Author’s response to reviews

Title: Sequencing and curation strategies for identifying candidate glioblastoma treatments

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Reviewer reports:

Mauro Biffoni (Reviewer 1): The authors describe the results of a comparative evaluation of different methods to identify actionable targets in a therapy-orphan, aggressive malignancy. They show that in 30 glioblastoma patients all but one tumors carried a genetic alteration suitable for targeted therapy when analysed by whole genome sequencing (WGS). Whole exome sequencing (WES) produced results with a good correlation with WGS in the evaluation of variant allele frequency. The two techniques allowed the identification of at least one actionable target in all patients' samples. In a limited number of cases information from WGS/WES caused a therapeutic decision by the clinician, the time to have the information available for clinician was identified as a critical step for usefulness. By comparing sequencing with gene panels analyses they found that WGS was not able to identify a potential target in 2.5% of the cases whereas panels in 39.5%. Automated results curation was correlated with manual one but required few minute instead of months. The authors conclude that WGS/RNA sequencing will be reasonably a potential routine tool provided that costs and efficiency improve and automatic curation of results is used. The manuscript is well written and only minor modifications are needed:

Authors should explain all acronyms the first time they are used.

The manuscript was reviewed for acronyms used and explained all first use.

The identification of Caris Molecular Intel as Panel 6 is missing in the section Patients and Methods-Comparison of Targeted Panels (page 11, row 34).
The identification of this panel (Panel 6) is added.

Page 13, row 20, reasons for exclusion of 6 patients should be described also in the text.

Reasons for exclusion of 6 patients were also added to the text.

In Table 1 age should be expressed as median (range) rather than mean (SD), total number of patients indication would ease the reading.

The age is now expressed as median (range) and the total number of patients is included.

Page 13, rows 47-52, the ploidy of four remaining tumors should be indicated, e.g. 2 were hypoploid and 2 hyperploid.

Four samples were hyperploid and three were hypoploid. This is added to the text.

Some typing errors are present, e.g. EFGR for EGFR at page 15 row 46, vemurafinib for vemurafenib at page 17 row 29.

Typing errors are corrected.

Michael Berens (Reviewer 2): Summary

Frank et al describe consenting, collection, genomic analysis (whole genome sequencing and RNA Seq), gene-drug matching analytics and tumor board reporting to physicians for 30 GBM cases enrolled from 7 clinical sites in New York. Tumor (80X) and matched germline (40X) WGS were used to estimate chromosome, gene, and allele copy numbers, along with mutations, intronic and exonic splice variants; SNVs were assigned to Tiers 1 – 4 as previously published (citation #18 needs to be fully annotated). RNA Seq was used to support WGS variant calls as well as to report level of expression of each variant; by comparing against the gene expression of 169 GBM samples (TCGA), a modified z-score for each gene was calculated and used as a proxy of differential gene expression. Genomic reporting was compared between manual (expert) curation and Molecular Profiling Analysis (MPA) WGA (IBM Research proof-of-concept environment of Watson for Genomics) as published. MPA results were used to ascribe direct and indirect therapeutic options, selecting from molecularly-targeted therapies only. WGA updates with each addition of a new clinical sample, as all previous WGS and RNA Seq data of prior samples become the reference set against which z-scores were derived. WGS data were compared with findings reported on Targeted Panel findings for a subset of GBM cases. Time from sample receipt to tumor board averaged 4.5 months, with 1.9 months consumed in scheduling the tumor board after the pipeline analysis was completed. The results from 30 GBM cases uncovered 44 genes with targetable SNVs; all but one sample had CNVs that were
considered targetable. Three (of 30) GBM cases sequenced led to a change in the therapeutic strategy in patient care.

Critique

The influence of tumor purity on sequencing analysis warrants comment.

We have previously shown that >95% variant calling sensitivity with at least 20% tumor purity. Both SNV/Indel and CNV sensitivity began to be compromised below 30% purity resulting in loss of identification of sub-clonal heterogeneity and copy number deletions.[1] In this current study, tumor purity ranged from 15 to 95%. In the 2 samples with tumor purity <20% (15 and 19%), it is possible that not all variants present were detected. Despite this, it was possible to make clinically actionable and/or significant calls in all samples. The number of samples with purity <20% is added to the text and this is added to the discussion.

