DURATION: Droplet digital PCR – Using chromosome Rearrangements As Tumour-specific markers In disease mONitoring for lung cancer

Protocol Version: 1.0
Dated: 16th July 2018

Investigators

Chief Researcher:

Associate Professor Alexander Dobrovic

Co-ordinating Principal Investigator:

Associate Professor Gavin Wright

Co-investigators:

Associate Professor Thomas John

Associate Professor Benjamin Solomon

Dr Hongdo Do

Associate Professor Paul Mitchell

Professor Anthony Papenfuss

Dr Prudence Russell

Associate Professor Sarah-Jane Dawson

Mr Stephen Barnett

Mr Naveed Alam

TABLE OF CONTENTS

Section **Page**

LIST OF ABBREVIATIONS……………………………………………………….4

1.0 PROTOCOL SUMMARY…………………………………………………………..5

2.0 BACKGROUND…………………………………………………………………….7

2.1 Lung Cancer………………………………………………………………..7

2.2 DNA-based biomarkers for the detection of circulating tumour DNA …..8

2.3 Personalised analysis of rearranged ends (PARE)……………………….8

2.4 Preliminary ctDNA data……………………………………………………...10

2.5 Preliminary ctDNA using the PARE approach…………………………….10

2.6 Building a matched DNA tumour resource…………………………………11

3.0 RESEARCH PLAN……………………………………………………………….12

3.1 Purpose………………………………………………………………………..12

3.2 Patient recruitment into the study……………………………………….12

3.3 Inclusion criteria………………………………………………………………13

3.4 Exclusion criteria……………………………………………………………...13

3.5 Plasma DNA isolation and storage…………………………………………13

3.6 Identification of chromosome rearrangement markers for ctDNA………14

3.7 Clinical Follow-up……………………………………………………………..16

3.8 Objectives……………………………………………………………………….16

4.0 TIMELINES…………………………………………………………………….…..18

5.0 SCHEDULE OF ASSESSMENTS………………………………………………..19

6.0 OVERALL OUTCOMES AND SIGNIFICANCE…………………………………….20

7.0 ETHICAL CONSIDERATIONS……………………………………………………20

8.0 STATISTICAL CONSIDERATIONS………………………………………….……..21

9.0 RECORD KEEPING PROCEDURES AND STORAGE OF DATA……………...21

10.0 PRIVACY……………………………………………………………………….…21

11.0 REFERENCES…………………………………………………………………...22

Appendix 1 – Blood and Tissue Handling Instructions………………………………24

LIST OF ABBREVIATIONS

ALK	Anaplastic lymphoma kinase
BC	Buffy Coat
BRAF	Human oncogene that codes for a protein called B-Raf
CAPP-Seq	CAncer Personalized Profiling by deep Sequencing
CEA	Carcinoembryonic antigen
CML	Chronic myeloid leukaemia
Co-I	Co-investigator
CT	Computed tomography
cfDNA	Circulating free Deoxyribonucleic acid
ctDNA	Circulating tumour Deoxyribonucleic acid
ddPCR	droplet digital polymerase chain reaction
DNA	Deoxyribonucleic acid
dsDNA	Double stranded Deoxyribonucleic acid
EGFR	Epidermal growth factor receptor
EML4-ALK	Fusion Gene EML4 (echinoderm microtubule-associated protein-like 4) fused to ALK (Anaplastic lymphoma kinase)
FF	Fresh frozen tumour
FFPE	Formalin-fixed paraffin-embedded tumour
GRIDSS	Genome Rearrangement IDentification Software Suite
HRM	High resolution melting
KRAS	Human oncogene coding for a protein called Kirsten RAt Sarcoma virus
MRD	Minimal residual disease
NGS	Next generation sequencing
NSCLC	Non-small cell lung cancer
NTC	No template control
NTRK	neurotropic tyrosine kinase
ONJCRI	Olivia Newton-John Cancer Research Institute
ONJCWC	Olivia Newton-John Cancer Wellness and Research Centre
PARE	Personalised analysis of rearranged ends
PCR	Polymerase chain reaction
PI	Principal Investigator
RET	Oncogene coding for the Rearranged during transfection protein
RNA	Ribonucleic acid
ROS1	Receptor tyrosine kinase encoded by the ROS Proto-Oncogene 1, Receptor Tyrosine Kinase gene
RT-PCR	Reverse transcription polymerase chain reaction
SV	Structural variant
TAM	Tagged-Amplicon
TAM-seq	Tagged-Amplicon Sequencing
TCGA	The Cancer Genome Atlas
TGEG	Translational genomics and epigenomics
VCCC	Victorian Comprehensive Cancer Centre
WGS	Whole genome sequencing

