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

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Stem cell phenotype plays a role in an *in vitro* model of small cell lung cancer reflecting disease progression

*Submitted for publication*

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Abstract

Small cell lung cancer (SCLC) is characterized by a poor prognosis that is related to aggressive tumor growth, tendency to rapidly metastasize and acquisition of chemoresistance upon treatment. In this study the involvement of Epithelial to Mesenchymal transition (EMT) and cancer stem cells (CSCs) in resistance to therapy and metastasis was investigated in GLC14, GLC16 and GLC19 SCLC cell lines, representing an untreated, treated and progressing tumor from one patient during clinical follow-up, respectively. All cell lines expressed the epithelial markers EpCAM and E-cadherin and lacked the mesenchymal markers Vimentin and Fibronectin. GLC14, derived from a lymph node metastasis, demonstrated highest invasive capacity and spheroid forming potential compared to GLC16 and GLC19 both derived from the primary tumor. Expression of the CSC markers CD44 and SOX2 increased sequentially in GL14, GLC16 and GLC19, suggesting enhanced CSC properties during disease progression. CD44 sorted cells obtained from all cell lines were enriched for SOX2 expression and demonstrated enhanced spheroid forming potential. In conclusion, we provide evidence substantiating the involvement of CSCs during SCLC treatment and disease progression, whereas no involvement of EMT could be observed.
Introduction
Lung cancer is a devastating disease and the number of deaths by lung cancer alone exceeds that of all other types of cancer-related deaths combined [1]. Histologically lung cancer can be divided in two main types, non-small cell lung cancer (NSCLC) and small cell lung cancer (SCLC). SCLC is the most aggressive form of lung cancer, accounting for approximately 8-12% of all cases [2]. In most cases at the time of presentation in clinic the tumor is already metastasized to distant organs making curation virtually impossible. At initiation of therapy SCLC usually is sensitive to traditional chemo- and radiotherapy, however, it soon acquires resistance leading to 5-year survival rates of less than 10% of all cases [2].

The cancer stem cell (CSC) model proposes that a fraction of cells in the tumor, the CSCs, are the main drivers of tumor formation and progression, and are responsible for relapse of disease after treatment [3,4]. In lung cancer several markers for CSCs have been reported, including CD133, CD44 and aldehyde dehydrogenase (ALDH) activity [5,6]. Alterations in signaling pathways such as Notch, Hedgehog and WNT/β-catenin facilitate transformation of normal cells into cancer initiating cells and maintain stemness [5]. Additionally, specific transcription factors, such as SOX2, OCT4, NANOG and bone morphogenetic proteins (BMPs) were also linked to self-renewal potential in lung CSCs [5,6]. The stromal cell-derived factor-1 (SDF-1), also known as CXCL12 a ligand for the membrane receptor CXCR4, was identified as a CSC marker in NSCLC [7].

CSCs have been mostly studied in NSCLC, whereas the presence of CSCs in SCLC has been less well explored. CSCs in SCLC were reported to be enriched in the side population (SP) [8] and in CD133 positive cells [9,10]. The SP in SCLC displayed enhanced expression of SOX2 and NANOG [11]. CD133 as CSC marker in SCLC is somewhat controversial since both CD133+ and CD133− cells could form spheres in in vitro assays [9]. In another study CD44 and CD90 positive SCLC cells demonstrated enhanced CSC properties together with increased expression of the mesenchymal marker Vimentin [12].

Epithelial to Mesenchymal transition (EMT) has been identified as a crucial step in tumor invasion and metastasis in different types of solid tumors, including lung cancer [13]. EMT was originally described as a reversible process occurring in embryogenesis, wound healing and tissue repair, facilitating enhanced migratory potential of epithelial cells through the acquisition of mesenchymal properties [14].
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EMT is characterized by loss of epithelial markers, such as EpCAM and E-cadherin and a gain of mesenchymal markers, such as Vimentin and Fibronectin. Lung cancer is an epithelial tumor, however, a small fraction of cells may undergo EMT leading to expression of mesenchymal markers and attaining a fibroblast-like morphology [15]. EMT can be demonstrated most often in the advancing tumor front with cells invading the surrounding normal tissue [16]. EMT induction in several in vitro tumor models, including lung cancer, was shown to be mediated by EMT transcription factors such as ZEB1, SNAIL, SLUG and TWIST and EMT can be induced by exposure of cells to specific cytokines like TGF-β [17,18], however evidences of EMT occurring in SCLC are scarce.

