Comparing sequencing assays and human-machine analyses in actionable genomics for glioblastoma

**ABSTRACT**

**Objective:** To analyze a glioblastoma tumor specimen with 3 different platforms and compare potentially actionable calls from each.

**Methods:** Tumor DNA was analyzed by a commercial targeted panel. In addition, tumor-normal DNA was analyzed by whole-genome sequencing (WGS) and tumor RNA was analyzed by RNA sequencing (RNA-seq). The WGS and RNA-seq data were analyzed by a team of bioinformaticians and cancer oncologists, and separately by IBM Watson Genomic Analytics (WGA), an automated system for prioritizing somatic variants and identifying drugs.

**Results:** More variants were identified by WGS/RNA analysis than by targeted panels. WGA completed a comparable analysis in a fraction of the time required by the human analysts.

**Conclusions:** The development of an effective human-machine interface in the analysis of deep cancer genomic datasets may provide potentially clinically actionable calls for individual patients in a more timely and efficient manner than currently possible.

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**GLOSSARY**

CVN = copy number variant; EGFR = epidermal growth factor receptor; GATK = Genome Analysis Toolkit; GBM = glioblastoma; IRB = institutional review board; NLP = Natural Language Processing; NYGC = New York Genome Center; RNA-seq = RNA sequencing; SNV = single nucleotide variant; SV = structural variant; TCGA = The Cancer Genome Atlas; TPM = transcripts per million; VCF = variant call file; VUS = variants of uncertain significance; WGA = Watson Genomic Analytics; WGS = whole-genome sequencing.

The clinical application of next-generation sequencing technology to cancer diagnosis and treatment is in its early stages. An initial implementation of this technology has been in targeted panels, where subsets of cancer-relevant and/or highly actionable genes are scrutinized for potentially actionable mutations. This approach has been widely adopted, offering high redundancy of sequence coverage for the small number of sites of known clinical utility at relatively low cost.

However, recent studies have shown that many more potentially clinically actionable mutations exist both in known cancer genes and in other genes not yet identified as cancer drivers. Improvements in the efficiency of next-generation sequencing make it possible to consider whole-genome sequencing (WGS) as well as other omic assays such as RNA sequencing (RNA-seq) as clinical assays, but uncertainties remain about how much additional useful information is available from these assays.

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Aside from cost, a challenge of WGS or whole-transcriptome data is the expertise and time required to interpret the full spectrum of somatic mutations. To address this challenge, Watson for Genomics (Watson Genomic Analytics [WGA]), a cancer analytic tool, uses standard variant call files (VCFs), copy number variant (CNV), and differential gene expression data to return a list of recommended cancer drugs. Here, we present the results of a targeted cancer panel along with WGS and RNA-seq in a patient with glioblastoma (GBM). We also compare results of expert interpretation of the tumor genome by bioinformaticians and oncologists at New York Genome Center (NYGC) and at collaborating institutions with those generated by WGA.

METHODS Standard protocol approvals, registrations, and patient consents. This study was approved by multiple institutional review boards (IRBs), including Rockefeller University IRB and Biomedical Research Alliance of New York IRB. The study was registered in ClinicalTrials.gov (NCT02725684). Informed written consent was obtained from the participant.

Participant. This report describes the first participant in a multi-institutional study. NYGC-GBM-01 was a 76-year-old man with GBM. DNA and RNA were extracted from snap-frozen tissue. DNA from blood was obtained for comparison. The samples were analyzed by WGS and RNA-seq.

Single nucleotide variants and INDELs. Whole-genome libraries were prepared using the Illumina TruSeq Nano DNA Sample Prep Kit and were sequenced on Illumina HiSeq X instruments (Illumina, San Diego, CA). Paired-end 2 × 150 bp reads were aligned to the GRCh37 human reference (BWA aln v.0.7.8) and processed using a pipeline that includes marking of duplicate reads using Picard tools and realignment around INDELs and base recalibration using Genome Analysis Toolkit (GATK) version 2.7.4, muTect v1.1.4, LoFreq v2.0.0, Strelka v1.0.13, Pindel,11 and Scalpel12 used to return the union of variant calls. Variants were filtered out if they were at >1% frequency in the 1000 Genomes or ExAC data sets, had more than 2 alleles to remove artifacts, raw frequency in the tumor was lower than that in the normal, or matched a custom “blacklist” of known systematic errors generated by comparing normal germline replicates. Remaining single nucleotide variants (SNVs) and INDELs were annotated via snpEff,13 snpSift,13 and GATK VariantAnnotator using annotation from ENSEMBL,14 COSMIC,15 Gene Ontology,16 and 1000 Genomes.17

Structural variation. Structural variants (SVs), such as CNVs and complex genomic rearrangements, were detected by NBIC-seq,18 Delly,19 CREST,20 and BreakDancer.21 We prioritized SVs in the intersection of callers and those with additional split-read evidence via SplazerS.22 SVs with split-read support in the matched normal or annotated as known germline variants (1000 Genomes call set, Database of Genomic Variants) were removed as likely germline variants. The predicted somatic SVs were annotated with gene overlap (RefSeq, Cancer Gene Census) including prediction of potential effect on resulting proteins.

Tumor purity and ploidy. Tumor purity was calculated from WGS data using Titan.23 In addition, purity and ploidy were calculated from the Illumina OMNI 2.5M Array using ASCAT.24

RNA sequencing. We used the Illumina TruSeq stranded messenger RNA protocol and sequenced 100 million reads. Reads were aligned using STAR25 and Gencode genes were quantified using featureCounts.26 Ninety-five percent of reads mapped the reference genome. We normalized the counts with DESeq2 and adjusted the quantification to account for GC bias27 and batch effects28 between The Cancer Genome Atlas (TCGA) GBM RNA-seq and our sample. The normalized expression data are used to identify GBM subtypes.29

Therapeutic targets and drug recommendations. The NYGC uses the custom clinical Tier classification system for SNVs. Tier 1 variants are clinically important variants in the cancer type being studied (e.g., epidermal growth factor receptor [EGFR] T790M is known to be clinically important in lung cancer). The same variant observed in a cancer unknown to manifest this variant is classified as Tier 2 (e.g., the clinical importance of EGFR T790M is unknown in GBM). Tier 3 variants are in targetable genes; however, the specific variant is not known to be targetable (e.g., an unknown mutation in EGFR). Tier 4 variants are in genes cataloged by COSMIC cancer census and not included in Tiers 1–3. All other variants are in Tier 5 and considered variants of uncertain significance (VUS). Variants in Tiers 1–4 are considered potentially targetable. Variants were matched to potential treatments by identifying the most aberrant genes from a combination of SNV, INDEL, SV, and RNA-seq data and by searching the NYGC drug-to-gene database. Prioritization of potential treatments was based on further manual assessment including criteria such as strength of data supporting variants detected, FDA approval of drug in GBM or in another cancer type, current GBM trial for a drug, and successful use of the drug to target the variant identified to treat GBM or other cancer types.

