Multigene profiling to identify alternative treatment options for glioblastoma: a pilot study

Tania Tabone,[1] Hazem J Abuhusain,[2] Anna K Nowak,[3] Australian Genomics and Clinical Outcome of Glioma (AGOG) Network, Wendy N Erber,[1] Kerrie L McDonald[2]

▶ Additional material is published online only. To view please visit the journal online (http://dx.doi.org/10.1136/jclinpath-2014-202173).

[1]Translational Cancer Pathology Laboratory, School of Pathology and Laboratory Medicine, University of Western Australia, Crawley, Western Australia, Australia
[2]Cure Brain Cancer Neuro-oncology Group, Prince of Wales Clinical School, UNSW Australia
[3]Department of Medical Oncology, School of Medicine and Pharmacology, University of Western Australia, Sir Charles Gairdner Hospital, Nedlands, Western Australia, Australia

Correspondence to
Dr Kerrie L McDonald, Cure Brain Cancer Neuro-oncology Group, Prince of Wales Clinical School, Level 2, Lowy Cancer Research Centre, UNSW, Kensington, New South Wales 2052, Australia; k.mcdonald@unsw.edu.au

AGOG Network Authors are listed in online supplementary table S1.

Received 8 January 2014
Accepted 3 March 2014
Published Online First 2 April 2014

To cite: Tabone T, Abuhusain HJ, Nowak AK, *et al*. *J Clin Pathol* 2014;**67**:550–555.

ABSTRACT

Glioblastoma (GBM) is a highly aggressive malignancy and the most effective treatment regime has a high relapse rate. Increasingly, the development of therapies involves defining drug–diagnostic combinations where the presence of a molecular target or marker identifies patients who are most likely to respond to a specific therapy. Trials in other solid cancers have demonstrated clear utility in the incorporation of biomarkers to stratify patients to targeted treatment, however, there are no mutations that are currently used to inform treatment options for GBM.

Aims We piloted the use of high-throughput next-generation sequencing technology to identify genetic mutations in 44 GBM specimens that may be amenable to current or future targeted therapeutic strategies.

Method Somatic mutation profiling was performed using the AmpliSeq Cancer Hotspot Panel v2 and semiconductor sequencing technology.

Results A total of 66 mutations were detected in 35/44 (80%) patients. The number of mutations per tumour ranged from 0 to 4 (average per tumour=1.5). The most frequent mutations were in *TP53* (n=12), *PTEN* (n=9), *EGFR* (n=8) and *PIK3CA* (n=5). Clinically actionable somatic mutations were detected in 24/35 (69%) patients.

Conclusions This study demonstrates that the use of an 'off-the-shelf' oncogene primer panel and benchtop next-generation sequencer can identify mutations and potentially actionable targets in the majority of GBM patients. Data from this pilot highlights the potential for targeted genetic resequencing to identify mutations that may inform treatment options and predict outcomes.

INTRODUCTION

Identification of somatically acquired genetic mutations has proven useful in unravelling the pathogenesis of many cancers. Genetic mutations also serve as biomarkers for disease diagnosis, classification and stratification. Advances in throughput and sensitivity of mutation screening technologies now provide a platform to implement biomarkers to assess disease prognosis, identify therapeutic targets and monitor treatment response through detection of minimal residual disease.

Glioblastoma (GBM) is not preventable by any known lifestyle changes, and there has been no significant change in the 5-year relative survival (<5%) in over 20 years. There is currently no curative treatment for GBM and progression is usually rapid and aggressive, hence, this form of cancer is fatal and presents a significant burden in terms of years of life lost. GBM is particularly heterogeneous, and as a consequence, each therapeutic is only likely to be effective in a small proportion of patients. It has never been more clinically relevant to identify somatically acquired mutations in the hope of better matching therapeutic options available for patients with advanced disease.

