cfDNA analysis of BRAF mutation in melanoma patients

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Tweede Nederlandse cfDNA Themadag 2018
Beatrixgebouw, Utrecht
**Disclosure of speaker’s interest**

<table>
<thead>
<tr>
<th>Category</th>
<th>Manon Huibers</th>
</tr>
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<tbody>
<tr>
<td>No (potential) conflicts of interest</td>
<td>None</td>
</tr>
<tr>
<td>Relations that could be relevant for the meeting</td>
<td>None</td>
</tr>
<tr>
<td>• Sponsorship or research funds</td>
<td>None, related to this talk</td>
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<td>• Payment or other (financial) renumeration</td>
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<td>• Shareholder</td>
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<td>• Other relation, viz...</td>
<td></td>
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</tbody>
</table>
Liquid biopsies (vs tissue biopsies)

Tissue biopsy
- Invasive / painful
- Complications
- Limited timepoints
- Sometimes impossible..
- Sampling error?

Liquid biopsy
- Minimally invasive
- Multiple timepoints
- Always accessible
- No Sampling error?

http://www.clpmag.com/2015/05/liquid-biopsies-less/
### Table 1

Studies that show utility of ctDNA as a biomarker of disease status in metastatic melanoma.

<table>
<thead>
<tr>
<th>Author</th>
<th>Publication date</th>
<th>No. of patients</th>
<th>Stage</th>
<th>Mutations</th>
<th>Method</th>
<th>Analytical sensitivity</th>
<th>Treatment</th>
<th>Diagnostic sensitivity</th>
<th>Associated with or prognostic</th>
</tr>
</thead>
<tbody>
<tr>
<td>Ascierto et al.</td>
<td>2014</td>
<td>91</td>
<td>IV</td>
<td>(\text{BRAF}^{	ext{V600E}})</td>
<td>BEAMing</td>
<td>Not reported</td>
<td>Combination Dab + Tra</td>
<td>75%–89%</td>
<td>Tumour burden, ORR &amp; PFS</td>
</tr>
<tr>
<td>Bettegowda et al.</td>
<td>2014</td>
<td>20</td>
<td>IV</td>
<td>(\text{BRAF})</td>
<td>BEAMing PCR, ligation or SafeSegS</td>
<td>Not reported</td>
<td>—</td>
<td>85%</td>
<td>Not assessed</td>
</tr>
<tr>
<td>Lipson et al.</td>
<td>2014</td>
<td>12</td>
<td>IV</td>
<td>(\text{BRAF})</td>
<td>BEAMing &amp; targeted resequencing (TERT)</td>
<td>0.01%</td>
<td>Ipi/BMS-936559</td>
<td>50% (BRAF unbiased)</td>
<td>Not assessed</td>
</tr>
<tr>
<td>Sanmamed et al.</td>
<td>2015</td>
<td>20</td>
<td>IV</td>
<td>(\text{BRAF}^{	ext{V600E}})</td>
