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Characterization of DLL3-positive circulating tumor cells (CTCs) in patients with small cell lung cancer (SCLC) and evaluation of their clinical relevance during front-line treatment

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ABSTRACT

Objectives: The aim of the study was to characterize and evaluate the presence of DLL3-positive Circulating Tumor Cells (CTCs) in SCLC patients receiving front-line chemotherapy and assess their clinical relevance.
Materials and methods: Peripheral blood was obtained from treatment-naïve patients with SCLC (n = 108 patients), after one etoposide/platinum cycle (n = 68 patients) and on disease progression (n = 48 patients). Immunofluorescence staining using antibodies against the DLL3, cytokeratins (CK), CD45 and vimentin (Vim) was used for the detection and characterization of CTCs.
Results: Before treatment, 74.1% of patients had detectable $DLL3^+/CD45^-$ CTCs. One-treatment cycle significantly decreased both the detection rate ($p < 0.001$) and the absolute number ($p < 0.001$) of $DLL3^+/CD45^-$ CTCs. Triple immunofluorescence staining using anti-CK, anti-Vim and anti-DLL3 antibodies revealed an important CTC heterogeneity since DLL3 could be detected in Vim^+, Vim^-, CK^+ and CK^- CTCs. On disease progression, both the detection rate and the absolute number of $DLL3^+/CD45^-$ CTCs were significantly increased compared to post-1^{st} cycle values ($p < 0.001$ and $p = 0.002$, respectively). In addition, 22.7% of patients had detectable $DLL3^+/CD45^-$ cells which could not be captured by the CellSearch assay. In multivariate analysis, the detection of DLL3+/CD45- CTCs at baseline was significantly associated with decreased progression-free survival (HR = 10.8; $p = 0.005$) whereas their detection on disease progression was associated with decreased overall survival (HR: 28.2; $p = 0.016$).
Conclusions: These findings demonstrate an important heterogeneity of CTCs, based on the expression of CK, Vim and DLL3, in patients with SCLC and the changes of $DLL3^+/CD45^-$ CTCs during treatment seem to be a dynamic biomarker associated with patients' clinical outcome.

1. Introduction

Small-Cell Lung Cancer (SCLC) is a neuroendocrine tumor with poor prognosis accounting for about 13% of lung cancer cases [1–4]. The Delta-Notch pathway is an evolutionarily conserved signaling pathway which controls a broad spectrum of developmental processes such as cell fate determination, terminal differentiation and proliferation; this pathway acts as an oncogenic stimulus in some tumor types, including neuroendocrine tumors [5–7]. Notch receptors and ligands are transmembrane proteins belonging to the Epidermal Growth Factor (EGF)-like family of proteins including among others, the Notch 1–4 receptors and ligands delta-like 1–4 (DLL1, DLL2, DLL3, DLL4) [8]. DLL3 is normally overexpressed in fetal brain and plays a key role in somatogenesis by inhibiting activation of the Notch pathway whereas all other ligands activate Notch signaling [9–11].

DLL3 protein is expressed on the cell surface in distinct tumors. Recently, it was reported that tumor cells expressing DLL3 could be detected in 73% of patients with SCLC and large-cell neuroendocrine

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carcinoma (LCNEC) [9]. Since DLL3 is exclusively expressed on tumor but not normal cells, it can be considered as a Trojan horse for potent therapeutic implication [9]. Indeed, an anti-DLL3-drug conjugate antibody has shown promising efficacy against pulmonary patient-derived xenograft neuroendocrine tumors, including SCLC [10].

The presence of circulating tumor cells (CTCs) in the bloodstream represents an obligatory step towards the metastatic spread of a tumor. Previous studies have shown that relatively high numbers of CTCs are frequently detected in patients with SCLC [11]. Our group has demonstrated the phenotypic heterogeneity of CTCs in SCLC patients, based on the expression of TTF-1 and CD56 molecules [12,13]. Moreover, the heterogeneity of the disease is further documented by the different cell morphology, genetic changes, proliferative, apoptotic, anti-apoptotic and/or epithelial-to-mesenchymal transition (EMT) phenotype and sensitivity to chemotherapy [3,14–16]. In fact, during their dissemination from the primary tumor, the cells undergo epithelial-to-mesenchymal transition (EMT) which is characterized by loss of their epithelial phenotype and the overexpression of their mesenchymal phenotype [17,18]. Therefore, it seems that technologies, such as the FDA approved CellSearch platform, designed to detect CTCs based on the expression of epithelial markers, may fail to detect CTCs expressing an EMT phenotype. Moreover, this EMT transition could explain, at least in part, the high heterogeneity of CTCs which could be, probably, linked to their metastatic potential.

Given the difficulty to have available serial tissue material from the tumor, the current study was designed to investigate the real-time follow up expression of DLL3 on CTCs from patients with newly diagnosed SCLC and to evaluate their clinical relevance as a dynamic biomarker during front-line chemotherapy.

