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Original Research

Exploring the Feasibility and Utility of Exome-scale Tumour Sequencing in a Clinical Setting

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Abstract

Background:

Technology has progressed from single gene panel to large-scale genomic sequencing. This is raising expectations from clinicians and patients alike. The utility and performance of this technology in a clinical setting needs to be evaluated.

Aim: This pilot study investigated the feasibility of using exome-scale sequencing (ESS) to identify molecular drivers within cancers in real-time for Precision oncology in the clinic.

Methods:

Between March 2014 and March 2015, the Victorian Comprehensive Cancer Centre Alliance explored the feasibility and utility of ESS in a pilot study. DNA extracted from the tumour specimens underwent both ESS and targeted "hotspot" sequencing (TS). Blood was taken for germline analysis. A multi-disciplinary molecular tumour board determined the clinical relevance of identified mutations; in particular, whether they were "actionable" and/or "druggable".

Results:

Of 23 patients screened, 15 (65%) met the tissue requirements for genomic analysis. TS and ESS was successful in all cases. ESS identified pathogenic somatic variants in 73% (11/15 cases) versus 53% (8/15 cases) using TS. Clinically focussed ESS identified 63 variants, consisting of 30 somatic variants (including all 13 identified by TS) and 33 germline variants. Overall, there were 48 unique variants. ESS had a clinical impact in 53% (8/15 cases); 47% (7/15 cases) were referred to the familial cancer clinic, and "druggable" targets were identified in 53% (8/15 cases).

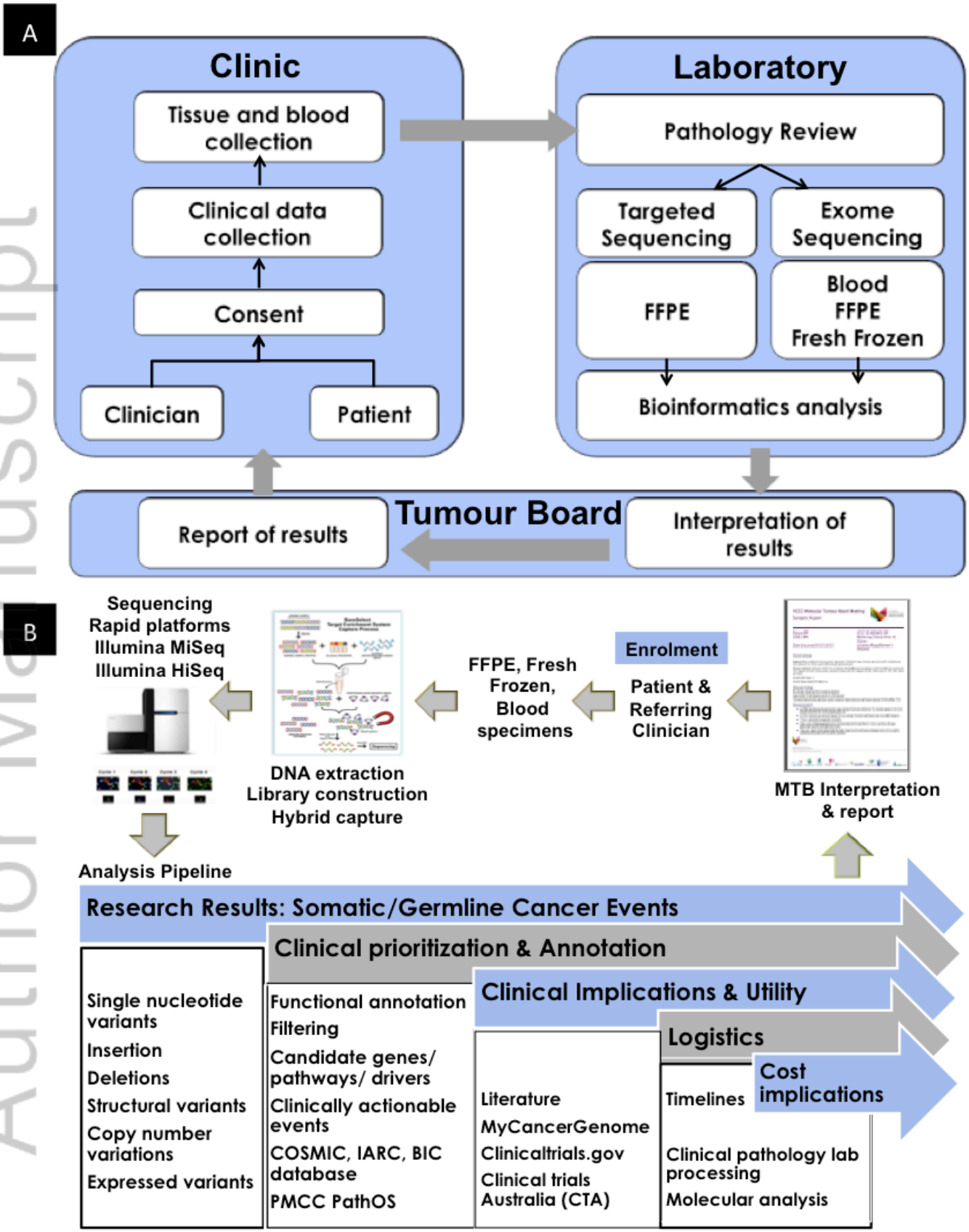
Conclusion:

Exome-scale sequencing of tumour DNA impacted clinical decision-making in 53%, with 20% more pathogenic variants identified through ES than TS. The identification of germline variants in 47% was an unexpected finding.

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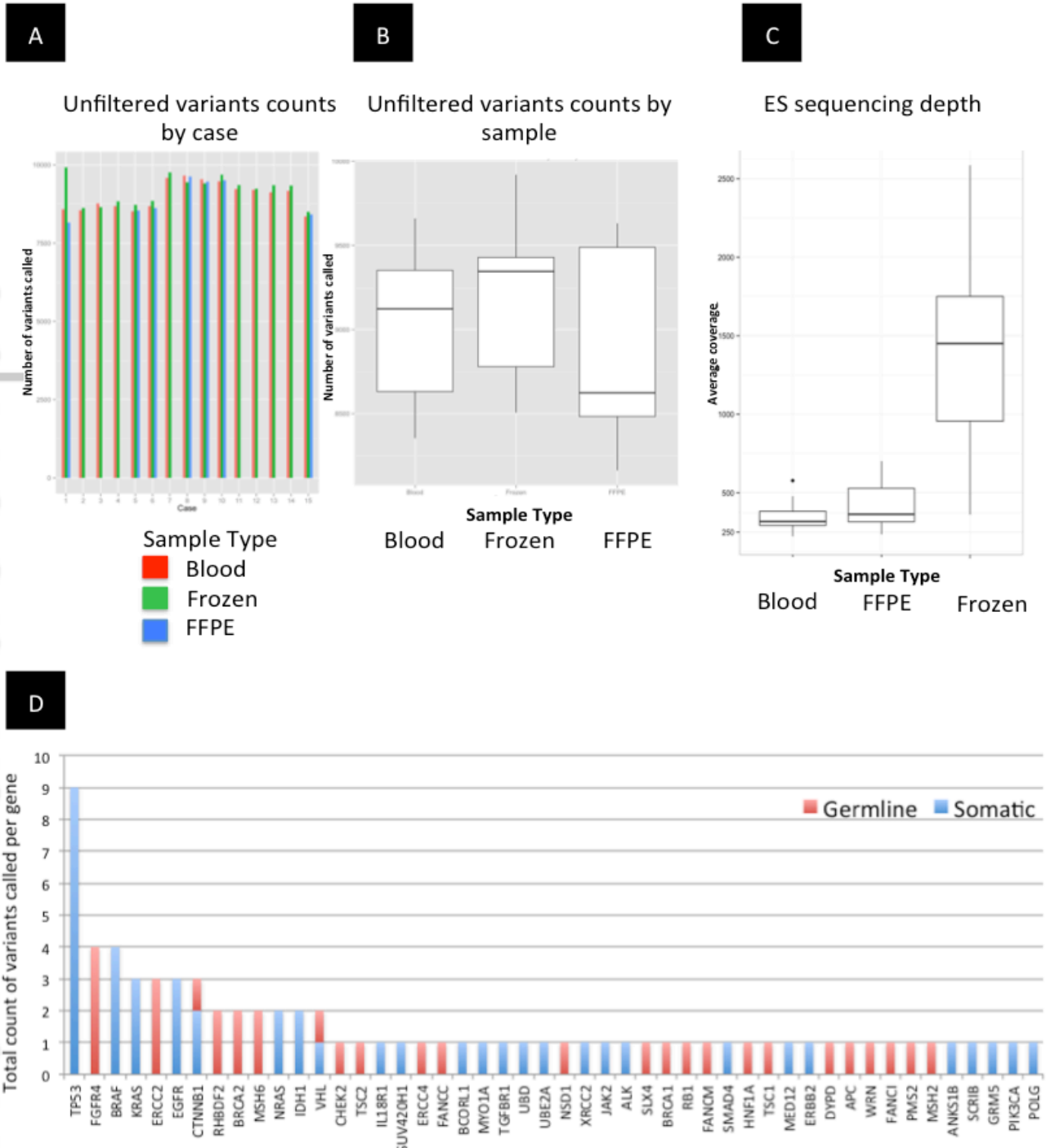