<td>BioRad ddPCR</td>
<td>0.005%</td>
<td>Vern and Dab</td>
<td>84%</td>
<td>Tumour burden, PFS &amp; OS</td>
</tr>
<tr>
<td>Tsao et al.</td>
<td>2015</td>
<td>6</td>
<td>IV</td>
<td>(\text{BRAF}^{	ext{V600E}}, \text{NRAS}^{Q61R})</td>
<td>BioRad ddPCR</td>
<td>Not reported</td>
<td>Dabrafenib and MK3475</td>
<td>Not assessed</td>
<td>Not assessed</td>
</tr>
<tr>
<td>Gray et al.</td>
<td>2015</td>
<td>48</td>
<td>IV</td>
<td>(\text{BRAF}^{	ext{V600E}}, \text{NRAS}^{Q61R})</td>
<td>BioRad ddPCR</td>
<td>Not reported</td>
<td>Vern, Dab, Combi, Pembro, Nivo and Ipi</td>
<td>73%</td>
<td>ORR &amp; PFS</td>
</tr>
<tr>
<td>Gonzalez-Cao et al.</td>
<td>2015</td>
<td>22</td>
<td>IV</td>
<td>(\text{BRAF}^{	ext{V600E}})</td>
<td>AS-PCR</td>
<td>0.005%</td>
<td>Vern and Dab</td>
<td>50%</td>
<td>PFS &amp; OS</td>
</tr>
<tr>
<td>Santiago-Walker et al.</td>
<td>2015</td>
<td>836</td>
<td>IV</td>
<td>(\text{BRAF}^{	ext{V600E}})</td>
<td>BEAMing</td>
<td>Not reported</td>
<td>Dabrafenib and/or Trametinib</td>
<td>88%</td>
<td>ORR, PFS &amp; OS</td>
</tr>
<tr>
<td>Grotti et al.</td>
<td>2015</td>
<td>214</td>
<td>II, III and IV</td>
<td>(\text{BRAF}^{	ext{V600E}}, \text{NRAS}^{Q61R}, \text{PBRCA}^{R530K})</td>
<td>WES, Targeted re-sequencing</td>
<td>Not reported</td>
<td>Vern, Dab, Combi, Pembro, Nivo and Ipi</td>
<td>88%</td>
<td>ORR &amp; PFS</td>
</tr>
<tr>
<td>Chang et al.</td>
<td>2016</td>
<td>31</td>
<td>IV</td>
<td>(\text{BRAF}^{	ext{V600E}}, \text{NRAS}^{Q61R}, \text{PBRCA}^{R530K})</td>
<td>BioRad ddPCR</td>
<td>Not reported</td>
<td>Combination Combi, BRAF inhibitor</td>
<td>Not assessed</td>
<td>OS</td>
</tr>
<tr>
<td>Knoś et al.</td>
<td>2016</td>
<td>38</td>
<td>IIIc, IV</td>
<td>(\text{BRAF}^{	ext{V600E}})</td>
<td>Therascreen BRAF RQ Kit (Qiagen)</td>
<td>Not reported</td>
<td>Ipi, Pembro and Nivo</td>
<td>Not assessed</td>
<td>PFS &amp; OS</td>
</tr>
<tr>
<td>Lee et al.</td>
<td>2017</td>
<td>76</td>
<td>IV</td>
<td>(\text{BRAF}^{	ext{V600E}}, \text{NRAS}^{	ext{K}70}, \text{TERT})</td>
<td>ddPCR</td>
<td>Not reported</td>
<td>MAIKi, Immunotherapy</td>
<td>Not assessed</td>
<td>Tumour burden, PFS</td>
</tr>
<tr>
<td>Wong et al.</td>
<td>2017</td>
<td>52</td>
<td>IV</td>
<td>(\text{BRAF}^{	ext{V600E}}, \text{NRAS}^{	ext{K}70}, \text{TERT})</td>
<td>dPCR, Targeted amplicon (TA) sequencing</td>
<td>0.1% 2%</td>
<td>—</td>
<td>Not assessed</td>
<td>—</td>
</tr>
</tbody>
</table>

