a highly tumorigenic subpopulation, are deemed responsible for causing tumor growth, therapy resistance and recurrence, and metastasis possibly through epithelial-to-mesenchymal (EMT) reprogramming. A substantial amount of evidence points to the involvement of CSCs in tumorigenesis and progression of lung cancer. In this review, we will critically discuss lung tumorigenesis and therapeutic opportunities based on the CSC concept also taking into account possible cellular and molecular similarity with normal lung tissue homeostasis.

Adult lung epithelial stem cells and tissue repair

Adult stem cells are key cellular components of mature tissues or organs that participate in replenishing damaged and aged cells to maintain tissue homeostasis. These multipotent stem cells are able to self-renew and are restricted in their lineage to a specific subset of cells, only capable of giving rise to cellular offspring functional in that particular organ. Long-lived tissue-specific stem cells have been identified in various organs, such as the intestinal and skin stem cells, which are dedicated to differentiate into lineage-specific progenitor cells to secure proper tissue formation and organ functioning⁵. The progenitor cells of those two organs are required to cycle frequently to balance-off the shedding-prone environment.

The lungs have been recognized to possess remarkable tissue repair capability. This allows the lungs to recover efficiently from injury in response to inhaled particles/irritants and from bacterial and viral infections. At steady state, unlike in skin and intestine, the lung

epithelia's turn-over rate is extremely low with half-cycling time averaging at 17 months, referring to the terminally differentiated ciliated cells' turn-over using lineage-labeling system⁶. For proliferation, they rely on signals originating from surrounding mesenchyme to initiate cell cycling⁷. The tissue homeostasis and mild injury are regionally maintained and fixed by resident epithelial cells acting as lineage-committed progenitors in respective proximodistal locations (see Table 1). Basal cells provide maintenance functions in proximal regions (close to trachea), whereas club and alveolar epithelial type II (AEC II) cells are at distal sites. AEC II cells are restricted to the alveolar region of the lungs. In response to severe injury, different sets of resident epithelia can dedifferentiate/differentiate and transdifferentiate into epithelial cells that aim to restore lung tissue homeostasis. In studies employing rodents lung injury models, lung resident epithelia such as basal cells, club cells, variant club cells and AEC II cells were found to serve facultative stem cell reparative activity, which has been extensively reviewed recently (see⁸). In brief, basal cells maintain and repair large airway epithelium, whereas secretogoblin, family 1A, member 1 (SCGB1A1)⁺ club cells are responsible for maintenance and repair of small airways under homeostatic conditions or conditions of mild injury^{9,10}. In the alveolar space, AEC II cells can proliferate and differentiate into AEC I cells during steady state and mild injury¹¹. Upon more severe injury, transformation related protein 63 (TRP63)⁺keratin 5 (KRT5)⁺KRT14⁺ basal cells were able to replenish naphthalene, SO₂ and H1N1 viral-induced damaged ciliated, club or even AEC I and AEC II cells. Recent studies demonstrated that mouse and human SRY-box 9 (SOX9)⁺ basal cells, presumably arising from SOX2⁺ progenitors, represent a rare subpopulation of basal cells that are able to regenerate alveolar and bronchial epithelium and restore lung function in orthotopic transplantation experiments^{12,13}. In addition, SOX2⁻ progenitors termed alveolar epithelial progenitors expressing transmembrane 4 L six family member 1 (TM4SF1) and AXIN2 also function both during normal homeostasis and acute alveolar injury¹⁴.

Table 1. Human lung resident epithelia's locations, functions and facultative stem activity.

Resident epithelium	Location	Proximodistal location	Function	Facultative stem activity
Basal cells	Trachea, bronchi and bronchioles (absent in respiratory bronchioles)	Proximal	Structural support and stromal interactions	Yes
Ciliated cells	Trachea, bronchi and bronchioles	Proximal and distal	Clearance of irritants with cilia	No
Goblet cells	Trachea, bronchi and bronchioles	Proximal	Clearance of irritants with mucus Secretory for protection like	No
Club cells	Bronchioles	Proximal and distal	secreting secretoglobin protein	Yes
Variant club cells	Neuroepithelial bodies and bronchoalveolar duct junctions	Proximal and distal	Not known, perhaps same as club cells	Yes
Neuroendocrine cells	Trachea, bronchi and bronchioles	Proximal and distal	Responders to neuronal signals by releasing hormone (receptosecretory)	No
Alveolar epithelial cell I (AEC I)	Alveoli	Distal	Facilitate gas exchange	No
Alveolar epithelial cell II (AEC II)	Alveoli	Distal	Provide secretory vesicles filled with surfactants	Yes

In addition to acquiring stem cell properties, EMT is known to play an important role in lung repair¹⁵. In general, EMT strips-off epithelial phenotypes and switches cells to possess mesenchymal phenotypes with migratory potential. This is modulated by transcription factors like snail family transcriptional repressor 1 (SNAIL), twist family bHLH transcription factor 1 (TWIST1) and zinc finger E-Box binding homeobox 1 (ZEB1) by reducing expression of epithelial and increasing mesenchymal proteins¹⁶. Diverse signaling pathways are able to activate these transcription factors to initiate EMT, including Notch, Sonic Hedgehog (SHH), Wnt/ β -catenin signaling, and tyrosine kinase receptors like EGFR, fibroblast growth factor receptor (FGFR), hepatocyte growth factor receptor (HGFR/MET), insulin-like growth factor receptor (IGFR) and platelet-derived growth factor (PDGF) as well as phosphoinositide 3-kinase (PI3K)/AKT, and the most studied transforming growth factor β (TGF β /SMAD pathway (see also Figure 1)¹⁶. Lung repair involves migration of cells to the damaged region that, for example, was shown to involve Wnt/FGF10-dependent enhanced expression of SNAIL, vimentin (VIM) and alpha-smooth muscle actin (SMA, presently known as actin, alpha 2 (ACTA2)) in mouse models¹⁷. Overall, it appears that epithelial cell plasticity is regulated in a tightly controlled spatial and injury-dependent manner in the adult lung with the aim of maintaining airway homeostasis and repair (see also Figure 2). This view of facultative stem cell-mediated lung repair is increasingly recognized and substantially different from the dedicated stem cell-mediated repair that is seen, for example, in hematopoiesis and small intestine's regeneration⁵.

Figure 1. Schematic relationship of signaling pathways regulating epithelial, mesenchymal and stem cell phenotypes. Diverse array of signaling pathways are able to induce the reprogramming process towards mesenchymal phenotype through epithelial-to-mesenchymal transition (EMT), characterized by among others downregulating ECAD/CDH1 and upregulating NCAD/CDH2 expression. Each signaling pathway is known to interact with different EMT transcription factors like TWIST, SNAI and ZEB as depicted, all of which support mesenchymal phenotypic induction. Most signaling pathways are able to activate SNAI to induce EMT. SNAI is also able to activate the other transcription factors for EMT induction. EMT is proposed to also encourage stem cell properties induction. Indeed, several signaling pathways are known as embryonic signaling pathways like Notch, SHH and Wnt, whereas others have been associated with cancer stem cells (CSC) as indicated by the red solid lines (also discussed in main text). Solid black line indicates pathways activating SNAI, dashed black line indicates pathways activating ZEB, dotted black line indicates pathways activating TWIST and solid red line indicates pathways associated to increased stemness.

Figure 2. Cellular components of lung tissue and lung cancer, and the microenvironment. Lung cancer, like normal tissue, consists of heterogeneous cellular components leading to complex cellular and molecular interactions. Across the proximodistal locations, three major histologically different tumor types can arise from respective regional facultative stem cells, except for the least known facultative stem potential of neuroendocrine cells. The figure depicts mirror image of normal lung epithelia and tumor lung epithelia and their respective microenvironments. The capability of those cells to undergo hyperplasia bridges their facultative stem potential with cancer stem cell (CSC) transformation. Cytokines secretions directed from stromal cells may also contribute to the stem cell phenotypes maintenance aside of their internal regulations (see text for more detail).

CSCs in lung cancer

The origin of CSCs in the lung

The concept of attributing tumor-initiating and tumor expansion activity to a malignant stem cell compartment has been proposed almost two centuries ago¹⁸. This notion gave birth to the CSC theory that postulates the presence of cells possessing stem cell properties in tumors, which are crucial for tumorigenesis and progression. In analogy to normal stem cells, CSCs also undergo symmetric and asymmetric cell division to self-renew and to give rise to more differentiated offspring. Moreover, there is likely not only one specific CSC population, but multiple, each being generated from both overlapping and independent carcinogenic events. This concept is different to what is accepted in the conventional idea of potency in long-lived-normal stem cells. Nonetheless, CSCs should be defined by their functional capabilities, not by their supposed origin e.g., mutated normal stem cells¹⁹. Thus, heterogeneity represents not only vertical hierarchical relationship between parental cells and progenies, but also horizontal relationship among inter-clonal CSCs.

By considering the stem cell properties of lung CSCs and the likelihood of stem cell activities being triggered in lungs mostly through injury–repair responses, lung CSCs may have initially arisen from proliferative cells of the lung epithelia, i.e., the facultative stem cells. Different histological lung cancer subtypes are suggested to originate from different facultative stem cells. Weeden and colleagues provided an association between injury–repair responses of lung facultative stem cells and tumorigenesis where they demonstrated that TRP63⁺KRT5⁺ basal cells preferentially repaired DNA damage by the notoriously unreliable non-homologous end joining (NHEJ) repair mechanism, which may lead to genetic instability in basal cells and onset of oncogenic transformation²⁰. Basal cells were suggested to be the origin of CSCs in SCC due to similarities of molecular markers and their spatial origin in proximal lung. In addition, the requirement of SOX2 to restrict speciation to proximal origin and development of SCC further suggests close correlations between the two^{21–23}.

In the distal lung of *KRAS* mutant mice models, AEC II cells have been exclusively shown to progress to ADC, whereas club cells became hyperplastic in bronchoalveolar duct junctions²⁴. *KRAS* and *EGFR*'s involvement to AEC II cells self-renewal during injury has been proposed as triggers for continuous proliferation of ADC CSCs²⁵. It was proposed that the normal involvement of *EGFR* and *KRAS* pathways in AEC II cells stemness is hijacked during oncogenesis leading to malignant stem cell activity. The likelihood of club cells to progress to ADC has not been demonstrated, which may be caused by elevated expression of SOX2 during injury²⁴. Indeed, when NOTCH1 intracellular domain (ICD) was overexpressed, SOX2 levels were reduced and club cells in bronchioles developed into ADC in a *KrasG12D* background, implicating SOX2 as a suppressor of tumor formation in club cells²⁶.

For SCLC, pulmonary neuroendocrine cells (PNECs) were shown to be the candidate of transformation upon inactivation of *Trp53*, Rb transcriptional corepressor 1 (*Rb1*) and phosphatase and tensin homolog (*Pten*) in adult mouse lung²⁷. The triple mutations in differentiated PNECs led to hyperplastic lesions, whereas mutations in proliferating PNECs resulted in tumor formation and invasion in mice.

Taken together, current findings show that chronic injury is a tumor-initiating event in lungs and that the origin of lung CSCs is highly dependent on the type of injury inflicted and epithelial cell type affected (see also Figure 2).

Identification of lung CSCs

Pioneering studies of CSCs in lung cancer have shown certain subpopulations displaying self-renewal, differentiating and tumor-regenerative ability. Those include cells expressing putative CSC cell surface markers, cells excluding Hoechst 33342 dye (termed side population (SP)) and cells surviving chemotherapies (for recent review see²⁸). Upregulation of several proteins, such as aldehyde dehydrogenase (ALDH), ATP binding cassette subfamily G member 2 (ABCG2), CD44, CD117/KIT, CD133/prominin 1 (PROM1), Nanog homeobox (NANOG) and POU class 5 homeobox 1 (POU5F1, formerly known as OCT3/4) have been extensively characterized to be associated with stem cell properties in lung cancer and have been used to identify CSC populations^{28,29}. It should be noted that thus far no marker is deemed robust and reproducible enough to detect specific sets of CSCs in lungs. Regardless, the existence of lung CSCs according to both marker expression and functional properties has been clearly demonstrated.

Lung CSCs have some striking similarities with their normal counterparts, particularly reflecting their epithelial origin. The PNECs are similar to CSCs of SCLC that favor Notch inhibition to prevent differentiation upon injury and acquire a proliferative state³⁰. SOX2 expression is needed for the emergence of proliferating basal cells upon injury as well as maintenance of CSCs in SCC^{21,22}. Requirement of SOX9 to generate functional alveoli suggests that AEC II facultative stem cells in distal airways are akin to the SOX9-expressing CSCs giving rise to ADC^{12,31}. Together this suggests that there are close resemblances between tumor and normal stem cells in terms of their pathways and transcription factor utilization.

On the other hand, variable stem cell marker expression may be dependent on the tissue and cellular context in which the CSCs develop. For example, progression of club cells or AEC II cells to ADC CSC might be expected when the right combinations of genetic alterations are given. However, in *Pten*^{-/-};*Cdkn2ab*^{-/-};*Sox2* lung cancer mice models, Ferone and colleagues showed that SOX2 not only defines the oncogenic switch of proximal basal cells to SCC, but also allows SCC progression in distal club and AEC II cells²³. This further points to an important role of the stem cell transcription factor SOX2 in lung cancer progression, although independent of cell type of origin and tissue location. However, the role and interactions between SOX2 and SOX9 in lung cancer progression warrant further study to better understand their involvement in lung cancer development along the proximodistal axis and in different altered genetic backgrounds.

Lung CSCs regulation, cellular plasticity and the tumor microenvironment

Signaling pathways in lung CSCs

Considering the cellular resemblance of lung CSCs with facultative stem cells, it is not surprising that lung CSCs also make use of signaling pathways regulating self-renewal and differentiation processes in normal tissue. In general, signaling pathways involved in CSCs' perpetuation are Janus family tyrosine kinase (JAK)/signal transducer and activator of

transcription (STAT), nuclear factor kappa B (NFκB), Notch, PI3K/AKT serine/threonine kinase, SHH and Wnt/β-catenin pathways. An overview is provided in Figure 3. The general consensus on how these pathways work has been reviewed elsewhere³².

Figure 3. Currently known molecular pathways regulating stemness of different histological subtypes of lung cancer. The figure depicts molecular mechanisms implicated in regulating lung CSCs that originate from their respective facultative stem cells, namely lung squamous cell carcinoma (SCC), lung adenocarcinoma (ADC) and lung small cell carcinoma (SCLC). SCC and ADC are collectively depicted as lung non-small cell carcinoma (NSCLC) as the information for the two are still poorly segregated. Signaling pathways are distinctly employed by NSCLC in contrast to SCLC for their stemness maintenance. Several pharmacological agents (see also main text) are developed to target lung CSCs at various molecular levels covering signaling pathways, genetic/epigenetic modulators, transcription factors, cell reprogramming system and cytokines/growth factors secretions from stromal cells.

In response to injury, SHH pathway is deactivated to promote proliferation of adjacent mesenchyme which encourages the proliferation of normal lung epithelium, possibly through paracrine signaling that enforces suppression of SHH signaling⁷. The Notch pathway has a role in promoting differentiation of PNECs, maintaining club cells identity, and driving proliferation and maturation of AEC II cells^{30,33}. Wnt/β-catenin pathway is associated with epithelia hyperplasia and regeneration during injury, and more specifically, AEC II stem cell identity and proliferative potential^{34,35}. The above mentioned embryonic signaling pathways were similarly found to be relevant in lung CSCs maintenance, likely being rooted from their respective cell of origins³⁶. Activation of the JAK/STAT, PI3K/AKT and NFκB pathways are linked with extrinsic factors derived from chronic inflammatory conditions. This resembles the

situation in chronic obstructive pulmonary disease (COPD) in which inflammation often leads to activation of these pathways in response to inflammatory cytokines³⁷. Lung CSC maintenance and COPD are perhaps sustained by the same injury-dependent inflammatory processes.

Lung CSC plasticity

High cellular plasticity is a characteristic for lung malignancies. This is exemplified by Akunuru and colleagues who characterized different subpopulations of CSCs in human ADC expressing SP, CD133⁺, ALDH^{high} and CD24^{low}CD44^{high}³⁸. When comparing SP, CD133⁺ and ALDH^{high} populations with marker negative populations, they noticed differential expression of self-renewal-related genes such as HES family bHLH transcription factor 1 (*HES1*), *NANOG*, *NOTCH1*, *POU5F1*, *SHH* and *SOX2*, and metastasis-related genes like C-X-C motif chemokine receptor 4 (*CXCR4*), tumor necrosis factor (*TNF*), homeobox B9 (*HOXB9*), *TGFB1*, vascular endothelial growth factor A (*VEGFA*), interleukin 1 beta (*IL1B*) and *IL6*. They also found that EMT reprogramming by *TGFB1* dynamically changed the proportion of the CSCs whereby SP cells decreased, and CD133⁺, ALDH^{high} and CD24^{low}CD44^{high} cells proportions increased. Concomitantly, the expression of self-renewal-related and metastasis-related genes was increased in primary ADC cells. In addition, stem cell marker negative populations could also give rise to CSC marker-positive population suggesting plasticity of the non-CSCs population. Finally, the observed inter-convertibility of lung CSCs may not be surprising considering high plasticity in lung epithelia as discussed earlier above. Together, this illustrates that cellular plasticity in normal lung has been replicated by lung cancers in which interactions with the tumor microenvironment play an important role.

Lung CSCs and the tumor microenvironment

Tumor microenvironment (TME) is a crucial immediate extrinsic factor that influences how CSCs behave. Stromal cells located in the surroundings of tumor are often observed to assist CSCs to thrive by affecting their cellular machinery. As mentioned earlier, this is especially true when inflammation is sustained in the course of injury when stromal cells are recruited to facilitate the healing process. Those cells include cancer associated fibroblasts (CAFs) and infiltrating immune cells, particularly the tumor associated macrophages (TAMs). Such assistance can also be recruited from distant sites such as the mesenchymal stem cells (MSCs) from bone marrow. Much has been revealed on the role of TME, including hypoxia, and its players in lung cancer development^{39,40}. Some evidence related to CAFs, TAMs and MSCs will be highlighted below.

Located in the connective tissue filled with extracellular matrix, fibroblasts are the closest stromal cells to epithelial cells. Under normal conditions, fibroblasts function to secrete matrices and soluble secreted factors to provide anchorage support and survival cues for epithelial cells. However in TME, fibroblasts are reprogrammed to support CSCs. In a report, CAFs have been identified to highly express ACTA2 and SNAI1 that are uniquely present if compared to normal fibroblasts⁴¹. When co-cultured with NSCLC cells, CAFs were able to stimulate a more migratory and invasive phenotype as well as EMT properties signified by elevation of VIM, TWIST1, SNAI1, ZEB1, matrix metalloproteinase 2 (MMP2) and MMP9

expressions through micro RNA (miR)-33b downregulation. MiRs are non-coding oligonucleotides that perform regulatory functions of gene expression and are known as dual players in both stimulating and suppressing tumor growth. An important role for miR-16 was demonstrated in regulating the FGFR1-dependent production of HGF by CAFs⁴². MiR-16-mediated inhibition of HGF production reduced the migration of adjacent lung cancer cells. It was proposed that low miR-16 levels in CAFs from smokers may be linked with tumor progression. In another study the production of IL6 by CAFs was shown to enhance NSCLC invasiveness via JAK2/STAT3 signaling⁴³. Paracrine effects by CAFs was also shown to involve IGF2-IGF1R by maintaining stemness of CSCs characterized by high expression of NANOG and POU5F1⁴⁴. Recently, CAF heterogeneity was demonstrated by variable expression of the cell surface markers CD10 and G protein-coupled receptor 77 (GPR77). Both were found to promote tumor formation and chemoresistance⁴⁵. GPR77-dependent IL6 and IL8 production were found to provide a CSC supportive niche and, moreover, targeting GRP77 with a neutralizing antibody restored chemosensitivity and reduced tumor growth in NSCLC models.

During tissue regeneration and inflammation, macrophages with distinct phenotypes actively partake in all stages of inflammatory response and healing processes. Upon injury, resident and bone-marrow-derived macrophages arising from monocytes are recruited to mediate pro-inflammatory functions upon receiving appropriate stimuli like damage-associated molecular patterns (DAMPs), pathogen-associated molecular patterns (PAMPs), cytokines, growth factors and other molecular mediators⁴⁶. M1 and M2 macrophages alternatively mediate pro- and anti-inflammatory responses to facilitate tissue remodeling and repair. Tissue destructive M1 subset releases pro-inflammatory mediators like TNF, interferon gamma (IFNG) and IL12, whereas M2 subset releases anti-inflammatory mediators like IL4, IL10, TGFB1, HGF, IGF1, PDGF and VEGFA⁴⁶⁻⁴⁸. A recent study assessing the effect of different macrophages subsets revealed different consequences to NSCLC progression. The pro-inflammatory M1 subset suppressed tumorigenicity of A549 ADC cells by increasing cisplatin sensitivity, inducing apoptosis and enhancing senescence⁴⁹. M2a and M2c subsets promoted invasion and increased tumorigenicity. Therefore, M2 macrophages are associated with pro-tumorigenic behavior. Indeed, IL10-producer TAMs were detected at higher percentage in more advanced stages of NSCLC⁵⁰. Moreover, high IL10 expression by TAMs was associated with poorer overall survival (OS). Cytokines released by TAMs like TGFB1, was characterized to contribute in EMT reprogramming through downregulation of miR-138 with consequent enrichment of CD44⁺CD90⁺ lung CSCs and a concurrent increase in colony-forming ability⁵¹. Mechanistically, TAMs may also modulate stemness of NSCLC through elevated expression of ubiquitin specific peptidase 17-like family member 9, pseudogene (USP17L9P, formerly known as USP17)⁵². This can be achieved by stimulation of wide array of cytokines, namely TNF, IL1B, IL4, IL6, IL8, IL10, CXCL12, C-C motif chemokine ligand 18 (CCL18) and CCL22 through the binding of hypoxia inducible factor 1 subunit alpha (HIF1A), STAT3, STAT6 and NFkB1 transcription factors. Elevated of USP17L9P expression consequently led to higher cancer intrinsic inflammation as indicated by the production of TNF, IL1B, IL6, IL8, IL12 and IL23, and increased stemness indicated by higher

expression of stem cell markers like ABCG2, ALDH1, CD44, CD117, CD133, kruppel like factor 4 (KLF4), MYC, NANOG, POU5F1 and SOX2.

Not much information has been obtained for the involvement of MSCs towards stemness maintenance of lung CSCs. One study showed that they modulated stemness of ADC CSCs through IL6/JAK2/STAT3 pathway⁵³. Co-culture of MSC with lung ADC promoted sphere formation and increased expressions of NANOG, POU5F1 and SOX2.

Taken together, the role of CAFs, TAMs and MSCs within TME is to provide stimulatory molecules that directly or indirectly enhance stem cell properties of lung CSCs.

EMT cellular reprogramming

EMT has also been proposed to encourage stem cell maintenance. For example, Chang and colleagues has provided a clear link between mesenchymal reprogramming and stemness induction through Wnt signaling in NSCLC⁵⁴. They showed that exposure of serially selected low and high motility A549 sphere-derived cell clones to EMT inducers alone is not sufficient to promote stemness. In fact, the cells have to be sufficiently mesenchymal at the beginning (low E-cadherin (ECAD) expression) in order to be susceptible to WNT3A-induced EMT. Increased stemness of high motility mesenchymal cell clones in response to WNT3A stimulation was demonstrated by elevated sphere-forming ability, higher number of SP and elevated expression of a number of mesenchymal, self-renewal, CSC-associated and ABC transporters genes. Chromatin immunoprecipitation (ChIP) experiments indicated that EMT helps switching in transcriptional activity involving catenin beta 1 (CTNNB1)/ECAD/SOX15 to CTNNB1/TWIST1/Transcription Factor 4 (TCF4) complex. The authors identified five gene signatures consisting of nuclear CTNNB1^{high}/nuclear TWIST1^{high}/ECAD^{low}/SOX15^{low}/CD133^{high} to be associated with disease progression and metastasis of lung cancer.

A relationship between EMT and expression of CSC marker CD44 has been reported by Su and co-workers⁵⁵. The authors demonstrated that CD133⁺ cells co-expressing CD44 displayed higher migration and invasion capability when compared to CD44⁻CD133⁺ lung cancer cells. Only the double positive cell exhibited increased sphere formation and *in vivo* tumor regeneration. WNT3A was able to increase CTNNB1 and forkhead box M1 (FOXM1) expression in CD133⁺CD44⁻ population mimicking the phenotype of CD133⁺CD44⁺ population. This indicates that Wnt/ β -catenin pathway is active in CD133⁺CD44⁺ CSCs. Knock-down and overexpression of *FOXM1* gene revealed that increased metastatic phenotype is mediated through TWIST in a FOXM1-dependent mechanism.

A different and striking way by which lung cancer cells can obtain mesenchymal properties has been reported to involve cell fusions between epithelial cancer cells and bone marrow-derived mesenchymal cells. Apparently, spontaneously formed hybrids between lung cancer cells and stromal MSCs was found to result in an EMT programmed cell characterized by reduced ECAD and pan-cytokeratins expression, increased VIM, ACTA2 and fibronectin 1 (FN1) expression together with increased motility and invasiveness⁵⁶. Moreover, the hybrid became more stem-like indicated by elevated expression of stem cell markers like ALDH1, BMI1, NANOG, NOTCH1, POU5F1 and SOX2, and increased sphere-forming ability. The hybrid also displayed enhanced *in vivo* tumorigenic potential. The authors further speculated

that the hybrid formation is stimulated during inflammation, thus providing a new link between inflammation and tumorigenesis. Furthermore, it appeared that the hybrids were rather instable and tend to revert back to epithelial phenotype, which would facilitate secondary colonization upon metastasis. Hybrid stability was found to be regulated by grainyhead like transcription factor 2 (GRHL2), miR-145 and Ovo like transcriptional repressor 1 (OVOL1) and regulation of the miR-200/ ZEB1 balance that controls EMT⁵⁷.

Cell intrinsic regulation of lung CSCs

Aside from modulation of stem cell properties from external stimuli as described above, cell intrinsic genetic alterations are able to directly affect molecular mechanisms controlling stemness and boost tumor aggressiveness. Several examples of such molecular alterations covering genetic, epigenetic and other regulatory components in lung cancer are described below.

NSCLC

The accumulation of DNA damage is one of the main triggers of tumorigenesis leading to various genetic aberrations such as single nucleotide polymorphism (SNP), deletions, chromosomal translocations or breaks, aneuploidization and polyploidization. Commonly observed genetic aberrations in NSCLC include *BRAF*, *EGFR*, erb-b2 receptor tyrosine kinase 2 (*ERBB2*), *KRAS*, *MET*, *ROS1* and *ALK*-echinoderm microtubule associated protein-like 4 (*EML4*) gene fusion⁵⁸. For some of these genetic alterations, involvement in stem cell regulation has been suggested. As discussed earlier under 3.1., *KRAS* and *EGFR* have been implicated in self-renewal during lung injury and it has been proposed that their continuous activation may lead to ADC CSCs. In addition, constitutively activated *EGFR* mutant cells were shown to increase the expression of spalt like transcription factor 4 (*SALL4*), a zinc finger transcription factor that is known to interact with *KLF4*, *POU5F1* and *SOX2*. This could lead to the maintenance of stem cell properties like spheroid formation and pluripotency genes expressions in lung ADC⁵⁹.

Recently, a novel fusion gene was identified in invasive mucinous subtype of lung ADC⁶⁰. Being mutually exclusive with currently known mutations like *KRAS*, *EGFR*, *BRAF* and *ERBB2*, the *CD74-NRG1* fusion was further characterized. *CD74-NRG1* contains transmembrane domain of *CD74* and EGF-like domain of neuregulin 1 (*NRG1*), which is a ligand for *ERBB2/ERBB3*. *ERBB3* stimulation resulted in *NFκB* activation and enhanced secretion of *IGF2* and spheroid growth in lung cancer cells⁶¹. Remarkably, lung cancer possessing this gene fusion is self-sustaining *in vitro*, independent of growth factors commonly supplied to enrich CSCs. This finding demonstrates yet another example that lung CSCs can develop a self-reliance mechanism to maintain stem cell phenotype through genetic alteration.

TM4SF4 expression, a protein within the same family as the marker of alveolar epithelial progenitor cells known to be involved in normal lung homeostasis as mentioned earlier (see section 2), was found elevated in *ALDH*⁺ CSCs and in γ -irradiation resistant ADC cells and was associated with both enhanced stem cell properties and EMT phenotypes⁶². *TM4SF4* stimulates the release of osteopontin, an extracellular matrix protein involved in the regulation of many processes in various tissues including bone formation, tissue repair and

immune reaction. In A549 ADC cells, TM4SF4 via IGF1RB-dependent glycogen synthase kinase 3 beta (GSK3B)/CTNNB1 activation led to osteopontin production that in an autocrine loop, was able to stimulate migration, invasion, sphere formation and expression of EMT- and CSC-associated genes, including neuronal cadherin (NCAD or presently known as cadherin 2 (*CDH2*)), *VIM*, *POU5F1* and *SOX2*. More in depth analyses revealed that the EMT and CSC phenotypes were regulated by osteopontin-dependent activation of CD44 and integrins, and subsequent activation of JAK2/STAT3 and focal adhesion kinase (FAK)/STAT3 pathways. In addition, osteopontin expression was shown to be increased in tumor specimens compared to normal lung tissue and correlated with poorer survival. This study highlights the intricate measures taken by CSCs to sustain their growth using feedback loop mechanism to stimulate common pathways like STAT3 for stemness maintenance.

MiRs also have been reported to regulate lung CSCs. Recent years of studies in lung cancer have revealed that miRs impact on multiple CSC components by post-transcriptionally regulating genes including stem cell genes, EMT machinery and epigenetic mechanisms^{29,63,64}. For instance, overexpression of miR-708-5p was found to lead to DNA hypomethylation by promoting the downregulation of DNA methyltransferase 3 alpha (DNMT3A) in NSCLC cell lines⁶⁵. This resulted in re-expression of ECAD, which consequently reduced the stem cell properties and tumor-generating capacity of CSCs in NSCLC through reduction of Wnt/ β -catenin pathway activation. MiR-708-5p expression correlated with higher OS and relapse-free survival of NSCLC patients. Even though not discussed by the authors, it can be deduced that abolishing the mesenchymal phenotype due to restoring ECAD expression may lead to reduction of CSC properties. Moreover, this finding illustrates that the CSC phenotype is controlled at different levels involving a miR, epigenetic alterations via DNMT3A and EMT reprogramming. Another miRNA, miR-150, was shown to promote cell invasion and metastasis in NSCLC⁶⁶. This was achieved by inhibiting production of forkhead box O4 (FOXO4), a negative regulator of cell proliferation, resulting in increased anchorage-independent growth, migration and invasion that involved triggering of EMT through NF κ B/SNAI1/YY1/Raf kinase inhibitor protein (RKIP) circuitry. Clinical data showed that miR-150 expression was associated with metastatic NSCLC. Although a more direct relation between miR-150 and CSC properties was not explored, its oncogenic and EMT activating properties are suggestive for such a role.

A novel class of non-coding RNAs called P-element induced wimpy testis (Piwi)-interacting RNAs (piRs) has recently gained attention for its role in tumorigenesis. PiRs are able to silence mobile elements like transposons, particularly in germ line cells, and the association between piRs and NSCLC has been examined by Peng and colleagues where they assessed tumor-suppressive function of piR-55490⁶⁷. PiR-55490 was shown to be downregulated in human lung carcinoma specimens that involved inhibition of mechanistic target of rapamycin (mTOR) at transcriptional level, rendering it incapable of supporting proliferation of lung cancer. In the same year, Li and co-workers investigated oncogenic piR-651's effect on NSCLC⁶⁸. First, they confirmed the clinical relevance of piR-651 by showing elevated expression in NSCLC tumor samples when compared to adjacent normal tissues. Although the CSC compartment was not directly studied, the authors identified significant correlation of cyclin D1 (*CCND1*) and cyclin dependent kinase 4 (*CDK4*) expressions with

piR-651, indicative of either higher proliferative or self-renewal potential of cells in NSCLC upon piR-651 upregulation. Since the exploration of the role of piRs in CSCs maintenance is still in its infancy, further study is warranted.

SCLC

In SCLC, inactivating mutations in tumor protein 53 (*TP53*) and *RBI* are major contributors to tumorigenesis, whereas tumor maintenance and progression are attributed to mutations in *NOTCH* and *PTEN*, amplification of proto-oncogene *MYC* family members, activation of FGFR pathway and hypermethylation activity such as by enhancer of zeste 2 polycomb repressive complex 2 subunit (*EZH2*)^{3,69}. Of note, the SCLC progression genes have all been implicated in regulating (cancer) stem cells. Different with NSCLC, SCLC does not seem to favor NFκB activation to develop, but instead blocking NFκB with dominant negative form of inhibitor of kappa B alpha (IκBα) accelerated tumor progression of SCLC in an inducible lentiviral SCLC mouse model⁷⁰. SCLC appears to depend more on protein kinase A (PKA)-dependent activation of cyclic AMP response element-binding protein (CREB) pathway for mediating tumor growth and maintenance of a neuroendocrine phenotype.

As mentioned, amplification of *MYC* family members is commonly associated with SCLC tumors. Chen and co-workers recently identified a new target gene of MYCN and achaete-scute homolog 1 (*ASCL1*) transcriptional activity of a transcriptional repressor called *INSM1* critical for neuroendocrine differentiation⁷¹. SHH signaling was found to act upstream of MYCN activation and SHH inhibitors reduced MYCN and *INSM1* activation leading to inhibition of lung cancer growth. The two studies described above suggested that CSCs of SCLC heavily rely on signaling pathways that favor neuroendocrine phenotype maintenance. In addition, recently *ASCL1* was identified as the primary activator of MYCL1, nuclear factor I B (NFIB), RET and SOX2, and for tumor formation in mouse models⁷². Different from the majority of SCLCs with high neuroendocrine features, recently a subtype with a low neuroendocrine phenotype was identified, characterized by EMT reprogramming, activated Hippo, Notch and TGFβ pathway, MYC amplification and overexpression under influence of RE1 silencing transcription factor (REST), mechanisms known to contribute to stemness⁷³.

Taken together, CSCs in both NSCLC and SCLC are regulated through cell autonomous molecular mechanisms also including intrinsic genetic aberrations that have been linked with tumorigenesis and tumor progression in these malignancies.

Therapy resistance, relapse and lung CSCs

CSCs are deemed to be resistant for standard therapies according to the concept. Therapeutic resistance has been associated with expression of several CSC phenotypes which indirectly links CSCs involvements in resistance to therapies as also reviewed recently⁷⁴. For example, Wang and colleagues showed the involvement of CSCs in therapeutic resistance frequently seen in the clinic in lung cancer cell lines⁷⁵. In A549 and NCI-H460 cell lines, pre-treatment with cisplatin resulted in increased colony formation and sphere formation in the surviving fraction, while paclitaxel or doxorubicin pre-treatments did not. The increase in stemness correlated with upregulation of several pluripotency-related genes like NANOG, POU5F1 and SOX2, and several ALDH isoforms that are linked with multidrug resistance.

Aberrant activation of the CCAAT enhancer binding protein B (CEBPB)/Tribblespseudokinase 1 (TRIB1)/histone deacetylase (HDAC)/TP53 axis was identified to induce cisplatin resistance. Knock-down of TRIB1 and chemical inhibition of HDAC restored sensitivity to cisplatin. In addition, TRIB1 was found to be clinically relevant as its expression is NSCLC-specific and elevated in cisplatin-treated NSCLC patients and correlated with poor treatment outcome and OS. With this finding, one wonders whether cisplatin usage as first line treatment is the best treatment option. This finding also emphasizes the importance of epigenetic mechanism such as histones acetylation in tumorigenic gate-keeping and specifically the targeting of lung CSCs.