The Cancer Genome Atlas (TCGA) has generated an unprecedented amount of multidimensional data and is providing a global description of the genetic abnormalities that are present in GBM. An average of 47 mutations per GBM have been identified, although far fewer were candidates to be driver mutations.[1] Candidate driver mutations are most frequent in the TP53 pathway, RB1 pathway, and the PI3K/PTEN pathway. Mutations in these pathways are generally mutually exclusive, suggesting that they are key to tumourigenesis, and functionally equivalent.[2] Several genetic alterations operative in the development of GBM have been considered prognostic, including amplification of *EGFR*,[3–6] *TP53* mutations[1 7] and *PTEN* mutations.[8 9] At least two distinct cluster profiles have been identified: proneural and mesenchymal-angiogenic signatures, which differ in survival and response to treatment.[10 11] Proneural tumours are more commonly *IDH1* mutated, and a better prognostic group.[12 13]

The scale of sequencing undertaken by the TCGA could never be replicated for every patient's tumour in the domestic pathology laboratory. However, targeted resequencing of cancer consensus genes has potential for implementation in a routine clinical setting. This method is rapid and cost effective compared to multiple individual diagnostic tests, and the data output is bioinformatically manageable compared to whole genome sequencing approaches. Multigene mutational profiling proves a wealth of information to the treating clinician to identify the best treatment options for individual patients, so that these can be administered in a timely manner for better outcome. Herein, we screened DNA from 44 GBM patients for somatic mutations in 50 oncogenes and tumour suppressor genes using semiconductor sequencing.

MATERIALS AND METHODS

Cohort

Fresh-frozen GBM tumour samples (n=44) were identified for this study from the prospectively collected Australian Genomics and Clinical Outcome of Glioma (AGOG) Biospecimen Resource. Human Research Ethics Committee approval was obtained for the collection and use of freshly frozen human GBM tissue for this project; all participants had previously provided written informed consent for

tissue biobanking and research use. Only newly diagnosed, untreated GBM specimens were included in this study. Patients were selected based on a diagnosis of primary (de novo) GBM and to incorporate a range of common survival outcomes from <6 months to >24 months. Patient age, gender, tumour location and other genetic markers (ie, MGMT methylation status and *EGFR* amplification) were randomised across the survival groups. A summary of the clinical features for each tumour is provided in table 1. No germline DNA was available for this study. All patients were *IDH1* wild type at position 132 according to previous immunohistochemical (IHC) study data. DNA from 50 mg of snap-frozen tissue was extracted and purified using the QIAamp DNA Mini Kit as per protocol (Qiagen). The purified DNA was quantified using the Qubit 2.0 Fluorometer and Qubit dsDNA HS Assay Kit (Life Technologies).

MGMT methylation analysis

Bisulfite modification followed by CpG pyrosequencing was performed to assess the percentage level of *MGMT* promoter methylation for each tumour specimen. Chemically methylated or unmethylated universal human genomic DNA (Millipore) controls were included. In brief, tumour DNA (500 ng) was bisulfite modified using the EZ DNA methylation kit (Zymo Research) according to the manufacturer's recommendations. The CpG pyrosequencing methylation assay was performed with the PyroMark MGMT kit (Qiagen) on a pyrosequencer (PSQ96 MA System, Qiagen) MA system (Qiagen), according to the manufacturer's protocol and as published previously.[14]

Detection of EGFR gene amplification

EGFR amplification was reported in the patient pathology reports by PathWest Laboratory Medicine WA, using the Multiplex Ligation-dependent Probe Amplification (MLPA, MCR-Holland) assay as described previously.[15]

AmpliSeq Library preparation

Amplicon libraries for individual patient DNA samples were prepared using the Ion AmpliSeq Ready-to-use Panel to amplify the target regions of 50 oncogenes and tumour suppressor genes implicated in solid tumours (see online supplementary table S2). Target regions were amplified from 10 ng of genomic DNA in a single multiplex PCR reaction using the premixed Ion AmpliSeq Cancer HotSpot Panel v2 and the AmpliSeq HiFi Master Mix (Ion AmpliSeq Library Kit 2.0). The resulting 207 multiplexed amplicons were treated with FuPa Reagent (Life Technologies) to partially digest the primer sequences and phosphorylate the amplicon ends. Sequencing adapters with unique barcodes (Ion Xpress Barcode Adapters 1–96, Life Technologies) were ligated to the amplification products and purified using Agencourt AMPure XP Reagent (Beckman Coulter) according to the manufacturer's instructions. The amplicon libraries underwent a second round of amplification to quantify and visualise the library fragments using the Agilent 2100 Bioanalyser and Agilent High Sensitivity DNA Kit (Agilent Technologies) according to the manufacturer's instructions. The final library concentrations were standardised to 100pM in low Tris-EDTA (TE) buffer (Life Technologies).