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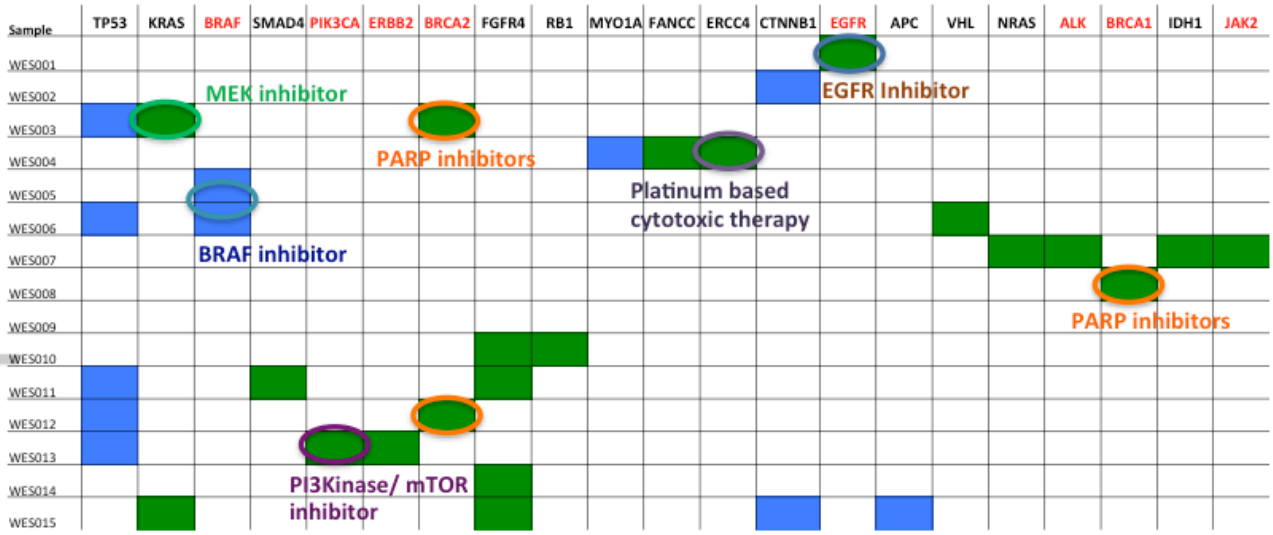
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Known Driver mutations or Implicated in key tumorgenesis pathway

