The expression of fetal oncogene 5T4 in CTCs obtained from NSCLC patients is discordant with the expression measured in the tumor.

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Introduction

The fetal oncogene 5T4 is a cell surface protein, with over-expression observed in a variety of cancers as compared to normal adult tissue. Recent studies have shown that expression of 5T4 appears to be associated with the undifferentiated state and the epithelial-mesenchymal transition (EMT), and may be associated with a more invasive phenotype (1). We have developed assays to measure the expression of the fetal oncogene, 5T4, in formalin fixed paraffin embedded (FFPE) tumors and in the circulating tumor cells (CTCs)(2). These assays were then used to investigate 5T4 expression in a small cohort of samples from patients with NSCLC. We obtained matched primary tumor and blood samples, with the blood being obtained prior to resection of the primary tumor. The expression of 5T4 was found to be robust and measurable in both the FFPE tumors and CTCs. However, we observed no concordance between the degree of 5T4 expression in the tumor and the CTCs. These assays will both be used to measure 5T4 expression in patients receiving anti-5T4 therapy (3) in upcoming clinical trials in order to assess their predictive utility.
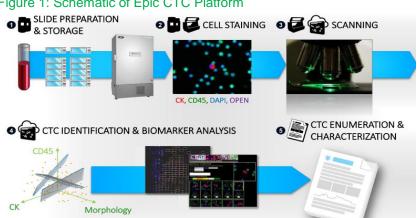
Methods

Patient Samples: FFPE NSCLC tissue was obtained prospectively (ConversantBio, Huntsville, AL), from 25 adencarcinoma and 10 squamous cell carcinoma therapy naïve patients at initial diagnosis. All sample collection was appropriately consented. A matched blood sample was obtained from each patient immediately prior to biopsy. Each sample was accompanied by a pathology report giving details of indication, stage and grade.

Tissue IHC: Tissue sections (4μm) were obtained from each block. Paraffin was removed and samples were re-hydrated using standard xylene/ethanol immersion. The slides were subjected to antigen retrieval and blocked using industry standard procedures. Slides were stained using an monoclonal antibody against 5T4. Primary antibody was detected and slides were counterstained using the Leica Bond refine detection kit. Stained sections were assessed and scored by an anatomic pathologist to generate H-scores.

CTCs (Figure 1): (1) Nucleated cells from blood sample were placed onto slides and stored at -80C. (2) Slides were stained with Cytokeratin (CK), CD45, DAPI and 5T4 (utilizing a polyclonal anti-5T4 antibody). (3) Slides were scanned in 4 channels (4) Pathology algorithm identified CTC candidates confirmed by human reader. (4) 5T4 characterized on each CTC
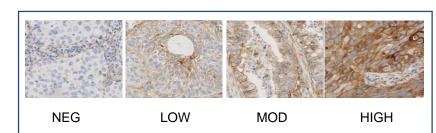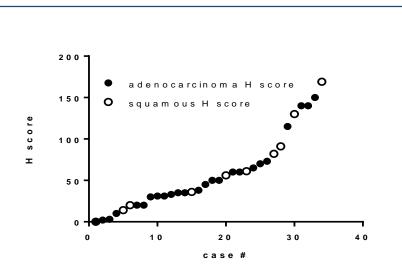
Figure 1: Schematic of Epic CTC Platform

Results
IHC staining of NSCLC samples

Figure 2: examples of negative, low moderate and high staining
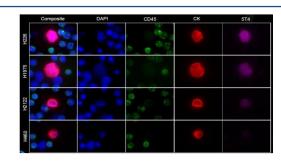

NEG   LOW   MOD   HIGH

NSCLC Xenograft models orthogonally characterized for 5T4 expression by qRT-PCR and western blot analysis. Note increased membrane staining of target as expression levels increase.
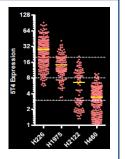
Figure 3: H-scores of NSCLC tissue stained for 5T4

H score values for 5T4 in NSCLC samples ranged from 0-169. Only 2 samples had no staining. The average H score across the sample set was 56.1±7.7. There was no significant difference in 5T4 expression between adeno-carcinoma and squamous cell carcinoma sub-types.
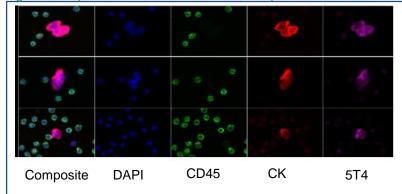
Figure 4: Cell line 5T4 expression

Cell line controls were utilized for 5T4 CTC assay development and to establish the 5T4 expression threshold for CTCs in patient samples. Cells are stained with antibodies against CK (red), CD45 (green), 5T4 (magenta) and counterstained with DAPI (blue).

Figure 5: 5T4 expression on CTCs in NSCLC patients

Dot Plot of CTCs enumerated and characterized from representative patient samples. Each dot represents a CTC and the position on the y-axis corresponds to level of 5T4 expression. Green dots denote clusters of CTCs, Blue dots denote single CTCs.

Figure 6: Representative CTCs from NSCLC patients

Composite   DAPI   CD45   CK   5T4

Three representative 5T4+ CTC/CTC Clusters found in patient samples. Cells are stained with antibodies against CK (red), CD45 (green), 5T4 (magenta) and counterstained with DAPI (blue).

Table 1: Metrics for 5T4 expressing CTCs in blood from NSCLC patients

	CTC/mL	CTC H-Score:	Cluster-Weighted H-Score:	5T4 Burden/mL	Cluster Weighted 5T4 Burden/mL
Adeno-carcinoma	35±9	42±6	53±7	100±29	287±104
squamous cell carcinoma	80±19	33±5	46±6	182±32	407±77

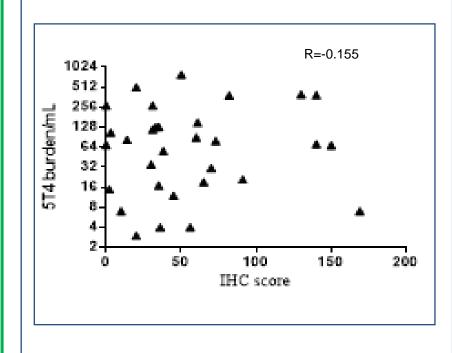**CTC/mL:** All the CTCs in a sample, regardless of 5T4 expression levels.
CTC H-score: Is calculated using the following equation; (% cell expressing no 5T4)+ (% cells expressing 1+ 5T4)+ % cells expressing 2+ 5T4) + (% cells expressing 3+ 5T4).
5T4 Burden: Is the sum of 5T4 signal intensities from all positive cells found in each patient.
Cluster Weighted Scores: Account for the number of CTCs in a cluster rather than treating the cluster as a single data point.

There is no correlation between degree of 5T4 expression in tumor sample and CTCs

Figure 7: Lack of correlation between IHC score in the tumor and 5T4 burden/mL in the CTCs

R=-0.155

Conclusions

- We have developed robust assays to measure 5T4 expression in the tumor and in the CTCs of NSCLC patients
- There is robust expression of 5T4 in NSCLC patients.
  - In the tumor as measured by IHC
  - In the CTCs as measured by IF
- There are samples which are positive for 5T4 in both tumor and CTCs
  - There is no correlation in the degree of 5T4 expression between the tumor and CTCs
- Both of these assays will be used to correlate 5T4 expression and response to anti-5T4 ADC in upcoming clinical trials

References

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2. Marrinuci et al. Arch Pathol Lab Med 2009 133 1468-71
3. Sapra et al Mol Cancer Ther 2013 12 38-47