A Wide Spectrum of EGFR Mutations in Glioblastoma is Detected by a Single Clinical Oncology Targeted Next-Generation Sequencing Panel

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Abstract

With the advent of large-scale genomic analysis, the genetic landscape of glioblastoma (GBM) has become more clear, including characteristic genetic alterations in EGFR. In routine clinical practice, genetic alterations in GBMs are identified using several disparate techniques that consume already limited amounts of tissue and add to overall testing costs. In this study, we sought to determine if the full spectrum of EGFR mutations in GBMs could be detected using a single next generation sequencing (NGS) based oncology assay in 34 consecutive cases. Using a battery of informatics tools to identify single nucleotide variants, insertions and deletions, and amplification (including variants EGFRvIII and EGFRvV), twenty-one of the 34 (62%) individuals had had at least one alteration in EGFR by sequencing, consistent with published datasets. Mutations detected include several single nucleotide variants, amplification (confirmed by fluorescence in situ hybridization), and the variants EGFRvIII and EGFRvV (confirmed by multiplex ligation-dependent probe amplification). Here we show that a single NGS assay can identify the full spectrum of relevant EGFR mutations. Overall, sequencing based diagnostics have the potential to maximize the amount of genetic information obtained from GBMs and simultaneously reduce the total time, required specimen material, and costs associated with current multimodality studies.

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INTRODUCTION

Glioblastoma (GBM) is the most common primary malignancy of the central nervous system in the adult population, corresponding to a World Health Organization (WHO) Grade IV tumor. Recent large-scale genomic efforts have helped to define the mutational landscape of GBM, as well as providing insight to clinically relevant mutations and molecular subtypes (Brennan et al., 2013; Frattini et al., 2013; Parsons et al., 2008; Sturm et al., 2012; TCGA, 2008; Verhaak et al., 2010). Key genetic drivers involved with core pathways are variably associated with specific GBM subtypes, and include isocitrate dehydrogenase (IDH), NF1, TP53, telomerase reverse transcriptase (TERT) promoter region, phosphatase and tensin homolog (PTEN), cyclin dependent kinase 2A/B (CDKN2A/B), platelet derived growth factor receptor A (PDGFRA), and epidermal growth factor receptor (EGFR) (Brennan et al., 2013; Frattini et al., 2013; Killela et al., 2013; Nonoguchi et al., 2013; Ozawa et al., 2010; Parsons et al., 2008; Sturm et al., 2012; TCGA, 2008; Verhaak et al., 2010; Yan et al., 2009). GBMs contain a spectrum of mutations within these genes including single nucleotide variations (SNVs), gene amplifications, insertions and deletions (indels), and structural gene rearrangements. In GBM, EGFR is commonly found to encompass the spectrum of these types of mutations, requiring testing by a multitude of molecular oncology assays to ascertain the full spectrum of mutations (Brennan et al., 2013; TCGA, 2008).

Although limited clinical efficacy has been demonstrated so far, amplified and variant EGFR is currently being investigated as a therapeutic target in GBM (Gan et al., 2013; Reardon et al., 2013; Roth and Weller, 2014; Schulte et al., 2013). The EGFR variant, EGFRvIII (genomic deletions of exon 2 through 7), is a constitutively active form of EGFR that has attracted much attention as a GBM therapeutic target. While testing for EGFRvIII is not performed during routine neuropathological workup of GBM, mutational status is used by some clinicians for the inclusion of ongoing vaccine or antibody trials targeting EGFRvIII (Gan et al., 2013; Roth and Weller, 2014).