Watson Genomic Analytics. WGA, an IBM research proof-of-concept environment of Watson for Genomics,31 is a cognitive system built on several different predictive models to analyze up to whole-genome scale molecular data. VCFs, CNV, and gene expression data are input to WGA. The VCF file provided to WGA contains the union from 3 calling algorithms each for SNVs and INDELs specified in the Methods section. CNV data are inputted as copy number log2 (T/N) ratio values per gene. Modified Z-scores of RNA-seq normalized expression data per gene are used as proxy for differential gene expression. Modified z-score per gene is calculated by subtracting the median transcripts per million (TPM) value (over the TCGA GBM cohort) to this sample’s TPM and dividing by the TCGA SD. With this input, WGA leverages a comprehensive database of structured (20+ sources include DrugBank, NCI, COSMIC, ClinVar, and 1000 Genomes) and unstructured (evidence extracted from literature using Natural Language Processing [NLP]) biological and medical data. To date, WGA processed abstracts from PubMed and where possible, began analyzing full-text articles. In addition, the NLP engine is being trained to understand the approximate clinical trials at ClinicalTrials.gov. It is from the unstructured sources that WGA maintains a current repository of drug resistive or sensitizing markers for the drug of interest, and relevant clinical trials.
RESULTS  Case report. NYGC-GBM-01 was a 76-year-old man who presented with headache and difficulty with ambulation. CT of the brain revealed a mass in the left parietal region. He underwent initial resection for which pathology revealed a GBM, negative for the following: EGFR amplification by in situ hybridization fluorescence, EGFReIII RNA expression, IDH1 R132H by immunostaining, 1p36/19q13 deletion by fluorescent in situ hybridization analysis, and MGMT methylation. Postsurgically, he had right-sided hemineglect and right/left confusion. He became somnolent and required a re-resection.
the functional catalytic protein, PIK3CA. Activating mutations of PIK3CA are known cancer drivers, as are loss-of-function mutations of PIK3R1. Functional studies have shown that PIK3R1 amino acid D560 is involved in hydrogen binding with PIK3CA and is an essential amino acid in regulating the activity of the catalytic subunit. The mutation identified here is in the same helical inhibitory (iSH2) domain of PIK3R1. The variant binds but fails to inhibit PIK3CA, leading to enhanced cell survival, Akt activation, anchorage-independent cell growth, and oncogenesis.35,36 Furthermore, analysis of the crystal structure (figure 2) supports the conclusion that this mutation would inhibit the functional interaction between the 2 proteins, specifically through N345 of PIK3CA, resulting in PIK3CA activation.

A recent cell line study identified a synergistic effect between MET exon 14–skipping variants and a PIK3CA E545K oncogenic variant, in which a combination of an MET inhibitor and a PIK3CA inhibitor showed better sensitivity than single therapy.37 The PIK3CA E545K variant also activates PIK3CA. Taken together, these findings led us to suggest combinatorial INC280 (MET inhibitor) and BKM120 (PIK3CA inhibitor) therapy for potential clinical consideration, and this suggestion would have made the patient eligible for a clinical trial assessing efficacy of this combination (NCT01870726).

Watson for Genomic analysis. Data for NYGC-GBM-01 were input into WGA, which produced a report summarizing actionable variants and a list of associated drugs, including some based on a pathway target analysis. WGA identified 6 actionable alterations, 14 associated drugs, 9 VUS (including FGFR3), copy number losses in Chr 9 (focal), 10 (armscale), and 11 (focal), and gain on Chr 7 (armscale). Both the

