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

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1 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.

2 Aim

We have established a new ctDNA technology based on circulating nucleoprotein structure and DNA sequence, in which cfDNA library preparation, NGS and bioinformatics are obviated to facilitate a low cost, rapid, automatable, high throughput ctDNA technology. Our method, based on TF ChIP-Seq with CCCTC-binding factor protein (CTCF) as candidate, allows a physical ctDNA fragments isolation from total cfDNA by removal of background cfDNA fragments 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

We then identified gain of occupancy sequences characterized by CTCF binding site sequences that were presents in the CTCF isolates of cancer subjects but absent from those subjects without cancer. **We considered these sequences as tumor specific.**

3 Method

Our first proof-of-concept was based on leukemia patients. We isolated CTCF-cfDNA from plasma using magnetic beads coated with anti-CTFC antibodies. Immunoprecipitated cfDNA was extracted, amplified and sequenced on an Illumina platform. Frequency size analysis showed the presence of a short DNA peak (35-100bp) for samples with high cfDNA (or circulating nucleosome level (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). Moreover, as shown by V-plots, the CTCF binding sequences were located centrally within CTCF bound cfDNA fragments (Figure 1c).

This method allowed us to identify 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 (2-qPCR assay: Sensitivity 74%, Specificity 96%).

Due to these results, we decided to reproduce our method on another type of cancer : 4 liver cancers and 4 intra hepatic bile duct cancers were selected. The CTCF-cfDNA was isolated from plasma by immunoprecipitation. After high throughput sequencing, the normalized coverage showed, as previously, the presence of CTCF DNA binding site on DNA short fragment. This presence is specific to the CTCF immunoprecipitation and is not present with unspecific antibody immunoprecipitation (figure 3a).

The combined analysis of DNA binding sites present in liver cancers compared to healthy patients are represented in the Volcano plot. We found 843 CTCF-DNA binding sites linked to the presence of liver or bile cancer (figure 3b). These sites have a False Discovery Rate < 0,05 with a p-value <0,05.

