Molecular Subtyping of Circulating Tumor Cells in Patients with Small Cell Lung Cancer

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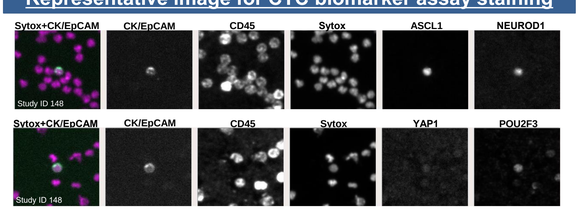
INTRODUCTION

- Small Cell Lung Cancer (SCLC) comprises approximately 10-15% of all lung cancers[1].
- Most patients are diagnosed with metastatic/extensive stage disease where median survival is just 1 year, and 5-year overall survival rate is < 2%[1].
- While SCLC is initially sensitive to conventional platinum doublet chemotherapy, most patients subsequently relapse[1].
- Unlike NSCLC, tumor mutational profiling and the rational implementation of targeted therapies into the care of the patients with SCLC has not yet proven effective in this disease.
- Barriers to the study of SCLC stem from limited amounts of tumor from biopsy samples and limited access to longitudinal tumor samples to understand tumor evolution.
- Recently, a new molecular classification of SCLC, defined by expression of four key transcription regulators – ASCL1, NEUROD1, YAP1 and POU2F3 – has been proposed[2].
- Of these four transcription factors, ASCL1 and NEUROD1are classified as neuroendocrine markers (NE) and YAP1 and POU2F3 as non-neuroendocrine markers (non-NE).
- These molecular subtypes may confer differential biology and therapeutic vulnerabilities, however, studies to date have been constrained by the paucity of available longitudinal tumor biopsy samples obtained from patients with SCLC[3].
- Circulating tumor cells (CTCs) were prospectively evaluated in the CONVERT trial (n=75 unique patients) in patients with limited stage SCLC patients, where CTC counts of 2,15 and 50 all significantly correlated with progression free survival (PFS) and overall survival(OS)[4].

OBJECTIVES

- To quantify CTC numbers in patients with SCLC at initial diagnosis and throughout their treatment course, in order to monitor therapeutic response/resistance and progression of disease.
- To determine subtype marker expression on CTCs at the time of diagnosis and to evaluate for dynamic changes in marker expression throughout treatment course expression during treatment in patients with SCLC.

Representative image for CTC biomarker assay staining



Representative image for CTC biomarker assay staining. Top Panel: Shows the NE CTC biomarker assay with CTCs identified as CK/EpCAM positive, CD45 negative and positive Sytox stain indicating nucleus and NE markers ASCL1 and NEUROD1. Bottom Panel: Shows the non-NE CTC biomarker assay with CTCs identified as in top panel and non-NE markers YAP1 and POU2F3. Magnification is 20X.

METHODS

- Informed consent was obtained from patients with SCLC using an IRB approved protocol (IRB#030763).
- We collected blood samples from 28 patients, including treatment naïve samples from the entire cohort as well as on-treatment and relapsed samples for processing using the RareCyte® platform (median of 4 collections per patient).
- The RareCyte® rare-cell analysis platform combines density-based collection of nucleated blood cells with automated staining, high-resolution digital microscopic imaging, and image analysis to quantify and isolate CTCs[5].
- CTCs were detected based on their expression of CK/EpCAM positive and CD45 negative staining[6,7].
- Each sample was also evaluated for expression of neuroendocrine markers (ASCL1, NEUROD1) and non-neuroendocrine markers (YAP1, POU2F3). Each set of neuroendocrine and non-neuroendocrine markers were co-stained along with the CTC markers.
- Antibodies were validated using SCLC cell lines known to be positive and negative for each of the markers and cell line spike-in in normal blood was used to mimic the clinical samples.
- Mean fluorescence intensities of each marker were extracted using a python program, and intensity cut-offs for positive expression was based on the cell line data.

Figure 1: CTC counts prior to the start of first line treatment

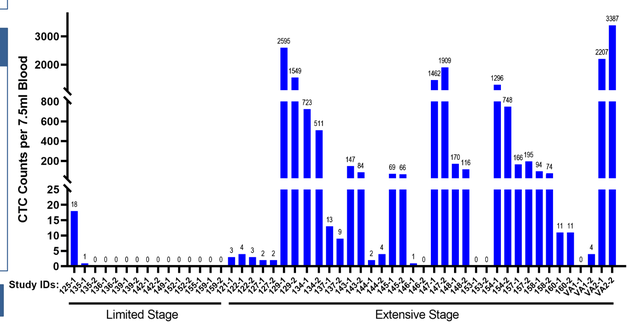


Figure 1: CTC counts prior to the start of first line treatment. Two blood collection tubes each containing 7.5ml were collected and processed to obtain CTC counts using the RareCyte® Liquid Biopsy platform. Each tube was processed to make 8 slides, and each slide was then stained using the Leica® automated stainer and scanned using the CyteFinder® system. CTC counts were confirmed based on CK/EpCAM positive stain and CD45 negative stain along with positive nuclear stain. Since two tubes of blood were obtained from each patient, we counted CTCs from both samples.

