Physical isolation of tumour associated ctDNA fragments for novel AML liquid biopsy

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Introduction

The core issue all tumour 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 foundation of ctDNA assays comprises of extracting and sequencing all plasma cfDNA including background. The key to tackle the low detection levels of ctDNA, is through in silico robust bioinformatic analysis of sequence data.

We believe that a better result would be to identify tumour associated ctDNA fragment sequences after physical isolation from normal cfDNA fragments with the same sequence based on nucleoprotein structure using wet chemistry.

We have created novel method for ctDNA analysis in which circulating cell free CTCF-DNA nucleoproteins (cfCTCF-DNA) of tumour origin are chemically isolated from non-tumour nucleoproteins comprising cfDNA of the same sequences by chromatin immunoprecipitation (ChIP) (figure 2).

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 multitude of cfCTCF-DNA cancer associated gain of occupancy binding site sequences that were present in the CTCF plasma isolates of cancer patients, but not present in isolates from healthy subjects or subjects with inflammatory conditions (where the sequences occur as nucleosomes and had been removed).

We figured that any DNA fragment involving a cancer associated CTCF gain of occupancy binding site sequence in a CTCF-ctDNA fragment isolated from plasma by Chromatin Immunoprecipitation (ChIP), must be tumour associated.

Conflict of Interest

All authors are employees of Volition.

J Micallef and M Herzog are shareholders of Volition.

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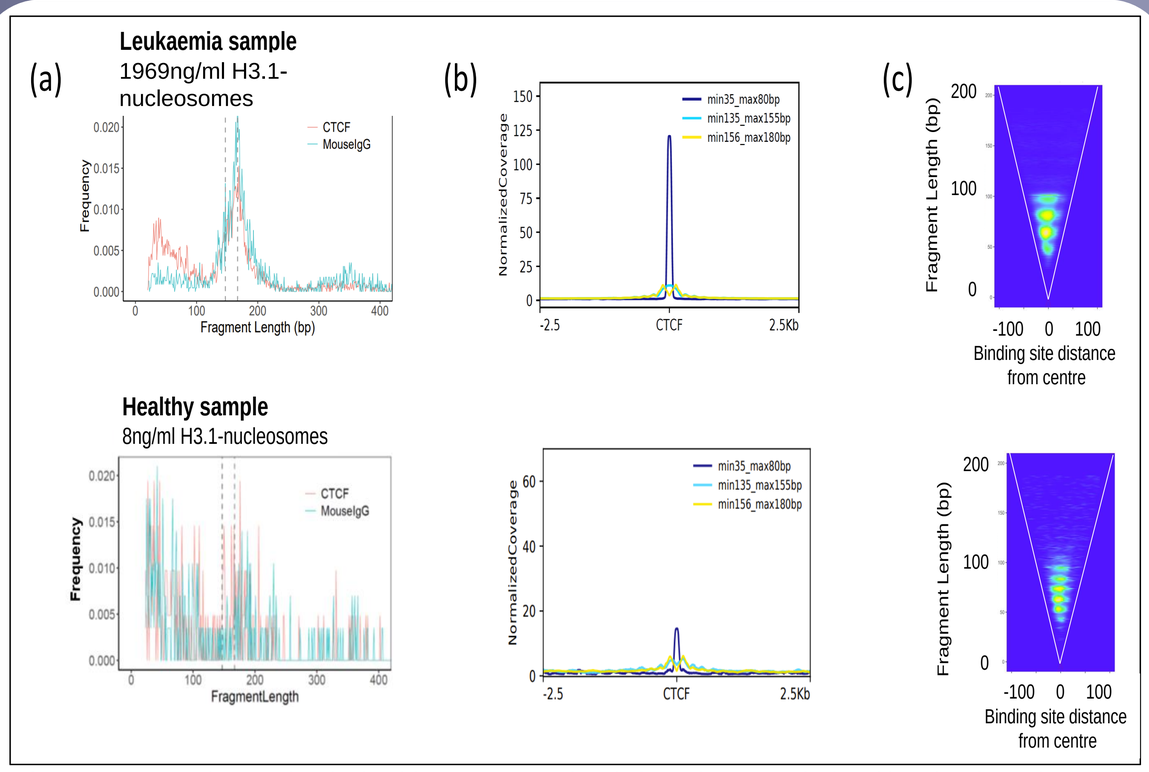