4 Results

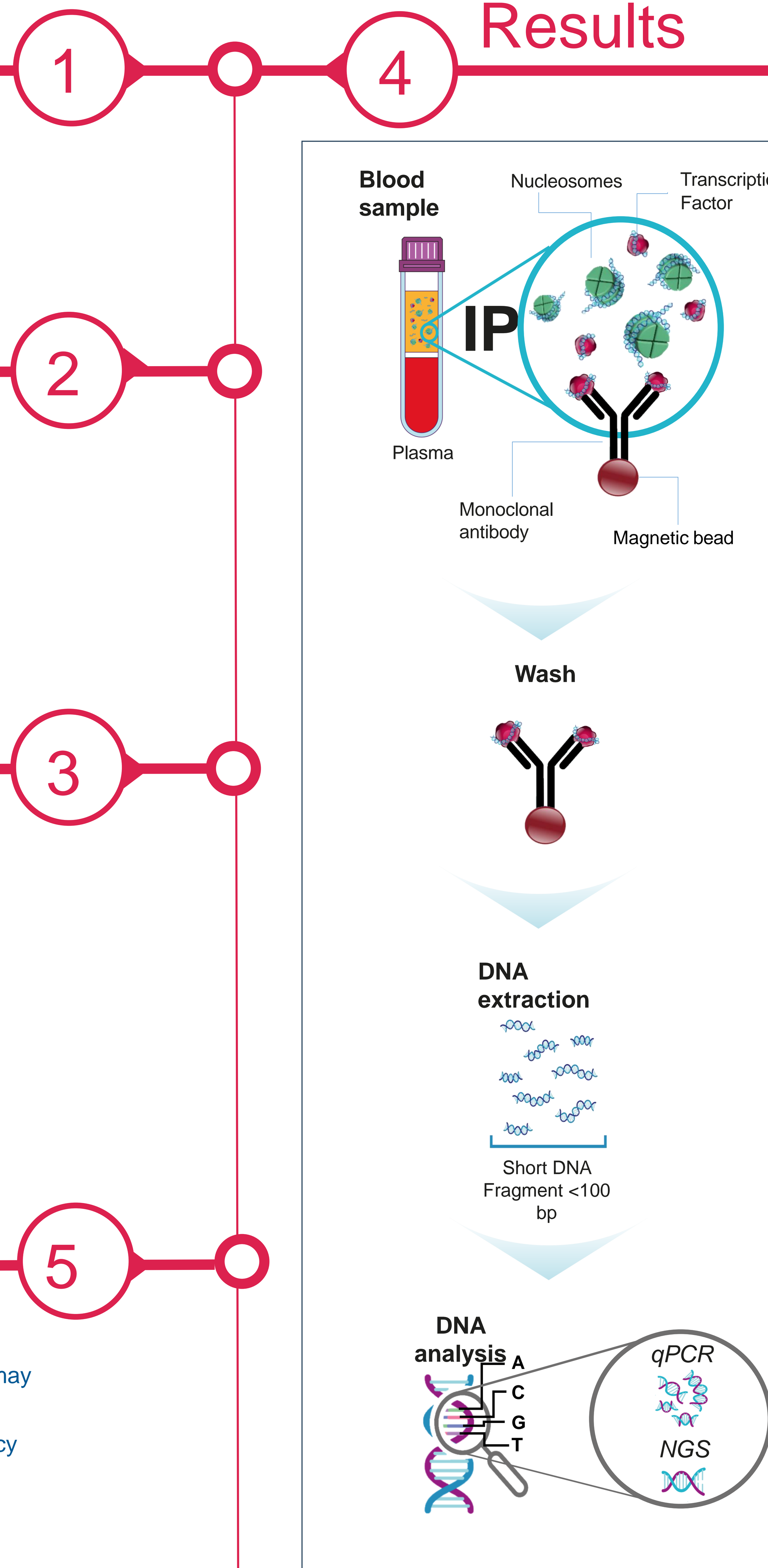

Figure 1: Schematic process to isolate and analyze the complex Transcription Factor – TF DNA binding site. We immunoprecipitated TF protein linked to a corresponding DNA binding site using a magnetic monoclonal antibody. We then isolated and purified the TF-associated DNA for analysis by next generation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

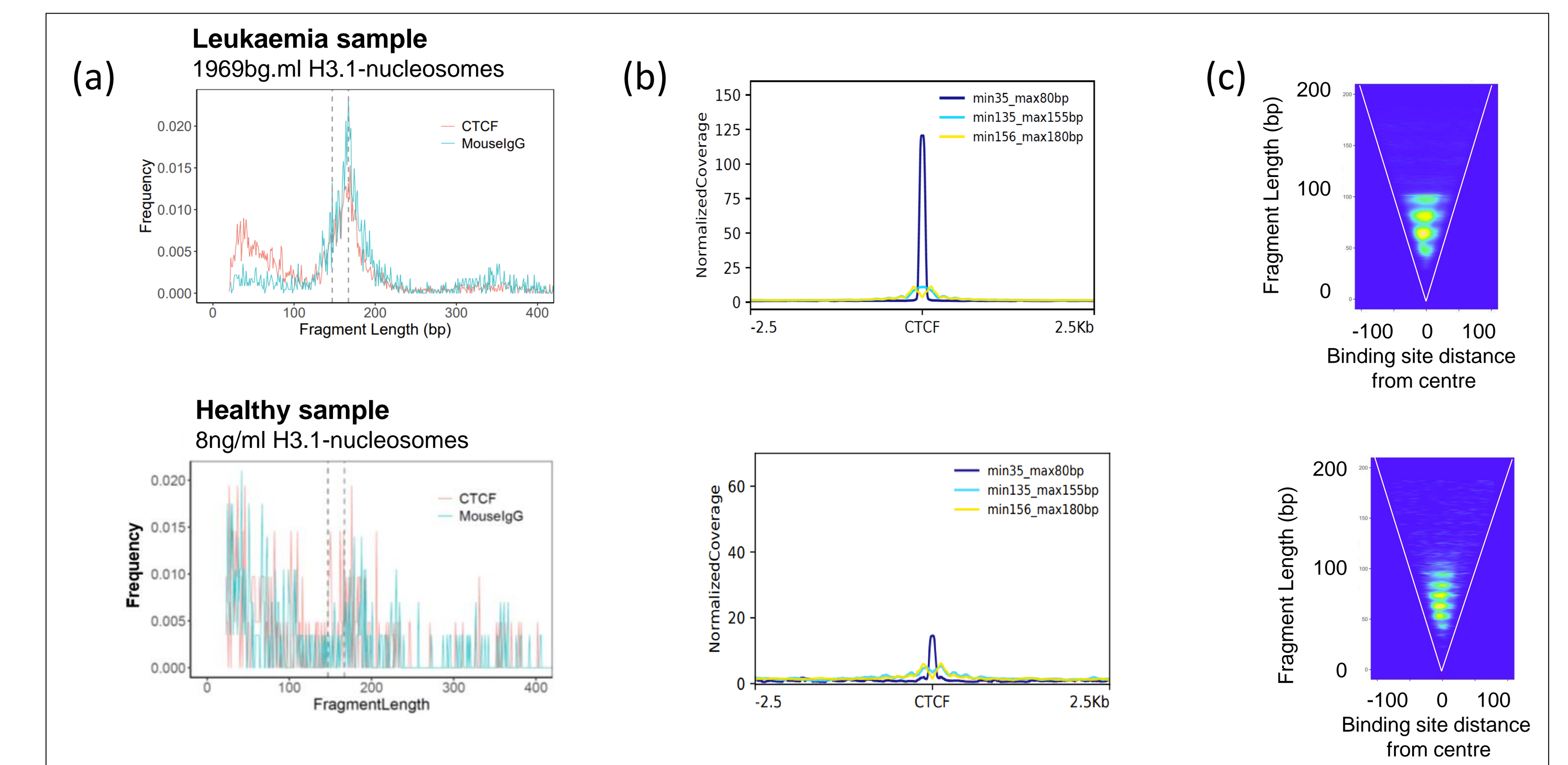

Figure 2: Sequencing results obtained for CTCF associated cfDNA fragments isolated by ChIP from plasma samples collected from a leukemia patient with a high circulating H3.1-nucleosome level and a healthy subject with a low circulating H3.1-nucleosome level: (a) cfDNA fragment size profiles; (b) Normalized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