Emulsion PCR and semiconductor sequencing

Eight individual patient barcoded libraries (100pM each) were pooled, and the final concentration adjusted to 9pM in nuclease-free water. Multiplexed barcoded libraries were then clonally amplified with biotinylated primers onto Ion Sphere Particles (ISPs; Ion Xpress Template Kit 2.0) by emulsion PCR

Table 1 Clinical data for GBM patients divided into specific survival times

	Survival group 1 <6 months	Survival group 2 6–12 months	Survival group 3 12–24 months	Survival group 4 >24 months
Patient numbers	8	13	15	8
Median survival (months) (95% CI)	4.0 (3.7 to 4.1)	10.5 (7.2 to 13.9)	14.2 (13.8 to 14.6)	30.3 (27.7 to 32.9)
Median age (years) (range)	66 (51–85)	65 (38–76)	60 (45–77)	59 (24–70)
Location in the brain				
Frontal	3	6	2	4
Temporal	3	4	7	2
Parietal	–	3	3	1
Occipital	1	–	1	1
Multifocal	–	–	1	–
Unknown	1	–	1	–
Extent of surgery				
Total resection	8	12	14	8
Biopsy	0	1	1	0
Received concurrent treatment				
Yes	3	12	15	8
No	5	1	0	0
MGMT promoter methylation				
Yes	2	1	3	3
No	6	11	9	2
Not tested	0	1	3	3
EGFR amplification				
Yes	1	2	4	1
No	2	2	3	2
Not tested	5	9	8	5

GBM, glioblastoma.

(emPCR) using the OneTouch 2 System (Life Technologies). Following emPCR, the ISPs were recovered by centrifugation and template-positive ISPs were isolated by binding to streptavidin-coated beads (Ion OneTouch 2 Kit). A sequencing primer was ligated to the enriched ISPs before loading onto an Ion 316 Chip for single-end sequence analysis. Semiconductor sequencing was performed on an Ion Personal Genome Machine (PGM) Sequencer using the Ion PGM 200 Sequencing Kit (Life Technologies).

Coverage and data analysis

Torrent Suite V.3.6.2 Software (Life Technologies) was used to parse barcoded reads, align reads to the reference genome (human genome build 19; hg19), base call and to generate run metrics, including chip loading efficiency, total read counts, quality and total coverage. ANOVAR (Biobase) and Oncomine Gene Browser (Compendia) were used to identify variants and predict amino acid changes and clinical significance. The Integrative Genomics Viewer (IGV) was used to visualise the read alignment and the presence of variants against the reference genome and to confirm the integrity of variant calls by detecting possible strand bias and sequencing errors.

RESULTS

Clinicopathological correlation

The study population consisted of 44 GBM patients with a higher ratio of males to females (30:14). The median age at diagnosis was 63.3 years (range 24–85 years). The median survival rate was 12.0 months (range 3.7–41.5 months) (table 1). The majority of tumours were localised to the frontal (15 patients, 34%) and temporal lobes (16 patients, 36%). Seven patients had GBM arising in the parietal lobe region, three patients had GBM arising in the occipital lobe, and one patient's GBM was multifocal. The tumour location was unknown for two patients. The majority of patients had total (greater than 95%) tumour resection during their first surgery and two patients received biopsies only. Following surgery, 38 out of 44 patients received combined radiotherapy and chemotherapy (temozolomide (TMZ)).[16] *MGMT* promoter methylation results were available for 37 of the 44 patients. The percentage methylated of those tested was 24%. *EGFR* amplification is not routinely requested as a pathology test, thus, results were only available for a small subset of the cohort (19 out of 44). *EGFR* amplification was detected in almost half the tumours tested (9 out of 19).

The 44 GBM patient cohort was divided into four groups according to survival outcomes: Group 1 (n=8): survival <6 months; Group 2 (n=13): survival 6–12 months; Group 3 (n=15): survival 12–24 months and Group 4 (n=8): survival >24 months (table 1; figure 1). Age of the patients did not significantly differ between the four groups (p value: 0.151). A high percentage of patients who did not receive radiotherapy or any adjuvant chemotherapy showed very poor overall survival (Group 1).