May predict sensitivity or resistance to targeted therapy

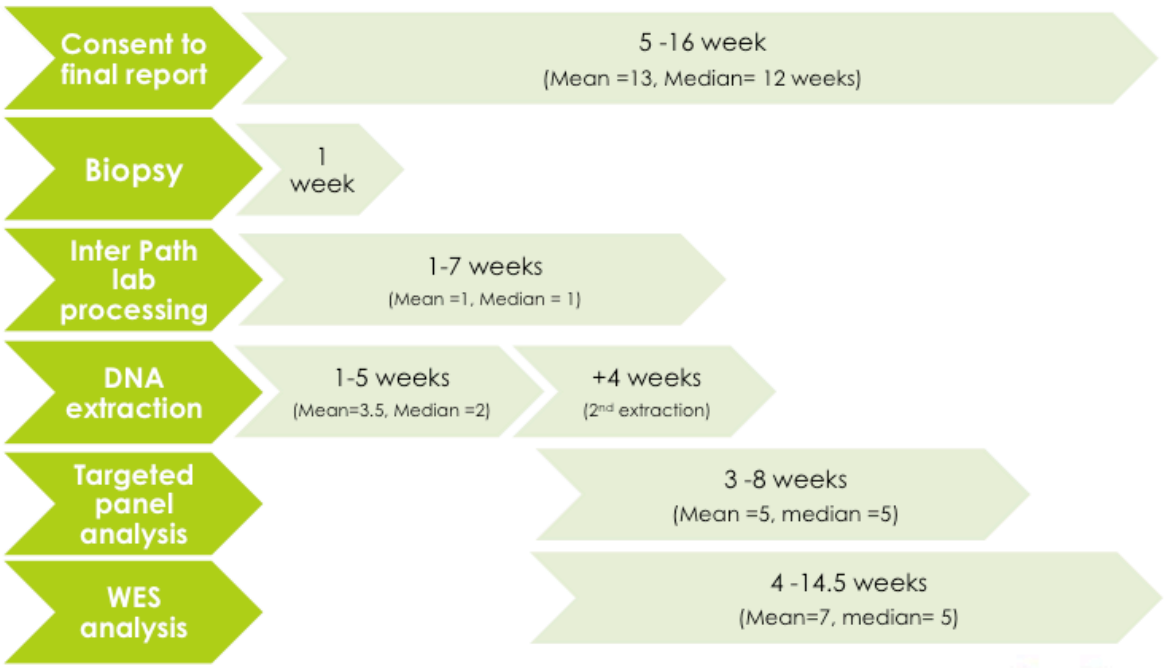
FDA approved drug available

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Table 1. Summary of Participants characteristics

Number screened	23
Number enrolled	15
Reasons for screening failure Lack of tumour in sample Insufficient tumour tissue Age of archived tissue >6 months Fresh frozen sample compromised	 2 4 1 1
Characteristics of participants enrolled	N=15 (%)
Age	Median [38] 59 (1.5- 72) years
Gender Male Female	 6 (40%) 9 (60%)
History of cancer in the family*	3 (20%)
Prior identified molecular marker	3 (20%)
Prior line of treatment	2 (1-5) lines

*None of these involved familial cancer syndromes

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Table 2. Summary of primary tumour, sequencing results and outcomes

Patient	Age	Primary Tumour	Exome Sequencing		Targeted Sequencing	Clinical Impact
			Germline variants	Somatic variants	Targeted Results	
WES01	61	Lung	Nil	EGFR x2	EGFR x1	N
WES02	60	Ovarian	CHEK2 TSC2	CTNNB1 IL18R1 SUV420H1	CTNNB1	Y
WES03	41	Pancreas	BRCA2	KRAS TP53	Nil	Y
WES04	58	Uterine	ERCC4 FANCC	BCORL1 MYO1A TGFBR1 UBD UBE2A	Nil	Y
WES05	16	Brain	NSD1 XRCC2	BRAF	BRAF	Y
WES06	67	Thyroid	ERCC2 VHL	BRAF TP53	BRAF VHL TP53	Y
WES07	53	Melanoma	Nil	NRAS IDH1 JAK2 ALK	IDH1 NRAS	N
WES08	66	Renal	BRCA1 SLX4	Nil	Nil	N
WES09	21	Skin	RHBDF2	Nil	Nil	N
WES10	31	Muscle	RB1 FGFR4	Nil	Nil	N
WES11	68	Prostate	FANCM FGFR4	TP53 x2 SMAD4	TP53 x2	N
WES12	72	Uterine	BRCA2 HNF1A MSH6 RHBF2 TSC1	MED12 TP53	TP53	Y
WES13	70	Uterine	Nil	ERBB2 TP53	PIK3CA	Y
WES14	1.5	Liver	FGFR4 DYPD	Nil	Nil	N
WES15	65	Ovarian	FGFR4 ERCC2 x2 APC WRN	KRAS ANKS1B SCRIB GRM5	KRAS	Y

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			FANCI CTNNB1 PMS2 MSH6 MSH2	POLG				
Comparison of Exome Sequencing vs. Targeted Sequencing Results by participant					ES Number (%)		TS Number (%)	
Cases with variants identified					15/15	(100%)	9/15	(60%)
Somatic pathogenic variants identified					11/15	(73%)	8/15	(53%)
Germline pathogenic variants identified					7/15	(47%)	n/a	n/a
Somatic variants of unknown clinical significance					4/15	(27%)	1/15	(7%)
Germline variants of unknown clinical significance					9/15	(60%)	0/15	(0%)
Clinical Outcomes								
Referred to the Familial Cancer Centre (FCC)					7/15	(47%)	n/a	n/a
Druggable targets identified					8/15	(53%)	6/15	(40%)
Changed to matched treatment					3/15	(20%)	2/15	(13%)
Overall informed cancer management					8/15	(53%)	6/15	(40%)

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Original Research

Exploring the Feasibility and Utility of Exome-scale Tumour Sequencing in a Clinical Setting

Introduction

The rapidly increasing knowledge of tumour biology, an increasing use of next generation sequencing (NGS) in the clinic, and a plethora of molecularly targeted agents in early clinical development has facilitated the application of Precision Oncology. (1-4) Molecular profiling is becoming more accessible to patients; patients can now have their tumour profiled, either through commercial platforms or in house molecular profiling programs. Despite these advancements, the practice of Precision Oncology remains in its infancy and largely restricted to large drug development programs with access to early phase clinical trials of novel molecularly targeted therapies. Since 2006, only 24 therapeutic agents with a molecular companion diagnostic have been approved by the FDA.