The possible role of CSC in NSCLC in contributing to tyrosine kinase inhibitors (TKIs) resistance has been explored by various investigators. *EGFR* mutant NSCLC cells selected for gefitinib resistance demonstrated high expression levels of stem cell markers such as ALDH1A1, CD133, CXCR4, POU5F1 and SOX2 and, moreover, were highly tumorigenic *in vivo* suggesting that lung CSCs are refractory for this treatment⁷⁶. Others pre-treated NSCLC cells with different TKIs consisting of gefitinib, regorafenib and sorafenib and found an increase of stem-like phenotypes, which included colony formation, sphere formation, ALDH activity and the expression of POU5F1 and SOX2⁷⁷. The pre-treatments also evoked higher expression of mesenchymal markers like NCAD, VIM and MMP2 concomitantly with higher migratory potential. Further experiments employing transcriptional profiling identified the activation of AKT/FOXM1/stathmin 1 (STMN1) pathway in mediating resistance. Pharmacological inhibition of AKT restored TKI sensitivity. Furthermore, both STMN1 and FOXM1 appeared to be associated with unfavorable prognosis and survival outcome of NSCLC patients.

On the other hand, EMT that forms close association with stemness may also influence resistance phenotype. EMT induction was shown to mediate resistance towards EGFR inhibition in *EGFR*-mutant NSCLC through ZEB1 expression⁷⁸. ZEB1 was found to directly cause transcriptional repression of BIM (presently known as BCL2 like 11), rendering insensitivity to EGFR inhibitor-induced apoptosis due to diminished levels of this pro-apoptotic protein. In other situation, embryonic signaling pathways that support CSC development like Notch may also confer resistance. High NOTCH1 expression in gefitinib-resistant lung cancer cell line was found to promote EMT phenotypes, whereas silencing it with small interfering RNA (siRNA) reversed the phenotypes in addition to restoring sensitivity to gefitinib⁷⁹. Silenced NOTCH1 also reduced colony formation of the gefitinib-resistant lung cancer cell line, indicating reduced CSC potential due to Notch abolishing.

The increase in CSC properties of therapy-resistant lung cancer cells will obviously favor relapse and progression of disease. However, direct evidence supporting therapeutic resistance and relapse due to increased tumoral stemness, for example by comparing biopsies before and after relapse has not been formally presented. Interestingly, a recent study in mice has linked the reactivation of latent/dormant tumors to altered activity of CD133⁺ lung CSCs⁸⁰. Li and co-workers showed that latent NSCLC xenografts in nude mice could be reactivated by co-implanting fresh NSCLC material, which was associated with a shift in asymmetric - to symmetric cell division in the CD133⁺ lung CSC compartment. Further investigation revealed that this transition was initiated by IGF1 signaling involving the PI3K/AKT/ β -catenin axis.

The expansion of the lung CSC fraction subsequently stimulated angiogenesis further fueling tumor relapse. Although this study supports a proposed major role of lung CSCs in tumor relapse, more definitive proof in longitudinal biopsies from patients is currently missing. Use of liquid biopsies represented by circulating tumor cells (CTCs) will greatly facilitate the analyses of identity and levels of CSC markers during treatment and progression.

Overall these studies provide substantial evidence for the involvement of lung CSCs and EMT in drug resistance in NSCLC.

Pharmacological targeting of lung CSCs

Current knowledge of the molecular mechanisms that sustain lung CSCs, either being TME-derived or cell autonomous, has enabled studies to explore their value as therapeutic targets. Several examples are described below and summarized in Table 2.

Table 2. Examples of pharmacological targeting of lung cancer stem cells and their tumor microenvironment.

Pharmacological drug	Target(s)	Clinical study	Reference
Targeting tumor microenvironment			
Apatinib	VEGFR2 and HGF/MET	NCT02515435	85,86
CBP501	calmodulin	NCT00942825	84
Nintedanib	VEGFR, FGFR and PDGFR	NCT00806819 , NCT00979576, NCT02231164, NCT02300298, NCT01441297	81,82
NSC12	FGF2	-	83
Targeting lung CSC directly			
ABBV-399	MET	NCT02099058, NCT03574753, NCT03539536	97,98
Anlotinib	VEGFR1/2/3, FGFR1/2/3, PDGFRA/B, MET and CD117	NCT01924195, NCT02388919	99
AZD9150	STAT3	NCT02983578, NCT03421353	-
CUDC-101	HDAC, EGFR and HER2	NCT01171924, NCT01702285	92
Defactinib	FAK	NCT01943292, NCT01951690	94
DSF/Cu complex	ALDH1A1	-	100
MK-2206	AKT	NCT01294306	96
Napabucasin	STAT3	NCT01325441, NCT02826161	95
Omomyc	MYC	-	90
Pazopanib	VEGFR1/2/3, PDGFR and CD117	NCT01262820, NCT01027598, NCT00871403	-
RO4929097	Gamma-secretase/Notch	NCT01193881, NCT01193868, NCT01217411	-
Sulforaphane	MYC	-	89
TMU-35435	HDAC	-	93
TTI-101	STAT3	NCT03195699	-

Targeting the TME

As pointed out earlier the TME has an important contribution in CSC maintenance in lung cancer. Several studies have explored the possible therapeutic benefit of targeting specific components of the TME. A multikinase receptor inhibitor named nintedanib was found to have selective inhibitory effect on CAFs assisting ADC, but not CAFs assisting SSC⁸¹. Nintedanib was able to prevent TGF β -dependent activation of fibroblasts into CAFs and consequently suppressed tumor growth and invasion *in vitro*. This study revealed the importance of preventing activation of CAFs, hence eliminating the stromal support to cancer cells. Despite its promise in preclinical studies, nintedanib did not perform as well as expected in patients. Several clinical trials evaluating its efficacy in combination with chemotherapeutic drugs reported lack of efficacy or development of adverse events (NCT00806819, NCT00979576, NCT02231164 and NCT02300298). Nonetheless, new clinical trials have been initiated for evaluating combinatorial response with chemotherapies in SCLC patients even though limited activity was seen in recent phase II study in patients with relapsed SCLC (NCT01441297)⁸².

FGF ligands produced by both stromal and tumor cells contribute to tumor aggressiveness and stemness, providing interesting targets for therapy. In addition to FGFR receptor inhibitors, recently a FGF-ligand trap was developed based on the antagonistic effect of pentraxin 3 (PTX3) on FGF signaling leading to anti-tumor effects in NSCLC⁸³. Using pharmacophore modeling analyses of the PTX3-FGF binding site allowed the development of an acetylated pentapeptide named NSC12 that sequesters FGFs and effectively inhibited angiogenesis and tumor growth in both murine and human lung cancer cell lines. Although not explored, eliminating FGF from external environment would potentially limit CSC growth in both NSCLC and SCLC.

Previously shown to increase the uptake of cisplatin to cancer cells, the calmodulin inhibitor CBP501 was also found to have anti-tumor effects by targeting the TME involving suppression of lung CSC activity⁸⁴. CBP501 could suppress the lipopolysaccharide (LPS)-induced production of IL6, IL10 and TNF by macrophages and subsequently resulted in reduction of stem cell properties in lewis lung carcinoma mouse model including decreased expression of the drug resistance molecule ABCG2, decreased spheroid formation and tumor initiation capacity, and reduced metastatic potential. Evaluation in clinic (NCT00942825), however, did not see additional benefit when CBP501 was employed along with cisplatin and pemetrexed as compared to cisplatin and pemetrexed regimen only. Progression free survival (PFS) was observed at 140 days vs. 165 days, respectively.

The VEGFR2 inhibitor apatinib has recently been explored for its effect towards EMT⁸⁵. It was reported that apatinib reduced macrophage-induced EMT reprogramming in NSCLC cell lines. Pre-treatment of apatinib to polarized macrophages led to inability of macrophage's secretion to induce migration and invasion. In-depth investigation led to the finding that apatinib blocked HGF and MET expression that are probably utilized by NSCLCs for EMT reprogramming. The efficacy of this therapeutic modality proves that diminishing the secretory support within TME is important to attenuate EMT induction that may also affect stemness maintenance of lung CSCs, although not examined. Several clinical trials evaluating the efficacy of apatinib have been initiated for NSCLC and SCLC. An active phase II trial

(NCT02515435) in pre-treated advanced non-squamous NSCLC patients reported a clinical endpoint with median OS and PFS of 7.69 and 3.06 months, respectively⁸⁶.

Together these studies suggest that targeting of the fibroblast and macrophage components of the TME may have therapeutic value although likely more relevant targets in the TME need to be identified. Whether these strategies also target lung CSCs remains to be demonstrated.

Targeting molecular features of lung CSCs

Targeting intricate mechanisms that maintain the undifferentiated stem cells state of lung CSCs is obviously an attractive therapeutic approach. Below several examples are discussed particularly those that have undergone clinical testing.

Based on the involvement of the Notch pathway in CSC maintenance, two clinical trials were initiated to explore its possible therapeutic value in NSCLC. RO4929097, a gamma-secretase inhibitor, was used in phase I dose-escalation study (NCT01193881) with erlotinib combination in stage IV or recurrent NSCLC patients and in a phase II efficacy study (NCT01193868) in chemotherapy-treated advanced NSCLC patients. Both studies have since been terminated due to halt in production of RO4929097. The phase II trial, however, revealed poor drug efficacy indicated by observation of progressive disease in all five patients. The same drug was also evaluated in clinical trial (NCT01217411) in combination with radiotherapy on patients with extensive SCLC with brain metastasis. Low accrual and discontinued drug production led to premature termination of the study.

Inhibition of embryonic signaling pathways including Notch may sound attractive, but there is a risk of normal homeostasis disruption since those pathways are still operating in normal adult lung. Besides, it is important to realize that when such pathways are acting as morphogenetic gradients, inhibiting one might risk in promoting the other subtype of lung cancers along the proximodistal axis, e.g., NFκB and Notch pathways contradicting activation in SCLC and NSCLC. Indeed, progression of NSCLC to SCLC has been observed during therapeutic resistance in the clinic, in which lung ADC in some patients transformed into SCLC displaying high grade neuroendocrine phenotypes after TKI treatment⁸⁷. The opposite transformation was seen when chemotherapy was employed to treat SCLC patients; Notch pathway in this case was proposed to be the double-edge sword that facilitated the resistance⁸⁸.

MYC represents another attractive target for therapy although direct therapeutic targeting of transcription factors remains challenging. The phytochemical compound, sulforaphane, was found to induce apoptosis and suppress CSC properties in NSCLCs⁸⁹. Sulforaphane accomplishes this inhibition through the modulation of miR-214 and subsequent transcriptional repression of MYC. Interestingly, in addition to MYC, also CTNNB1, CCNE1, EZH2 and survivin are targets of miR-214 and reduced by sulforaphane treatment. Furthermore, chemotherapeutics like cisplatin and doxorubicin could induce MYC expression leading to higher sphere-forming ability and proportion of CD133⁺ and ALDH⁺ cells, which could be reversed by sulforaphane and miR-214 mimic treatments. In addition, sulforaphane was able to sensitize NSCLC to chemotherapy both *in vitro* and *in vivo*. Its multi-targeting properties make sulforaphane a promising compound for further evaluation in clinical studies. A more targeted approach has been developed by overexpressing a dominant negative MYC,

named Omomyc, and recently its therapeutic impact was tested in SCLC. Omomyc was determined to inhibit cell growth and induce cell death in SCLC cell lines with or without amplification of *MYC*, *MYCL* and *MYCN*, all with genetically silenced *TP53* and *RBI*⁹⁰. Although effective in preclinical studies, application in the clinic is more difficult with respect to drug delivery issues. Regardless, targeting MYC family members that play an important role in the maintenance of lung CSCs provides a promising therapeutic option that remains to be further explored.

Epigenetic alterations have been connected with stemness in lung cancer. Preclinical studies with HDAC inhibitors, chemotherapy and targeted therapies have demonstrated efficacy in therapy-resistant NSCLCs^{75,91}. Several phase I/II clinical trials have employed HDAC inhibitors as single or combined treatments both in SCLC and NSCLC. CUDC-101, a multi-targeting inhibitor for HDAC, EGFR, and HER2 was evaluated in preclinical and clinical settings⁹². *In vitro* and *in vivo* studies showed anti-tumor activity in multiple tumor types, notably in erlotinib-resistant lung cancer models. A phase I clinical trial (NCT01171924) indicated the drug is well-tolerated when administered intravenously. Oral administration of the compound was also studied in phase I clinical trial (NCT01702285), however the study was recently terminated with unknown reason. A novel HDAC inhibitor named TMU-35435 has also been developed aiming to interfere with Wnt/ β -catenin pathway⁹³. TMU-35435 induces histone acetylations thus altering gene expression including negative regulators of Wnt/ β -catenin pathway that were reactivated such as *CDH1*, secreted frizzled related protein 4 (*SFRP4*), beta-transducin repeat containing E3 ubiquitin protein ligase (*BTRC*), *AXIN2*, retinoic acid receptor beta (*RARB*) and catenin beta interacting protein 1 (*CTNNBIP1*). Combined treatment with the demethylation agent 5-aza-dC synergized the anti-cancer effects both *in vitro* and *in vivo*. Usage of epigenetic modifying drugs leading to alterations in gene expression including those that control lung CSCs may have use in clinical settings.

To counter the effect of FAK in a self-sufficient CSC feedback loop, FAK inhibitor, VS-6063 (defactinib) was examined for efficacy. A phase I trial (NCT01943292) in non-hematologic malignancies and phase II trial (NCT01951690) in KRAS mutant NSCLC have been conducted to assess safety, tolerability, OS and PFS. The phase I trial concluded well-tolerable treatment with reversible adverse events like fatigue and hyperbilirubinemia⁹⁴. Results of the phase II trial have not yet been reported.

STAT3 is recognized as an attractive molecular target due to its frequent involvement in CSC maintenance. Napabucasin (BBI608), advocated as the first-in-class CSC inhibitor that blocks the transcriptional activity of STAT3 has been tested in combination with paclitaxel in previously treated patients with metastatic squamous or non-squamous NSCLC in a still-ongoing phase Ib/II clinical trial (NCT01325441)⁹⁵. The study concluded with well-tolerable and manageable adverse events with encouraging signs of anti-cancer activity. Due to more favorable observation in patients with non-squamous NSCLC, it was quickly brought to phase III clinical trial (NCT02826161) to compare its combination with paclitaxel and paclitaxel alone in this patient group. However, the study was prematurely terminated due to change of treatment landscape and evolving standard of care. It is not clear if the study will be continued, but meanwhile, three clinical trials aiming at evaluating different STAT3 inhibitors (AZD9150 and TTI-101) are still recruiting patients (NCT02983578, NCT03195699 and NCT03421353).

Targeting of the PI3K/AKT pathway has also been explored. The pan-AKT inhibitor, MK-2206, has been evaluated in both preclinical and clinical studies. In an *in vitro* study involving various erlotinib-sensitive and -resistant NSCLC cell lines, MK-2206 sensitized erlotinib-resistant NSCLC cell line to erlotinib treatment and inhibited the growth by inhibiting MET/HGF-dependent resistance mechanism, rendering them incapable of sphere-forming⁹⁶. The same concept was tested in the phase II clinical trial (NCT01294306) evaluating efficacy and tolerability in advanced NSCLC patients. No additional benefit was observed as patients with EGFR-mutated tumors had 10.6 months median OS compared to patients with EGFR wild-type tumors who had 11.1 months median OS.

In an effort to block aberrant signaling by MET receptor due to *MET* amplification and MET overexpression, ABBV-399, a MET antibody–drug conjugate was developed making use of the cytotoxic drug monomethylauristatin E (MMAE)⁹⁷. High efficacy of this therapeutic relies on certain threshold overexpression of MET as examined in NSCLC cell lines and patient-derived xenografts (PDXs). A recent ongoing phase I clinical trial (NCT02099058) revealed that monotherapy of the antibody–drug conjugate or combination with erlotinib was well-tolerated and exhibited anti-tumor activity with three MET⁺ treated patients of 16 displayed partial response⁹⁸. Phase II clinical trial (NCT03574753) evaluating overall response in patients with MET⁺ stage IV or recurrent SCC is still ongoing and future phase II clinical trial (NCT03539536) to determine best-suited population for the therapy will be performed.

Pharmacological drugs targeting CD117/KIT aiming at inhibiting the receptor tyrosine kinase activity have been explored. Three multikinase inhibitors that also exert inhibitory effect towards CD117 have entered clinical evaluations, namely anlotinib, famitinib and pazopanib. Anlotinib (AL3818) targets VEGFR1/2/3, FGFR1/2/3, PDGFRA/B, MET and CD117 and has been tested in phase II and III clinical trials (NCT01924195 and NCT02388919) addressing efficacy in patients with advance and previously treated NSCLC. Studies have been completed and reported superior performance of anlotinib over placebo group with median OS and PFS of 9.63 vs. 6.30 and 5.37 vs. 1.40 months, respectively⁹⁹. The encouraging results have resulted in new clinical studies also exploring the possibility of its incorporation into first and second line therapies in both NSCLC and SCLC. Pazopanib, with similar targets as anlotinib, also has been tested in NSCLC and most encouraging result were obtained in clinical evaluation for stage IIIB/IV NSCLC patients with progressive disease upon first line treatment of bevacizumab (NCT01262820). In the single intervention study, median PFS and OS were obtained at 10.9 and 24.1 weeks, respectively. Pazopanib has also been tested in combination with erlotinib (NCT01027598) and pemetrexed (NCT00871403) in both cases showing benefits for PFS but not OS.

The ALDH enzyme, often linked with CSCs, has also been evaluated for therapeutic value in preclinical studies. Liu and colleagues used disulfiram (DSF)/copper (Cu) complex for targeting ALDH1A1 positive lung CSCs¹⁰⁰. The complex was able to reduce the proportion of ALDH⁺ population in NSCLC cell lines and upon implantation as xenografts in nude mice. This was associated with treatment induced reduction of expression of NANOG, POU5F1 and SOX2, colony formation and invasion *in vitro* as well as decreased tumor size *in vivo*.

Lung CSCs are proposed to be less immunogenic and recent successes obtained with immune checkpoint inhibitors suggest that despite of this feature, lung CSC are likely

eliminated or kept in check. For example, a 1-year survival rate of as high as 51% in patients treated with nivolumab was observed who were previously treated with doublet platinum-based chemotherapy¹⁰¹. Although not previously evaluated in lung cancer, some targeting methods may be potentially interesting. For example, bifunctional immune checkpoint-targeted antibody–ligand traps have been produced that aside from targeting immune checkpoints, also can sequester cytokines suppressing immune responses. Ravi and colleagues generated an antibody against cytotoxic T-lymphocyte associated protein 4 (CTLA4), programmed cell death 1 (PDCD1, formerly known as PD1) or CD274 (formerly known as PDL1) fused to the ectodomain of TGFBR2 that is capable of sequestering TGFBI ligands¹⁰². Since within the milieu of lung tumor, TGFBI cytokines also control EMT reprogramming, hence this modality could be very potent.

Overall, studies to assess the possible clinical benefit of targeting molecular mechanism driving lung CSCs are limited. Thus far, no large clinical success has been reported and the value of this therapeutic approach remains to be demonstrated.

Conclusions and future directions

In this review, we aimed to provide a concise and comprehensive overview on the existence and therapeutic value of lung CSCs, both in NSCLC and SCLC, and also discussed the role of EMT in this context. Their origin and molecular mechanisms involved have been highlighted, providing evidence for the view that the occurrence and activation of facultative stem cells in the lung resembles the way in which their malignant counterparts arise. Epithelia within the lungs are not frequent cell-cyclers and only tissue injury is capable of triggering stem cell activity as well as EMT that are required for tissue repair. Upon continued injuries by inhaling DNA damaging/cytotoxic agents, the likelihood of sustained DNA damage and associated inflammation increases. Both genetic alterations and inflammatory conditions are the main inducers of malignant transformation of normal facultative stem cells into lung CSCs and their maintenance. However, direct evidence linking these processes is still largely missing and requires further research. As discussed, cellular context appears to be an important determinant in CSC development and different cells of origin have been proposed for NSCLC and SCLC, also providing an explanation for differences in molecular properties and behavior. The existence of lung CSCs has been demonstrated by multiple research teams based mainly on biomarkers expression alone or in combination with functional assays. However, the notorious unreliability of biomarkers and high plasticity of lung CSCs, as well as the existence of multiple CSC populations, continues to complicate a more precise identification. Moreover, the plasticity, the multiple regulatory mechanisms that can sustain lung CSC phenotypes and the likelihood of parallel existence of different lung CSC types provide a great challenge for developing effective targeted therapy. Both cell extrinsic signals, provided by the TME, as well as cell intrinsic molecular mechanisms have been found to regulate lung CSCs that at least partially overlap with the mechanisms relevant for maintaining normal lung homeostasis. Whether targeting TME may be sufficient to disrupt CSC maintenance is questionable considering the self-sufficient nature of CSCs. Indeed, lung CSC targeted therapies thus far tested in the clinic did not show encouraging results. This is in contrast to promising findings in preclinical models. A likely cause of the discrepancies of outcome between preclinical and

clinical studies can be attributed to the preclinical models used. Since CSCs and EMT regulations depend considerably on tissue context and signals derived from stromal components, frequently used long-term *in vitro* 2D-cultured established cell lines seem to be poor models. Also use of more direct patient-derived tumor cells alone in the absence of a stromal compartment or xenograft mouse models lacking a fully functional immune compartment lacks context that is relevant for studying CSC features. Although inevitably being only surrogates from reality, tumor-stroma co-culture models and genetically-engineered mouse models (GEMMs) likely provide better models to study lung CSC complexity and develop therapeutic approaches that may have more success in the clinic. Alternatively, human organ chip models using microfluidic technology that recapitulate the systemic set-up of an organ including breathing mechanics and microenvironment interactions may serve as a useful tool for drug discovery¹⁰³.

Conflict of interest statement

The authors declare that they have no conflict of interest.

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Chapter 3

A unique small cell lung carcinoma disease progression model shows progressive accumulation of cancer stem cell properties and CD44 as a potential diagnostic marker

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Abstract

Objectives: Cancer stem cells (CSCs) have been implicated in disease progression of aggressive cancers including small cell lung carcinoma (SCLC). Here, we have examined the possible contribution of CSCs to SCLC progression and aggressiveness.

Materials and methods: GLC-14, GLC-16 and GLC-19 SCLC cell lines derived from one patient, representing increasing progressive stages of disease were used. CSC marker expressions was determined by RT-qPCR and western blotting analyses, and heterogeneity was studied by CSC marker expression by immunofluorescence microscopy and flow cytometry. Colony formation assays were used to assess stem cell properties and therapy sensitivity.

Results: Increasing expression of stem cell markers MYC, SOX2 and particularly CD44 were found in association with advancing disease. Single and overlapping expression of these markers indicated the presence of different CSC populations. The accumulation of more homogeneous double- and triple-positive CSC populations evolved with disease progression. Functional characterization of CSC properties affirmed higher proficiency of colony forming ability and increased resistance to γ -irradiation in GLC-16 and GLC-19 compared to GLC-14. GLC-19 colony formation was significantly inhibited by a human anti-CD44 antibody.

Conclusion: The progressive increase of MYC, SOX2 and particularly CD44 expression that was accompanied with enhanced colony forming capacity and resistance in the *in vitro* GLC disease progression model, supports the potential clinical relevance of CSC populations in malignancy and disease relapse of SCLC.

Keywords: small cell lung cancer, *in vitro* tumor progression model, cancer stem cells, tumor heterogeneity, CD44

Introduction

Small cell lung carcinoma (SCLC) is a histological subtype of lung carcinoma with a 5 year survival rate of only 6% after diagnosis¹. Although only representing about 10% of total lung cancer incidence, SCLC has the highest mortality rate. SCLCs can be categorized into two cellular subtypes based on the presence (classic SCLC) or absence (variant SCLC) of neuroendocrine (NE) markers like neural cell adhesion molecule (NCAM), synaptophysin (SYP) and chromogranin A (CHGA)^{2,3}. However, recent understandings of the molecular biology of SCLC suggest that these subtypes maybe present as a subset of cells within one patient and as such constitute intratumoral heterogeneity⁴. Currently, SCLC patients are treated based on the extent of disease in accordance to tumor, node and metastasis (TNM) staging⁵. Early stage patients (T1–T3 with or without nodal involvement) undergo concurrent chemoradiotherapy consisting of cisplatin–etoposide regimen and high dose radiation to known tumor locations⁵. Later stage patients (also known as extensive disease, i.e., metastasized disease) receive platinum-based chemotherapies in combination with etoposide as first line treatment, and single agent topotecan in the second line and beyond⁵. Unfortunately, patients with SCLC are typically diagnosed with extensive disease and after initial good responses to chemotherapy, disease relapses due to therapy resistance. Novel treatments are highly warranted to improve the prognosis of this deadly lung cancer type.

The cancer stem cell (CSC) theory postulates that a subpopulation of cells possessing stem cell properties are capable and responsible for tumor maintenance and hence therapeutic resistance and metastasis⁶. This concept has been explored in SCLC towards some extent, for example by the identification of specific CSC subpopulations characterized by specific stem cell markers expression or features such as aldehyde dehydrogenase 1 (ALDH1), CD44, Prominin-1 (PROM1/CD133), POU domain, class 5, transcription factor 1 (POU5F1), SALL-like protein 4 (SALL4), SRY-box transcription factor 2 (SOX2), Hoechst dye exclusion side populations and exhibition of resistance to therapies⁷. Recent comparative transcriptomics of small cell neuroendocrine cancers from multiple epithelial origins, including SCLC, with adult stem cells revealed overlapping gene signatures that are associated with advanced tumors and poor clinical outcomes⁸. This suggests that SCLC displays stem cell properties, indicative of the presence and possible relevance of CSCs for disease development and progression.

In order to obtain more direct evidence of presence of CSCs in SCLC, we used our previously described SCLC *in vitro* progression model. This model consists of three SCLC cell lines named GLC-14, GLC-16 and GLC-19 that were isolated from three consecutive biopsies acquired during a clinical follow-up of an SCLC patient [9]. GLC-14, GLC-16 and GLC-19 represent different stages of disease, i.e., the tumor at diagnosis, first treatment-relapse and treated-relapsed-progressed cell line model, respectively. In the present study, expression analysis of a selected panel of CSC-related genes was performed in this unique model and several CSC characteristics were evaluated, including clonogenic growth and γ -irradiation sensitivity. In addition, the therapeutic value of CD44 was examined.

Methods

Cell culture

The Groningen Lung Carcinoma (GLC) cell lines were previously described by Berendsen *et al.*⁹. GLC-14, GLC-16 and GLC-19 were routinely maintained in RPMI 1640 medium (Thermo Fisher Scientific, Breda, The Netherlands) supplemented with 10% fetal calf serum (FCS, Bodinco B. V., Alkmaar, The Netherlands) in a 37°C humidified incubator under atmospheric condition with supplementation of 5% CO₂.

Irradiation and generation of resistant cells

All three cell lines were prepared for irradiation according to standard protocols for suspension cells. An IBL 637 Cesium-137 γ -ray source (CIS-BioInternational, France) was used to irradiate cells at 1, 2 or 4 Gy. To generate irradiation-resistant GLC-16 cells, cells were subjected to progressively increasing irradiation doses of 2, 4 and 6 Gy after each initial expansion and with at least two week time intervals.

RNA isolation, cDNA synthesis and real-time quantitative PCR analysis

Total RNA was isolated using RNeasy mini kit (Qiagen, Qiagen Benelux B. V., Venlo, The Netherlands) and reverse-transcribed into cDNA using an iScript cDNA synthesis kit (Bio-Rad, Bio-Rad Laboratories B. V., Veenendaal, The Netherlands). Prior to reverse transcription, RNA quality was confirmed with gel electrophoresis and Nanodrop 1000 (Thermo Fisher Scientific, Breda, The Netherlands) absorbance measurement at A230, A260 and A280. cDNA

(10 ng/reaction) was used as template for real-time qPCR analysis using iTaq Universal SYBR green supermix (Bio-Rad) on a CFX384 real-time PCR detection system (Bio-Rad). Forward and reverse primers (see supplementary Table 1) were used at 500 nM. PCR reactions were performed at 95°C for 3 min followed by 40 cycles of 95°C for 10 s and 60°C for 30 s. The quantification cycle (C_q) were calculated and relative gene expression was analyzed after normalizing for HPRT1 and GAPDH as house-keeping genes to yield $-\Delta C_q$. Data were normalized to the lowest $-\Delta C_q$ mean value. Three biological replicates of mRNA were collected for quantitative comparisons.

SDS-PAGE and western blotting

Protein was isolated by lysing cells using Pierce RIPA buffer (Thermo Fisher Scientific) supplemented with 1% of 100X Halt Protease Inhibitor (Thermo Fisher Scientific) and 1% of 100X Halt Phosphatase Inhibitor (Thermo Fisher Scientific). Protein lysates were quantified using Pierce BCA protein assay kit (Thermo Fisher Scientific) in accordance to manufacturer protocol using an iMark microplate reader (Bio-Rad). Protein lysates (20 μ g/lane) were separated by SDS-PAGE (8–15%) and transferred onto PVDF membranes. Membranes were blocked in 5% bovine serum albumin (BSA) prepared in Tris-buffered saline (TBS) containing 0.05% Tween-20 (TBST) and incubated overnight at 4°C with primary antibody at 1:1000 dilution. Primary antibodies used were: mouse anti- β -catenin 1 (610154) from BD Biosciences (Becton, Dickinson B. V., Vianen, The Netherlands); rabbit anti-MYC (5605), rabbit anti-MYCN (84406), mouse anti-SOX2 (4900) and rabbit anti-SOX9 (82630) from Cell Signaling Technology Inc. (Bioke, Leiden, The Netherlands). Membranes were washed with TBST and subsequently incubated with polyclonal HRP-conjugated goat anti-rabbit (P0448, Dako, Agilent Technologies Netherlands B. V., Amstelveen, The Netherlands) or rabbit anti-mouse (P0260, Dako) secondary antibodies at 1:2000 for 1 hr at room temperature (RT). Lumi-light plus western blotting substrate (Roche, Roche Diagnostics Nederland B. V., Flevoiland, The Netherlands) was used to establish chemiluminescent signal for protein bands detection in a ChemiDoc MP imaging system (Bio-Rad). B-actin (mouse anti-actin, 8691002, MP Biomedicals, Bio-Connect B. V., Huissen, The Netherlands) was used as loading control at 1:10000.

Immunofluorescence microscopy

Cells (2×10^5 cells/mL) were cytospun in a Shandon Cytospin 3 centrifuge (Thermo Fisher Scientific) onto the poly-L-lysine coated slides. Cells were fixed with 2% paraformaldehyde (PFA) for 15 min at RT followed by permeabilization using PBS containing 0.5% Tween-20 (PBST) for 15 min at RT. Blocking was performed with PBST containing 2% BSA and 1:50 normal goat serum (Dako) for 1 hr at RT followed by incubation with primary antibodies dilutions for 1.5 hr at RT. Primary antibodies consisting of rat anti-CD44 (103002, BioLegend, Amsterdam, The Netherlands), rabbit anti-MYC (5605) and mouse anti-SOX2 (4900) from Cell Signaling Technology Inc., were diluted in blocking buffer at 1:50. Polyclonal fluorophore-conjugated goat secondary antibodies consisting of anti-rat-Alexa Fluor 488 (A-11006), anti-mouse-Alexa Fluor 488 (A-11001), anti-mouse-Alexa Fluor 568 (A-11004) and anti-rabbit-Alexa Fluor 568 (A-11011) from Thermo Fisher Scientific were prepared in

blocking buffer at 1:200 and incubated for 1 hr at RT. DAPI (2 mg/mL, Sigma-Aldrich, Sigma-Aldrich Chemie B. V., Zwijndrecht, The Netherlands) was used at 1:1000 to stain nuclei. Isotype non-targeting antibodies were used as negative staining controls, namely rat IgG2b κ (400601, BioLegend), Pharmingen mouse IgG1 κ (554721, BD Pharmingen Inc., Becton, Dickinson B. V., Vianen, The Netherlands) and rabbit IgG (3900, Cell Signaling Technology Inc.). Slides were washed thrice with PBST after each staining step. Mounting medium (5% gelatin, 50% glycerol, pH 7.0) was applied along with coverslip and allowed to dry overnight. Stainings were visualized using the EVOS FL cell imaging system (Thermo Fisher Scientific).

Flow cytometry

Cells were fixed with 2% PFA for 15 min at RT. Permeabilization was performed with PBST for at least 15 min at RT. Cells were centrifuged and resuspended with PBST containing 10% FCS. Primary antibodies were diluted as described above for immunofluorescence staining at 1:500, 1:100 and 1:200, respectively for anti-CD44, anti-MYC and anti-SOX2, and incubated for 1.5 hr at 4°C. CD44 surface staining was performed on non-fixed and non-permeabilized cells for 30 min at 4°C. Polyclonal fluorophore-conjugated goat secondary antibodies consisting of anti-rat-Alexa Fluor 488 (A-11006), anti-mouse-Alexa Fluor 488 (A-11001), anti-mouse-Alexa Fluor 647 (A-21235) and anti-rabbit-Alexa Fluor 647 (A-21244) from Thermo Fisher Scientific were prepared in PBST containing 10% FCS at 1:200, and incubated for 1 hr (or 20 min for CD44) at 4°C. For triple stainings, goat anti-rat-Alexa Fluor 488 (A-11006, Thermo Fisher Scientific), goat anti-mouse-PE (1010-09, SouthernBiotech, Sanbio, Uden, The Netherlands) and goat anti-rabbit-Alexa Fluor 647 (A-21244, Thermo Fisher Scientific) were used at 1:200. Isotype non-targeting antibodies were used as negative staining controls as described in immunofluorescence microscopy. Washing was performed twice with PBST after each staining step. For CD44 stainings and washing steps, PBS was used instead of PBST. Finally, cells were resuspended into PBS containing 10% FCS and analyzed using the Accuri C6 (BD Biosciences) or FACSCalibur (BD Biosciences) flow cytometer.

Colony forming assays

For the Calcein–Hoechst staining colony forming assay, 6-well plates were coated with 1:30 medium-diluted Matrigel for at least 30 min at 37°C. Cells were seeded at 2×10^4 cells/well. After two weeks of incubation, the cells were stained with 0.1 $\mu\text{g/mL}$ of Hoechst 33342 (Sigma-Aldrich) and 0.1 μM of Calcein-AM (BioLegend) for 30 min at 37°C.

For soft agar assays in 6-well plates, 3% solutions of agar (Merck Life Science, Amsterdam Zuidoost, The Netherlands) and low melting temperature SeaPlaque agarose (Lonza, Breda, The Netherlands) were prepared separately in demineralized water and autoclaved. Medium consisting of 1:1 mixture of F-12 Nutrient Mix (Thermo Fisher Scientific) and DMEM low glucose (1 g/L) (Thermo Fisher Scientific) supplemented with 20% FCS was used to dilute the top agarose layer (0.3% (w/v)) and bottom agar layer (0.5% (w/v)). Cells were gently dissociated with a needle 20G x 1.5 inches (Microlance 3, BD Biosciences) and suspended in the top layer at 2×10^4 cells/well. After two weeks of incubation with or without irradiation, the cells were stained with 0.1 $\mu\text{g/mL}$ of Hoechst 33342 dye in PBS for 30 minutes at 37°C. Four non-overlapping micrographs were acquired for each well using EVOS FL.