Sequence coverage

Analysis of our sequencing data showed a mean coverage depth of 1600 reads per nucleotide position within the target region. The 1×, 10× and 100× coverage were 100%, 100% and 99%, respectively. Sequence coverage was assessed from the number and distribution of reads across target amplicons. An average of 5.3 million of the total 6.3 million addressable wells in the Ion 316 Chip were consistently loaded with ISPs, and after subtracting poly clones (multiple-templated beads), low quality sequence reads (<Q20) and primer dimer, 3.3 million (62.2%) of these particles contained library templates. The individual samples averaged 373 784 mapped sequence reads (range 312 271–653 751), with a mean read depth of 1809 per variant. The distribution of reads across the 207 amplicons was consistent across sample, with an average uniformity of amplicon coverage of 95.41% and 95.92% of the sequence reads mapped to targeted gene regions (aligned to hg19). The mean read length was 111 bp (range 105–114 bp).

Sequence validation

A minimum of 300 000 reads with a quality score of AQ20 (one misaligned base per 100 bases) was used as the measure of successful sequencing of a sample. For a variant to be considered true, sequencing coverage of 1000× and a variant frequency of at least 10% in a background of wild-type allele were used as a minimum requirement in this study.

Variants detected

Overall, 17 288 299 nucleotides were interrogated across the 44 GBM cases analysed. A total of 644 mutations in 32 genes were identified, with a mean of 14 variants per patient (range 7–21 variants). Since constitutional DNA was not available to deduce germline polymorphisms, a stringent mutation detection criterion was applied in order to identify somatically acquired mutations. Mutations present in the population with a minor allele frequency greater than 5% according to the 1000 Genomes Project, intronic mutations and synonymous exonic mutations were removed. Therefore, a total of 66 variants were predicted to cause non-synonymous coding changes in 18 different genes, with an average of 1–2 variants detected per patient (range 0–4 variants) (table 2; figure 1). Of these, 48 variants were unique in the study cohort with 39 variants not previously described in dbSNP, 1000 Genomes Project or the published literature. Using this stringent mutation detection approach, there were nine GBM cases in which no significant mutations were detected.

The non-synonymous mutations detected are summarised in online supplementary table S3 and graphically represented in figure 1. In the poorest survival group (Group 1: survival <6 months, n=8), two patient tumours' harboured no non-synonymous mutations. For the remaining patients in Group 1, the most commonly mutated gene was *PTEN* found in two out of the six patients, however, the mutation position differed. Patient 17 harboured 2 *PTEN* mutations (p.Q171R and p.F241S), while patient 15 had a stop-gain mutation (p.R233X). Five of the six patients harboured potentially treatable mutations. Two patients were methylated at the *MGMT* promoter, however, these two individuals were not treated with temozolomide.

For the Group 2 patients (survival 6–12 months, n=13), three patients harboured multiple mutations (≥2). The *EGFR* mutation, p.A289 V, located in the extracellular domain (ED) region of *EGFR* was detected in four patients. Eighty-two per cent of the mutations detected in Group 2 were potentially amenable to drugs, which is clinically interesting given that the majority of the patients (92%) were unmethylated at the *MGMT* promoter. Patients belonging to group 3 demonstrated a higher survival time (survival 12–24 months, n=15) compared to the cohort survival median time (12.0 months (range 3.7–41.5 months). Forty-seven per cent of the tumours were located in the temporal region of the brain (7 out of 15). Multiple non-synonymous mutations (≥2) were seen in six patients. Mutations in *TP53* were common (50%). Mutations in the