PROTOCOL SUMMARY

Trial name	*DURATION: Droplet digital PCR – Using chromosome Rearrangements As Tumour-specific markers In disease mONitoring for lung cancer*
Hypothesis	This study will evaluate the practicality of digital droplet PCR (ddPCR) assays based on chromosome rearrangements to detect circulating tumour DNA (ctDNA) especially for longitudinal assessment of disease in a cohort of lung cancer patients with early stage tumours that initially undergo surgically resection. The hypothesis is that ctDNA can be detected at an unprecedented level of sensitivity and accuracy by using (ddPCR) assays that use patient tumour-specific rearrangements as highly specific (individualised) tumour biomarkers.
Objectives	Primary Objective 1: To assess the presence of circulating DNA (ctDNA) pre- and post-surgical resection as a predictor of relapse/disease-free survival in surgically treated lung cancer patients. Primary Objective 2: To monitor ctDNA levels post-surgery with longer-term serial sampling of plasma. Secondary Objective 1: To build a matched DNA tumour resource to facilitate future DNA and liquid biopsy research Secondary Objective 2: To facilitate HREC approved retrospective studies and translational studies using clinical and molecular data from this resource.
Outcomes	Objective 1 Outcome: Determine the utility of ctDNA post-surgery as a marker of metastatic or persistent disease at one month and 6 months after surgery. Objective 2 Outcome: Confirm that ctDNA levels post-surgery with longer-term serial sampling of plasma predict or correlate with disease relapse (6 monthly to 36 months).
Subject Enrolment	120 patients in multiple centres across the Victorian Comprehensive Cancer Centre (hospital partners).
Subjects Follow-up	Participants will be followed up at one month post-surgery and then 6-monthly post-surgery for 36 months, with a clinical review, CT scan and blood testing for ctDNA at each time point. Standard care follow-up (CT and clinic review) will continue annually for up to 5 years.
Inclusion/exclusion criteria	Inclusion criteria: 1. Patient with suspected or proven stage I – IIIA NSCLC requiring surgical resection. 2. Availability of tumour tissue for the purpose of DNA analysis, (either prior to or during surgery) with enough tumour material of at least 50% tumour purity to yield sufficient DNA (1-2ug) for whole genome sequencing 3. Age ≥18 Exclusion criteria: 1. Persons with a cognitive impairment, intellectual disability or mental illness who are not competent to consent. 2. Patients unfit for lung cancer resection.

Sample size	In order to show a clinically important and statistically significant difference to usual clinical follow-up (Type I error probability p=0.05 and power of 80%) we would require 100 patients. Allowing for a 20% attrition rate, we would seek to enrol 120 patients over 2 years.

2 BACKGROUND

2.1 Lung Cancer

The standardised incidence of non-small cell lung cancer (NSCLC) in Victoria since 2000 has plateaued to around 30 per 100,000 population, making it the 4th most common solid cancer diagnosis in the State (Cancer Council Victoria Interactive Statistics Website: 1982-2016). However, more deaths are attributable to NSCLC than any other cancer (Siegel 2012) and as symptoms occur late, the majority of patients are diagnosed with locally advanced or metastatic disease.

In patients whose disease is potentially curable, either through surgery or radical chemo-radiotherapy, the major determinant of remission remains pathological stage, with more advanced disease being associated with increased rates of relapse and subsequent death. However even for early stage patients undergoing surgical resection with curative intent, subsequent relapse and death rates are high. Some cohorts of stage I disease with larger tumours have approximately 40-50% chance of disease relapse within 3 years. While the use of adjuvant chemotherapy has improved the rates of cure, improvements are modest with an absolute benefit of 5% (Pignon 2008), due partly to the lack of a predictive biomarker for benefit.

Therapeutic options other than chemotherapy for advanced disease have recently appeared, including therapy with tyrosine kinase inhibitors for ALK or ROS1 rearranged and *EGFR* or *BRAF* mutant tumours, and immunotherapy using checkpoint inhibitors. These offer the promise of more effective and durable disease control and possibly even cure of low volume relapsed or recurrent disease.

Currently there are no evidence-based guidelines for the clinical follow up of NSCLC patients after surgical resection. Moreover, in lung cancer, CT scanning is the only widely available method to monitor for relapse. CT scans result in radiation exposure to the patient and a financial burden on the healthcare system as each scan costs ~$400. Several studies have failed to show that more intense surveillance with post-operative CT scans alters outcome, indicating that by the time that recurrence is detectable on CT scan, the cancer is often incurable.

On the other hand, regular monitoring for ctDNA, can be done at low cost (around $300 for ctDNA extraction and droplet digital PCR for samples) once the initial testing has been performed, with no health risk to the patient. Monitoring of ctDNA been shown to be superior to

CT scans in other tumour types (e.g. our work in melanoma; Tsao 2015, Chia 2016). Patients can be monitored much more closely and with greater sensitivity, resulting in timely treatments and lower burden to the health care system.

2.2 DNA-based biomarkers for the detection of circulating tumour DNA

Blood-based biomarkers used in routine clinical practice for monitoring response of solid tumours to therapy have been generally based on non-molecular technologies e.g. immunoassays for serum tumour marker such as carcinoembryonic antigen (CEA) for colorectal cancer and CA19-9 for pancreatic cancer. All have major limitations especially with regard to sensitivity and specificity, and often, limited relationship to disease burden. **In addition, there are currently NO sensitive or specific blood-based biomarkers in clinical use for lung cancer.** Other biomarkers based on methodologies such as circulating tumour cells (including our work published in Hardingham 1993) and proteomic biomarkers (Hanash 2008) have not become routinely used due to methodological constraints and sensitivity issues.

Circulating tumour DNA (ctDNA) is a component of the circulating free DNA (cfDNA) in the blood of cancer patients. ctDNA is thought to derive from apoptosis and necrosis of tumour cells (Jahr 2001). The potential of ctDNA disease monitoring is clear. Diehl et al. (2008), using mutation analysis in colorectal cancer patients, reported that ctDNA levels accurately reflected response to treatment. ctDNA levels were lower in patients after surgery or chemotherapy. This has been supported by many other studies (Schwarzenbach 2011; Ignatiadis & Dawson, 2014).

ctDNA can not be distinguished from wild type DNA without suitable cancer specific DNA biomarkers. Tumour-specific changes can be used as molecular markers to detect minimal amounts of ctDNA, and hence the presence of tumour, in a background of wild type DNA (Leary 2010; Dawson 2013). Until now, most studies have used tumour specific mutations to identify ctDNA in cancer patients as in our recent studies using *BRAF* mutations in melanoma (Tsao 2015) and *EGFR* mutations in lung cancer (Chia et al. 2016; Thai et al 2017).