ctDNA as an indicator of emerging resistance to targeted therapy. Serial ctDNA level measurement can be used to determine the exact point of emergence of acquired resistance to targeted therapy. At the point of emerging relapse, the tumour burden, and consequent ctDNA levels are low, therefore immediate change in treatment strategies may provide optimal clinical benefit in melanoma patients.
Schematic representation of pseudoprogression in patients undergoing immunotherapy. ctDNA levels measurement can be used to distinguish between true progression (high) and pseudo-progression (low).

ctDNA in melanoma to predict recurrence

• Differentiate between patients who will recur or those who are cured by surgery.
• ctDNA predicts for relapse and survival and could aid selection of patients for adjuvant therapy.\(^2\)
  – 11-14% ctDNA detected in blood after surgery

Better...
- Disease free survival
- Distant metastasis free
- Survival

2. Annals of Oncology 29: 490–496, 2018
Mutations – detection in cfDNA

• **Single mutation**
  – Droplet digital PCR
    • MYD88 p.L265P
    • EGFR p.T790M
    • BRAF p.V600E
    • EGFR p.L858R
    • EGFR exon 19 deletions
    • EGFR other mutations
    • CD79B mutations
    • RET mutations
    • KRAS mutations
    • Etc...

• **Multiple mutations**
  – Targeted NGS panel (e.g. full TP53 or multiple hotspots)
    • Avenio ROCHE workflow (Wendy de Leng)
    • Nanopore sequencing (Wigard Kloosterman)
  – Whole Exome Sequencing (e.g. NIPT)
Diagnostic workflow UMC Utrecht

- cfDNA analysis request from clinician
  - Hix/GLIMS
    - Under LKCH requests “tumor markers”
  - Blood withdrawal in cfDNA tube (ROCHE) at poli-25 (or clinical department)
  - Blood tube transported (by ‘bode’) to LKCH lab
  - LKCH lab distributes request to “speciel lab”
  - Pre-analytical work up by “speciel lab” (2x/week)
    - spin protocol (double spin)
    - 2ml plasma input for cfDNA isolation (QiaCube)
  - cfDNA measurement in ng/ul (Qubit)
  - Report cfDNA concentration in GLIMS (ng/ul, 2 decimals)

- cfDNA analysis report back to clinician
  - Pathology report is visible in Hix
  - Report result in PALGA/UDPS
    - System includes known tumor information
  - When result is difficult/interesting:
    - Discuss case in Molecular tumor board (1x/2weeks)
  - Data analysis ddPCR (Quantasoft and report form)
  - cfDNA mutation analysis with ddPCR (2x/week)
  - Print at Molecular Pathology lab with cfDNA request
    - Barcode patient information
    - Gene of interest
    - DNA concentration (ng/ul)
Biorad – QX200

1. Make droplets

2. PCR DNA in droplets

3. Read and analyze results

Since 2015
Manual droplet generator

Since 2018
Automated droplet generator
BRAF exon 15
c.1799T>A; p.V600E

Amplicon length = 91nt

BRAF mutant

FW Primer
FAM
Mutant Probe
T
DNA

RV Primer
A

BRAF wildtype

FW Primer
HEX
Wildtype Probe
A
DNA

RV Primer
T

Fluorescence amplitude

mutant

‘empty’ droplets

wild type

Fluorescence amplitude
3. Liquid biopsy applications in oncology

- **Diagnosis**
  - Lymphoma – MYD88 L265P

- **Treatment choice**
  - Lung Cancer – EGFR T790M

- **Monitoring of disease (and diagnosis of brain metastasis)**
  - Melanoma – BRAF V600E

- **Relapse detection**
  - Head & Neck oncology – TP53 mutations
Melanoma – BRAF V600E

- BRAF mutations in melanoma: 37-50%
  - V600E mutation among BRAF-mutated melanomas: 80-90%
- Melanoma patients with BRAF mutation
  - Increased sensitivity for BRAF inhibitors (vemurafenib)

https://www.mycancergenome.org/content/disease/melanoma/braf/54/
Lito et al, Nature [2014]: The MEK-ERK pathway. (A) normal situation (B) V600E mutation in BRAF
Validation of BRAF V600E ddPCR assay

• Negative controls
  – Water
  – Negative FFPE samples (based on NGS)
  – cfDNA of healthy individuals
• Positive control:
  – Horizon reference sample
• FFPE controls
  – Melanoma + and – (also Lung and colon)
• Samples of interest
  – cfDNA from patients suspected to be positive for mutation
BRAF- results negative controls

- Cut off value is 2 mutant droplets
- Every sample above 2 mutant droplets is stated positive for BRAF V600E mutation

<table>
<thead>
<tr>
<th>Sample\droplets</th>
<th>Mutant droplets</th>
<th>WT droplets</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>amount</td>
<td>minimum</td>
</tr>
<tr>
<td>Water control</td>
<td>N=26</td>
<td>0</td>
</tr>
<tr>
<td>FFPE “healthy”</td>
<td>N=4</td>
<td>0</td>
</tr>
<tr>
<td>FFPE Melanoma neg.</td>
<td>N=10</td>
<td>0</td>
</tr>
<tr>
<td>FFPE Lung neg.</td>
<td>N=10</td>
<td>0</td>
</tr>
<tr>
<td>FFPE Colon neg.</td>
<td>N=10</td>
<td>0</td>
</tr>
<tr>
<td>cfDNA “healthy” MDD</td>
<td>N=8</td>
<td>0</td>
</tr>
</tbody>
</table>
BRAF- Horizon reference control

- Commercial available control, contains multiple mutations
- Contains 10% BRAF V600E mutation