In the clinical setting, most molecular testing is performed using NGS and cancer panels. Prior to the advent of NGS, tumour genotyping was performed only on specific genomic loci. Current assays now take a targeted gene panel approach analysing a selected number of genes. Improvements in NGS technology have made it possible to sequence to an exome-scale, which can encompass the whole exome or whole genome if so desired, in a shorter timeframe and for lower cost. The major advantage of an exome-scale approach is to identify alterations in tumour suppressors, or in the increasingly complex area of "long tail' hot spot alterations. (5, 6) However, there are several trade-offs when the portion of genome sequenced is increased to an exome-scale. These include, 1) loss of depth, which may limit the ability to confidently call a variant of low allele fraction, 2) the requirement of germline sequencing to enhance the identification of true somatic variants, which may uncover incidental clinically relevant inherited disorders and 3) the increased bioinformatics analyses. (7, 8) Subsequently, there is continued debate around which type of sequencing (targeted gene panel versus exome-scale) would greater facilitate precision oncology and provide greater benefit to patients within the clinic; currently it remains unclear whether exome-scale

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sequencing is feasible in the clinical setting and whether the additional depth and breadth of molecular information provided by exome-scale sequencing (ESS) has any clinical impact.

Through the Victorian Comprehensive Cancer Centre Alliance, we completed a pilot study investigating the feasibility of performing real-time clinically focused ESS on freshly obtained tumour specimens from 15 patients with advanced cancer. We also compared the performance of ESS against a targeted sequencing (TS) panel, focusing on the additional information provided by ESS and the impact this had on patient care.

Methods

The primary objective of this study was to demonstrate feasibility of ESS. Feasibility was assessed on the following criteria: patient acceptability (recruitment of > 50% of patients approached), successful biopsy of tumour specimens (sufficient tumour specimens for sequencing in >50% of patients biopsied), successful sequencing analysis (completed ESS in >80% of patients sequenced), turn-around times (results within median 12 weeks) and clinical utility (actionable genomic results identified in >30% of patients sequenced). An exploratory objective was to identify the additional molecular information provided by ESS compared to TS and determine the proportion of patients where this resulted in changes to management. A sample size of 15 patients was selected to demonstrate feasibility. Approval from the Human Research Ethics Committee (HREC) of Peter MacCallum Cancer Centre was obtained.

Eligibility

Suitable patients with advanced solid malignancies were recruited from six institutes from the Victorian Comprehensive Cancer Centre (VCCC) alliance - (Peter MacCallum Cancer Centre, Royal Children's Hospital, Royal Melbourne Hospital, Royal Women's Hospital, St Vincent's Hospital, and Western Health). Patients were required to have a histological diagnosis of malignancy; biopsiable disease; be suitable for systemic treatment and a life expectancy of >4 months. Written informed consent was obtained from patients prior

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to collection of the paired samples. Only those with sufficient tumour from freshly obtained specimens were eligible for inclusion in this study.

Study procedures

Consented patients provided baseline clinical data including participant and disease characteristics, and current and intended future treatments prior to tumour sequencing. A disease site amenable to biopsy was identified and patients underwent biopsy once deemed safe to do so. Patients also underwent a peripheral blood sample collection for germline DNA. Specimens collected from biopsy were divided in two, with one portion being snap frozen and the other undergoing fixation. DNA was extracted from both the fresh frozen (FF) and formalin-fixed paraffin embedded (FFPE) specimens, followed by ESS and TS on both. TS were performed by a NATA accredited laboratory, while ESS was performed by a research laboratory. Genetic aberrations identified by either ESS or TS were presented at a multi-disciplinary molecular tumour board (MTB) comprising of scientist, pathologists, bioinformaticians, geneticists, and clinicians. Results were reviewed to determine the biological and clinical relevance of identified mutations and whether they were "actionable" and/or "druggable". These results were fed back to the treating clinician, and the impact of the results on patient management was recorded. (Figure 1. Study Schema and Work Flow process)

Laboratory procedures:

Following the tumour biopsy, each case underwent pathological review to assess the suitability of the tissue specimen, cellularity and percentage of tumour. Samples with insufficient tumour content were deemed ineligible for ESS and TS. Where there was sufficient tumour for analysis, DNA was extracted using the DNeasy Blood & Tissue kit (Qiagen, Hilden, Germany), and evaluated for quality and quantity by Qubit fluorimetry and Q-PCR. Where possible, both ES and TS were performed on the paired FF and FFPE specimens.