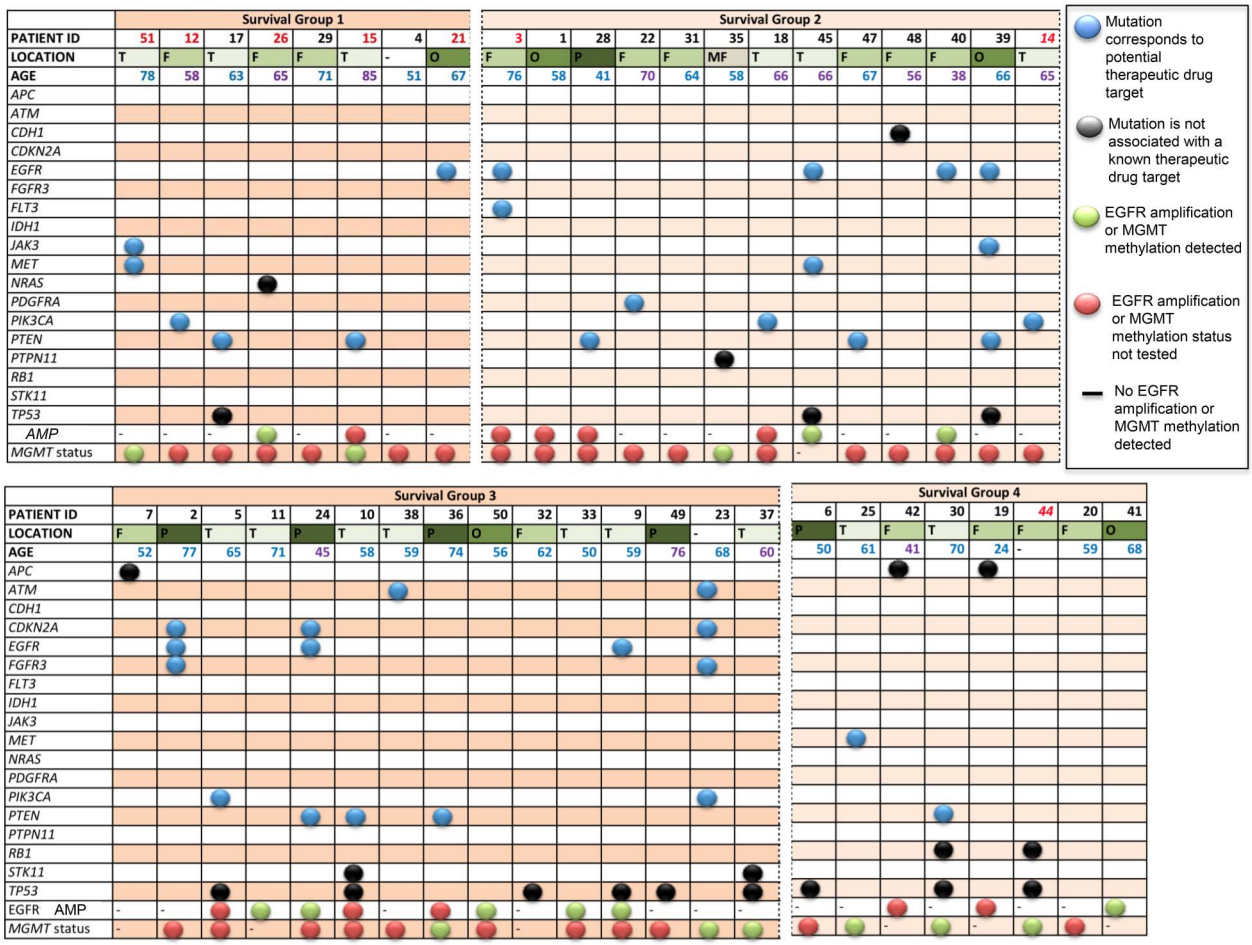

Figure 1 Distribution of mutations in patients categorised by overall survival times. The mutational profile of 18 genes is illustrated. Patients are listed in order of increasing time of survival and grouped into 4 survival groups: Group 1: survival <6 months; Group 2: survival 6–12 months; Group 3: survival 12–24 months and Group 4: >24 months. *Blue spot*: mutation corresponding to a potential therapeutic drug target; *Black spot*: mutation is not associated with a known therapeutic drug target; *Green spot*: EGFR amplification or MGMT methylation detected; *Red spot*: no EGFR amplification or MGMT methylation detected; (–) represents EGFR amplification or MGMT methylation status not tested. Tumour location is indicated at the top of the figure where: T, temporal; F, frontal; O, occipital; P, parietal; MF, multifocal. Age is given in years and the blue font represents male and the purple font represents female.

EGFR gene were also detected, and although localised to the ED, they were all located in different positions (p.G392V, p. R108K and one tumour had both p. A289V and p.V729M mutations). Amplification of the *EGFR* gene was also a common event in this group with five of the eight tested showing amplification. Three patients had *PTEN* mutations, while two had mutations in *CDKN2* and *STK11*. Promoter methylation of the *MGMT* gene was again uncommon (25%). Sixty-seven per cent of patients harboured mutations that were potentially treatable with targeted agents.

