Establishment and Characterization of a Pair of Patient-derived Human Non-small Cell Lung Cancer Cell Lines from a Primary Tumor and Corresponding Lymph Node Metastasis

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Abstract. Background: Non-small lung cancer is the leading cause of cancer-related mortality worldwide. For a deeper understanding of tumor biology, we established a pair of cell lines derived from a primary tumor and a corresponding lymph node metastasis. Material and Methods: The cell line BC4323 from the primary tumor (PT) and a mediastinal lymph node metastasis (LN) were derived from an adenocarcinoma (pT2, pN2, G3, UICC stage IIIa) in a 47-year-old female patient. Comparative characterization was performed by in vitro analysis. A murine xenograft was established for analysis of in vivo behavior. Results: Chromosomal aberrations were detected in multiple chromosomal sections throughout the entire genome, with only a few differences between PT and LN cells. High-level Kirsten ras oncogene homolog (KRAS) mutation and amplification were seen based on a chromosomal translocation and novel assembled chromosome. In contrast to the genomic level, at the mRNA and protein levels, multiple differences were detectable, in particular in markers for cell adhesion [e.g. epithelial cell adhesion molecule (EpCAM), CD44, P-selectin binding, epidermal growth factor receptor (EGFR) and integrin alphaV] and the epithelial–mesenchymal transition. Due to accelerated tumor growth in vivo by the PT cells, a shortened overall survival was seen (60 vs. 101 days, p=0.005). Conclusion: We provide detailed analysis of a cell line derived from a primary tumor and a corresponding LN metastasis. This unique feature allows further investigative analysis of the differences and regulatory processes underlying the metastatic process during tumor progression in non-small cell lung cancer.

Lung cancer is the leading cause of cancer-related mortality worldwide (1). Non-small cell lung cancer (NSCLC) accounts for 80% of all lung cancer-related deaths. Patients with NSCLC often experience relapse and develop metastases after initial surgery; this results in an approximate 5-year survival rate of only 15% (2). NSCLC commonly metastasizes to distant organs such as brain, adrenal glands, liver and bones, and this is generally considered a sign of dismal prognosis and poor clinical outcome (3). Important driver mutations in the carcinogenesis of NSCLCS have been identified that have radically altered treatment pathways for NSCLC (4, 5). New drugs have been developed that specifically target key mutated proteins such as epidermal growth factor receptor (EGFR), and Kirsten ras oncogene homolog (KRAS) and anaplastic lymphoma kinase (ALK) mutations (6-8). However, the overall survival (OS) of patients is still unsatisfactory as the aforementioned agents often do not have the expected success in clinical practice. These aspects show the importance of an improved understanding of the pathophysiological processes in tumor cells that promote local aggression and recurrence in NSCLC, and in particular, the need for understanding the metastatic process, which despite its clinical importance is still poorly understood (9). Cell lines established from NSCLC are widely used for in vitro and in vivo analyses of carcinogenesis, as well as in the development of new targeted therapies and...
conventional chemotherapy (10). However, these cell lines are derived from primary tumors in the majority of cases and therefore the obtained findings are of limited clinical relevance, since a comparison between the primary tumor and a corresponding metastasis is not possible.

Herein, we established a pair of cell lines from a NSCLC from the primary tumor and a corresponding LN metastasis and characterized these cells extensively in vitro and in vivo with respect to differences in gene expression and the biological behavior of the cells, providing a tool for a deepened analysis of the metastatic process.

Materials and Methods

Patient and surgical procedure. A female patient was operated on in September 2004 at the Department for General, Visceral and Thoracic Surgery at the University Medical-Center Hamburg Eppendorf. The patient was 47 years old at the time of the operation and underwent a right-sided pneumectomy with locoregional and mediastinal lymphadenectomy. The patient presented with a history of 60 pack-years of smoking before surgery and a distant metastasis was found 8 weeks after the initial operation. She received four cycles of palliative chemotherapy with carboplatin/vinorelbine and finally died 5.5 months after initial diagnosis. The final histopathological examination revealed an adenocarcinoma of the lung, pT2, pN2, G3, UICC stage IIIa. Written-informed consent by the patient for investigational purposes was obtained before surgery.