The influence on sample preservation method on sequencing analysis warrants comment.

Previous studies have shown that sequencing coverage is higher and more uniform with fresh frozen versus paraffin-embedded samples. Nonetheless, there was 71% agreement for SNVs and 0.44 median correlation for CNVs, resulting in the detection of 98% of actionable variants seen in fresh frozen samples in FFPE samples. This suggests that FFPE samples perform similarly for this purpose.[2] We’ve noted this in the discussion as a limitation.

The detailed molecular models of PIK3R1 and PIK3CA (Figure 1), indicating the SNVs detected seems a curious component of the manuscript. This figure is minimally referenced in the Results, and not commented on in the Discussion. Eliminate Figure 1.

The figure is removed.

The utility of including of reporting the 3 patients for whom two samples were sequenced is unclear. If these findings are to be included, the patients should be noted in Table 2, and the implications of the findings warrant comment in the Discussion.

We removed the section on the results of 2 samples sequenced from each of three patients from the results.

Why were RNA Seq data available from only 26 samples? The source of the “fail” warrants notation.

Of 30 samples, sufficient RNA quantity for library preparation was extracted from 27 and sufficient coverage and high quality sequences were obtained from 26. This is edited in the text.
The correlation of VFA called by DNA and RNA sequencing (fig 3) is only minimally discussed. Did the correlation coefficient track better or worse for specific genes?

Because we had a limited number of samples with multiple variants per gene, we could not perform a gene-specific correlation analysis of DNA and RNA allelic frequencies. Some genes in Fig 3 only have one variant per gene. Therefore we combined all Tier 1-3 variants to illustrate the correlation. A larger sample size may show correlations in allelic frequencies in overamplified genes and highly expressed genes where read count tends to be higher than the mean in both DNA and RNA sequencing. We have included this observation in the RNA analysis section on page 16.

No mention is made of the allele abundance from the RNA Seq analysis.

In our analysis, we did not consider allele-specific expression. Our focus was on using RNA-Seq to confirm somatic variants detected in WGS, count the number of reads supporting each variant and compared with the number of reads supporting the germline allele (in addition, we used RNA-Seq to identify fusion genes and measure gene expression). It would be interesting to analyze this and other datasets in search for allele specific expression, or regulated (up- or down-regulated) expression of alleles somatically altered, taking into account copy-number changes etc. It is, however, beyond the scope of this current study and would presumably require a much larger sample size (in order to accumulate recurrent events).

The Splice Variant reporting (Figs 4 and 5) are of interest, and warrant elaboration in respect to driver events in oncogenesis and/or drug resistance. Would a more granular expansion upstream and downstream of some splice variants (transcriptional regulation, network pathway signaling, etc) warrant calling out for future improvements?

We thank the reviewer for this comment. We include these examples to illustrate the importance of RNASeq in this type of analyses. In this particular example, EGFRvIII is a known and well-document driver event. In other case of splicing aberrations the interpretation is more complex and much harder to automate, in particular, when these events are not recurrent. We have included a note related to this point in the discussion.

Which samples had high mutation burden? Indicate these in Table 2.

Two samples had high mutation burden: GBM 8 and GBM 25 had mutations in MSH2 and in POLE and POLA1 respectively. This is added to Table 2.

The description of “…potentially synergistic combination therapy options in seven patients” warrants some explanation of the strength of the assignment of “synergy”.
While we did not measure the strength of synergy, we used the term to describe treatment options that targeted multiple mutations occurring in the same pathway or those that targeted multiple arms of the same pathway. For example, in one patient, we identified a MET gain, PI3KR1 activating variant, and PTEN loss. These three genes are part of the same pathway, that eventually leads to AKT activation and gene transcription affecting proliferation, invasiveness, and cell survival. We identified drugs to target two of these genes, a MET inhibitor for MET gain, and PI3K inhibitor for the PI3KR1 activating variant. We clarified what we meant by synergistic combination therapy options in the manuscript.