2.3 Personalised analysis of rearranged ends (PARE)

The concept of using rearrangements for monitoring disease goes back to the use of recurrent chromosome rearrangements in haematological malignancies. In addition to the comparatively rare recurrent mutations such as *EML4-ALK*, each epithelial tumour harbours a “personal” set of chromosomal rearrangements, which can be identified by whole genome sequencing. These

personal rearrangements can be used as markers to identify the presence of tumour DNA. The personalised analysis of rearranged ends (PARE) approach was established in a proof-of-concept study using two colorectal and two breast tumours (Leary 2010). The key advantage of this approach relies on the nature of chromosomal rearrangements. As rearrangements are tumour specific, only DNA containing the specific rearrangements will be amplified using PCR assays flanking the rearrangement junction. These assays are able to reach exquisite sensitivity and specificity in determining the presence of ctDNA (Leary 2010, Do 2016).

Several key improvements will be used by us to advance the personal rearrangement approach to accurately measure the amount of ctDNA: (i) cost-efficient low-pass (30x) whole genome sequencing on the Illumina HiSeq X Ten; (ii) a new bioinformatic pipeline for identification of rearrangements following WGS (Cameron et al, 2017) and (iii) droplet digital PCR to count the number of translocation templates in plasma to monitor changes during treatment. The WGS will be performed at an accredited genomic research laboratory. The bioinformatic analysis will be performed using the GRIDSS methodology developed by Tony Papenfuss (co-investigator; published in Cameron 2017) in PI Dobrovic's laboratory. The primer design, library preparation and droplet digital PCR will be performed in PI Dobrovic's laboratory. **This approach has been validated in a pilot study (Do 2016)**

We aim to identify up to 5 discrete chromosomal rearrangements per patient and choose the best 3 for routine testing. Preference will be given to high copy number rearrangements as (i) they are more likely to be drivers or truncal alterations and thus retained during tumour evolution and (ii) they effectively increase the sensitivity of the assay due to their higher copy number/cancer genome. If known driver mutations such as those involving ALK, ROS1, RET and NTRK fusions are identified, they will be chosen.

The disadvantage of the personal rearrangement approach is the initial cost for whole-genome sequencing (WGS), which we minimise by using the Illumina HiSeq X Ten in optimal batching runs. The apparent disadvantage of this approach is more than amply compensated for by (i) the uniform workflow, (ii) the low cost of the PCR assays, and (iii) the exquisite sensitivity of detection of rearrangements using relatively simple PCR methodologies. This can be contrasted with alternative approaches using next generation sequencing (NGS) such as TAM-seq (Dawson 2013) and CAPP-Seq (Newman 2014). These studies use tagged-amplicon (TAM) and capture-based (CAP) approaches that require NGS **at each time point**. Logistically the latter is high cost, particularly when batching of samples is limited, or not possible due to clinical demands for rapid turnaround time. In contrast, our digital PCR approach is both low

cost and rapid turnaround facilitating rapid clinical decision-making. There are also additional benefits to running WGS of tumour DNA including copy number information and mutation detection and enumeration.

2.4 Preliminary ctDNA data

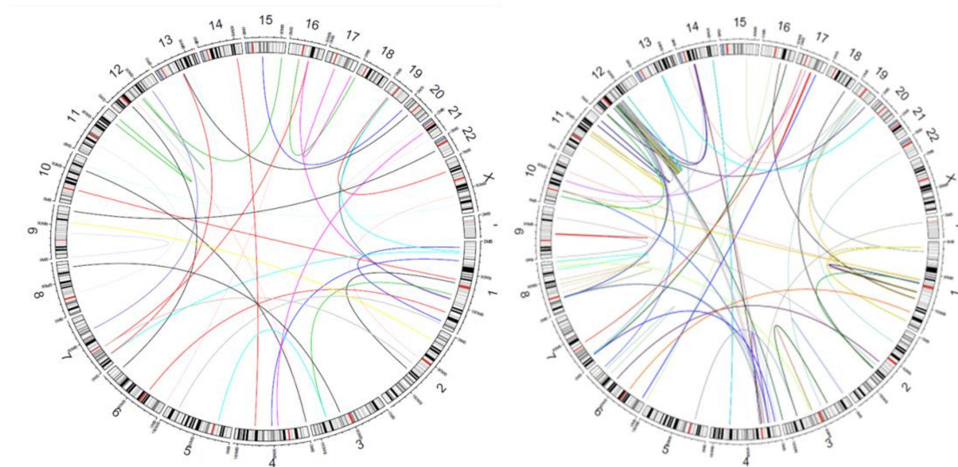
PI Dobrovic has had considerable experience in designing assays for rearrangements both at the DNA and at the RNA level. Identification of mutations in ctDNA from metastatic cancer patients has been performed by his team for melanoma (Tsao 2015) and lung cancer (Michael 2014; Chia 2016; Thai 2017). As part of a small clinical trial (Michael 2014), tumours and ctDNA from 23 NSCLC patients were scanned for mutations in exon 2 of *KRAS* and exons 18-21 of *EGFR* by high resolution melting. Six patients had mutations in their tumours. In four of those patients, identical mutations were detected in the plasma. These results show that for late stage cancer patients, even relatively insensitive methods (e.g. Standard High Resolution Melting sensitivity is around 5%) may be sufficient for ctDNA detection. However, for early stage patients and for MRD studies it is necessary to utilize assays that are appropriately sensitive. Highly sensitive and specific mutation assays can be difficult to optimise and may require blockers to inhibit amplification of wild type DNA (Zapparoli 2013).

PI Dobrovic's laboratory has expertise with the droplet digital PCR technology using the Bio-Rad QX200. They have developed assays for mutation and methylation detection on good quality as well as FFPE-derived DNA. They have extensive experience in using the *BRAF* V600E and the *EGFR* T790M ddPCR assays (Tsao 2015). Our ctDNA protocols based on droplet digital PCR have been accredited by NATA and we are using these testing protocols in diagnostic testing for AstraZeneca.