2. Patients and methods

2.1. Patient samples and cytospin preparations

Peripheral blood (20 ml in EDTA and 7.5 ml in CellSearch Save preservative tubes; Menarini) was obtained from newly-diagnosed and histologically documented patients with SCLC before the initiation of etoposide/platinum treatment (n = 108 patients; baseline sample), after one chemotherapy cycle (n = 68 patients; post-1st cycle sample) and at the time of disease progression, before the initiation of second line treatment (n = 48 patients; disease progression sample). All blood samples were obtained at the middle of vein puncture, after the first 5 ml were discarded to avoid contamination with epithelial cells from the skin. Patients with extensive disease received up to 6-cycles of etoposide/cisplatin, while patients with limited disease were treated with concurrent etoposide/cisplatin and radiotherapy, as well as prophylactic cranial irradiation (PCI) according to national guidelines. Staging of the disease was performed by CT scan of thorax, abdomen and CNS as well as whole body bone scan before the initiation of systemic treatment; moreover, further studies with MRI were performed if required. The study has been approved by the Ethics and Scientific Committees of the University General Hospital of Heraklion and all patients signed a written informed consent.

Peripheral blood mononuclear cells (PBMCs) were isolated by Ficoll–Hypaque density (d = 1077 g/mL; Sigma-Aldrich, GmbH, Germany) gradient centrifugation at 1800 rpm, for 30 min. Centrifugation and cytospins were prepared as previously described [19,20]. In brief, aliquots of 5×10^5 PBMCs were cytocentrifuged at 2000 rpm for 2 min on glass microscope slides. Cytospins were air dried and stored at −80 °C, until use. Two slides (10×10^5 PBMCs) from each patient were analyzed at each time point.

2.2. Detection of CTCs using the CellSearch assay

For the enumeration of CTCs, blood samples were maintained at ambient temperature and processed within 72 h using the CellSearch assay (Menarini, Italy) according to the instructions of the manufacturer [21]. CTC morphology was confirmed in all cases and analysis was performed with the CellTracks Analyser II by an experienced biologist (EP). Results are expressed as number of CTCs/7.5 ml of blood. Patients with > 5CTC/7.5 ml of blood were considered as positive whereas patients with < 5CTCs/7.5 ml of blood were considered as negative according to previous reports Hou et al. [11].

2.3. Double and triple immunofluorescence assays

DLL3 was detected by double immunofluorescence staining using monoclonal antibodies against DLL3 (anti-rabbit: Thermo Fisher Scientific, Fremont, CA, USA) and CD45 (anti-mouse: DAKO, Agilent Technologies, Denmark) or the mouse anti-A45-B/B3 (anti-cytokeratins 8, 18, and 19; Micromet, Munich, Germany) as previously described [22]. In brief, PBMC cytospins were fixed with ice-cold acetone:methanol 9:1 (v/v) for 20 min. The incubation period was 1 h for all primary and secondary antibodies. DLL3 was labelled with Alexa 555 (Molecular Probes, Invitrogen, CA, USA) whereas CK and CD45 with Alexa 488 (Molecular Probes). The omission of the first antibody in each staining has been used as a negative control. Finally, 4′,6-diamidino-2-phenylindole (DAPI)-antifade (Molecular Probes) was added to each sample for nuclear staining. Slides were analyzed using a fluorescence microscope (Leica DM 2500, Heidelberg, Germany). Results are expressed as number of CTCs/10^6 PBMCs.

For triple immunofluorescence staining, cytospins from six selected patients (three with ≥5CTCs/7.5 mL and three with 0 CTCs/7.5 ml by CellSearch at baseline), were stained with the anti-DLL3, the mouse anti-A45-B/B3 and the rabbit anti-vimentin (Santa Cruz Biotechnology, CA) antibodies. Briefly, fixed PBMCs were incubated with blocking buffer (PBS/2% FBS) for 1 h, washed with PBS and Zenon technology (Molecular Probes) was used for vimentin detection; subsequently, cells were stained with anti-DLL3 and Alexa 555. Afterwards, cells were stained with anti-A45-B/B3 (Micromet) and Alexa 633 (Molecular Probes). Finally, DAPI-antifade reagent (Molecular Probes) was added to each sample for nuclear staining. The incubation period for all primary and secondary antibodies was 1 h. Negative controls were prepared by omitting the corresponding primary antibody.

2.4. Determination of immunofluorescence sensitivity

The human H209 and HeLa cell lines were obtained from the American Type Culture Collection (ATCC, Manassas, VA, USA) and used as positive controls, as follows: cyto-centrifuged H209 cells were used as positive controls for CK and DLL3 expression; HeLa cells were used as positive controls for CK and vimentin expression. In addition, all cell lines were double stained with anti-CD45 and either anti-DLL3 or anti-Vim antibodies in order to exclude possible ectopic expression in tumor cells from these cell lines. To determine the sensitivity of the method, all cell lines were spiked in peripheral blood obtained from healthy blood donors and the PBMCs, obtained after Ficoll-Hypaque density centrifugation, were used to prepare cytospins, as per patients' samples. H209 cells were cultured in RPMI (Gibco-BRL Life Technologies, Rockville, USA) supplemented with 25 mM glucose, 1 mM sodium pyruvate, 1gr/L sodium bicarbonate, 2 mM L-glutamine (Gibco-BRL), 1-mM Hepes (Gibco-BRL), 50 mg/mL penicillin/streptomycin (Gibco-BRL) and 10% FBS (Gibco-BRL). HeLa cells were cultured in 1:1 (vol/vol) DMEM (Gibco-BRL) supplemented with 10% FBS (Gibco-BRL), 2 mmol L-glutamine (Gibco-BRL) and 50 mg/mL penicillin/streptomycin (Gibco-BRL). All cells were maintained in a humidified atmosphere of 5% CO_2 in air. Sub-cultivation of all cell lines was performed using 0.25% trypsin and 5mMol ethylenediamine-tetra-acetic acid (EDTA; Gibco BRL). All experiments were performed during the logarithmic growth phase of the cell lines.