While anatomic neuropathological examination has long been necessary for the diagnosis and clinical management of GBM, advances in the understanding of underlying molecular alterations and subsequent molecular testing is evolving to further classify GBM subtypes and guide personalized care for individuals with GBM (Mrugala, 2013). Routine neuropathological testing has begun to incorporate several disparate ancillary testing strategies such as Fluorescence In Situ Hybridization (FISH), immunohistochemistry (IHC), and various types of polymerase chain reaction (PCR), and sequencing, that may consume otherwise limited biopsy or surgical material as well as increase total testing costs (Appin and Brat, 2015; Huse and Aldape, 2014; Louis et al., 2014). Therefore, for molecular GBM workup, it would be beneficial to incorporate a single genetic test capable of detecting the spectrum of disease causing mutations (amplifications, indels, structural rearrangements, and SNVs) across multiple genes of interest, such as EGFR. Next-generation sequencing (NGS) is a powerful tool for the detection of cancer related somatic mutations that harbor...
diagnostic, prognostic, or predictive utility. Emerging NGS clinical oncology targeted panels have are typically limited to the detection of SNVs and small indels (Cottrell et al., 2014; Marrone et al., 2014; Pritchard et al., 2014). While there are important SNVs found in gliomas, such as those occurring in IDH1 and IDH2, other mutations of interest include gene copy number variations, structural rearrangements, and specific deletion mutations such as EGFRvIII. Although detection of structural variants and amplification of EGFR has been demonstrated by whole genome sequencing, the capability of detecting multiple mutation types including differential amplification of EGFR in targeted NGS brain tumor panels has not been addressed (Yang et al., 2013). In this study, we sought to determine the spectrum of mutations that can be identified by a clinical oncology targeted NGS panel as a way to streamline diagnostic testing and effectively utilize tissue that may be limited due to the nature of stereotactic biopsies often performed for brain tumor diagnostics.

**MATERIAL AND METHODS**

**Case Selection**

The use of human subject material was performed in accordance with guidelines set by the Human Research Protection Office of Washington University. Thirty-six consecutive cases of glioblastoma that underwent clinical next-generation targeted sequencing at the Washington University Genomics and Pathology Services (GPS) using a comprehensive oncology panel containing either 25 genes (V1 panel, n=17 cases) or 151 genes (V2 panel, n=19 cases) (Supplementary Tables 1 and 2), were selected for analysis.

**Next-Generation Sequencing (NGS)**

Oncologist directed NGS was performed as previously described (Cimino et al., 2014b). Between 300 and 750 ng of total genomic DNA (as determined by Qubit, Life Technologies, Grand Island, NY) was extracted from formalin-fixed paraffin-embedded (FFPE) tissue from each of the cases and was first fragmented to approximately 200–300 base pairs using a Covaris E210 instrument (Covaris Inc, Woburn, MA). Each sample was then end-repaired, A-tailed, ligated adapters using the SureSelect library kit (Agilent Technologies, Santa Clara, CA), and amplified via limited cycle PCR. DNA libraries were captured using either the GPS V1 or V2 Comprehensive Cancer Gene Set probes (Washington University, St. Louis, MO), amplified with custom indexing primers and sequenced on a single HiSeq2000 lane. Base calls and quality scores were generated by the included Casava software (v1.7 or v1.8).

**Analysis of Sequencing Reads**

Reads were first mapped to the human genome (NCBI build 37.2; hg19) using Novoalign (Novocraft, Selangor, Malaysia) with default paired-end parameters. Sequence data was ‘cleaned’ to remove duplicate reads, re-calibrate quality scores, and re-align around known polymorphisms using the Genome Analysis Toolkit (GATK v1.6) (http://www.broadinstitute.org/gatk) and picard tools (v1.88) MarkDuplicates (http://sourceforge.net/projects/picard/files) according to the GATK best practices guidelines (DePristo et al., 2011; McKenna et al., 2010; Van der Auwera et al., 2013). Quality metrics were calculated using the BEDTools and Samtools software packages (http://...
Single nucleotide variants and small indels were called using the GATK unified genotyper (DePristo et al., 2011; McKenna et al., 2010; Van der Auwera et al., 2013). Sequence variants present as constitutional polymorphisms in dbSNP (http://www.ncbi.nlm.nih.gov/projects/SNP) were documented. Larger indels were called using Pindel (https://trac.nbic.nl.pindel) with default parameters (Ye et al., 2009). Copy number changes were called using the laboratory CopyCAT2, written by our laboratory to call CNVs from targeted clinical oncology assays, (https://github.com/abelhj/cc2/) with default parameters (Sehn et al., 2014). Single nucleotide somatic variants were compared to published datasets from The Cancer Genome Atlas (TCGA) Research Network (http://cancergenome.nih.gov/) and Catalogue Of Somatic Mutations In Cancer (COSMIC) (http://cancer.sanger.ac.uk/cancergenome/projects/cosmic/) (Forbes et al., 2015; TCGA, 2008). Somatic mutations were assessed for damaging effects using PolyPhen-2 (http://genetics.bwh.harvard.edu/pph2/) (Adzhubei et al., 2010). Variants with a mean allele frequency (MAF) >0.01 in any population according to 1000 Genomes project (http://www.1000genomes.org/) or Exome Variant Project (http://evs.gs.washington.edu/niehsExome/) were flagged as constitutional polymorphisms (Abecasis et al., 2010).

