Chemotherapy-induced Enrichment of Cancer Stem Cells in Lung Cancer

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Abstract
Small cell lung cancer (SCLC) is a neuroendocrine tumor of the lung that is clinically characterized by early recurrence after initial complete response to combination chemotherapy, resulting in dismal prognosis. Cancer stem cells (CSCs) are suspected to survive the initial cycles of treatment and trigger relapse in form of a chemoresistant tumor. Whereas a profound amount of results reporting on CSCs exists for non-small cell lung cancers (NSCLC), far less data which are restricted to a few cancer cell lines and CD133-positive subpopulations of fresh tumor tissues are available for SCLC. Enrichment of CSCs in response to chemoradiotherapy and in recurrent tumors would prove their clinical relevance. The GLC14, GLC16 and GLC19 series of SCLC cell lines established from serial biopsies of one patient before, during and after therapy, respectively, provides an opportunity to investigate the phenotypical changes during chemo-radiotherapy. We demonstrated by whole genome expression analysis that CSC markers such as CD44, CD133, CD47, ALDH1A1, AKR1C and members of the WNT and notch pathways are increasingly expressed after chemo-radiotherapy in GLC16 and GLC19, compared to the treatment-naive GLC14 SCLC cells. These findings in addition to the available literature suggest a role for CSCs in SCLC tumor recurrence and in the resistant phenotype. Thus, targeting of CSCs may constitute a promising approach to delay or prevent recurrences that would eventually result in treatment failure and poor survival rates in SCLC patients.

Keywords: Small-cell lung cancer; Chemotherapy; Cancer stem cells; Microarray; Chemo-resistance

Small Cell Lung Cancer
Lung cancer is the leading cause of cancer death due to inadequate therapeutic options, thus equaling morbidity with mortality rates and causing an unsolved health problem [1,2]. Among the neuroendocrine tumors that account for approximately 20% of lung cancers, SCLC comprises the majority of cases [3]. This highly malignant tumor originates from neuroendocrine cells (Amine Precursor Uptake and Decarboxylation/APUD cells) in the bronchus called Feyrter cells and in consequence of its rapid growth, early dissemination and progression to drug resistance after successful first-line therapy treatment of SCLC has limited efficacy [4-7]. Lung cancer is the most preventable cancer worldwide owing to the fact that the predominant risk factor is tobacco consumption and women are increasingly affected [8]. Non-neuroendocrine lung tumors which are subsumed as non-SCLC (NSCLC), including adenocarcinoma, squamous cell carcinoma and large cell carcinoma, differ by a lower growth fraction and aggressiveness from SCLC. Without treatment, SCLC has an aggressive clinical course, with median survival times from diagnosis of only several months [7]. Because the majority of patients with SCLC present with disseminated disease, localized therapy is not possible in most cases. The current chemotherapy regimens prolong survival, nevertheless, the overall survival at 5 years is only 5 to 10% [5-7]. Patients with SCLC confined to the hemithorax or mediastinum, or the supraclavicular lymph nodes are classified as having limited-stage disease with an expected median survival of 16 to 24 months and the other patients with extensive-stage disease have a median survival of only 6 to 12 months [5-7].

In patients with limited-stage SCLC, combination chemotherapy produces results that are clearly superior to single-agent treatment with overall objective response rates of 65 to 90% and complete response rates ranging from 45 to 75%. The combination of etoposide and cisplatin/carboplatin chemotherapy with concurrent chest irradiation is standard and achieves median survivals of 18 to 24 months and 40% to 50% 2-year survival with less than a 3% treatment-related mortality [5-7]. The prognosis for patients with relapsing SCLC is exceedingly poor, with expected median survival between 2 to 3 months. Topotecan is the single approved chemotherapeutic drug for second line treatment of SCLC prolonging live for several months [9]. In summary, the treatment of SCLC is characterized by a lack of progress and extremely short survival rates in advanced disease, that have been not seen much improvement in the past decades. There are many proposals as to why this may be, including the presence of resistant CSCs, accelerated development of acquired resistance, redundancy in cell survival/signalizing pathways, etc., but the underlying cause remains unclear so far.