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TS were performed on the Miseq system v2 (Illumina, San Diego, USA). Amplification of mutational hotspots in 48 cancer-related genes in 212-targeted amplicons was performed using the TruSeq Amplicon Cancer Panel, (TSACP; Illumina, San Diego, USA) and customised amplicon panels developed at PMCC. Enseml Variant Effect Predictor (9) and additional in-house plugins were used to enrich annotations. All variants identified were loaded into a relational database staging area and linked with external variant databases including COSMIC (10), dbSNP, BIC, and IARC. The variants were placed within an ORM (object relational mapping) framework; and curated, explored and annotated with the PathOS Variant Management System (11) an in-house web application designed for the PMCC. Amplicons with less than 100 aligned reads were not analysed.

ESS was performed on the HiSeq 2500 (Illumina, San Diego, USA), using the Trusight One sequencing panel (Illumina, San Diego, USA) targeting 4,813 genes associated with known clinical phenotypes across 64,454 regions, spanning approximately 11.8Mb to a mean depth of coverage of 100x for blood, 500x for FFPE and 1000x for FF. Whilst this panel did not cover the entire exome, it represents approximately 25% of the coding exome and is considered to be on a clinically focused exome-scale level. Variants from germline DNA identified from the peripheral blood specimen were removed when tumour exome analysis was performed.

One Hiseq 2500 (Illumina, San Diego, USA) run was performed per case with 8% dedicated to DNA extracted from blood, 67% from FF and 25% from FFPE. GATK best practice and VarScan2 were used for variant calling. Only variants that pass the GATK quality filter and VarScan2 false-positive filter were included in further analysis. The Variant Effector Predictor (VEP) tool employing the dbSNP, ExAC 1000 genomes and COSMIC databases as well as SIFT and PolyPhen pathogenicity prediction scores was used to annotate variants.

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Germline variants were filtered based on pre-determined inherited cancer gene lists. (Appendix Table 1) Somatic variants were initially filtered based on a pre-determined gene list applicable to the clinical presentation. This was followed by more extensive analysis of all variants. Pathogenicity was assigned to variants based on the standards and guidelines for the interpretation of sequence variants recommended by the American College of Medical Genetics and Genomics and the Association for Molecular Pathology. (12) Variants were categorised as (C5) pathogenic, (C4) likely pathogenic, (C3) uncertain clinical significance –likely pathogenic (C2) uncertain clinical significance likely benign, or (C1) benign. The final annotated list of variants per sample was prioritised according to their annotation and presented for curation, interpretation and discussion at the MTB. (Figure 1B. Work Flow process)

Results

Between March 2014 and March 2015, 23 participants were approached for the study with 100% providing written informed consent. Of these, greater than 50% (15 of 23) had sufficient tumour available for sequencing and subsequently, only these 15 were eligible for further analysis. Each of these 15 patients had advanced stage cancer with a median age of 59 years, and a median of 2 prior lines of systemic therapy. Nine were female. The distribution of tumours and baseline characteristics are described in tables 1 and 2 (Table 1-2).

From the 15 eligible patients, average tumour purity of 65% was achieved in FFPE and 20% in FF specimens. Both TS and ESS were successful on all 15 patients. ESS was performed at a median depth of coverage of 341x for blood, 585x for FFPE and 1537x for FF specimens. (Figure 2C) There was high accuracy and concordance found between the number of unfiltered variants called from the FFPE and FF, however in 8/15 cases FFPE samples were inadequate to produce a sequencing library. (Figure 2A) Each case generated over 8,000 variants of sufficient experimental quality to analyse. The mean number of unfiltered variants called was 9,124 (Standard deviation (SD) 438.3) in blood, 9346 (SD 449.7) in FF tissue and 8625 (SD 619.8) in FFPE tissue. (Figure 2B) The list of variants was reduced to a list of highly clinically significant variants to enable clinical interpretation (Figure 2D).

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The time taken from consent to issuing of final report ranged from 5-16 (median: 12) weeks, with a median of 1 (range: 1-7) weeks for inter-laboratory specimen transfer, median 2 (1-5) weeks for DNA extraction from the trio of bio-specimens (blood, FF & FFPE), and a median of 5 (range: 3-8) weeks from sequencing to reporting for both ES and TS. If a 2nd extraction was needed this took an additional 4 weeks (Figure 4).