Here, we investigated a possible involvement of EMT and CSCs during SCLC disease progression in a unique longitudinal SCLC cell culture model.

Materials and Methods

Cell lines and cell culture

GLC14, GLC16 and GLC19 cells were derived from the same SCLC patient treated at the University Medical Center Groningen in 1984-1986 as described previously [19] (see also Figure 1A). Briefly, GLC14 was derived from a resected supraclavicular right lymph node (LN) metastasis prior to any systemic treatment. After treatment with 5 cycles of cyclophosphamide, doxorubicin and etoposide (CDE) a complete response was observed. In July 1985 the tumor relapsed locally and 4 cycles of CDE treatment were given. A partial response was observed and GLC16 was derived from an endobronchial biopsy. Radiotherapy was given to the region of the primary tumor to control tumor growth. In February 1986 the tumor relapsed and GLC19 was derived from an endobronchial biopsy. The cell lines were cultured in RPMI-1640 medium (Life Technologies, Bleiswijk, The Netherlands) with 10% FCS (Bodinco, Haarlem, The Netherlands) and maintained at 37°C in a humidified incubator supplied with 5% CO2. Spheroid cultures were in Neurobasal media (NBM) (Life Technologies) supplied with 2% of B27 supplement (Life Technologies), 20ng/ml EGF (R&D systems, Abingdon, UK) and 10ng/ml b-FGF (Millipore, Amsterdam, The Netherlands). The human NSCLC cell line A549 treated with TGF-β (Peprotech, London, UK) was used as a positive control.

MTS Chemosensitivity assays

Linearity between cell numbers and absorption and growth curves were determined first to optimize MTS (3-(4,5-dimethylthiazol-2-yl)-5-(3-carboxymethoxyphenyl)-2-(4-
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sulfophenyl)-2H-tetrazolium) conditions for the cell lines. For chemosensitivity studies GLC14, GLC16 and GLC19 were plated at 12,500, 25,000 and 45,000 cells, respectively, per well in triplicate in 96-wells microtiter plates. After 24h cells were incubated with adriamycin (Doxorubicin®, Pharmachemie BV, Haarlem, the Netherlands), cisplatin (Accord Healthcare BV, Rijsbergen, The Netherlands), etoposide (Vepesid®, Teva Pharma BV, Haarlem, The Netherlands) and taxotere (Docetaxel®, Sanofi-Aventis, Gouda, The Netherlands) for 96h after which MTS (Promega, Leiden, the Netherlands) was added and absorbance was measured at 490 nm.

Invasion assay
Transwell chambers with 6.5mm, 8.0µm pore polycarbonate membrane inserts (3422, Corning, Amsterdam, The Netherlands) were used. Inserts were coated with collagen type 1 (BD Biosciences, Erembodegem, Belgium) at 37°C and after removal blocked with 1% BSA in PBS overnight at 37°C. Cells were harvested and dissociated in serum-free RPMI-1640 (RPMI-1640 + 0.1% FCS) and 50,000 cells were seeded in the upper chamber. RPMI-1640 + 10% FCS was used as chemoattractant in the lower chamber. RPMI-1640 + 0.1% FCS was used as a negative control. After 6 hours incubation at 37°C membranes were fixed in 75% methanol/15% acetic acid, and subsequently stained with Coomassie-blue in 45% methanol/10% acetic acid for 10 minutes and invasive cells were quantified by microscopic counting. For each condition, cells in at least three independent fields were counted and the average was calculated.

Western blotting
Western blot analysis was performed as described before [20]. The following primary antibodies and dilutions were used: mouse monoclonal E-cadherin 1:1000 (clone 36/E-cadherin, BD Biosciences), rabbit polyclonal N-cadherin 1:500 (Cell Signaling, Bioke, Leiden, The Netherlands), mouse monoclonal Fibronectin 1:1000 (clone 10/Fibronectin, BD Biosciences), mouse monoclonal Vimentin 1:500 (sc-6260, Santa Cruz biotechnology, Bioconnect, Huissen, The Netherlands), mouse monoclonal SLUG 1:500 (sc-166476, Santa Cruz biotechnology, Bioconnect), goat polyclonal ZEB1 1:200 (sc-10572, Santa Cruz biotechnology, Bioconnect), rabbit polyclonal TWIST 1:2000 (Abcam, Cambridge, UK), rabbit polyclonal CXCR4 1:1000 (ab2090, Abcam), rabbit polyclonal OCT4 1:1000 (ab19857, Abcam), mouse monoclonal SOX2 1:1000 (L1D6A2, Cell Signaling, Bioke), mouse monoclonal β-catenin 1:500 (clone 14/Beta-Catenin, BD Biosciences). Equal loading of protein was confirmed by
β-Actin staining using 1:10000 (clone C4, ICN Pharmaceuticals, Zoetermeer, The Netherlands). The membranes were incubated with primary antibodies at 4°C overnight and next day washed with Tris-buffered Saline with 0.1% Tween-20 and incubated with the appropriate horseradish peroxide (HRP)-labelled secondary antibodies (all 1:1000, all from DAKO, Glostrup, Denmark) for 1 hour at room temperature. Proteins were visualized by chemiluminiscence using BM chemiluminiscence detection kit (Roche Applied Science, Almere, The Netherlands).