Cancer Stem Cells Concept
According to a popular newer concept, CSCs are a rare population of undifferentiated cells driving tumor initiation, maintenance and spreading [10,11]. This contrasts or expands the former model of cancer development as a stochastic process with outgrowth of well-adapted tumor cell clones. CSCs display unlimited proliferation potential, ability to self-renew and capacity to generate a progeny of differentiated cells, recapitulating the major tumor populations. CSC-like cells have been characterized from leukemia, and a range of solid tumors, including melanoma, glioblastoma as well as breast, prostate, pancreatic, and colon carcinomas [10-12]. These cells can be expanded in vitro as tumor spheres, i.e. mamma- and neurospheres, and produce...
original tumors in immunodeficient mice at lower cell numbers than the unfractionated tumor cell population. Brain, hematopoietic, prostate and colon cancer CSCs feature the membrane antigen CD133, whose expression is shared by normal stem cells of different lineages [13]. In a combined model of complex tumor development integrating the stochastic and CSC model, genetically distinct CSCs exist on top of each heterogeneous tumor subclones [11]. Most therapies are directed at the bulk of rapidly dividing tumor cells but not the slow dividing CSCs. CSCs are resistant to both radiation and chemotherapy, and, therefore, this subpopulation persists following therapy and seems to be responsible for relapses which occur in some cases after an extended period of dormancy of cells residing in a CSC niche [14,15]. Resistance to therapy is believed to be a complex, multistep process that begins with the short-term, inherent plasticity of subpopulations of cancer cells. The cytotoxic stimuli resulting from inhibiting a target can result in enhanced expression/activity of stemness factors in persisting subpopulations of cells [16]. Expression of these stemness factors results in genetic plasticity that allows these cells to remain in a dormant, drug-tolerant state. Since the role of CSCs is still not firmly established, these cells are also alluded to as tumor initiating cells [TICs], cancer initiating cells [CICs] and tumor propagating cells [TPCs]. Recently, independent studies using lineage-tracing technique in mouse models have corroborated the existence of CSCs in brain, skin and intestinal tumors [17,18]. Eradicating CSCs, in addition or instead of the fast growing tumor mass seems to constitute a promising approach to achieve a long-lasting response and thereby to improve cancer therapy.

Although work and publications on CSCs mounted in recent years, a stringent definition of CSCs in solid tumors and specific markers for CSCs in distinct types of tumors are still lacking that some phenotypic and functional features of CSCs can reversibly turn on and off constituting a transient state rather than a hierarchically determined characteristic of rare CSCs [20]. CSCs may be defined by their high tumorigenicity in vivo, but in practice they are usually detected by the presence of combinations of various antigens such as CD24, CD44, CD133, EpCAM, CD166, Lgr5, CD47, and ALDH activity in various malignant tumors [10,20]. In the CSC model, targeting embryonic progenitor markers β-tubulin-3 and glial fibrillary acidic protein. These MTC cell lines [26]. The CD133+ cells could be expanded by sphere formation assay, passaged multiple times, and expressed neural progenitor markers β-tubulin-3 and glial fibrillary acidic protein. These data support the existence of CSC-like cells in MTC, which exhibit the features of self-renewal and of multiple lineage differentiation that is dependent on ret proto-oncogene receptor activity.

Accumulating evidence point to the occurrence of CSCs in neuroendocrine lung tumors and this data on SP cells, CD133-, CD166-, CD44- and ALDH1-expressing cell subpopulations, as discussed in the following sections, are beginning to clarify the true phenotype of the lung CSCs [21].

**Lung Cancer and CSCs**

In comparison to the rapidly advancing research on breast and colon cancer CSCs, investigations on this cell subpopulation in the area of lung cancer is lagging behind. Identifying these stem cells in lung cancer and finding ways for their elimination may lead to new clinical approaches to delay or prevent disease recurrence [27-30].