2.5 Preliminary ctDNA using the PARE approach

We have performed whole genome sequencing and bio-informatic analysis of two lung tumour samples. The genomic sequence was aligned to the human reference genome (hg19. Rearrangements were identified using GRIDSS (Figure 1). It identified 44 and 106 potential genomic rearrangements, respectively, in two tumours. Each of these rearrangements could be used as a biomarker to detect ctDNA. We chose 5 chromosomal rearrangements per patient to design primers and then chose the 3 with the best performance for testing.

Figure 1: Circos plots for two patients demonstrating multiple chromosome rearrangements from their lung tumours, which were part of our pilot study. Each interior-line in the middle of the Circos plot represents one chromosomal rearrangement. These are then detected in the plasma by ddPCR.



Droplet digital PCR (ddPCR), the key technique for this proposal, is designed to overcome this major limitation of standard PCR; the inability to provide quantitative information. Each PCR is split into approximately 15,000-20,000 droplets. Usually, each droplet contains either 0 or 1 amplifiable DNA templates for a given PCR. Counting positive droplets allows accurate counting of tumour cell derived DNA by using Poisson statistics. A control assay is performed to count the total amount of genomes. Thus, by using ddPCR, we are able to accurately count tumour derived DNA templates and hence monitor tumour burden.

Another issue for DNA-based assays is that of tumour heterogeneity. By careful analysis of macro-dissected NSCLC tumours, we showed that *KRAS* and *BRAF* (but not *EGFR*) mutations were often heterogeneously distributed (Wright et al. 2014). This is consistent with those mutations often not being driver mutations in NSCLC. For this reason a mutational assay for *KRAS* may not be a reliable indicator of MRD. Whereas the same argument might be directed against rearrangement-based assays, we propose to overcome this by using the three best rearrangement assays for each patient in the current proposal.

2.6 Building a matched DNA tumour resource

The general area of research in which this study resides is characterised by rapidly evolving new discoveries. Stored serial blood samples with matched tumour DNA are an invaluable resource that would be costly to replicate, an inconvenience for additional patients and would result in lost time to new clinically important findings. As many of the newer targets in both

immunotherapy and in genomic therapies could be identified in these samples, we plan to store these samples indefinitely (or until exhausted) to facilitate HREC approved retrospective studies and translational studies using clinical and molecular data. All samples will be stored in the laboratory at Olivia Newton-John Cancer Research (ONJCRI) Institute in a re-identifiable format. Data will be stored at the VCCC sites and at the ONJCRI. The custodian of the code to re-identify this data will be the Coordinating Principal Investigator.

Use of these may include extracting biomarkers not involved in this study from the source specimens (plasma and tumour tissue) or from the genome sequencing already performed on these specimens.

No attempt will be made in this or future studies to look for inheritable or unrelated DNA traits other than for the diagnosis or treatment of lung malignancy.

3.0 RESEARCH PLAN

3.1 Purpose

The primary aim of this study is to evaluate the practicality of droplet digital PCR (ddPCR) assays based on chromosome rearrangements to detect circulating tumour DNA (ctDNA) especially for longitudinal assessment of disease in a cohort of lung cancer patients with early stage tumours that initially undergo surgically resection. Our pilot data show that ctDNA can be detected at an unprecedented level of sensitivity and accuracy by using ddPCR assays that use patient-specific rearrangements as highly specific tumour biomarkers (Do et al. 2016).

The secondary aim of the study is to build a matched DNA tumour resource to facilitate future HREC approved DNA and liquid biopsy research.

3.2 Patient recruitment into the study

Patients will be recruited from Victorian Comprehensive Cancer Centre hospital partners with high numbers of lung cancer resections, represented by clinician Co-Investigators at St. Vincent's Hospital, Royal Melbourne Hospital, Peter MacCallum Cancer Centre, and Austin Health. The VCCC Parkville Precinct, consisting of Peter MacCallum and Royal Melbourne Hospital, has the highest number, and St Vincent's and Austin Hospitals the next highest number of lung cancer resections in Victoria, respectively. Each site has more than 100 Stage I - IIIA NSCLC cases undergoing lung

cancer resections per year that could be potentially recruited into the study. Because our methodology allows us to detect a unique signature (chromosome rearrangements) from a patient's tumour, we will be able to recruit patients that would ordinarily be excluded from many clinical trials, e.g. those with neo-adjuvant therapies, or those treated previously for other cancers, including indolent metastatic cancers. As there is no intervention or confounder other than routine review and blood draw, there should be no issue with competition with other trials and we would request a multiple trial consent approach to ensure rapid recruitment.

The Investigator at the site will approach eligible patients who they deem competent to provide an informed consent and invite them to participate in the study. Both verbal and written information will be given to the patient and they will be able to discuss the study, ask questions and be invited to consult significant others in their decision to participate.

Participants will be enrolled based on the following: participant provides written informed consent, availability of tumour tissue for DNA analysis, and the successful collection of a pre-surgery blood sample. There should be enough tumour material with at least 50% tumour purity to yield sufficient DNA (1-2 ug) for whole genome sequencing.

3.3 Inclusion criteria

1. Patient with suspected or proven stage I – IIIA NSCLC requiring surgical resection.
2. Availability of a tumour specimen for the purpose of DNA analysis, (either prior to or during surgery) with enough material with at least 50% tumour to yield sufficient DNA (1-2ug) for whole genome sequencing
3. Age ≥18

3.4 Exclusion criteria

1. Persons with a cognitive impairment, intellectual disability or mental illness who are not competent to consent.
2. Persons unfit to proceed with lung cancer resection.

3.5 Plasma DNA isolation and storage/ tumour sampling and storage

Forty (40) ml of peripheral blood will be collected in Streck tubes or equivalent, which stabilize nucleic acids for transport within 5 days (Blood will be collected in EDTA tubes at ONJCRI) on day of surgery, day 4 (+/- 1 day), 30 days (+/- 1 week) post –

operatively, and then every 6 months (+/- 1 month) post–operatively for 3 years. Blood samples will be sent in a re-identifiable format as outlined in Appendix 1.