**Fluorescence in situ hybridization (FISH)**

Interphase FISH for EGFR was performed on FFPE tissue sections cut at a thickness of five μm on positively charged microscope slides as previously described (Cimino et al., 2014a). The paraffin was removed from the sections with three washes of five minutes each in CitriSolve. The slides were then hydrated in two washes of absolute ethanol for one minute each and allowed to air dry. The slides were processed through a pretreatment solution of sodium thiocyanate which had been pre-heated to 80°C. After a three minute wash in distilled water, the tissue was digested in protease solution (pepsin in 0.2N HCl) for 15 minutes at 37°C, followed by another three-minute wash in distilled water. The slides were allowed to air dry after which they were dehydrated by passing through consecutive 70%, 85%, and 100% ethanol solutions for one minute each. The slides were again allowed to air dry before applying prepared probe mixture. Probes used were purchased from Abbott Molecular (Des Plaines, IL) and included Vysis LSI EGFR (7p12) SpectrumOrange Probe and Vysis CEP 7 (7p11.1-q11.1 Alpha Satellite DNA) SpectrumGreen FISH Probe Kit (Catalog #05J48-001). Probes were diluted at a concentration of 1:50 in tDenHyb-2 hybridization buffer (Insitus Biotechnologies, Inc., Albuquerque, NM) and well-mixed. Next, probe in buffer was applied to the appropriate slide to cover the tissue section and the section was coverslipped. Co-denaturation was achieved by incubating the slides at 73°C for five minutes in a slide moat. Hybridization occurred by transferring the slides to a 37°C slide light-shielded, humid slide moat overnight. Post-hybridization, the coverslips were removed and the slides immersed in 75°C wash solution (2XSSC/0.3%NP40) for two minutes followed by a one minute wash in jar containing the same solution at room temperature. The slides were allowed to air dry in the dark and were then counterstained with 10μl of DAPI II (Abbott Molecular, Inc.). Slides were examined using an Olympus BX60 fluorescent microscope with appropriate filters for SpectrumOrange, SpectrumGreen, and the DAPI counterstain. The signal patterns were documented using a CoolSnap camera and CytoVision Imaging System. FISH for EGFR amplification was considered positive based on published guidelines (Varella-Garcia et al., 2009).
Multiplex Ligation-Dependent Probe Amplification (MLPA)

For each case with available material, between 50 to 200 ng of total genomic DNA was extracted from FFPE. MLPA reactions were performed using the SALSA MLPA P315 EGFR probemix commercially available from MRC Holland (Amsterdam, Netherlands), according to the manufacturer’s instructions for the general MLPA protocol for the detection and quantification of nucleic acid sequences. PCR and ligation reactions were run on a MJ Research PTC-100 programmable thermocycler. Resultant PCR products were sized by capillary electrophoresis using an ABI sequencer 3130xl (Life Technologies, Grand Island, NY). Capillary electrophoresis data was analyzed using the freely available software Coffalyser.NET provided by MRC Holland (http://www.mlpa.com) [Accessed September, 2014].