ImageJ was used to obtain threshold of colony detection and to automatically count the colonies that were $\geq 0.01 \text{ mm}^2$ in size¹⁰. The counts from representative micrographs were used to extrapolate the colony forming efficiency percentage by multiplying the size factor of the well and divided by the number of cells initially seeded (2×10^4 cells/well). Cells were also stained with crystal violet (0.1% (w/v)) to obtain qualitative visualization of each well and captured by AID vSpot Spectrum plate reader. For CD44 blocking assay, GLC-19 cells were pre-treated for 24 hr with human anti-CD44 (550988, BD Pharmingen Inc.) and seeded for soft agar growth.

Cell viability assays

Control and irradiated GLC-14, GLC-16 and GLC-19 cells were cultured for 96 hr prior to cell viability assessment using MTS assay (Promega, Promega Benelux, Leiden, The Netherlands) according to manufacturer instructions. MTS absorbance was measured with a Multiskan Sky (Thermo Fisher Scientific) microplate reader at 490 nm. For assessment of human anti-CD44 antibody anti-proliferative effects in GLC-19, cells were seeded into opaque-well black 96-well plate and treated as indicated. The Synergy 2 multi-mode microplate reader (BioTek, BioSPX, La Abcoude, The Netherlands) was used to measure the Calcein signal at 488_{Ex}/520_{Em}.

Statistical analysis and graph/diagram plotting

Data were expressed as mean $\pm$ standard deviation (SD) or median $\pm$ interquartile range (IQR). Experiments were performed in three independent experiments unless otherwise stated. Statistical significance was evaluated by using ANOVA in GraphPad Prism 5 software (GraphPad software Inc., California, USA). Dunnett's or Tukey's multiple comparison test was used for Post-hoc test. Statistical significance was expressed as ***, $P < 0.001$; **, $P < 0.01$; *, $P < 0.05$. Graphs were plotted using GraphPad Prism 5 software. Venn diagrams were either plotted using Venn Diagram Plotter or eulerAPE¹¹.

Results

The GLC cell lines as SCLC progression model

Fig. 1A illustrates the generation of GLC-14, GLC-16 and GLC-19 cells during disease progression, representing tumor status at diagnosis and after first and second relapse after indicated treatments, respectively. GLC-14 was derived from a lymph node biopsy, whereas the other cells were obtained from the primary site, providing a temporally progressive, but not spatially similar model. The GLC cell lines grow as non-adherent aggregates with GLC-14 showing spheroid growth, GLC-16 displaying irregular aggregates and GLC-19 forming loose grape-like aggregates. When cultured on Matrigel-coated surfaces partially adherent growth and aggregates were formed (Fig. 1B).

Figure 1. The GLC small cell lung carcinoma cell line disease progression model. (A) The three cell lines, GLC-14, GLC-16 and GLC-19, were isolated at different stages of SCLC disease and treatments as indicated in the timeline. CDE is cyclophosphamide, doxorubicin and etoposide treatment regimen. Phase contrast micrographs depict spheroid/aggregate morphologies of *in vitro* cultures (white scale bar is 400 μ m). (B) GLC-14, GLC-16 and GLC-19 grown on Matrigel-coated surfaces formed partially adherent colonies with some structural variations consisting of attached aggregates as depicted in representative micrographs (white scale bar is 1000 μ m). Calcein (green), live-cells and Hoechst (blue), nuclei.

Expression of CSC-related markers in GLC cell lines

The expression of CSC-related markers was examined in the GLC model, anticipating an increase in marker expression during progressive disease. RT-qPCR was performed to evaluate the mRNA levels of a panel of CSC markers consisting of genes associated to drug resistance such as ATP binding cassette subfamily G member 2 (*ABCG2*) and aldehyde dehydrogenase family 1 member A3 (*ALDH1A3*), the stem cell transcription factors Myc proto-oncogene (*MYC*), *SOX2* and *SOX9*, and CSC-linked cell surface receptors *CD24*, *CD44* and integrin subunit alpha 6 (*ITGA6*). *MYC* and *CD44* transcript levels appeared to be strongly elevated in the more advanced disease models GLC-16 and GLC-19, for *CD44* especially in GLC-19 (Fig. 2A). The upregulation of *MYC* and cell surface *CD44* expression were also confirmed at the protein level (Fig. 2B and 2C). Notably, among the CSC transcription factors that are active in lung tissue, *MYCN* expression was only detected in GLC-14 consistent with earlier reported chromosomal amplification¹². Furthermore, an increased expression was seen for *SOX2* at the protein level, and *SOX9* and β -catenin 1 expression increments were especially

higher in GLC-16 (Fig. 2B). Overall, CD44, MYC and SOX2 are most consistently progressively enriched in the GLC model.

Figure 2. Expression of CSC-related markers in the GLC cell lines. (A) Graph showing relative mRNA levels of a panel of CSC-related genes using RT-qPCR. (B) Western blots showing expression of indicated stem cell transcription factors. (C) CD44 cell surface expression determined by flow cytometry. The percentage of CD44 positive cells in each cell line is indicated. Three independent experiments were performed. Data is expressed as mean $\pm$ SD.

CSC markers are heterogeneously expressed and progressively enriched in GLC cells

Since CD44, MYC and SOX2 were progressively enriched in the GLC disease progression model, we examined their expression patterns in more detail. Immunofluorescence microscopy analyses of co-stained GLC cells for SOX2/MYC, CD44/SOX2 and CD44/MYC confirmed progressive expression patterns and, moreover, showed heterogeneous staining patterns for all three markers (Fig. 3A–C). Both single and overlapping staining patterns were detected. GLC-14 cells were predominantly positive for SOX2 with lower MYC levels, and levels increased in GLC-16 cells. In GLC-19 cells, levels of SOX2 and MYC further increased and most notably levels of CD44 strongly increased. The expression of these CSC markers was further examined and quantified by flow cytometric analyses for more precise estimation of population size of the observed heterogeneity. Double-staining patterns for SOX2/MYC, CD44/SOX2 and CD44/MYC were generated and extrapolated to an overall expression pattern of all three markers for each individual cell per GLC cell line, which is proportionally visualized in Fig. 4A (see also Supplementary Fig. S1). The largest fraction of cells is positive for SOX2 in GLC-14 and GLC-16 cells, followed by the MYC positive subpopulation that is largely overlapping with SOX2 positive cells, although also a single positive MYC population is detectable. CD44 expression is low in GLC-14 and increases in GLC-16 cells, showing overlapping expression with SOX2 together with an increasing subpopulation of cells triple positive for SOX2/MYC/CD44. Whereas GLC-14 and GLC-16 are more heterogeneous for

expression of these CSC markers, interestingly, GLC-19 cells are predominantly triple positive or double positive for CD44/SOX2 with relatively smaller subpopulations of single positive cells. To further corroborate this, triple stainings were performed on GLC-19 cells, showing a similar marker distribution pattern with a large subpopulation of triple positive cells and CD44/SOX2 double positives, and a minority of cells being single positive, predominantly for CD44 (Fig. 4B). Furthermore, the mean expression level of the individual markers in each GLC cell line based on the fluorescence intensity in flow cytometric analyses showed largely similar levels of MYC and SOX2 per cell, whereas CD44 levels per cell increased in GLC-16 and GLC-19 (Fig. 4C). The detection of MYC in GLC-14 was not consistent with the data obtained in western blotting and immunofluorescence using the same antibody and may be due to cross-detection of MYCN under flow cytometry conditions.

Thus, the proportion of cells expressing all three CSC markers increased over time in the disease progression model and particularly CD44 expression was gradually enriched both at individual cell level as well as in the proportion of positive cells. The increment of the subpopulation positive for all three markers eventually results in a more homogeneous population of cells particularly in GLC-19 representing the most advanced stage of disease.

Figure 3. CD44, MYC and SOX2 expression in the GLC cell lines. (A–C) Immunofluorescence microscopy on SOX2/MYC, CD44/SOX2 and CD44/MYC double stained GLC cell lines. Single and overlapping stained cell subpopulations as indicated were detected. DAPI counterstaining visualized the nuclei. Scale bars represent 200 μm .

Figure 4. Quantification of CSC marker expression indicating a gradual increase of marker expression and occurrence of a triple positive CD44, MYC and SOX2 subpopulation in GLC-19 cells. (A) Flow cytometric analyses of SOX2/MYC, CD44/SOX2 and CD44/MYC double stained GLC cell lines was performed. The relative proportions of single, double and triple positive cells are also indicated in Venn diagrams. (B) Co-stainings of combinations of CD44, MYC and SOX2 were performed and expression was quantified by flow cytometry and visualized in a Venn diagram. (C) The expression level of each marker per positive individual GLC cell line was quantified by flow cytometry and depicted as mean fluorescence intensity. Data was collected from three independent experiments and is expressed as mean $\pm$ SD. Statistical significance is expressed as **, $P < 0.01$.

Colony forming efficiency and radiotherapy sensitivity of the GLC cell lines

Next, we examined colony forming potential of the GLC cell lines in soft agar plates, which can be taken as a measure for stemness. The basal levels of colony formation (colony forming efficiency) and colony size were determined. GLC-19 had the highest colony forming efficiency followed by GLC-16 and GLC-14 (GLC-19 > GLC-16 > GLC-14, $P < 0.001$, Fig. 5A and 5B left panel). The median colony size of the GLC cell lines appeared similar although the colony size varied more in GLC-16 and GLC-19 (Fig. 5B right panel). We continued by exposing cells to irradiation that is part of the SCLC treatment regimen. Cells exposed to mock, 1 or 2 Gy of γ -irradiation showed a dose-dependent reduction of colony forming efficiency (Fig. 5C), with GLC-14 showing lowest colony forming efficiency after irradiation, whereas GLC-16 and GLC-19 displayed around equal colony forming efficiencies (1 Gy, GLC-14 < GLC-16 and GLC-19, $P < 0.001$; 2 Gy, GLC-14 < GLC-16, $P < 0.05$; Fig. 5C and Supplementary Fig. S2). Average colony size was not affected (Fig. 5C).

We also tested irradiation sensitivity in short-term MTS cell viability assays. Somewhat unexpected, almost no reduction of cell viability/cell growth of GLC-14 and GLC-16 cells at all doses of irradiation was detected (Fig. 5D). GLC-19 was the most sensitive cell line displaying significant dose-dependent reduction of cell viability.

Together, these findings indicate that colony forming efficiency is more potent in GLC-16 and GLC-19, which also demonstrated higher resistance to radiotherapy. On the contrary, in MTS

assays GLC-19 cells were most sensitive, illustrating that colony formation potential and short-term cell viability determine different cellular properties.

Figure 5. Colony forming capability and radiotherapy resistance of the GLC cell lines. Dissociated GLC cells were suspended in soft agar plates and cultured for two weeks, and colony forming efficiency and colony size

were determined. (A) Basal levels of colony formation of the GLC cell lines are shown, after seeding 2×10^4 cells/well. (B) Quantified basal levels of colony numbers and sizes for the indicated cells are depicted. (C) Mock (control), 1 or 2 Gy γ -irradiated cells were seeded in soft agar and colonies numbers and sizes are depicted in graphs. (D) MTS assays were used for measuring cell viability of GLC cell lines 96 hr post γ -irradiation exposure at different doses. Colony forming efficiency is indicated as mean $\pm$ SD, and colony size as median $\pm$ IQR. Graphs are representative data from three independent experiments. Percentage annotated is the percentage of colony forming efficiency relative to the untreated control after pre-treatments. Statistical significance is expressed as ***, $P < 0.001$; **, $P < 0.01$; *, $P < 0.05$.

Efficacy of CD44 targeted therapy

As described above, we found that CSC markers CD44, MYC and SOX2 were associated with disease progression in the GLC model. An anti-CD44 blocking antibody was associated with disease progression in the GLC model. An anti-CD44 blocking antibody was examined for ability to inhibit clonal growth in GLC-19 cells with high levels of CD44¹³. GLC-19 cells were pre-treated with anti-CD44 antibody or an IgG control for 24 hr prior to seeding in soft agar. Colony forming efficiency was reduced with little impact on colony size (Fig. 6A and 6B). On the other hand, the anti-CD44 antibody did not affect cell viability of GLC-19 cells in short-term experiments (Supplementary Fig. S3). These data suggest that inhibiting CD44 may impact on CSC functioning and therefore limit their long-term survival and growth.

Figure 6. Targeting of CD44 in GLC as potential therapeutic strategy. Targeting of CD44 with human anti-CD44 antibody was examined for suppressing clonal growth. (A) Graphical representation shows colonies of GLC-19 grown in soft agar and after staining with crystal violet in response to 500 and 1000 ng/mL of anti-CD44 antibody (B) Representative graphs depict colony forming efficiency and the difference in size of colony formed in GLC-19 in response to control and anti-CD44 antibody at 500 and 1000 ng/mL. Data is expressed as mean $\pm$ SD (colony forming efficiency) or median $\pm$ IQR (colony size). Graphs are representative data from three independent experiments. Percentage of colony forming efficiency relative to the untreated control after pre-treatments is indicated. Statistical significance is expressed as ***, $P < 0.001$.

Discussion

In the present study, we examined the involvement of cancer stem cells (CSCs) in SCLC disease progression using the unique GLC cell model representing different stages of SCLC. Our findings show that *MYC*, *SOX2* and particularly *CD44* are progressively enriched in this *in vitro* disease progression model and are correlated with functional CSC properties such as increased colony formation and therapeutic (γ -irradiation) resistance supporting the CSC hypothesis.

The current CSC theory assumes that CSCs are the most malignant tumor cells and share characteristics with normal stem cells such that both are arranged in the apex of a hierarchical organization of multiple cell types that possess different proliferative and differentiation potencies¹⁴. Just like normal stem cells, CSCs are at the top of the hierarchy and function to replenish exhausted tumor (tissue for normal stem cells) through asymmetric cell divisions, creating a heterogeneous cellular tumor composition. Also, similar to normal stem cells, CSCs may exist in small numbers in early tumorigenesis, therefore representing a small subset in the population. However, as the disease and malignant properties progress, likely also as result of therapeutic pressure, the CSC proportion increases due to an aberrant shift to favor symmetric divisions that would only generate aggressive CSCs^{15,16}. In our GLC progression model, we could confirm such a pattern of progressive accumulation of CSC traits based on both marker expressions and functional assays (see Fig. S5). Multiple CSC populations gradually emerged in the GLC cell lines as evident by *CD44*, *MYC* and *SOX2* marker expressions which developed into a more homogeneous triple positive population at the most advanced stage. For instance, we noted that largely *SOX2*⁺ GLC14 cells had only a small *CD44*⁺ subpopulation that at later stages expanded to yield largely *CD44*⁺ GLC-19 cell populations, mostly triple positive. Since *CD44* is largely absent in GLC-14 cells, this marker is associated with later stages of disease progression. Consistent with the CSC theory, our functional characterizations of the GLC cells suggest an acquisition of irradiation resistance phenotypes during advanced disease. Together, these findings link disease progression of SCLC with progressively higher expression of CSC-related markers and CSC properties including therapy resistance.

SOX2 expression was detected in all GLC cell lines, suggesting its relevance for initiation and progression of SCLC disease. This is consistent with the known key role of *SOX2* in lung development and homeostasis, which is the initiation and identity maintenance of proximal epithelia. During lung development, *SOX2* is important for two crucial processes involving branching morphogenesis and epithelial cell differentiation, including the putative origin of SCLC—the pulmonary neuroendocrine cells (PNECs)¹⁷. During normal homeostasis maintenance, the proximal airway marker *SOX2* is responsible for differentiation of bronchiolar epithelium as evident by the lacking of differentiation when *Sox2* was deleted in murine progenitor cells¹⁸. The importance of *SOX2* in SCLC was highlighted by genomic analyses of primary SCLC tumors and cell lines that revealed *SOX2* DNA amplification in approximately 27% of the assessed samples¹⁹. Recent evidence from a SCLC mouse model with *Sox2* deletion suggested *SOX2*'s role as oncogenic driver of SCLC²⁰.

Previous characterization by Kok and colleagues showed that GLC cell lines possess DNA amplification and transcripts expression of *MYC* genes family consisting of N-myc proto-oncogene (*MYCN*) in GLC-14 and *MYC* in GLC-16 and GLC-19¹². The present study

confirmed their expression at the protein level. The *MYC* gene is well-known for regulating growth control of normal stem cells and CSCs²¹. DNA amplification of *MYC* is often considered an indication for tumor progression as it is frequently detected in SCLC cell lines derived from advanced disease²². Indeed, both GLC-16 and GLC-19 that were retrieved from biopsies isolated from treated and relapsed primary tumors showed high *MYC* levels. Moreover, *MYC* overexpression was suggested to drive SCLC progression from the typical classic subtype with high NE phenotypes to the variant subtype characterized by low NE phenotypes²³. In addition, while double/triple negative (*TP53*, *RBI* with/without *PTEN* deletions) often are sufficient to generate SCLC, overexpression of *MYC* was found to accelerate progression, i.e., from a differentiated cell into high NE SCLC and further into low NE SCLC or even non-NE SCLC. This phenomenon may be reflected in our progression model. Morphologically, there is progressive transition from classic SCLC with spheroid structure in GLC-14, semi-classic SCLC with irregular compact structure in GLC-16 to variant SCLC with loose aggregates in GLC-19, consistent with the morphologies described by Zhang and colleagues when culturing microdissected tumors from a *MYC*-driven genetically engineered mouse model (GEMM)³. On the other hand, amplification of *MYCN* is likely to occur initially at the early stage of tumor development to drive the maintenance of neuroendocrine phenotype before metastatic dissemination, as observed in our treatment-naïve model GLC-14^{21,24}. However, judging on the exclusivity of the DNA amplification of *MYC* and *MYCN* in our disease progression model, a common ancestor may have branched out before the occurrence of amplifications and assumes different fates at later stage.

CD44 expression is a rare occurrence in SCLC whether it is in cell lines or resected tumor tissues^{25,26}. However, data from our GLC cell lines shows a progressive enrichment of CD44 expression to become the major population in time. Preliminary data from our laboratory suggests that CD44 populations are selected under therapeutic pressure as stepwise increasing doses of γ -irradiation in GLC-16 cells is accompanied by gradual increased CD44 expression (Supplementary Fig. S4).

Colony forming efficiency was highest in GLC-19, followed by GLC-16 and GLC-14, in agreement with an increasing CSC phenotype during disease progression. Sensitivity to γ -irradiation was also in line with this, GLC-14 being the most sensitive, and GLC-16 and GLC-19 showing more equal levels of sensitivity. In contrast, GLC-19 was most sensitive to γ -irradiation in short-term cell viability assays, although it should be noted that this assay does not discriminate between CSC and non-CSC. Although speculative, this may be in part related to an observed somewhat higher proliferative activity of GLC-19 cell cultures. DNA repair capability may not be the major contributor to these differences because our preliminary observations demonstrated that all GLC cell lines were capable of repairing irradiation-induced DNA damage at similar rates (data not shown).

The CSC markers *MYC*, *SOX2* and particularly CD44 may have diagnostic and predictive values for SCLC patients. Although oncogenic *MYC* has been linked with progressive stages of SCLC, studies evaluating *MYC*'s predictive value in SCLC are absent from literature. However, genetically engineered SCLC mice models with *Rbl^{fl/fl}Trp53^{fl/fl}Myc^{LSL/LSL}* genotype had significantly higher mortality as compared to *Rbl^{fl/fl}Trp53^{fl/fl}Pten^{fl/fl}* mice, indicating a more aggressive phenotype of *MYC* overexpressing

tumors²³. In a previous study, CD44 and SOX2 did not have predictive value for overall survival in a small cohort of 38 SCLC patients²⁷. In contrast, another study reported that higher SOX2 expression correlated with poor prognosis of disease in a larger cohort of SCLC patients²⁸. Furthermore, loss of CD44 has been associated with poor prognosis in SCLC, contrary as to what might be expected from our *in vitro* data²⁹. However, it should be taken into account that evaluation of CSC biomarker expression by immunohistochemistry (IHC) in patient samples may be difficult due to possible specific niche localization of CSCs and/or to the unknown relevance of smaller or larger CSC populations with overlapping or distinct marker expression for overall malignancy.

Our study highlighted the need to develop inhibitors for progressively enriched CSC fractions which may contribute to therapeutic resistance and tumor recurrence. Inhibition of CD44 suggested a potential reducing effect of CSC activity. CD44 blockade is still not well-studied thus far. However several options may be employed, including monoclonal antibodies anti-CD44, synthetic peptides, aptamers, natural compounds, ligand–drug conjugates and CD44 decoys³⁰. Whether targeting MYC and SOX2 may have therapeutic value remains to be further tested, although therapeutic targeting of transcription factors still forms a challenge. Apart from BET inhibitor JQ1 to inhibit MYC transcription, natural compound sulforaphane and transduction with dominant negative MYC called Omomyc may be employed¹⁴. Specific vulnerability to aurora kinase inhibitors in *MYC*-amplified SCLC has received attention lately and needs further testing²³. Attempts to therapeutically block SOX2 remains difficult and is underexplored. Recently developed synthetic DNA-binding inhibitor of SOX2 may be further tested for its efficacy³¹.

The GLC disease progression model is not without limitations. Firstly, the GLC cell lines were obtained from only one patient with SCLC, limiting generalizability of our data. Secondly, GLC-14 was isolated from a lymph node biopsy and being a metastatic lesion likely has different properties than GLC-16 and GLC-19. A different tumor microenvironment may shape epigenomes of cells thus modifying the gene expression profiles. Thirdly, the ancestry of the three cell lines is not known and the assumption that they emerged from a common ancestor during tumor initiation remains speculative. Finally, *in vitro* culturing poses several caveats including selection of subclones, absence of microenvironment interactions and absence of physiological changes—all of which will influence the progression of disease. Despite this, the GLC model illustrates a parallel increase in CSC properties and disease progression.

In conclusion, by employing a unique *in vitro* SCLC disease progression model, we have obtained supportive evidence for the involvement of enriched different populations of CSC in disease progression. The therapeutic benefit of targeting identified CSC regulators, such as CD44, remains to be further examined.

Disclosures

The other authors have stated nothing to disclose.

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CRediT authorship contribution statement

Win Sen Heng: Conceptualization, Methodology, Validation, Formal analysis, Investigation, Data curation, Writing - original draft, Writing - review & editing, Visualization, Project administration. **Milind Pore:** Conceptualization, Investigation, Writing - review & editing. **Coby Meijer:** Conceptualization, Methodology, Writing - review & editing, Supervision. **T. Jeroen N. Hiltermann:** Conceptualization, Writing - review & editing. **Shiau-Chuen Cheah:** Writing - review & editing, Supervision. **Reinoud Gosens:** Methodology, Supervision, Writing - review & editing. **Frank A.E. Kruyt:** Conceptualization, Methodology, Resources, Writing - review & editing, Supervision, Funding acquisition.

Declaration of Competing Interest

The authors report no declarations of interest.

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Supplementary data

Supplementary Table 1. The primer sequences of genes used in RT-qPCR experiment.

Gene	Primer Sequence
HPRT1	F: 5'-CTCCGTTATGGCGACCC-3' R: 5'-CACCCTTTCCAAATCCTCAG-3'
GAPDH	F: 5'-CCTGCACCACCAACTGCTTA-3' R: 5'-GCAGTGATGGCATGGACTGT-3'
ABCG2	F: 5'-GGGTTCTCTTCTTCCTGACGACC-3' R: 5'-TGGTTGTGAGATTGACCAACAGAC-3'
SOX2	F: 5'-GGGAAATGGGAGGGGTGCAAAGAGG-3' R: 5'-TTGCGTGAGTGTGGATGGGATTGGTG-3'
CD24	F: 5'-ACAGCCAGTCTCTTCGTGGT-3' R: 5'-CCTGTTTTTCCTTGCCACAT-3'
CD44	F: 5'-CGGACACCATGGACAAGTTT-3' R: 5'-CCGTCCGAGATGCTGTAG-3'
ALDH1A3	F: 5'-AAAAAGAGCGAATAGCACCG-3' R: 5'-GCATAGAGGGCGTTGTAGCA-3'
SOX9	F: 5'-GCTCTGGGAGACTTCTGAACGA-3' R: 5'-CCGTTCTTCACCGACTTCCT-3'
ITGA6	F: 5'-CTGTGCTTGCTCTACCTGTCTG-3' R: 5'-CTCCCGTTCTGTTGGCTCTC-3'
MYC	F: 5'-GGAAAACAGCAGCCTCCCGC-3' R: 5'-ACGGCTGCACCGAGTCGTAGT-3'

A unique small cell lung carcinoma disease progression model shows progressive accumulation of cancer stem cell properties and CD44 as a potential diagnostic marker

Supplementary Figure S1. Graphical representation of CD44, MYC and SOX2 expressions in GLC cell lines. (a) SOX2/MYC, (b) CD44/SOX2 and (c) CD44/MYC were co-stained and 1 x 10⁴ events were included in the gating and analysis. Respective isotype controls / were used to gate the quadrant.

Supplementary Figure S2. Graphical representations of GLC cell lines colonies growth in soft agar after radiotherapy treatments. GLC cell lines were exposed to irradiation at indicated doses. Colonies were stained with crystal violet. Quantified data are shown in Fig. 5.

Supplementary Figure S3. Graph showing cell viability of GLC-19 cell lines in response to anti-CD44 treatment. GLC-19 cells were seeded overnight before being exposed to control IgG or anti-CD44 antibody for 96 hr and measured for cell viability with Calcein-AM. Data is expressed as mean $\pm$ SD.

A unique small cell lung carcinoma disease progression model shows progressive accumulation of cancer stem cell properties and CD44 as a potential diagnostic marker

Supplementary Figure S4. Graph showing CD44 expressions in irradiation-resistant clones of GLC cell lines. The percentage of CD44 expression was measured with flow cytometry and compared across different irradiation-resistant clones of GLC-16 cell line exposed to indicated doses sequentially. Data is expressed as mean $\pm$ SD. Statistical significance is expressed as **, $P < 0.01$; *, $P < 0.05$.

A

B

Supplementary Figure S5. Qualitative visualization of temporal characteristics of GLC cell lines. The measurement of (A) CSC marker and (B) functional phenotypes were scored at three levels, i.e., 0, 1, and 2, based on the relative comparison of respective phenotypes among GLC cell lines. Phenotypes with similar pattern may be categorized as interdependent phenotypes.

Chapter 4

Chelerythrine Chloride Downregulates β -Catenin and Inhibits Stem Cell Properties of Non-Small Cell Lung Carcinoma

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Abstract

Plant secondary metabolites have been seen as alternatives to seeking of new medicines for treating various diseases. Phytochemical scientists remain hopeful that compounds isolated from natural source could help alleviate the leading problem in oncology—the lung malignancy that kills an estimated two million people annually. In the present study, we characterized a medicinal compound benzophenanthridine alkaloid called chelerythrine chloride for its anti-tumorigenic activities. Cell viability assays confirmed its cytotoxicity and anti-proliferative activity in non-small cell lung carcinoma (NSCLC) cell lines. Immunofluorescence staining of β -catenin revealed that there was reduction of nuclear content as well as overall cellular content of β -catenin after treating NCI-H1703 with chelerythrine chloride. In functional characterizations, we observed favorable inhibitory activities of chelerythrine chloride in cancer stem cell (CSC) properties which include soft agar colony forming, migration, invasion, and spheroid forming abilities. Interesting observations in chelerythrine chloride treatment noted that its action abides to a certain concentration-specific-targeting behavior in modulating β -catenin expression and apoptotic cell death. The downregulation of β -catenin implicates to downregulate CSC transcription factors like SOX2 and MYC. In conclusion, chelerythrine chloride has the potential to mitigate cancer growth due to inhibitory actions towards tumorigenic activity of CSC in lung cancer and it can be flexibly adjusted according to concentration to modulate specific targeting in different cell lines.

Keywords: alternative medicine; natural compound; herbal compound; lung cancer; apoptosis; Wnt inhibitor; dosing; benzophenanthridine alkaloid.

Introduction

In the 21st century, we still very much rely on the testing of phytochemical compounds to facilitate drug discovery. It is not surprising that the next wonder drug hailed by Paul Ehrlich as a “magic bullet” was born through the search from nature. Growing interest sparks on plant-derived compounds because of their medicinally valuable bioactivities^{1,2}. Until today, plant products have been used in various aspects of life, most importantly for our daily needs that directly contribute to our well-being. Besides fulfilling our needs in foods and beverages, plant products have historically been used for herbs and remedies for various ailments. Phytochemical compounds are known as plant secondary metabolites. They are plants’ secondary needs that are not involved in their primary metabolism. For this reason, they exist in small concentrations and are unique in molecular structure. This can be useful in two ways in pharmacology: it can be used directly to treat diseases or it can serve as inspiration for novel drug discovery³. In addition, natural products have been used for consumption for as long as humans could try and experiment throughout history. This has endowed us with knowledge on which are safe to use and which may be harmful to consume.

Malignancy is not a new problem for mankind. The rate of incidence and death remains high every year, especially for lung cancer—the leading cause of cancer-related death. Statistically, lung cancer comprises 80%–85% incidence of NSCLC and 15%–20% of small cell lung carcinoma (SCLC); those numbers combined summed to around 2.1 million cases based on 2018’s estimation⁴. Although significant advancements have been observed over the

years for NSCLC disease management, some problems remain to be addressed, which include therapeutic resistance, toxicity, suitability, and cost-effectiveness⁵⁻¹⁰. From a cellular perspective, CSC's existence has been proposed to be the underlying factor for therapeutic resistance, relapse, and metastasis due to its role in maintaining and perpetuating the disease¹¹. The riddance of CSC may be an essential step toward tumor eradication.

Chelerythrine is a type III benzophenanthridine alkaloid isolated from herbal plants like *Chelidonium majus* and *Macleaya cordata*¹²⁻¹⁴. Those herbs have been utilized in folk medicine for various discomforts such as inflammation, pain, and cough, and recently isolated alkaloids including chelerythrine have been demonstrated to possess anti-bacteria, anti-fungal, and anti-tumor activities. At present, chelerythrine is most recognized as a potent inhibitor of protein kinase C (PKC) for isoform α and β ¹⁵. One of the earliest studies to demonstrate chelerythrine's anti-tumor activity was its cytotoxicity to a wide range of different histologies of cancer cell lines¹⁶. Studies over the years have successfully established the apoptotic-inducing ability of chelerythrine in a variety of cancer cells, namely in uveal melanoma, leukemia, prostate cancer, hepatoma, and renal cancer¹⁷⁻²¹. The latest reports suggest apoptosis is mediated through reactive oxygen species (ROS)-dependent endoplasmic reticulum (ER) stress induction and possibly through signal transducer and activator of transcription 3 (STAT3) inactivation^{22,23}. Studies also showed that chelerythrine was able to inhibit the growth of cisplatin-resistant NSCLC and triple-negative breast cancer cells as well as sensitizing them to chemotherapies^{24,25}. All of these suggest that chelerythrine has the potential to target CSC for thorough riddance of tumors.

Wnt/ β -catenin is one of the evolutionary-conserved signaling pathways in metazoan that is responsible for regulating cellular processes like proliferation, survival, motility, differentiation, and apoptosis²⁶. The pathway is activated upon the binding of Wnt ligand to Frizzled and low-density lipoprotein receptor-related protein 5/6 (LRP5/6) receptors. This association activates a sequential signaling cascade that involves an intracellular transducer like Disheveled to inactivate the constitutively functioning β -catenin degradation complex that constantly targets β -catenin for proteosomal recycling. In the absence of degradation complex activity, free β -catenin molecules translocate to the nucleus to associate with various adaptor proteins and transcription factors, and together they bind onto Wnt responsive elements to transcribe Wnt target genes for regulating the aforementioned cellular processes²⁷. During lung development and morphogenesis, the Wnt/ β -catenin pathway acts as the speciation control for distal epithelium differentiation²⁸. In adult lungs, its role in homeostasis maintenance of the distal epithelium remains consistent as indicated by the responsiveness of AXIN2⁺ alveolar progenitors of the lung toward Wnt signal and the requirement of Wnt signaling in epithelial differentiation of alveolar epithelial type II cells (AEC II) to terminally differentiated AEC I cells^{29,30}.

In NSCLCs, the Wnt/ β -catenin pathway may be aberrantly activated to support tumorigenesis^{31,32}. For instance, NSCLCs may self-sustain themselves by overexpressing Wnt ligands. Constitutive expression of β -catenin variants coupled with aberrant cytoplasmic stabilization could increase the activity of β -catenin-mediated transcriptions. In some other case, loss of heterozygosity or hypermethylation of adenomatous polyposis coli (APC) as part of the β -catenin destruction complex, or the presence of its variant, could also contribute to

hyperactivation of the Wnt/ β -catenin pathway. Alternatively, intracellular Wnt antagonists' expressions may be reduced or lost³³. Sustained canonical Wnt activity in NSCLCs leads to maintenance of the CSC component of the tumor, which consequently drives the progression of the disease³⁴. Hence, we seek to find out the growth-inhibiting mechanism of chelerythrine in NSCLCs by exploring its inhibitory action on the Wnt/ β -catenin pathway. Our findings suggested that chelerythrine chloride indeed possessed some inhibitory activity toward the Wnt/ β -catenin pathway through β -catenin downregulation. This inhibition is associated with reduced CSC properties that may potentially lead to improved CSC targeting in the future.

Results

Chelethyrtine Chloride Inhibited the Growth of NCI-H1703, SK-LU-1, and Human Lung Cancer Stem Cells (HLCSC)

To check whether the growth-inhibitory effect of chelerythrine chloride is applicable to major histological cell types of NSCLC, we used three cell lines consisting of squamous cell carcinoma, adenocarcinoma, and lung CSC cell lines. Using a real-time cell analyzer (RTCA) that evaluates cell viability based on impedance to detect cell attachment, we evaluated chelerythrine chloride's growth-inhibitory kinetic in NCI-H1703, SK-LU-1, and HLCSC. HLCSC was certified by endorsing companies to exert cancer stem cells properties, including markers expressions (CD133, aldehyde dehydrogenase (ALDH), stage-specific embryonic antigen 3/4 (SSEA3/4), alkaline phosphatase, octamer-binding transcription factor 4 (OCT4) and CD43), and in vivo tumorigenicity (<1000 cells). Figure 1 shows dose–response kinetic curves and dose–response curves for 24, 48, and 72 h treatment of chelerythrine chloride in the three cell lines. The treatment kinetic of chelerythrine chloride always started with rapid initial cytotoxicity indicated by curves with negative gradient, and this cytotoxic effect was more pronouncedly seen in mid-range (6.25 and 12.5 $\mu\text{g/mL}$) to high-treatment concentrations (25 and 50 $\mu\text{g/mL}$) in all three cell lines (Figure 1A–C). Growth arresting activity (flat curve) was observed as the after-effect of treatment in mid-range to high concentrations. A closer look at specific time-points after 24 h treatment intervals, NCI-H1703, SK-LU-1, and HLCSC were almost equally sensitive toward chelerythrine chloride at all treatment concentrations (Figure 1D–F). However, minor sensitivity observation from the dose–response curves deduced that NCI-H1703 was slightly more sensitive to 1.56 $\mu\text{g/mL}$ of chelerythrine chloride when compared to SK-LU-1 and HLCSC. This difference can be more clearly seen in differences of IC_{20} , IC_{50} , and IC_{80} values of chelerythrine chloride among the cell lines (Table 1). Interestingly, NCI-H1703 was slightly more resistant at higher treatment concentrations indicated by slightly higher IC_{80} values. All in all, chelerythrine chloride is a potent growth inhibitor of major histologies of NCLSC.