2.5. DLL3 expression on primary tumor cells

Immunohistochemical analysis of DLL3 protein expression was performed in 20 patients, for whom available tissue material from

formalin-fixed paraffin-embedded (FFPE) biopsies could be retrieved from the archives of the Pathology Department of University General Hospital of Heraklion. Sections of 4 μ thickness were cut and mounted in charged glass slides, which were then stained with anti-DLL3 mouse monoclonal antibody [Ventana DLL3 (SP347) Assay, Roche Diagnostics Germany] on BenchMark IHC/ISH instruments using the OptiView DAB IHC Detection Kit. The analysis was performed under a 20X and 40X magnification. The percentage of tumor cells, positive (cytoplasmic or membranous) at any staining intensity, among total neoplastic cells was determined. When at least 50% of cells expressed DLL3, a sample was scored as DLL3-high, whereas, in DLL3 expression in fewer than 50% of cells, the sample was scored as DLL3-low [23]. Experiments in which the primary antibody was omitted were used as negative controls.

2.6. Study design and statistics

This is a prospective, translational research, single institution study, investigating the expression of DLL3 and vimentin on CTCs from consecutive patients with SCLC in order to define whether their changes during front-line chemotherapy could be of clinical relevance. Because of the observational nature of the study it was not possible make a clear statistical hypothesis to estimate the appropriate number of patients to be enrolled in the study. The evaluation of CTCs was done blindly to clinical data. Progression-free survival (PFS) was calculated for all patients from the day of treatment initiation until treatment discontinuation due to disease progression or death. Overall survival (OS) was calculated for all patients from the start date of therapy to the date of death due to any cause. The association of risk factors with PFS and OS was analyzed using the log-rank test and the Kaplan–Meier method. Qualitative factors were compared by Pearson's Chi-square test or Fisher's exact test whenever appropriate. Pearson's correlation coefficient was used to evaluate the correlation between the Cellsearch and immunofluorescence. Univariate and multivariate Cox proportional hazards regression models with hazard ratios (HR) and 95% CIs were used to evaluate the predictive and prognostic relevance of CTCs. Statistical significance was set at $p = 0.05$. All statistical analysis was performed using the SPSS v.20 software (IBM Corp. Armonk, NY, USA).

3. Results

3.1. DLL3 and Vimentin expression in cancer cell lines and PBMCs of healthy individuals

Immunofluorescence studies revealed that 85% ± 5%/10^6 H209 and 95% ± 5%/10^6 HeLa cells (mean+/-SD values from 5 experiments) were $CK^+/DLL3^+$ and CK^+/Vim^+, respectively. Spiking experiments with 1-1000 H209 and HeLa cells/10^6 PBMCs from blood donors demonstrated that immunofluorescence could detect up to 1 H209 or1 HeLa cell/10^6 PBMCs. Control experiments performed in 26 healthy blood donors could not detect $DLL3^+/CD45^-$ PBMCs. Moreover, although vimentin was found to be highly expressed in healthy blood donors' PBMCs, no CK^+/Vim^+ cells could be detected in any of the tested subjects (n = 10; data not shown).

3.2. Patients' characteristics

Between 11/2010 and 05/2015, 108 patients with newly diagnosed SCLC were enrolled in the study in the Department of Medical Oncology of University General Hospital of Heraklion, Crete and had available cytospins from PBMCs for immunofluorescence staining. The patients' characteristics are listed in Table 1. The median age was 66 years, 91 (84.3%) patients were males, 63 (58.3%) had a PS (ECOG) of 0–1, and 71 (65.7%) had extensive disease. Moreover, 79 (73.1%) patients had lactate dehydrogenase (LDH) serum levels above the upper normal limit, 12 (11.1%) had brain, 40 (37.0%) liver and 32 (29.6%) bone metastases at the time of diagnosis. Thirty-seven (34.3%) patients received concurrent chemo-radiotherapy for limited disease and 30 of them PCI. Seventy-seven (71.3%) patients achieved an objective response (CR/PR) (Table 1). Eighty-three (76.9%) patients were evaluable for CTC enumeration using the CellSearch assay before treatment initiation (data presented in 16; Suppl. Fig. 1). Briefly, CTCs (≥5CTCs/7.5 ml) could be detected in 50 (60.2%) patients with a median number of 14 CTCs/7.5 ml (range, 0–10.000). The detection of CTCs, using the CellSearch assay, was correlated with PS, disease stage, LDH levels, liver or bone metastases and response to treatment (data reported in ref. [16]].

Table 1
Patients' demographics.