Establishment of cell lines BC4323PT and BC4323LN. Small fragments of the tumor (BC4323PT) and the mediastinal LN metastasis (BC4323LN) (1 mm³) were enzymatically disaggregated after incubation with 0.5% collagenase IV (Sigma Aldrich, Steinheim, Germany) at 37°C in a rotary shaker for 45 min. After centrifugation at 700 x g for 5 min, the cell pellet was collected, washed twice in cell-culture medium (RPMI-1640; Invitrogen, New York, NY, USA) and re-suspended in cell culture medium (RPMI-1640 supplemented with 10% fetal calf serum (FCS) and 200 IU/ml penicillin-streptomycin) then plated in 75 cm² cell-culture flasks (Sarstedt, Darmstadt, Germany). The cells were cultured under standard conditions at 37°C with 5% CO₂. Cell-culture medium was replaced every 3-5 days, depending on the confluence of the cells.

The cells were cultivated for 25 passages to eliminate fibroblasts and confirm malignant immortal cell growth of the cells. For passaging, the cells were treated with 0.05% Trypsin-EDTA (Invitrogen, Darmstadt, Germany) for 3 minutes at 37°C; the enzymatic reactions were stopped with cell-culture medium and the cells were transferred into new culture flasks.

Animal experiments. Animal experiments were conducted according to the United Kingdom Co-Ordinating Committee on Cancer Research for the welfare of animals in experimental neoplasia (11). The experiment was approved and supervised by the Institutional Animal Welfare Officer and approved by the local licensing authority.

For the subcutaneous tumor model, 1x10⁶ BC4343PT and BC4323LN tumor cells were injected into the right scapula region in 8- to 12-week-old C57BL/6N Pfp⁻/⁻/Rag2⁻/⁻ double-knockout mice, respectively. Animals were sacrificed when primary tumors exceeded 2 cm³ or ulcerated the mouse skin. The mice were terminally narcotized and sacrificed by cardiocentesis.

The left lungs were homogenized in a sample disruptor (TissueLyser II; Qiagen, Hilden, Germany) and subjected to DNA-isolation (QIAamp DNA Mini Kit; Qiagen). Bone marrow was collected by flushing the left femoral and tibial bones with 1 ml of 0.9% NaCl. Two hundred microliters of blood and the bone marrow DNA suspensions were subjected to DNA isolation using QIAamp DNA Blood Mini Kit.

DNA concentrations in all the samples were quantified using a NanoDrop spectrophotometer (Peqlab, Erlangen, Germany). As the content of detectable Alu-sequences in the subsequent quantitative polymerase chain reaction (qPCR) would have been affected by varying the DNA concentration, all lung and bone marrow DNA samples were normalized to 30 ng/µl using AE buffer (Qiagen). The concentrations of blood DNA were quite similar in all samples (approx. 10 ng/µl) and were therefore not normalized. qPCR was performed with established human-specific Alu-primers (12). Two microliters of total DNA (i.e. 60 ng lung or bone marrow DNA; 20 ng blood DNA) were used for each qPCR. Numerical data were determined against a standard curve as described elsewhere (12). For each sample, analyses were performed in duplicate and as independent experiments at least twice.