**Flow cytometry and Fluorescent Associated Cell Sorting (FACS)**

Cells were repeatedly pipetted to generate single cell suspensions. Cells were washed with FACS buffer (PBS at pH 7.2 with 0.5% BSA and 2mM EDTA) and pelleted by centrifugation. Cells were resuspended in 100µl of FACS buffer and the indicated antibodies were added and incubated on a rotary shaker in the dark at 4°C for 30 minutes. Cells were washed with cold FACS buffer, centrifuged and resuspended in FACS buffer for analysis by flow cytometry FACSCalibur, BD Biosciences). The antibodies used were: mouse monoclonal CD326 -APC 1:10 (HEA-125, Miltenyi Biotech, Leiden, The Netherlands), mouse monoclonal CD133-PE 1:10 (AC-133, Miltenyi Biotech), mouse monoclonal CD24-FITC 1:10 (ML5, BD Biosciences) and mouse monoclonal CD44-PE 1:10 (G44-26, BD Biosciences). Corresponding isotype-matched antibodies directly labeled with PE, FITC or APC and unstained cells were used as negative controls. Data were analyzed with FlowJo software (Tree Star, Ashland, USA).

For CD44 sorting, cells were stained with CD44-PE as described above and treated with DAPI (Life Technologies) for 5 minutes prior to sorting. Unstained cells and PE isotype-matched antibody were used as a negative control. Viable CD44+ and CD44- cells were sorted for determination of SOX2 expression using MoFlo XDP (Beckman Coulter Nederland, Woerden, The Netherlands). An additional second sorting with CD44+ and CD44- cells was performed to further enrich for high CD44+ and high CD44- cells to evaluate spheroid forming potential.

**Spheroid forming assay**

To compare spheroid forming potential, cells were harvested and stained with DAPI for 5 minutes prior to sorting. Viable cells were sorted using MoFlo XDP (Beckman Coulter Nederland) in 96 well plates at 10, 20, 50 and 100 cells per well containing 100µl NBM with supplements in triplicates. After 7 days additional fresh NBM with supplement was added (100µl) and cells were incubated further for two weeks. The
number of spheroids was quantified by counting spheroids with approximately 3-5mm diameter in each well using 10X magnification of light microscope. The spheroid forming potential was calculated as the average number of spheroids formed per number of seeded cells per well. Similarly spheroid forming potential of high CD44+ and high CD44- sorted cells were determined.

Immunohistochemistry and immunofluorescent staining
Immunocytochemistry was performed as described previously [21]. The following primary antibodies were used for staining: mouse monoclonal E-cadherin 1:100 (24E10, Cell Signaling, Bioke), mouse monoclonal EpCAM 1:500 (VU1D9, Cell Signaling, Bioke), mouse monoclonal Cytokeratins 8-18-19, 1:100 (clone 2A4, Abcam), mouse monoclonal SOX2 1:600 (L1D6A2, Cell Signaling, Bioke) and rat monoclonal CD44 1:100 (IM7, Biolegend, London, UK). For every staining corresponding isotype-matched IgG antibody and PBS was used as negative controls. Corresponding secondary and tertiary antibodies conjugated to HRP (all from DAKO) were used in 1:100 dilutions. Diaminobenzidine (DAB) was used as a chromogen and hematoxyline was used for counterstaining. Images were obtained with a Leica DM 300 (Leica, Rijswijk, The Netherlands).

For immunofluorescent staining sorted CD44+ and CD44– cells were cytopspined on glass slides and processed for SOX2 staining (monoclonal antibody, L1D6A2, 1:100, Cell Signaling, Bioke). Rabbit anti-mouse Alexa 488 antibodies 1:100 (Life Technologies) was used as secondary antibody and nuclei were stained with DAPI 1:500 (Life Technologies). Images were taken by immunofluorescence microscope equipped with Leica Application Suit software (Leica DM 6000B, Leica).

