High-Resolution Profiling of EGFR Mutations in Glioblastoma Patients using an Ultrasensitive Digital PCR Approach

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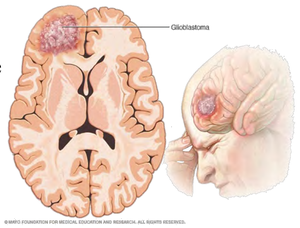
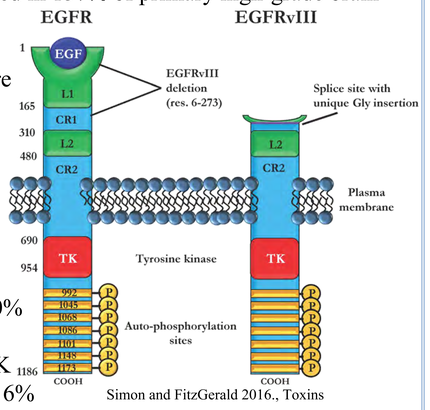
Introduction

Glioblastoma multiforme (GBM) is the most aggressive type of adult brain cancer. The average survival time after GBM diagnosis is 14.6 months even with current tri-modality therapy. Epidermal growth factor receptor (EGFR) is amplified in 157% of primary high-grade brain tumor GBM. Mutations in EGFR lead to more aggressive tumor progression and diminished survival. EGFR variant III occurs in 50-60% of EGFR and is present in up to 30% of gliomas, with A289V and R108K present in 3% and 6% of all gliomas, respectively. EGFRvIII results in an in-frame deletion of the extracellular domain (exons 2-7). We are in dire need of a molecular assay that rapidly profiles these alterations in EGFR since other assays currently available clinically, like next-generation sequencing, may take up to 4 weeks due to the batching of samples in current workflows.



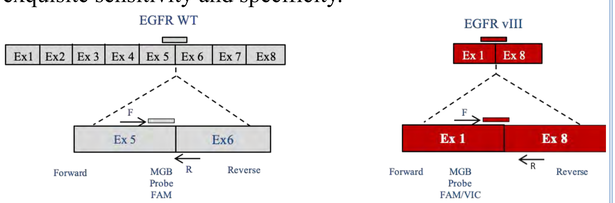
Glioblastoma multiforme. From Mayo Clinic



Simon and FitzGerald 2016., Toxins

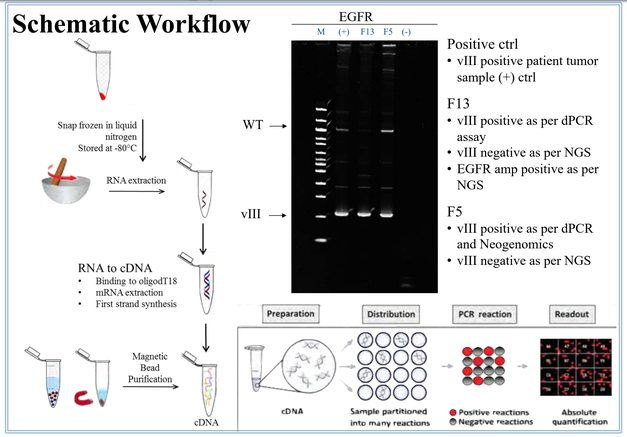
Methods

Our lab has established a very sensitive and novel digital polymerase chain reaction (dPCR) assay that detects EGFRvIII in patient tumors within 24 hours of resection. This dPCR assay utilizes RNA extracted from microgram quantities of resected tumors from GBM patients, which is then converted to complementary DNA (cDNA). cDNA is then pre-amplified and subjected to the dPCR assay using specific primers and probes for EGFRvIII and EGFR WT. The assay is multiplexed with an internal reference control, RNaseP. The same starting material can be used to detect the presence or absence of two other mutations, R108K and A289V, with exquisite sensitivity and specificity.