Statistics

Comparisons made between groups were performed using the R statistics package (R, version 2.15.1, R Project for Statistical Computing, http://www.r-project.org/). Plots of 95% confidence intervals (CI) were created in R. Two-tailed Student’s T-tests were performed using GraphPad Prism software (Graph-Pad Software Inc., San Diego CA).

RESULTS

Demographics and Sequence Metrics

A total of 36 consecutive cases of glioblastoma, representing 34 individuals, underwent institutional testing by a clinical oncology targeted NGS panel utilizing formalin-fixed paraffin-embedded tissue derived from surgical biopsy or resection material. Demographic characteristics of the individuals are summarized in Table 1. The average individual age at time of diagnosis was 52.6 years with a range of 18 to 74 years. Of the 34 individuals, 20 were male and 14 were female. Each case averaged a total number of sequencing reads of 9,794,370 (V1 panel = 4,975,456 and V2 panel = 14,613,285), the average unique, on target coverage was 731x (V1 panel = 631 and V2 panel = 830), and the percentage unique on-target reads was 53.7% (V1 panel = 61.0% and V2 panel = 46.4%).

Spectrum of EGFR Mutations

Twenty one of the 34 (62%) individuals with GBM had at least one alteration in EGFR by sequencing (Table 1), consistent with published datasets (Brennan et al., 2013; TCGA, 2008). Various EGFR SNVs, including SNPs, were identified in 17 of the 34 (50%) individuals. There were nine cases with the same known EGFR polymorphism (p.R521K) contained in dbSNP (rs2227983). Only one case had a synonymous mutation (c.474C>T) in EGFR. Nine total cases harbored nine unique non-SNP gene variants, including and 1 truncating mutation (Table 1). Of these nine non-synonymous mutations, five (56%) were also found to occur in the TCGA Research Network GBM dataset. The four EGFR somatic mutations not found in the TCGA Research Network datasets include p.F254I, p.C558Y, p.Y610C, and p.C333Lfs*6 (truncating mutation). As paired normal samples were not sequenced along with the glioblastoma samples, we cannot definitively classify these four somatic mutations as either non-pathogenic ‘personal single nucleotide polymorphisms’

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(low mean allele frequency variants specific to an individual and not present in public databases) or true somatically acquired tumor specific variants. However, these four mutations were predicted to be deleterious by PolyPhen-2. By NGS, EGFR amplification was identified in 20 of 34 (59%) individuals. Of those 20 cases, 15 (75%) were called positive for EGFR amplification by FISH (mean NGS copy number 30.57), 4 were considered negative for EGFR amplification all of which had low-level amplification by sequencing (mean NGS copy number 3.00), and 1 case was equivocal for EGFR amplification by FISH (NGS copy number 13.71). The mean NGS EGFR copy number for the 15 concordant cases (30.57) and the 4 discordant cases (3.00) was statistically significant ($P=0.0141$, 95% CI=6.130 to 49.02). One of the cases with EGFR amplification also was found to harbor the EGFRvIII mutation (deletion of exons 2 to 7) (Figure 1) and 1 additional case had the EGFRvV (deletion of exons 25 to 27) mutation. Seventeen of the 36 cases had additional material available for further MLPA analysis, and this confirmed that one case had the EGFRvIII mutation (Figure 2).

**DISCUSSION**

Whole scale genome analysis has been successful in discovering and identifying many mutations related to GBM. However, the utility of whole genome or exome sequencing in the molecular oncology laboratory is not necessarily practical. Targeted molecular oncology panels now offered by many institutions and companies offer a more efficient alternative to such testing. To expand upon the current clinical capabilities of this type of assay, we used GBM to investigate the spectrum of detectable mutations. In this study, we have successfully shown that a single NGS assay consisting of a targeted cancer panel, using EGFR as a model gene, can detect a wide spectrum of mutations in GBM, including SNVs, gene amplification, and the deletion variants EGFRvIII and EGFRvV.