Statistics
All experiments were performed independently at least three times. Statistical analysis was performed for chemosensitivity, invasive capacity, CSC surface marker expression and spheroid forming potential using a double sided, paired or unpaired Student t-test. A p-value < 0.05 was considered significant.

Results
Cell lines
GLC14, GLC16, and GLC19 represent different stages of disease progression during treatment [19] (Figure 1A). Of note, GLC14 was derived from a LN biopsy, whereas the other cell lines were derived from primary tumor biopsies. Cells grew in
clumps upon cell culturing in regular cell culture medium. GLC14 formed more regular, densely packed and somewhat bigger clumps, whereas GLC16 and GLC19 grew in irregular, loosely packed and smaller clumps (Figure 1B). These growth properties are similar as described previously [19]. The three cell lines could also be maintained in EGF and b-FGF-supplemented serum-free NBM that is known to induce spheroid growth and to facilitate growth of stem-like cells in other tumor models [22]. Under these conditions GLC14 cells formed tightly packed regular spheroids, whereas the other cell lines formed more irregular, less condensed spheroids (Figure 1B).

Figure 1. Generation and morphological characteristics of the GLC cell lines in culture.
(A) Timeline of the establishment of the GLC cell lines derived from one SCLC patient at different stage of disease progression. (B) GLC cell lines grown in RPMI-1640 + 10% FCS or serum-free NBM with supplements.

**Chemosensitivity and invasive capacity**

Sensitivity of GLC14, GLC16 and GLC19 for Adriamycin, cisplatin, etoposide and taxotere was tested. Overall no significant differences in sensitivity to the drugs could be observed (data not shown).

The invasive capacity was studied using collagen-coated transwells (Figure 2). GLC14 displayed significantly the highest level of basal invasive capacity (approximately 30 cells per membrane), whereas GLC16 and GLC19 had lower and similar levels of invasive capacity (around 3 to 5 cells per membrane). The addition
of 10% FCS as chemoattractant resulted in a significant 2- to 3-fold increase in invasive capacity in all three cell lines.

![Figure 2. Invasive capacity of the GLC cell lines.](image)

Invasive capacity was examined by transwell assays. GLC14 has significantly higher invasive capacity than GLC16 (P<0.001) and GLC19 (P<0.001). All three cell lines displayed significantly higher invasive capacity when using 10% FCS as chemoattractant when compared to 0.1% FCS; GLC14 (P<0.001), GLC16 (P<0.05) and GLC19 (P<0.05). Data represent the mean ± SD of three independent experiments.

**EMT characteristics**

Western blot analysis showed that all three cell lines expressed the epithelial marker E-cadherin; expression was somewhat stronger in GLC16 and GLC19 (Figure 3A). High and comparable expression of EpCAM was observed in all three cell lines as determined by flow cytometry (Figure 3B). Similar levels of EpCAM and cytokeratins 8-18-19 could also be demonstrated by immunohistochemistry (Figure 3C). Analyses of expression of the mesenchymal markers showed expression of N-cadherin at moderate levels in all three cell lines, whereas no expression of Fibronectin and Vimentin was observed (Figure 3A). As control, these markers were clearly expressed in A549 cells treated with TGF-β, a cytokine known to potently induce EMT in these cells [23]. Analyses of EMT transcription factors showed high levels of SLUG and TWIST and low levels of ZEB1 in all three cell lines (Figure 3D). Overall, the cell lines expressed mainly epithelial markers and no clear differences in EMT status were observed between the cell lines.
Figure 3. EMT properties in the GLC cell lines.

(A) Protein expression of E-Cadherin, N-Cadherin, Fibronectin and Vimentin was measured by Western blotting. All three cell lines expressed E-cadherin with highest expression in GLC16 and GLC19. From the mesenchymal markers examined, only N-Cadherin was detected. A549+TGF-β served as a positive control for EMT marker expression. (B) Cell surface expression of EpCAM measured by flow cytometry was detected in all cells with no significant differences. Data represent the mean percentage expression ± SD of three independent experiments. (C) EpCAM and Cytokeratins 8-18-19 expression determined by immunocytochemistry, showing high levels in all three cell lines. (D) Expression of EMT transcription factors SLUG, ZEB1 and TWIST examined by Western blotting revealing no clear differences in expression. β-actin was used as loading control.