Cell-free plasma will be isolated using a double spin procedure. Plasma samples will be then stored at the ONJCRI laboratory at -80°C until DNA extraction. ctDNA will be extracted at the ONJCRI laboratory using the QIAamp Circulating Nucleic Acid Kit (the accepted standard for ctDNA extraction) and quantified using the dsDNA kit on the Qubit fluorimeter (Invitrogen). The extracted DNA will be stored at -80°C to minimise degradation.

Tumour biopsies will be taken on the day of surgery and processed and shipped as outlined in Appendix 1. (See Appendix 1 for details of blood and tissue handling)

3.6 Identification of chromosome rearrangement markers for ctDNA

Currently most lung cancer patients in the VCCC public hospitals in this study undergo testing for *EGFR* mutations. Those with identified exon 19 deletions will be analysed with primers that specifically amplify the deletion, as these aberrations are effectively a driver gene rearrangement. The ONJCRI laboratory has primers to cover the most common deletions of the *EGFR* gene.

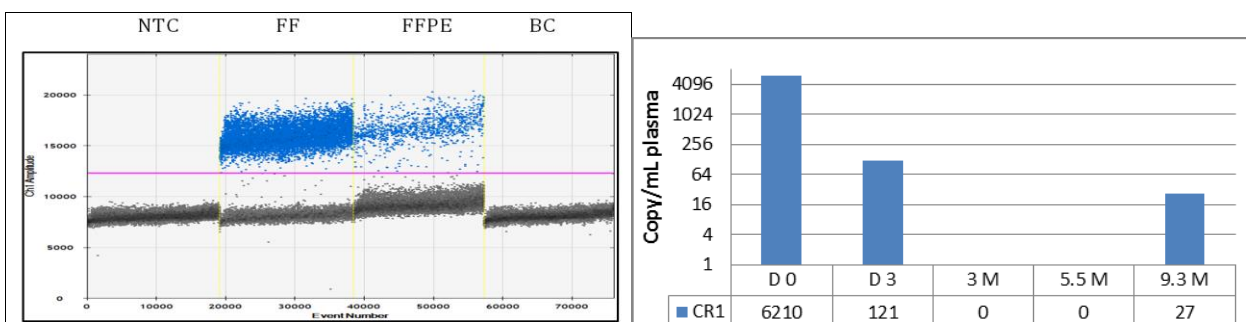
For tumours without *EGFR* exon 19 mutations, DNA from the tumour will be quantified using fluorimetry and fragmentation assessed using a Perkin Elmer LabChip GX analyser. Only DNA of suitable quality and quantity (optimally 1-2 ug total DNA) will be used. DNA will be sequenced by whole-genome shotgun sequencing utilising the Illumina platform.

GRIDSS (Genome Rearrangement IDentification Software Suite) is a structural variant (SV) caller developed by Co-I Papenfuss that performs whole genome breakpoint assembly using a novel positional de Bruijn graph algorithm, and combines breakpoint assembly, split read, and read pair evidence using a probabilistic variant scoring framework (Cameron et al. *2017*). GRIDSS halves the false discovery rate compared to other recent methods. GRIDSS is high speed, prioritises SV calls and stratifies into high and low confidence sets, and detects micro-homologies and untemplated sequence. We aim to use at least 3 and up to 5 high-confidence chromosomal rearrangements identified by GRIDSS analysis in the primary tumour. We expect to find a sufficient number of rearrangements in every tumour based on previously

published data from the TCGA lung cancer data (TCGA 2014; Imielinski 2012). Priority will be given to driver rearrangements e.g. EML4-ALK, when present, and to rearrangements that are within amplified regions as indicated by a greater number of reads. Amplified regions should release more DNA templates/genome into the bloodstream, hence increasing the accuracy of identification of ctDNA present at lower levels.

After successful identification of suitable rearrangements, primers specific to the rearrangements (i.e. spanning the DNA breakpoint) will be designed and tested on primary tumour DNA as well as normal white blood cell DNA to confirm the translocation to be somatic and not germline. We will test DNA extracted from 4 ml of plasma to increase the total amount of templates. Primers will amplify regions of 70-100 base pairs. ddPCR will be performed on the QX200 digital droplet system enabling direct counting of translocated DNA fragments (Figure 2).

Figure 2. We have demonstrated proof of principle in an example of tumour recurrence 9 months post-surgery. The ddPCR results (left) show a somatic genomic rearrangement. Serial monitoring of the genomic rearrangement in the patient's blood showed a fall within 3 months of surgery, then a rise at 9.3 months (right). NTC: No template control, FF: fresh frozen tumour, FFPE: formalin-fixed tumour, BC: buffy coat.



Successful assays will then be used to detect circulating tumour DNA. Each patient will have at least 3 rearrangements assessed in each ctDNA sample. The tumours will also have been screened for *EGFR* mutations. Where they are positive for L858R or G719X, plasma DNA will also be screened by ddPCR for those mutations. This will give a relative sensitivity for the mutational and chromosome rearrangement assays.

EGFR Exon 19 deletions will be included as one of the 3 gene rearrangements if they are present in the tumour.

Following completion of the testing required for this study, tumour and blood samples will remain stored indefinitely (or until exhausted) at the ONJCRI for future HREC approved DNA and liquid biopsy research.

3.7 Clinical Follow-up.

Participants will be followed up with a clinic review including physical examination at 30 days (+/- 1 week) post–operatively, and then every 6 months (+/- 1 month) post–operatively with a clinic review including physical examination and CT scan for 3 years. Participants will then be followed annually with clinic review including physical examination and CT scan for up to 5 years. They may also require an additional review at time of relapse if clinically indicated. This follow-up schedule is standard of care.

Participation in this study does not preclude participation in other clinical trials.