While many types of mutations were detected with this single oncology assay, the current assay is unable to detect certain structural rearrangements, such as the gene fusion EGFR-SEPT14 (Frattini et al., 2013). This is because the genomic breakpoints occur within intronic sequences of EGFR, which are not targeted by our assay, and therefore are not identifiable. Inclusion of targeted intronic sequences in future targeted NGS panels or sequencing mRNA may aid in overcoming this current limitation. Another limitation of the current targeted NGS method is the inability to definitively distinguish EGFR amplification as a result of double minute chromosomes versus intra-chromosomal 7 amplification (Lopez-Gines et al., 2010). However, amplifications via double minute chromosomes tend to occur at a higher level than those by intra-chromosomal amplification (Lopez-Gines et al., 2010). While coverage reads provided by NGS do allow for the discrimination of relative EGFR copy numbers, the possible utility of EGFR amplification as a surrogate marker for amplification mechanism may be confounded by sampling bias and tumor heterogeneity (Snuderl et al., 2011). In addition to tumor heterogeneity of EGFR amplification, GBM also demonstrates heterogeneity of EGFR gene variants, which includes the presence of EGFRvIII, as detected by single-cell NGS (Francis et al., 2014). Samples that contain only very minor tumor components involving EGFRvIII may not be detected by the current assay.
Conclusions

Here we demonstrate the utility of targeted oncology sequencing panels to identify multiple classes of mutations common in GBMs. Further, we demonstrate that exon-level CNVs such as EGFRvIII can be detected in targeted sequencing data. As brain biopsy tissue is often very limited, using a single assay to replace FISH, Sanger based sequencing, and MLPA analysis has obvious advantages in the clinical molecular oncology laboratory.

Supplementary Material

Refer to Web version on PubMed Central for supplementary material.

Acknowledgments

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References


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HIGHLIGHTS

- Glioblastomas cases were sequenced by a clinical oncology next-generation panel
- \( EGFR \) was used as a model to demonstrate a variety of detectable mutations
- Mutation types included SNVs, CNVs, and indels (including the \( EGFRvIII \) variant)
- CNV detection was validated by FISH and MLPA
Figure 1.
Glioblastoma Case 6 with EGFRvIII mutation. A) Hematoxylin and eosin stained section showing histological features of a high grade diffusely infiltrative astrocytoma with microvascular proliferation and pseudopalisading necrosis. B) Interphase FISH highlighting EGFR amplification (Red-EGFR, Green-CEP 7). C) NGS coverage plot of chromosome 7 showing increased read coverage of the EGFR gene with a relative decrease in coverage in exons 2 through 7 corresponding to the vIII mutation. D) MLPA electropherogram for EGFR confirming decreased copy numbers of exons 2 through 7, as indicated by arrows.
Figure 2. 
MLPA results validating NGS detection of EGFRvIII. Seventeen cases of glioblastoma were analyzed by MLPA for EGFR regional loss and normalized copy numbers of exons are plotted with the average values and 95% CI. Red triangles indicate data points for case 6, which show significantly lower relative copy numbers of exons 2 through 7, corresponding to the EGFRvIII mutation.
Table 1

Demographic and molecular information for the cases of glioblastoma.

<table>
<thead>
<tr>
<th>Sample</th>
<th>Age (Years)</th>
<th>Sex</th>
<th>Diagnosis (WHO Grade IV)</th>
<th>EGFR SNV by NGS</th>
<th>EGFR CNV by NGS</th>
<th>EGFR CNV by FISH</th>
<th>EGFR Indel by NGS</th>
<th>EGFR Indel by MLPA</th>
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<td>M</td>
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<td>Polymorphism p.R521K</td>
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<td>74</td>
<td>M</td>
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<td>Polymorphism p.R521K</td>
<td>Not amplified</td>
<td>Non-informative</td>
<td>None</td>
<td>N/A</td>
</tr>
</tbody>
</table>


* Same patient.
** Same patient.

Underlined - Somatic GBM mutations also found in TCGA Research Network dataset.