The tumorigenic cells in SCLC and NSCLC were first characterized as a rare population of undifferentiated cells expressing CD133, a marker of normal and CSCs of the hematopoietic, neural, endothelial and epithelial lineages [31]. Lung cancer CD133+ cells were able to grow indefinitely as tumor spheres in serum-free medium containing epidermal growth factor (EGF) and basic fibroblast growth factor (bFGF). The injection of a low number of lung cancer CD133+ cells in immune-compromised mice generated xenografts matching the original tumor. Upon differentiation, lung cancer CD133+ cells acquired the specific lineage markers and lost CD133 expression as well as tumorigenicity. Furthermore, a panel of 15 primary lung cancer cell lines representing SCLC, large cell, squamous cell carcinoma and adenocarcinoma, comprised a small population of cells strongly positive for CD44 with coexpression of CD90 in the spheroid-forming cell fraction [32]. These CD44+/CD90+ cells revealed increased expression of mesenchymal-benignous stem cell-related markers and resistance to irradiation, indicative of CSCs, in case of SCLC and large cell carcinoma. The description of different stem cell markers for the same tumor entities demonstrate that this subject is not settled so far for lung cancer.
NSCLC and CSCs

CD44, CD133 and CD90 have been widely accepted for isolation of CSCs in human hematological malignancies as well as in solid tumors [32]. The most prominent CSC marker found in NSCLC lung cancer tissues is CD133, commonly associated with the maintenance, metastasis and drug-resistance of tumor cells [33,34]. The different approaches to identify CSCs in NSCLC are discussed below.

Sphere formation of NSCLC CSCs: Sphere formation in tissue culture is particularly useful to enrich the potential CSC subpopulations when specific CSC makers have not been defined [35,36]. To obtain tumor cell spheres, culture conditions have to be adjusted to serum-free media, supplemented with several growth factors, such as EGF, bFGF, leukemia inhibitory factor (LIF) and various other additives, upon which the spheroid growth of subpopulations of the tumor cell lines which display expression profiles consistent with CSCs is favored. Furthermore, sphere cells exhibit increased expression of CSC surface markers and oncogenes compared to adherent cultures and are associated with enhanced tumorigenicity and chemoresistance.

In a study by Eramo et al. 0.6 to 22.0% of SCLC and NSCLC tumor cells were capable of forming spheres with varying frequency of CD133 expression [32]. Lung cancer CD133+ cells were able to grow indefinitely as tumor spheres in serum-free stem cell selection media. These CD133+ lung cancer sphere showed expression of stemness genes such as Oct-4 and NANOG, self-renewal potential, chemotherapeutic resistance, and ability to recapitulate tumor heterogeneity in vivo. Sphere growth from 10 NSCLC patient samples and five NSCLC lung cancer cell lines sorted for CD133+ expression displayed higher Oct-4 expression and the multiple drug-resistant marker ABCG2 in conjunction with significant resistance to chemotherapeutic agents (i.e., cisplatin, etoposide, doxorubicin, and paclitaxel) and radiotherapy [37]. The treatment of Oct-4 siRNA could specifically block the capability of CD133+ to form spheres and enhanced response to chemoradiotherapy. Sphere growth was obtained for 11 out of 15 lung adenocarcinoma malignant pleural effusion patient samples in another study [38]. Compared to adherent cells, these sphere cells were associated with enhanced ALDH1 expression and Oct-4, Nanot3, and Stat3 mRNA expression. In summary, according to their sphere-forming capability and specific markers, NSCLC specimens contain a subpopulation of cells with CSC characteristics. The NCI-H1915 large cell NSCLC line forms typical CSC tumor spheres and recapitulates original tumor histologies in brain xenotransplantation models [39]. Furthermore, brain metastases of patients also expressed CD15 and CD133, markers suggestive of a stem-like population.

