Enabling Sequence-Specific Stabilisation of Circulating Tumour DNA Using Xeno Nucleic Acids

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Background

Circulating tumour DNA (ctDNA) is a type of cell-free DNA (cfDNA) that is released by tumours into the bloodstream. ctDNA is a diverse biomarker used for minimally invasive diagnosis, profiling and treatment monitoring[1]. However, the major challenges in leveraging ctDNA for this purpose are the low abundance of cfDNA in the bloodstream and the low proportion of ctDNA within the cfDNA pool[2]. Recent methods, such as those introduced by Martin-Alonso et al. have shown promise in enhancing the overall quantity of cfDNA by reducing its clearance[3], but there remains a significant knowledge gap in strategies aimed at increasing the low proportion of ctDNA within the cfDNA pool.

To address this gap in knowledge, we propose to develop a method to stabilise ctDNA in a sequence-specific manner using Xeno Nucleic Acids (XNAs), which are synthetic nucleic acid analogs capable of binding to DNA. By forming stable complexes with ctDNA, XNAs would protect it from degradation by circulating nucleases, thereby enhancing stability and increasing detectability in blood samples.

Aims

1. Determine the fraction of tumours detectable by a fixed XNA panel
2. Establish and validate an experimental *in vitro* system of cfDNA degradation
3. Investigate the ability of XNAs to stabilize target sequences

Materials and Methods

Aim 1

Mutational data was acquired using the TCGAmutations R data package by PoisonAlien. The dataset was split into a discovery set containing 70% of patients in the TCGA cohort and a test set containing the remaining 30% of patients. The most common mutations from the discovery set were cross-validated using Monte-Carlo cross validation. The final panel was then applied to the test set to determine the number of patients with mutations that will be detected by the XNA panel. All data analysis was done in R.

Aims 2 & 3

Cell culturing and supernatant processing

HepG2 cells were seeded at a cell count that corresponds to ~50% confluency. After 24h, the media was removed, and the cells were washed with PBS. Fresh complete media was added to the cells along with the XNAs, which were then allowed to grow for 4h, 8h, 24h. At those time points, the media was collected and centrifuged twice at 2500 rpm for 10 minutes. For samples that underwent cell-free incubation, the collected cell free media was further incubated in the water bath at 37 °C for 24h and 48h. Samples were stored at -80 °C until extraction.

cfDNA extraction and quantification

cfDNA extraction was performed using QIAmp Circulating Nucleic Acid Kit. Size separation was performed using ProNex Size-Selective Purification System. Quantification of DNA was performed using Qubit fluorometer 4.0. Quantification and size distribution of cfDNA was determined using Agilent 2100 Bioanalyzer using Agilent DNA 1000 Kit. For aim 3 experiments, ddPCR was performed using Bio-Rad's QX200 ddPCR system. Droplets for the ddPCR were generated by Bio-Rad Automated Droplet Generator, PCR amplification was done using Bio-Rad C1000 Touch Thermal Cycler and the droplets were read by Bio-Rad QX200 Droplet Reader.

Results

Fraction of tumours detectable by a fixed XNA panel

Mutational data from The Cancer Genome Atlas (TCGA) was used to estimate the coverage of fixed XNA panels[5]. After cross-validation, the performance of the pan-cancer panel was rather suboptimal (~50% coverage while targeting 100 mutations), which indicates that pan-cancer early detection just using XNAs may not be a viable approach. However, for certain cancer types such as Uveal Melanoma (UVM), Low Grade Glioma (LGG) and Colon Adenocarcinoma (COAD), coverage can be >80% making fixed XNA panels a viable option (Fig. 1, top). It is worth noting that the TCGA data is obtained from 100X whole exome sequencing of tumors, instead of whole genome sequencing, which may omit some mutational hotspots that are not in the exome. However, it has been shown that most mutational hotspots are in the exome[5], hence we thought that this was still a very good estimate of XNA coverage. There is quite a wide spread of mutations across all cancer types and shared mutations are rather scarce, which contributes to the low coverage of the XNA panels (Fig. 1, bottom).