Figure 1: Sequencing results obtained for CTCF associated cfDNA fragments isolated by ChIP from plasma samples collected from a leukaemia patient with a high circulating H3.1-nucleosome level and a healthy subject with a low circulating H3.1-nucleosome level: (a) cfDNA fragment size profiles; (b) Normalized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

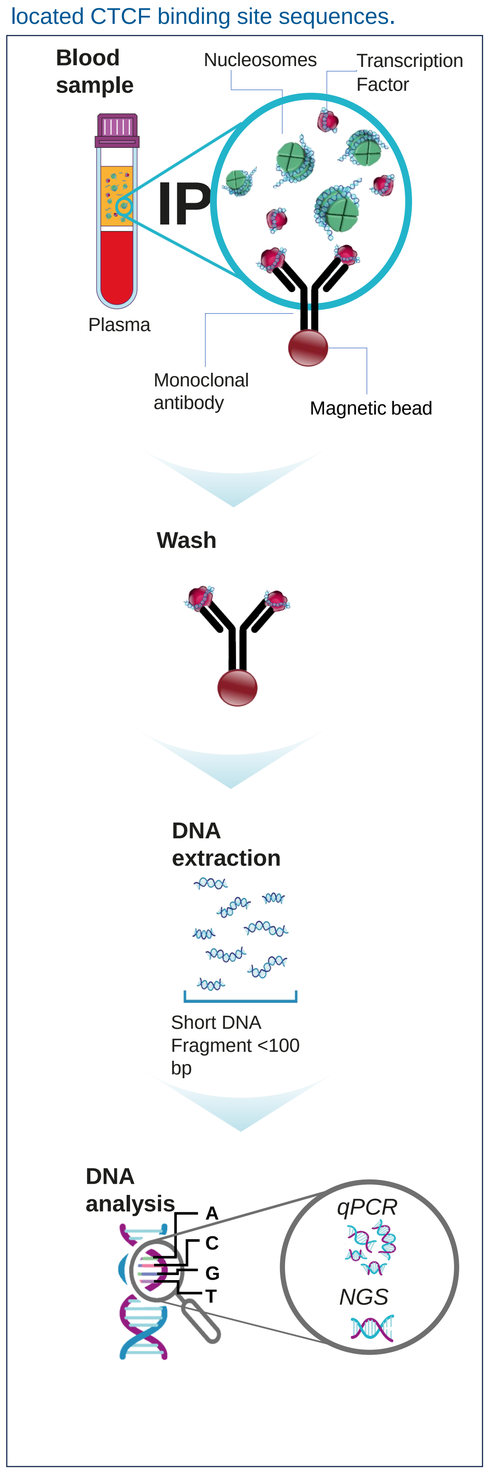

Figure 2: Schematic process to isolate and analyze the complex Transcription Factor – TF DNA binding site. We immunoprecipitated TF protein linked to a corresponding DNA binding site using a magnetic monoclonal antibody. We then isolated and purified the TF-associated DNA for analysis by next generation sequencing to identify genomic binding site locations specifically occupied in only in AML samples. qPCR methods will be developed to detect specifically this disease.

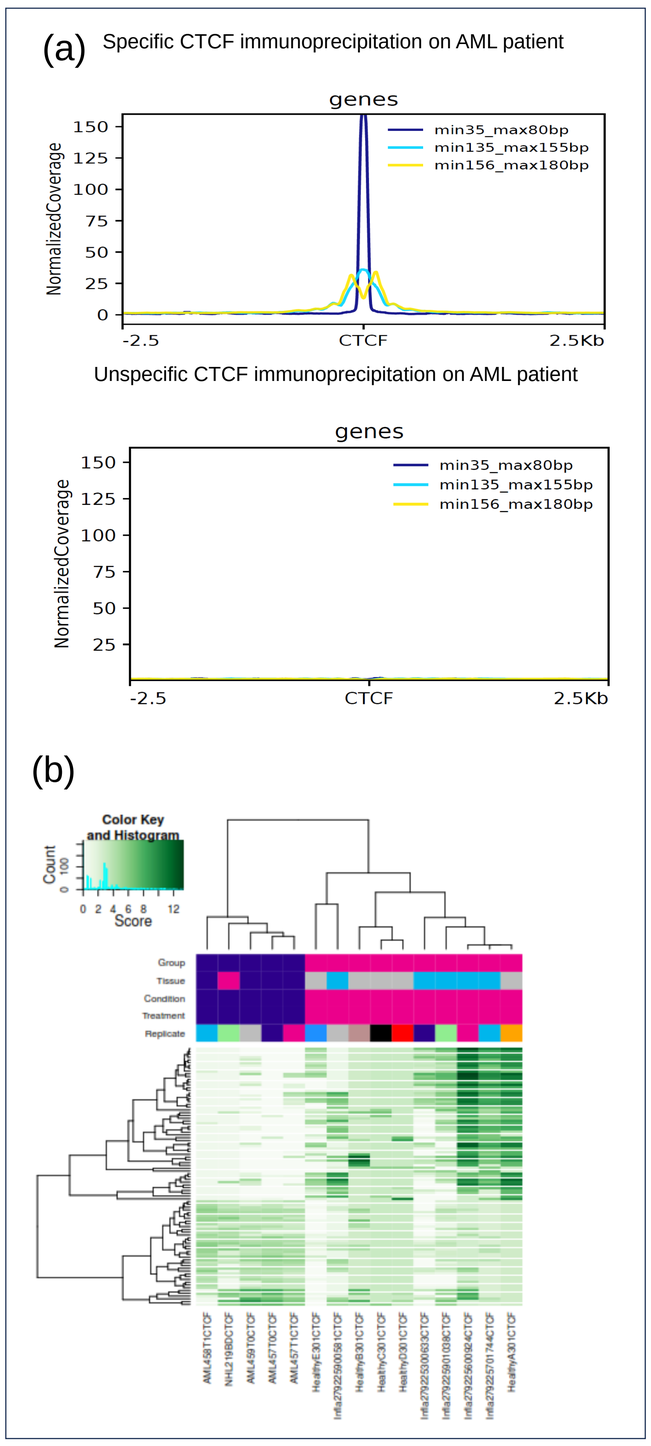

Figure 3: Sequencing results obtained for CTCF associated cfDNA fragments isolated by ChIP from plasma samples collected from an AML patient : (a) Normalized coverage of annotated CTCF binding site loci sequences by 35-80bp cfDNA fragments and nucleosome size fragments after specific and unspecific CTCF immunoprecipitation; (b) Heat map showing CTCF-DNA binding site associated or not with AML presence compared to healthy patients. This combination allows to discriminate AML and healthy in 2 populations.

CTCF Isolation

Our initial proof-of-concept was completed on acute myeloid leukaemia (AML) patients as they were considered to have elevated ctDNA levels in circulation. We isolated CTCF-cfDNA nucleoproteins from plasma with magnetic beads coated with anti-CTFC antibodies. After which immunoprecipitated cfDNA was extracted, amplified and sequenced on an Illumina platform. The presence of a short DNA peak (35-100bp) in samples with elevated nucleosome/cfDNA levels was depicted by frequency analysis plots (figure 1a). 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 1b). V-plots exhibit that the CTCF binding sequences were centrally located within CTCF bound cfDNA fragments (figure 1c).

Biomarker Discovery

Anti-CTCF ChIP-Seq was performed on 4 patients diagnosed with AML, 5 patients with inflammatory conditions and 5 healthy volunteers. We identified 29 CTCF-binding site sequences (figure 3b) present in the ChIP isolate from cancer patients that were absent from the non-cancer patients.

We then developed qPCR assays for 10/29 CTCF binding site gain of occupancy sequences selectively occupied in cancer.

qPCR Assays

The 10 qPCR assays were investigated as liquid biopsy assays for detection of AML in a preliminary proof-of-concept study. ChIP isolates from plasma samples obtained from AML patients (n=31) and from control subjects that were either healthy (n=35) or had an inflammatory condition (n=15) were tested for the presence of the 10 CTCF binding site sequences selectively occupied in cancer.

Preliminary Results

The 10 qPCR assays for cfCTCF-DNA gain of occupancy biomarkers were effective for detection of AML. Using a simple qPCR cutoff, a single qPCR assay detected 61% of AML cases at **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 74% of AML cases at **96% specificity.**

Conclusions

- cfCTCF-DNA occupancy biomarkers represent a new class of untapped cancer biomarkers for cancer detection.
- ChIP/PCR of plasma nucleoproteins is rapid, low cost, suitable for automation and may provide a useful novel liquid biopsy method.
- We have shown a clinical proof-of-principle in haematopoietic cancer patients.
- The full potential of this method may allow tumour cell of origin analysis in the future.
- Further clinical studies to ascertain the clinical accuracy of the method are required.

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