3.8 Objectives

1. **To assess the presence of ctDNA pre- and post-surgical resection as a predictor of relapse/disease-free survival in surgically treated lung cancer patients.**

(*Research question: Is persistent ctDNA useful to determine the success of surgical resection ± adjuvant chemotherapy?)*

We plan to assess ctDNA as a marker of MRD in plasma samples 0 days prior to surgery, 3-5 days after surgery, and one month post-surgery (stage I-III, prior to any chemotherapy) and 6 months (i.e. after any adjuvant chemotherapy is delivered, if indicated). It should be noted that adjuvant chemotherapy is indicated in all fit Stage II and IIIA (and some Stage IB) patients. As adjuvant chemotherapy is usually commenced beyond the first month post-surgery, and lasts for 4 three-week cycles, the 6-month plasma sample will be analysed in addition to the 1-month sample to determine the immediate effect of chemotherapy. As such, compared to pre-operative ctDNA levels, the 1-month (and/or 6-month) time point ctDNA level could be used to define complete, partial, or absence of molecular response to therapy. The presence of ctDNA at the 1-month time point likely indicates the presence of metastatic (or persistent loco-regional) disease and should predict early relapse. Disappearance of the ctDNA (complete molecular

response) after adjuvant chemotherapy would be an early marker of treatment success that until now has been impossible to determine until later relapse of disease.

The statistical significance of the difference in cumulative survival of ctDNA positive and negative samples will be estimated using the Cox proportional hazards model. Analysis will be performed at the end of the funding period by which time a substantial number of patients would be expected to have relapsed and/or commenced new treatment. This will answer the question of whether persistent ctDNA after curative intent surgery correlates with residual disease.

2. **To monitor ctDNA levels post surgery with longer-term serial sampling of plasma.**

(Research Question: Does an increase in ctDNA levels pre-date and predict the onset of overt disease relapse?)

Levels of ctDNA will be related to the timing of radiological relapse and other clinical parameters to evaluate ctDNA not only as a sensitive relapse marker but also as a marker of response to subsequent systemic therapy.

In patients that have a complete molecular response, emergence of detectable ctDNA will likely provide an early indication of imminent overt relapse (The ctDNA data for our pilot study (Figure 2) is an example). Stage II-IIIA patients have a much higher risk of relapse, and all these patients and some stage IB patients (if medically fit) are offered adjuvant chemotherapy. This provides an opportunity to track levels of ctDNA over the natural history of the lung cancer, as well as the molecular response to subsequent interventions. Patients who relapse after surgery can undergo multiple therapeutic paths; e.g. radiotherapy, chemotherapy, targeted therapy, or immunotherapy. Patients with high mutation rates identified by WGS are likely to be entered into immunotherapy trials (as was the case with patient PL2 after relapse). We plan to follow each patient from Objective 2 (up to 120 patients total) for up to 3 years, even if they have relapsed, as we will be able to determine their response to all subsequent therapies such as chemotherapy, radiotherapy, targeted therapy and immunotherapy. Participants will donate 40 ml of blood at 6, 12, 18, 24, 30 and 36 months (+/- I month). Follow-up for clinical relapse and overall survival will continue for up to 5 years.

3. **Secondary objectives :To build a matched DNA tumour resource to facilitate future DNA and liquid biopsy research to facilitate HREC approved retrospective studies and translational studies using clinical and molecular data from this resource.**

Tumour and liquid biopsies will be stored in the Translational Genomics and Epigenomics Laboratory at the ONJCRI. The custodian of these samples will be Associate Professor Alexander Dobrovic. Only investigators on this study will have access to the samples. New investigators will have future access if an HREC approved study is actioned in collaboration with the investigators on this study. The custodian of the code for the identity of these samples will be Associate Professor Gavin Wright and trial coordinator Jane Mack.

4.0 TIMELINES

We expect to recruit 120 patients for the study within the first two years. We anticipate enrolling 60-80 patients within the first year and the remaining patients by the middle of year two of the study. Given the short time frame of Objective 1; involving only a post-therapy sample one month after surgery, and a post-therapy sample after any adjuvant chemotherapy (mainly stage II and III patients), we anticipate to have the Objective 1 laboratory analysis completed by the end of year 2.

For Objective 2, longitudinal sampling will finish early in the second half of year 5. The last time point we propose to monitor is at 36 months post surgery and the last patients enrolled in mid-year 2 should be finished by then. Routine clinical follow up will continue to end of the study. Data analysis will be done in year 5, prior to publication of the data.

For the secondary objectives, tumour and liquid biopsies will be kept indefinitely (or until exhausted) at the ONJCRI for use in future HREC approved retrospective studies.

5.0 SCHEDULE OF ASSESSMENTS

Procedures / Evaluations	In-hospital Phase			Follow-up Phase				
	Baseline (Pre-op)	**Day of Surgery**	**Day 4** (± 1 day)	**30-Day Clinic Visit** (± 1 week)	**6-36 Months** (± 1 month)	**4 years**	**5 years**	**Relapse**[c]
Eligibility assessment	X							
Informed consent	X							
Patient demography	X							
Clinical Review including Physical Exam	X			X	X	X	X	X[c]
Medical/surgical history	X							
CT Scan	X[a]				X	X	X	X[c]
Tumour Biopsy	X[b]	X[b]						
Blood/Serum Samples		X	X	X	X			

a) Within 90 days of informed consent.
b) Tumour biopsy may be taken pre-operatively (within 90 days) OR after induction of anaesthesia on day of surgery if not obtained prior.
c) If clinically indicated.

.