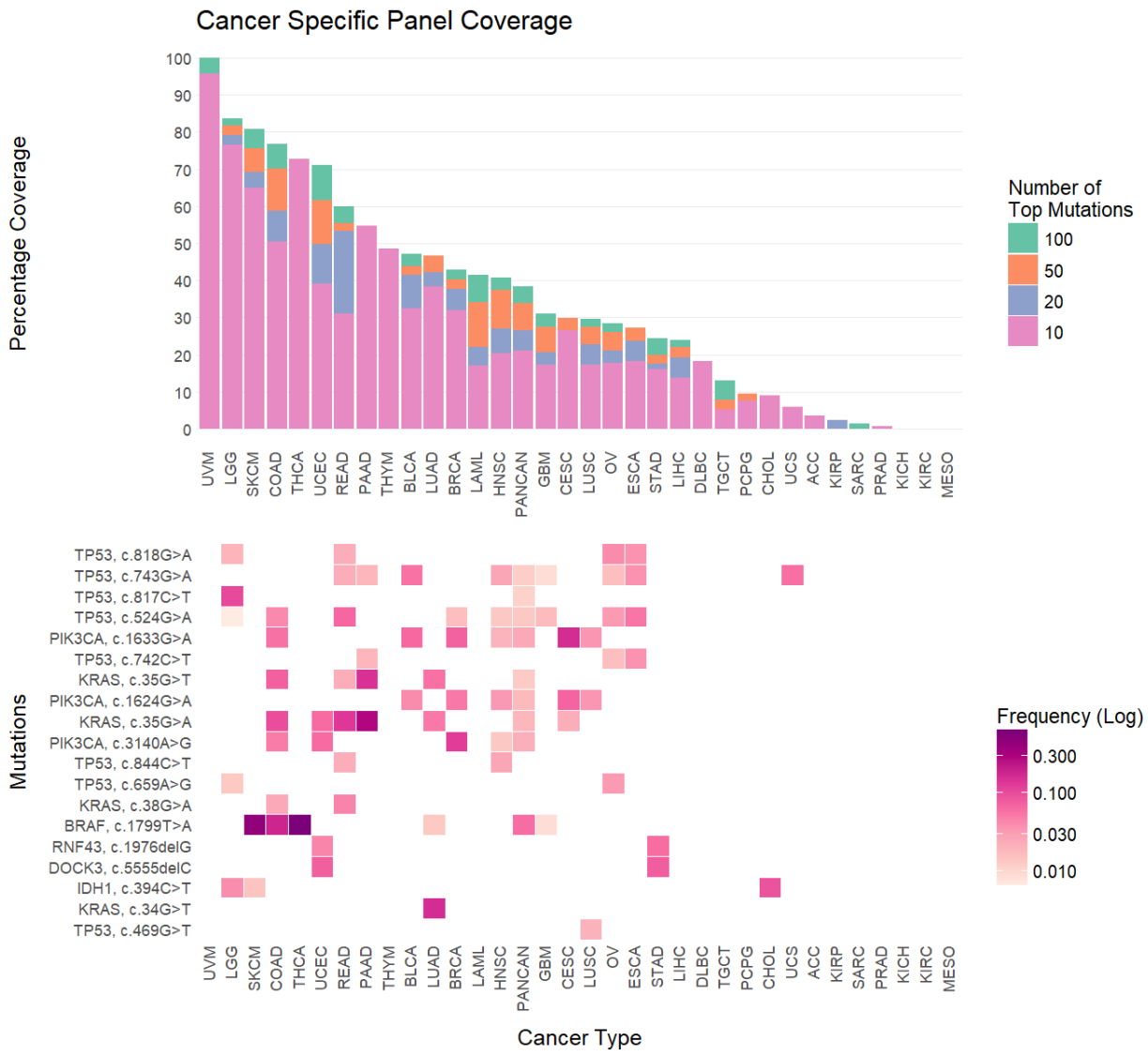

Fig. 1. Top: Estimated performance of a fixed XNA panel on patients in the TCGA cohort. Bottom: Heatmap of the most common nucleotide mutations that contribute to the top mutations.

