Novel ctDNA technology for early cancer detection by immunoprecipitation of tumor associated ctDNA fragments and analysis by qPCR

Abstract Presentation Number: 2428

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Introduction

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

Current ctDNA methods involve extraction of all plasma cfDNA and next generation sequencing (NGS). This results in the sequencing of all non-specific background plasma cfDNA which results in severe assay interference. ctDNA detection therefore requires comprehensive in silico bioinformatic analysis of cfDNA sequence data.

A better resolution would be to pinpoint tumor associated ctDNA fragment sequences once physically isolated from normal cfDNA fragments with the same sequence based on nucleoprotein structure using wet chemistry.

We have cultivated a novel ctDNA analysis method in which classes of tumor derived ctDNA are isolated, with removal of all non-tumor cfDNA of the same sequences, for analysis by qPCR. (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 plethora of CTCF genomic binding loci sequences that are particularly occupied by CTCF isolates of cancer subjects and show as CTCF-ctDNA nucleoprotein complexes in cancer patients' plasma. However, they fail to arise in those characterized as healthy subjects and subjects with inflammatory conditions.

We rationalized that any DNA fragment encompassing a cancer associated CTCF gain of occupancy binding site sequence in a CTCF-ctDNA fragment isolated from plasma by Chromatin Immunoprecipitation (ChIP), compared to control must be tumor associated.

Declaration of Interest

All authors are employees of Volition.
J Micallef, M Herzog and T Bygott are shareholders of Volition.

This research was funded by the Walloon Region (convention #8515)

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

Our first POC was executed on acute myeloid leukemia (AML) patients due to their elevated ctDNA levels in circulation. We isolated CTCF-cfDNA complexes from plasma with magnetic beads coated with anti-CTFC antibodies. Immunoprecipitated cfDNA was extracted, amplified and sequenced on an Illumina platform. Frequency analysis plots demonstrated the presence of a short DNA peak (35-100bp) in samples with elevated nucleosome/cfDNA levels (figure 2a). Normalized coverage of CTCF binding site loci showed an elevated level of small 35-100bp cfDNA fragments but an absence of nucleosome sized fragments for all samples including healthy samples (figure 2b). The CTCF binding sequences were centrally positioned within CTCF bound cfDNA fragments as depicted in the V-plots. (figure 2c). These fragments were also noticeably evident even in healthy subjects with minimal H3.1-nucleosome levels (figure 2c).

Using 15 samples from subjects with AML, healthy and inflammatory conditions. We discovered 29 CTCF-binding site sequences present in the ChIP isolate from cancer subjects that were absent from those subjects without cancer. qPCR assays developed for 10/29 CTCF binding site loci demonstrated to be effective for AML cancer detection with 1-qPCR assay: **Sensitivity 61%, Specificity 98%** and with 2-qPCR assays: **Sensitivity 74%, Specificity 96%.**

Application to Pan-Cancer

We reproduced our method using samples from solid cancer subjects. The 10 qPCR assays were evaluated on 134 subjects including 50 control subjects comprised of healthy and inflammatory conditions. All 10 qPCR assays were effective in detecting various cancers (figure 3). Some required more PCRs than others for detection. As the qPCR target sequences were selected based on an AML model, we saw high detection levels as expected for AML. On the other hand, we believe the detection levels for the various solid cancers to be encouraging for this preliminary experiment.

Conclusions

- We have combined the isolation of plasma cfCTCF-DNA nucleoproteins with PCR analysis of cancer associated CTCF gain of occupancy binding site sequences which is a novel ctDNA analysis technology that may provide the basis of a useful, rapid, low-cost, detection method of cancer.
- This strategy provides access to an entirely new class of hitherto unknown potential plasma biomarkers accessible to analysis without NGS.
- This could be an automated liquid biopsy method, as the first step is a basic immunoprecipitation assay, and the hematopoietic second step uses qPCR.
- This method was worked up using cancer samples; the biomarkers tested also gave positive results for solid cancers. The full potential of this method may allow tumor cell of origin analysis in the future.
- We are currently investigating using disease selective biomarker discovery (e.g., discovery using breast or liver subjects for biomarkers to detect breast or liver cancer) rather than an AML model with the objective to further improve the sensitivity and specificity profiles.