		$DLL3^+/CD45^-$ (%) (n = 108)		p
	n (%)	+ve	-ve	
Age	Median 66 (range, 44 - 82)			
Gender				
Male	91 (84,3)	68 (63,0)	23 (21,3)	0,721
Female	17 (15,7)	12 (11,1)	5 (4,6)	
PS				
0-1	63 (58,3)	38 (35,2)	25 (23,1)	< 0,001
≥2	45 (41,7)	42 (38,9)	3 (2,8)	
Stage				
LD	37 (34,3)	19 (17,6)	18 (16,7)	< 0,001
ED	71 (65,7)	61 (56,5)	10 (9,3)	
LDH				
High	79 (73,1)	65 (60,2)	14 (13,2)	0,001
Low	27 (25,0)	13 (12,0)	14 (13,2)	
Unknown	2 (1,9)	1 (0,9)	1 (0,9)	
Liver Metastases (at diagnosis)				
Yes	40 (37,0)	39 (36,1)	1 (0,9)	< 0,001
No	66 (61,1)	39 (36,1)	27 (25,0)	
Unknown	2 (1,9)	2 (1,9)	0 (0,0)	
CNS (at diagnosis)				
Yes	12 (11,1)	11 (10,2)	1 (0,9)	0,111
No	92 (85,2)	62 (57,4)	27 (26,0)	
Unknown	4 (3,7)	4 (3,7)	0 (0,0)	
Bone Metastases (at diagnosis)				
Yes	32 (29,6)	29 (28,2)	3 (2,8)	0,01
No	71 (65,7)	48 (46,6)	23 (21,3)	
Unknown	5 (4,6)	4 (3,7)	1 (0,9)	
Response				
CR/PR	77 (71,3)	54 (50,0)	23 (21,3)	0,048
SD	11 (10,2)	7 (6,5)	4 (3,7)	
PD	14 (13,0)	14 (13,0)	0 (0,0)	
Unknown	6 (5,6)	5 (4,6)	1 (0,9)	

3.3. Detection of DLL3 in primary tumor cells and CTCs ($DLL3^+/CD45^-$ CTCs) before treatment initiation

Before treatment initiation, double immunofluorescence revealed the presence of $DLL3^+/CD45^-$ CTCs (Fig. 1) in 80 (74.1%) out of 108 patients (Table 2). There was a significant association between the detection of $DLL3^+$/CD45- cells and PS (ECOG) ($p < 0.001$), disease stage ($p < 0.001$), LDH levels ($p = 0.001$), the presence of liver ($p < 0.001$) or bone ($p = 0.041$) metastases and response to treatment ($p = 0.009$) (Table 1). The median number of $DLL3^+/CD45^-$ CTCs was 7/10^6 PBMCs (range, 0–299).

Immunohistochemical staining of the primary tumor cells revealed that 14 (70%) of 20 patients with available tissue material were considered as DLL3-high since DLL3 protein could be detected in > 50% of the tumor cells (Suppl. Fig. 2 and Suppl. Table 1). Among the DLL3-high patients, $DLL3^+/CD45^-$ CTCs could be detected in 12 (85.7%) of them with a median of 77 CTCs/10^6 PBMCs (range 2–289); similarly among the DLL3-low patients, $DLL3^+/CD45^-$ CTCs were detected in 5 (83.3%) of them with a median of 7 CTCs/10^6 PBMCs (range 0–24).

