Cancer stem cells and epithelial-to-mesenchymal transition in lung cancer

Pore, Milind M.

Chapter 1

General introduction

Lung cancer

Lung cancer is one of the most frequent malignant tumors in Europe [1] and the United states [2]. It is the leading cause of all cancer related deaths worldwide [3]. Each year approximately 1.8 million people are diagnosed with lung cancer and over 1.6 million people succumb to this disease. Lung cancers arise from the respiratory epithelium and on the basis of histological features can be subdivided into two main subtypes non-small-cell lung cancer (NSCLC) and small-cell lung cancer (SCLC). About 80-85% of all lung cancer cases are NSCLC, which is further classified into adenocarcinoma (ADC), squamous cell carcinoma (SCC) and large-cell lung cancer (LCLC) [4]. The remaining 15-20% of lung cancer are SCLC that display a neuroendocrine differentiation [5]. SCLC is clinically the most aggressive type of lung cancer and is often centrally located and spreads readily to adjacent lymph nodes and is associated with early extrathoracic metastases [6].

The predominant risk factor for lung cancer is smoking [7]. Other risk factors of lung cancer are exposure to asbestos, radon, arsenic, chromium, nickel, vinyl chloride, and ionizing radiation [8,9]. Most of the lung cancer patients are diagnosed with advanced disease where conventional therapies are modestly effective and no curative treatments are available as yet. Because of this the overall 5-year survival in NSCLC patients is poor, being less than 15% for all disease stages combined [3]. SCLC has an even worse prognosis but is usually initially responsive to chemotherapy. However, SCLC recurs rapidly and 5-year survival is therefore only 5% [10]. For the treatment of NSCLC in localized disease surgical resection is preferred, however, in advanced stage NSCLC a multimodality approach is preferred that might include chemotherapy, radiotherapy, sometimes surgery and palliative care.

More recently new classes of drugs have been introduced that specifically target certain molecular pathways. For example, small molecules that specifically inhibit tyrosine kinase receptor activity of the epidermal growth factor receptors (EGFR), such as gefitinib and erlotinib also known as Tyrosine Kinase Inhibitors (TKI) are available to treat a subpopulation of NSCLC patients with mutated EGFR gene [11]. For SCLC little progress has been made in the treatment of this disease in the past several years. The treatment usually includes platinum-based combination chemotherapy, combined with hyperfractionated thoracic radiation and prophylactic cranial irradiation depending on disease stage, response to therapy and of course treatment wish of the patient [12].

Most of the lung cancer patients harbor tumor suppressor p53 mutations, hence preventing the tumor cell apoptosis (programmed cell death) [13]. Currently targeting of the apoptotic pathway is being studied in several preclinical and clinical studies in lung cancer that aim to selectively trigger the activation of caspases and proteases that form the central executioners of apoptosis [14]. This includes pro-apoptotic approaches such as the targeting of tumor necrosis factor (TNF)-related apoptosis-inducing ligand (TRAIL) receptors that leads to activation of the so-called extrinsic or death receptor apoptotic pathway [15]. Another strategy is neutralizing anti-apoptotic proteins, such as inhibitor of apoptosis proteins (IAPs) that suppress caspase activity or the mitochondrial apoptosis inhibitor BCL-2, that can enhance the efficacy of chemo- and radiotherapy [16].

Cancer Stem Cells (CSC)

Novel discoveries in the field of cancer cell biology have provided exciting new insights in the mechanisms that drive tumor initiation and progression such as the identification of CSCs and the process of Epithelial to Mesenchymal transition (EMT) [17]. Tumor cells are highly heterogeneous in nature and two main models have been proposed to explain this heterogeneity [18]. The stochastic clonal selection model suggests that mutant tumor cells with growth advantage are selected and expand during tumor progression. The cells in the dominant population have a similar potential for (re)generating tumor growth. The CSC hypothesis suggests that tumor cells are heterogeneous in nature and hierarchically ordered. At the top of the hierarchy a small subpopulation of cancer cells exist that have stem cell properties such as high self-renewal ability multilineage differentiation, and resistance to therapy. These cells are therefore termed CSCs or tumor initiating cells [18]. The earliest reports on CSCs came from leukemia in which a rare population characterized by high CD34 and low CD38 expression upon transplantation in mouse generated leukemia displaying the same disease properties as observed in the patient [19]. The first evidence of CSCs in solid tumors was reported by Al Hajj et al. in breast cancer [20]. In this study CD44 high and CD24 low breast cancer cells were found to enrich for cells with tumor initiating capacity in mouse models. Thereafter CSC populations were identified in other solid tumors such as brain, colorectal, head and neck, pancreatic, prostate, ovarian, skin squamous cell carcinoma (SCC) as well as lung cancer [21]. Histologically lung cancer is divided according to the site of its origin. Recently normal stem cells that make the lung epithelium at trachea, bronchus and alveoli have been identified [22]. It is considered most likely that CSCs arise from normal stem cells that have long life

spans and able to accumulate oncogenic mutations over time.

Several methods have been employed to isolate lung CSCs from cell lines and primary tumor material. Specific culture conditions can be applied to select for stem/progenitor cells such as culturing under non-adherent serum-free conditions in tightly defined medium leading to spheroid growth [23,24]. Furthermore, several markers or properties specific for CSCs are used to enrich for this cell population. For example, the side population (SP) is widely used to isolate the CSC population by FACS on the principle that CSCs have the ability to effectively efflux the dye Hoechst 33342 due to high expression levels of the ATP binding cassette transporter superfamily member-G2 (ABCG2), as was also described for lung cancer cell lines and primary tumor material [25]. Other methods such as the selection of therapy resistant cell populations [26], cells exhibiting aldehyde dehydrogenase activity (ALDH) [27], or the use of cell surface markers thought to be selective for lung CSCs such as CD44, CD133, and CXCR4 are often used to enrich for lung CSC populations [26]. In addition, stem cell pluripotency markers such as OCT4, SOX2 and NANOG have been used as CSC markers in lung cancer [28]. However, there are large discrepancies in CSC marker expressions in lung cancer; for example SP cells do not always express other cell surface CSC markers and vice versa [26]. Thus, the applicability of CSC markers is often depending on the cell culture model used and there is a need to identify more common "bonafide" CSC markers in lung cancer for isolation purposes and to study their molecular biology for developing novel therapeutic approaches.

Epithelial to Mesenchymal transition (EMT)

EMT is a complex cellular and molecular program in which epithelial cells lose their phenotype by shedding cell-to-cell adhesion molecules, such as desmosomes, tight- and gap junctions, lose apical-basal polarity and attain frontal-rear polarity and acquire a mesenchymal phenotype that is associated with high motility and invasive properties [29]. EMT has been classified in three different categories according to the biological context in which they occur [30]. Type 1 EMT is associated with embryogenesis and organogenesis, Type 2 EMT is associated with wound healing, tissue regeneration and organ fibrosis, whereas Type 3 EMT has been associated with neoplastic cells that promote tumor progression [30]. Cells undergoing EMT can be identified by loss of epithelial markers such as E-cadherin, EpCAM and cytokeratins and gain of mesenchymal markers like Fibronectin, Vimentin and N-cadherin. In the activation of EMT several nuclear transcription factors, signal

transduction pathways and external stimuli including the extracellular matrix (ECM) are involved. Examples of nuclear transcription factors are ZEB1, SNAIL, SLUG, TWIST, KLF8, and E47. These transcription factors directly or indirectly suppress E-cadherin expression and are instrumental in EMT induction. The external stimuli that regulate EMT are derived from the microenvironment involving the ECM, stromal cells and macrophages [31]. Several key triggers for EMT that contribute to tumorigenesis have been identified including WNT, NOTCH and TGF-β signaling [32]. For example, activation of TGF-β receptor-1 leads to phosphorylation and activation of SMAD2 and SMAD3 and subsequently the activation of EMT transcription factors and EMT [29]. TGF-β exposed NSCLC A549 cells are a well known model to study EMT leading to altered morphology and loss of epithelial markers like E-cadherin and EpCAM and gain of mesenchymal markers such as Fibronectin and Vimentin and was first described by Kasai and coworkers [33]. In addition to TGF-β, several tyrosine kinase receptors such as fibroblast growth factor (FGF), insulin growth factor (IGF), epithelial growth factor (EGF) family members and platelet derived growth factor (PDGF) receptors play important roles in the regulation of EMT [34]. EMT has been extensively studied in preclinical models and also has been demonstrated to occur at the tumor/ host tissue interface in patient tumor samples [33,35,36]. Markers of EMT have been associated with clinical outcome in lung cancer, for example loss of E-cadherin has been correlated with worse outcome in NSCLC [37]. EMT also plays a role in acquired resistance towards targeted therapies, e.g. NSCLC cell lines with epithelial characteristics were significantly more sensitive to gefitinib when compared to mesenchymal NSCLC cell lines and, moreover, exogenous expression of E-cadherin sensitized the mesenchymal NSCLC cells towards gefitinib treatment [38]. EMT also has been associated with acquired resistance to EGFR TKIs in laboratory models in lung cancer [39,40]. Interestingly, the induction of EMT has been associated with CSCs, which was first discovered in breast cancer [41]. The possible link between CSCs and EMT has thus far not been extensively studied in lung cancer.

Circulating Tumor Cells (CTCs)

Recent evidence suggests that the metastatic spread from the primary tumour site may be an early event in cancer progression and is not its late consequence as previously thought [42]. Even before the primary tumor is detected cancer cells can invade to adjacent tissues from where they can travel to the lymphatic and blood circulation. Tumor cells in blood are known as CTCs [43]. A proportion of CTCs are able to extravasate and colonize distant sites by forming micrometastasis; some of

them remain dormant, however, many ultimately result in metastatic disease. Several methods have been developed to detect and isolate CTCs from peripheral blood from patients with solid tumors [44]. CTC numbers in blood appeared to be variable also depending on the tumor type studied. However, large numbers of CTCs have been detected in breast (upto 30,000 CTCs/7.5 ml blood) and SCLC (upto 10,000 CTCs/7.5 ml of blood) [45,46]. Most of the CTC detection methods are based on enrichment of CTCs from vast numbers of blood cells and additional detection with tumor specific marker [47]. The enrichment of tumor cells can be achieved by the virtue of physical properties such as size, density and charge or specific biological markers expression on CTCs that are absent on blood cells. Currently, the CellSearch (Veridex LLC, Raritan, NJ, USA) system is the only FDA approved CTC detection method thus far. CTCs are captured by ferromagnetic beads coupled with the epithelial cell adhesion molecule (EpCAM) and are subsequently analyzed by immunohistochemistry for presence of cytokeratin 8,18,19 and absence of the leukocyte specific marker CD45 [47]. A major disadvantage of this method is that particularly epithelial CTCs are isolated. Therefore CTC detection methods that are based on size, density or charge are marker independent and are becoming more popular and are used in parallel to CellSearch in various studies [44]. Detection of CTCs is relatively simple and provides a non-invasive method with great implications for determining the prognosis and predicting the response of therapy of patients with solid tumors including lung cancer. Overall high CTC counts were correlated with worse outcome whereas patients, with lower CTC counts lived longer. For example, in breast cancer 5 CTCs/7.5 ml of blood was used as a threshold to determine the prognostic value which was also used in many subsequent studies [48]. In SCLC presence of ≥ 2 CTCs/7.5 ml of peripheral venous blood was found in 75% of patients with limited and extensive disease and used as a threshold for low or high CTC numbers [49,50]. In parallel these studies also showed the value of using CTC detection for predicting treatment outcome.