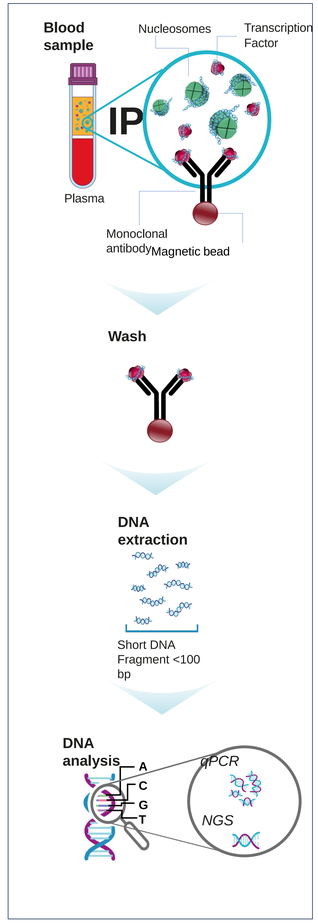

Figure 1: Schematic process to isolate and analyze Transcription Factor – TF DNA binding site complexes. We immunoprecipitated TF protein linked to a corresponding DNA binding site using a magnetic monoclonal antibody. We then isolated and purified the TF-associated DNA for analysis by next generation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

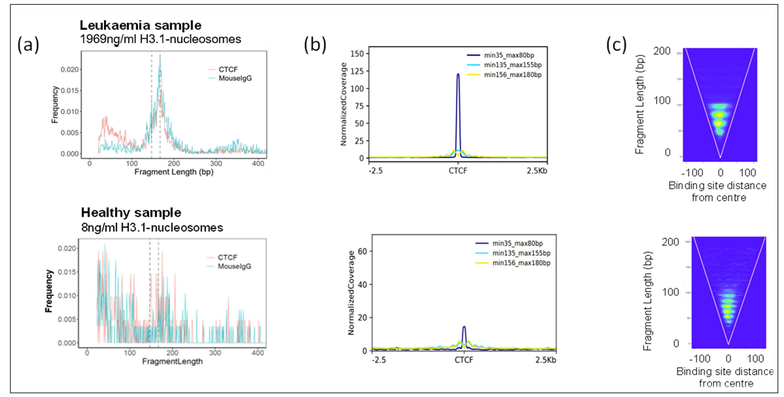

Figure 2: Sequencing results obtained for CTCF associated cfDNA fragments isolated by ChIP from plasma samples collected from a leukemia patient with a high circulating H3.1-nucleosome level and a healthy subject with a low circulating H3.1-nucleosome level: (a) cfDNA fragment size profiles; (b) Normalized 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.

Cancer	Subjects	Positive	Sensitivity	Specificity	PCRs
AML	31	19	61%	98%	1
CRC	13	10	77%	92%	2
Breast	10	4	40%	92%	1
Prostate	10	7	70%	90%	2
Liver	10	7	70%	92%	1
Lung	10	4	40%	96%	3

Figure 3: qPCR results obtained for CTCF gain of occupancy sites discovered using an AML model. CRC – Colorectal Cancer. Using a simple cutoff where a qPCR result exceeding the cutoff was positive, a single qPCR assay detected 19/31 AML cases (61%) with 1 false positive result among 50 control samples (98% specificity). Addition of a second qPCR assay to make a 2-member qPCR panel, where a result exceeding cutoff in either or both assays was classified as positive resulted in the detection of CRC and prostate. For lung cancer a 3-member qPCR panel was used with the same cutoff methods as described above.

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