Figure 1. Dose–response curves of chelerythrine chloride treatments in NCI-H1703, SK-LU-1, and human lung cancer stem cells (HLCSC). Representative dose–response curves show the kinetic response of (A) NCI-H1703, (B) SK-LU-1, and (C) HLCSC toward chelerythrine chloride in a span of 72 h treatments. Curves show dose–response of NCI-H1703, SK-LU-1, and HLCSC toward chelerythrine chloride after (D) 24, (E) 48, and (F) 72 h treatment. Dose–response kinetic curves in (A–C) were plotted from duplicate data obtained in a representative of two independent experiments. Dose–response curves in (D–F) were derived from means of CI at specified time-points from two independent experiments.

Table 1. IC₂₀, IC₅₀, and IC₈₀ values of chelerythrine chloride.

Cell lines	IC ₂₀ (µg/mL) ¹		
	24 h	48 h	72 h
NCI-H1703	1.85 ± 0.04	1.81 ± 0.03	1.89 ± 0.05
SK-LU-1	2.35 ± 0.02	2.17 ± 0.04	2.18 ± 0.04
HLCSC	2.54 ± 0.08	2.73 ± 0.05	3.04 ± 0.08
Cell lines	IC ₅₀ (µg/mL) ²		
	24 h	48 h	72 h
NCI-H1703	3.30 ± 0.08	3.16 ± 0.05	3.15 ± 0.08
SK-LU-1	3.14 ± 0.03	3.17 ± 0.06	3.19 ± 0.06
HLCSC	3.20 ± 0.10	3.48 ± 0.06	3.88 ± 0.10
Cell lines	IC ₈₀ (µg/mL) ¹		
	24 h	48 h	72 h
NCI-H1703	5.87 ± 0.14	5.48 ± 0.09	5.23 ± 0.13
SK-LU-1	4.19 ± 0.04	4.62 ± 0.08	4.65 ± 0.09
HLCSC	4.04 ± 0.12	4.43 ± 0.08	4.94 ± 0.13

¹ IC_{20/80} values were estimated using the formula $IC_F = \left(\frac{100 - F}{F}\right)^{1/H} \times IC_{50}$, whereby F is the desired percentage of maximal inhibition, i.e., 20% or 80%, and H is hill slope/gradient of the non-linear regression curve. ² IC₅₀ data were based on mean values obtained from duplicated dose–response curves in two independent experiments.

Chelerythrine Chloride Reduces the Global Expression and the Proportion of Cells with Positive Nuclear β -Catenin

Due to its importance in lung development and homeostasis, as well as its aberrancies during tumorigenesis, the Wnt/ β -catenin pathway may be a lucrative target for inhibiting CSCs to ultimately mitigate therapeutic resistance, recurrence, and metastasis^{28,30,32,34}. In order to determine whether chelerythrine chloride treatment would impact the signaling of the Wnt/ β -catenin pathway, NCI-H1703, SK-LU-1, and HLCSC were treated with chelerythrine chloride and checked for β -catenin expression with immunofluorescence staining. This would not only check the expression level of β -catenin, but also its localization since its nuclear retention is necessary for transduction of Wnt/ β -catenin signaling^{35,36}. Prior to chelerythrine chloride treatment, GSK3i was added at 4 μ M to NCI-H1703 and at 8 μ M to SK-LU-1 and HLCSC. GSK3i inhibits GSK3 β —the negative regulator of β -catenin degradation machinery to stimulate β -catenin cytoplasmic stabilization and thus enhances nuclear localization. This mimics the phenomenon for negative regulators deregulations and β -catenin nuclear localization and retention during canonical Wnt activation. Visualization of immunofluorescence stainings clearly demonstrated some morphological changes as well as changes in expression level and localization status of β -catenin after chelerythrine chloride treatment (Figure 2A–C). DNA stains (DAPI or Hoechst 33342) revealed that chelerythrine chloride's treatment may have induced cell shrinkage and chromatin condensation as early signs of apoptotic cell death³⁷. These effects were seen at least in two treatment concentrations in all cell lines, namely at 1.5 and 3 μ g/mL. Moreover, the stretching pattern of healthy adherent NCI-H1703 and SK-LU-1 cells became more oval-shaped after the treatments. Not only was the average β -catenin cellular expression reduced, but the treatments also diminished the localization of β -catenin in the nucleus. This is indicated by the complete absence between overlapping colors of β -catenin (green or orange) and nuclear site (blue). Statistically, the reduction of cell number with positive nuclear β -catenin was observed in NCI-H1703 at 0.75 and 1.5 μ g/mL, in SK-LU-1 at 1.5 and 3 μ g/mL, and in HLCSC at 3 μ g/mL of chelerythrine chloride treatments ($p < 0.001$, Figure 2D). NCI-H1703 is interestingly more responsive to low concentrations of chelerythrine chloride and due to that we decided to focus on this cell line for downstream experiments. Western blotting on several CSC-related transcription factors including β -catenin revealed that there were downregulations of MYC, SOX2, and β -catenin upon chelerythrine chloride treatments, further suggesting the potential of chelerythrine chloride in impacting CSC activities (Figure 2E). In conclusion, chelerythrine chloride can be specifically concentration-adjusted to inhibit Wnt/ β -catenin in different lung cancer cells from different histological origins through β -catenin downregulation. The downregulation may negatively impact CSC properties and induce apoptosis.

Figure 2. Molecular implications of chelerythrine chloride treatment in lung cancer cell lines. NCI-H1703, SK-LU-1, and HLCSC were treated with GSK3i for 24 h and sequentially treated with various concentrations of chelerythrine chloride for 24 h. Subsequently, immunofluorescence stainings of β -catenin were compared among the cell lines. (A) Representative micrographs show immunofluorescence stainings for chelerythrine chloride treatment in NCI-H1703. (B) Representative micrographs show immunofluorescence stainings for chelerythrine chloride treatment in SK-LU-1. (C) Representative micrographs show immunofluorescence stainings for chelerythrine chloride treatment in HLCSC. Scale bars represent 100 μ m. (D) Representative quantification shows cell number with positive nuclear β -catenin in an independent immunostaining experiment. Error bars are expressed as mean $\pm$ SD. Most responsive chelerythrine chloride-treated cell lines—NCI-H1703's—protein lysates were resolved using Western blotting. (E) Representative Western blots show the effect of chelerythrine chloride treatment toward the expression of CSC-related transcription factors, namely β -catenin, MYC, and SOX2. Statistical significance was expressed as ***, $p < 0.001$.

Chelerythrine Chloride Partly Induces Apoptosis and Inhibits the Functions of CSC

The potential of compounds to inhibit CSC activities is best shown by demonstrating its ability to induce apoptosis and its inhibitory action toward CSC functions. ApoTox-Glo triplex

assay was first employed to measure cell viability, cytotoxicity, and late apoptotic activity (caspase-3/7 activity) from the same chelerythrine chloride-treated sets of NCI-H1703 cells. Significant reduction of cell viability was observed in 3 and 6 $\mu\text{g/mL}$ ($p < 0.05$ and $p < 0.01$, respectively) of chelerythrine chloride-treated cells (Figure 3A). In parallel with these, cytotoxicity was highly induced in the same treatment groups ($p < 0.001$). Apoptosis signal was detected in both 1.5 and 3 $\mu\text{g/mL}$ treatment groups, but interestingly, only 3 $\mu\text{g/mL}$ -treated cells displayed an elevated level of apoptosis ($p < 0.001$). This suggests that chelerythrine chloride may induce other forms of cell death in the 6 $\mu\text{g/mL}$ treatment group.

The most essential trait of CSCs to perpetuate tumors is by endowing themselves with self-renewal capability. This property can be demonstrated in vitro in soft agar colony-forming assay that determines the efficiency of colony formation under anchorage-independence growth condition. Figure 3B shows visual representations of NCI-H1703 colonies grown in soft agar after being treated with chelerythrine chloride at 1.5, 3, and 6 $\mu\text{g/mL}$. Generally, chelerythrine chloride induced dose-dependent inhibitory effect on colony-forming efficiency and complete inhibition was observed in the 6 $\mu\text{g/mL}$ treatment group. Concentration that has very little impact on NCI-H1703's cell viability, i.e., 1.5 $\mu\text{g/mL}$, also repressed soft agar formation. Similarly, the dose-dependent inhibitory effect was also observed in cells grown as anchorage-independent multicellular tumor spheroids—a micrometastasis model as shown in Figure 3C. Healthy spheroids grow as compact aggregates, whereas spheroids with compromised health appear loosely packed. Spheroid cell viability assay that is based on ATP production capacity demonstrated a dose-dependent reduction of cell viability in the spheroids upon chelerythrine chloride treatment at increasing concentrations (Figure 3D). Concentration at 1.5 $\mu\text{g/mL}$ again does not appear to interfere with cell growth during the treatment duration of 24 h. However, when estimating cell viability of monolayer NCI-H1703 cells using the same cell viability assay, the 1.5 $\mu\text{g/mL}$ treatment group appears to be more cytotoxic than 3 $\mu\text{g/mL}$. The discrepancy of this with the estimations from RTCA and ApoTox-Glo cell viability assay may suggest that 1.5 $\mu\text{g/mL}$ of chelerythrine chloride probably interrupts cell ATP production machinery without compromising cell attachment (RTCA) and membrane integrity (ApoTox-Glo cell viability). The effect in spheroid may in general be delayed by a slower diffusion rate of low-concentration chelerythrine chloride.

Besides colony formation, migration and invasion are two of the utmost important traits of surviving CSCs for distant secondary colony formation during therapeutic resistance. Hence it is imperative to determine whether chelerythrine chloride inhibitory effect also affects these properties. Significant decrement of migration and invasion of NCI-H1703 cells was seen after 16 h incubation of chelerythrine chloride at 1.5 $\mu\text{g/mL}$ (migration, $p < 0.05$; invasion, $p < 0.001$) (Figure 3E). Treatment at 3 $\mu\text{g/mL}$ effectively induced complete abolishing of both migration and invasion of NCI-H1703 (migration, $p < 0.001$; invasion, $p < 0.001$). After taking into account the cytotoxic effect of each treatment concentration, chelerythrine chloride retarded approximately 40% of both migration and invasion ability of NCI-H1703 in aforementioned treatment concentrations, respectively (Figure 3E and 3F).

Chelerythrine Chloride Downregulates β -Catenin and Inhibits Stem Cell Properties of Non-Small Cell Lung Carcinoma

Figure 3. Chelerythrine chloride induces apoptosis and inhibits CSC functions. (A) Parallel estimations of cell viability, cytotoxicity and apoptosis were performed after 24 h treatment of chelerythrine chloride in NCI-H1703 at indicated concentrations using ApoTox-Glo triplex assay. (B) Representative micrographs show three weeks grown soft agar colonies of NCI-H1703 upon treatment of chelerythrine chloride at indicated concentrations. Scale bars represent 1000 μm . (C) Representative micrographs show spheroids' morphological characteristics after 24 h treatment of chelerythrine chloride at indicated concentrations. Scale bars represent 200 μm . (D) Dose-response bar graph show comparison of cytotoxicity of various concentration of chelerythrine chloride between monolayer and spheroid models of NCI-H1703. (E) Estimations of migration and invasion of NCI-H1703 after 16 h of chelerythrine chloride treatment at indicated concentrations using real-time cell analyzer (RTCA). (F) Effect of corresponding treatment concentrations in (E) to cell viability is shown in bar graph. All data were obtained from mean of three independent experiments. Error bars are expressed as mean $\pm$ SD. Statistical significance was expressed as ***, $p < 0.001$; **, $p < 0.01$; *, $p < 0.05$.

Discussion

In this epoch of rapid technological advancement, a portion of the drug development research is still fueled by discoveries from natural resources. From safety perspective, naturally-derived phytochemical compounds are deemed superior, especially when acquired from those which have been incorporated into diet as foods, beverages, spices and even folk medicines for hundreds if not thousands of years³⁸. In oncology, some established chemotherapeutic drugs were also derived directly or indirectly from plant secondary metabolites, including paclitaxel, vinorelbine and topotecan³⁹. Many phytochemical compounds have been discovered hitherto and characterization works are still ongoing⁴⁰.

In the present study, chelerythrine chloride—a benzophenanthridine alkaloid was characterized for its potential to inhibit CSCs and their commonly found properties in NSCLCs. Kinetic profiles of the chelerythrine chloride's dose–response in three cell lines tested showed an overall similar pattern of cytotoxicity and growth arrest throughout the tested concentrations. We also checked the growth inhibition of chelerythrine chloride in a representative normal cell line—the lung fibroblast IMR-90 cell line, but we did not see selective inhibition of chelerythrine chloride toward cancer cell line (refer to Figure S4 and S5). However, extrapolations of IC₂₀ and IC₈₀ values from IC₅₀ values revealed interesting behavior of NCI-H1703 toward chelerythrine chloride treatment, i.e., a slight shift to higher sensitivity at low-concentration treatment (<IC₅₀ value) and slight shift to higher resistance at high concentration treatment (>IC₅₀ value) when compared to the other two cell lines. We also observed similar pattern of higher sensitivity with the significant reduction of β -catenin expression when treated with low, but not high concentrations of chelerythrine chloride—a dose-independent phenomenon that was also seen in the SK-LU-1, but may or may not be the case for HLCSC. Two plausible reasons may be able to explain this specific sensitivity. One reason is that the expression of WNT1 and WNT2 in NCI-H1703 confer dependence toward Wnt/ β -catenin signaling for growth^{41,42}. However, our immunofluorescence stainings revealed undetectable β -catenin expression in the absence of GSK3i treatment in NCI-H1703, further questioning how Wnt/ β -catenin signaling works in NCI-H1703. The other reason explains that NCI-H1703's squamous cell carcinoma–basal cell cancer identity predisposes sensitivity to SOX2 downregulation as part of the response we saw for chelerythrine chloride treatments^{43–45}. Co-downregulation, or rather co-upregulation of SOX2 and β -catenin is not a common sight in squamous cell lung carcinoma since they have contradicting regulating behavior in respect to their proximal and distal speciation functions^{28,43,46}. Nevertheless, chelerythrine chloride was able to inhibit the growth of lung cancer cell lines from different proximal/distal origins, the β -catenin nuclear localization in several lung histologies, and β -catenin and perhaps SOX2 and MYC expression regardless of the tissue origin. This wide applicability of chelerythrine chloride favors its development as cancer therapy. Furthermore, its sub-to-non-toxic specific targeting potentially offer a new therapeutic window for cancer therapy.

We also saw that only treatment at 3 μ g/mL of chelerythrine chloride was able to induce significant increase in apoptotic activity. Albeit insignificant, treatment at 1.5 μ g/mL was observed to induce apoptotic cell death as indicated by a two-fold increase of caspase-3/7 activity. Absence of apoptotic activity induction in 6 μ g/mL treatment group presumably indicates the occurrence of necrotic cell death as treatment of chelerythrine chloride at 6 μ g/mL

or higher witnessed a rapid decrease of viable cells. In addition, necrosis is commonly referred as default pathway to cellular death when apoptosis is inhibited, thus making it more likely to be the case⁴⁷. The observation of bimodal cell death occurrence due to chelerythrine treatments is not new as it has been reported by others in leukemia and uveal melanoma^{17,18}.

Cell death and ROS are two interrelated components. Decades ago, ROS was thought to be insignificant metabolic byproduct that is harmful if not cleared-off or neutralized in timely manner. However, current understandings highlighted the involvement of ROS as an important second messenger for signaling that regulates not only cellular process like cell death, but also proliferation⁴⁸. Modest production of ROS aids in normal functioning of cells, whereas excess amount may lead to lethal oxidative stress. Cellular intrinsic antioxidative machinery normally balances the destructive effect of excessive ROS⁴⁹. In the case of chelerythrine chloride treatment, there could be involvement of ROS generation beyond what the intrinsic cellular antioxidants could handle, thus leading to oxidative stress and cell death. Indeed, several studies including in NSCLC, prostate cancer and renal cell carcinoma confirmed that chelerythrine-induced cell death was mediated through ROS generation and was reversible upon pre-treatment of the ROS scavenging agent—*N*-acetyl-L-cysteine (NAC)^{22,23,50}. Different type of cell death can ensue depending on the amount of ROS generated. Apoptosis, autophagic cell death and necrosis are generally induced by low, moderate and high amount of ROS, respectively⁴⁸. In the present study, we observed induction of apoptosis in low concentration of chelerythrine chloride, but not in high, suggesting chelerythrine chloride might dose-dependently generate ROS. This observation was also reported by other investigators²³.

It was hypothesized that during therapeutic resistance, CSCs survive the therapeutic challenge and repopulate tumors locally and perhaps distantly¹¹. Equipped with self-renewal ability, they re-establish themselves and the tumor bulk. With their migration and invasion ability, they travel to distant organs as micrometastases to establish a secondary colony. In the present study, chelerythrine chloride was observed to inhibit these multi-steps of relapse and metastasis as shown by the reduction of soft agar colony formation, inhibition of spheroid growth, and reduction of both migration and invasion ability. This suggests that chelerythrine chloride potentially exhibited wide array effects of inhibitory to signaling that regulates cellular processes, in this case including signaling that uses β -catenin, MYC, SOX2, and possibly EMT machinery, which are commonly associated with proliferation, motility, differentiation, and survival.

Chelerythrine chloride treatment modulated great molecular responses in NCI-H1703 even at non-lethal concentrations. Because chelerythrine chloride is a PKC inhibitor, the observed molecular responses may be modulated through PKC inhibition. Indeed, PKC was identified as an additional component of the Wnt/ β -catenin pathway that acts to augment GSK3 β inhibition by canonical Wnt signaling⁵¹. Therefore, chelerythrine chloride plausibly abolished GSK3i-induced- β -catenin expression through PKC inhibition. This reasoning is further strengthened by an in vitro observation that confirmed the high affinity of pharmacological targets of chelerythrine chloride (PKC- α and - β) with GSK3 β as an inactivating phosphorylation target⁵². The only caveat to this explanation points to the conflicting response of MYC expression as a canonical Wnt downstream activating target⁵³. Although GSK3i treatment mimicked the cytoplasmic accumulation and nuclear localization of

β -catenin, MYC expression was not upregulated upon treatment. Furthermore, NCI-H1703 already possessed high MYC expression prior to the treatment. Hence, it is not clear whether complete abolishment of MYC is directly influenced by β -catenin downregulation.

As a matter of fact, we are currently still facing a major shortcoming in cancer treatments due to general toxicities. Whether it is due to an excipient or its non-specific cellular targets, even plant-derived chemotherapeutic drugs are still associated with a certain extent of toxicity. Traditional chemotherapy like paclitaxel works by enhancing microtubules' polymerization and stabilization, hence inducing cytotoxicity through disruption of the normal cell cycle progression during mitosis⁵⁴. The idea of targeting the machinery used during mitosis to induce cytotoxicity is the underlying reason for its non-exclusive targeting to cancer since cell division occurs also in normal cells for homeostasis maintenance⁵⁵. To improve cancer targeting using phytochemical compounds, the targeting may have to be specific, but at the same time flexible. In the present study, we assessed chelerythrine chloride's effect toward Wnt/ β -catenin signaling and we found that chelerythrine chloride can be flexibly adjusted according to concentration to trigger desired specific targeting in different cell lines. Nuclear localization of β -catenin was differently modulated by chelerythrine chloride at different concentrations in different cell lines. This suggests that using the correct concentration, chelerythrine chloride can be used to improve pathway targeting. This specific targeting may not significantly reduce cell viability, but is capable of diminishing self-renewal capacity—a desirable CSC targeting.

A recent study revealed the potential synergistic effect of increasing pathways targeting coverage in combinatorial regimens consisting of phytochemical compounds and chemotherapy⁵⁶. Phytochemical compounds also have the potential to alleviate toxicity induced by chemotherapy, further justifying their usage along with the traditional treatment modalities⁵⁵. Chelerythrine chloride has been thus far combined with erlotinib and cisplatin in pre-clinical studies involving NSCLCs, and respective authors have concluded the desirable synergistic or additive effect of the combined treatment^{24,57}.

Materials and Methods

Cell Culture

Human lung squamous cell carcinoma cell line NCI-H1703 and human lung adenocarcinoma cell line SK-LU-1 were purchased from American Type Culture Collections (ATCC, Manassas, Virginia, USA). Human lung cancer stem cells (HLCSCs) primary cell line was purchased from Celprogen, (Torrance, California, USA). NCI-H1703 and SK-LU-1 were routinely maintained in Gibco's Dulbecco's modified Eagle's medium (DMEM) with 4.5 g/L D-glucose (Thermo Fisher Scientific, Waltham, Massachusetts, USA) supplemented with 1% (v/v) of 100 mM Gibco's sodium pyruvate, 1% (v/v) of 10,000 U/mL Penicillin-10,000 μ g/mL Streptomycin (Pen-Strep), and 10% (v/v) of Gibco's fetal bovine serum (FBS). HLCSC was routinely maintained in human lung cancer stem cell complete growth medium with serum (Celprogen). All cell cultures were maintained in 5% CO₂ humidified atmospheric condition CO₂ incubator at 37°C. Passaging was performed regularly with Sigma-Aldrich's (St. Louis, Missouri, USA) 0.25% (w/v) trypsin-Ethylenediaminetetraacetic acid (EDTA) solution.

Chemicals

Chelerythrine chloride was purchased from Chromadex (Los Angeles, CA, USA). Dimethyl sulfoxide (DMSO, Sigma-Aldrich, St. Louis, Missouri, USA) was used to dissolve chelerythrine chloride and GSK-3 inhibitor X (Calbiochem, San Diego, California, USA) into 10 mg/mL and 1 mg/mL stock solution, respectively.

Cell Viability, Cytotoxicity and Anti-Proliferative Assay

Real-time cell analyzer (RTCA) was performed using an E-plate 16 on xCELLigence RTCA-Dual Purpose (DP) platform (ACEA Bioscience, San Diego, California, USA). Briefly, 50 μ L of complete medium was added into each well for 30 min incubation before acquiring background measurements. Optimized cell density (1×10^4 cells/well for SK-LU-1; 1.25×10^4 cells/well for HLCSC and NCI-H1703) was seeded upon background measurement and was incubated 30 min at room temperature in the biological safety cabinet in order to facilitate cells settling before putting back onto the RTCA-DP platform for overnight incubation. Subsequently, chelerythrine chloride was added to each well and incubated at various concentrations for up to 72 h. RTCA software 2.0 was used to generate kinetic dose-response curve by normalizing the cell index (CI) to the time when the compound was added. IC₅₀ values were estimated by plotting CI against concentration using algorithm for sigmoidal dose-response curve with variable slope.

CellTiter-Glo 3D cell viability assay (Promega, Madison, Wisconsin, USA) was used to evaluate the growth of spheroid culture (refer to spheroid generation section) along with the parallel run of monolayer culture (seeded at 1×10^4 cells/well). After 24 h treatment with chelerythrine chloride at indicated concentrations, each of the spheroids was transferred to a single well of an opaque-white plate for cell viability measurement according to the manufacturer's protocol. Monolayer culture was directly seeded in the opaque-white plate. Luminescence was measured as relative luminescence unit (RLU) by using FLUOstar Omega microplate reader (BMG LABTECH, Ortenberg, Germany). Each sample was normalized against respective vehicle control. GraphPad Prism v5.01 software (San Diego, California, USA) was used to plot concentration against the percentage of viability.

ApoTox-Glo triplex assay (Promega, Madison, Wisconsin, USA) was used to confirm cell viability along with cytotoxicity and apoptosis measurement. Apoptosis was measured by monitoring caspase-3/7 activity. Briefly, NCI-H1703 cells were seeded overnight in opaque-walled white microplate at 1×10^4 cells/well before administering chelerythrine chloride at indicated concentrations. After 24 h treatment, the plate was treated according to the manufacturer's protocol. Sequentially, cell viability and cytotoxicity were evaluated by measuring fluorescence at 400_{Ex}/505_{Em} and 485_{Ex}/520_{Em}, respectively, by using FLUOstar Omega microplate reader. Luminescence for caspase-3/7 activity measurement was then measured upon adding appropriate reagent.

Immunocytochemistry

NCI-H1703, SK-LU-1, and HLCSC were first treated with glycogen synthase kinase 3 inhibitor X (GSK3i) for 24 h upon growing overnight in order to artificially activate the Wnt/ β -catenin pathway before the onset of chelerythrine chloride treatment. Immunofluorescence was

performed by using Cellomics® Beta-Catenin Activation Kits (Thermo Scientific, Waltham, Massachusetts, USA) in accordance with the manufacturer's protocol or by staining with mouse anti-beta catenin 1 (cat# 610154, BD Biosciences, Franklin Lakes, New Jersey, USA) and counter-stained with anti-mouse-Alexa Fluor 488 (A-11001, Thermo Fisher Scientific) and DAPI (1:1000 of 2 mg/mL, Sigma-Aldrich). Five random, non-overlapping frames were captured from each well by using Axio Vert.A1 inverted microscope equipped with HXP-120V light source and Axiocam MR R3 camera (Carl Zeiss, Oberkochen, Germany) or EVOS FL cell imaging system (Thermo Fisher Scientific). Cells harboring nuclear β -catenin were manually counted by using Image J's cell counter plugin and the positive counts were divided by the respective total number of cells in the captured frames to obtain the percentage of cells with positive nuclear β -catenin⁵⁸. Treated samples were compared with untreated Wnt-activated negative control.

SDS-PAGE and Western Blotting

NCI-H1703 was treated with or without GSK3i for 24 h and administered with chelerythrine chloride for the following 24 h. Cells were harvested with rubber policeman and lysed with a mixture of Pierce RIPA buffer (Thermo Fisher Scientific), 1% of 100X Halt Protease Inhibitor (Thermo Fisher Scientific), and 1% of 100X Halt Phosphatase Inhibitor (Thermo Fisher Scientific). Protein quantification was performed using BCA protein assay kit (Thermo Fisher Scientific) following the manufacturer's protocol and measured using iMark microplate reader (Bio-rad, Basel, Switzerland) at 595 nm. Protein lysate was loaded into SDS-PAGE at 20 μ g and transferred onto a PVDF membrane. The membrane was blocked with 5% bovine serum albumin (BSA) prepared in Tris-buffered saline (TBS) containing 0.05% Tween-20 (TBST). Primary antibodies mouse anti-beta catenin 1 (cat# 610154, BD Biosciences), rabbit anti-MYC (cat# 5605, Cell Signaling Technology Inc., Danvers, Massachusetts, USA), and mouse anti-SOX2 (cat# 4900, Cell Signaling Technology Inc.) were applied overnight at 4°C at 1:1000. Upon incubation, TBST was used to wash unbound antibodies and subsequently applied with polyclonal HRP-conjugated goat anti-rabbit (cat# P0448, Dako, Jena, Germany) or rabbit anti-mouse (P0260, Dako) secondary antibodies at 1:2000 for 1 h at room temperature. Chemiluminescent signal was resolved using lumi-light plus Western blotting substrate (Roche, Basel, Switzerland) and visualized using ChemiDoc MP imaging system (Bio-rad). Mouse anti-actin (cat# 8691002, MP Biomedicals, Santa Ana, California, USA) was used as loading control at 1:10000.

Soft Agar Colony Formation Assay

The assay was performed as described previously with some modifications⁵⁹. Briefly, top/bottom agar was established at 0.35%/0.6% (w/v) with noble agar (BD Biosciences, Franklin Lakes, New Jersey, USA) in a 6-well plate. NCI-H1703 cells were suspended within the top agar at 5×10^4 cells. Treated and untreated groups were kept wet with either medium or medium containing chelerythrine chloride at adjusted concentrations by a renewal of every three days. After three weeks, the plate was collected and stained with 0.01% (v/v) of crystal violet for 30 min at room temperature. The plate was then washed three times with PBS. SZ51 zoom stereo microscope (Olympus, Shinjuku, Tokyo, Japan) was used to visualize the colonies,

and toupcam microscope digital camera (ToupTek, Zhejiang, China) equipped with Toupview software (ToupTek, Zhejiang, China) was used to capture the micrographs.

Spheroid Generation

NCI-H1703 was seeded at 400 cells/well in a Costar ultra-low attachment microplate (Corning, New York, NY, USA) by using the same DMEM medium used for monolayer culture. After an overnight incubation, matrigel (final concentration at 2.5% (v/v)) was added gently toward the center of the well to facilitate the compact formation of a spherical structure. The spheroid was optimized to grow to 300–400 μ m in diameter four days post-generation. The diameter of the spheroid was measured by using Zen 2 pro software (Carl Zeiss, Oberkochen, Germany).

Migration and Invasion Assay

RTCA-DP system was similarly used for migration and invasion evaluation. CIM-plate 16 that consists of two chambers was used for this purpose. Overnight serum-starved 4×10^4 NCI-H1703 cells were seeded with serum-free medium at the upper chamber upon background measurement, whereas medium containing 10% (v/v) of FBS was added to the lower chamber to serve as chemoattractant. Serum-free medium in the lower chamber served as control. For migration, no matrigel coating is necessary, but for invasion, 1:30 matrigel was used to coat the upper chamber 4 h prior to cell seeding. Chelerythrine chloride was added at the indicated concentration upon seeding. The migration–invasion was monitored for up to 24 h. RTCA software 2.0 was used to generate dose–response kinetic curve of both migration and invasion of NCI-H1703 by expressing CI against time.

Statistical Analysis

All data were expressed as mean $\pm$ standard deviation (SD). Statistical significance was evaluated by using one-way analysis of variance (ANOVA) in GraphPad Prism v5.01 software. Dunnet's multiple comparison test was used for post-hoc. Statistical significance was expressed as ***, $p < 0.001$; **, $p < 0.01$; *, $p < 0.05$. All data were collected from three independent experiments unless otherwise specified.

Conclusions

With our characterizations, we have confirmed that chelerythrine chloride exhibits cytotoxicity and an anti-proliferative effect in lung cancer cell lines. A specific effect like rapid cell death, which implies necrotic default cellular death, is typically induced at high concentrations. At lower concentrations, apoptosis might ensue. Concentration modulation also shifts sensitivity toward certain pharmacological actions such as the reduction of nuclear β -catenin localization observed in our study. Such concentration modulation is cell line-dependent. The current view for the usage of phytochemical compounds is in combination with chemotherapy. The usage of phytochemical compounds in cancer therapy will need to focus on manipulating their minimum effective dose and maximum tolerable dose. In pharmacology, dosing may be the key in improving the pharmacokinetics issue, but the current study also highlights that it can also enhance pharmacodynamics, particularly on how specific targeting is

desired. With this specific targeting possibility, dosing in pharmacokinetics and pharmacodynamics becomes a compounded challenge. Further study thus needs to embark on how dosing can be manipulated through these two pharmacological aspects.

Author Contributions

Conceptualization, W.S.H. and S.-C.C.; methodology, W.S.H. and S.-C.C.; formal analysis, W.S.H.; investigation, W.S.H.; resources, S.-C.C.; writing—original draft preparation, W.S.H.; writing—review and editing, W.S.H. and S.-C.C.; visualization, W.S.H.; supervision, S.-C.C.; project administration, W.S.H.; funding acquisition, S.-C.C. All authors have read and agreed to the published version of the manuscript.

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Conflicts of Interest

The authors declare no conflict of interest.

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Supplementary data

Figure S1. Kinetic dose–response curve of NCI-H1703 in independent experiment 2.

Figure S2. Kinetic dose–response curve of SK-LU-1 in independent experiment 2.

Figure S3. Kinetic dose–response curve of HLCSC in independent experiment 2.

Figure S4. Kinetic dose-response curve of IMR-90 in independent experiment 1.

Figure S5. Kinetic dose-response curve of IMR-90 in independent experiment 2.

Figure S6. Compilation of western blots of MYC, β -catenin and SOX2 in NCI-H1703 after chelerythrine chloride treatment.

Chapter 5

Identification of Phytochemical-Based β -Catenin Nuclear Localization Inhibitor in NSCLC: Differential Targeting Population from Member of Isothiocyanates

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Abstract

Decades of research has convinced us that phytochemical compounds contained within the plant products are the real deal, and they provide benefits such as health maintenance and cure to illnesses. One of the deadliest noncommunicable diseases today is lung cancer, hence its disease management still deserves attention. Wnt/ β -catenin pathway activation conferring cancer stem cell (CSC) activities to non-small cell lung carcinomas (NSCLCs) may explain why the disease is still difficult to cure. In the present study, we assessed several representatives of phytochemical categories consisting of alkaloids, chalcones and isothiocyanates for their inhibitory activity to nuclear localization of β -catenin—an important event for Wnt/ β -catenin pathway activation, in lung cancer cell lines. Real-time cell analyzer confirmed that evodiamine (EVO), chelidonine (CHE), isoliquiritigenin (ISO), licochalcone-A (LICO), benzyl isothiocyanate (BI) and phenethyl isothiocyanate (PI) exhibited anti-proliferative activities and cytotoxicities to adenocarcinoma cell line SK-LU-1 and human lung CSC primary cell line (HLCSC). Immunofluorescence assay identified that CHE, ISO, LICO, BI and PI were capable of reducing the number of cells harboring β -catenin within the nuclei of these cells. We extended the characterizations of BI and PI in Wnt-dependent squamous cell carcinoma cell line NCI-H1703 on several CSC functions and found that BI was better at inhibiting soft agar colony formation as an output of self-renewal ability, whereas PI was more effective in inhibiting the growth of multicellular tumor spheroid model mimicking micrometastases. Both however were not able to inhibit migration and invasion of NCI-H1703. In conclusion, BI could potentially be used as a safer alternative to target undifferentiated CSCs as adjuvant therapy, whereas PI could be used as chemotherapy to remove bulk tumor.

Keywords: alternative medicine; natural compound; lung cancer; β -catenin nuclear localization inhibitor; alkaloid; chalcone; isothiocyanate

Introduction

Plants and their products have been indispensable source of living for many if not all organisms. The live support that they provide for mankind is most notably demonstrated by their use for shelter, clothing, food and health-promoting remedies. In particular, the benefits offered for health are accounted by plant secondary metabolites in foods and herbs called phytochemicals that still present as important source of drug candidates today mainly due to their highly variable chemical structures that can be used to prevent or treat illnesses, directly or indirectly^{1,2}.

Lung cancer is still among the deadliest noncommunicable disease and malignancy today with around two million annual new cases based on an estimation from 2018³. Non-small cell lung cancer (NSCLC) hugely contributes to lung cancer's high incidence annually at approximately 85% of total lung cancer cases. In contrast to small cell lung cancer (SCLC) subtype that only accounts for 15% of total lung cancer incidence, NSCLC, which is mainly classified as adenocarcinoma (ADC), squamous cell carcinoma (SCC) and large cell carcinoma (LCC), grows and spreads more slowly, hence has better prognosis⁴. NSCLC also has better treatment options available which include radiotherapy and surgery for early stage disease (I or II) and (neo)adjuvant chemotherapy and immunotherapy for late stage disease (III or IV)^{4,5}.

Some patients presenting with mutated biomarkers may benefit from the use of tyrosine kinase inhibitor (TKI) drugs^{4,5}. Although immunotherapy consisting of immune-checkpoint inhibitors has made some breakthrough in recent years, some challenges remain to be addressed, including suitability, toxicity and responsiveness⁶⁻⁸. Nevertheless, NSCLC patients' five-year survival rate for early, locally advanced and advanced stage of disease is still lower than 18%⁴.