In the longest survival cohort, Group 4 (>24 months, n=8), no mutations were detected in two of the patients. Mutations in the *TP53* gene were detected in four out six patients. Two patients harboured mutations in *RB1* and another two patients had mutations in the *APC* gene. These mutations were not present in the patients belonging to the other three survival groups. Unfortunately, there are no known therapeutics to target *TP53*, *RB1* and *APC*. Therefore, four out of the six patients in this survival group would not benefit from gene-targeted therapy. Incidentally, *MGMT* promoter methylation was detected in three out five of the tested patients.

DISCUSSION

Diagnosis and management of many solid tumours now relies on the integration of genomic information into the routine pathology workflow and the consolidation of a range of testing to a single platform is desirable. For a complex disease such as GBM, a single platform and a single assay may never be achieved because of the different testing modalities needed to detect methylation, chromosomal loss, gene amplification and deletion and protein expression. However, mutations in key genes such as *TP53*, *EGFR*, *PTEN* and *IDH1* are not currently being tested for in routine pathology, and the field would significantly benefit from further development of multigene mutational profiling.

A significant unmet need is to discover new treatments to improve the standard-of-care therapy (chemoradiotherapy: radiation therapy plus temozolomide followed by six cycles of adjuvant temozolomide). *MGMT* promoter methylation is a strong predictor of benefit with chemoradiotherapy.[17–20] This predictive role was prospectively confirmed by the Radiation Therapy Oncology Group (RTOG) study 0525.[21] *MGMT* promoter methylation status is also a useful predictive marker for benefit

Table 2 Summary of sequencing data

Total number of genes mutated	32
Total number of mutations:	644
UTR3	87
Intronic	89
Splicing	46
ncRNA, intronic	3
Exonic, synonymous	279
Exonic, non-synonymous	138
Exonic, frameshift	3
Exonic, stopgain	5
Exonic, stoploss	1
Total number of genes mutated with a MAF <5%	24
Total number of mutations with a MAF <5%:	113
UTR3	2
Intronic	30
Exonic, synonymous	15
Exonic, non-synonymous	58
Exonic, frameshift	2
Exonic, stopgain	5
Exonic, stoploss	1
Total number of genes with a non-synonymous mutation	17
Total number of non-synonymous mutations	66

MAF, minor allele frequency; ncRNA, non-coding RNA; UTR, untranslated region.

from TMZ in patients older than 70.[17 18 22] There is very little progress or improvement for the management of GBM patients who are MGMT unmethylated and do not derive survival benefit from TMZ. Targeted inhibitors have been added to TMZ to improve efficacy, notably temsirolimus (an inhibitor of PTEN) and irinotecan (CPT-11; topoisomerase I inhibitor).[23] The trials were not positive, and no rationalisation of the patient cohort by testing the treatments' companion biomarker was conducted. Testing a larger number of genes of which could harbour clinically actionable somatic mutations is urgently needed for GBM, in particular, for the survival Groups 2 and 3, where MGMT methylation was rarely detected.

We have demonstrated in a pilot study of GBM that a 'personalised medicine' approach using an off-the-shelf gene panel and a bench-top sequencer had usefulness in identifying biomarkers for targeted drug therapy. We found mutations in *EGFR*, *TP53* and *PTEN* genes were frequent, occurring in 18%, 27% and 20% of patients, respectively. A recent study by Ciriello *et al*[24] also found similar mutational frequencies in their analysis of TCGA data with mutations detected in *EGFR* (12%), *TP53* (31%) and *PTEN* (20%). Mutations of potential therapeutic significance were detected in 24/35 (69%) patients.

As this was a pilot study to demonstrate that we could detect gene mutations for targeted drug therapy, the study was not adequately powered to detect mutations with prognostic or predictive utility. A significant challenge of this data was the number of different missense mutations found within each gene, and interpreting the clinical value of such mutations. *EGFR* mutations were all localised to the ED region.[25] The most common mutation identified was A289 V (4 out of 8 GBM) and single tumours with the mutation R108K and G598V. One study has reported that the ectopic expression of these mutations (A289V, R108K and G598V) in NIH-3T3 fibroblasts conferred anchorage-independent colony formation, and formed tumours in vivo demonstrating that the *EGFR* ED mutants are oncogenic.[26] The same study also demonstrated a greater sensitivity to erlotinib in patients harbouring ED mutations.[26] Furthermore, targeted therapy has been used effectively for patients with non-small cell lung cancer (NSCLC) with responsiveness to the small molecule EGFR tyrosine kinase inhibitors (TKIs), erlotinib and gefitinib, tightly linked to the presence of missense mutations in the *EGFR* kinase domain. While current clinical trials with these TKIs have failed to show any response in GBM clinical trials, the presence or absence of mutations in *EGFR* were not taken into consideration in these studies.[27 28] A restored hope for targeted EGFR therapy for GBM has been generated from the development of second-generation irreversible EGFR inhibitors such as dacomitinib (PF-00299804).[29] Dacomitinib has a higher affinity for the *EGFR* ED and irreversible blockade may result in a longer suppression of EGFR.