Chemotherapeutic selection of NSCLC CSCs: According to the CSC hypothesis, chemotherapeutic preferentially targets the rapidly dividing mass of differentiated cells in tumors and spares the slowly growing, chemoresistant CSC cells. Vice versa it should be possible to enrich CSC cells in vitro by cultivation of cell lines in the presence of chemotherapeutics. This was demonstrated in drug-selected cells (doxorubicin, cisplatin, or etoposide) of the human lung cancer cell line H460, which displayed spheroid formation, stem cell characteristics, self-renewal capacity and ability to differentiate, as well as high tumorigenic and metastatic potential [40]. Inherent and acquired cisplatin resistance reduces the effectiveness of this agent in the treatment of NSCLC. An isogenic model of cisplatin resistance was generated in a panel of NSCLC cell lines (A549, SKMES-1, MOR, H460) by selecting cisplatin resistant sublines over a period of twelve months [41]. Resistant cells accumulated in the G1/G0 phase of the cell cycle and showed enhanced clonogenic survival. Moreover, these cells displayed a CSC-like signature with increased expression of CD133+/CD44+ Nanog, Oct-4 and SOX-2 markers, increased ALDH activity and upregulation of the epithelial-mesenchymal transition (EMT) markers, namely c-Met and β-catenin. Concordantly, these resistant sublines demonstrated decreased uptake of cisplatin and reduced cisplatin-GpG DNA adduct formation compared to the parental cell lines. In a similar experimental approach, cisplatin treatment of lung cancer cells in vitro resulted in enrichment of the CD133+ cell fraction, both after acute cytotoxic exposure and in cells with a stable cisplatin-resistant phenotype [42]. Subpopulations of CD133+ABCG2+ and CD133+CXCR4+ cells were spared in vivo cisplatin treatment of lung tumor xenografts established from primary tumors.

Cell surface markers of NSCLC CSCs: CD133+ cells sorted from patient lung cancer tissue samples, displayed enhanced expression of Oct-4, enhanced self-renewal ability, increased expression of ABCG2, enhanced resistance to chemoradiotherapy, increased invasiveness and tumorigenicity, as well as increased spheroid formation compared to CD133- cells [37]. In a similar study, Bertolini et al. reported enhanced tumorigenic potential in vivo, elevated expression of ABCG2, CXCR4, ITG6A, Oct-4, and NANOG as well as cisplatin resistance in vitro and in vivo of CD133+ NSCLC cells [42]. The CD133+ and epithelial-specific antigen-positive (CD133+ESA+) subpopulation is increased in primary NSCLC compared with normal lung tissue. It is distinguished by expression of stemness genes, adhesion, higher motility and drug efflux than the CD133- counterpart and has higher tumorigenic potential in immune-compromised mice [42]. Accordingly, a tendency toward shorter progression-free survival was observed in CD133+ NSCLC patients treated with platinum-containing regimens. In tumor specimen of 45 patients with respectable NSCLC, expression of CD133 showed a higher correlation with deceased patients and a strong significant association with patients exhibiting progressive disease [43]. As further CSC marker, expression of CD44 on 62-96% of tumor cells was described for 6 out of 10 human NSCLC lines. Compared to CD44- cells, CD44+ cells displayed spheroid formation, resistance to cisplatin treatment in vitro, enhanced tumorigenicity in vivo, and elevated expression of stemness genes Oct-4, NANOG, and SOX2 [44].