Tumor recurrence and metastasis are two clinical phenomena that were proposed as consequences associated to cancer stem cell (CSC) activities. Within tumor bulk, this subpopulation is therapeutically resistant and capable of reprogramming itself through epithelial-to-mesenchymal transition (EMT) into metastatic cancer cell⁹. Therefore, the clinical aim of cancer treatments is both tumor bulk eradication and CSC elimination. In NSCLC, CSC maintenance and perpetuation rely on Wnt/ β -catenin pathway aberrant activation, which is mostly contributed by changes of methylation status of Wnt/ β -catenin pathway negative regulators leading to pathway deregulation^{10,11}. The negative regulators of Wnt/ β -catenin work by preventing intracellular transducer β -catenin to localize within nucleus for Wnt target genes transcriptions¹⁰.

Alkaloids, chalcones and isothiocyanates are among the most studied phytochemical groups in NSCLCs in the last ten years. However, their specific effect in targeting nuclear localization of β -catenin—an event that positively affects the activation of canonical Wnt pathway—is not well-studied. Moreover, the majority of the studies only focused in characterizing the most commonly used adenocarcinoma cell line A549. Hence, in the present study, we studied alkaloids represented by chelidonine (CHE) and evodiamine (EVO), chalcones represented by isoliquiritigenin (ISO) and licochalcone-A (LICO) and isothiocyanates represented by benzyl isothiocyanate (BI) and phenethyl isothiocyanate (PI) in lung cancer cells. We initially used real-time cell analyzer (RTCA) to obtain growth inhibitory profiles and IC₅₀ values of the selected phytochemicals in ADC cell line SK-LU-1 and CSC primary cell line human lung CSC (HLCSC) and evaluated their inhibitory activity towards nuclear localization of β -catenin. Subsequently, we further examined phytochemicals capable of inhibiting nuclear localization of β -catenin with CSC functional assays in SCC cell line NCI-H1703. Interestingly, we found that phytochemicals belonging to the same group tend to have similar cell line-specific cytotoxic profiles with one or another displaying higher activity towards either of the tested cell lines. This difference was consistently observed when we zoomed in to see BI and PI's effects towards CSC functions.

Results

The Growth Inhibitory Profiles and IC₅₀ Values of Alkaloids, Chalcones and Isothiocyanates

To obtain the growth inhibitory profiles of selected phytochemicals, we used real-time cell analyzer (RTCA) that measures impedance difference to assay cell attachment-dependent cell viability. The dose-response profiles are capable of revealing whether the assessed compounds exhibit anti-proliferative activity and cytotoxicity. We used SK-LU-1 as representative of differentiated ADC tumor cell line and HLCSC as representative of undifferentiated CSC cell line. HLCSC was characterized by endorsing company to enrich with CSC properties, including markers expressions (CD133, aldehyde dehydrogenase (ALDH), stage-specific embryonic antigen 3/4 (SSEA3/4), alkaline phosphatase, octamer-binding

transcription factor 4 (OCT4) and CD43) and in vivo tumorigenicity (< 1000 cells). Figure 1 shows the growth inhibitory profiles of representatives of alkaloid, chalcone and isothiocyanate in SK-LU-1 and HLCSC. Phytochemicals exhibited dose-dependent cytotoxicity and anti-proliferative activity which were most consistently seen at ≤ 6.25 $\mu\text{g/mL}$ treatments. A two-way repeated measures ANOVA was used to statistically analyze the effects of time, treatment concentrations and the interactions of those two variables towards the growth of SK-LU-1 and HLCSC. Table 1 shows the summary of the analyses. Treatments variable always had the largest effect size towards response difference displayed in the curves, except for alkaloids treatments in HLCSC, in which time had a larger effect size than the other variables. Phytochemicals in the same category noticeably have more similar growth inhibitory profiles in a cell line-specific manner. For instance, both EVO and CHE had early cytotoxicity (negative gradient) and late growth arrest (flat curve) in SK-LU-1 at all tested concentrations, except 1.56 and 3.13 $\mu\text{g/mL}$ treatments for which some recovery (positive gradient) was seen ($p < 0.001$). In HLCSC, EVO and CHE similarly induced multiple growth arrest occasions transitioning in between recoveries ($p < 0.001$); however, at least two tested concentrations (1.56 and 3.13 $\mu\text{g/mL}$) of EVO and CHE did not evoke significant reduction of growth ($p > 0.05$). BI and PI were highly cytotoxic, killing SK-LU-1 and HLCSC at an early time-point at treatment concentration of ≥ 6.25 $\mu\text{g/mL}$ ($p < 0.001$). They were also anti-proliferative towards HLCSC at early time-point of 1.56 and 3.13 $\mu\text{g/mL}$ treatments. As expected, PI did not significantly induce HLCSC cell killing at these concentrations, whereas BI's was only unable to reduce significant growth at 1.56 $\mu\text{g/mL}$ treatment ($p > 0.05$). LICO and ISO are the least similar group and two of the least effective phytochemicals tested. They induced a complete cell killing in SK-LU-1 and HLCSC at 25 and 50 $\mu\text{g/mL}$ treatments (only at 50 $\mu\text{g/mL}$ for ISO in SK-LU-1; $p < 0.001$). In LICO- and ISO-treated SK-LU-1, at least three concentrations (1.56, 3.13 and 6.25 $\mu\text{g/mL}$) failed to induce significant inhibition, whereas this occurred in HLCSC for at least the two lowest mentioned concentrations ($p > 0.05$). None of the phytochemical compounds tested was as effective as positive control compound SAL that induced either cytotoxicity or growth arrest in SK-LU-1 and HLCSC at all treatment concentrations, except in 1.56 $\mu\text{g/mL}$ -treated SK-LU-1 (Figure S1; $p < 0.001$).

Identification of Phytochemical-Based β -Catenin Nuclear Localization Inhibitor in NSCLC: Differential Targeting Population from Member of Isothiocyanates

Figure 1. Dose-response curves depicting growth inhibitory kinetics of phytochemicals representatives of alkaloids, chalcones and isothiocyanates in SK-LU-1 and HLCSC. Various phytochemicals were administered at indicated concentrations after initial overnight growth, and the growth was continuously monitored for at least 72 h post-treatment. Curves presented are representative curves from two independent experiments. Error bars are expressed as mean $\pm$ SD from duplicated wells.

Table 1. Summary of the two-way repeated measures ANOVA analysis results of alkaloids, chalcones and isothiocyanates in SK-LU-1 and HLCSC.

Phytochemical Category	Phytochemical Compound	Cell Line	Independent Variables	Results
Isothiocyanate	BI	SK-LU-1	Time	F (184, 1288) = 992.6, $p < 0.0001$, $R^2 = 0.08$
			Treatment	F (6, 1288) = 1095, $p < 0.0001$, $R^2 = 0.79$
			Interaction	F (1104, 1288) = 250.8, $p < 0.0001$, $R^2 = 0.13$
		HLCSC	Time	F (184, 1288) = 352.5, $p < 0.0001$, $R^2 = 0.12$
			Treatment	F (6, 1288) = 301.2, $p < 0.0001$, $R^2 = 0.65$
			Interaction	F (1104, 1288) = 105.6, $p < 0.0001$, $R^2 = 0.22$
	PI	SK-LU-1	Time	F (184, 1288) = 195.1, $p < 0.0001$, $R^2 = 0.03$
			Treatment	F (6, 1288) = 1584, $p < 0.0001$, $R^2 = 0.85$
			Interaction	F (1104, 1288) = 116.9, $p < 0.0001$, $R^2 = 0.12$
		HLCSC	Time	F (184, 1288) = 4931, $p < 0.0001$, $R^2 = 0.16$
			Treatment	F (6, 1288) = 777.7, $p < 0.0001$, $R^2 = 0.67$
			Interaction	F (1104, 1288) = 851.8, $p < 0.0001$, $R^2 = 0.17$
Chalcone	LICO	SK-LU-1	Time	F (184, 1288) = 362.4, $p < 0.0001$, $R^2 = 0.11$
			Treatment	F (6, 1288) = 180.9, $p < 0.0001$, $R^2 = 0.78$
			Interaction	F (1104, 1288) = 56.6, $p < 0.0001$, $R^2 = 0.10$
		HLCSC	Time	F (184, 1288) = 1362, $p < 0.0001$, $R^2 = 0.27$
			Treatment	F (6, 1288) = 201, $p < 0.0001$, $R^2 = 0.55$
			Interaction	F (1104, 1288) = 149.5, $p < 0.0001$, $R^2 = 0.18$
	ISO	SK-LU-1	Time	F (184, 1288) = 365.3, $p < 0.0001$, $R^2 = 0.12$
			Treatment	F (6, 1288) = 307.5, $p < 0.0001$, $R^2 = 0.75$
			Interaction	F (1104, 1288) = 62, $p < 0.0001$, $R^2 = 0.13$
		HLCSC	Time	F (184, 1288) = 1985, $p < 0.0001$, $R^2 = 0.31$
			Treatment	F (6, 1288) = 362.8, $p < 0.0001$, $R^2 = 0.52$
			Interaction	F (1104, 1288) = 175.1, $p < 0.0001$, $R^2 = 0.17$
Alkaloid	EVO	SK-LU-1	Time	F (184, 1288) = 151.7, $p < 0.0001$, $R^2 = 0.08$
			Treatment	F (6, 1288) = 52.7, $p < 0.0001$, $R^2 = 0.76$
			Interaction	F (1104, 1288) = 47.9, $p < 0.0001$, $R^2 = 0.15$
		HLCSC	Time	F (184, 1288) = 1929, $p < 0.0001$, $R^2 = 0.48$
			Treatment	F (6, 1288) = 206.3, $p < 0.0001$, $R^2 = 0.33$
			Interaction	F (1104, 1288) = 130.5, $p < 0.0001$, $R^2 = 0.19$
	CHE	SK-LU-1	Time	F (184, 1288) = 59.4, $p < 0.0001$, $R^2 = 0.06$
			Treatment	F (6, 1288) = 38.9, $p < 0.0001$, $R^2 = 0.74$
			Interaction	F (1104, 1288) = 31.4, $p < 0.0001$, $R^2 = 0.18$

Table 1. Summary of the two-way repeated measures ANOVA analysis results of alkaloids, chalcones and isothiocyanates in SK-LU-1 and HLCSC (continued).

Phytochemical Category	Phytochemical Compound	Cell Line	Independent Variables	Results
Alkaloid	CHE	HLCSC	Time	$F(184, 1288) = 328.3, p < 0.0001, R^2 = 0.49$
			Treatment	$F(6, 1288) = 121.8, p < 0.0001, R^2 = 0.34$
			Interaction	$F(1104, 1288) = 17.2, p < 0.0001, R^2 = 0.16$
Positive control	SAL	SK-LU-1	Time	$F(184, 1288) = 71.5, p < 0.0001, R^2 = 0.04$
			Treatment	$F(6, 1288) = 80.9, p < 0.0001, R^2 = 0.79$
			Interaction	$F(1104, 1288) = 47.1, p < 0.0001, R^2 = 0.16$
		HLCSC	Time	$F(184, 1288) = 933.5, p < 0.0001, R^2 = 0.15$
			Treatment	$F(6, 1288) = 591.4, p < 0.0001, R^2 = 0.65$
			Interaction	$F(1104, 1288) = 205.4, p < 0.0001, R^2 = 0.20$

The growth inhibitory effectiveness of phytochemicals was also determined by evaluating IC_{50} values from specific time-intervals of dose–response curves. Table 2 summarizes the IC_{50} values of respective phytochemicals in SK-LU-1 and HLCSC at 24, 48 and 72 h post-treatment. As similarly shown by the dose–response curve, the IC_{50} values indicate that SAL was more superior than all tested phytochemicals with especially greater potency towards HLCSC. PI, EVO and CHE showed similar activity as SAL towards SK-LU-1 cells (8–19 μM), but none was as effective as SAL against HLCSC. It is worth noting that, from each category of phytochemicals, BI, LICO and EVO were the members which were relatively more potent in inhibiting HLCSC’s growth in isothiocyanate, chalcone and alkaloid, respectively.

Table 2. IC_{50} values of alkaloids, chalcones and isothiocyanates in SK-LU-1 and HLCSC.

		IC_{50} Values (mean $\pm$ SD)					
Phytochemical Category	Phytochemical Compound	SK-LU-1					
		24 h		48 h		72 h	
		$\mu g/mL$	μM	$\mu g/mL$	μM	$\mu g/mL$	μM
Isothiocyanate	BI	3.96 ± 0.27	26.54 ± 1.80	4.54 ± 1.35	30.39 ± 9.05	4.99 ± 1.04	33.41 ± 6.97
	PI	2.68 ± 0.01	16.39 ± 0.04	2.69 ± 0.00	16.48 ± 0.00	2.81 ± 0.04	17.18 ± 0.22
Chalcone	LICO	12.75 ± 0.38	37.68 ± 1.13	13.96 ± 1.41	41.24 ± 4.16	16.28 ± 1.79	48.09 ± 5.29
	ISO	10.93 ± 2.28	42.63 ± 8.91	16.73 ± 0.40	65.29 ± 1.55	17.11 ± 0.79	66.75 ± 3.06
Alkaloid	EVO	5.71 ± 0.69	18.81 ± 2.26	5.41 ± 1.06	17.83 ± 3.50	4.33 ± 0.36	14.26 ± 1.19
	CHE	4.32 ± 0.21	12.21 ± 0.58	2.86 ± 0.55	8.09 ± 1.56	2.74 ± 0.47	7.74 ± 1.34
Positive control	SAL	10.40 ± 2.33	13.84 ± 3.10	9.96 ± 1.10	13.26 ± 1.46	10.18 ± 0.33	13.56 ± 0.43

Table 2. IC₅₀ values of alkaloids, chalcones and isothiocyanates in SK-LU-1 and HLCSC (continued).

		IC ₅₀ Values (mean ± SD)					
Phytochemical Category	Phytochemical Compound	HLCSC					
		24 h		48 h		72 h	
		µg/mL	µM	µg/mL	µM	µg/mL	µM
Isothiocyanate	BI	2.88 ± 0.24	19.30 ± 1.61	2.80 ± 0.11	18.73 ± 0.71	3.14 ± 0.23	21.04 ± 1.52
	PI	5.14 ± 0.20	31.49 ± 1.21	6.10 ± 0.07	37.37 ± 0.43	6.27 ± 0.16	38.38 ± 1.00
Chalcone	LICO	8.27 ± 1.49	24.42 ± 4.41	8.65 ± 0.63	25.55 ± 1.86	12.01 ± 0.59	35.49 ± 1.76
	ISO	22.25 ± 0.67	86.81 ± 2.62	23.01 ± 0.12	89.76 ± 0.47	23.52 ± 0.45	91.77 ± 1.74
Alkaloid	EVO	4.71 ± 1.49	15.53 ± 4.90	7.72 ± 0.72	25.45 ± 2.38	9.13 ± 0.54	30.10 ± 1.77
	CHE	8.2 ± 0.44	23.21 ± 1.24	7.86 ± 0.04	22.23 ± 0.10	12.57 ± 2.72	35.57 ± 7.68
Positive control	SAL	3.46 ± 0.35	4.61 ± 0.47	4.42 ± 1.90	5.89 ± 2.52	2.57 ± 0.38	3.42 ± 0.50

Chalcones and Isothiocyanates Modulated Reduction of β-Catenin Nuclear Localization

The nuclear localization of β-catenin is considered to be the hallmark activation event of Wnt/β-catenin pathway and therefore inhibition of this event could potentially limit downstream pro-tumorigenic effect of the pathway. In addition to the cytotoxicity and growth arrest capability of phytochemicals that we have shown above, we were interested to characterize the ability of these phytochemicals to disrupt the activity of Wnt/β-catenin signaling by visualizing the nuclear localization activity in response to phytochemicals treatment. SK-LU-1 and HLCSC were first pre-treated with GSK3i at 8 µM for 24 h to maximize the nuclear localization of β-catenin before administering phytochemicals for another 24 h. At least three phytochemicals in each cell lines tested could inhibit localization activity of β-catenin indicated by increased number of cells that only stained blue for nuclei, regardless of whether the cytoplasm stained orange for β-catenin. In SK-LU-1, BI (4 µg/mL, $p < 0.001$), PI (3 µg/mL, $p < 0.001$), LICO (13 µg/mL, $p < 0.001$) and CHE (4 µg/mL, $p < 0.01$) significantly inhibited the localization of β-catenin to nucleus (Figure 2A,B). On the other hand, BI (3 µg/mL, $p < 0.01$), PI (1.25 µg/mL, $p < 0.05$; 2.5 µg/mL, $p < 0.001$; 5 µg/mL, $p < 0.001$) and ISO (22 µg/mL, $p < 0.001$) inhibited the localization in HLCSC (Figure 3A,B). All of these indicate that isothiocyanates (BI and PI) and chalcones (ISO and LICO) may be potent inhibitors of β-catenin nuclear localization as either one or both of the respective phytochemical members exhibited their effects in both examined cell lines. Due to their wider applicability and lower IC₅₀ values to inhibit β-catenin nuclear localization, we selected BI and PI for further characterizations.

Figure 2. Immunofluorescence detecting localization behavior of β -catenin in phytochemicals-treated SK-LU-1. SK-LU-1 was pre-treated with GSK3i at 8 μ M for 24 h followed by phytochemicals treatments for 24 h. (A) Representative micrographs depicting immunofluorescence staining of β -catenin (orange) counterstained with Hoechst 33342 (blue) to indicate nuclei in phytochemicals-treated SK-LU-1. White circles highlight cells with diminished β -catenin localization in nucleus. Scale bars represent 100 μ m. (B) Graph representing percentage of SK-LU-1 with nuclear β -catenin calculated in response to treatment at $\frac{1}{4}$ IC₅₀, $\frac{1}{2}$ IC₅₀ and IC₅₀ concentrations of respective phytochemicals. The quantification data were derived from a representative immunostaining experiment. Error bars are expressed as mean $\pm$ SD from triplicate data in an independent experiment. Statistical significance is expressed as *** $p < 0.001$; ** $p < 0.01$.

Both Isothiocyanate Members were Equally Potent to Inhibit the Growth of Wnt-Dependent Cell Line NCI-H1703 Although with Differing Sensitivity in Inhibiting Undifferentiated and Differentiated Lung Cancer Cells

To focus observations on canonical Wnt-dependent growth, we used NCI-H1703 for downstream experiments. NCI-H1703 was previously identified to depend its survival on the expressions of WNT1 and WNT2 ligands^{12,13}. Furthermore, NCI-H1703 is a lung SCC cell line, therefore characterization in this cell line can add to our observation in another lung carcinoma histological subtype for the selected phytochemicals.

Growth inhibitory profiles of BI and PI were similarly obtained in NCI-H1703 by using RTCA. The dose–response curves indicate that the isothiocyanate compounds could dose-dependently inhibit NCI-H1703 (Figure S2). Based on the curve, we determined the IC₅₀ values and used them to extrapolate IC₂₀ and IC₈₀ values. These values enabled us to see phytochemicals relative inhibitory effectiveness among different cell lines. Tables 3–5 show IC₂₀, IC₅₀ and IC₈₀ values of BI, PI and reference compound SAL, respectively, for the evaluation of inhibitory effectiveness in NCI-H1703, SK-LU-1 and HLCSC. NCI-H1703 shows similar sensitivity to BI and PI throughout the three measured time-points across three different IC values. This is judged relatively to other tested cell lines consisting of SK-LU-1 and HLCSC. Table 3 shows that NCI-H1703 and HLCSC were relatively more sensitive to BI compared to SK-LU-1, whereas Table 4 demonstrates that NCI-H1703 and SK-LU-1 were relatively more sensitive to PI, but HLCSC was relatively more resistant to it. As expected, only HLCSC consistently showed higher sensitivity to SAL (Table 5). All of these suggest that BI and PI could more effectively target either undifferentiated (e.g., HLCSC) or differentiated (e.g., SK-LU-1) lung cancer cells, but both could be equally potent in inhibiting the growth of Wnt-dependent cells NCI-H1703.

Table 3. IC₂₀, IC₅₀ and IC₈₀ values of BI.

Cell Lines	IC ₂₀ (μg/mL) ¹		
	24 h	48 h	72 h
NCI-H1703	1.49 ± 0.08	1.72 ± 0.07	1.83 ± 0.07
SK-LU-1	1.87 ± 0.13	2.91 ± 0.87	3.46 ± 0.72
HLCSC	1.88 ± 0.16	2.65 ± 0.10	2.91 ± 0.21
Cell Lines	IC ₅₀ (μg/mL) ²		
	24 h	48 h	72 h
NCI-H1703	2.90 ± 0.16	2.93 ± 0.13	2.95 ± 0.11
SK-LU-1	3.96 ± 0.27	4.54 ± 1.35	4.99 ± 1.04
HLCSC	2.88 ± 0.24	2.80 ± 0.11	3.14 ± 0.23
Cell Lines	IC ₈₀ (μg/mL) ¹		
	24 h	48 h	72 h
NCI-H1703	5.65 ± 0.30	5.01 ± 0.22	4.73 ± 0.17
SK-LU-1	8.41 ± 0.57	7.08 ± 2.11	7.19 ± 1.50
HLCSC	4.41 ± 0.37	2.95 ± 0.11	3.38 ± 0.24

¹ IC_{20/80} values were estimated using the formula $IC_F = \left(\frac{100-F}{F}\right)^{1/H} \times IC_{50}$, where F is the desired percentage of maximal inhibition, i.e., 20% or 80%, and H is hill slope/gradient of the non-linear regression curve. ² IC₅₀ data were based on mean values obtained from duplicated dose–response curves in two independent experiments.

Table 4. IC₂₀, IC₅₀ and IC₈₀ values of PI.

Cell Lines	IC ₂₀ (μg/mL) ¹		
	24 h	48 h	72 h
NCI-H1703	1.74 ± 0.14	1.81 ± 0.14	1.99 ± 0.01
SK-LU-1	1.81 ± 0.00	2.14 ± 0.00	2.03 ± 0.03
HLCSC	3.81 ± 0.15	5.78 ± 0.07	5.89 ± 0.15
Cell Lines	IC ₅₀ (μg/mL) ²		
	24 h	48 h	72 h
NCI-H1703	2.82 ± 0.22	2.78 ± 0.21	2.92 ± 0.01
SK-LU-1	2.68 ± 0.01	2.69 ± 0.00	2.81 ± 0.04
HLCSC	5.14 ± 0.20	6.10 ± 0.07	6.27 ± 0.16
Cell Lines	IC ₈₀ (μg/mL) ¹		
	24 h	48 h	72 h
NCI-H1703	4.55 ± 0.35	4.26 ± 0.32	4.29 ± 0.02
SK-LU-1	3.96 ± 0.01	3.38 ± 0.00	3.87 ± 0.05
HLCSC	6.94 ± 0.27	6.44 ± 0.07	6.66 ± 0.17

¹ IC_{20/80} values were estimated using the formula $IC_F = \left(\frac{100-F}{F}\right)^{1/H} \times IC_{50}$, where F is the desired percentage of maximal inhibition, i.e., 20% or 80%, and H is hill slope/gradient of the non-linear regression curve. ² IC₅₀ data were based on mean values obtained from duplicated dose–response curves in two independent experiments.

Table 5. IC₂₀, IC₅₀ and IC₈₀ values of SAL.

Cell Lines	IC ₂₀ (μg/mL) ¹		
	24 h	48 h	72 h
NCI-H1703	0.85 ± 0.31	0.60 ± 0.03	0.54 ± 0.05
SK-LU-1	2.50 ± 0.56	1.22 ± 0.13	1.13 ± 0.04
HLCSC	1.02 ± 0.10	0.71 ± 0.30	0.26 ± 0.04
Cell Lines	IC ₅₀ (μg/mL) ²		
	24 h	48 h	72 h
NCI-H1703	11.03 ± 4.03	6.00 ± 0.28	4.96 ± 0.42
SK-LU-1	10.40 ± 2.33	9.96 ± 1.10	10.18 ± 0.33
HLCSC	3.46 ± 0.35	4.42 ± 1.90	2.57 ± 0.37
Cell Lines	IC ₈₀ (μg/mL) ¹		
	24 h	48 h	72 h
NCI-H1703	143.92 ± 52.59	60.36 ± 2.78	45.37 ± 3.82
SK-LU-1	43.17 ± 9.66	80.94 ± 8.91	91.89 ± 2.94
HLCSC	11.79 ± 1.20	27.64 ± 11.85	25.32 ± 3.70

¹ IC_{20/80} values were estimated using the formula $IC_F = \left(\frac{100-F}{F}\right)^{1/H} \times IC_{50}$, where F is the desired percentage of maximal inhibition, i.e., 20% or 80%, and H is hill slope/gradient of the non-linear regression curve. ² IC₅₀ data were based on mean values obtained from duplicated dose–response curves in two independent experiments.

BI and PI Inhibited the Growth of NCI-H1703's Soft Agar Colony and Spheroid, But not Migration and Invasion

Wnt/β-catenin signaling is associated with increased CSC functions that include proliferation, survival and motility. To determine whether BI and PI's difference in inhibiting differentiated and undifferentiated lung cancer cells also affects their abilities in inhibiting these CSC functions, we used NCI-H1703 cell line to evaluate both phytochemicals inhibitory activities towards CSC properties such as soft agar colony formation, spheroid growth, migration and invasion. Both isothiocyanate compounds were observed to exhibit similar inhibitory effect towards NCI-H1703's growth and nuclear localization of β-catenin at IC₅₀ concentrations. Hence, we picked IC₅₀ values, as well as concentrations two-fold lower and two-fold higher than IC₅₀ values, for soft agar colony treatments. Figure 4 shows inhibitions of

BI and PI towards the aforementioned CSC properties. Long-term colony formation that relies on the viability of CSC was more effectively inhibited by BI at 1.5 and 3 $\mu\text{g/mL}$ compared to PI where no inhibition was observed at the same concentrations (Figure 4A). On the other hand, PI was more potent in inhibiting the growth of multicellular tumor spheroid (MCTS) model of NCI-H1703 in dose-dependent manner (Figure 4B). Spheroids treated with increasing concentrations of BI and PI may appear as compact structure, which indicate intact and viable structure, whereas dying spheroids are morphologically loose, hence appear bigger in size (Figure S3). Both BI and PI induced a loose appearance to spheroids when treated with ≥ 6.25 $\mu\text{g/mL}$ as also depicted in Figure 4B with their low cell viability. Figure 4C depicts migration and invasion capability of NCI-H1703 in response to BI and PI treatments. Sixteen hours after the onset of treatments, there was significant reduction of percentage of migration and invasion's CI in BI- and PI-treated NCI-H1703 relative to control. However, dose-response of BI and PI at the concentrations that had significant reduction of migration and invasion signal suggested that the reduction solely was due to reduction of cell viability. Therefore, the measured activity was an output of cytotoxicity, but not migration and invasion activity decrement.

Figure 4. Soft agar colony formation, multicellular tumor spheroid growth, migration and invasion in BI- and PI-treated NCI-H1703. (A) Representatives micrographs of colony formation of NCI-H1703 in soft agar after BI and PI treatment at indicated concentrations. Scale bars represent 1000 μm . (B) Graphs presenting cell viability of parallel set-up of monolayer and spheroid models that were treated with BI and PI at indicated concentrations. (C) Graphs presenting migration and invasion of NCI-H1703 (left) after BI and PI treatments and dose-response graphs (right) of respective phytochemicals at indicated concentrations. Error bars in spheroid cell viability, migration and invasion data were expressed as mean $\pm$ SD from three independent experiments. Error bars in dose-response of viable cells data were expressed as mean $\pm$ SD from two independent experiments. Statistical significance is expressed as *** $p < 0.001$; ** $p < 0.01$; * $p < 0.05$.

Discussion

In the present study, we examined dietary and herbal phytochemicals for their inhibitory effects in NSCLCs. Among phytochemicals representatives of alkaloids comprising of CHE and EVO, chalcones comprising of ISO and LICO and isothiocyanates comprising of BI and PI, we found that members of isothiocyanates exhibit mutual inhibitory activity towards the activation of Wnt/ β -catenin pathway, but further characterization showed different targeting preference between undifferentiated lung CSC (HLCSC) and differentiated lung cancer cells (SK-LU-1). By using SAL as positive control compound against CSCs on the basis of IC₂₀, IC₅₀ and IC₈₀ values, we confirmed that BI was more potent against HLCSC compared to SK-LU-1, whereas PI was more desirable to inhibit SK-LU-1. Interestingly, these patterns were replicated in assays such as soft agar colony formation assay where BI was seen to be more effective in inhibiting colony growth and MCTS assay where PI reduced the number of viable cells more effectively.

Wnt/ β -catenin pathway is a conserved embryonic signaling that is still actively used by adult lung tissue. For instance, alveolar epithelial cells (AECs) II can constitutively activate canonical Wnt pathway to maintain stemness with Wnt stimulation from adjacent fibroblast cells and they can switch on Wnt-autocrine signaling during severe injury¹⁴. Wnt-fibroblast growth factor (FGF) signaling is utilized by mesenchymal cells in the airway to support epithelial cells during injury¹⁵. NSCLCs in respective tissue origins (i.e., SCC from the airway and ADC from air sacs) use the same Wnt/ β -catenin mechanism, but with some deregulations such as through aberrant inhibition or decreased expression of Wnt negative regulators, and increased expression of Wnt ligands or transducers such as Dishevelled (DVL) or β -catenin^{10,11,16}. Catenin B1 gene (*CTNNB1*) may be mutated in ADC leading to evasion of proteosomal degradation and constitutive activation of Wnt pathway¹⁷. Cells with increased nuclear accumulation of β -catenin, for example, enrich for CSC traits such as higher expression of nuclear Nanog in NSCLCs, which predicts for poor prognosis of NSCLC¹⁸. CSC enrichment or inability to kill CSC population was deemed to be the root of therapeutic resistance problem in clinics today, including for NSCLCs⁹.

Hence, in the present study, we looked at potential phytochemicals which are able to diminish localization of β -catenin to nucleus and found that chalcone and isothiocyanate phytochemicals were able to decrease this hallmark activation event. Interestingly, other chalcone and isothiocyanate members have also been found to inhibit Wnt/ β -catenin pathway. The chalcone cardamomin has been demonstrated to reduce nuclear β -catenin localization in triple negative breast cancer cell line BT-549¹⁹. Other chalcones including derricin and derricidin have also been reported to possess such effect when treated to HCT116 colon cancer cell line²⁰. An isothiocyanate compound, sulforaphane, was reported to inhibit canonical Wnt transcriptional activity in two separate studies, as demonstrated by TCF/LEF reporter assays in breast CSCs and colorectal cancer cell lines SW480, DLD1 and HCT116^{21,22}. In this study, while isothiocyanate members consistently exhibited this effect, chalcones did not. It is not clear what caused the inconsistency, but concentration-specific targeting may result in concentration-independent dose-response. Our previous study on chelerythrine chloride identified this specific-targeting dose-independent effect of nuclear localization of β -catenin in NCI-H1703 and SK-LU-1²³. At low concentration, the effect was higher. In this case, inhibitory

effect towards nuclear localization of β -catenin for ISO in SK-LU-1 and LICO in HLCSC may be exhibited at either lower or higher concentration than those tested in this study. Future study is warranted to confirm how widespread the dose-independent specific-targeting phenomenon is.

The most important CSC function which confers on CSC the ability to perpetuate and maintain tumor is the self-renewal ability²⁴. This ability can be demonstrated through soft agar colony-forming assay *in vitro*. Only CSC possessing self-renewal ability is capable of forming visible long-term colony in soft agar culturing under an anchorage independence situation²⁴. On the other hand, MCTS is a three-dimensional (3D) tumor model that mimics the structure of avascular micrometastases *in vivo*²⁵. Besides that, its multilayered cells architecture also simulates the *in vivo* physiological condition of tumor bulk that is often presented with complex relationships among chemical gradients, inner hypoxia, cell–cell interactions and cell–matrix interactions that ultimately affect drug response²⁶. In addition, due to its layering barrier by differentiated cells, drug penetration is an important factor considered when using this drug testing model since it may provide physical protective-barrier for CSC that may lie inside²⁷. As such, BI at 1.5 and 3 $\mu\text{g/mL}$ may be specifically targeting CSCs leading to lower capability of NCI-H1703 cells to form larger colonies in higher density. PI may majorly rely on its differentiated cells cytotoxicity to decrease the number of viable cells in NCI-H1703 MCTS model that mainly consist of differentiated cells maintained by small number of CSCs. Penetration may not be a differing factor since BI and PI have approximately the same molecular size (149.21 vs. 163.24 g/mol, respectively).

To assess the selectivity of phytochemicals towards cancer growth inhibitions, we performed dose–response assessment of phytochemicals in normal lung fibroblast cell line IMR-90. However, the results suggest that IMR-90 cells were as susceptible as other lung cancer cell lines used in the present study (Figure S4). Although we did not confirm the selectivity of growth inhibitory effect towards cancer cells, phytochemicals assessed in this study have been widely incorporated in products used for daily life. LICO and ISO are compounds isolated from licorice, a natural sweetener ingredient for some snacks and drinks²⁸. Isothiocyanates can be obtained from cruciferous vegetables such as cabbage, broccoli and cauliflower²⁹. EVO is found in evodia fruits commonly used as traditional medicine in China³⁰. CHE is the major alkaloid derived from the *Chelidonium majus* medicinal plant used in Europe and China³¹. With all these histories of consumptions by human, the described phytochemicals are undoubtedly safe at low concentration. Besides that, we noticed a therapeutic window for selective CSC inhibition whereby at a concentration that modulated reduction of colony formation, BI was not cytotoxic to the 3D model that better replicates multicellular conformation of tumor bulk—a model that perhaps also better mimics the architecture of normal epithelial cells *in vivo* when compared to monolayer 2D model.

Materials and Methods

Cell Culture

Human lung ADC cell line SK-LU-1 and human lung SCC cell line NCI-H1703 were purchased from American Type Culture Collections (ATCC, Manassas, VA, USA). Human lung cancer stem cell (HLCSC) primary cell line was purchased from Celprogen (Torrance,

CA, USA). SK-LU-1 and NCI-H1703 were routinely maintained in Gibco's Dulbecco's modified Eagle's medium (DMEM) with 4.5 g/L D-glucose (Thermo Fisher Scientific, Waltham, MA, USA) supplemented with 1% (v/v) of 100 mM Gibco's sodium pyruvate, 1% (v/v) of 10,000 U/mL Penicillin–10,000 μ g/mL Streptomycin (Pen-Strep) and 10% (v/v) of Gibco's fetal bovine serum (FBS). HLCSC was routinely maintained in human lung cancer stem cell complete growth medium with serum (Celprogen). All cell cultures were maintained in 5% CO₂ humidified atmospheric condition CO₂ incubator at 37 °C. Passaging was regularly performed with Sigma-Aldrich's (St. Louis, MO, USA) 0.25% (w/v) trypsin–ethylenediaminetetraacetic acid (EDTA) solution.

Chemicals

Benzyl isothiocyanate (BI) and licochalcone-A (LICO) were obtained from Sigma-Aldrich. Chelidonine (CHE), evodiamine (EVO), isoliquiritigenin (ISO) and phenethyl isothiocyanate (PI) were obtained from Chromadex (Los Angeles, CA, USA). Salinomycin (SAL) was obtained from AdooQ Bioscience (Irvine, CA, USA). Glycogen Synthase Kinase-3 inhibitor X (GSK3i) was purchased from Calbiochem (San Diego, CA, USA). All phytochemicals were prepared by dissolving in dimethyl sulfoxide (DMSO, Sigma-Aldrich) at 10 mg/mL stock.

Cell Viability, Cytotoxicity and Anti-Proliferative Assay

Real time cell analyzer (RTCA) was performed as described previously using an E-plate 16 on xCELLigence RTCA-Dual Purpose (DP) platform (ACEA Bioscience, San Diego, CA, USA)²³. Briefly, 50 μ L of complete medium were added into each well for 30-min incubation before acquiring background measurements. Optimized cell density (1×10^4 cells/well for SK-LU-1 and 1.25×10^4 cells/well for HLCSC and NCI-H1703) was seeded upon background measurement and was incubated for 30 min at room temperature in the biological safety cabinet in order to facilitate cells settling before putting back onto the RTCA-DP platform for overnight incubation. Subsequently, phytochemicals were added to each well and incubated at various concentrations for up to 72 h. RTCA software 2.0 was used to generate kinetic dose–response curve by normalizing the cell index (CI) to the time when the phytochemical was added. IC₅₀ values were estimated by plotting CI against concentration using algorithm for sigmoidal dose–response curve with variable slope.

CellTiter-Glo 3D cell viability assay (Promega, Madison, WI, USA) was used to evaluate the growth of spheroid culture (refer to spheroid generation section) along with the parallel run of monolayer culture (seeded at 1×10^4 cells/well). After 24 h treatment with selected phytochemicals at indicated concentrations, each of the spheroids was transferred to a single well of an opaque-white plate for cell viability measurement according to the manufacturer's protocol. Monolayer culture was directly seeded in the opaque-white plate. Luminescence was measured as relative luminescence unit (RLU) by using FLUOstar Omega microplate reader (BMG LABTECH, Ortenberg, Germany). Each sample was normalized against respective vehicle control. GraphPad Prism v5.01 software (San Diego, CA, USA) was used to plot concentration against percentage of viability.

Immunocytochemistry

Immunofluorescence assay was performed as described previously²³. SK-LU-1 and HLCSC were first treated with GSK3i for 24 h upon growing overnight in order to optimally increase β -catenin localization before the onset of phytochemicals treatments. Immunofluorescence was performed by using Cellomics® Beta-Catenin Activation Kits (Thermo Scientific, Waltham, MA, USA) in accordance with the manufacturer's protocol. Five random, non-overlapping frames were captured from each well by using Axio Vert.A1 inverted microscope equipped with HXP-120V light source and AxioCam MR R3 camera (Carl Zeiss, Oberkochen, Germany). Cells harboring nuclear β -catenin were manually counted by using ImageJ's cell counter plugin and the positive counts were divided by the respective total number of cells in the captured frames to obtain percentage of cells with positive nuclear β -catenin³². Treated samples were compared with untreated Wnt-activated negative control.

Soft Agar Colony Formation Assay

The assay was performed as described previously with some modifications^{23,33}. Briefly, top/bottom agar was established at 0.35%/0.6% (w/v) with noble agar (Becton Dickinson, Franklin Lakes, NJ, USA) in a six-well plate. NCI-H1703 cells were suspended within the top agar at 5×10^4 cells. Treated and untreated groups were kept wet with either medium or medium containing selected phytochemicals at adjusted concentrations by renewal of every three days. After three weeks, the plate was collected and stained with 0.01% (v/v) of crystal violet for 30 min at room temperature. The plate was then washed for three times with PBS. SZ51 zoom stereo microscope (Olympus, Shinjuku, Tokyo, Japan) was used to visualize the colonies and Toupcam microscope digital camera (ToupTek, Zhejiang, China) equipped with Toupview software (ToupTek, Zhejiang, China) was used to capture the micrographs.

Multicellular Tumor Spheroid Generation Assay

NCI-H1703 was seeded at 400 cells/well in a Costar ultra-low attachment microplate (Corning Inc., Corning, NY, USA) by using the same DMEM medium used for monolayer culture. After an overnight incubation, Matrigel (final concentration at 2.5% (v/v), Corning Inc.) was added gently towards the center of the well to facilitate compact formation of spherical structure. The spheroid was optimized to grow to 300–400 μ m in diameter four days post-generation. The diameter of spheroid was measured by using Zen 2 pro software (Carl Zeiss).

Migration and Invasion Assay

RTCA-DP system was similarly used for migration and invasion evaluation. CIM-plate 16 that consists of two chambers was used for this purpose. Overnight serum-starved 4×10^4 NCI-H1703 cells were seeded with serum-free medium at the upper chamber upon background measurement, whereas medium containing 10% (v/v) of FBS was added to the lower chamber to serve as chemoattractant. Serum-free medium in the lower chamber serves as control. For migration, no Matrigel coating is necessary, but, for invasion, 1:30 Matrigel was used to coat upper chamber 4 h prior to cell seeding. Selected phytochemicals were added at indicated concentrations upon seeding. The migration–invasion was monitored for up to 24 h. RTCA

software 2.0 was used to generate dose–response kinetic curve of both migration and invasion of NCI-H1703 by expressing CI against time.

Statistical Analysis

All data were expressed as mean $\pm$ standard deviation (SD). Statistical significance was evaluated by using one-way analysis of variance (ANOVA) in SPSS 22.0 software (IBM SPSS, Chicago, IL, USA). Dunnett's multiple comparison test was used for Post-hoc. The effect of time and treatment concentrations as well as the interactions of both towards cell growth in dose–response curve was statistically analyzed by using two-way repeated measures ANOVA in GraphPad Prism v5.01 software. Simple main effect analysis of treatment concentrations was analyzed by using Bonferroni test. Mean difference for a particular treatment concentration was considered significant if the majority observed time-points had statistical significance when compared to those of untreated control. Statistical significance was expressed as ***, $p < 0.001$; **, $p < 0.01$; *, $p < 0.05$. All data were collected from three independent experiments unless otherwise specified.

Conclusions

Through our characterizations, we confirmed that alkaloids EVO and CHE, chalcones LICO and ISO and isothiocyanates BI and PI exhibited decent anti-proliferative and cytotoxic effects. These effects may in part be due to inhibition towards β -catenin nuclear localization such as those observed in chalcones and isothiocyanates treatments. Our study and literature search suggest that the chalcones and isothiocyanates' Wnt/ β -catenin inhibitory activity may be something generalizable for their classes since other members of chalcone and isothiocyanate were also found to have similar effect. Further characterizations interestingly revealed that isothiocyanate members BI and PI had different potency in targeting different cellular components judging from the sensitivity of undifferentiated and differentiated lung cancer cells in response to the treatments, even though they only differ by an extra methylene group in PI's chemical structure. This hidden effect was further proven in the soft agar CSC assay, where CSC is enriched, and the MCTS model, where CSC is present in minute number. As constituent phytochemicals that are present in dietary vegetables, BI and PI may offer an alternative for cancer prevention. Therapeutically, BI and PI combinations or BI used as adjuvant therapy with chemotherapy may be seen as ideal composite therapy for CSC eradication and tumor bulk clearance. Future study may need to assess their potential use further in clinics as β -catenin nuclear localization inhibitors.

Author Contributions

Conceptualization, W.S.H. and S.-C.C.; methodology, W.S.H. and S.-C.C.; formal analysis, W.S.H.; investigation, W.S.H.; resources, S.-C.C.; writing—original draft preparation, W.S.H.; writing—review and editing, W.S.H. and S.-C.C.; visualization, W.S.H.; supervision, S.-C.C.; project administration, W.S.H.; and funding acquisition, S.-C.C. All authors have read and agreed to the published version of the manuscript.

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Conflicts of Interest

The authors declare no competing conflicts of interest.

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Supplementary data

Figure S1. Kinetic dose–response curves of SAL in SK-LU-1 and HLCSC.

Figure S2. Kinetic dose–response curves of BI, PI and SAL in NCI-H1703.

Figure S3. Representative micrographs of multicellular tumor spheroid model of NCI-H1703 treated with various concentrations of BI and PI.

Figure S4. Kinetic dose–response curves of phytochemicals in IMR-90.

Chapter 6

Understanding Lung Carcinogenesis from a Morphostatic Perspective: Prevention and Therapeutic Potential of Phytochemicals for Targeting Cancer Stem Cells

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Abstract

Lung cancer is still one of the deadliest cancers, with over two million incidences annually. Prevention is regarded as the most efficient way to reduce both the incidence and death figures. Nevertheless, treatment should still be improved, particularly in addressing therapeutic resistance due to cancer stem cells—the assumed drivers of tumor initiation and progression. Phytochemicals in plant-based diets are thought to contribute substantially to lung cancer prevention and may be efficacious for targeting lung cancer stem cells. In this review, we collect recent literature on lung homeostasis, carcinogenesis, and phytochemicals studied in lung cancers. We provide a comprehensive overview of how normal lung tissue operates and relate it with lung carcinogenesis to redefine better targets for lung cancer stem cells. Nine well-studied phytochemical compounds, namely curcumin, resveratrol, quercetin, epigallocatechin-3-gallate, luteolin, sulforaphane, berberine, genistein, and capsaicin, are discussed in terms of their chemopreventive and anticancer mechanisms in lung cancer and potential use in the clinic. How the use of phytochemicals can be improved by structural manipulations, targeted delivery, concentration adjustments, and combinatorial treatments is also highlighted. We propose that lung carcinomas should be treated differently based on their respective cellular origins. Targeting quiescence-inducing, inflammation-dampening, or reactive oxygen species-balancing pathways appears particularly interesting.

Keywords: lung carcinogenesis; phytochemicals; cancer stem cell; epigallocatechin-3-gallate; sulforaphane

Introduction

Lung cancer is a malignant disease comprising two major histological categories known as non-small cell lung carcinoma (NSCLC) and small cell lung carcinoma (SCLC). In NSCLC, the major subtypes include adenocarcinoma (ADC), squamous cell carcinoma (SCC), and large cell carcinoma (LCC). A great proportion of cancer mortality today is still accounted for by

lung cancer-related deaths (mainly NSCLC), representing 18.4% of all cancer deaths¹. If proper preventive measures are not taken, about 1 in 5 people in countries with poor access to healthcare will die due to cancer by 2025². Some of the promoted cancer preventive measures include cessation of tobacco product and alcohol usage, pollution reduction, sufficient physical activity, and a healthy diet³. Among the proposed preventive solutions, eating a healthy diet that is mainly plant-based should be of primary concern, especially in developed countries, considering its huge contribution to overall health⁴. However, poor commitment due to various reasons such as high cost, poor taste, and low obtainability often renders this less achievable.

We generally acquire our nutrients from food in the form of macronutrients (i.e., carbohydrates, proteins, and fats) and micronutrients (i.e., vitamins and minerals). A plant-based diet that mainly consists of vegetables and fruits is considered good for well-being, mainly because it is enriched with vitamins, minerals, and dietary fibers. However, the contribution of micronutrients in vegetables and fruits to lowering the risk of lung cancer is controversial due to some inconsistent associations between the two variables^{5,6}. Dietary fiber mainly helps in controlling other noncommunicable diseases such as cardiovascular disease, diabetes, and obesity⁷. Phytochemical compounds—the additional phytonutrients in vegetables and fruits—were found to have preventive and therapeutic potential for lung cancer as evidently shown by numerous *in vitro* and *in vivo* studies⁸. This suggests that phytochemical compounds may play a role in providing a plant-based diet's added health benefits.

Cancer stem cell (CSC) theory postulates that there exists a tumor subpopulation for tumor maintenance, such as normal stem cells maintaining tissue steady state. CSC is the tumorigenic subpopulation capable of generating both tumorigenic and less tumorigenic offspring in the tumor bulk and is deemed responsible for the initiation and propagation of malignant diseases, including lung cancer⁹. This subpopulation is the reason why lung cancer gains resistance towards therapy over time and disease recurs in patients after initial remission¹⁰. Therefore, it is imperative to develop new therapies to target this subpopulation in order to achieve complete remission.

Hence, it is our goal to review the most recent findings on the benefits offered by plant-derived compounds regarding their potential to prevent or treat lung cancer. The mechanisms of action known thus far are discussed, such as modulation of inflammatory responses, oxidative stress, and CSCs. Firstly, we provide a comprehensive overview of lung functioning and relate this information with the pathological dysregulations of lung cancer at molecular and cellular levels. Subsequently, we focus on the relevant molecular pathways and relate them to potential phytochemical compounds, and we make comments based on the usefulness of targeting.

A Close Connection between Normal Lung Tissue Repair Mechanism and Carcinogenesis

The close resemblance of normal stem cells and CSCs is increasingly being recognized¹¹. Both compartments are located at the apex of the hierarchy and possess high potency to proliferate and differentiate, thus making them capable of symmetric and asymmetric divisions. Both compartments also make use of respective stem cell niches for expansion cues and utilize similar signaling pathways in a tissue-specific manner^{12,13}. Recently, we highlighted that lung progenitor cells could be the cells of origin of CSCs for different

histological types of lung carcinomas, both of which are triggered to proliferate extensively in response to chronic injury⁹. In brief, basal cells, pulmonary neuroendocrine cells (PNECs), and alveolar epithelial cells II (AECs) are considered to be the cell origins of CSCs for SCC, SCLC, and ADC, respectively. It is not completely clear how these cells could evade a carcinogenic transformation and in what conditions these cells eventually become transformed. Nonetheless, these cells fulfill several criteria that accurately define how CSCs behave. In the sections below, the lungs and their resident epithelial cells, and how carcinogenesis can be triggered in those cells are briefly described, followed by a comprehensive description of how each resident cell proliferates and differentiates based upon the signaling pathways and chemical gradients they use during homeostasis maintenance and injury repair. This is important to recognize tissue- and cell-specific behavior that can be directly reflected towards the understandings of specific lung carcinomas.

Lung Tissue, Progenitor Cell, Cancer Stem Cell, and Injury-Triggered Carcinogenesis

The lung tissue consists of respiratory epithelia that line the trachea, bronchi, bronchioles, and alveoli for gas conduction and exchange (see **Figure 1** for schematic representation). Major resident epithelial cells, such as goblet cells, ciliated cells, basal cells, and innervated PNECs, are found in the pseudostratified columnar ciliated layer of the epithelia, whereas club cells are found in the bronchiolar region^{9,14}. Variant club cells that are resistant to the cytotoxic effects of naphthalene reside in the respiratory bronchioles along with PNECs clustered as the neuroepithelial bodies (NEB) and in the bronchioalveolar duct junctions^{9,14}. AEC I and AEC II cells are restricted to the alveolar space^{9,15}. Because the lungs are constantly exposed to pathogens and irritants via respiration, the resident epithelial cells have to ensure clearance of contaminants for homeostasis maintenance and inflammation evasion.

Figure 1. Schematic diagram showing the histological and cytological variations in different locations of the lung. In each tissue, there is at least one type of progenitor cell that is capable of proliferation, facilitating tissue repair in case of injury. In lung injury-triggered carcinogenesis, repeatedly induced inflammation accompanied by tissue repair could potentially lead to a chronic state that changes various extracellular and intracellular modulators and consequently encourages the emergence of lung cancer stem cells (CSCs) from progenitor cells. CSCs and tissue resolution (TR) are two sides of the same coin. AEC, alveolar epithelial cell; PNEC, pulmonary neuroendocrine cell; ROS, reactive oxygen species.

When the lungs suffer an injury, several lung resident cells such as basal cells, club cells, variant club cells, and AEC II cells could act as facultative stem cells to repair damage by repopulating lost cells⁹. PNECs can also take part in repopulating ciliated and club cells in naphthalene-induced injury¹⁶. These cells' activity is lineage-committed, which means that their renewal function is induced at the respective location where they are residing. However, recent progress in the identification of progenitor cell candidates for lung injury repair points to the involvement of other cells, such as the lineage-negative epithelial progenitor (LNEP) cell, a basal cell progenitor termed the bronchial epithelial stem cell (BESC), and a subset of the AEC II cell population termed alveolar epithelial progenitor (AEP), in repairing distal lung epithelium^{17–19}. These cells together with the well-studied facultative stem cells are key candidates for the emergence of CSCs because their proliferative activity is mostly injury-triggered and their activity coincides with other tumor-supporting events that occur after injury consisting of DNA damage and tissue repair-associated inflammation. Given the unique cellular turnover rate of lung epithelium and potent repair capacity during injury, these cells fulfill several premises to be defined as lung CSCs: (1) they are long-lived; (2) they are mostly quiescent; (3) they can survive mild or severe injury; (4) they are known to have the potency to differentiate and proliferate for both homeostasis and injury repair⁹. Based on the similarities in the location and protein markers, lung progenitors as key players for tissue repair have clear

connections with CSCs of lung carcinomas, thus explaining the overlapping between tissue repair and carcinogenesis.

Continuous lung injury disrupts the normal physiology of the lungs by impairing the morphostatic field through systemic response such as chronic inflammation, which also damages DNA and proteins. Morphostatic components may include epigenetic sets of pre-programmed modules that determine cell fate decisions and tissue organization, including chromatin modifications, bioelectric events, mechanical stress, and chemical gradients²⁰. Chronic inflammatory microenvironment may eventually result in wounds that do not re-pattern properly during wound repair, which is assumed to non-autonomously encourage CSC development and support tumor initiation²¹. Intracellular damage such as in DNA can be induced by genotoxic agents or through excess reactive oxygen species (ROS) generation also produced by inflammatory cells during injury-associated lung inflammation²². Prolonged DNA damage may lead the progenitor cells to accumulate genetic aberrations over time which could lead to tumorigenesis, and together with the epigenetic components, they may shape how DNA aberrations are recurring in a tissue-specific manner to maintain stemness, which also non-autonomously enhances tumor progression²¹. Thus, the emergence of CSCs is achieved through a series of processes that arrest tissue repair resolution and disrupt the morphostatic field, which enables the continuous proliferation of unregulated progenitor cells.

Normal Physiological Responses for Homeostasis and Injury Repair in Lung Tissue

To further understand how CSCs hijack the normal stem cell machinery for proliferation and differentiation, below we comprehensively describe the processes of homeostasis and how injury repair activities differ across different progenitor cells within the lung epithelia. The focus of the description is on morphostatic signaling pathways that regulate repair processes to maintain homeostasis, and their potential to control CSC activity.

At steady state, the lung tissue is actively maintained by the Hedgehog (Hh) pathway to remain in a quiescent state. During acute lung tissue injury, Sonic Hedgehog (SHH) protein is downregulated in SHH-expressing cells including club, ciliated, and AEC II cells, which consequently induces proliferation of adjacent GLI1⁺ SHH-responsive mesenchymal cells through a self-sufficient Platelet-Derived Growth Factor, B polypeptide (PDGFB)-dependent mechanism²³. The absence of SHH paracrine signaling from lung epithelia removes the restriction to feed proliferative signals, i.e., FGF10 for lung epithelium regeneration²⁴.

Basal Cell

The basal cell is the most essential facultative stem cell marked by sex-determining region Y-box transcription factor 2 (SOX2), tumor protein 63 (TP63), and keratin 5 (KRT5) expressions. It is able to differentiate into ciliated, club, AEC I, and AEC II cells for homeostasis maintenance and injury repair⁹. A study in mice that combined long-term lineage tracing, biophysical modeling, and single-cell molecular analyses revealed that there were two pools of basal cells, comprising stem cell and committed progenitor pools²⁵. SOX2 is required for basal cell specification from naïve epithelial cells as evident by transcriptional targeting of SOX2 on TP63 basal cell marker expression²⁶. Besides TP63, the transcriptional coactivator YAP1 functions to maintain basal cell stemness by directly interacting with TP63 and

regulating common target genes²⁷. SOX2 is also used to maintain the basal cell's identity and promote its self-renewal through Fibroblast Growth Factor Receptor 2 (FGFR2) signaling involving the Phosphoinositide 3-Kinase (PI3K)/AKT pathway^{18,28}.

During homeostasis maintenance, the basal cell is stochastically fated to differentiate into luminal lineages, and basal cell loss is compensated through duplication of neighboring basal cells²⁹. The basal cell accomplishes club cell differentiation by generating the NOTCH3⁺ parabasal cell, which activates NOTCH1 and NOTCH2 by jagged 1 (JAG1) and JAG2 ligands³⁰. Activation of Janus Kinase/Signal Transducer and Activator Transcription 3 (JAK/STAT3) signaling by interleukin 6 (IL6) from surrounding mesenchyme inhibits Notch signaling and directs the basal cell towards ciliated cell differentiation³¹. BMP ligand secretions from surrounding mesenchyme also reduce basal cell proliferation as shown by higher BMP antagonist Follistatin (FST) expression during regeneration³².

Club Cell

The club cell is a prominent facultative stem cell within the bronchioles³³. This cell is marked with SCGB1A1 marker expression and generated by basal cells and variant club cells during homeostasis and injury repair^{34,35}. Similar to a basal cell, the club cell bears a proximal identity of SOX2 expression along with ciliated and goblet cells³⁶. A club cell can also be regenerated by a variant club cell (SCGB1A1⁺cytochrome P450 2F2 (CYP2F2)⁺) through WNT7B-triggered paracrine stimulation of mesenchymal FGF10 resulting in simultaneous activation of Wnt/ β -catenin and Notch pathways and transient Epithelial-to-Mesenchymal Transition (EMT) reprogramming in the epithelia, which in turn initiates repair³⁷.

For homeostasis and repair, the club cells are able to differentiate into ciliated cells by acting as transit-amplifying progenitor cells in the trachea and long-term progenitor cells in the bronchioles³⁸. Alternate NOTCH2-JAG1-mediated lateral inhibition in neighboring cells directs to ciliated cell differentiation³⁹. A recent lineage tracing assay and in vitro differentiation assay of purified club cells demonstrated wider club cells' functional capability in repairing injured lung epithelia outside of the bronchiolar region by dedifferentiation into basal cells and transdifferentiation into AEC II cells to repair respective regional damage^{18,40}. Interestingly, tumor protein 53 (TP53) also affects club cell proliferation and differentiation by keeping epithelial quiescence at steady state⁴¹. Loss of expression of TP53 leads to poorer differentiation of club cell to ciliated cell but higher dedifferentiation potential, e.g., self-renewing or dedifferentiating to a more naïve cell fate such as TP63⁺KRT5⁻ progenitor cells, which can normally be inhibited through contact inhibition imposed by a neighboring basal cell^{41,42}. YAP1 overexpression phenocopies TP53 loss in a club cell²⁷. This may suggest that Hippo/YAP1 signaling and TP53 are somehow interacting to achieve balance to maintain lung epithelial quiescence by controlling the tendency to proliferate and differentiate⁴³.

PNEC

The PNEC is marked by Achaete-Scute homolog 1 (ASCL1), calcitonin gene-related peptide 1 (CALCA), synaptophysin (SYP), chromogranin A (CHGA), and ubiquitin carboxy-terminal hydrolase isozyme L1 (UCHL1)¹⁶. While it is not previously known to be derived from any progenitor cell in the lung, a PNEC can be generated by a SHH-irresponsive Twist-related

protein 2 (TWIST2)⁺ mesenchymal cell on the occasion when the lung epithelia suffer naphthalene or lipopolysaccharide-induced injury⁴⁴. Apart from renewing themselves, PNECs can repopulate club and ciliated cells in dire situations¹⁶. UCHL1⁺ PNECs can serve to repopulate club cells when wiped out by naphthalene treatment through a NOTCH1-dependent Notch signaling induction⁴⁵. Transdifferentiation of PNEC to the ciliated cell uses the histone-lysine N-methyltransferase EZH2-IL6-STAT3 pathway⁴⁶. A recent study discovered that a small number (2–4 cells) of differentiated PNECs in a cluster of NEB (a group of 20–30 PNECs) possess stem cell function, thus representing dedicated stem cells that are capable of self-renewal and differentiation under the strict guidance of genes such as transformation-related protein 53 (*Trp53*) and retinoblastoma (*Rb*) (quiescent signals) to ensure injury-dependent proliferation and control of subsequent sensitivity to dispersal and transdifferentiation signals⁴⁷.

AEC II

AEC II is a prominent facultative stem cell in the peripheral lung epithelia. This cell is marked with the expression of pulmonary Surfactant-associated protein C (SFTPC) and mainly only self-renews and differentiates into Homeodomain-only protein (HOPX)⁺Podoplanin (PDPN)⁺Aquaporin 5 (AQP5)⁺ AEC I for homeostasis maintenance¹⁵. AEC II is known to have restricted regenerative capacity that only generates residents within the alveolar compartment for homeostasis and mild injury repair. To maintain the integrity of the alveolar compartment, neo-basal/basal-like cells could be generated by several precursors or intermediate progenitor cells to repair severe injury, including LNEPs, BSCs, SOX2⁺ progenitor cells, and intrapulmonary bronchial progenitor cells^{17,18,48,49}. In contrast to the basal cell in the proximal region, neo-basal/basal-like cells generated by LNEPs are maintained by hypoxia-inducible factor 1- α (HIF1A)-mediated Notch activity and differentiate into AEC II upon Wnt/ β -catenin-driven Notch blockade^{17,50}. On the other hand, BSCs are also capable of differentiating to neo-basal/basal-like cells that further differentiate into AEC II or club cells in response to bleomycin-induced severe lung injury¹⁸.

FGF10-FGFR2B signaling strength controls whether the BSC-generated neo-basal/basal-like cells are expanded or transdifferentiated to AEC II; a sufficiently high level of FGF10 biases towards AEC II differentiation, whereas subsequent FGF10 supply termination leads to AEC I differentiation¹⁸. The sufficiency of FGF10 feed is controlled by epithelial WNT7B feedback to airway smooth muscle cells (ASMCs), which is negatively controlled by the Integrin-Linked Kinase (ILK)-Hippo pathway for quiescence maintenance⁵¹. Apart from being responsive to FGF10 signaling, AEC II also proliferates in response to EGFR and GTPase KRAS signaling⁵². A single Platelet-Derived Growth Factor Receptor Alpha (PDGFRA)⁺ fibroblast can serve as the stem cell niche for an AEC II by supplying Wnt ligands that allow the cell to proliferate extensively with appropriate signals and maintain its stem cell identity, which would otherwise be differentiated to AEC I upon exiting the niche⁵³. This Wnt active population (AXIN2⁺Transmembrane 4 L6 family member 1 (TM4SF1)⁺) is a distinct smaller AEC II cell population termed AEPs¹⁹. Under acute injury response, AEPs become Wnt autocrine-proficient, thus enabling them to regenerate lost AEC I and AEC II⁵³. Like how BMP signaling influences the basal cell, the stable expression of phosphorylated mothers against

decapentaplegic homolog 1/5/8 (pSMAD 1/5/8) of BMP signaling in AEC I and AEC II maintains cellular quiescence, whereas expression of antagonist FST promotes AEC II proliferation⁵⁴.

The signaling pathways regulating self-renewal and overall interactions of lung epithelial residents for proliferation and differentiation are simplistically summarized in **Figure 2**.

Figure 2. Schematic diagram illustrating self-renewal and the interactions of lung epithelial residents with mesenchyme to trigger proliferation or to maintain tissue quiescence. In the different proximodistal locations of lung epithelia, signaling pathways are used differently for differentiation, whereas similar signals are used for proliferation and lung tissue quiescence in progenitor cells of respective lung tissue locations. FGF10 or WNT from mesenchyme consequently triggers self-renewal of progenitor cells if differentiating and tissue quiescence signals (–|sign) are missing during tissue repair or homeostasis. Green signals favor proliferation, whereas red signals favor tissue quiescence. AEC, alveolar epithelial cell; AEP, alveolar epithelial progenitor; BESC, bronchial epithelial stem cell; LNEP, lineage-negative epithelial progenitor; PNEC, pulmonary neuroendocrine cell.

Dysregulations and Aberrations Supporting Lung Carcinogenesis

Chromosomal aberrations and genetic mutations have been very well-explored as common features of malignancies, including the lung cancers. Contributions of the microenvironment have also been fairly well-documented along with the key players consisting of immune cells, mesenchymal stem cells, and fibroblasts⁹. These intrinsic and extrinsic modulators, bridged by chronic inflammation, are involved in the intricate mechanisms that underlie the lung carcinogenesis that comprises the emergence and perpetuation of CSCs. Representative molecular aberrations that cover different aspects of carcinogenesis, including survival, stemness maintenance, and evasion of quiescence/differentiation, are briefly described below. The potential impact of chronic inflammation on extracellular modulators that shape the morphostatic instructions is also concisely delineated. Additionally, the involvement of EMT in supporting carcinogenesis is also discussed. **Figure 3** summarizes the variety of aberrations and dysregulations that support the transformation of progenitor cells into CSCs.

Figure 3. Schematic diagram depicting various molecular aberrations and dysregulations supporting the development of CSCs. The microenvironmental components are influenced by the recurrently induced inflammation and, in turn, feed the negatively affected morphostatic instructions to epithelial cells leading to aberrant changes of genetic and epigenetic components. This consequently enables favorable behavioral changes that facilitate the cellular transformation of CSCs. EMT, epithelial-to-mesenchymal transition.

Molecular Aberrations Driving Tumor Initiation and Lung CSC Maintenance

The increased understanding of the molecular processes involved in programming normal lung tissue into desired lung carcinoma, irrespective of cell origin, has provided the knowledge required for dissecting molecular aberrations for the development of lung carcinomas. A mouse model with phosphatase and tensin homolog gene (*Pten*) and cyclin-dependent kinase inhibitor 2a and 2b genes (*Cdkn2ab*) deletions in addition to SOX2 overexpression in tracheobronchial basal cells, and other peripherally located club cells and AEC II cells, has been shown to induce lung SCC⁵⁵. On the other hand, common sets of oncogenic drivers consisting of dominant negative TP53, myristoylated AKT serine/threonine kinase 1 (myrAKT1), retinoblastoma-associated protein-short hairpin RNA (*RBI*-shRNA), MYC proto-oncogene protein (MYC), and B-cell lymphoma 2 apoptosis regulator protein (BCL2) were demonstrated to transform normal human bronchial epithelial (NHBE) cells into small cell neuroendocrine cancer that had a similar transcriptional and chromatin accessibility landscape to SCLC⁵⁶. For ADC, Notch signaling activation was shown to generate a tumor in addition to GTPase KRas activation in AEC II cells; forced activation of Notch signaling, likely

NOTCH2, to block SOX2 activity was shown to transform club cells that were not previously known to progress to ADC^{39,57}. Usually, these molecular aberrations act to maintain potency to proliferate, arrest differentiation, prevent quiescent restoration, and increase survival in the CSC compartment. In agreement with how the normal lung tissue operates, several tumor suppressor genes that are involved in quiescence maintenance such as *CDKN2A* that codes for p16 protein, *RBI*, *PTEN*, and *TP53* are commonly lost in lung carcinomas⁵⁸. Also consistent with the regulators of proliferation we described above, some common gene amplification or overexpression targets found in lung carcinomas are *SOX2*, *MYC*, phosphatidylinositol-4,5-bisphosphate 3-kinase catalytic subunit alpha (*PIK3CA*) that codes for a subunit of PI3K, *EGFR*, and *KRAS*⁵⁸. Many more molecular aberrations have recently been discovered to help us understand further how tumorigenesis in the lung can be evoked and maintained.

An example of dysregulation of an important cell oxidant-protective mechanism was demonstrated recently to promote lung tumorigenesis. Mutations in Kelch-like ECH associated protein 1 (*KEAP1*) were shown to activate the KEAP1/Nuclear Factor erythroid -related factor 2 (NRF2) pathway and subsequently promote cellular detoxifying and antioxidant activities which contributed to tumor development⁵⁹.

Nuclear Factor kappa-light-chain-enhancer of activated B cells (NF- κ B) is also one of the overexpressed protein targets in NSCLCs. In a study employing CSC-enriched cisplatin-resistant NSCLCs, NF- κ B expression could be reduced using a small molecule inhibitor dehydromethylepoxyquinomicin (DHMEQ) to restore sensitivity towards cisplatin treatment⁶⁰. This indicates that the NF- κ B pathway may be involved in the stemness maintenance of NSCLCs.

YAP that is constitutively inhibited by the Hippo pathway in lung progenitor cells was lately found to be overexpressed in lung ADC⁶¹. YAP overexpression was associated with proliferation and autophagy in lung ADC cell lines A549 and H1299 and induced activation of the AKT/mammalian Target of Rapamycin (mTOR) pathway by PTEN suppression. Thus, lung CSCs can breach the Hippo pathway's cell-intrinsic quiescence regulating function by overexpressing YAP protein.

CREB-binding protein (*CREBBP*) is a gene encoding an acetyltransferase that is often lost in SCLC^{58,62}. Such loss accelerated neuroendocrine tumors in autochthonous mouse models, including SCLC. Mechanistically, *Crebbp* was found to positively regulate cadherin 1 (CDH1) expression by acetylating histone residues, which suppressed tumorigenesis through inhibition of proliferation. Hence, CREBBP is an important negative regulator of proliferation to limit the emergence of CSC in the lung tissue.

Serine/threonine kinase 11 (*STK11*) is also one of the most frequently mutated genes in lung ADC⁵⁸. Interestingly, mutations in this particular gene often co-occur with mutations of *KRAS* in NSCLCs⁶³. Whether *STK11* was presented as single or double mutations with *KRAS*, it was associated with worse overall survival (OS) and progression-free survival (PFS) in metastatic NSCLCs. In a separate study, STK11 was identified as the normal program of ciliated differentiation in the airways which functions by suppressing Extracellular signal-regulated Kinase 1/2 (ERK 1/2) activity in mediating inactivation of pRB⁶⁴. The loss of the differentiation program by mutations in NSCLCs explains how differentiation can be arrested in CSCs.

Dysregulations of Signals from Microenvironment during Lung Tissue Repair

Persistent airway inflammation observed in smoking patients with chronic obstructive pulmonary disease (COPD) and lung cancer is related to continuous tissue repair and perhaps coupled with continuous damage induction, which can be from the original inducer, e.g., cigarette smoking, or from chronic inflammation^{65,66}. Chronic inflammation is a detrimental event because it can cause excessive tissue damage and disrupt the process of tissue repair. For example, cigarette smoke's ROS might act as a second messenger signal for intracellular signaling such as the NF- κ B pathway that induces inflammation or indirectly by imposing tissue damage that would also trigger inflammation as a tissue repair mechanism⁶⁷. Cigarette smoking guarantees repeated exposure of the lung to toxic particulates and ROS, which ensures sustained inflammation. Indeed, a study found that cigarette smoke could delay the spontaneous death of neutrophils, which prolongs the tissue destruction and pathogen-killing phase through more enzymes and ROS release⁶⁸. On the other hand, cigarette smoke can also exhibit an immunosuppressive effect on the activity of inflammatory cells responding to tissue damage⁶⁷. Although they are increased in number, alveolar macrophages with cigarette smoke exposure have been documented to have impaired function particularly in resolving repair, thus prolonging inflammation⁶⁹. Moreover, cigarette smoke can hinder tissue repair activity by impairing the proliferation of fibroblasts, hence compromising their tissue regenerative support function⁷⁰. Regardless of the effect from cigarette smoke, progenitor cells need to communicate to the inflammatory cells that they are ready to expand themselves or otherwise inflammation cannot be resolved. This necessity is evident in mice with the absence of Yap/tafazzin (Taz) in the Hippo pathway; the mice displayed a delay of regenerative proliferation in AEC II and inability to upregulate NF- κ B inhibitor alpha (NFKB1A) to inhibit NF- κ B-mediated inflammatory response causing prolonged infection-induced inflammation⁷¹.

Chemical signals originating from the extracellular milieu may also be negatively perturbed by chronic inflammation. Upon recurrent cycles of acute inflammation, proliferative progenitor cells of the lung in the resolution phase may be irresponsive to external quiescence and/or differentiation signals such as BMP ligands (quiescence signal), Notch ligands, and IL6 (differentiation signal) due to desensitization of receptors that transduce the signals⁴⁷. On the other hand, it is plausible that the source of external signals, i.e., stromal cells, may be damaged in the same way as parenchymal cells by lung cancer risk factors or chronic inflammation itself, hence producing dysfunctional ligands that are incapable of activating differentiation or restoring quiescence. The BMP pathway noted as a quiescence maintaining-pathway is likely dysregulated in lung carcinomas in this way. Indeed, when the recombinant proteins of BMP6 and BMP7 ligands were treated in NSCLC and SCLC cell lines, respectively, proliferation was inhibited in those cells^{72,73}. Alternatively, repeated or continuous chemokine and cytokine secretions at the inflammatory site could dilute other signals derived from the microenvironment. Chronic inflammation may also, through acute cytokine secretion such as IL1B, maintain stemness by inducing chromatin modification in *CDH1* and subsequent EMT reprogramming⁷⁴.