| Table 3 | List of variants identified as actionable by 3 different platforms |
|---------|---------------------------------------------------------------|
| Gene    | Variant           | Identified variant | Identified associated drugs | NYGC | WGA | FO | NYGC | WGA | FO |
|---------|-------------------|--------------------|------------------------------|------|-----|----|------|-----|----|
| CDKN2A  | Deletion          | Yes                | Yes                          | Yes  | Yes | Yes| Palbociclib, LY2835219 | Palbociclib LY2835219 | Clinical trial |
| CDKN2B  | Deletion          | Yes                | Yes                          | Yes  | Yes | Yes| Palbociclib, LEE001     | Palbociclib LEE002  | Clinical trial |
| EGFR    | Gain (whole arm)  |Yes                 | —                            |—    |—   |—  | Cetuximab                   |—                   |—               |
| ERG     | Missense P114Q    |Yes                |—                            |Yes  |Yes |    | RI-EIP                      |RI-EIP              |—               |
| FGFR3   | Missense L49V     |Yes                | VUS                          |—    |—   |   | TK-1258                     |—                  |—               |
| MET     | Amplification     |Yes                |Yes                          |Yes  |Yes |Yes| INC280                      |Crizotinib, cabozantinib |Crizotinib, cabozantinib|
| MET     | Frame shift R755fs|Yes                | —                            |Yes  |Yes |Yes| INC280                      |—                  |—               |
| MET     | Exon skipping     |Yes                | —                            |—    |—   |   | INC280                      |—                  |—               |
| NF1     | Deletion          |Yes                |—                            |Yes  |Yes |Yes| MEK162                      |MEK162, cobimetinib, trametinib, GDC-0994 |Everolimus, temsirolimus, trametinib |
| NF1     | Nonsense R461     |Yes                |Yes                          |Yes  |Yes |Yes| MEK162                      |—                  |—               |
| PIK3R1  | Insertion R562-M563ins|Yes|Yes|—|BKM120|BKM120, LY3023414|—|
| PTEN    | Loss (whole arm)  |Yes                | —                            |—    |—   |   | Everolimus, AZD2014            |—                  |—               |
| STAG2   | Frame shift R1012 fs|Yes|Yes|Yes|Veliparib, clinical trial|—|Olaparib|
| DNM3A   | Splice site 2083-1G->C|—|—|Yes|—|—|—|
| TERT    | Promoter-146C>T   |Yes                |—                            |Yes  |—   |   |—                  |—                  |—               |
| ABL2    | Missense D716N    |Germine            | NA                          |VUS  |—   |   |—                  |—                  |—               |
| mTOR    | Missense H1687R   |Germine            | NA                          |VUS  |—   |   |—                  |—                  |—               |
| NPM1    | Missense E169D    |Germine            | NA                          |VUS  |—   |   |—                  |—                  |—               |
| NTRK1   | Missense G18E     |Germine            | NA                          |VUS  |—   |   |—                  |—                  |—               |
| PTCH1   | Missense P1250R   |Germine            | NA                          |VUS  |—   |   |—                  |—                  |—               |
| TSC1    | Missense G1035S   |Germine            | NA                          |VUS  |—   |   |—                  |—                  |—               |

Abbreviations: FO = FoundationOne; NYGC = New York Genome Center; RNA-seq = RNA sequencing; WGA = Watson Genomic Analytics; WGS = whole-genome sequencing.

Genes, variant description, and, where appropriate, candidate clinically relevant drugs are listed. Variants identified by the FO as variants of uncertain significance (VUS) were identified by the NYGC as germline variants.
NYGC and WGA identified 5 actionable alterations (in genes \textit{NF1}, \textit{MET}, \textit{CDKN2A}, \textit{CDKN2B}, and \textit{PIK3R1}; table 3). WGA reported an \textit{NF1} SNV and annotated the variant as inactivating but did not deem copy number change to be sufficient for calling this or \textit{EGFR} and \textit{PTEN}. A 1-copy gain of \textit{EGFR} was below WGA’s threshold for classification as a targetable variant. Furthermore, this variant was shown to be negative for amplification by in situ hybridization fluorescence. However, the NYGC decided to list it as potentially targetable, given it is a known actionable variant in GBM. Similarly, a 1-copy \textit{PTEN} loss is reported by the NYGC but not by WGA or by the FoundationOne. The NYGC reported this variant because of its clinical implications; it is associated with resistance to EGFR tyrosine kinase inhibition via AKT/mTOR pathway activation and is linked to cetuximab resistance, but can be targeted by mTOR inhibitors.\textsuperscript{38} For \textit{PIK3R1}, the NYGC identified BKM120 as a potential therapeutic option based on additional RNA-seq evidence of overexpression of \textit{PIK3CA}. WGA identified \textit{PIK3R1} as a relevant variant via SNV data by WGS and used RNA-seq information in pathway and drug analysis to also recommend BKM120. The \textit{MET} amplification and associated drugs are reported by both platforms; however, WGA had 2 drugs for \textit{MET} amplification, whereas the NYGC prioritized 1 therapeutic option, INC280, based on GBM trial data availability. The NYGC reported 8 clinical trials associated with 5 genes. WGA found 10 clinical trials that may be relevant across 6 actionable alterations.