Scope and aim of the thesis

A better understanding of the proposed roles of CSCs and EMT in lung cancer progression and treatment is important for the development of better prognostic markers and targeted therapies. Moreover, the detection of CTC levels in lung cancer patients may provide a novel and non-invasive diagnostic and prognostic method to predict disease outcome and response to therapy, and EMT occurring in the tumor may play a role in facilitating intravasation and the occurrence of CTCs in

the blood. Research described in this thesis primarily focuses on investigating the role of CSCs and EMT on lung cancer progression.

Outline of thesis

In **chapter 2** we studied the possible involvement of CSCs and EMT in resistance to therapy and metastatic spread by employing an *in vitro* model consisting of 3 SCLC cell lines, GLC14, GLC16 and GLC19, representing an untreated, treated and progressing tumor from one patient during clinical follow-up, respectively. We hypothesized that enrichment of CSCs and induction of EMT is associated with disease progression and would be reflected in this unique longitudinal SCLC model. Therefore the cell lines were evaluated for the expression of several CSC and EMT markers. Their invasive and spheroid forming potential was also tested.

In **chapter 3** we used the TGF-β-inducible NSCLC A549 EMT model to study the possible effects of EMT on chemosensitivity, migration potential, invasive capacity and cancer stem cell properties. Also the effect of TGF-β on the expression of several CSC markers was studied and in parallel their capacity to grow as spheroids was evaluated as a measure of a CSC phenotype. To confirm *in vitro* findings, an orthotopic mouse model was set up to explore the metastatic spread of transpleurally injected parental and TGF-β-induced mesenchymal luciferase transfected A549 cells by bioluminescent imaging (BLI) and pathological analyses. Also an attempt was made to monitor CTC levels in this model.

In **chapter 4** we describe a clinical study in which we aimed to investigate the prognostic value of CTCs in SCLC patients and whether changes in CTC number are predictive for response to chemotherapy. For this multicenter prospective study blood samples were obtained from 59 patients with SCLC before, after one cycle, and at the end of chemotherapy. CTCs were measured using Veridex CellSearch systems. CTC numbers and disease state (limited or extensive disease) was compared with therapy response and overall survival in patients. SCLC is a neuroendocrine tumor that is characterized by high numbers of CTCs as determined by the CellSearch system.

In **chapter 5** we used primary diagnostic biopsy material obtained from SCLC patients that enrolled in the CTC study described in chapter 4. The aim of this study was to investigate the expression of CSC and EMT markers and their association with CTC levels and overall survival in these patients. The biopsies available from

38 patients were immunohistochemically stained for the CSC markers CD44 and SOX2, the epithelial markers E-cadherin, EpCAM and Cytokeratin 8,18,19 and the mesenchymal markers c-MET and Vimentin. Individual and combination of CSC and EMT markers expression were compared to previously determined CTC levels and overall survival of patients.

In **chapter 6** we aimed to study CSCs in the esophageal adenocarcinoma (EAC) cell lines OE19 and OE33. Cells were grown as adherent monolayers supplemented with FCS and as spheroids in serum free Neurobasal media (NBM) supplemented with epidermal growth factor (EGF), basic Fibroblast Growth Factor (bFGF), known to enrich for stem cell properties. CSC characteristics were studied by determining CSC marker expression, spheroid forming capacity and chemosensitivity. Tumor forming capacity was determined by subcutaneous injection of monolayer or spheroid cultured cells in NOD/SCID mice. Finally, transcriptional profiles of monolayer and spheroid cultured OE19 and OE33 cells and their respective xenografts were compared using Illumina platform to identify differences in gene expression.

In **chapter 7** we reviewed current and novel methods of targeting apoptosis pathways in lung cancer. This includes targeting tumor necrosis factor (TNF)-related apoptosis-inducing ligand (TRAIL) receptors, BCL-2 family members and apoptosis-inhibitory proteins (IAPs). Preclinical studies are discussed along with results from early clinical trials and future perspectives of apoptosis targeted strategies in lung cancer are sketched.

In **chapter 8** the experimental results of this thesis are summarized, followed by a summarizing discussion on CSCs, EMT and CTCs and the clinical implications as well as future directions for research.

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