REFERENCES

[1]Rudin, CM et al., Nat Rev Dis Primers 7, 3 (2021)
[2]Rudin, CM et al., Nat Rev Cancer 19, 289–297 (2019)
[3]Schwendenwein, A et al., Mol Ther Oncolytics Feb 6;20:470-483 (2021)
[4]Tay, RY et al., Ann Oncol. Jul 1;30(7):1114-1120 (2019)
[5]Campton De et al., BMC Cancer 15:360 (2015)
[6]de Wit S et al., Scientific Reports volume 5, Article number: 12270 (2015)
[7]Togo S et al., Oncotarget May 23; 8(21): 34884–34895 (2017)

Workflow for quantification of CTCs from blood samples

Workflow for quantification of CTCs from blood samples. Each blood collection tube with 7.5ml of blood is transferred to the AccuCyte® blood separation tube and centrifuged at 3000g for 25 min. Plasma is separated and displacement fluid is added along with the buffy coat isolation tube and centrifuged at 1000g for 20 min. Transfer fluid is used to resuspend the isolated buffy coat and 8 slides are made for every blood collection tube. These slides are then stained using the CTC biomarker staining kits using a Leica® automated stainer. Stained slides are banked at -20˚C or scanned using the CyteFinder® system and CTC are quantified using the CyteFinder® software. (Image source: www.rarecyte.com and www.leicabiosystems.com)

Figure 2: Quantitation of biomarker expression on CTCs prior to first line treatment

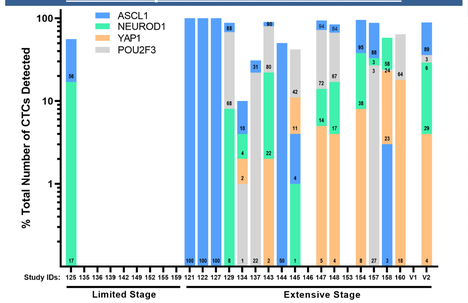


Figure 2 : Quantitation of biomarker expression on CTCs prior to first line treatment. Expression of each marker (ASCL1, NEUROD1, YAP1, POU2F3) was quantified as described in the methods. Marker expression is represented as percentage of CTC positive for each marker in total number of CTCs detected.

Table 1: Quantitation of CTC counts and corresponding biomarker expression

Study ID	CTC #s		Subtype Markers			
	Set-1	Set-2	ASCL1 %	NEUROD1 %	YAP1 %	POU2F3 %
121	3	na	100	0	na	na
122	4	3	100	0	0	0
125	18	na	56	17	na	na
127	2	2	100	0	0	0
129	2595	1549	88	8	0	68
134	723	511	10	4	2	1
135	1	0	0	0	0	0
136	0	0	0	0	0	0
137	13	9	31	0	0	22
139	0	0	0	0	0	0
142	0	0	0	0	0	0
143	147	84	58	4	2	6
144	2	4	50	0	0	0
145	69	66	4	1	11	42
146	1	0	0	0	0	0
147	1462	1909	94	14	5	72
148	170	116	84	17	4	67
149	0	na	0	0	na	na
152	0	0	0	0	0	0
153	0	0	0	0	0	0
154	1296	748	86	20	8	8
155	0	na	0	0	na	na
157	166	195	88	33	0	27
158	94	74	3	58	23	24
159	0	0	0	0	0	0
160	11	11	0	0	18	64
V1	0	4	0	0	0	0
V2	2207	3387	89	29	4	36

Table 1: Quantitation of CTC counts and corresponding biomarker expression for CTC cohort prior to first line treatment. For each patients, CTC count and biomarker expression were determined as detailed in the methods section. Biomarker expression is represented as percentage of CTCs positive with a given biomarker in total number of CTC detected. Key: 'na' indicates sample was not available.

Figure 3: Longitudinal monitoring of CTCs

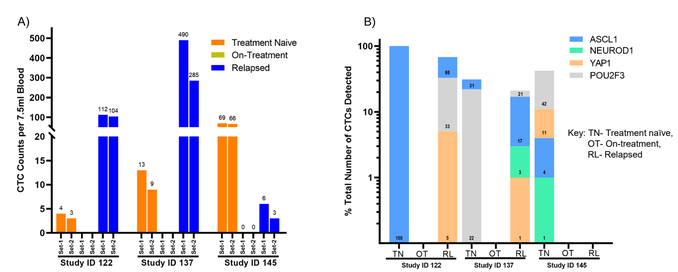


Figure 3: Longitudinal monitoring of CTCs. A)For the indicated study IDs, CTC counts have been plotted at treatment naïve, on-treatment and relapsed timepoints in each patient's disease course. On-treatment refers to blood samples obtained prior to the start of the third cycle of first line platinum/etoposide/atezolizumab therapy. B) Dynamic changes in biomarker expression with disease progression. Biomarker expression as percentage CTCs positive in total CTCs detected is plotted for each time point for the indicated patients. For study IDs 122 and 137 there was no On-treatment sample available.

SUMMARY

- We developed an assay to evaluate the expression of NE (ASCL1, NEUROD1) and non-NE (YAP1, POU2F3) markers on CTCs.
- We collected blood samples from 28 SCLC patients and obtained data on CTC counts at different treatment naïve, on-treatment and at relapsed timepoints.
- CTCs were detected in 20/28 (71%) patients. Of these, ASCL1 was positive in 16/20 (80%), NEUROD1 was positive in 11/20 (55%), YAP1 was positive in 9/18 (50%) and POU2F3 was positive in 12/18 (67%) of samples.
- Of the three relapse samples analyzed to date, 2/3 had increase in CTC counts and decrease in ASCL1 expression from treatment naïve timepoint to relapsed timepoint. The third sample had decrease in CTC count and lost the expression of all biomarkers in the few CTC detected at the relapsed timepoint.

FUTURE DIRECTIONS

- We will correlate the expression of biomarkers on CTCs with the tumor biopsy tissues from the same cohort.
- We will correlate the biomarker expression on CTCs and biopsy tissue with treatment outcomes and evolution of disease for the entire cohort.
- We have n=28 SCLC patients with longitudinal blood collections that have been processed and slides banked (sample size=136, median=4) that are in the process of being analyzed.

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