Flow cytometry. Cultured cells were trypsinized, washed and stained on ice with either phycoerythrin (PE)-, or fluorescein-isothiocyanate (FITC)-, or allophycocyanin (APC)-conjugated primary antibodies without prior fixation. The following antibodies were used according to the manufacturers’ recommended concentrations: activated leukocyte cell adhesion molecule (AICAM; 1:100 IgG1 PE; ebioscience, Frankfurt, Germany), epithelial cell adhesion molecule (EpCAM; 1:100 IgG1 PE; R&D Systems, Minneapolis, MN, USA), CD44 (1:100 IgG1 FITC; R&D Systems), CXC-motif-chemokine receptor 4 (CXC4; 1:100 IgG2a, APC; R&D Systems), carbohydrate-antigen 19-9 (CA19-9; 1:100 IgM APC; R&D Systems), CD15s (1:100 IgM APC; R&D Systems), P-selectin (1:100 P-Selectin chimera APC; R&D Systems), E-selectin (1:100 E-selectin chimera APC; R&D Systems), epidermal growth factor receptor (EGFR; 1:100 IgG2b PE; ebioscience), vascular endothelial growth factor (VEGF; 1:100 rabbit polyclonal IgG PE; ebioscience), CD24 (1:100 IgG1 PE; R&D Systems), CD133 (1:100 IgG2b PE; ebioscience), L1 cell adhesion molecule (L1-CAM; 1:100 IgG2a PE; ebioscience), carcinoembryonic antigen-related cell adhesion molecule (CEACAM1; 1:100 IgG2b PE; R&D Systems), CEACAM6 (1:100 IgG2a APC; R&D Systems), integrin alpha V (1:100, IgG2a FITC; Santa Cruz, Dallas, TX, USA), integrin beta 1 (1:100 IgG1 FITC; Santa Cruz), and integrin beta 4 (1:200, IgG1, Alexa Fluor 660; ebioscience). Corresponding isotype controls were used as negative controls.

For intracellular protein detection, cells were fixed with 2% formaldehyde for 20 min at room temperature. After washing with PBS, cells were permeabilized with 1% saponin (Sarstedt) for 20 min at room temperature, after which the cells were again centrifuged for 5 min, the cell pellet was collected, washed twice in cell-culture medium (RPMI-1640; Invitrogen, New York, NY, USA) and re-suspended in cell culture medium (RPMI-1640 supplemented with 10% fetal calf serum (FCS) and 200 IU/ml penicillin-streptomycin) then plated in 75 cm² cell-culture flasks (Sarstedt, Darmstadt, Germany). The cells were cultured under standard conditions at 37°C with 5% CO₂. Cell-culture medium was replaced every 3-5 days, depending on the confluence of the cells.

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were purified over Amicon Ultra 30k filters (Millipore, Billerica, MA, USA), S100 calcium-binding protein A 4 (S100 A4; clone A5114; Dako), HIF-1α (H1667; Sigma-Aldrich, Hamburg, Germany), zinc finger E-box-binding homeobox 1 (ZEB1; polyclonal, Sigma-Aldrich), ZEB2 (polyclonal; Sigma-Aldrich), twist-related protein 1 (TWIST; polyclonal; Abcam), and Ki67 (clone MIB-1; Dako).

Array-based comparative genomic hybridization, KRAS and EGFR exon analysis. Array-based comparative genomic hybridization (CGH) was performed using the Agilent Human Genome Microarray Kit 2x400K (Agilent Technologies, Santa Clara, CA, USA), a high resolution 60-mer oligonucleotide-based microarray with a median overall probe spacing of about 5.3 kb. Prior to DNA extraction, the cells were treated with 100 nM Torin1 (R&D Systems, Wiesbaden-Nordenstadt, Germany) for 5 h to provide reliable profiles of rapidly dividing cells in the subsequent array-CGH analyses. Labelling and hybridization of genomic DNA was performed according to the protocol provided by Agilent. Briefly, 0.75 μg of DNA was labelled by random priming using the Agilent Genomic DNA Labelling Kit and Cy3-dUTP and reference DNA (pool of five sex-by random priming using the Agilent Genomic DNA Labelling Kit provided by Agilent. Briefly, 0.75 μg of DNA was labelled with Cy5-dUTP. Labeled products were subjected to sequencing with the BigDye Terminator Cycle Sequencing Kit (Applied Biosystems) and analyzed on an ABI 3100 genetic analyzer.