In vitro cell free DNA model

Prior to investigating the ability of XNAs to stabilize target sequences, it was necessary to characterize the *in vitro* system of cfDNA degradation on the cell lines used. cfDNA is usually found as ~160 bp fragments *in vivo*, which corresponds to the length of DNA wrapped around one histone[1]. We found that the supernatant from cell culture media containing 10% Fetal Calf Serum (FCS) produced a low fraction of cfDNA relative to genomic DNA. Increasing the FCS concentration (and hence increasing the nuclease concentration in the media) not only increased the fraction of cfDNA, but also increased the concentration of cfDNA in the media (Fig. 2A).

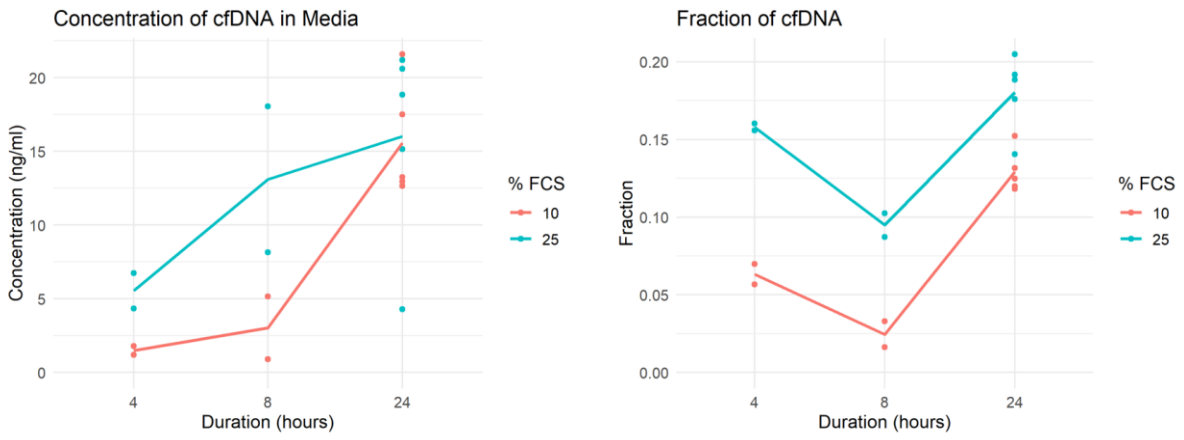

Fig. 2A. Concentration and Fraction of cfDNA after 4h, 8h and 24h with 10% and 25% FCS.

However, the fraction of cfDNA was still quite low, especially when compared to the high ratio of cfDNA to genomic DNA observed *in vivo*. Hence, two approaches were taken to increase the recovery of cfDNA: size-selection purification and cell-free incubation. Size-selection purification using the ProNex Size-Selection Purification Kit managed to effectively separate the cfDNA from the genomic DNA, but the yield of the cfDNA was significantly lower due to losses during the purification procedure (Fig. 2B). On the other hand, cell-free incubation yielded better recovery of cfDNA compared to size selection similar concentrations to cfDNA in untreated media, all while maintaining a high fraction of cfDNA (Fig. 2C).

