Immunoprecipitation of tumor associated ctDNA fragments for novel lung cancer liquid biopsy

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Introduction

The main issue all tumor associated ctDNA assays have is a high background of normal cfDNA of near identical sequences. This issue is greatest in early stage I/II cancer patients that tend to have plasma that is >99.9% background normal cfDNA.

Currently, the basis of ctDNA analysis consists of extracting and sequencing all plasma cfDNA including background. In silico comprehensive bioinformatic analysis of sequence data is used to combat the low detection levels of ctDNA.

A better solution could be to identify tumor associated ctDNA fragment sequences after physically isolation from normal cfDNA fragments with the same sequence based on nucleoprotein structure using wet chemistry.

We have developed a new physical ctDNA isolation method which separates specific circulating nucleoprotein - DNA structures from the typical cfDNA background with same sequence. Our method, based on TF ChIP-Seq with CCCTC-binding factor protein (CTCF) as target, allows physical ctDNA fragment isolation from total cfDNA background in circulation in plasma. (figure 1)

The method combines 2 elements:

1. Identification of nucleoprotein structures in which the combination of protein and nucleic acid sequence occurs exclusively in the circulation of cancer patients
2. Physical isolation and detection of those chromatin fragments from plasma

Using a novel biomarker discovery method, we identified a magnitude of gain of occupancy sequences characterized by CTCF binding site sequences that were present in the CTCF plasma isolates of cancer subjects but absent from those subjects without cancer. We considered these sequences to be tumor specific.

We believed that any DNA fragment containing a cancer associated CTCF gain of occupancy compared to control must be tumor associated.

Declaration of Interest

D Pamart is an employee of Volition.

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Proof-of-concept: Isolation of CTCF-cfDNA nucleoproteins from plasma of AML patients

Our first POC was performed on acute myeloid leukemia (AML) patients as they were considered to have high ctDNA in circulation. We isolated CTCF-cfDNA complexes from plasma with magnetic beads coated with anti-CTFC antibodies. Immunoprecipitated cfDNA was then extracted, amplified and sequenced on an Illumina platform. Frequency analysis plots proved the presence of a short DNA peak (35-100bp) in samples with high nucleosome/cfDNA levels (figure 2a). Normalized coverage of CTCF binding site loci showed a high level of small 35-100bp cfDNA fragments but an absence of nucleosome sized fragments for all samples including healthy samples (figure 2b). V-plots demonstrate that the CTCF binding sequences were located centrally within CTCF bound cfDNA fragments (figure 2c).

We identified 29 CTCF-binding site sequences present in the ChIP isolate from cancer patients that were absent from other patients. Specific qPCR assays developed to these sequences showed good performance for AML cancer detection with 1-qPCR assay: **Sensitivity 61%, Specificity 98%** and with 2-qPCR assays: **Sensitivity 74%, Specificity 96%.**

Application to Lung Cancer

We reproduced our method using samples from 10 lung cancer patients. The CTCF-cfDNA was isolated from plasma by immunoprecipitation and after Next Generation Sequencing, the normalized coverage showed the presence of CTCF DNA binding site on DNA short fragment. This presence is specific to the CTCF-DNA complex immunoprecipitation and is not present with unspecific antibody immunoprecipitation (figure 3a). Using a 3-qPCR assay based off an AML model we detected lung cancer (all stages): **Sensitivity: 40%, Specificity: 96%**

We found 113 candidate CTCF-DNA binding sites selectively present in plasma samples of lung cancer patients (figure 3b). These sites have a False Discovery Rate < 0,05 with a p-value <0,05. We will develop PCR assays for selected candidates.

Conclusions

- We have isolated cancer associated cfDNA from normal cfDNA of the same sequence by wet chemistry for detection by simple PCR. This strategy provides access to an entirely new class of hitherto unknown potential plasma biomarkers accessible to analysis without NGS.
- We have demonstrated a novel ctDNA analysis technology that may provide the basis of rapid, low-cost detection of early-stage cancer.
- This could be an automated liquid biopsy method, as the first step is a basic immunoprecipitation assay, and the second step uses qPCR.
- This method was worked up using haemopoietic cancer samples; the biomarkers tested also gave positive results for lung cancer. The full potential of this method may allow tumor cell of origin analysis in the future.
- We are currently investigating using lung cancer models rather than an AML model with the objective to further improve the sensitivity and specificity profiles.