The genomic findings from four patients (NYGC-GBM7, NYGC-GBM17, NYGC-GBM25, and NYGC-GBM12) are reported in some detail. What was the reason to discuss these cases specifically in the Results section?

These four patients were specifically discussed because treatment decisions were made or changed as a direct result of the WGS/RNA-seq done in this study. This is now stated in the text to clarify this point.

The description of the “Concordance of WGS/RNA Seq and panel-based diagnostic reports” is a strength of the manuscript. The results are succinctly and clearly described. Figure 6 is a helpful rendering of the comparison. Calling out the significance of the germline reference to make somatic SNV calls is done well.

Thank you for the comment.

The detailed rendering of the Concordance of Drug recommendations between NY-GGC and WGA” is a strength of the manuscript. Page 22 notes “(see Appendix)”, but no appendix is provided.

The appendix existed in a prior version of the manuscript. The content of that manuscript has since been incorporated into the Methods section of the manuscript. Mention of the appendix is now removed.

As cytotoxic therapy (temozolomide, specifically) remains a standard of care for GBM patients, and other traditional agents (platinum drugs, cell cycle disruptors, antimetabolites, etc), the limitations of the study using targeted therapeutics only warrant deeper discussion, and recommendation for how to improve the WGA and the NY-CCG.

The purpose of WGS in this study was to produce a personalized annotation of variants present in each individual for identification of targeted therapies. In addition, tumor mutation burden offers another metric for consideration of less targeted therapies such as immunotherapies. Any targets and therapeutics identified for consideration is in parallel with or in addition to and not in place of the standard of care that includes cytotoxic and other traditional agents. We envision on
this technology as a way to identify adjuvants to these standard of care therapies. Patients in this study all received or were receiving standard of care therapies such as radiation and temozolomide and only considered targeted therapies as an addition. We clarify this in the discussion.

The manuscript warrants a listing of strategies and risks to bridge the gap between the final (present) iteration of NY-GGC or WGA for patient treatment planning decisions and what would be a "routine" approach to using genomics as a mainline treatment resource. Especially, the enrollment of 30 patients by 7 institutes over 16 months seems to be an opportunity to discuss clinical adoption barriers, which the coauthors of the manuscript could articulate.

In addition to lowered cost and increasingly automated interpretation of sequencing data as mentioned earlier in the discussion, other strategies towards improved implementation of WGS/RNA-seq include submission of samples as soon as possible after resection so that results are available and can be considered at the time treatment decisions are being made. This requires both clinician education about the utility of such an assay as well as the availability of clinically approved tests. To this end, NYGC has recently obtained such approval; it will be interesting to see how this influences adoption of WGS/RNA-seq for somatic variants in the coming months in New York. This has been added to the discussion.

A comment on the interval between resection to sample submission (shown as a median of 67 days) should be commented upon.

Samples were submitted at a median of 67 days from resection in this current study. Initial resections were generally received sooner (median 41 days) while resections of recurrences were received at a median of 353 days. Clinician perception of the usefulness and relevance of the sequencing data may have been affected by the time between resection and submission. This is added to the discussion.

This Discussion should also address what are the currently known challenges in regulatory approval for such technology.

Recently, New York Genome Center became the first institution in New York to obtain approval from New York State Department of Health to offer WGS/RNA-seq for tumor normal pairs as a clinical test. One of the biggest challenges in obtaining regulatory approval in WGS was in demonstrating reproducibility at far less depth (80X/40X for tumor normal pair) than depths of 100-200X for exomes and 500X for panels. Specifically, true negatives remain uncertain even with very high depths due to the nature of oncology variant callers which look for variants and not for correct base calls and remain a limit of this assay. This point is added to the discussion.
Minor points

Page 16, line 7. …We also discovered a CHST11-PKP fusion. Should be: CHST11-PKP2 fusion

This is corrected.

Reference #18 is incomplete.

The reference is corrected.

References:
