A novel immunoprecipitation/PCR method for detection of plasma cfDNA fragments selectively occupied by CTCF in cancer

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Introduction

The central problem faced by all tumor associated ctDNA assays is a high background of normal cfDNA of near identical sequence. The problem is greatest in early stage I or II cancer patients whose plasma may comprise >99.99% background normal cfDNA.

All current ctDNA assays involve extraction and sequencing of all plasma cfDNA including background. The solution to the detection of low levels of ctDNA is provided in silico through the use of sophisticated bioinformatic analysis of sequence data.

We hypothesized that a better solution would be to identify tumor associated ctDNA fragment sequences that can be physically isolated from normal cfDNA fragments with the same sequence based on nucleoprotein structure using wet chemistry.

We have developed a novel ctDNA method which avoids the normal cfDNA problem by physically isolating ctDNA fragments with complete removal of background cfDNA fragments of the same sequence.

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 number of CTCF genomic binding loci sequences that are selectively occupied by CTCF in cancer and appear as CTCF-ctDNA nucleoprotein complexes in the plasma of cancer patients, but which occur as nucleosomes in the plasma of healthy subjects and subjects with inflammatory conditions

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

Declaration of Interest

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

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Isolation of CTCF-cfDNA nucleoproteins from plasma

Isolation by ChIP of transcription factor bound DNA from plasma has not previously been reported. We classified a cfDNA fragment as CTCF associated if (i) it was present in an anti-CTCF ChIP isolate, (ii) its sequence aligned to a characterized CTCF binding site locus and (iii) it was <100bp in length.

We isolated CTCF-cfDNA from plasma using magnetic beads coated with anti-CTFC antibodies. The presence of CTCF protein in the isolate was confirmed by Western Blot. Immunoprecipitated cfDNA was extracted, amplified and sequenced on an Illumina platform. CTCF-cfDNA levels in healthy subjects were too low to visualize in a frequency plot, but a 35-100bp peak was visible for plasma samples with elevated circulating chromatin as measured using the Nu.Q®H3.1 assay (Figure 1a). Normalized coverage of annotated CTCF binding site loci was high for small 35-100bp cfDNA fragments but absent for nucleosome sized fragments and this coverage was detectable for all samples including healthy samples (Figure 1b). The CTCF binding sequence was located centrally within CTCF bound cfDNA fragments as shown by V-plots, and these fragments were also clearly detectable even in healthy samples with low H3.1-nucleosome levels (Figure 1c). We reproducibly observed multiple discreet CTCF associated cfDNA sizes (Figure 1c). These may be degradation products or represent multiple CTCF binding site classes.

Biomarker Discovery for CTCF cfDNA gain of occupancy binding site sequences

We performed anti-CTCF ChIP-Seq on plasma samples obtained from 5 healthy subjects, 5 subjects with inflammatory conditions and 5 patients diagnosed with a hematopoietic cancer. We discovered 29 CTCF-binding site sequences present in the ChIP isolate from cancer patients that were absent from patients with inflammatory conditions or from healthy subjects.

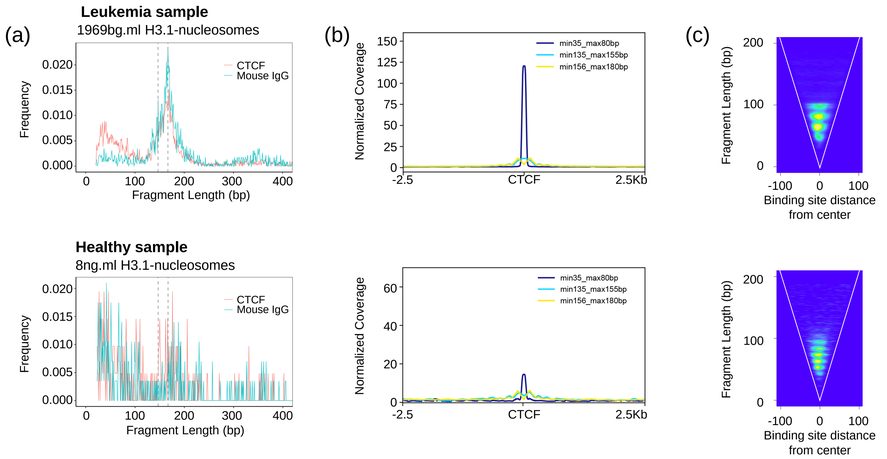

Figure 1: Sequencing results obtained for CTCF associated cfDNA fragments isolated by ChIP from plasma samples collected from a leukemia patient with a high circulating H3.1-nucleosome level and a healthy subject with a low circulating H3.1-nucleosome level: (a) cfDNA fragment size profiles; (b) Normalised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

qPCR assays for CTCF cfDNA binding site gain of occupancy biomarkers

qPCR assays were developed for 10 of the 29 CTCF gain of occupancy binding site sequence biomarkers identified by biomarker discovery. Simple cut-offs were used to determine whether the result of a qPCR assay was positive or negative.

All the qPCR assays for the 10 CTCF binding site loci tested were effective for the detection of hematopoietic cancers. The assays also detected solid cancers tested. However, the various qPCR assays had widely varying sensitivity for different solid cancers. This result indicates that identification of tumor type may also be possible, and we are investigating this by further biomarker discovery focused on particular solid cancer diseases.

Preliminary patient sample results for qPCR ctDNA liquid biopsy assay

The 10 qPCR assays were tested on samples collected from 74 subjects with a cancer including leukemia (n=31), breast (n=10), prostate (n=10), liver (n=10) or colorectal cancer (n=13) and 50 control subjects including 15 subjects with an inflammatory condition. All 10 qPCR assays were effective and individually showed selectivity for different cancer types. The results of this preliminary experiment were encouraging for the detection rate of liquid and solid cancers, including stage I cancers. A single qPCR assay detected 61% of leukemias at 98% specificity. Using different 2-qPCR assays with simple cutoffs we observed:

Detection of leukemia with a 2-qPCR assay: Sensitivity 74%, Specificity 96%

As expected, high detection rates were observed for leukemia. However, the qPCR biomarkers also detected solid cancers. We expect improved accuracy for all solid cancer types in future by targeted discovery using appropriate disease samples.

Detection of common solid cancers (all stages): Sensitivity 58%, Specificity 90%
Detection of common solid cancers (stage I): Sensitivity 44%, Specificity 90%

As an example, we used two qPCR assays that performed well for CRC to show the potential for increasing detection of particular tumor types, even with biomarkers discovered for leukemia. We will also investigate new biomarkers for further improvement.

Detection of CRC (all stages) with a 2-qPCR assay: Sensitivity 77%, specificity 92%

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 entire new class of hitherto unknown potential plasma biomarkers accessible to analysis without NGS.
- We have demonstrated a potential clinical effect in a pilot patient sample experiment. Investigation in larger clinical cohorts is required.
- Although discovered using haemopoietic cancer samples, the biomarkers tested also gave positive results for solid cancers – although at a lower sensitivity.
- Sensitivity and specificity for solid cancers may be improved by disease selective biomarker discovery (e.g., discovery using CRC samples for biomarkers to detect CRC). We are currently investigating this.
- Tumor cell of origin analysis may be possible in the future.
- The multiple discreet cfDNA fragment sizes observed in Figure 1c may relate to degradation products or may represent variable co-factor binding in nucleoproteins.
- CTCF-ChIP/qPCR shows promise for the accurate, rapid, low-cost detection of early-stage cancer. The method may also be suitable for automation.

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