Aside from ligand-induced signaling through, for example, WNT7B and FGF10, cells can communicate via bioelectric signals that involve the cellular influx and efflux of H⁺, Na⁺, K⁺, Ca²⁺, and Cl⁻ ions regulated by ion channels and cause changes in membrane potential⁷⁵.

Cells in a specific region can present a spatiotemporal distribution of voltage gradients that confer a physiological state or phenotype. Bioelectric events involving changes in ion flows, pH, membrane potentials, and electric fields in the extracellular site affect morphogenetic and morphostatic instructions of tissue and regulate proliferation, differentiation/dedifferentiation, apoptosis, and migration/orientation^{75,76}. In general, depolarization of cells from the initial steady state of quiescent cells causes activation of signaling pathways that can modulate various cellular processes⁷⁶. Bioelectric signals also may be used for cell–cell communication tools through intercellular gap junctions and provide positional information and cell identity in tissue regeneration⁷⁷.

How chronic inflammation can lead to perturbation of this signaling remains unclear. However, voltage gradients that regulate tissue organization may be modified during chronic inflammation to result in hyperproliferation, which enables tumorigenic transformation. For instance, ROS from inflammatory cells such as second messenger hydrogen peroxide (H_2O_2) also regulates transepithelial potential and electric field to stimulate regeneration. In ROS-depleted condition by inhibition of Nicotinamide Adenine Dinucleotide Phosphate (NADPH) oxidase-mediated ROS production, regeneration was reduced in a tail-amputated *Xenopus laevis* tadpole but could be rescued by exogenously added H_2O_2 that was proposed to act upstream of voltage-gated Na^+ channels to modulate regeneration⁷⁸. This shows that chronic inflammation might amplify the regenerative activity of progenitor cells due to multiplied ROS production. Such ROS-dependent induction of regeneration may be related to an evolutionary-conserved phenomenon termed apoptosis-induced proliferation (AiP), which is a compensatory mechanism of undead or dying cells to stimulate proliferation either through ROS-tumor necrosis factor (TNF)-c-Jun N-terminal kinase (JNK)-dependent pathway or secretion of mitogenic signals such as WNT and SHH from dying cells, respectively⁷⁹.

In another instance, when apoptotic neutrophils are not cleared off by efferocytosis during chronic inflammation, their cellular contents including cytotoxic proteases, caspases, and ROS could leak to the inflammatory site⁶⁹. Leaked ROS could be a charged molecule such as superoxide anion ($\text{O}_2^{\cdot-}$), which could result in unintended changes in epithelial transmembrane potential or even trans-epithelial potential⁸⁰. Changes in transmembrane potential at the individual cell level can encourage tumorigenesis, and this is exemplified by cell depolarization in *Xenopus laevis*'s embryonic stem cells that can stimulate melanocytes to adopt a hyperproliferative invasive phenotype, which suggests a neoplastic-like transformation⁸¹. Indeed, one study documented that persistent inflammation could increase Gamma-Aminobutyric Acid (GABA)-induced depolarization of rat cutaneous dorsal root ganglion neurons in vitro⁸². On the contrary, hyperpolarization renders the cells resistant to malignant transformation even with oncogenes expression⁸³. Future works need to confirm whether chronic inflammation can contribute to the increased depolarization of lung epithelial cells and the mechanism behind this route of tumorigenesis.

Additionally, due to the damaging nature of chronic inflammation, surrounding supporting structures such as the nerve, which functions to communicate long-range bioelectric positional and identity cues, could be cut off, which leads to interruption of the communication. Indeed, as reported in the insect *Leucophaea maderae*, severance of recurrent nerves led to a

high incidence of tumor development⁸⁴. In the lung, this situation can be especially relevant for PNECs as they are known to be innervated.

Chronic inflammation is undoubtedly involved in some dysregulations in the repair of lung epithelia during injury. Dysregulations of extracellular signaling cues partially or completely disconnect the communication effort among the regenerative players within the microenvironment, thus the tissue never gets properly repaired and leads to wounds that do not heal.

Cellular Reprogramming Involving Transitioning to Mesenchymal Phenotype

EMT is a normal cellular reprogramming pathway that also occurs during tissue repair, especially at the proliferative phase to encourage regenerations³⁷. Our previous review has discussed the evidence of stemness induction in lung CSCs through the EMT process⁹. EMT switches on the mesenchymal phenotype with the expression of cadherin 2 (CDH2/N-cadherin) and switches off the epithelial phenotype by downregulating CDH1 as the hallmarks of activation. Many signaling pathways have been characterized to induce EMT by activating one of the three EMT-associated transcription factors consisting of zinc finger protein SNAIL, TWIST, and Zinc finger E-box-binding homeobox (ZEB); however, only Transforming Growth Factor Beta 1 (TGFB1) induction through the TGFβ pathway has the versatility to activate all three transcription factors⁹. Due to this versatility, TGFB1 can be used to abuse EMT reprogramming by CSCs because TGFB1 is one of the cytokines that are freely available during the regenerative phase⁸⁵. Although EMT is usually transiently activated during tissue repair by chromatin remodeling that silences *CDH1*, partial EMT induction is a common sight in many cases of carcinomas, which means that it could be very well-sustained⁸⁶. Moreover, EMT “memory” can be created by acute exposure of proinflammatory cytokine IL1B to induce sustained EMT and stemness maintenance which supports CSCs’ activity in disease progression, hence leading to common clinical outcomes such as therapeutic resistance, metastasis, and even immune evasion^{74,86}.

Current Use of Phytochemical Compounds in Treating Lung Cancer

Phytochemical compounds have been used in cancer treatment since the inception of chemotherapy. Vinca alkaloids were some of the earliest developed phytochemical-based compounds, including vinorelbine that was isolated from rosy periwinkle, first produced in 1979 and approved by the Food and Drug Administration (FDA) in 1994 to treat NSCLC patients^{87–89}. In the 1960s, a new diterpenoid phytochemical called paclitaxel was discovered and isolated from the bark of Pacific yew and was approved for the use of NSCLC patients in 1998⁹⁰. Soon after due to compound scarcity and difficulties in isolation, a semi-synthetic drug called docetaxel was made and isolated from the needle of European yew, and it was approved in 1999 to manage NSCLC patients^{87,91}. Etoposide, which was approved along with cisplatin by the FDA as a first-line therapy for SCLC in 1985, was discovered as a semi-synthetic compound of podophyllotoxin from American mandrake^{92,93}.

Both vinorelbine and the taxanes (paclitaxel and docetaxel) work by affecting the dynamics of microtubule polymerization. Apart from having different binding sites, they are in huge contrast to each other in affecting the polymerization of microtubules. Vinorelbine binds

to tubulin dimers (α - and β -tubulin) and inhibits their polymerization into microtubules required for mitotic spindle formation^{88,89}. The taxanes bind to β -tubulin and enhance the polymerization into stable microtubules thereby preventing microtubule depolymerization^{90,91}. The interrupted dynamics of microtubules polymerization by those compounds leads to mitotic arrest at metaphase which results in cell death. Etoposide is known as a topoisomerase poison and it works by binding to topoisomerase II enzyme–DNA complex preventing religation of double-strand breaks (DSBs) and effectively trapping the intermediate reaction at enzyme–DNA complex during the unwinding function of topoisomerase II causing inhibition in replication fork progression and lethal DSBs⁹⁴.

These phytochemical-derived chemotherapy drugs have two major limitations. Firstly, these drugs have unwanted side effects of not only impairing DNA replication and transcription in cancer cells but also proliferative normal cells, such as hair follicle cells, gastrointestinal cells, and hematocytes leading to hair loss, weight loss, fatigue, neutropenia, leukocytopenia, thrombocytopenia, nausea, and vomiting^{88,89,91}. Secondly, drug resistance is observed for these drugs mediated by expression of efflux pumps such as ATP Binding Cassette (ABC) transporter protein, defects of apoptotic pathways, mutations of drug targets that alter drug–target binding affinity, and higher DNA repair efficiency in vinorelbine, etoposide, and paclitaxel-resistant lung cancer cells⁹⁵. These two main problems may be recurring problems for new drug development, so the underlying factors causing these should be taken into account.

The Potential Use of Phytochemical Compounds for Prevention and Targeting CSCs in Lung Cancer

Phytochemical compounds still provide good sources for drug candidates due to their limitless biodiversity, although the limitations noted above can be recurring. The formulation of the drugs can be optimized, and the active component can be used as a pure compound or be produced as a semi-synthetic or synthetic drug. Coupled with better targeting strategies, expected limitations can be overcome. To illustrate the cell-protective and anticancer activities of phytochemicals, nine phytochemical compounds will be discussed with the most active research in the field in the last decade, namely curcumin, resveratrol, quercetin, epigallocatechin-3-gallate, luteolin, sulforaphane, berberine, genistein, and capsaicin. The phytochemical compounds' effects on signaling pathways are summarized in **Table 1**.

Table 1. Summary of the activity of listed phytochemical compounds on targeting signaling pathways.

Phytochemical	Antioxi dant	Anti- inflam matory	Signaling pathway target								
			Wnt	Notch	Hh	JNK	JAK/ STAT3	PI3K/ AKT	NF-κB	RTK	TGFβ/ BMP
Curcumin	Yes	Yes	↓	↓	↓	-	↓	↓	↓	↓	-
Resveratrol	Yes	Yes	-	-	-	-	↓	↓	↓	↓	↓
Quercetin	Yes	Yes	-	-	-	-	↓	↓	↓	↓	-
EGCG	Yes	Yes	↓	-	↓	↓	-	↓	↓	↓	↓
Luteolin	Yes	Yes	-	-	-	↑	↓	↑↓	↓	↑↓	↓
Sulforaphane	Yes	Yes	↓	↓	↓	-	-	↓	↓	↑↓	-
Berberine	Yes	Yes	-	-	-	-	↓	↓	↓	↑↓	↓
Genistein	Yes	Yes	-	-	-	-	-	↓	↓	↓	-
Capsaicin	Yes	Yes	-	-	-	-	-	↓	↓	-	-

Curcumin

Curcumin is a polyphenolic compound isolated from the rhizome of the plant *Curcuma longa* (also known as turmeric) that is widely used as a cooking spice in Southeast Asia, India, and China. Being one of the most actively tested phytochemical compounds in the field, curcumin has been known to be an anti-inflammatory, antioxidant, and anticancer agent⁹⁶.

Curcumin’s chemopreventive effect can be linked with its antioxidant properties in vivo by boosting the expression of cytoprotective enzyme heme oxygenase 1 (HO1) that leads to the catalysis production of antioxidants⁹⁷. Additionally, curcumin prevents inflammation by blocking cadmium-induced NF-κB-dependent IL6 and ERK-dependent IL8 epithelial secretions⁹⁸. On the other hand, curcumin can exert its anticancer property through the inhibition of several signaling pathways. Curcumin induces apoptosis and autophagy in NSCLC by inhibiting the PI3K/AKT/mTOR pathway that is known as a downstream target of FGF signaling⁹⁹. Hepatocyte Growth Factor (HGF)-induced EMT can also be abolished through inhibition of this pathway¹⁰⁰. Wu and co-authors reported that curcumin is capable of inhibiting lung cancer growth through NOTCH1 inhibition¹⁰¹. The JAK2/STAT3 pathway was also reported to be curcumin’s target¹⁰². Besides targeting the PI3K/AKT pathway, curcumin can also target another more universally used paracrine or autocrine pathway in lung tissue, i.e., the Wnt/β-catenin pathway¹⁰³. The same study also suggested SHH pathway inhibition as curcumin’s anti-CSC mechanism.

Curcumin has been associated with some limitations, including low oral bioavailability and genotoxicity when a high dose was given¹⁰⁴. Systemic treatment may not be an option considering its multiple pathway-targeting activities. Low dose curcumin even for the long-term, has been found to eliminate curcumin’s carcinogenic problem and helped to alleviate therapy resistance opening the possibility for combination with other treatments^{105,106}. To solve the bioavailability issue, a technique employing inhalable curcumin composite particles for direct lung delivery was recently introduced¹⁰⁷.

Resveratrol

Resveratrol is a polyphenolic stilbene compound belonging to the phytoalexin class that is derived from grape seeds and skin, and its products such as red wines. This well-studied

phytochemical compound is known to exhibit antioxidant, anti-inflammatory, and anticancer activities¹⁰⁸.

Resveratrol's antioxidant activity is related to its ability to decrease the oxidative status (lipid peroxidation, myeloperoxidase (MPO), xanthine oxidase (XO) activities, and 8-hydroxydeoxyguanosine level) while it enhances enzymatic and non-enzymatic antioxidants (catalase (CAT), superoxide dismutase (SOD), glutathione peroxidase (GPX) activities, total oxidant status, and thiol level) in nicotine-treated rats¹⁰⁹. Its chemopreventive anti-inflammatory activity was shown in the same report by its ability to reduce IL2, IL6, and TNF release. Suppression of inflammation through the NF- κ B pathway may be related to its ability to decrease the phosphorylation of AKT¹¹⁰. Resveratrol's anticancer activity was shown by its ability to perturb STAT3 signaling and to block TGFB1-induced EMT^{111,112}.

Due to its mild nature, resveratrol is often used in combination with other drugs; curcumin with its wide medicinal properties may potentially be used together with resveratrol to modulate the biophysical property of cancer cells, i.e., by inducing hyperpolarization¹¹³. Enhanced killing of etoposide to NSCLC cells was modulated by resveratrol through downregulation of ERK1/2 and AKT pathways that promote DNA damage repair¹¹⁴. At present, resveratrol is still presented with similar limitations with other phytochemical compounds, such as some general toxicity when used excessively in high doses, genotoxicity, and low oral bioavailability¹⁰⁸. To counter those problems, resveratrol must be delivered at the right concentration, and this has been addressed in the recent development of nanoparticles or nanocomposite delivery methods¹¹⁵.

Quercetin

Quercetin is a flavonol commonly found in food ingredients such as broccoli, onions, tea, berries, and citrus fruits. It is known as a phytochemical compound that possesses antioxidant, anti-inflammatory, and anticancer properties¹¹⁶.

Quercetin's chemopreventive anti-inflammatory effect was shown by its activity in reducing benzo[a]pyrene diol epoxide (BPDE)-induced NHBE cells transformation through inhibiting IL6-dependent STAT3 activation of the epithelium and BPDE-stimulated IL6 secretion by inhibiting the NF- κ B and ERK pathways in the human lung fibroblasts¹¹⁷. Quercetin's antioxidant activity may not have been reported in the last decade in lung cancer, but it is widely known that quercetin has a positive impact on the level of glutathione (GSH) and other enzymatic antioxidants, which potentially leads to a chemopreventive effect against oxidant-induced lung cancer¹¹⁸. The proliferation of NSCLC can be hampered by quercetin through inhibition of pAKT expression by quercetin leading to cell cycle arrest¹¹⁹.

Having a similar situation with resveratrol, the mild effect of quercetin requires potentiation of other drugs or therapy to work optimally. For example, a combination strategy that employed quercetin and curcumin was able to increase acetylation of TP53 which would otherwise be depressed due to benzo[a]pyrene (BP) treatment, suggesting the potential of reactivation of suppressed quiescence or apoptotic module with the phytochemical compounds¹²⁰. With the common shortcoming of low oral bioavailability, inhalable nanoemulsion was developed to improve the delivery of quercetin to the lung tissue¹²¹.

Epigallocatechin-3-gallate

Epigallocatechin-3-gallate (EGCG) is a polyphenolic compound belonging to the catechins grouping that is most abundantly found in green tea. EGCG is yet another phytochemical compound that is known to have antioxidant, anti-inflammatory, and anticancer functions¹²².

Antioxidant activity of EGCG has been ill-reported in lung cancer in the last decade. However, EGCG is well-recognized as an antioxidant phytochemical compound due to its reducing phenol rings. EGCG's anti-inflammatory property was demonstrated by its effect in alleviating paraquat-induced acute lung injury by inhibiting the NF- κ B pathway and downstream cytokine production¹²³. The preventive mechanism in smoking-associated NSCLC was evident by a study showing EGCG's action in downregulating pro-inflammatory cyclooxygenase 2 (COX2), AKT, ERK, and HIF1A in nicotine-treated cells¹²⁴. Evidence of EGCG's anticancer mechanisms is based on several lines of study. Importantly, EGCG was able to modulate microRNA expressions to inhibit the activity of several signaling pathways such as MAPK, PI3K, Wnt, SHH, and TGF β pathways to inhibit NSCLC's growth and proliferation¹²⁵. Downregulation of Wnt/ β -catenin activation decreased CSC marker expression, suppressed proliferation, and evoked apoptosis¹²⁶. In another study, EGCG was found to downregulate the pEGFR, pAKT, pERK1/2, and p-mTOR pathways¹²⁷. The JNK pathway was found to be another target of EGCG to induce cell cycle arrest¹²⁸. EGCG exerted pro-oxidative property by modulating ROS level to inhibit ERK1/2 pathway¹²⁹.

Apart from the activities of EGCG as a single targeting agent, the activity of DNA methyltransferase (DNMT) and histone deacetylase (HDAC) could be hampered by EGCG and cisplatin co-treatment resulting in increased expression of genes involved in maintaining cellular quiescence¹³⁰. Combination with quercetin was reported to potentially increase the cellular absorption of EGCG into NSCLC cells¹³¹. EGCG is currently presented with the common low oral bioavailability problem; thus, a technique has been developed to improve its concentration in the lung tissue using nanoemulsion¹³².

Luteolin

Luteolin is a polyphenolic compound commonly found in tea, celery, broccoli, and green pepper. This phytochemical compound also exhibits wide medicinal value, including anti-inflammatory, antioxidant, and anticancer activities¹³³.

To counteract inflammation, luteolin was found to decrease HIF1A and COX2 promoter activities; reduce IL1B, IL6, IL8, and TNF secretions; and inhibit the AKT, MAPK, NF- κ B, and STAT3 pathways, which prevented the malignant transformation of human lung epithelial cells treated with hexavalent chromium (Cr(IV))¹³⁴. The antioxidant effect of luteolin was shown by its ability to improve the level of both enzymatic antioxidants such as SOD, CAT, GPX, and glutathione-s-transferase (GST), and non-enzymatic antioxidants such as GSH, vitamin E, and vitamin C through NF- κ B pathway blockade which otherwise were decreased in BP-fed mice¹³⁵. For its anticancer mechanism, luteolin activated the MEK-ERK and AKT pathways to exert its anti-proliferative and apoptotic activities¹³⁶. In other situations, luteolin pre-treatment diminished TGF β 1-dependent EMT induction through the inhibition of PI3K-

AKT-NFKB1A-NF- κ B-SNAIL pathways¹³⁷. In an apparent ROS modulation situation, luteolin activated the JNK pathway to generate O_2^- to mediate cytotoxic killing of NSCLC¹³⁸.

Like EGCG, luteolin can be used to inhibit HDAC activity to potentiate cisplatin cytotoxicity in lung cancer cells¹³⁹. A combination of low-dose EGCG and luteolin could synergistically increase apoptosis of lung cancer cells¹⁴⁰. To improve cellular uptake and targeted delivery, luteolin has been encapsulated into a model of delivery such as liposomes¹⁴¹.

Sulforaphane

Sulforaphane is an isothiocyanate commonly obtained from cruciferous vegetables such as broccoli, cauliflower, cabbage, and kale. Like many others, this phytochemical compound also has antioxidant, anti-inflammatory, and anticancer properties¹⁴².

As a chemopreventive agent, sulforaphane was able to inhibit tumor formation induced by tobacco smoke through disruption of IL6-dependent activation of the Notch pathway and increment of CSC properties such as deltaNP63alpha, CD133, and POU domain, class 5, transcription factor 1 (POU5F1)¹⁴³. In its anticancer response, sulforaphane activated ERK1/2 to promote the downregulation of a pro-survival cue in NSCLC called BCL2-like protein 11 (BCL2L11), resulting in its proteasomal degradation and apoptotic induction¹⁴⁴. Anoikis resistance and anchorage-independent growth were inhibited by inactivating the AKT pathway and downregulation of Catenin Beta-1 (CTNNB1)¹⁴⁵. Apoptosis can be induced by stimulating the generation of ROS; however, such an effect was lower in high-EGFR-expressing lung cancer cells¹⁴⁶. Thus, this indicates that sulforaphane may need to be combined with TKIs or other phytochemical compounds targeting RTK such as EGFR. In another instance whereby NHBE cells were transformed by cadmium, sulforaphane was able to exert its anti-inflammatory effect by reducing ROS level which would otherwise trigger TNF release, NF- κ B pathway activation, and downstream expression of COX2¹⁴⁷. Sulforaphane's anticancer mechanism was also found to be mediated by inhibition of DNMT and HDAC activities¹⁴⁸.

Sulforaphane was reported to sensitize and decrease the resistance of NSCLC towards the currently used TKIs such as gefitinib¹⁴⁹. Sole administration of sulforaphane or combination with gefitinib was able to inhibit the Hh pathway by downregulating SHH, Smoothed homolog (SMO), and GLI1¹⁴⁹. Good synergism in inducing cell cycle arrest and apoptosis was also observed in the treatment of NSCLC using sulforaphane and allyl isothiocyanate, suggesting the potential of combinatory exploitation of the two isothiocyanates in lung cancer therapy¹⁵⁰.

Berberine

Berberine is an isoquinoline alkaloid compound isolated from the rhizome of the herbal plants *Coptis chinensis* (Chinese goldthread) and *Hydrastis canadensis* (goldenseal). This medicinal compound is also known to exert antioxidant, anti-inflammatory, and anticancer effects¹⁵¹.

As part of berberine's anti-inflammatory mechanisms, it suppressed multiple pathways in NSCLC including NF- κ B/COX2, PI3K/AKT, vascular endothelial growth factor (VEGF)/HIF1A, and MERK/ERK¹⁵². Berberine's anticancer mechanism is mediated by the downregulation of CD142 (tissue factor) in NSCLC that resulted in MAPK signaling inhibition

and apoptotic induction¹⁵³. Derivative berberine hydrochloride may also inhibit proliferation and apoptosis by abolishing the activation of JAK2 and NF- κ B pathways in NSCLC¹⁵⁴. Chromatin remodeling is in part involved in the berberine-dependent induction of growth arrest and apoptosis which is by suppression of HDAC activities¹⁵⁵. Berberine activated the p38 MAPK pathway and dispensable ERK1/2 pathways to increase TP53 and Forkhead box protein O3 (FOXO3) expressions, which accentuated growth arrest and apoptotic inductions¹⁵⁶. Berberine could inhibit TGFB1-induced EMT in NSCLC, which could potentially support CSC maintenance¹⁵⁷. At a low dose, berberine induced transient cellular change that was similar to cellular quiescence¹⁵⁸.

In some preclinical studies, berberine combined with doxorubicin was found to have a good synergistic effect against lung cancer cells¹⁵⁹. Interestingly, berberine was able to sensitize lung cancer cells to doxorubicin's cytotoxic activity by inhibiting doxorubicin-induced STAT3 activation¹⁵⁹. For improving the targeted delivery of berberine, hyaluronate/lactoferrin layer-by-layer lipid was utilized¹⁶⁰.

Genistein

Genistein is an isoflavone compound that is ubiquitously found in soy and soy-based food products such as soymilk, tofu, and tempeh. Like all other phytochemical compounds listed here, genistein is blessed with antioxidant, anti-inflammatory, and anticancer properties¹⁶¹.

Genistein was reported to mediate proliferative inhibition and apoptotic induction in NSCLC through suppressive activities towards the PI3K/AKT/HIF1A/VEGF and NF- κ B/COX2 signaling pathways, which enhanced the production of ROS¹⁶². Other reports included Hepatocyte Growth Factor Receptor (HGFR) as a target for the apoptotic induction and indicated that TP53 intracellular stability was enhanced by genistein treatment^{163,164}.

In some preclinical studies, genistein was assessed with other therapy forms, such as trichostatin A (HDAC inhibitor) and all-trans retinoic acid (ATRA, differentiation therapy), to synergistically enhance apoptosis in NSCLC^{165,166}. To co-deliver genistein with other potential drugs, an aptamer-hybrid nanoparticle has been developed and tested in NSCLC¹⁶⁷.

Capsaicin

Capsaicin is a homovanillic acid derivative that can be obtained from chili pepper—a very popular food spice consumed by people from all over the world. Although it is not well-studied yet in recent years, some evidence indicates that capsaicin may possess favorable antioxidant, anti-inflammatory, and anticancer activities¹⁶⁸.

Limited evidence is available on capsaicin's activity towards lung cancer in the last decade. In BP-treated mice, capsaicin was able to prevent the development of lung cancer by inhibiting TNF and IL6 secretions, NF- κ B pathway activation, and COX2 expression¹⁶⁹. Capsaicin modulated the TP53 reactivation in NSCLC to degrade HIF1A, which negatively impacted the VEGF expression, therefore discouraging tumor growth¹⁷⁰. Interestingly, capsaicin was also found to activate transient receptor potential vanilloid 6 (TRPV6) of cation-channel receptor to activate the calcium-mediated calpain pathway, which consequently induced apoptosis in SCLC¹⁷¹.

Capsaicin has been tested along with other TKIs such as erlotinib and gefitinib in some preclinical findings where the combinations were able to enhance cytotoxicity towards NSCLC by inactivating the AKT pathway and restoring enzymatic antioxidant activities such as SOD and CAT^{172,173}. To improve targeting, nanoparticles have also been developed for capsaicin delivery¹⁷³.

Discussion and Future Perspective

Malignant diseases including lung cancer have mostly been viewed as diseases at the cellular level. In this review, we attempted to view lung cancer as a tissue-level deviation that fails to adhere to morphostatic instructions as part of the larger goal of homeostasis maintenance. Then, we described studies that may point to the potential use of phytochemicals not only for prevention but also treatment, with a particular focus on CSCs. By considering the low turnover rate of lung epithelia and how injury can trigger stem cell activity, CSCs can be transformed from the proliferative residents of the epithelia in response to repeated injury induction and chronic inflammation. Molecular aberrations are undoubtedly very prevalent in lung carcinomas, and tissue-specific programs related to proliferation, quiescence, differentiation, and survival are usually preferentially selected through the cancer evolution creating versatile CSCs. These cellular processes represent important targets for CSCs.

Conventional chemotherapy and TKIs that target proliferation are not able to effectively eliminate CSCs due to therapeutic resistance leading to tumor recurrence⁹. Differentiation therapy using retinoids has also been documented to face the same resistance problem, which limits its usage¹⁷⁴. Although therapies that can restore quiescence such as CDK inhibitors have led to some disappointment in lung cancer, they are to be explored further including other alternative quiescence inducers^{175,176}.

With the role of ROS and inflammation in CSC emergence and maintenance being recognized, phytochemical compounds that have preventive or cell-protective activities including anti-inflammatory and antioxidant activities can potentially be utilized as prevention and therapy candidates for tackling the inflammatory and ROS imbalanced microenvironment that perpetuates CSCs, thus helping to eliminate the source of disease. Increasing evidence has demonstrated that anti-inflammatory drugs may be used as adjuvants for chemosensitization and chemoprotection against conventional therapy's side effects¹⁷⁷. It is worth mentioning that therapeutic modulating of ion channels using phytochemical compounds in lung cancer could be a future endeavor because modulating hyperpolarization to resting membrane potential can effectively restore CSCs to their quiescence¹⁷⁸. Recent mounting evidence has shown that phytochemical compounds have the potential to be used as an alternative to immune checkpoint inhibitors against Programmed cell death 1 ligand 1 (PDL1) such as the cases of EGCG and berberine^{179,180}. Future study is still necessary to demonstrate whether the utilization of phytochemical compounds can help alleviate the resistance challenge and side effects of immunotherapy.

While embryonic signaling pathways such as Wnt/ β -catenin, Notch, and Hh, and pro-mitotic signaling pathways such as PI3K/AKT, RTKs, JNK, and JAK/STAT3 can each be a decent target for inhibiting CSCs, it is good to realize that cancers are tissue-specific diseases and therefore should be treated contextually as signaling pathways behave differently in a

tissue-specific manner. For instance, the ability of curcumin and sulforaphane to downregulate the Notch pathway in lung tissue can be contradictory to the lung cancer inhibitory goal. NOTCH1 is an instructive signal for differentiation of club cells from basal cells; thus, enforced Notch deactivation may arrest differentiation and help to maintain CSCs of SCC. In the distal lung, its inhibition is likely oncogenic because Notch inhibition activates the AEC II cell differentiation program and therefore risks supporting the CSCs of ADC. Perhaps due to these reasons, Notch inhibition has achieved little success in clinical trials⁹.

Moreover, inhibiting JAK/STAT3 may not be as favorable as originally thought. Half a dozen of the phytochemical compounds listed above can inhibit the JAK/STAT3 pathway. As reported in a study for curcumin, targeting the JAK/STAT3 pathway may only be suitable for ADC¹⁰². Like Notch, the JAK/STAT3 pathway is a less unifying pathway in lung epithelia. Inhibiting JAK/STAT3 might result in a ciliated cell differentiation blockade which enables CSCs derived from basal cells, PNECs, and club cells to maintain stemness.

Another unfavorable target in lung tissue is the Hh pathway. Although it is a unifying pathway across proximodistal regions, the Hh pathway using SHH as a ligand is a negative regulatory pathway for lung epithelial proliferation exerted from the surrounding mesenchyme; thus, once inhibited, it would result in the unfavorable therapeutic outcome of unregulated FGF10-dependent proliferation of CSCs.

Since lung tissue is mostly quiescent, targeting lung CSCs with the inhibition of locally unifying signaling pathways should not cause many issues. The Wnt/ β -catenin pathway is generally used for paracrine signaling between epithelial and stromal cells and sometimes can be used in an autocrine manner. FGF2 and other RTKs that activate the downstream PI3K/AKT pathway are the response that bridges the communication between lung epithelial cells and mesenchyme. Thus, targeting the Wnt/ β -catenin and PI3K/AKT pathways may yield favorable outcomes and little toxicity. NF- κ B universally controls inflammation and is usually downregulated in preparation for regeneration, whereas EMT reprogramming is usually transiently activated for regeneration; therefore, they should be inhibited to restore tissue quiescence. However, one study found that inhibiting NF- κ B activity may moderately promote acceleration of SCLC development, thus suggesting that targeting NF- κ B may not be an option for treating SCLC patients¹⁸¹. Besides inhibiting these stemness maintaining pathways, phytochemical compounds should preferably restore the activation of signaling pathways that are active at the steady state to maintain quiescence of lung tissue, namely the Hh, BMP, and Hippo pathways. These pathways should be the preferred target of restoration since they are the inherent negative regulatory pathways of quiescence of the lung tissue. However, the stimulatory activity of the listed phytochemical compounds towards those pathways is not well-studied and hence requires further investigation.

Due to phytochemical compounds' pleiotropic effects and complex chemical structures, their usage requires special attention. With the desire to improve them as drug candidates, several approaches for improvement can be taken. Firstly, analogs of phytochemical compounds can be synthesized by structural modifications of the original phytochemical structure to simplify the overall structure to have smaller size without unnecessary off-target functional groups, increase efficacy, increase selectivity, or improve physicochemical properties¹⁸².

Secondly, phytochemical compounds can be loaded into drug vehicles to facilitate a more targeted transport to lung tissue. The vehicles or carriers could take several forms, including large porous microparticles, polymeric nanoparticles, micelles, liposomes, or polyethylene glycol (PEG)-conjugated agents, among others¹⁸³. Phytochemical compounds loaded into carriers can be directly administered to the lungs by inhalation. Inhalation ensures quick response and good local bioavailability besides eliminating several challenges in drug delivery such as absorption, stability, clearance, and off-target side effects due to systemic treatment, like when using oral and intravenous administrations¹⁸⁴. Besides, data obtained by *in vitro* testing may be more relevant to the situation during inhalation delivery.

Thirdly, efficacy and toxicity are balanced by dosing strategy. With inhalation delivery in place of the current standard drug administration, a lower dose can be administered to achieve similar efficacy without evoking toxicity in other systems. Our study revealed that the phytochemical compound chelerythrine chloride can significantly inhibit β -catenin nuclear localization at minimally cytotoxic concentrations, thus indicating that pathway activity targeting is achievable at a low dose¹⁸⁵. Dosing can be adjusted to an even lower level when phytochemical compounds are co-delivered to the site of the target, e.g., EGCG and luteolin combinations could produce a synergistic effect when co-treated at a low dose¹⁴⁰. Indeed, a combinatory regimen is preferred as demonstrated by how mixtures of herbal ingredients are prescribed by physicians in traditional medicine even though only a single ailment is treated¹⁸⁶.

Utilizing phytochemical compounds as drug candidates is associated with some practical advantages and disadvantages. Firstly, natural products including plants and their derived phytochemical compounds have been subjected to animal or self-experimentation and use for centuries, and hence there is little concern over the safety or side effects of the consumed plant products¹⁸⁷. Some might even be made into a tonic for regular consumption over a long time. Secondly, phytochemical compounds are structurally diverse and unique, making them very likely to have a new chemical entity that could serve as a reference for innovative drug discovery. Thirdly, many phytochemical compounds are derived from dietary vegetables and fruits, thus providing an inexpensive supply of starting biomaterial for drug production—such as those that we have listed in this review.

However, disadvantageously, finding novel chemical entities can be laborious and time-consuming¹⁸⁷. Given the time and resources needed to isolate, purify, and then examine their therapeutic value after a novel encounter, it may end up being too expensive to produce. The cost of production and availability may also depend on the rarity of the plant species or scarcity of obtainable amount from where the phytochemical compound was isolated. Fortunately, with today's advancement of scientific knowledge, desired phytochemical compounds can be produced by bioengineering so that they are available in excess from plant sources or by bioengineering at microorganism-dependent biochemical factories to meet the economic standards of drug production^{188,189}.

In conclusion, plant-based diets provide added health benefits due to their extra constituent of phytochemical compounds. According to recent research, these compounds possess anticancer, antioxidant, and anti-inflammatory activities that may not only be useful in the prevention but also in the treatment of lung cancer. Besides these aspects, potential targets that should be explored further include ion channels and quiescence pathways. When targeting

signaling pathways, it should either be tissue-specific or more locally unifying, depending on the target carcinoma. Although lung cancer treatment with phytochemical compound-derived chemotherapies has had some downsides, with better strategies and targets, newer phytochemical-derived drugs can be developed into successful therapies.

Author Contributions

Conceptualization, W.S.H.; writing—original draft preparation, W.S.H.; writing—review and editing, W.S.H., F.A.E.K., and S.-C.C.; visualization, W.S.H.; supervision, F.A.E.K. and S.-C.C.; project administration, W.S.H.; funding acquisition, F.A.E.K. and S.-C.C. All authors have read and agreed to the published version of the manuscript.

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Conflicts of Interest

The authors declare no conflict of interest.

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A stylized illustration of human lungs in shades of blue and teal. The left lung (viewer's right) has a prominent red, irregularly shaped mass in its lower lobe, representing a tumor. The trachea and bronchial tree are visible. The background is a solid teal color.