**Comparison with a panel.** Table 3 also compares all variants and drugs identified by the NYGC and FoundationOne. NYGC analysis identified 8 unique variants not found by the FoundationOne, including an exon-skipping event. The NYGC identified drugs for 10 targets, while FoundationOne identified drugs for 4. Furthermore, 6 of the variants reported as of unknown significance occurring in the tumor by FoundationOne were germline variants. One variant (\textit{DNMT3A} splice site 2083-1G>C) was called by FoundationOne, but the position and base change were different from a nearby variant identified by the NYGC.

**DISCUSSION** The NYGC is undertaking a WGS research study in patients with GBM to investigate the efficiency and feasibility of WGS to inform therapeutic options. Here, the results of NYGC WGS and RNA-seq were compared with a clinical panel assay. Also, in collaboration with IBM, the NYGC examined the therapeutic options identified by WGA based on WGS and RNA-seq data. Genomic results from this patient clearly displayed the diversity of driver events typically seen in GBM. Of interest, we identified mutations in targetable genes that were not precise matches to known specific targetable variants, and which nonetheless suggested potential therapeutic options.
Multimodal analysis (WGS and RNA-seq) increased confidence in the identification of the MET mutation; analysis of the literature of prior MET exon-skipping events suggested the plausibility of considering a tyrosine kinase inhibitor that could target MET. Similarly, manual literature search of the PIK3CA E545K oncogenic variant led to the conclusion that this was likely an activating mutation. Moreover, manual database searches resulted in the suggestion of a combinatorial treatment with a MET inhibitor and a PIK3CA inhibitor, which made logical sense and also made the patient eligible for a clinical trial for this combination (NCT01870726). None of these observations were evident from the panel. This suggests that pursuing a more extensive comparison of panel and deeper sequencing (e.g., WGS and RNA-seq) will be of interest. An added point, previously noted by others, is that the sequencing of both germline and tumor DNA not only heightened our sensitivity for what variants might be tumor drivers but was able to rule out a number of germline variants called by the FoundationOne as not likely to be primary drivers of this patient’s GBM.

Although we conducted WGS of this sample at roughly twice the cost of WES, the primary analysis was performed on the protein-coding region of the genome. There may be technical advantage to WGS even for assaying targeted regions. WES relies on hybridization capture of specific genes which introduces intrinsic bias for each gene as a function of GC/AT content, while WGS relies more simply on mechanical shearing of DNA prior to sequencing. Previous studies have found that for disorders caused by constitutional mutations, WGS is more sensitive than WES for variant detection. To assess whether WGS could detect variants not identified by WES to justify the added cost, it would require a direct comparison of the assays on the same sample. We are undertaking a study to address this question.

This patient died approximately 8 months from the time of initial resection falling short of the median survival time for GBM. The oncologist recommended enrollment in a clinical trial targeting PIK3 and MET alterations on recurrence on adjuvant temozolomide. However, the patient’s clinical decline eliminated his ability to participate in trials. This highlights one of the challenges of the clinical application of precision medicine technology. The identification of targets and potentially useful drugs in a timely manner is only the first step. Drug and drug trial access is crucial to determine the benefit of this approach in cancer management.

Another key observation was that the WGA analysis vastly accelerated the time to discovery of potentially actionable variants from the VCF files. As previously reported, we found that WGA was able to provide reports of potentially clinically actionable insights within 10 minutes, while human analysis of this patient’s VCF file took an estimated 160 hours of person-time. This is critical if sequencing is to be brought out of the research arena and into the scaled, real-world clinical realm. This study is an important step forward promoting human-machine interface as a way to address a key bottleneck in cancer genomics.