Cellular activities of NSCLC CSCs: CSC cells may be detected as side cell (SP) subpopulation by assay of the Hoechst 33342 efflux which is mainly effected by the ATP-cassette-binding ABCG2 [23]. Dye efflux assays were employed to isolate SP cells were from six human NSCLC cell lines (H460, H23, HTB-58, A549, H441, and H2170) [45]. Xenograft experiments in immune-compromised mice showed that these SP cells were enriched in tumor-initiating and disseminating capability, displayed elevated expression of ATP-binding cassette transporters (ABCG2) and chemoresistance. In this case, unlimited proliferative potential was conferred by high expression of human telomerase reverse transcriptase (hTERT) and the G1 quiescent state indicated by a lower expression of minichromosome maintenance (MCM)-7 protein. In another study, sixteen clinical lung cancer samples also displayed a smaller but persistent SP population [46]. However, expression of CD133 was restricted to the SCLC line H446 in contrast to the NSCLC lines A549, H157, H226, Calu-1 and H292, as identified by real-time PCR and fluorescence-activated...
The measurement of ALDH1 activity and expression represents a universal marker for the identification and isolation of CSCs from multiple sources [47]. Functional tests for this marker CSCs rely on the fluorescence-activated cell sorter analysis [48]. In human lung cancer cell lines ALDH1 activity was associated with self-renewal and differentiation, resistance to chemotherapy, expression of CD133 and enhanced tumorigenicity, as well as ability to recapitulate the original tumor heterogeneity in vivo [49]. From 303 clinical patient specimens and controls, overexpression of ALDH1 was positively correlated with stage and grade of the tumor and associated with poorer prognosis for patients with early-stage lung cancer.

**SCLC and CSCs**

For the characterization of putative CSCs in SCLC only a limited number of studies have become available so far [50].

**Sphere formation of SCLC CSCs**

Stem-like cells were enriched from the SCLC cell line H446 by growing them as spheres in a defined serum-free medium [51]. These cells showed increased *in vitro* clonogenic and *in vivo* tumorigenic potential as well as drug resistance and contained a higher proportion of cells expressing the stem cell surface markers CD133 and urokinase-type plasminogen activator receptor (uPAR), when compared with unfractionated cells. However, both CD133+ and CD133− cell fractions were capable of forming spheres, whereas only cells derived from the uPAR+ fraction exhibit this capability. Moreover, formation of transplantable tumors was restricted to uPAR+ cells which could also differentiate into CD56− cells, CK+/cytokeratin-positive cells, and uPAR cells. These data indicated the existence of a population of tumor sphere-forming cells with CSC properties in the H446 SCLC cell line. A similar enrichment of SCLC stem-like cells by isolation of sphere-forming cells from SCLC cell lines was applied for screening differentially expressed miRNAs [52]. Of the five downregulated miRNAs (let-7, miR-20, 21, 27a and 30b), miR-27a was found to enhance the stem-like properties of SCLC cells *in vitro* and to constitute critical factor in maintaining a stem cell function in SCLC.

**Chemoresistance of selected SCLC cell populations**

The relationship between expression of the CSC markers CD133 and uPAR/CD87 was investigated in six SCLC cell lines [53]. The SBC7 cell line showed the highest expression levels of both CD133 and CD87 and was used to isolate distinct cellular subsets. Both CD133+/CD87− and CD133+/CD87+ subpopulations showed a higher resistance to etoposide and paclitaxel as well as greater repopulating ability than the CD133−/CD87− subpopulation. CD133+/CD87− cells contained more G0 quiescent cells than CD133+/CD87+ cells, although CD133+/CD87+ cells showed the highest tumorigenic potential.

**Cell surface markers of SCLC CSCs**

SCLC and NSCLC with neuroendocrine features express achaete-scute complex homologue 1 (ASCL1), where the factor may play a role in the primitive neuroendocrine phenotype of these tumors. Knockdown of ASCL1 in cultured SCLC resulted in inhibition of soft agar clonogenic capacity and induction of apoptosis [54]. Two stem cell markers, namely CD133 and ALDH1A1, are directly regulated by ASCL1 in SCLC. In SCLC xenografts, a relatively abundant CD133 (high)-ASCL1 (high)-ALDH1(high) subpopulation is associated with markedly enhanced tumorigenicity compared with cells with weak CD133 expression. This finding suggests that a broad range of SCLC cells has tumorigenic capacity rather than a small discrete population. The urokinase plasminogen activator (uPA) and its receptor uPAR/CD87 are major regulators of extracellular matrix degradation and are involved in cell migration and invasion. The uPAR-positive cells in six SCLC lines demonstrated multidrug resistance, high clonogenic activity and coexpression of CD44 and MDR1, putative cancer stem cell markers [55].