Variants were reported only for cancer related genes. TS identified 13 somatic variants. Clinically focussed ESS identified a total of 63 variants, consisting of 30 somatic variants (including all 13 identified by TS) and 33 germline variants. Overall, there were 48 unique variants. These were classified as pathogenic (n=17), possibly pathogenic (n=9), possibly benign (n=5) and of unknown clinical significance (n=32). Table 2 details the variants identified in individual patients, while Appendix Table 1 lists the variants by gene, alongside their clinical significance. ESS identified pathogenic somatic variants in 11/15 cases (73%) compared to 8/15 cases (53%) using TS; both well above the feasibility endpoint of 30%. The 33 germline variants were identified in 9 of the 15 cases (60%) and classified as pathogenic (n=2), possibly pathogenic (n=7), possibly benign (n=5), and of unknown clinical significance (n=19) (Table 2). The pathogenic and possibly pathogenic germline variants occurred in 7 cases.

TP53 was the most frequently mutated gene studied (9/63 variants), and occurring in 30% (5/15) of cases (Figure 2B). When clustered by gene function, somatic variants within DNA damage repair, and MAPK-RAS pathways were the most commonly affected (Appendix Table 2).

The clinical and actionable relevance of each variant identified was analysed and interpreted by a panel of experts at the multidisciplinary molecular tumour board meetings (Figure 3). Overall, findings informed cancer management in 10 cases (66%) with 7 cases being referred to FCC and 3 cases receiving a molecularly matched therapy. An additional 5 cases with druggable targets identified have not required

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matched treatment at the time of analysis (24 months post sequencing). When compared to TS, ESS resulted in changed management in 8 additional patients (7 referrals to a familial cancer centre, and 1 matched treatment), with another 2 cases not yet requiring matching. (Appendix Figure 1)

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Discussion:

To date, the use of ESS in oncology has been largely limited to the discovery space. Improvements in technology have now made it possible to explore the use of ESS in the clinic. This pilot study set out to assess the feasibility of performing real-time ESS in patients with advanced cancer and explore its potential for additional clinical utility when compared to TS. Based upon predefined endpoints, our pilot study has demonstrated that ESS is feasible in the clinical setting, and can provide additional benefit to patients.

Our study met all the feasibility endpoints. It was a surprise to us that all patients approached consented to participation in the study. Our initial concerns were that the requirement of a fresh biopsy specimen would deter many patients. While the 100% acceptability rate is very impressive, we do suspect that it merely represents a selection bias, i.e. those who were approached were those with disease suitable for biopsy and those who were thought most likely to accept a biopsy to facilitate personalized medicine. In regards to successful biopsies, we set the bar relatively low (at 50%), as we had concerns over the amount and quality of tumour tissue that would be required for ESS. The 65% success rate is fairly consistent with previous reports from phase I studies where paired biopsies have been required for PD studies.

Generating results in a timely manner was challenging. As this was our first foray into ESS, the pilot was seen as an opportunity to fine tune processes which had only just been developed. Accordingly, our patients were selected with this in mind, i.e. they were not requiring results urgently, reflected in the median lines of treatment being 2. It is generally accepted within the precision oncology community that results from molecular profiling should be produced within 3 weeks. This time frame allows treatment/trial decisions prior to patient deterioration, and is the target of most commercialised profiling services and academic profiling studies. In our study, ESS required a median of 12 weeks, just meeting our feasibility endpoint. Further analyses revealed that the batching for DNA extraction, sequencing and the manual curation represented a substantial proportion of this time. The experiences gained from this pilot have enabled us to generate protocols and standard operating procedures to meet the 3-week target in the future.

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The feasibility endpoint of clinical utility was easily achieved by our pilot. Previous studies of TS have generally demonstrated that actionable results can be identified in 30-50% of patients tested. Our aim of >30% was met, with ESS identifying an actionable somatic result in 73% of patients, also comparing favourably to TS (53%). An additional and unexpected benefit was the identification of possibly pathogenic germline variants in 46% of patients. Although small, our pilot does suggest ESS provides additional clinical utility above and beyond TS.

The field of precision oncology and the use of molecular profiling have evolved rapidly in a short time. Only 5 years ago, a study compared targeted sequencing using PACBIoRS to mutation genotyping using Sequenom MASSArray; at the time, both platforms were at the cutting edge of high throughput genetic testing in the clinic, whereas, now, both are rarely mentioned let alone used. While not the first, our pilot study is amongst a series of studies which have tested the clinical use of ESS. (13-16) As with the other studies, our study suggests ESS can be used effectively in a clinical setting.

While it is clear that ESS can identify enormous amounts of genomic data, there remain questions around whether more data actually results in clinical benefit. A pilot study conducted by the British Colombia Cancer Agency (BCCA) involved highly complex genomic testing, including whole genome analysis (WGA) and RNA-sequencing, to inform treatment decisions. Extensive testing of 100 patients resulted in 1 patient being enrolled onto a clinical trial and receiving a molecularly matched treatment, highlighting that more complex molecular testing in isolation may not facilitate precision oncology.(17) Clearly, all requisite components of a precision medicine program – molecular profiling and access to targeted agents must be available to enable this to be successful, a point which the authors acknowledge.