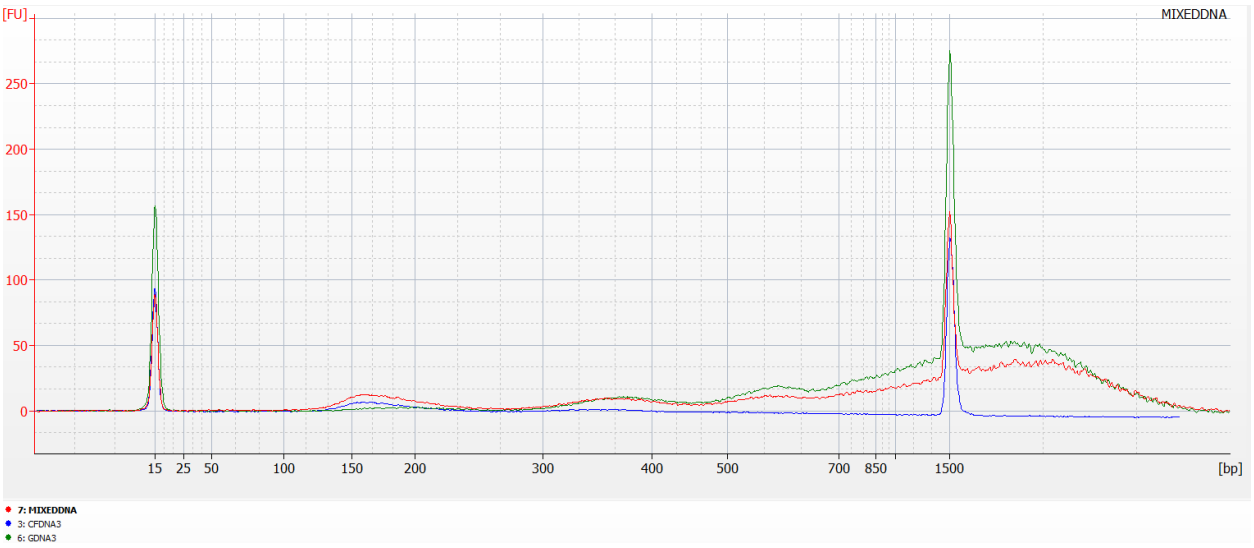

Fig. 2B. Bioanalyzer traces of size separated cfDNA using ProNex Size-Selection Purification Kit. Red trace represents control DNA. Green trace represents genomic DNA obtained from size selection. Blue trace represents cfDNA obtained from size selection.

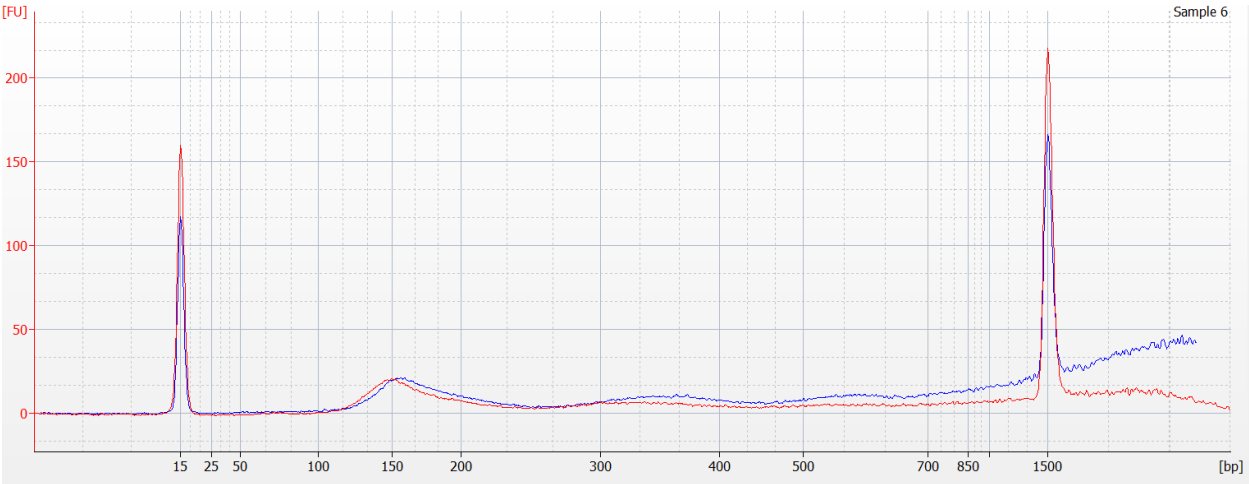

Fig. 2C. Bioanalyzer traces of cfDNA after cell-free incubation. Blue trace represents control DNA extracted from untreated media. Red trace represents DNA extracted from media incubated without cells for 24h.