**Cellular activities of SCLC CSCs**

SP cells in SCLC cell line H446 account for approximately 5% of the whole cell population, have a stronger capability of forming tumor spheres and exhibit increased mRNA expression levels of ABCG2, CD133, and nucleostemin [56]. *In vivo*, SP cells showed better proliferative ability, tumorigenesis and higher drug resistance as well as differentiation into non-SP cells. Rare SP cells (<1%) with high proliferative capacity *in vitro*, efficient self-renewal and reduced cell surface expression of the neuronal differentiation markers, CD56 and CD90, were described for several SCLC cell lines [50]. As few as 50 SP cells from H146 and H526 SCLC cell lines rapidly reconstituted tumors in mice and these cells overexpressed many genes associated with CSCs, such as ABCG2, FGFI, IGFI, MYC, SOX1/2, WNT1, as well as genes involved in drug resistance, angiogenesis, Notch and Hedgehog pathways.

The expression of ALDH1A1 and ALDH3A1 was investigated in patients with lung cancers: squamous cell cancer, adenoscarcinoma, and SCLC [57]. These aldehyde dehydrogenases were significantly expressed in NSCLC and at lower levels in SCLC. Atypical pneumocytes demonstrated significantly higher levels of expression than normal pneumocytes, pointing to an upregulation during malignant transformation. Further characterization of the ALDH+ cells is not available so far.