6.0 OVERALL OUTCOMES AND SIGNIFICANCE

The study is expected to:

- Assess the practicalities of the implementation of ctDNA markers into patient management.
- Clarify the utility of gene rearrangements to generate specific markers for each patient's cancer to detect ctDNA.
- Indicate if ctDNA monitoring is superior to the radiological tests in current practice by predicting relapse well in advance.
- Systematically use biomarkers other than point mutations to determine MRD and monitor early stage patients for tumour recurrence for 3 years
- Generate data that will be published in high impact peer-reviewed journals and presented at national and international conferences.
- Open the way to clinical studies that can address the question if patients with MRD identified by ctDNA would benefit from adjuvant treatment after surgery.
- Build a matched DNA tumour resource to facilitate future DNA and liquid biopsy research.

Despite current curative approaches for NSCLC involving surgery, 30-80% of patients will relapse with incurable disease, depending on stage at diagnosis. This high rate of relapse is also coupled with high mortality as most relapses are detected once tumours have already metastasised and median survival with advanced disease is less than 12 months. **Patients with early-stage primary tumours are the group in which appropriately timed therapeutic intervention is most likely to make a clinical difference.** The study will assess ctDNA in a large cohort of patients with early-stage primary tumours that are being treated with the primary aim of cure.

7.0 ETHICAL CONSIDERATIONS

As part of our identification process we will match the discovered gene rearrangements against the participant's own germline DNA. We do not otherwise read or interpret the germline DNA, therefore we will not be able to, nor intend to, identify any genetic disorders outside of the tumour itself. Occasionally we will identify a potential targeted treatment for an individual participant's cancer. In our pilot study, a cryptic *EGFR* exon 19 deletion was identified which made the patient eligible for treatment with tyrosine kinase inhibitors. In a second patient, an elevated rate of coding mutations led to the patient being chosen for treatment with checkpoint inhibitors and the patient returned to

and remains in molecular remission. Such information will be passed on to the participant's treating physician once it is identified. It should be noted that the timing of any actionable genomic findings will be determined by the batched testing and analysis that necessarily will be performed as part of this protocol.

For future studies, if an actionable DNA or biomarker finding is made, this will be communicated to the participant's physician. However, as this will not occur in real time, the chances of changing an individual participant's therapy is very small.

8.0 STATISTICAL CONSIDERATIONS

Based on prospective databases of patients surgically treated with curative intent at the Austin and St Vincent's Hospitals, 45-55% will have clinically detected relapse within 3 years. Usual clinical follow-up for relapse consists of patient-reported symptoms, regularly scheduled physical examination and CT scans. Assuming ddPCR can detect at least two-thirds of these relapses immediately post completion of therapy, we would need 100 patients to show a clinically important and statistically significant difference to usual clinical follow-up (Type I error probability $p=0.05$ and power of 80%). Allowing for 20% attrition rate, we would seek to enroll 120 patients over 2 years.

9.0 RECORD KEEPING PROCEDURES AND STORAGE OF DATA

Data will be stored securely in locked research offices at each VCCC site or the ONJCRI, and on password protected databases. Access to information stored on database will only be available to approved research personnel.

10.0 PRIVACY

Personal information, such as health information will remain privileged and confidential, except as required by law. Information collected for this project will be identifiable (that is, information will be linked to name or hospital medical record number). We are required to keep this information secure and confidential in order to protect participant's privacy. Identifiable information will not be given to anyone outside this study without written consent of the participants. Information provided to laboratory researchers will be de-identified (re-identifiable), meaning that personal information are removed and replaced with a unique code known only to authorised project staff.

11.0 REFERENCES

Bettegowda, C., M. Sausen, R. J. Leary, et al. (2014). Detection of circulating tumor DNA in early- and late-stage human malignancies. Sci Transl Med 6: 224ra224.
Cameron D, Schroeder J, Penington JS, **Do H**, Molania R, **Dobrovic A**, Speed TP,

Papenfuss AT. GRIDSS: sensitive and specific genomic rearrangement detection using positional de Bruijn graph assembly Nature Methods, under review
Chia et al. 2016

Dawson, S. J., N. Rosenfeld, C. Caldas (2013). Circulating tumor DNA to monitor metastatic breast cancer. N Engl J Med 369: 93-94.
Diehl, F., K. Schmidt, M. A. Choti, et al. (2008). Circulating mutant DNA to assess tumor dynamics. Nat Med 14: 985-990.

Do H, Krypuy M, **Mitchell PL, Dobrovic A (**2008). High resolution melting analysis for rapid and sensitive EGFR and KRAS mutation detection in formalin fixed paraffin embedded biopsies. BMC Cancer 8:142.

Do H, Dobrovic A (2009). Limited copy number-high resolution melting (LCN-HRM) enables the detection and identification by sequencing of low level mutations in cancer biopsies. Mol Cancer 8: 82.

Do H., Dobrovic, A. (2015). Sequence artifacts in DNA from formalin-fixed tissues: causes and strategies for minimization. Clinical Chemistry 61:64-71

Do H, Cameron D, Molania R, Thapa B, Rivalland G, **Mitchell PL**, Murone C**, John T, Papenfuss A, Dobrovic A.** (2016). Digital PCR of genomic rearrangements for monitoring circulating tumour DNA. Circulating Nucleic Acids in Plasma and Serum

Dobrovic A, Morley AA, Seshadri R et al. (1991). Molecular diagnosis of Philadelphia negative CML using the polymerase chain reaction and DNA analysis: clinical features and course of M-bcr negative and M-bcr positive CML. Leukemia 5: 187-190.

Fan, H. C., Y. J. Blumenfeld, U. Chitkara, et al. (2008). Noninvasive diagnosis of fetal aneuploidy by shotgun sequencing DNA from maternal blood. Proc Natl Acad Sci U S A 105: 16266-16271.

Hanash SM, Pitteri SJ, Faca VM (2008). Mining the plasma proteome for cancer biomarkers. Nature 452: 571-579.

Hardingham, J. E., D. Kotasek, R. E. Sage, **Dobrovic A.** (1993). Molecular detection of residual lymphoma cells in peripheral blood stem cell harvests and following autologous transplantation. Bone Marrow Transplant 11: 15-20.