Cytogenetic and fluorescence in situ hybridization (FISH) analysis. Chromosomal preparation and fluorescence-R banding were performed using standard procedures for the KRA5 locus (BlueFISH, RPI1-707G18; BlueGene, Illumina, Inc., San Diego, CA, USA) (16). For EGFR and Kras exon mutation analysis, 10 ng of DNA extracted from PT and LN cells were subjected to PCR reactions using AmpliTaq GOLD PCR master mix (Applied Biosystems). EGFR exons 18, 19, 20 and 21 were analyzed with respect to single nucleotide polymorphisms. The primer sequences used have been published before (13) For KRAS exon analysis, two primers (forward 5’-GCTGCTGAAAATGACTGAA-3’ and reverse 5’-AGTAAGGTCTGCACACGAA-3’) were used. PCR products were subjected to sequencing with the BigDye Terminator Cycle Sequencing Kit (Applied Biosystems) and analyzed on an ABI PRISM 3100 genetic analyzer (Applied Biosystems).

Results

Two bronchial adenocarcinoma cell lines designated BC4323PT and BC4323LN were obtained from a resected PT and a corresponding mediastinal LN metastasis. Within the first 2 weeks, tumor cell clusters adhered to the surface of the cell-culture flasks and gradually formed cell colonies. Initially, contaminating fibroblastic cells proliferated in between and surrounded the tumor cell colonies. During serial passages, the number of fibroblasts gradually decreased and they were replaced entirely by tumor cells. Tumor cells exhibited adherent growth at the bottom of the cell-culture flasks and presented a monolayer growth pattern (Figure 1A and B). At first sight, cells derived from the primary tumor did not differ in shape, size or growth pattern compared to those derived from the LN metastasis.

BC4323PT cells exhibited an increased cell proliferative capability compared to BC4323LN cells, as determined by XTT assays (Figure 1C). Treatment with different chemotherapeutic agents showed, in general, a higher chemosensitivity for BC4323LN cells than BC4323PT cells.
Figure 1. Primary tumor (BC4323PT) (A) and lymph node metastasis (BC4323LN) (B) cells show adherence to the bottom of cell culture flasks. BC4323PT cells exhibited increased proliferative ability compared to BC4323LN cells as determined by the XTT assay (C). Cytotoxic proliferation tests showed, in general, greater sensitivity to cytotoxic drugs for BC4323LN cells than BC4323PT cells (D-G). *Difference between cell lines significant at p<0.05 by the Wilcoxon test.
that was reflected by decreased proliferation at equivalent concentrations of chemotherapy agents (Figure 1D-G). The largest differences between BC4323PT and BC4323LN cells were found for treatment with cisplatin and docetaxel.

**Cytogenetics and FISH.** As analyzed by array-CGH, chromosomal amplifications and deletions were found in multiple chromosomal sections throughout the entire genome (Figure 2). There were only minor differences in chromosomal aberrations between the BC4323PT and BC4323LN cells, as depicted in Figure 3A. Chromosomal gains in BC4323PT cells compared to BC4323LN were seen in chromosomes 1q and 2p. Losses were observed more frequently (4q, 4p, 8p, 9p, 11q and Xq). Amplifications found in BC4323LN were in 5q, 5p and 12q, while deletions were not detectable in either cell line. High-level amplifications were found in both BC4323PT and BC4323LN cells on chromosome 12p (Figure 3A). Array-CGH showed a massive chromosomal gain of the p-arm of chromosome 12. The KRAS gene is located within the amplified gene region, as shown in Figure 3A. Classical banding analysis showed a tetraploid complex karyotype with numerical and structural aberrations. An additional FISH analysis for the KRAS locus on metaphases showed four regular signals for KRAS located in 12p12, as well as an amplified signal for KRAS in a derivative chromosome 4 and a high-level amplification in a marker chromosome (Figure 3B and C). KRAS mutation analysis revealed a mutation in exon 2, type c.35G>C p. (G12A). The mutation was found to be probably homozygous. No mutations were found in any of the analyzed EGFR exons. Responses to tyrosine kinase inhibitors were not expected and therefore not tested for (data not shown).