**GLC14/16/19 series of SCLC cell lines and CSC markers**

Longitudinal biopsies, viable cells or cell lines collected before and during chemotherapy are rarely available for SCLC patients. Due to the aggressive clinical course and disseminated disease at presentation, these patients rarely undergo tumor resection and the diagnosis is performed thin needle biopsy specimen, leaving hardly tissue samples for further investigations [58]. However, one series of three biopsies that were used to constitute the corresponding cell lines GLC14, GLC16 and GLC19, were described for a single SCLC patient by Berendsen et al. in 1988 [59]. In detail, the GLC14 cell line was established using a biopsy from a right supraventricular node of this 55-yr-old woman revealing SCLC extended disease. Treatment was started with cyclophosphamide, doxorubicin and etoposide (CDE), resulting in complete response after five cycles. Prophylactic cranial irradiation (10 times, 30 Gy) was given. After three months the chest X-ray showed SCLC recurrence which was treated by reinduction chemotherapy with CDE. After four cycles of chemotherapy a partial response was observed and the GLC16 cell line established from a biopsy specimen of the remaining tumor. After further radiotherapy the chest X-ray normalized. However, the tumor
engagement of CD47 is associated with an induction of downstream associated increase in the intensity of NOTCH2 staining [61]. Immunohistochemical analysis demonstrated a chemotherapy- or the Notch developmental pathways, particularly NOTCH2 [60]. Chromosomal aberration frequently found in lung cancer affects the tumor-initiating (TIC) or chemotherapy-induced cell populations. A tumors [27]. The other markers are characteristics of CSC-related, CD133 and ALDH1A1 constitute classical cancer stem cell markers and together with Oct-4, Klf4 and c-Myc, is sufficient to artificially reprogram normal cells into stem-like cells [66]. SOX2 is linked to stem cells, self-renewal, and embryonic development [11,30]. Among the most significantly mutated genes in SCLC, SOX2 and Notch pathways, has also been identified in lung tumor cells and Notch signaling pathway, as well as receptors for the growth factors FGFs and EGF. Thus, GLC19 cells are distinguished from GLC14 by a reduced growth rate, expression of CSC markers, increased expression of components of the Wnt and Notch signaling pathway, which are only partially overlapping with those found for GLC16 cells, most likely due to the different therapy regimen used in this later phase of treatment. A comparative analysis of the overexpressed gene transcripts using GLC14 versus GLC16 and GLC19 cells was performed employing the Applied Biosystems Human Genome Survey Microarray V2.0 (Figure 1). Uregulated molecular pathways in the GLC16/19 cell lines compared to the GLC14 cells are expected to be associated with tumor progression and chemoresistance in this case of SCLC. In GLC16 cells, transcripts for the stem cell markers CD133, ALDH1A1, WNT16, NOTCH2 and CD47 were significantly upregulated (> 2.5fold) compared to chemonaïve GLC16 cells. Furthermore, increased expression of c-KIT (CD117), fibroblast growth factor receptor 1 (FGFR1) and integrin alpha-6(ITGA6) seems to supplement this cancer stem cell phenotype. CD133 and ALDH1A1 constitute classical cancer stem cell markers found expressed in a wide range of critical subpopulations of solid tumors [27]. The other markers are characteristics of CSC-related, tumor-initiating (TIC) or chemotherapy-induced cell populations. A chromosomal aberration frequently found in lung cancer affects the 1p region and comprises alterations in genes belonging to the Wnt or the Notch developmental pathways, particularly NOTCH2 [60]. Furthermore, during the search for CSC-related gene expression in residual tumor cells of neoadjuvant-treated gastric cancer patients immunohistochemical analysis demonstrated a chemotherapy-associated increase in the intensity of NOTCH2 staining [61]. Engagement of CD47 is associated with an induction of downstream signaling via G-proteins, leading to the activation of the PI3K/Akt survival pathway [20]. For the case of invasive bladder cancer, a subpopulation of CD44+CK5-CK20 cells was identified as the TIC subpopulation, distinguished by expression of CD47. Moreover, high tumorigenic and metastatic properties of lung NSCLC CSCs are associated with expression of the stem cell factor (SCF) and its receptor c-kit (CD117) play likewise an important role in survival and proliferation of lung CSCs [28]. A highly tumorigenic ITGA6+ subpopulation of cancer cells was recently been identified in primary and metastatic breast cancer samples [62]. This subpopulation is capable of growth as spherical organoids, displays resistance to proapoptotic agents and high tumorigenicity in immunodeficient mice depending on the expression of this antigen. In summary, the GLC16 cell line obtained after CDE chemotherapy exhibits expression of classical CSC markers and of a number of additional antigens which are involved in tumor initiation and dissemination. Consistent with the exposure to cyclophosphamide GLC16 cells express high levels of DNA repair protein O6-methylguanine-DNA-methyltransferase (MGMT) transcript, known to be involved in chemotherapy resistance, particularly resistance of in CD133+ gliblastomamultiforme stem cells to the alkylating agent temozolomide [63]. Similarly, genome-wide gene expression of GLC19 cells was compared to GLC14 cells [64]. Putative classical markers of cancer stem cells overexpressed in GLC19 cells included CD44, ALDH1A1 and the aldoketoreductase family 1, members C1/13 (AKR1C1 and AKR1C13). Again, several mediators upregulated in GLC19 cells belong to the Wnt and Notch signaling pathways. They comprise secreted frizzled-related protein 1 (SFRP1), WNT5B, coactivator mastermind-like 2 (MAML2), transducin-like enhancer of split 2 (E(sp1)) homolog (TLE2) and Notch receptor ligands jagged 1/2 (JAG1 and JAG2). GLC19 cells are distinguished from GLC14 by a reduced growth rate, expression of CSC markers, increased expression of components of the Wnt and Notch signalling pathway, as well as receptors for the growth factors FGFs and EGF. Thus, GLC19 reveals overexpression of CSC markers which are only partially overlapping with those found for GLC16 cells, most likely due to the different therapy regimen used in this later phase of treatment.