Ignatiadis, M., **Dawson, S.J.** (2014). Circulating tumor cells and circulating tumor DNA for precision medicine: dream or reality? Ann Oncol. 25:2304-13.

Imielinski, M., A. H. Berger, P. S. Hammerman, et al. (2012). Mapping the hallmarks of lung adenocarcinoma with massively parallel sequencing. Cell 150: 1107-1120.

Jahr, S., H. Hentze, S. Englisch, et al. (2001). DNA fragments in the blood plasma of cancer patients: quantitations and evidence for their origin from apoptotic and necrotic cells. Cancer Res 61: 1659-1665.

Leary, R. J., I. Kinde, F. Diehl, et al. (2010). Development of personalized tumor biomarkers using massively parallel sequencing. Sci Transl Med 2: 20ra14.

Michael M, White SC, Abdi E, Nott L, Clingan P, Zimet A, Button P, Gregory D, **Solomon B, Dobrovic A**, **Do H**, Clarke S. A multi-Center Randomized, Open-Label Phase II Trial of Tarceva in Sequential Combination with Gemcitabine Compared to Gemcitabine Monotherapy as First-Line Therapy in Elderly or ECOG PS of 2 Patients with Advanced NSCLC. Asia Pacific J. Clin. Oncol. 2014 11:4-14

Newman AM, Bratman SV, To J. et al. 2014. An ultrasensitive method for quantitating circulating tumor DNA with broad patient coverage. Nat Med 20:548.

Pignon JP, H. Tribodet GV, Scagliotti Jet al. (2008). Lung adjuvant cisplatin evaluation: a pooled analysis by the LACE Collaborative Group. J Clin Oncol 26: 3552-3559.
Schwarzenbach, H., D. S. Hoon and K. Pantel (2011). Cell-free nucleic acids as biomarkers in cancer patients. Nat Rev Cancer 11: 426-437

Siegel, R., D. Naishadham, A. Jemal (2012). Cancer statistics, 2012. CA Cancer J Clin 62:10-29.

TCGA (2012). Comprehensive molecular profiling of lung adenocarcinoma Nature 511:543-50.
Thai et al 2017

Tsao S C-H, Weiss J, Hudson C, Christophi C, Cebon J, Behren A, **Dobrovic** A. Monitoring response to therapy in melanoma by quantifying circulating tumour DNA with droplet digital PCR for BRAF and NRAS mutations. Scientific Reports 2015 5:11198

Wright GM, Do H, Weiss J, Alam NZ, **Rathi V**, Walkiewicz M, **John T, Russell PA**, **Dobrovic A.** Mapping of actionable mutations to histological subtype domains in lung adenocarcinoma: implications for precision medicine. Oncotarget. 2014 5:2107-15.

Zapparoli, G. V., R. N. Jorissen, C. A. Hewitt, **Dobrovic A.** (2013). Quantitative threefold allele-specific PCR (QuanTAS-PCR) for highly sensitive JAK2 V617F mutant allele detection. BMC Cancer 13: 206.

Appendix 1 Blood and Tissue Handling

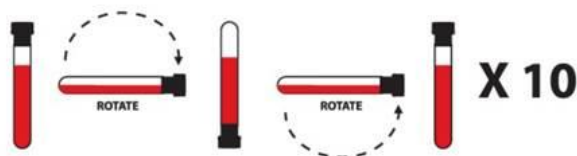
Sample requirements
Blood and tissue samples are to be collected at the participating sites and to be sent to the central laboratory for processing, storage and molecular testing.

Central laboratory details
Attn: Alexander Dobrovic/Hongdo Do
Translational Genomics and Epigenomics Lab
Olivia Newton-John Cancer Research Institute
Level 5, ONJCWC, 145 Studley Road, Heidelberg, Vic 3084 Australia

Sample types: Blood and Tissue samples

Blood samples

- Blood collection points: A total of 9 blood collections per patient; before surgery (baseline), day 4, 1 month, 6 months, 12 months, 18 months, 24 months, 30 months and 36 months.
- Blood collection tube: Streck tube or equivalent (10mL)
- Blood volume per collection: 40 mL (4 x 10mL Streck or equivalent tube). **IMPORTANT**: Fill the tube up to the top to ensure the blood volume is in the correct ratio with the preservative.
- Label: Each blood tube should be labelled with the following information; Patient Initial, Date of Birth, Collection Date/Time, Collection Time Point (e.g BL or D4 etc). Collection points are abbreviated as follows; before surgery (**BL**), day 4 (**D4**), 1 month (**1M**), 6 months (**6M**), 12 months (**12M**), 18 months (**18M**), 24 months (**24M**), 30 months (**30M**) and 36 months (**36M**).
- Instruction: Once blood is drawn, thoroughly mix the blood by gently inverting each Streck tube 10 times as shown below. Label each tube with all required information. Put the Streck tubes into 50mL Falcon tubes with tissue paper to provide protection during the shipping. Send the 50mL Falcon tubes to the central laboratory (TGEG, ONJCRI) by express post or by laboratory courier on a weekly basis.



  - Blood samples collected in Cell-Free RNA BCT (Streck tubes) are stable for up to 7 days when stored between 18 °C to 25 °C

Tissue sample

- Prepare **1 H&E** slide and **10 or 20x 5uM unstained tissue sections** mounted on uncoated slides. 10x 5uM sections for large tumour tissues (10mm x10mm) OR 20x 5uM sections for small tumour tissues (less than 10mm x10mm).
- A pathologist report to be provided for each case.
- Tissue samples can be batched for an interval of 6 months. Batched tissue samples are then to be sent to the central laboratory (TGEG, ONJCRI) along with the pathologist reports.
- Label: Each slide/pathology report should be labelled with the following information; Patient Initial, Date of Birth, Collection Date