Mutations in the *TP53* gene are more commonly associated with low-grade astrocytomas (50–60%) and secondary GBM (70%).[30] However, similar to the TCGA analysis of GBM,[1] we also found a high incidence of *TP53* in our GBM cohort. Thirty-eight per cent of the mutations found resided within the DNA binding domain (exons 4–8) of *TP53*. Missense mutations in this region, but not the transactivation domain (exons 1–3) or the oligomerisation domain (exons 9–11), influence the survival outcomes of patients with breast cancer.[31] Sequencing of *TP53* is not carried out in neuropathology. Rather, IHC detection of the mutant TP53 protein, based on a higher percentage staining reflective of the mutant protein's longer half-life, is conducted. It is not accurate and is not clinically relevant.

In summary, this pilot study of 44 *de novo* GBM demonstrates that the use of an 'off-the-shelf' oncogene primer panel and benchtop next-generation sequencer can identify mutations and potentially actionable targets in the majority of GBM patients. Although not shown here, the methodology is applicable to formalin-fixed paraffin-embedded tissue and, as such, it could be adopted into a clinical setting to accompany other routine tests. Further, the mutation yield could be further improved with a less 'cancer-generic' and more specific GBM primer

Take home messages

- Studies and trials in other solid cancers, including breast, pancreatic and non-small cell lung cancer (NSCLC) have demonstrated clear usefulness in drug–diagnostic combinations, however, there are no mutations that are currently used to inform treatment options for glioblastoma (GBM).
- GBM is heterogeneous and as a consequence, each therapeutic is only likely to be effective in a small proportion of patients.
- The sequencing of clinically actionable somatic mutations in GBM is highly relevant, but not conducted. This single platform assay could be developed preclinically with a view to be used clinically to inform drug trials exploring targeted agents to *EGFR*, *PTEN*, *PIK3CA* and *CDK*. Additionally, mutations in the *TP53* and *RB1* genes may influence response to a wide range of drugs targeted to the cell cycle of the cancer.
- Data from this pilot study highlights the potential for targeted genetic resequencing to identify mutations that may inform treatment options. Future large-scale trials will be required to validate and determine the true clinical utility of this approach for implementation into the clinic.

panel. There is an urgent need to find alternative therapies for MGMT unmethylated GBM and the development of therapies will no doubt involve defining drug-diagnostic combinations where the presence of a molecular target or marker identifies patients who are most likely to respond to a specific therapy.

This pilot study highlights the potential for targeted genetic resequencing to identify mutations that may inform treatment options. To complement this technology, patient-derived cell lines harbouring the same mutations would provide a valuable resource for high throughput testing of targeted drugs with a broad range of anti-tumour activity. Accurate diagnosis, which includes traditional histopathology and molecular histology, will lead to better treatment selection and improved survival times. Future large-scale studies will be required to determine the clinical usefulness of this approach for screening or monitoring purposes.

Acknowledgements For technical support and expertise we thank R Allcock.

Collaborators Australian Genomics and Clinical Outcomes of Glioma (AGOG) Network.

Contributors TT performed experimental work and data analysis. HJA and AN provided patient materials. WNE provided project leadership. KLM collected clinical data and patient materials and coordinated the study. All authors contributed to the final manuscript.

Funding This work was principally supported by the Olle Fund For Brain Cancer Research. This work was also supported by the Cancer Council NSW with assistance from Cancer Australia.

Competing interests TT was supported by a Cancer Pathology Fellowship from Cancer Council Western Australia. KLM was supported by the Cure Brain Cancer Foundation.

Ethics approval Human Research Ethics Committee South Eastern Sydney Local Health District Prince of Wales Hospital.

Provenance and peer review Not commissioned; externally peer reviewed.

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