Conclusion

The aggressive course of SCLC and the fast development of recurrent and resistant disease may be associated with the presence of tumor cells with CSC-like characteristics. Lung CSCs could derive from bronchioalveolar stem cells (BASCs) and alveolar epithelial type II cells (AEC2), as well as from cells which express the CSC marker glycoprotein CD133 or markers for Sp8 [29]. Normal stem cells that have undergone mutational events or transit-amplifying or differentiated cells subjected to dedifferentiation could be the source of CSCs [10]. Activation of pathways that normally regulate embryonic lung development and injury repair, including the Wnt, Hedgehog and Notch pathways, has also been identified in lung tumor cells [11,30]. Among the most significantly mutated genes in SCLC, SOX2 amplification was detectable in 27% of the samples [65]. Expression of SOX2 is linked to stem cells, self-renewal, and embryonic development and together with Oct-4, Klf4 and c-Myc, is sufficient to artificially reprogram normal cells into stem-like cells [66]. The cell surface marker CD133 has previously been identified as a reliable marker for CSCs in some of tumor entities [34,37]. However,
no mechanism has so far been proposed to link CD133 expression with the CSC phenotype [13]. The reliability of this marker as a CSC marker has recently been disputed [67]. In case of colon cancer CSCs, CD133 expression was found not to be restricted to intestinal stem or cancer-initiating cells, and that during the metastatic transition CD133+ tumor cells might give rise to a more aggressive CD133+ subset [68]. In NSCLC, both CD133+ and CD133− subpopulations were described to comprise CSCs [69]. It has been reported that CD133 may play a role in response to hypoxia, cell cycle regulation and proliferation of cells but not necessarily tumor initiation [33,67]. Independently of its role as marker of CSCs, immunohistochemical assessment of 161 NSCLCs surgically resected revealed CD133 expression was correlated with tumor stage and predictive of unfavorable prognosis in patients with stages II-IV NSCLC [70]. In contrast, characterization of the chemoresistant residual NSCLC tumor cells which cause tumor regrowth, so-called tumor reinitiating cells (TRICs), were studied in xenograft and genetically engineered mouse models, revealing no consistent enrichment of CSC marker-positive cells, but expression of EMT characteristics [34,71]. Furthermore, the NCI-H446 cell line used in characterization of CSCs in an SCLC cell line actually represents biochemical and morphological variant of SCLC with an atypical morphology.

Genome-wide expression analysis of the GLC14/16/19 series of SCLC revealed the increased expression of CSC features after chemoradiotherapy. Although at this time chemotherapy consisted of cyclophosphamide, doxorubicin and etoposide instead of the current standard therapy combining cisplatin/etoposide and etoposide, these findings are expected to apply for both protocols. Upregulation of the transcription for CD133, ALDH1A1, WNT16, NOTCH2 and CD47 in the GLC16 cells are consistent with a regrowth of CSC-like populations in this early relapsing SCLC. Interestingly, after partial response to chemotherapy, the remaining tumor cells (GLC19) exhibited a different CSC-like gene expression pattern including CD44, ALDH1A1 and AKR1C1/13 in combination with several mediators belonging to the WNT and Notch signaling pathways. These results demonstrate a chemoradiotherapy-triggered increase in CSC markers in tumor cell populations of this SCLC patient, suggesting a role for this kind of tumor cells in resistance and recurrence in vivo. An increase in the CSCs population after primary systemic therapy was found to present a poor prognostic factor in breast cancer [72]. Thus, recurrent SCLC seems to express the same markers, namely CD133, CD44, and ALDH1 that were published to predict relapse of lung adenocarcinoma (Hazard Ratio> 3.6) [73].

The CSC model also would offer an explanation for relapses of SCLC after a complete response to initial therapeutic interventions, and cancer therapy failures with a wide range of new therapeutics tested clinically [74-76]. CSC markers in SCLC seem to match those observed in other solid tumors and, therefore, the drugs showing preferential activity against CSCs of other tumors should be tested preclinically for the elimination of these resistant cells in SCLC to open the way to tackle the refractoriness of this tumor entity by radically different approaches [77,78]. In terms of the rapid recurrence of SCLC after primary therapy, this tumor entity would be ideally suited to test clinically the efficacy of CSC-directed therapies in combination with the elimination of the tumor bulk using conventional chemotherapy.

Conflict of Interest Disclosure and Acknowledgment

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