AUTHOR CONTRIBUTIONS
Kazimierz O. Wrzeszczynski: analysis and interpretation of the data and drafting and revising the manuscript. Mayu O. Frank: design and conceptualization of the study, interpretation of the data, and drafting and revising the manuscript. Takahiko Koyama: analysis and interpretation of the data and revising the manuscript. Kahn Rhrissorrakrai: analysis and interpretation of the data and revising the manuscript. Nicolas Robine: analysis and interpretation of the data and revising the manuscript. Filippo Utro: revising the manuscript. Anne-Katrin Emde, Bo-Juen Chen, Kanika Arora, Minita Shah, Vladimir Vacic, Raquel Noerel, Ezhana
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Lasman has served on the scientific advisory boards of AstraZeneca, AbbVie, Sapience Therapeutics, Genentech, Biodinamica, VBI Vaccines, Cortice Biosciences, Oxiogene, Regeneron, and Novocure; has received travel funding/speaker honoraria from prIME Oncology and has served on the editorial boards of Neuro-Oncology and the Journal of Neurooncology; has been a consultant for WebMD, the American Society of Clinical Oncology, and the American Academy of Neurology; has been a grant reviewer for the Italian Association for Cancer Research; and has received research support from the NCI, the Radiation Therapy Oncology Group Foundation, and the James S. McDonnell Foundation. P. Canoll has served on the editorial boards of Planes and GLIA and has received research support from NIH/National Institute of Neurological Disorders and Stroke, NCI, NIDA, and the James F. McDonnell Foundation (Swanson). C. Grommes has received research support from Pharmacies. S. Harvey reports no disclosures. L. 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Royyuru holds patents for Mixed polynucleotide and forming method thereof, DNA sequencing using multiple metal layer structure with different organic coatings forming different transient bondings to DNA, Fabrication of tunneling junction for nanopore DNA sequencing, Verification of complex workflows through internal assessment or community based assessment, Field effect based nanosensor for biopolymer manipulation and detection, DNA sequence using multiple metal layer structure with different organic coatings forming different transient bondings to DNA, Integrated nanowire/nanosheet nanogap and nanopore for DNA and RNA-seq, Field effect based nanosensor for biopolymer manipulation and detection, Integrated nanowire/nanosheet nanogap and nanopore for DNA and RNA-seq, Electron beam sculpting of tunneling junction for nanopore DNA sequencing, Charged entities as locomotive to control motion of polymers through a nanochannel, Molecular dispensers (2), Nanopore capture system, Protein structure analysis (2), Method of identifying robust clustering, Hydrophobic moment of multi-domain proteins (3), System and program storage device of object classification utilizing optimized Boolean expressions, Apparatus, method, and product of manufacture for transforming supply chain networks using pair-wise nodal analysis, Method and apparatus for protein structure analysis, Techniques for reconstructing supply chain networks using pair-wise correlation analysis, and Object classification using an optimized Boolean expression; is an employee of IBM Corporation; is a member of the Industry Advisory Board of International Society for Computational Biology; and has received research support from Pfizer and CHDI. R.B. Darnell serves on the Scientific Advisory Board at the New York Genome Center, Roundtable on Translating Genomic-Based Research for Health at National Academy of Medicine, and Stanford Medicine Board of Fellows; holds patent nos. 7,989,203, 6602790, 6,750,029, 7,928,190, and 14/104,581; holds a patent (pending) for Use of BET inhibitors to treat neurodevelopmental disorders and epilepsy; has received honoraria from Lennart Philipson Memorial Lecture, Uppsala University, Sweden; has received research support from IBM, Sohn New York City Collaboration for Pediatric Cancer Research, Starr Cancer Consortium, and NIH; is a Howard Hughes Medical Institute investigator; and owns stock in Amgen, Illumina, and Agios. Go to Neurology.org/ng for full disclosure forms.

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