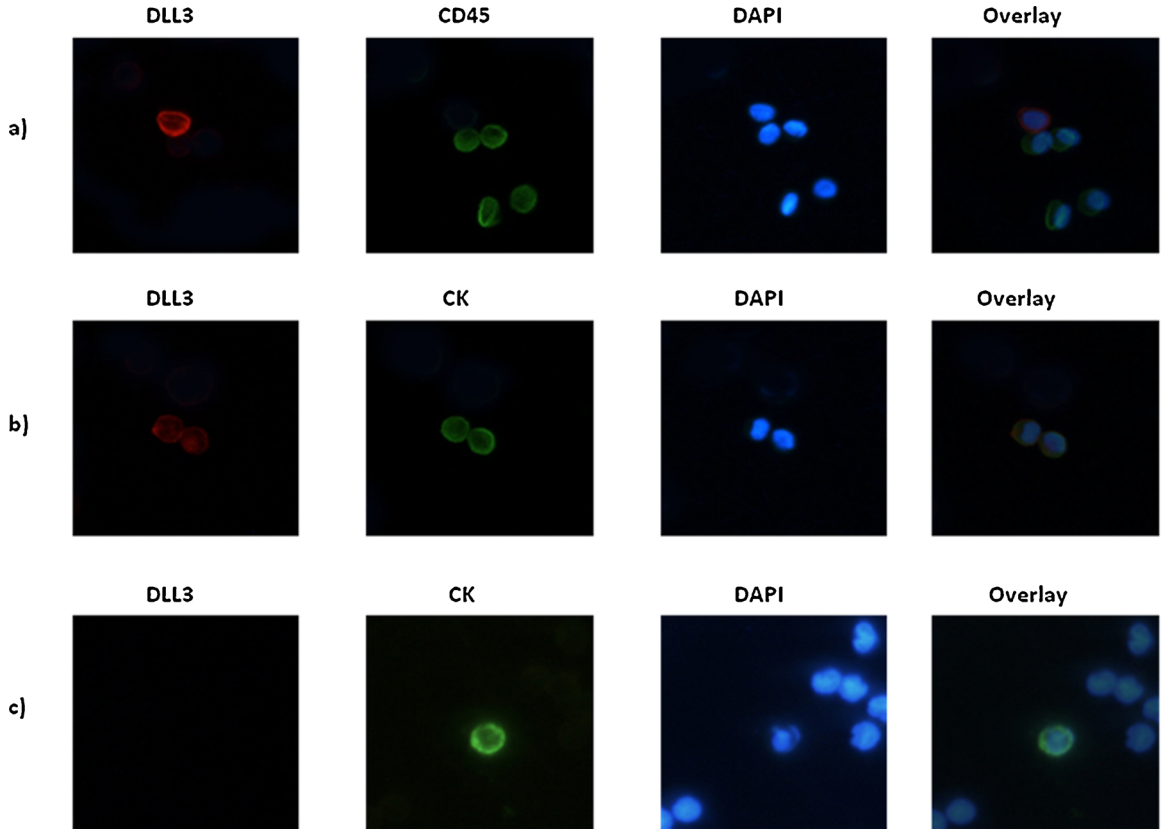

Fig. 1. a) DLL3$^+$/CD45$^-$, b) CK$^+$/DLL3$^+$, c) CK$^+$/DLL3$^-$ by immunofluorescent staining.

3.4. Detection of DLL3$^+$/CD45$^-$ CTCs in patients with and without detectable CTCs by the CellSearch

Forty-five (90.0%) out of 50 patients, who had detectable (≥5CTCs/7.5 ml) CTCs at baseline by CellSearch, also had detectable DLL3$^+$/CD45$^-$ CTCs by immunofluorescence. There was a significant correlation between the detection of CTCs by CellSearch at baseline and the DLL3$^+$/CD45- subpopulation (Pearson's correlation coefficient, r = 0.640; $p < 0.001$).

In 22 (20.4%) patients, no CTCs could be detected by CellSearch (0 CTCs/7.5 ml) at baseline; however, immunofluorescence revealed the presence of DLL3$^+$/CD45$^-$ CTCs in five (22.7%) of them (Table 2).

3.5. Detection of DLL3 in vimentin-positive CTCs

Since the Notch pathway is associated with the stem cell and EMT phenotype of the cells [24,25], we further investigated, in a selected number of patients, DLL3 co-expression with vimentin, which is considered as a mesenchymal marker [19]. For this purpose, six patients (3 patients with and 3 patients without detectable CTCs by CellSearch) were selected for triple immunofluorescence staining using anti-CK, anti-vimentin and anti-DLL3 antibodies. Five (83.3%) out of six patients had detectable CK$^-$/vim$^+$/DLL3$^+$ CTCs irrespectively of the detection of CTCs by CellSearch (Suppl. Table 2). It is interesting, that an important phenotypic heterogeneity was revealed since CK$^+$/DLL3$^+$, CK$^+$/DLL3$^-$, CK$^+$/Vim$^+$/DLL3$^+$, CK$^+$/Vim$^-$/DLL3$^+$, CK$^+$/Vim$^+$/DLL3- but not CK$^-$/vim$^-$/DLL3$^+$ CTCs could be detected in these patients (Suppl. Table 2, Figs. 1 and 2).

3.6. Detection of DLL3$^+$/CD45$^-$ CTCs after one chemotherapy cycle and at relapse

In 68 (63.0%) patients, a second blood sample was available after one treatment cycle for the detection of CTCs. The detection rate of DLL3$^+$/CD45$^-$ CTCs was significantly decreased after one treatment cycle (n = 30 patients; 44.1%) compared to the corresponding baseline values (Table 2). Chemotherapy also resulted to a significant decrease of the absolute number of CTCs ($p < 0.001$) compared to baseline values (Table 2). This was also the case for patients with < 5CTCs/7.5 ml ($p = 0.008$) but not for those without detectable CTCs (0 CTCs/ 7.5 ml), using the CellSearch assay, at baseline.

At the time of disease progression, a blood sample was available

Table 2
Detection of CTCs by CS and IF during front-line chemotherapy.

	Baseline		After one treatment cycle		Disease Progression	
	N (%)	Median (range)	N (%)	Median (range)	N (%)	Median (range)
CellSearch (≥5 CTCs/7.5 ml)	50/83 (60,2)	14 (0-10000)				
DLL3$^+$/CD45$^-$ (all patients)	80/108 (74,1)	7 (0-299)	30/68 (44,1)[a]	0 (0-101)[b]	42/48 (87,5)[e]	12 (0-197)[f]
DLL3$^+$/CD45$^-$ (patients with < 5 CTCs/ 7.5 ml in CellSearch)	13/33 (39,4)	0 (0-19)	3/24 (12,5)[c]	0 (0-7)[d]	9/13 (69,2)[g]	4 (0-16)[h]
DLL3$^+$/CD45$^-$ (patients with 0CTCs/ 7.5 ml in CellSearch)	5/22 (22,7)	0 (0-7)	1/16 (6,3)	0 (0-1)	4/7 (57,1)	2 (1-7)

p value: Baseline vs Post 1 st: a: < 0,001; b: < 0,001; c:0.008; d:0.008.
p value: Post 1 st vs Progression:e: < 0,001; f:0,002; g:0.014; h:0.014.

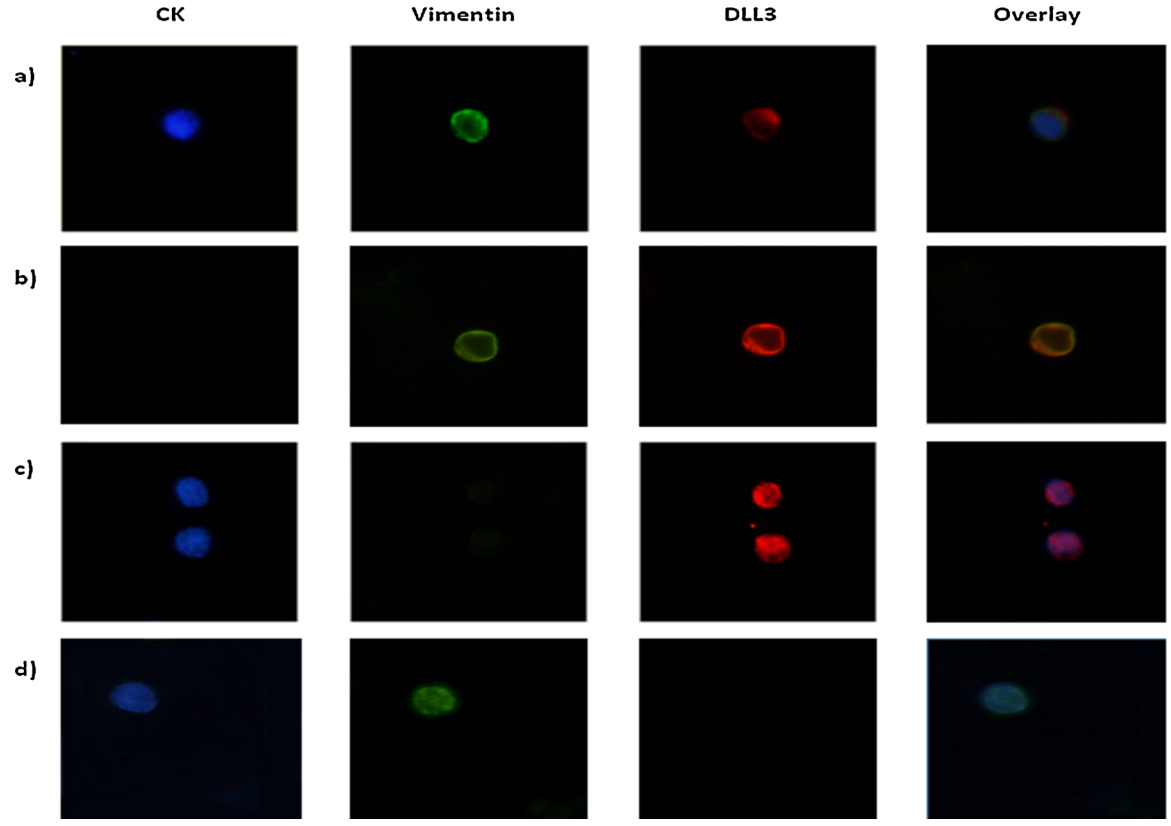

Fig. 2. a) $CK^+/Vim^+/DLL3^+$, b) $CK^-/Vim^+/DLL3^+$, c) $CK^+/Vim^-/DLL3^+$ and d) $CK^+/Vim^+/DLL3^-$ by immunofluorescent staining.

Table 3
Univariate and multivariate Cox Regression analysis.

	Univariate Analysis				Multivariate Analysis			
	PFS		OS		PFS		OS	
	HR (95%CI)	Sig.	HR (95%CI)	Sig.	HR (95%CI)	Sig.	HR (95%CI)	Sig.
PS (> 2 vs 0-1)	2,2 (1,5-3,4)	< 0,001	2,7 (1,7-4,3)	< 0,001	3,3 (1,0-11,5)	0,057	9,7 (1,6-60,4)	0,015
ED vs LD	3,2 (2,0-5,2)	< 0,001	3,7 (2,1-6,5)	< 0,001	2,6 (0,8-8,6)	0,125	8,7 (1,4-52,3)	0,018
LDH (High vs Normal)	2,1 (1,3-3,4)	0,006	3,1 (1,7-5,9)	< 0,001	1,8 (0,8-4,7)	0,212	1,5 (0,4-6,3)	0,547
Liver Metastases (Yes vs No)	2,2 (1,4-3,4)	< 0,001	2,4 (1,5-3,8)	< 0,001	1,1 (0,4-3,5)	0,032	7,8 (1,4-42,9)	0,018
Bone Metastases (Yes vs No)	2,4 (1,5-3,8)	< 0,001	3,2 (1,9-5,3)	< 0,001	4,7 (1,6-13,7)	0,004	3,5 (0,6-20,2)	0,156
Response (PD vs SD vs CR/PR)	1,2 (0,6-2,7)	< 0,001	1,4 (0,6-3,3)	< 0,001	7,8 (1,8-33,6)	0,006	4,5 (2,8-7,1)	0,007
$DLL3^+/CD45^-$ CTCs at baseline	6,0 (2,9-12,7)	< 0,001	8,8 (3,2-24,3)	< 0,001	10,8 (2,1-56,4)	0,005	1,8 (0,1-25,5)	0,661
$DLL3^+$/CD45- CTCs post-1^{st} cycle	2,1 (1,2-3,5)	0,01	2,1 (1,2-3,9)	0,011	1,8 (0,6-5,4)	0,282	28,2 (2,0-39,1)	0,016

from 48 patients. The number of patients with detectable $DLL3^+/CD45^-$ CTCs was significantly higher compared to the post-1^{st} cycle values, ($p < 0.001$); similarly, the absolute number of $DLL3^+/CD45^-$ CTCs was also significantly increased ($p = 0.002$; Table 2). Again, this was also the case for patients with < 5CTCs/7.5 ml ($p = 0.014$) but not for those without detectable CTCs (0 CTCs/ 7.5 ml) at baseline.

3.7. Detection of $DLL3^+/CD45^-$ CTCs and clinical outcome

Patients who experienced disease progression had a significantly higher detection rate of $DLL3^+/CD45^-$ CTCs at baseline and after one-treatment cycle compared to patients without disease progression at the time of analysis (74.1% vs 0.0%: $p < 0.001$; 44.1% vs 0.0%: $p < 0.001$, respectively) (Suppl. Table 3). The median PFS for the whole group of patients was 6.8 months (95% CI: 6.2–7.5). In patients with and without detectable $DLL3^+/CD45^-$ cells at baseline, the median PFS was 6.0 and 28.8 (95% CI: 5.3–6.7 and 1.4–56.2) months, respectively ($p < 0.001$; Suppl. Figure 3a), as well as after one treatment cycle ($p = 0.008$; Suppl. Figure 3b). Accordingly, the median OS for the whole group of patients was 10.8 months (95% CI: 8.8–12.8); the median OS was significantly different in patients with and without detectable $DLL3^+/CD45^-$ cells at baseline ($p < 0.001$; Suppl. Figure 3c) and after one-treatment cycle ($p = 0.009$; Suppl. Figure 3d) but not on disease progression (data not shown).

3.8. Univariate and multivariate analysis

Univariate analysis revealed that PS (ECOG), disease stage, LDH levels, organ metastases, response to treatment and increased numbers of $DLL3^+/CD45^-$ CTCs at baseline and after one-treatment cycle were significantly associated with a shorter PFS and OS (Table 3). In multivariate analysis, liver and bone metastases, response to treatment and the detection of $DLL3^+$/CD45- CTCs at baseline emerged as independent factors associated with a decreased PFS (Table 3); moreover, PS, disease stage, liver metastases, response to treatment, and the detection of CTCs by immunofluorescence at the time of disease

progression, emerged as independent prognostic factors associated with a decreased OS (Table 3).

4. Discussion

DLL3 is an intracellular protein which could be overexpressed and detected in the cell surface in tumor cells; immunocytochemistry has revealed that tumor cells from about 73% of patients with SCLC and LCNEC NSCLC express DLL3 on their cell surface [9]. The current study demonstrated, for the first time, that CTCs from most of patients with newly diagnosed SCLC also express DLL3. Moreover, the detection of $DLL3^+/CD45^-$ CTCs was independent on the expression of DLL3 in primary tumor cells. The incidence of detection as well as the number of $DLL3^+/CD45^-$ CTCs was significantly decreased after one-chemotherapy cycle but significantly increased at the time of disease progression, suggesting a role of dynamic biomarker for this CTC subpopulation in patients with SCLC receiving front-line treatment. This hypothesis is in line with previous studies which have documented the prognostic and predictive value of CTCs expressing various epithelial, neuroendocrine and other markers in different tumor types [12,13,21,26–30].

In the current study, the phenotypic characterization of CTCs was performed in a large cohort of SCLC patients using double and triple immunofluorescence staining with an anti-DLL3 antibody. Moreover, high expression of DLL3 in primary tumor cells was detected in 70% of the tested patients as already has been reported [9,10,23,31]. The clinical significance of high or lower DLL3 expression on primary tumor cells is not yet known. However, the observation that the detection rate of DLL3-positive CTCs was higher in patients with DLL3-high primary tumors than in patients with DLL3-low tumors seems to indicate a preferential migration capacity of these $DLL3^+/CD45^-$ CTCs.

The cytological characterization of CTCs, based on the expression of DLL3, Vim and CK, revealed an impressive phenotypic heterogeneity in patients with SCLC in agreement with previous studies [12,13,15]; indeed, various subpopulations of CTCs ($DLL3^+/CD45^-$, $CK^+/DLL3^+$, $CK^+/DLL3^-$, $CK^+/Vim^+/DLL3^+$, $CK^+/Vim^-/DLL3^+$, $CK^+/Vim^+/DLL3^-$) could be detected before the initiation of systemic treatment. Unfortunately, this study failed to follow the changes of the various phenotypically different subpopulations of CTCs during the evolution of the disease for reasons related to the availability of enough back-up slides corresponding to the different time points during treatment. It is noteworthy, that almost all tested patients had detectable $CK^-/Vim^+/DLL3^+$ CTCs in their blood, even in the absence of detectable CTCs by the CellSearch assay; this cell phenotype should not be attributed to normal hemopoietic cells since it was not possible to be detected by triple immufluorescence staining of PBMCs from normal blood donors. The $CK^-/Vim^+/DLL3^+$ CTCs probably represent a CTC subpopulation undergoing EMT which has down-modulated the expression of EpCAM and/or of CKs and, thus, cannot be captured by the CellSearch platform [20,21]. Therefore, it is reasonable to hypothesize that the different detected CTC subpopulations, based on the combined expression of CKs, DLL3 and vimentin, may represent a continuum of phenotypic changes from an epithelial to a mesenchymal status during the metastatic process. However, the biological role of the different CTC subpopulations as well as their clinical relevance during the evolution of the disease should be evaluated in a subsequent prospective study.

The presented data clearly indicate that even one-chemotherapy cycle significantly reduces the detection rate and the absolute number of CTCs, as has already been reported for other CTC subpopulations [12,13,15]. Moreover, the effect of treatment on $DLL3^+/CD45^-$ CTCs was an early event, since it was observed after one treatment cycle. It is important to note that the effect of chemotherapy on the decrease of $DLL3^+$ CTCs after one-treatment cycle was correlated with increased PFS and OS. Multivariate analysis demonstrated that the detection of $DLL3^+/CD45^-$ CTCs subpopulation at baseline was emerged as an independent factor associated with poor PFS.

1The detection of $DLL3^+/CD45^-$ CTCs was performed, in the current study, using immunofluorescence staining of PBMCs isolated after Ficoll-Hypaque centrifugation of peripheral blood which is a very simple and cheap procedure available, practically, in all diagnostic laboratories; this procedure does not require specialized infra-structure and equipment in contrast to the CellSearch assay which uses an expensive equipment and consumables. In addition, the possibility to efficiently detect $CK^-/DLL3^+/Vim^+$ CTCs in patients without detectable CTCs by the CellSearch platform represents an additional advantage of the used immunofluorescence assay for the longitudinal study of different CTCs' subpopulations during the clinical evolution of patients with SCLC. Therefore, since DLL3 is not expressed in normal tissues during the adult life, it could be considered as an ideal biomarker to follow treatment efficacy based on the dynamic changes of $CK^+/DLL3^+/Vim^+$ CTCs; on the other hand, $CK^+/DLL3^+/Vim^+$ CT could be considered as a potent treatment target. This is further supported by the fact that both the detection rate and the absolute number of $DLL3^+/CD45^-$ was significantly higher in patients with extended disease compared to limited disease suggesting a clear correlation with the tumor load.

Studies in mouse xenograft models, with transplanted tumors from patients with SCLC and LCNEC NSCLC, showed that treatment with the antibody-drug conjugate (ADC) rovalpituzumab tesirine (Rova-T or SC16LD6.5; AbbVie) was effective in 58.3% of the cases both in terms of tumor progression and recurrence prevention; moreover, Rova-T efficacy was significantly correlated with DLL3 expression, as determined using an FFPE-compatible anti-DLL3 antibody [9]. Recently, Rudin et al reported the results of a first-in-human, first-in-class phase I study of Rova-T in recurrent SCLC which demonstrated that 91% of the patients with DLL3-overexpressing tumors achieved a clinical benefit; this study further supports that DLL3 expression on tumor cells could be revealed as a potential biomarker identifying patients who are more likely to achieve a response and better long-term outcome with Rova-T treatment [23]. Since important molecular changes could occur during the evolution of a malignant tumor [32–34] and re-biopsies are not always feasible for their monitoring, CTCs could be an alternative source of biologic material because they could be released by both the primary tumor and the metastatic lesions. Despite the fact that the evaluation of the effect of Rova-T on DLL3-positive CTCs was beyond the objectives of the present study, our findings strongly support the hypothesis that the detection of $DLL3^+$ on CTCs could represent a useful biomarker for SCLC patients' selection as well as for the early evaluation of anti-DLL3 therapy. However, prospective studies that will include staining of NOTCH1 both in CTCs and the primary tumor could be helpful to define the significance of DLL3 expression in tumor cells and CTCs. Moreover, further evaluation of DLL3 expression in cells from new metastases developed during the disease progression and its correlation with DLL3 expression in CTCs would provide some valuable information on the molecular evolution of tumor cells.

In conclusion, these observations further highlight the prognostic importance of $DLL3^+/CD45^-$ CTCs in patients with SCLC, confirm their phenotypic heterogeneity and strongly suggest that the enumeration and characterization of $CK^+/DLL3^+/Vim^+$ CTCs might be of clinical relevance; therefore, the current study may be considered as a proof of principle that the expression of DLL3 can be used as a reliable dynamic real-time biomarker in SCLC patients receiving front-line treatment.

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Appendix A. Supplementary data

Supplementary material related to this article can be found, in the online version, at doi:https://doi.org/10.1016/j.lungcan.2019.06.025.

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