CSC properties

All three cell lines expressed the CSC markers β-catenin, CXCR4 and OCT4 at similar levels and, interestingly, SOX2 expression progressively increased from GLC14 to GLC16 and GLC19 cells (Figure 4A). FACS analyses of CSC specific cell surface markers, such as CD44, CD133 and CD24, showed no significant differences in the levels of CD133 and CD24 expression between the cell lines (Figure 4B). However, CD44 expression was significantly enhanced in GLC19 compared to the low levels in GLC14 and moderate level in GLC16 cells. GLC16 demonstrated significantly higher CD44 expression than GLC14 (Figure 4B). Testing of the spheroid forming potential of the cell lines revealed GLC14 to be most potent (~11%) followed by GLC19 (~6%) and GLC16 cells (~3%). Spheroid formation was proportional to the number of seeded cells for each cell line (Figure
4C). Amongst GLC16 and GLC19, the latter showed significantly higher spheroid forming potential.

**Figure 4. CSC characteristics of the GLC cell lines.**

(A) β-catenin, CXCR4, OCT4 and SOX2 expression was evaluated by Western blotting. β-actin was used as loading control. Increasing levels of SOX2 were seen. (B) Cell surface expression of CD44, CD133 and CD24 was measured by flow cytometry. GLC19 showed significantly higher expression of CD44 compared to GLC14 (P<0.05) and GLC16 (P<0.05). GLC16 cells had significantly higher expression of CD44 compared to GLC14 (P<0.05) Data represents mean percentage expression ± SD of three independent experiments. (C) Spheroid forming potential of GLC cell lines. GLC14 cells displayed highest spheroid forming potential when compared with GLC16 (P<0.01) and GLC19 (P<0.05). GLC19 showed significantly higher spheroid forming potential compared to GLC16 cells (P<0.01). Data represent mean ± SD of three independent experiments.

**Association of CD44 and SOX2 expression with increased spheroid forming potential**

The relation between CD44 and SOX2 expression and spheroid forming potential was further investigated. Cells positive for CD44 (CD44+) and negative for CD44 (CD44-) were sorted (Figure 5A) and stained for SOX2 expression. The CD44+ cells were mainly associated with SOX2 expression in all cell lines, as shown for GLC16.
cells (Figure 5B). Furthermore, sorted CD44+ cells from all three cell lines showed enhanced spheroid forming potential compared to CD44- cells. In GLC14 approximately 8% of CD44+ cells formed spheroids compared to ~4% in CD44- cells. Similarly in GLC16 approximately 7% CD44+ cells formed spheroids compared to ~4% in CD44- cells. In GLC19, approximately 6% of CD44+ cells formed spheroids compared to only ~1.5% in CD44- cells. Overall, CD44+ cells have a 2- to 4-fold increase in spheroid formation potential compared to CD44- cells (Figure 5C).

Figure 5. Association of CD44 and SOX2 expression, and spheroid forming potential of CD44+ sorted GLC cells.

(A) CD44 + and CD44- cells were sorted using CD44-PE and DAPI to differentiate between viable and dead cells. Upper panel: representative picture of gating and sorting of CD44+ and CD44- cells from GLC16 cells. Lower panel: an additional second sorting of CD44+ and CD44- cells was performed for spheroid forming potential assay. (B) Double fluorescent staining for CD44 and SOX2 by immunofluorescent microscopy. CD44+ cells show higher SOX2 expression than CD44- cells (representative pictures of GLC16). (C) Spheroid forming potential of sorted CD44+ cells of GLC14, GLC16 and GLC19 showed a significant two- to four-fold higher spheroid forming potential when compared to CD44- cells (P<0.01, P<0.05 and P <0.001 respectively). Data represent average percentage of spheroid forming potential ± SD from three independent experiments.
Discussion
In the present study a unique model consisting of three consecutive SCLC cell lines derived from one patient during treatment and clinical follow-up was used to explore the possible involvement of EMT and CSCs in SCLC disease progression. During this period the tumor changed from sensitive to clinically completely resistant to (chemo)therapy. GLC14, GLC16 and GLC19 were previously shown to match the corresponding patient biopsies with respect to their morphological, biochemical and immunohistochemical characteristics [19]. However, in agreement with previous studies [19,24] overall no significant differences in chemosensitivity were observed between the cell lines in vitro. Thus, the cell lines derived from sequential biopsies at different stages of disease progression did not reflect the resistance profile seen in the clinic.