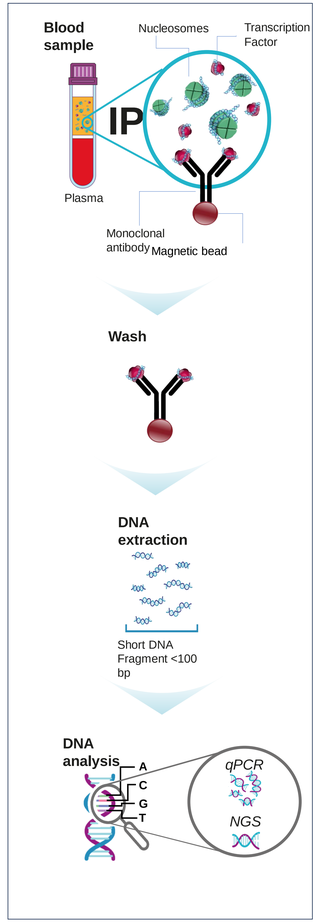

Figure 1: Schematic process to isolate and analyze the complex Transcription Factor – TF DNA binding site. We immunoprecipitated TF protein linked to a corresponding DNA binding site using a magnetic monoclonal antibody. We then isolated and purified the TF-associated DNA for analysis by next generation sequencing to identify genomic binding site locations specifically occupied in lung cancer. qPCR methods will be developed to detect specifically this disease.

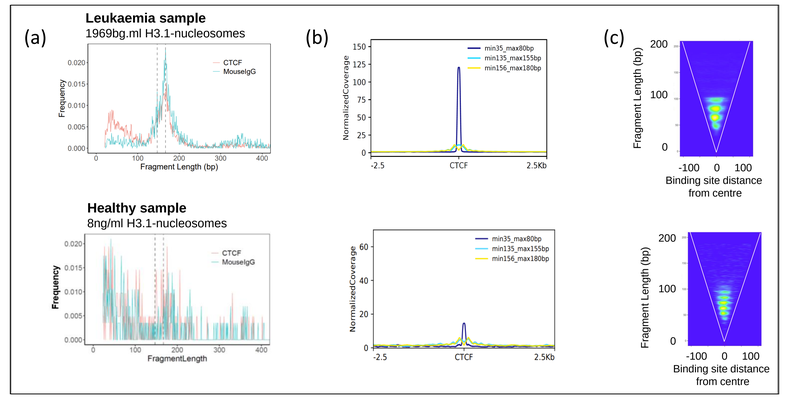

Figure 2: Sequencing results obtained for CTCF associated cfDNA fragments isolated by ChIP from plasma samples collected from a leukemia patient with a high circulating H3.1-nucleosome level and a healthy subject with a low circulating H3.1-nucleosome level: (a) cfDNA fragment size profiles; (b) Normalized coverage of annotated CTCF binding site loci sequences by 35-80bp cfDNA fragments and nucleosome size fragments; (c) V-plots showing CTCF associated cfDNA fragment sizes in the 30-100bp range with centrally located CTCF binding site sequences.

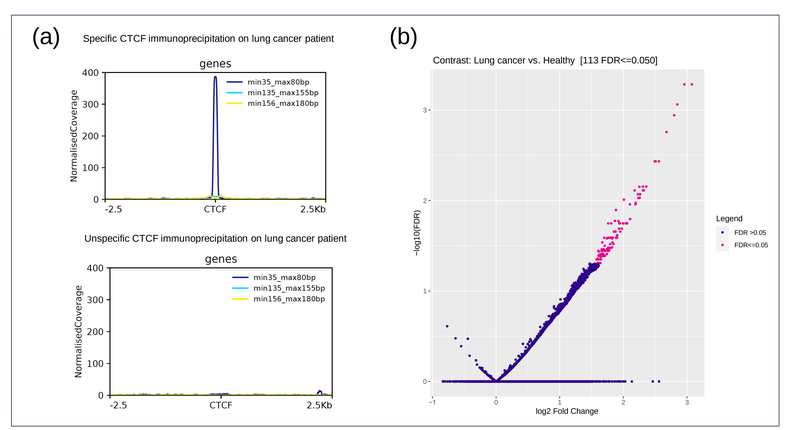

Figure 3: Sequencing results obtained for CTCF associated cfDNA fragments isolated by ChIP from plasma samples collected from a lung cancer patient : (a) Normalized coverage of annotated CTCF binding site loci sequences by 35-80bp cfDNA fragments and nucleosome size fragments after specific and unspecific CTCF immunoprecipitation; (b) Vulcano plot showing CTCF-DNA binding site associated with lung cancer presence compared to healthy patients.

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