**PCR expression analysis.** BC4323PT and BC4323LN cells were analyzed with respect to 180 genes correlated with EMT and extracellular matrix proteins. A total of 42 genes were found to be differentially regulated between the two cell lines. The highest up-regulation [fold-change (FC) >5] in BC4323LN compared to BC4323PT was found for desmoplakin (FC=30.7, \( p < 0.001 \)), osteopontin (FC 25.0, \( p=0.04 \)), tissue factor pathway inhibitor 2 (FC=22.0, \( p<0.001 \)), collagen type XIV alpha 1 (FC=12.1, \( p<0.001 \)), guanine nucleotide binding protein (G protein) gamma 11 (FC=11.2, \( p<0.001 \)) and tenascin C (FC=9.8, \( p<0.001 \)). The most up-regulated (FC>5) genes in BC4323PT compared to BC4323LN were collagen type XV alpha 1 (FC=33.9, \( p<0.001 \)), integrin alpha 4 (FC=12.6, \( p=0.016 \)) and ZEB2 (FC=5.6, \( p<0.001 \)).

**Flow cytometry.** Differences in expression of surface proteins, as analyzed by flow cytometry were detectable between the primary tumor and LN metastasis for EpCAM, CD44, P-selectin binding, EGFR and integrin alpha V. The cells were highly positive for Alcam, CD24, CD44, integrin alpha1, beta1 and integrin alpha V, while a moderate surface presentation was found for numerous proteins (Figure 4).

**Murine xenograft model.** In order to assess the in vivo growth of the tumor cells, BC4323PT and BC4323LN cells were injected subcutaneously in immunodeficient Pfp−/−/Rag2−/− mice. Tumor take was seen in every single mouse (N=10 per group); however, mice with BC4323PT cells showed accelerated tumor growth that was reflected in a decreased
Figure 3. A: Genomic profiles of chromosome 12 from lymph node metastasis (BC4323LN) (upper profile) and primary tumor (BC4323PT) (lower profile) cells showing a high level 12p intrachromosomal amplification (iamp12p). The region of iamp12p is magnified to show gene resolution, showing kirsten rat sarcoma viral oncogene homolog (KRAS) within a region of highest amplification in BC4323PT and BC4323LN cells. Karyotyping (B) and fluorescence in situ hybridization analyses with a specific KRAS probe (C) showing high level amplification of the KRAS gene in a novel assembled marker chromosome (M1).
Figure 4. Flow cytometric data for numerous surface proteins. Black line shows the corresponding isocontrol, blue line the primary tumor cells and red line the lymph node metastasis cells. Activated leukocyte cell adhesion molecule (ALCAM), epithelial cell adhesion molecule (EpCAM), CXC-motif-chemokine receptor 4 (CXCR4), carbohydrate-antigen 19-9 (CA19-9), epidermal growth factor receptor (EGFR), vascular endothelial growth factor (VEGF), L1 cell adhesion molecule (L1CAM), carcinoembryonic antigen-related cell adhesion molecule (CEACAM) 1, 5 and 6.
OS in this group compared to the BC4323LN-injected group (Figure 5A). The median time of growth from tumor-cell injection until reaching the termination criteria was 60 days in the BC4323PT group and 101 days in the BC4323LN group (log-rank test \( p=0.005 \)). There were no differences in tumor weight between the groups (Figure 5B).

The disseminative potential of BC4323 cells was assessed by PCR analysis for specific human sequences in different compartments. Circulating tumor cells (CTCs) in the blood were detected in 4/10 animals in the BC4323PT-injected group and 5/10 mice in the BC4323LN-injected group. The median number of CTCs did not differ significantly (Figure 5C). Similar results were found for lung metastases as determined by PCR, with 4/10 mice showing evidence of lung metastasis in the BC4323PT-injected group and 5/10 mice in the BC4323LN-injected group (Figure 5D). No correlation was found between a high number of CTCs and a higher copy number of human DNA sequences in the lung (data not shown). The total number of lung metastases was rather low, and microscopically detectable metastases were not seen. Human DNA sequences representing bone marrow metastases were not detected in any group (data not shown).