Our study has demonstrated that a serious by product of ESS, is the unexpected germline findings, which have clinical significance. In our study, ES identified an average of 2 germline and 2 somatic variants per

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participant, compared with an average of 1 somatic variant per participant by TS. The challenges encountered through unexpected germline findings are serious. Patients must be prepared for the fact that known pathogenic germline variants can be identified during ESS conducted for precision oncology purposes, and be aware of the potential implications this may have for their family. The advent of PARP inhibitors and the correlative benefit seen in patients with DNA repair defects demonstrates that germline variants can also facilitate precision oncology. (18, 19) Thus, the role of geneticists and genetic counsellors is likely to become increasingly important, not only after the variants are found, but also before the testing is performed.

The ability to profile each cancer in an individual patient for actionable mutations provides unprecedented possibilities for matched targeted therapies in a selected patient population. Several proof-of-concept studies that pair drugs with molecularly eligible patients to assess the impact from the targeted therapy on outcomes are currently underway. (4, 20, 21) Global initiatives such as the 2016 $200 million NIH Precision Medicine Initiative, the 100,000 Genome Project and the Cancer Moon-shot 2020 project will undoubtedly help to accelerate genomic approaches to the design and testing of tailored cancer treatments. It is increasingly important that oncologists appreciate the fundamental considerations and implications surrounding genomic testing.

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Conclusion:

This study did not intend to establish fully validated ES. It was designed to identify gaps between the current state of genomic testing and requirements for future clinical implementation. This study demonstrates that exome-scale sequencing is technically feasible in routine practice and complements clinical decision-making. However, further development of integrative laboratory work processes in order to shorten the time to results is required, at least within our program. Over time, the continued collection of data through ESS will improve our understanding of the roles of variants in cancer and tip the balance towards increased utility and feasibility of wide-scale genomic sequencing.

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Figure Legends

Figure 1. Study Schema & Work Flow

Legend:
Figure 1A: Study Schema. Figure 1B: Work Flow process. ESS= Exome-scale sequencing; MTB= Molecular Tumour Board; FFPE= Formalin Fixed Paraffin Embedded tissue; FF= Fresh Frozen tissue; SV= structural variations; COSMIC= Catalogue of Somatic Mutations in Cancer; dbSNP= Single Nucleotide Polymorphism database; IARC= International Agency for Research on Cancer; BIC= Breast cancer Information Core database; PMCC= Peter MacCallum Cancer Centre.

Figure 2. Analyses of unfiltered and filtered variants identified via exome scale sequencing of tumour DNA from all 15 cases
Legend: Figures 2A-C: Unfiltered variant results

Figure 2A: Number of unfiltered variants called in each of the 15 cases, absence of blue column indicates FFPE samples were inadequate to produce library.

Figure 2B: Box and whiskers plot that summarises the distribution of called unfiltered variants for each sample type. Box represents the inter-quartile range (IQR) and tip of whiskers are at 1.5 x IQR. Median of number of unfiltered variants for blood, FFPE and frozen samples are 9124, 8625 and 9346 respectively.

Figure 2C: Box and whiskers plot that summarises the achieved sequencing coverage for each sample type. Median of average coverage for blood, FFPE and frozen tissue are 341, 585 and 1537 respectively.

Figure 2D: Total count of Filtered somatic and germline variants called by gene.

ES= exome sequencing; FFPE= Formalin Fixed Paraffin Embedded tissue specimen; Fresh= Fresh frozen tissue specimen

Figure 3. Analysis of Actionable and Potentially "Druggable" Variants identified with examples of therapeutic options

Figure 4. Overview of logistic feasibility, processing and turn-around times in a clinical setting

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Supplementary Tables and Figure

Appendix Table 1: Expanded Hereditary Cancer Panel

Appendix Table 2. Summary of Variants identified and the associated pathways

Legend: Pathogenicity C1=Benign; C2=probably benign; C3=unknown clinical significance; C4=probably pathogenic; C5=pathogenic; A=Actionable; D= Druggable; P= Prognostic

Appendix Figure 1. Comparison of key altered molecular pathways identified in WES study and molecularly targeted clinical trials available at time of data analysis

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Table 1. Summary of Participants characteristics

†None of these involved familial cancer syndromes

Table 2. Summary of primary tumour, sequencing results and outcomes

Table 3. Comparison of ESS and TS

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