Droplet Digital PCR Analysis of CSF for Diagnosis of DIPG Histone Gene $H3F3A$ K27M Mutation

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The authors have no disclosures to report.
Diffuse intrinsic pontine glioma (DIPG) is a childhood tumor with invariably poor prognosis. Treatment is increasingly targeted toward molecular characteristics of tumors, including the recurrent \( H3F3A \) K27M mutation, but biopsy remains controversial due to operative risk. Here, we utilize droplet digital PCR (ddPCR) to identify cell-free tumor DNA in the CSF as a new means to establish diagnosis without tissue biopsy.
A novel assay was designed for use with ddPCR consisting of fluorescent probes specific for wildtype and K27M mutant H3F3A sequences.

This assay was validated using DNA isolated from non-tumor CSF (normal pressure hydrocephalus) and a synthetic positive control.

Patient CSF and tissue samples were collected at the time of surgical intervention, autopsy, or by lumbar puncture and DNA isolated.

QuantaSoft Analysis Pro was used for analysis of ddPCR results and calculation of variant allele fraction (VAF), mutant copies per ng DNA, and mutant copies per mL CSF (attempts to quantify tumor “burden”.)

All sequencing was conducted through the Pediatric MI-ONCOSEQ study.

DIPG007 (H3K27M+) and astrocyte cell lines were grown in co-culture and treated with radiation; luminescence of DIPG007 (luciferase+) and confluence of total cell population were assessed, as well as H3K27M mutant DNA in the cell culture media.
Validation of H3F3A K27M ddPCR assay

Positive control: synthetic K27M oligo and DNA from non-tumor CSF

Negative control: DNA from non-tumor CSF only

CSF from a 14yo with DIPG

~4000 copies of H3K27M per mL CSF
<table>
<thead>
<tr>
<th>Sample</th>
<th>Diagnosis</th>
<th>Time point</th>
<th>Source</th>
<th>VAF by sequencing</th>
<th>VAF by ddPCR</th>
<th>Copies per ng DNA</th>
<th>Copies per mL CSF</th>
</tr>
</thead>
<tbody>
<tr>
<td>UMPED17</td>
<td>GBM</td>
<td>Autopsy</td>
<td>Cisterna magna</td>
<td>48%</td>
<td>37.5%</td>
<td>105.3</td>
<td>25,121.6</td>
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<tr>
<td>UMPED18</td>
<td>DIPG</td>
<td>Diagnosis</td>
<td>Placement of VP shunt</td>
<td>57%</td>
<td>2.9% (preamplified)</td>
<td>n/a</td>
<td>n/a</td>
</tr>
<tr>
<td>UMPED18B</td>
<td>DIPG</td>
<td>Autopsy</td>
<td>LP</td>
<td>55%</td>
<td>37.4%</td>
<td>57.9</td>
<td>2,780</td>
</tr>
<tr>
<td>UMPED18B</td>
<td>DIPG</td>
<td>Autopsy</td>
<td>Rickham reservoir</td>
<td>55%</td>
<td>43.4%</td>
<td>132.9</td>
<td>420,167.5</td>
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<tr>
<td>UMPED21</td>
<td>GBM</td>
<td>Diagnosis</td>
<td>EVD</td>
<td>37%</td>
<td>17.9%</td>
<td>51.7</td>
<td>22,997.9</td>
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<tr>
<td>UMPED25</td>
<td>DIPG</td>
<td>Recurrence</td>
<td>EVD</td>
<td>47%</td>
<td>64.7%</td>
<td>112.2</td>
<td>4,044.9</td>
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<td>UMPED30</td>
<td>DIPG</td>
<td>Diagnosis</td>
<td>Intracranial subarachnoid</td>
<td>68%</td>
<td>Unable to detect</td>
<td></td>
<td></td>
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<td>UMPED24</td>
<td>DIPG</td>
<td>Diagnosis</td>
<td>EVD</td>
<td>24%</td>
<td>Unable to detect</td>
<td></td>
<td></td>
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<tr>
<td>UMPED43</td>
<td>Diffuse midline glioma</td>
<td>Diagnosis</td>
<td>EVD</td>
<td>41%</td>
<td>Unable to detect</td>
<td></td>
<td></td>
</tr>
</tbody>
</table>
H3K27M mutant copies per ng DNA by ddPCR correlates with proximity to tumor

Lateral ventricle (CSF): 132 copies
DIPG (inferior): 69 copies
Lumbar puncture (CSF): 57 copies
DIPG (middle): 238 copies
DIPG (superior): 255 copies

UMPED18 DIPG cell line
Primary cell line (media): 151 copies
Lateral ventricle (CSF): 132 copies
DIPG (superior): 255 copies
DIPG (middle): 238 copies
DIPG (inferior): 69 copies
Lumbar puncture (CSF): 57 copies
In vitro tumor cell (DIPG007)/astrocyte co-culture model reveals pattern of H3K27M mutant tumor DNA release into media after radiation treatment. DIPG007 (H3K27M) + NHA co-culture: untreated. DIPG007 (H3K27M) + NHA co-culture: 4 Gy XRT. 10-fold increase in H3K27M copies in media as tumor cell population dies.
Discussion

• A novel assay for cell-free tumor DNA (cf-tDNA) was designed for use with ddPCR consisting of fluorescent probes specific for wildtype and K27M mutant H3F3A sequences.
• We surveyed 3 tumor samples from a DIPG with established H3F3A mutational status by tumor sequencing and found our assay to be 100% sensitive in tissue.
• In CSF, our assay was positive in roughly 60% of samples from patients with tumors with established H3F3A mutation, and in every sample at recurrence or autopsy.
• In one patient with multiple tumor and CSF samples from diagnosis, autopsy, and primary cell line, H3K27M mutant copies per ng DNA correlated with proximity to tumor.
• A novel *in vitro* DIPG tumor cell/astrocyte co-culture model revealed correlative increase in H3K27M mutant copies in cell-free media by ddPCR with tumor growth, in addition to a significant jump 72 hours after treatment with radiation.
Summary

• We present a novel assay which is highly specific for the detection and quantification of H3K27M mutation in the spinal fluid of pediatric brain tumor patients.
• From our preliminary data, we hypothesize that H3K27M cf-tDNA correlates with CSF source and tumor status, and may acutely rise with effective treatments.
• This is a major step towards less invasive molecular diagnostics in a patient population for which biopsy confers substantial risk.
• Future work will focus on increasing the repertoire of assays for mutation and amplification detection with the ultimate goal of use in initial tumor characterization, as well as monitoring tumor DNA in the CSF over time in response to treatment.