**Microscopy and Immunohistochemistry.** As depicted in Figure 6, the primary patient tumor and the subcutaneous tumor developed in the murine xenograft were highly similar on H&E staining. Both patient and mouse-grown tumors presented considerable central necrosis, with only a small rim of vital tumor tissue at the border between the tumor and normal tissue. The tumor cells exhibited a high level of proliferative activity, represented by numerous mitotic figures within the entire vital tumor area.

Immunohistochemical staining was performed on mouse-grown tumors for numerous cell-surface proteins. Differences in antigen expression levels between tumors from BC4323PT- and BC4323LN-injected mice as revealed by either qPCR or flow cytometry were validated by immunohistochemistry (Figure 7). Different expression levels were found for CD44, tenascin C, TWIST and ZEB2.

**Discussion**

Lung cancer remains one of the major causes of cancer-related mortality in the Western world (1). The mortality rate of lung cancer is high, even when novel multimodal therapeutic strategies are employed. Despite aggressive local tumor growth, the individual prognosis of the majority of patients is limited by the development of local recurrence and distant metastasis (17).

In the present study, we characterized a pair of cell lines from a primary tumor and its corresponding LN metastasis. As distant and LN metastasis are one of the major challenges
Figure 6. Hematoxylin and eosin staining of the primary patient tumor as primarily resected (5- and 20-fold magnification) (A). Mouse-grown tumors of primary tumor (BC4323PT) (B) and lymph node metastasis (BC4323LN) cells (C) exhibit similar growth patterns, with central necrosis and vital tumor tissue only in the outer tumoral regions (5- and 20-fold magnification).

Figure 7. Immunohistochemical staining for CD44, tenascin C, twist-related protein 1 (TWIST) and zinc finger E-box binding homeobox 2 (ZEB2) of mouse-grown primary tumor (BC4323PT) and lymph node metastatic (BC4323LN) tumors.
in treating NSCLC, understanding the biology of the underlying processes is of major importance. We focused on whether we could find patterns that identified metastatic processes or differences between the phenotypes of cells. Both cell lines had the capacity to establish primary tumors, as examined in a murine xenograft model. However, there were essentially no distant metastases seen, although small numbers of human tumor cells were detected by qPCR. The results have to be interpreted cautiously, as we were unable to confirm the presence of micrometastases or even disseminated tumor cells microscopically. Irrespective of the metastatic status and as already shown for ovarian cancer and other tumor entities, patient-derived cell line xenograft models recapitulate the biological processes of tumor progression in a quite consistent way and can therefore increase our understanding of carcinogenesis, therapy resistance and metastatic progression (18-20). However, xenograft models have limitations with respect to vascular, inflammatory and stromal interactions of murine tissue and the tumor cells (21). Additionally, as also seen in our model, spontaneous metastases are rare events and different means of application (e.g. intravenous, intracranial, intraperitoneal injection) are often required to provoke distant metastasis (22, 23).

The question arises as to why non-metastatic cell lines were established from a clinically malignant tumor associated with the onset of widespread metastases shortly after surgery. As the two cell lines were genetically remarkably similar (see below), epigenetic changes could be the key to the different metastatic behavior of the cell lines in comparison to the tumor in the patient. Nestor et al. showed rapid reprogramming of epigenetic and transcriptional profiles just 3 days after mouse fibroblasts had been put into cell culture (24). The observed hypermethylation of the genome resulted in an up-regulation of genes associated with cell adhesion and extracellular matrix organization. Hence, these epigenetic changes could account for the non-metastatic phenotype observed after initial cell culture of the carcinoma cells. This phenomenon has already been observed in murine Lewis lung carcinoma, which does form lung metastases after subcutaneous implantation (25). Culture of tumor cells from subcutaneous primary tumors or artificial lung metastases resulted in two cell lines from a total of 405 cell lines that were tumorigenic but not metastatic, while the rest were both tumorigenic and metastatic. Interestingly, the metastatic phenotype was restored in these two cell lines by treating them with the demethylating agent 5-azacytidine (26). It is tempting to speculate that similar processes were at play in our two cell lines, which are an ideal pair to investigate this question.