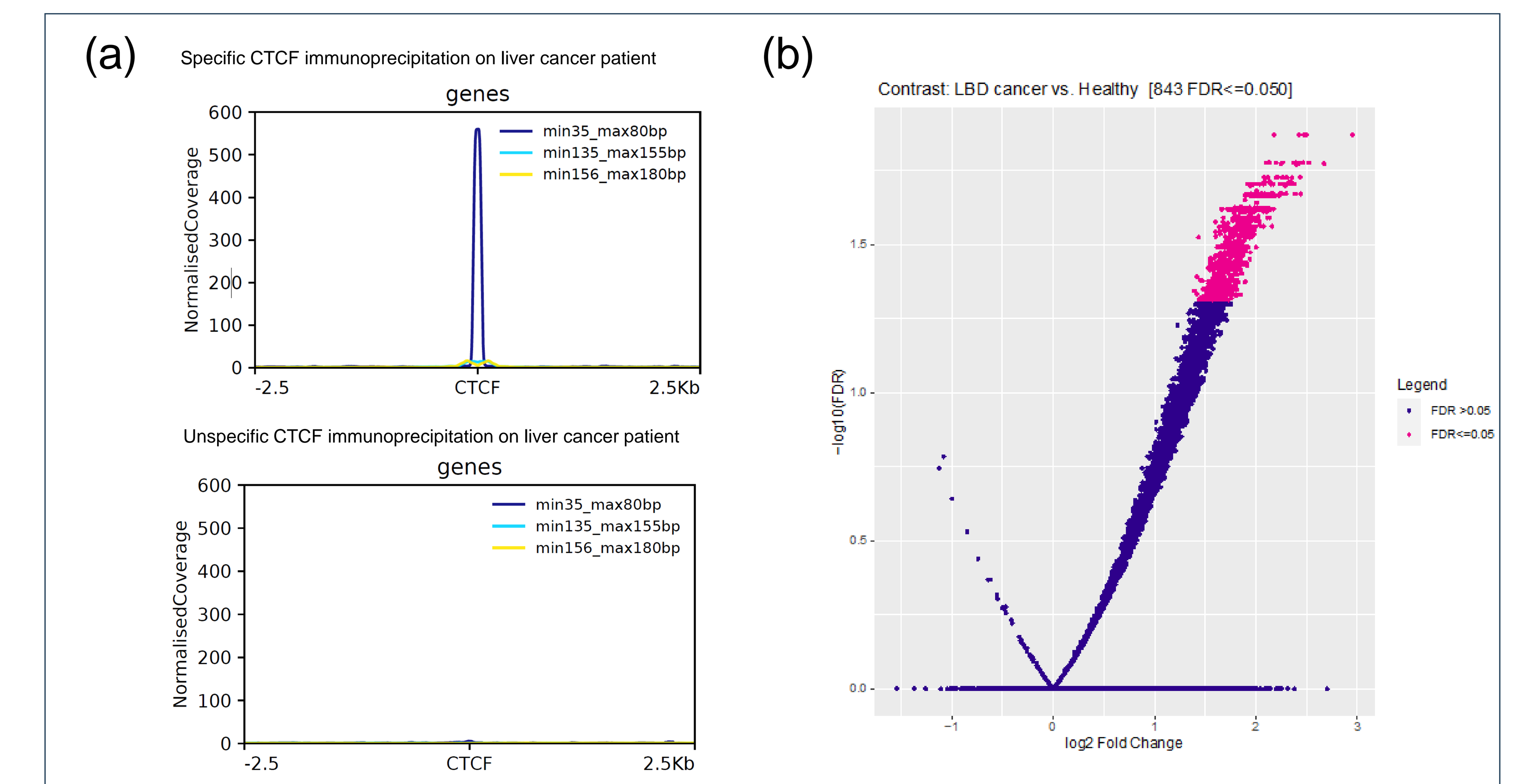

Figure 3: Sequencing results obtained for CTCF associated cfDNA fragments isolated by ChIP from plasma samples collected from a liver cancer patient : (a) Normalized coverage of annotated CTCF binding site loci sequences by 35-80bp cfDNA fragments and nucleosome size fragments after specific and unspecific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

5 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 clinical proof-of-principle in haemopoietic cancer patients and confirmed the principle for a new indication: liver cancer. The full potential of this method may allow tumor cell of origin analysis in the future.

CTCF-ChIP/qPCR shows promise for the accurate detection of leukemia by PCR, and we will next develop PCR liquid biopsy assays for liver and bile cancer to confirm the efficacy of CTCF-ChIP/qPCR in liver cancer.

These results offer renewed hope for rapid, low-cost detection of early-stage liver cancer.

6 Acknowledgements

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7 Contact information

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