Chapter 7

Summary, discussion, and future perspective

Summary

Lung cancer is a complex malignant disease that receives much attention due to its high prevalence among the world's populations with various demographic backgrounds. The demographic's variety often translates into the likelihood of exposure to different lung cancer risk factors. Although improvement in classifications succeeded in stratifying the disease into suitable treatment options, the current standing of disease incidence and death is still one of the highest of all cancers. As the 5-year overall survival rate of lung cancer remains low throughout the years, improvement in disease management is highly sought-after. In recent years, cancer stem cells (CSCs) have been found responsible for therapeutic resistance, tumor recurrence, and metastasis which contribute to the difficulty in treating this disease. Hence, this thesis aimed to explore the relevance of CSC targeting in treating lung cancer. In addition, for addressing toxicity problems and extending treatment applicability to patients, the thesis also characterized potential usage of phytochemicals as future drug candidates.

Chapter 1 provided an introduction on lung cancer including the history of how lung cancer became clinically significant, known risk factors, global statistics, diagnoses protocols, current therapies, and their limitations. Lung cancer was first linked with a behavioral tendency in 1761, i.e., smoking of tobacco products. While this was deemed as one of the risk factors for lung cancer, later many more risk factors were identified which are commonly associated with various occupations, behaviors, and genotypes. Due to the high prevalence of these risk factors, lung cancer is notoriously one of the cancers with the highest incidence in both sexes today. In addition, lung cancer is also known to have the highest rate of death among malignancies due to insufficient disease management despite histology- and cytology-based diagnosis routines that have enabled suitable stratification for therapeutic decisions. The CSC theory, which is the guiding concept of this thesis, was briefly introduced to explain its relevance for lung cancer from a clinical perspective by describing identification of CSCs based on different defining methods such as by clonogenic/tumorsphere growth, stem cell marker expression or dye-exclusive behavior combined with cell sorting, and chemotherapeutic resistance.

In **chapter 2**, we reviewed evidence pointing to the relevance of the CSC theory and of targeting CSCs in lung carcinoma, comprising of small cell lung carcinoma (SCLC) and non-small cell lung carcinoma (NSCLC). To put the CSC theory in perspective, the CSC component was discussed with the parallel existence of facultative stem cells in the normal lung. Based on the localization of major lung carcinomas and their putative cells of origin, facultative stem cells may be the candidates for aberrant oncogenic transformations. CSCs retain much of the properties of the facultative stem cells of the lung epithelia. For instance, CSCs use similar operating signaling pathways for their perpetuation, such as Wnt, Notch, Sonic Hedgehog (SHH), Janus Kinase/Signal Transducer and Activator of Transcription (JAK/STAT), Nuclear Factor kappa-light-chain-enhancer of activated B cells (NF- κ B), and Phosphoinositide 3-Kinase (PI3K)/AKT pathways. Like the plastic nature of facultative stem cells, non-CSCs can be converted to CSCs when needed. Tumor microenvironment (TME) components such as tumor-associated macrophages, cancer-associated fibroblasts, and mesenchymal stem cells support CSCs' growth as if it is part of the healing process through epithelial-to-mesenchymal (EMT) reprogramming. Cell-intrinsically, CSC maintenance can be supported by genetic aberrations, upregulated or downregulated expressions of stem cell genes, micro RNAs (miR),

piwi-interacting RNAs (piR), and hypo- or hypermethylation of regulatory genes. Consequently, maintenance of CSC phenotypes results in therapeutic resistance that facilitates tumor relapse including resistance to conventional chemotherapy and tyrosine kinase inhibitor (TKI) drugs. Novel pharmacological drugs based on CSC targeting thus becomes the effort of ongoing drug discovery research, which includes targeting of the TME and CSC's molecular features using antibodies, antibody–drug conjugates, small peptides, ligand trap, antisense oligonucleotides, nanoparticles, chemical/phytochemical compounds, dominant-negatives, and small-molecule inhibitors.

In **chapter 3**, the relevance of CSC populations and their properties was further experimentally demonstrated in a unique SCLC *in vitro* model. GLC cell lines derived from a single patient's biopsies obtained at different stages of SCLC disease were used to represent untreated (GLC-14), treated-relapsed (GLC-16), and treated-relapsed-progressed states (GLC-19) of SCLC. Firstly, RT-qPCR and western blotting were used to examine the expression levels of stem cell-related markers, especially those commonly expressed in lung epithelia. CD44, MYC, and sex determining region Y-box 2 (SOX2) were observed to accumulate progressively at gene and protein levels following the disease progression. Subsequently, immunofluorescence and flow cytometry were used to visualize their heterogeneous expression; the latter allowing quantification of CSC marker expression at the cellular and molecular levels. These stem cell markers displayed single and overlapping staining patterns in the GLC cells. Intriguingly, an evolution from initial SOX2⁺ cells as the major population in GLC-14 with smaller MYC⁺ and SOX2⁺MYC⁺ populations, to a considerably similar percentage of SOX2⁺, SOX2⁺MYC⁺, and SOX2⁺CD44⁺ populations in GLC-16, and largely SOX2⁺CD44⁺ and SOX2⁺MYC⁺CD44⁺ populations in GLC-19 was observed. Essentially, there was a progressive accumulation of the SOX2⁺MYC⁺CD44⁺ population forming a dominant more homogenous CSC population during SCLC disease progression. Increased CD44, MYC, and SOX2 expression correlated with higher soft agar colony-forming ability and resistance to γ -irradiation in the advanced disease models. Finally, assessment of CD44 blocking antibody towards CD44 revealed potential utilization of CD44 for CSC targeting.

In **chapter 4**, the opportunity to target CSCs in NSCLC was explored further by assessing a phytochemical compound called chelerythrine chloride in inhibiting the Wnt pathway, an important stem cell regulatory route. Anti-proliferative and cytotoxic activity of chelerythrine chloride was confirmed with a real-time cell analyzer (RTCA) in three different cell lines comprising of adenocarcinoma (ADC) cell line SK-LU-1, squamous cell carcinoma (SCC) cell line NCI-H1703, and the CSC cell line HLCSC. The Wnt-dependent cell line NCI-H1703 was noticed to be particularly sensitive for chelerythrine chloride. More in-depth molecular analyses demonstrated that chelerythrine chloride was capable of inhibiting the nuclear trans-localization of β -catenin in all three cell lines. Intriguingly, chelerythrine chloride was more effective in reducing β -catenin nuclear localization in NCI-H1703 at concentrations lower than IC₅₀ value, at which chelerythrine chloride downregulated important stem cell factors such as SOX2 and MYC. Apart from inducing apoptosis, chelerythrine chloride was capable of inhibiting soft agar colony formation, spheroid growth, migration, and invasion of NCI-H1703.

In **chapter 5**, more phytochemical compounds, alkaloids, chalcones and isothiocyanates, were studied for targeting lung CSCs similarly as in chapter 4. Firstly, alkaloids consisting of evodiamine and chelidonine, chalcones consisting of licochalcone-A and isoliquiritigenin, and isothiocyanates consisting of benzyl isothiocyanate and phenethyl isothiocyanate were assessed by RTCA to obtain dose–response profiles of SK-LU-1 and HLCSC. When assessing Wnt/ β -catenin pathway inhibition, chalcones and isothiocyanates were found to inhibit the nuclear localization of β -catenin. Further characterization of benzyl and phenethyl isothiocyanate showed that undifferentiated HLCSC and differentiated SK-LU-1 cells displayed different sensitivity to these phytochemical compounds, while having equal effectiveness in the Wnt-dependent cell line NCI-H1703. With functional assays, like soft agar colony formation and tumor spheroid assays, the differences were demonstrated with higher inhibition of colony formation displayed for benzyl isothiocyanate and higher inhibition of tumor spheroid growth for phenethyl isothiocyanate, indicating their preference for targeting different cell populations.

In **chapter 6**, we reviewed the similarity of key players involved in morphostasis (tissue homeostasis) of lung tissue and aberrations in lung carcinoma. In addition, the role of chronic inflammation herein and the potential of some well-studied phytochemical compounds to target CSC promoting mechanisms is discussed. For decades, CSCs have been observed to behave like tissue stem cells, hence were postulated as the root of malignant diseases. Both compartments are considered as the apex of cellular hierarchy which is capable of proliferation and differentiation. Mostly quiescent lung tissue can only become proliferative in a repair process in response to tissue injury. Cells involved in lung tissue repair such as basal cell, club cell, neuroendocrine cell, and alveolar epithelial cell type II (AEC II) become important candidates for CSC oncogenic transformations when repair process and inflammation is chronically induced. Genes such as tumor protein p53 (*TP53*), RB transcriptional corepressor 1 (*RBI*), phosphatase and tension homolog (*PTEN*), cyclin dependent kinase inhibitor 2A (*CDKN2A*), *SOX2*, *MYC* proto-oncogene, phosphatidylinositol-4,5-bisphosphate 3-kinase catalytic subunit alpha (*PIK3CA*), epidermal growth factor receptor (*EGFR*), and *KRAS* proto-oncogene are recurrently altered in tissue-specific manners depending on the type of lung carcinomas. Some gene mutations and alterations in protein expression involve antioxidant mechanisms, differentiation, inflammation, quiescence, and chromatin modification such as Kelch like ECH associated protein 1 (*KEAPI*), Serine/threonine kinase 11 (*STK11*), NF- κ B, yes-associated protein (YAP), and CREB-binding protein (*CREBBP*), respectively. These genes were recently recognized for their role in contributing to CSCs maintenance in lung carcinomas. Chronic inflammation mainly disturbs the microenvironment through damaging stromal cells, inflammatory cells, and extracellular matrices, and consequently disruption of external signals such as bioelectric and chemical signals. All of these deviations may either be prevented or treated with phytochemical compounds derived from plant products, including curcumin, resveratrol, quercetin, epigallocatechin-3-gallate, luteolin, sulforaphane, berberine, genistein, and capsaicin. These phytochemical compounds are known to exert antioxidant, anti-inflammation, anticancer activities which can inhibit CSC initiation and maintenance. Usage of phytochemical compounds may be improved by structural manipulations, targeted delivery, and concentration adjustments. Their therapeutic potential could be further examined as single

agents and upon combined use with standard therapy, which may solve current common therapeutic problems like resistance and toxicity.

Discussion and future perspective

Identification of lung CSCs and targeting their molecular aberrations

Early in the days when CSC's role to sustain tumors was just recognized, lung CSCs were identified in three different ways. In 2008, Eramo and colleagues used CD133 as a putative CSC marker to identify undifferentiated tumorigenic CSC population in lung cancer¹. Such population grew indefinitely as tumorspheres in serum-free medium supplemented with epidermal growth factor (EGF) and basic fibroblast growth factor (bFGF), generated tumor xenografts in immunocompromised mice, and lost CD133 expression upon differentiation. Later that year, Levina and colleagues demonstrated the use of chemotherapy drugs consisting of cisplatin, doxorubicin, and etoposide to select CSC population that lost cytokeratin 8/18 (CK 8/18) but expressed CD133, CD117, stage-specific embryonic antigen 3 (SSEA-3), podocalyxin like (PODXL), POU class 5 homeobox 1 (POU5F1), and nuclear β -catenin². Similar to the previously described population, these drug-surviving cells could grow as tumorspheres, self-renew, differentiate, and gave rise to a tumor in immunocompromised mice. Besides, these cells also displayed a side population (SP) phenotype that indicates Hoechst 33342 dye exclusive behavior, a property assigned to CSCs. Later in 2010, Salcido and colleagues made use of SP phenotype to isolate rare CSCs from SCLC cell lines³. These cells were not only capable of reconstituting tumors in mice but also overexpressed stem cell and drug resistance genes such as ATP binding cassette subfamily G member 2 (*ABCG2*), fibroblast growth factor 1 (*FGF1*), *MYC*, *SOX1/2*, and *WNT1*. Identifications of CSCs with putative CSC markers or SP characteristics tend to narrow down to a small subpopulation of about 1% or less of tumor bulk cells. However, chemotherapy drug selection tends to expand the originally rare population to take up a higher percentage². These pioneering studies have largely been used as the guiding reference for the effort to find better CSC targets. In chapter 2, we reviewed current evidence for the origin and maintenance mechanisms of lung CSCs and provided examples of targeted therapeutic strategies. In brief, these included targeting of lung CSC intrinsic – as well as extrinsic (TME-driven) regulatory mechanisms. Intrinsic targets include signal transducer and activator of transcription 3 (STAT3) inhibited by AZ9150, napabucasin and TT-101; *MYC* proto-oncogene by Omomyc and sulforaphane; and histone deacetylase (HDAC) by CUDC-101 and TMU-35435. Extrinsic targets comprise vascular endothelial growth factor receptor (VEGFR) inhibited by apatinib and nintedanib; calmodulin by CBP501; and FGF2 by NSC12. Success in preclinical studies had pushed some of these inhibitors into clinical trials, however they did not perform as well as expected. Both identification and therapeutic efforts are seemingly complicated by the plastic nature of CSCs, the multiple regulatory pathways of stemness, and the parallel existence of multiple CSC populations. The contradicting observation of efficacy between preclinical and clinical studies may be also (partly) due to the lacking of stromal components in preclinical models. To improve the correlation of preclinical and clinical testing, future studies assessing therapeutic candidates need to employ *in vitro* models that replicate the physiological situations as close as possible.

Today, there is still not a single stem cell and drug resistance-related protein marker that can be used universally to pin-point CSC population. One reason for the non-universality of the CSC marker is that CSCs develop based on tissue-specific morphostasis instructions, hence CSCs adapt tissue-specific CSC markers. Also, a single marker is definitely not sufficient to identify CSC populations. The usage of either one or combinations of CD44, MYC, or SOX2 protein as CSC markers in chapter 3 mapped out about 85% of total GLC populations and about 40% expressed all three markers in advanced disease model growing from just 2% in population of untreated SCLC model. On the other hand, finding the exact percentages of CSC populations is probably not useful knowing that there are likely multiple clones of CSCs that each require their own identification effort, further complicated by tissue plasticity that enables differentiated cells to also transform into CSCs. Further understanding of molecular drivers of plasticity, in which TME-derived signals also play an important role (see also chapter 2), is required to clearly determine the boundary between differentiated tumor cells and undifferentiated CSCs and to potentially identify more common mechanisms that can be therapeutically exploited.

Tumor evolution in disease progression of SCLC and implication for therapy

Tumor cells often develop resistance to therapy and the ability to metastasize during disease progression. CSCs were postulated to participate in the process both during tumor initiation and progression. The study described in chapter 3 further clarified that the fraction of the CSC population with the expressions of CD44, MYC, and SOX2 and its functional properties get progressively higher in time following the direction of disease progression. This is consistent with *in vitro* drug selection studies performed by others which demonstrated a higher percentage of drug-resistant CSCs in lung cancer cell lines after treatment with certain chemotherapy drugs^{2,4}. More importantly, the study in chapter 3 also highlighted that the initially heterogeneous populations can become more homogeneous due to progressive accumulation of the same CSC clone by symmetric divisions⁵. Also, the finding of high CD44 and MYC expression in advanced SCLC together with the cellular categorization of classic and variant SCLC could be of clinical importance. For example, variant SCLC that commonly has amplification/overexpression of MYC may display specific sensitivity to aurora kinase inhibitors^{6,7}. The preliminary data we obtained suggested that CD44 can potentially be targeted with anti-CD44 blocking antibodies to inhibit the CSC activity of advanced SCLC. Moreover, recent classifications have categorized SCLC into several subsets consisting of SCLC-A, SCLC-N, SCLC-Y and SCLC-P, which respectively is achaete-scute homolog 1 (ASCL1)^{high}, neurogenic differentiation factor 1 (NEUROD1)^{high}, transcriptional coactivator YAP1⁺ and POU domain, class 2, transcription factor 3 (POU2F3)⁺ SCLCs⁸. MYC was found to activate Notch and drive the temporal shift of classic SCLC-A to variant SCLC-Y and SOX2 participates in directing the distinct transcriptional programs of both subsets^{9,10}. As also proposed in chapter 3, CD44 may potentially be a diagnostic marker for variant/advanced SCLC. Our findings and others' have essentially highlighted the importance of targeting CD44, MYC, and SOX2 to control the disease progression in SCLC patients. Targeting CD44 in SCLC still awaits future study by using available tools such as monoclonal antibodies, synthetic peptides, decoys, and aptamers among others¹¹. For MYC, transduction by Omomyc, a

dominant-negative MYC variant expressing vector, and bromodomain and extra-terminal (BET) inhibitors like JQ1 can be further tested^{12,13}. The only available tool to target SOX2, a synthetic DNA-binding transcription inhibitor, still requires further study¹⁴.

CSC targeting in NSCLC using phytochemical compounds

Thus far, disease management has been more successful in NSCLC when compared to SCLC. The improvement in disease management was accounted for by the development of drugs including TKIs and immune checkpoint inhibitors (ICIs)¹⁵. Some drawbacks are still hindering the progress to achieve better curation in patients, including therapeutic resistance and toxicity (side effects) problems. Natural occurring phytochemical compounds may provide an alternative strategy to target lung CSCs. In Chapter 6, several dietary- and herbs-derived phytochemical compounds including curcumin, resveratrol, quercetin, epigallocatechin-3-gallate, luteolin, sulforaphane, berberine, genistein, and capsaicin, were highlighted for their abilities to target CSC-related signaling pathways that regulate cell growth, inflammation and oxidative-stress.

In chapter 4 and chapter 5, several phytochemical compounds were assessed for their potential to target the Wnt/ β -catenin pathway, a major CSC propagating pathway, based on their activities to inhibit β -catenin nuclear localization and a number of CSC properties. With the ability to inhibit β -catenin nuclear localization at non-toxic concentration of chelerythrine chloride, both therapeutic resistance due to CSC accumulation and toxicity problems can potentially be solved. This low dose non-toxic inhibition was seen in at least two different lung cancer cell lines. On the other hand, benzyl isothiocyanate and phenethyl isothiocyanate were found to target different populations, i.e., CSCs and differentiated tumor cells, respectively. This finding suggests optimal therapeutic effects by combining both isothiocyanates to eliminate both tumorigenic CSCs and less-tumorigenic bulk cancer cells and, in addition, may also have less side effects compared to conventional chemotherapy. Arguably, the finding in chapter 4 that emphasized concentration-dependent effects *in vitro*, will be difficult to reproduce *in vivo* or clinical settings because dose manipulations are usually complicated by pharmacokinetic and pharmacodynamic properties of the compounds. However, recent advancement in drug delivery enables direct delivery through for example inhalation of encapsulated drugs that essentially equals to sustained and immediate interactions of drugs, respiratory tissue, and its microenvironment excluding systemic metabolism¹⁶. This means research data obtained from monolayer 2D and multicellular tumor spheroid 3D cultures could be more relevant to predict the response of such delivery. In future work, various carriers could be loaded with the phytochemical compounds recommended in this thesis and tested for anti-lung cancer efficacy.

Relevance of the CSC theory for lung cancer eradication

Preclinical studies have shown that both NSCLCs and SCLCs constitute CSC subpopulations within the tumor bulk. Tumorspheres from resected tumor samples of NSCLC patients were demonstrated to exhibit stemness features, such as long-term self-renewal ability, high invasiveness, high survivability against conventional chemotherapies, and lower latency of tumor formation in immunocompromised mice when compared to regular adherent NSCLC

cultures¹⁷. These CSCs were identified by the overexpression of stem cell-related and invasion-associated genes like aldehyde dehydrogenase 1 family member A1 (*ALDH1A1*), *CD44*, integrin subunit alpha 6 (*ITGA6*), kruppel like factor 4 (*KLF4*), *NANOG* homeobox, and snail family transcriptional repressor 1 (*SNAIL*) among others. When transcriptional signatures of epithelial adult stem cells were analyzed and compared in various epithelial cancers, SCLC was found to enrich those gene signatures and their signature scores were significantly higher than those of lung ADC and lung SCC¹⁸. Moreover, besides CSCs enrichment by tumor sphere culturing, prolonged culturing on increasing doses of chemotherapies or TKIs can also select for CSCs, as also briefly mentioned by our review described in chapter 2. Our study in chapter 3, using an SCLC culture model reflecting progressive stages of disease, essentially demonstrated also enrichment of CSCs populations during occurrence of therapeutic resistance and worsening of disease. With all this evidence, theoretically, therapeutic candidates that reduce aforementioned traits should work effectively in regressing the tumor. However, therapeutic resistance that is proposed to be due to the presence of CSCs is still a major recurrent problem, although clinical trials have started to assess therapeutic potential of targeting CSC. The Notch pathway is currently the most frequently studied target of CSCs in clinical trials recruiting lung cancer patients. A phase I trial assessing RO4929097 (a γ -secretase inhibitor of Notch pathway) combined with erlotinib hydrochloride in stage IV or recurrent NSCLC patients (NCT01193881) was terminated for unknown reasons, whereas a phase II trial assessing the same drug in previously front-line chemotherapy-treated advanced NSCLC patients (NCT01193868) was also prematurely terminated due to drug production halt. Judging from the initial trial result, it appears that the drug did not perform as well as expected because all five patients enrolled had progressive disease. In a phase II trial assessing the combination of carboplatin, pemetrexed plus placebo or 1 or 2 truncated courses of demcizumab—a monoclonal antibody targeting delta like canonical Notch ligand 4 (*DLL4*) in non-squamous NSCLC patients (NCT2259582), as many as 14% to 17% patients in the different demcizumab-treated groups had progressive disease when compared to only 8% in the placebo group. On the other hand, patients receiving demcizumab showed around 50% stable disease, which was 10% higher compared to patients receiving the placebo. This indicates that Notch-targeting treatment is still far from desirable. Our review in chapter 6 briefly discusses how the Notch pathway is unsuitable for targeting lung CSCs because of tissue-specific behavior of the lung that mainly utilizes Notch as differentiating signals for many of the proposed cell origins of CSCs. Hence, it is still too early to conclude that the concept of targeting CSC is not effective; it still needs further testing perhaps by following what is recommended in chapter 6, including redefining better targets, perhaps the use of phytochemicals, developing better delivery/vehicle, and using better disease models for those assessments.

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Chapter 8

Nederlandse samenvatting (Summary in Dutch)

Samenvatting

Longkanker is een complexe kwaadaardige ziekte die veel aandacht krijgt vanwege de hoge prevalentie onder de wereldbevolking onafhankelijk van demografische achtergronden. De variëteit in demografie kan worden verklaard door blootstelling aan verschillende risicofactoren voor longkanker. Hoewel verbetering in classificaties van longkanker heeft geleid tot het beter stratificeren van de ziekte voor de meest geschikte behandeling, is de huidige status van ziekte-incidentie en sterfte nog steeds de hoogste van alle kankersoorten. Aangezien de totale 5-jaarsoverleving van longkanker door de jaren heen laag blijft, zijn betere behandelingen noodzakelijk. In de afgelopen jaren heeft onderzoek aangetoond dat kankerstemcellen (CSCs) verantwoordelijk kunnen zijn voor therapieresistentie, de terugkeer van tumoren na behandeling en uitgezaaide ziekte (metastases), en dus bijdragen aan de slechte prognose van longkankerpatiënten. In dit proefschrift is de relevantie van CSCs en doelgerichte therapie hiertegen onderzocht bij de behandeling van longkanker. Meer specifiek is er onderzocht of chemische verbindingen afkomstig van planten (fytotherapeutica) cytotoxisch zijn voor longkanker CSCs en eventueel als toekomstige geneesmiddelen gebruikt kunnen worden, waarbij toxiciteitsproblemen geassocieerd met standaard chemotherapie voorkomen kunnen worden.

Hoofdstuk 1 geeft een achtergrondinleiding over longkanker, inclusief de geschiedenis van hoe longkanker een klinisch probleem werd, bekende risicofactoren, globale statistieken, diagnoseprotocollen, huidige therapieën en hun beperkingen. Longkanker werd voor het eerst in verband gebracht met levensstijl in 1761, d.w.z. het roken van tabaksproducten. Later werden meer risicofactoren geïdentificeerd, sommige geassocieerd met verschillende beroepen, levensstijlfactoren maar ook genetische factoren. Door het vaak voorkomen van deze risicofactoren heeft longkanker tegenwoordig een van de hoogste incidenties van alle kankers bij beide geslachten. Anderzijds wordt het hoge sterftecijfer aan longkanker veroorzaakt door beperkt succes van de huidige behandelingen, ondanks dat op basis van histologie en cytologie specifieke en innovatieve therapeutische behandelingen mogelijk zijn. De CSC-theorie, die het leidende concept van dit proefschrift vormt, werd ook kort geïntroduceerd om de relevantie bij longkanker mede vanuit een klinisch perspectief te verduidelijken. De detectie van CSCs werd beschreven op basis van verschillende methoden, waaronder door expressie van stamcelmarkereiwitten en daarop gebaseerde isolatie (sortering), klonale-/tumorsfeergroei, chemotherapeutische resistentie en kleurstof-exclusie.

In **hoofdstuk 2** is het wetenschappelijk bewijs besproken dat wijst op de relevantie van de CSC-theorie en het doelgericht elimineren van CSCs in longcarcinoom, zowel het kleincellig longcarcinoom (SCLC) als het niet-kleincellig longcarcinoom (NSCLC). Om de CSC-theorie in perspectief te plaatsen, werden parallellen besproken tussen CSCs en facultatieve stamcellen die normaal aanwezig zijn in de longen. Op basis van de lokalisatie van longcarcinomen in het longepitheel, hetgeen aanwijzingen geeft voor de vermoedelijke cellen van tumorooorsprong, zijn verschillende typen facultatieve stamcellen geïdentificeerd die de basis vormen voor oncogene transformatie. CSCs behouden veel van de eigenschappen van de facultatieve stamcellen van het longepitheel. CSCs gebruiken bijvoorbeeld vergelijkbare operationele signaalroutes voor hun instandhouding, zoals Wnt, Notch, Sonic Hedgehog (SHH), Janus Kinase/Signal Transducer and Activator of Transcription (JAK/STAT), Nuclear Factor kappa-

light-chain-enhancer van geactiveerde B-cellen (NF-KB) en fosfoinositide 3-kinase (PI3K)/AKT-routes. De plastische eigenschappen van longepitheelcellen, die facultatieve stamcellen kunnen worden, lijken behouden te zijn in CSCs. Onderdelen van de tumormicro-omgeving, zoals tumor-geassocieerde macrofagen, -geassocieerde fibroblasten en mesenchymale stamcellen, ondersteunen de groei van CSCs door het normale genezingsproces na te bootsen door het activeren van o.a. epitheliaal-naar-mesenchymale (EMT) herprogrammering. Ook cel-intrinsieke factoren kunnen bijdragen tot CSCs als gevolg van genetische afwijkingen (mutaties), verhoogde of verlaagde expressies van stamcelgenen, micro-RNA's (miR), piwi-interactie-RNA's (piR) en hypo- of hypermethylering van stamcel regulerende genen. Het behoud van CSC-fenotypes draagt bij aan therapieresistentie, inclusief resistentie tegen conventionele chemotherapie en tyrosine-kinaseremmende (TKI)-geneesmiddelen, en terugkerende tumorgroei. Intensief onderzoek vindt plaats naar nieuwe geneesmiddelen op basis van het doelgericht elimineren van CSCs, waaronder moleculaire doelwitten van de tumormicro-omgeving, de tumor zelf en ook door gebruik te maken van tegen CSC-gerichte antilichamen, antilichamen gekoppeld aan een geneesmiddel, kleine moleculen/peptiden, antisense-oligonucleotiden, nanodeeltjes, en chemo/fytotherapie.

In **hoofdstuk 3** werd de relevantie van CSC populaties en hun eigenschappen verder experimenteel onderzocht en aangetoond in een uniek SCLC in vitro model bestaande uit cellen (GLC cellijnen) geïsoleerd uit biopsieën afkomstig van één patiënt tijdens verschillende stadia van de ziekte. Deze cellijnen zijn GLC-14, -16 en -19, respectievelijk verkregen uit tumorweefsel van de patiënt voor behandeling (onbehandeld), na behandeling bij terugkerende tumorgroei (behandeld-recidiverend) en na meerdere behandelingen bij progressieve tumorgroei (behandelde-recidiverend-progressieve SCLC). Eerst werden RT-qPCR en western blotting gebruikt om de expressieniveaus van stamcelgerelateerde markers te onderzoeken, met name markers die ook aanwezig kunnen zijn op normaal longepitheel. CD44, MYC en SOX2 bleken in toenemende mate te accumuleren in de ziekteprogressie modellen, zowel op gen- als eiwitniveaus. Vervolgens werden immunofluorescentie en flowcytometrie gebruikt om de heterogeniteit van marker expressie te onderzoeken en te kwantificeren op cellulair en moleculair niveau. Interessant was de bevinding dat GLC-14 bestaan uit voornamelijk SOX2+ cellen, met een kleine populatie MYC+ en SOX2+MYC+ cellen, GLC-16 een toename laat zien van de SOX2+MYC+ en SOX2+CD44+-populaties vergelijkbaar aan de grootte van de SOX2+ fractie. Ook bleek GLC-19 grotendeels te bestaan uit een SOX2+CD44+ en SOX2+MYC+CD44+ populatie. Er is dus een progressieve accumulatie van SOX2+MYC+CD44+ cellen hetgeen het ontstaan van een homogenere CSC-populatie weergeeft tijdens SCLC progressie, en die wellicht de meest kwaadaardige CSC populatie vertegenwoordigt. De progressieve cumulatieve expressies van CD44, MYC en SOX2 correleerden met een sterker kolonievormend (groei) vermogen en resistentie tegen radiotherapie (γ -bestraling) in de GLCcellen die gevorderde ziekte vertegenwoordigen. Het gebruik van een CD44-blokkerend antilichaam leidde tot verlaagde groei (kolonievorming) hetgeen suggereert dat doelgerichte therapie tegen CD44-positieve CSCs mogelijk therapeutisch voordeel kan opleveren.

In **hoofdstuk 4** werd de mogelijkheid onderzocht of van planten afkomstige fytochemische verbindingen gebruikt kunnen worden als therapie tegen NSCLC CSCs. Met name chelerythrinechloride werd onderzocht aangezien het een belangrijke stamcel route, Wnt,

kan remmen. De antiproliferatieve en cytotoxische activiteit van chelerythrinechloride werd bevestigd met een real-time cel-analysator (RTCA) in drie verschillende cellijnen, adenocarcinoom (ADC) cellijn SK-LU-1, plaveiselcelcarcinoom (SCC) cellijn NCI-H1703 en CSC-celijn HLCSC. De Wnt-afhankelijke cellijn NCI-H1703 bleek erg gevoelig te zijn voor chelerythrinechloride. Meer diepgaande moleculaire analyses toonden aan dat chelerythrinechloride de nucleaire translocatie van β -catenine in de drie cellijnen kon remmen, een belangrijke transcriptie activator van de Wnt signaalroute. Chelerythrinechloride bleek effectiever in het verminderen van de nucleaire translocatie van β -catenine in NCI-H1703 bij concentraties lager dan de IC50-waarde, waarbij ook de expressie van stamcellfactoren SOX2 en MYC verlaagd werd. Chelerythrinechloride behandeling verlaagde de kolonievorming/groei, sferoïde groei, migratie en invasie van NCI-H1703, hetgeen anti-CSC activiteit suggereert.

In **hoofdstuk 5** werden meer fytochemische verbindingen bestudeerd op hun anti-CSC activiteit in de longkankercellijnen op eenzelfde wijze als in hoofdstuk 4. De fytochemische verbindingen bestonden uit de alkaloiden, evodiamine en chelidonine, chalconen, licochalcone-A en isoliquiritigenine, en isothioylisocyanaten, benzylycynaat en fenethylisothiocyanaat. Alle verbindingen lieten celgroei-remmende activiteit zien op een concentratieafhankelijke manier met de RTCA in SK-LU-1 en HLCSC cellen. Het bestuderen van effecten op de Wnt/ β -catenine-route, gaf aan dat Chalconen en isothiocyanaaten het beste de nucleaire translocatie van β -catenine remmen. Ongedifferentieerde HLCSC- en gedifferentieerde SK-LU-1-cellen hadden een verschillende gevoeligheid voor benzylyl- en fenethylisothiocyanaat, terwijl beide verbindingen even effectief waren in de Wnt-afhankelijke cellijn NCI-H1703. Met functionele testen werd aangetoond dat benzylyisothiocyanaat een sterkere remming heeft op kolonievorming, en fenethylisothiocyanaat op sferoïdegroei, hetgeen er mogelijk op duidt dat deze verbindingen aangrijpen op verschillende (CSC) cel populaties.

In **hoofdstuk 6** hebben we de overeenkomst besproken tussen belangrijke mechanismen betrokken bij morfostase (weefselhomeostase) van longweefsel en afwijkingen hierin bij longcarcinoom in relatie tot CSCs. Hierbij is de belangrijke rol van chronische ontsteking belicht en de het mogelijke gebruik van fytochemische verbindingen voor kankerpreventie en als therapie voor longkanker. CSCs gedragen zich als weefselstamcellen en worden daarom beschouwd als de oorsprong van kanker. Beide typen stamcellen staan aan de top van de cellulaire hiërarchie, in staat tot proliferatie en differentiatie. Longweefsel dat grotendeels geen celdeling vertoont, kan worden geactiveerd tot cel proliferatie door weefsel schade en daardoor geïnitieerde herstelprocessen. De longcellen die hierbij betrokken zijn, zoals basale cellen, clubcellen, neuro-endocrine cellen en alveolaire epitheel type II cellen (AEC II), zijn belangrijke kandidaten voor oncogene transformatie tot CSCs wanneer het herstelproces en de ontstekingsreactie chronisch worden. Genen zoals tumorproteïne p53 (TP53), Retinoblastoma transcriptionele corepressor 1 (RB1), fosfatase en tensin homolog (PTEN), cycline-afhankelijke kinaseremmer 2A (CDKN2A), SOX2, MYC-proto-oncogen, fosfatidylinositol-4,5-bisfosfaat 3 -kinase katalytische subunit alfa (PIK3CA), epidermale groeifactorreceptor (EGFR) en KRAS-proto-oncogen zijn veelal weefsel specifiek gemuteerd, hetgeen het type carcinoom bepaalt. Sommige genmutaties of overexpressie van eiwitten welke onderdeel uitmaken van antioxidantmechanismen, differentiatie, ontsteking, niet-proliferatieve cel status

(zgn. *quiescence*) en chromatinemodificatie, zoals KEAP1, STK11, NF-KB, YAP en CREBBP, kunnen bijdragen aan het in stand houden van long CSCs. Chronische ontstekingen verstoren voornamelijk de micro-omgeving door het beschadigen van stromale cellen, de extracellulaire matrix en infiltratie van ontstekingscellen wat leidt tot verstoringen in bio-elektrische en chemische signalen. Het lijkt mogelijk deze voorstadia van kanker te voorkomen of tumor te behandelen met chemische verbindingen afgeleid van plantaardige producten, zoals curcumine, resveratrol, quercetine, epigallocatechine-3-gallaat, luteoline, sulforafaan, berberine, genisteïne en capsaïcine. Van deze fytotherapeutica is bekend dat ze antioxiderende, ontstekingsremmende en dus ook kanker bestrijdende activiteiten hebben die het ontstaan en in standhouden van CSC kunnen tegengaan. Het gebruik van fytotherapeutica als potentiële kankergeneesmiddelen kan worden verbeterd door structurele manipulaties, gerichte toediening, concentratie-aanpassingen en gecombineerd gebruik met bestaande behandelingen. Gebruik van fytotherapie kan wellicht ook veelvoorkomende problemen van huidige therapie verminderen, zoals resistentie en bijwerkingen. Verder onderzoek is noodzakelijk om de anti-CSC activiteit en effectiviteit van fytotherapeutica bij longkanker nader te bestuderen.

Appendix

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