Interestingly, only minor differences were detectable at the genomic, RNA and protein-expression levels. Array-CGH and FISH analysis of the genome revealed a highly amplified genomic region involving the KRAS gene, which is generally considered as a major driver mutation in carcinogenesis and might explain the highly aggressive behavior of this particular tumor in the clinical course of the patient (8, 27). The same result was found in both the primary tumor and LN metastasis. This finding was unexpected, as KRAS mutations in both primary tumors and LN metastases are rather infrequent, as reported by Sun and colleagues analyzing 80 patients with NSCLC (28). In the majority of patients, KRAS mutations were acquired during the metastatic process but not found in the primary tumor. Mutations in both the primary tumor and LN metastasis were found in only one out of 80 patients (1.3%) and this underlines their infrequent occurrence. Karyotyping including array-CGH, banding analysis and FISH showed a complex tetraploid karyotype due to a greatly increased genomic instability in the tumor cells, probably in combination with defects in double-strand repair mechanisms as reported for numerous tumor entities (29, 30). PCR KRAS exon analysis showed a rather uncommon homozygote mutation (type p.G12A) that can be explained by a breakage-fusion-bridge that leads to amplification of unstable chromosomal regions as previously described (31).

Other gene regions showed slight differences between the primary tumor and the LN metastasis, with inconclusive interpretation. Whether this is an effect of general genomic instability of the tumor genome or specific mutations promoting metastasis is not clear. As reported by Hoang and colleagues, differences in the mRNA profiles of NSCLC primary tumors and their corresponding LN metastases are much less frequent than initially expected (32). Samples from 11 patients revealed only minor differences in the expression profiles, making it difficult to identify a metastatic pattern explaining the lymphatic metastatic process. However, according to our results, cells from the primary tumor and LN metastasis exhibited a high level of homology that makes it almost impossible to differentiate between cells derived from the primary tumor and those from the LN metastasis. During the metastatic process, tumor cells generally undergo several morphological changes. In the so-called EMT, tumor cells change their phenotype from epithelial in the primary tumor to a mesenchymal phenotype (33). We therefore analyzed whether this pattern existed in the LN metastasis but, as shown, typical EMT markers (e.g. TWIST, and ZEB2) did not differ in such a way that we can predict whether the LN cells exhibited a more mesenchymal phenotype. This leads to the conclusion that tumor cells may have already undergone transition in the reverse direction (MET), which means that the cells at the site of metastasis had already re-established their epithelial phenotype. Another explanation could be that the lymphatic metastatic process differs from hematogenous distant metastasis (e.g. brain, and adrenal gland) in NSCLC. As lymphatic vessels are generally considered ‘leaky’ without tight junctions between the lymphatic endothelium, in contrast to blood vessels, the lymphatic system can be entered easily once
a tumor cell leaves the compact tumor formation. Therefore, the invasive and EMT programs of that particular tumor cell should differ between lymphatic and distant metastasis.

However, the cells derived from LN metastasis are, in this particular case, generally not that robust compared to the PT cells tumor, as seen in the chemosensitivity and proliferation assays, and the xenograft model. PT cells exhibited increased proliferation, both in vitro and in vivo. Possibly, cells that are able to establish distant metastases in LNs exhibit a high level of aggressiveness that is not reflected in high proliferative and growth rates but rather more in the way these cells have the ability to endure over a long period of time in a somewhat hostile environment.

In summary, we provide a unique tool for analyzing the ongoing processes during LN metastatic formation in NSCLC in a cell line model derived from patient material that could help improve our understanding of the metastatic process in NSCLC.

Conflicts of Interest
None.

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