XNAs interfere with ddPCR assay

The initial plan was to use ddPCR to detect the ctDNA enriched by the XNAs after an elution step, in which the XNAs will be removed using high pH. Unfortunately, our XNA only controls gave positive results in the ddPCR (Fig. 3). This complicated the analysis of the data obtained from the initial batch of samples as we were unable to separate the false positives generated by the XNAs and the true positives from the ctDNA.

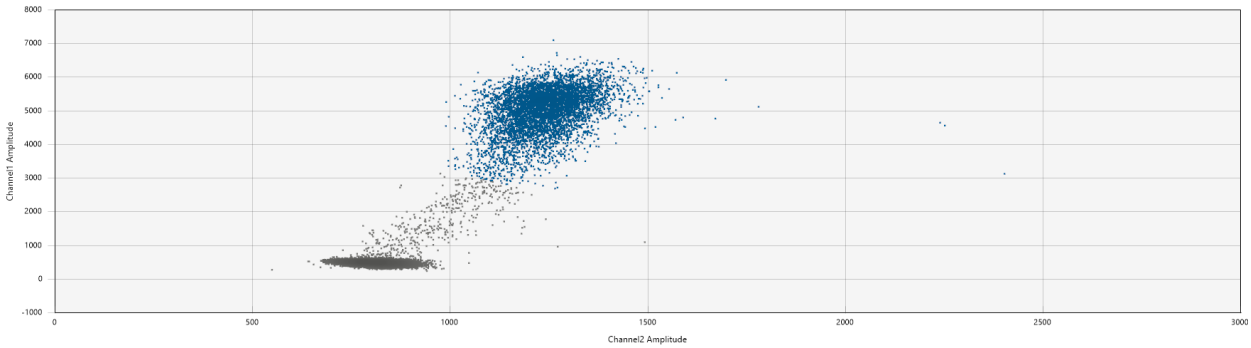

Fig. 3. ddPCR result of XNA only control.

Discussion and Future Directions

Due to the relatively poor performance of the pan-cancer panel, early detection using XNA panels may only be feasible for certain cancer types that have many common mutations. However, XNA panels will still be very useful for other applications such as monitoring minimal residue disease (MRD) due to its targeted nature.

The *in vitro* cfDNA model was successfully established, but it required a few modifications to make it more representative of in vivo cfDNA systems. Increased FCS concentration, size separation using ProNex Chemistry and cell free incubation all improved cfDNA recovery from the *in vitro* cfDNA model. Cell free incubation provided the least decrease in cfDNA yield and was the most physiologically relevant method of recovering cfDNA, hence this will be the approach we will be using moving forward.

The XNAs themselves seem to be able to produce a signal in the ddPCR. This is something that we did not expect as the XNAs that we used are unable to be amplified by conventional polymerases[6]. This phenomenon could be due to how short the XNAs are (<60 bp), hence the fluorescent probes in the ddPCR assay may have been cleaved when the polymerase binds to the XNA-DNA primer complex transiently, even though there may not be any amplification, resulting in false positives. Further experiments elucidating the behavior of XNAs in the ddPCR assay is required for accurate and reliable measurements of cfDNA enrichment. Another idea could be to use next-generation sequencing instead of ddPCR to quantify the number of mutant molecules that were enriched, because the XNAs should not be amplified during the pre-sequencing PCR amplification step.

Acknowledgements

During my time in the Frankell Lab, I got very comfortable with quite a few wet lab experimental techniques such as mammalian cell culture, cfDNA extraction and ddPCR. In addition, I had the opportunity to improve my skills in the dry lab by doing some bioinformatics and data analysis with R and Python. In terms of transferable skills, I was able to polish my organization, time-management and problem-solving skills. This studentship has reaffirmed my desire to pursue a career in academic research by opening my eyes to what a career in research truly entails.

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