Gene	Primers/MGB Probe	Sequence
EGFR WT	EGFR WT F Exon 5	5' AAG TGT GAT CCA AGC TGT CC 3'
EGFR WT	EGFR WT R Exon 6	5' TGC TGG GCA CAG ATG ATT T 3'
EGFR WT	EGFR WT MGB PROBE 5_6	5' 6FAM AGG AGA ACT GCC MGB NFQ 3'
EGFRvIII	EGFR vIII F exon 1	5' TCG GGC TCT GGA GGA AA 3'
EGFRvIII	EGFR vIII R exon 8	5' CTT CCT CCA TCT CAT AGC TGT C 3'
EGFRvIII	EGFRvIII MGB PROBE 1_8	5' 6FAM/VIC AAA GGT AAT TAT MGB NFQ 3'

Schematic Workflow



dPCR Values by Demographic

Sample	NGS vIII result	Sex	Age at Surgery (Years)	amp EGFR / control	EGFRvIII / control	amp EGFR vIII%
FFPE 1	ND	F	63	49.60	0.04	0%
FFPE 2	ND	M	64	2.22	0.00	0%
FFPE 3	95%	F	52	71.26	9.56	12%
FFPE 4	ND	M	56	74.87	0.00	0%
FFPE 5	80%	M	65	85.55	94.85	53%
FFPE 6	57%	F	52	92.85	22.65	20%
FFPE 7	ND	F	46	0.81	0.62	0%
FFPE 8	ND	M	53	22.17	0.00	0%
FFPE 9	ND	F	54	11.75	0.00	0%
FFPE 10	10%	M	60	96.97	0.00	0%
FFPE 11	41%	F	54	52.87	0.00	0%
FFPE 12	14%	F	76	147.90	0.00	0%
FFPE 13	ND	F	72	126.25	0.00	0%
FFPE 14	ND	F	22	11.38	0.00	0%

Analyses

EGFRvIII primers were further tested for specificity. dPCR assay was run on cDNA extracted from U87 WT and U87vIII cells following protocol (Picelli et al 2013). [cDNA] ranged from 1.25×10^{-3} ng/mL to 5×10^{-3} ng/mL. The assay was further validated when 25-30 cells were selected using Kuiqpick; we extracted 5 ng of cDNA for dPCR analysis.

Dilution of EGFR WT and vIII Plasmids

dPCR primers and MGB probes were tested on serial dilutions of plasmid cDNA of EGFR vIII ranging from 0.625×10^{-5} ng/mL to 5×10^{-5} ng/mL.

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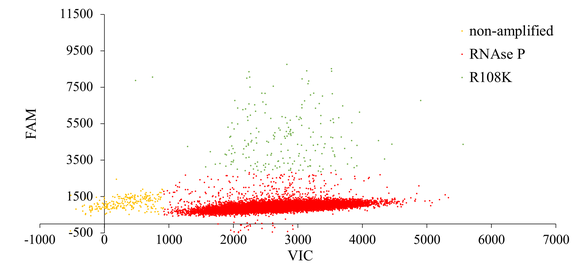
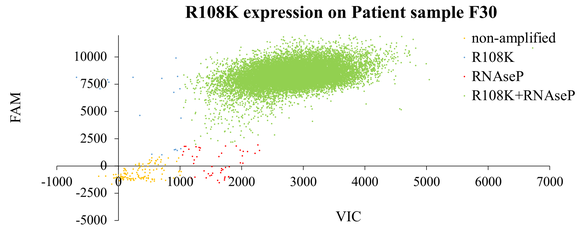
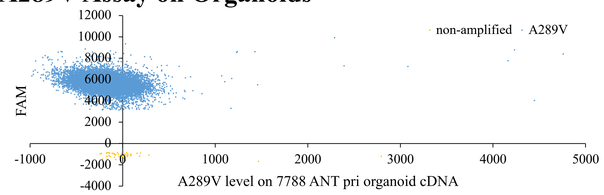
R108K assay on patient samples





Patient	R108K copies/uL	EGFRWT copies/uL	R108K %
F14	13.75	5953.3	0%
F30	9355.5	8017.7	46%

A289V Assay on Organoids



Sample	A289V copies/µL	RNA seq
7788 ANT Primary	3510.6	95%

Conclusions

- High-resolution assay identifies EGFRvIII in rare cell populations using unique primer sequences and MGB probe
- Sensitive (Limit of Quantification = 0.003%), specific, fast, robust, versatile, highly reproducible
- Can monitor disease progression and gauge therapeutic responsiveness in EGFRvIII patients

Future Directions

- Clinical trials
- Real-time liquid biopsy
  - Exosomes: cross BBB
  - Platelets: take up proteins and RNA from tumor
  - Circulating Tumor Cells

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