Interestingly, we found that pre-treatment LN derived-GLC14 cells showed significantly higher invasive capacity and enhanced spheroid forming potential, when compared to GLC16 and GLC19 derived from primary endobronchial tumor sites at later stages of disease. Of the different CSC markers that we examined in the GLC cell panel, CD44 and SOX2 showed distinct expression patterns. GLC14 expressed the lowest levels of CD44 and SOX2, GLC16 expressed intermediate levels and GLC19 expressed highest levels. This could be taken as evidence for the notion that according to the CSC model the number of CSCs in the tumor will increase as a result of high therapy resistance and failure to eliminate CSCs. However, as mentioned earlier, no difference in chemosensitivity between the cell lines was observed. This suggests that therapy resistance does not necessarily correlate with the presence of CSC properties in cells. In agreement with the CSC hypothesizes we observed stronger spheroid forming potential in primary tumor-derived GLC19 vs GLC16 cells. Surprisingly, metastatic LN-derived GLC14 cells had the highest spheroid forming potential and are likely driven by a different CSC population that is not characterized by CD44 and SOX2 expression. This is in accordance with the notion that heterogeneity also exists in the CSC compartment and the proposed existence of migrating/metastatic CSC that differ from the CSCs in the primary tumor [13,25]. Taken together, the increased levels of CD44 and SOX2 expressed in GLC19 cells compared to GLC16 may still be indicative for the acquisition of enhanced CSC properties during SCLC treatment in the primary tumor. Of note, previously SOX2 expression has been correlated with a worse prognosis in SCLC patients [26,27].
CD44+ sorted cells were associated with high SOX2 expression and enhanced spheroid forming potential in all three cell lines. From this, we may assume that GLC14 cells also contain a CSC population that is associated with CD44 and SOX2 expression. Indeed, CD44+ sorted cells from all three GLC cell lines had 2- to 4-fold enhanced spheroid formation potential when compared to CD44- counterparts. Our findings appear reminiscent to an earlier report on NSCLC cells, which showed that CD44+ NSCLC cells also are enrichment for SOX2 expression and display enhanced spheroid forming potential [28].

No evidence indicating a possible role for EMT in determining the invasive capacity of the cells was found in this study. GLC14, GLC16 and GLC19 showed similar expression patterns of epithelial and mesenchymal markers. Whereas evidence for the occurrence of EMT in association with local invasion and distant metastasis has been demonstrated in several tumor types [17, 29, 30] this has been hardly addressed in SCLC. Recently, EMT was reported to be induced in the human SCLC cell line NCI-H69 by HGF-mediated c-MET receptor activation, and treatment with the c-MET inhibitor crizotinib prevented EMT [31]. In addition, enhanced expression of Vimentin and SNAIL correlated with worse prognosis in a cohort of SCLC patients. In another study, subpopulations with varying EMT marker expression were found in NCI-H69 cells suggesting heterogeneity in the ability of tumor cells to undergo EMT [32]. Furthermore, high numbers of circulating tumor cells (CTCs) and circulating tumor microemboli (CTM) have been measured in blood of SCLC patients [33, 34] and analyses revealed cells with heterogeneous expression of EMT markers [35]. Further, it should be noted that most of the evidence for EMT in tumors relies on \textit{in vitro} investigations and correlation studies between EMT markers and clinical outcome in patients [15]. Unfortunately, biopsy material of which the GLC cell lines were derived is no longer available and therefore examination of the EMT status in the actual tumor samples is not possible. We found GLC14 cells to have the strongest invasive capacity in vitro and this may reflect their metastatic origin and associated genetic changes with such a phenotype that is independent of EMT.

Finally, it is likely that primary material derived cells upon culturing on plastic will acquire differences or select for a subset of cells and therefore not fully represent the original tumor. Therefore, the chemosensitivity profiles of the cell lines may not mimic the sensitivity seen in the clinic. Discrepancies may also involve the absence of stromal cells and microenvironment under cell culture conditions. The microenvironment is known to have an impact on drug sensitivity [36]. Additionally,
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EMT is mainly regulated by signals originating from the extracellular matrix and the microenvironment, which also play crucial roles in maintenance of the CSC population [37], which would be difficult to mimic in *in vitro* settings.

In conclusion, LN-derived GLC14 cells where most invasive and exhibited the highest spheroid forming potential. GLC16 and GLC19 derived from the primary tumor displayed progressive expression of CD44 and SOX2 and spheroid forming potential suggesting increased CSC properties of the tumor during progression of SCLC.

**Conflict of Interest**

There are no potential conflicts of interest.

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References


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