- FA of 9%
**BRAF- results**

BRAF V600E
pos. FFPE control confirmed with NGS

<table>
<thead>
<tr>
<th></th>
<th>Control</th>
<th>Pos. WT droplets</th>
<th>Pos. mutant droplets</th>
<th>Fractional Abundance (ddPCR)</th>
<th>Mutation Freq (NGS)</th>
</tr>
</thead>
<tbody>
<tr>
<td>1</td>
<td>3180</td>
<td>2497,5</td>
<td></td>
<td>43,35</td>
<td>45</td>
</tr>
<tr>
<td>2</td>
<td>1233</td>
<td>1594,5</td>
<td></td>
<td>56,65</td>
<td>58</td>
</tr>
<tr>
<td>3</td>
<td>1219</td>
<td>5416,5</td>
<td></td>
<td>84,25</td>
<td>82</td>
</tr>
<tr>
<td>4</td>
<td>3233</td>
<td>4525</td>
<td></td>
<td>59,8</td>
<td>60</td>
</tr>
<tr>
<td>5</td>
<td>4330,5</td>
<td>1328,5</td>
<td></td>
<td>21,35</td>
<td>21</td>
</tr>
<tr>
<td>6</td>
<td>1179</td>
<td>1153</td>
<td></td>
<td>49,7</td>
<td>51</td>
</tr>
<tr>
<td>7</td>
<td>1164,5</td>
<td>1172</td>
<td></td>
<td>50,1</td>
<td>53</td>
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<tr>
<td>8</td>
<td>2792</td>
<td>3931,5</td>
<td></td>
<td>59,6</td>
<td>57</td>
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<tr>
<td>9</td>
<td>435</td>
<td>276</td>
<td></td>
<td>39,25</td>
<td>37</td>
</tr>
<tr>
<td>10</td>
<td>2022,5</td>
<td>2582,5</td>
<td></td>
<td>56,7</td>
<td>51</td>
</tr>
</tbody>
</table>

**Correlation of melanoma FFPE pos.**

\[R = 0.9873\]

\[P < 0.0001\]
Follow up in time - BRAF p.V600E concordant cases with clinic

Patient A: stable on BRAF inhibitor

Constant amount of BRAF p.V600E mutant copies in blood cfDNA

Patient B: started on BRAF inhibitor

BRAF p.V600E mutant copies disappeared directly after treatment
Follow up in time - BRAF p.V600E discordant case with clinic

All tested samples are negative in plasma....

Clinical follow up:
- July 2016: skin metastasis - BRAF V600E mutation
- October 2017: progression based on radiology, leptomeningeal metastasis
- November/December 2017: progression based on radiology,

Questions:
1. What specific nucleotide change for BRAF V600E? Correct one!
2. No other metastasis, other than leptomeningeal; is mutation detectable in CSF and not in blood?
3. Biology of tumor is so, that ctDNA is not secreted in blood?
BRAF V600E mutation analysis on liquor

• 40-50% of the melanoma patients develops brain metastases.

• No proper diagnostic method yet
  – biopsy is not the preferred method (induces tumor growth, invasive, extinct and not always possible)
  – Cerebrospinal fluid (CSF, liquor) sample is cell poor

• mRNA expression levels and drug levels cannot be measured in a biopsy
  – Believed to correlate with tumor resistance
  – Develop a method to test for BRAF mutation status in CSF DNA and drug levels

1) Develop method to test for BRAF mutation status in CSF DNA

2) Develop method to test for BRAF mutation status in CSF RNA and drug levels

– Develop a method to monitor drug levels
**BRAF mutation analysis in CSF DNA**

- Detection of melanoma brain metastasis, without biopsy
- Same workflow as for MYD88 mutation analysis:
  - Waldeström (LPL located in brain) \(^1\)
  - Primary central nervous system lymphoma \(^2, 3\)
  - Etc...

- Work in progress... No positive sample in liquor requests

<table>
<thead>
<tr>
<th>PA-nummer</th>
<th>Mutatie gedetecteerd</th>
<th>vraagstelling</th>
<th>aantal wells</th>
<th>Opmerkingen</th>
</tr>
</thead>
<tbody>
<tr>
<td>1</td>
<td>NI, onvoldoende wt druppels</td>
<td>Metastase melanoom</td>
<td>6</td>
<td>~1,5ml (spijt LKCH) supernatant liquor, cfDNA</td>
</tr>
<tr>
<td>2</td>
<td>Nee, sensitiviteit 0,3%</td>
<td>Lymfoom / Langerhanscelhistiocytose</td>
<td>3</td>
<td>~350ul + 2 blanco’s, DNA uit celpellet</td>
</tr>
<tr>
<td>3</td>
<td>Nee/Ni, pellet onvoldoende wt, cfDNA 2% sensitiviteit</td>
<td>Langerhanscelhistiocytose</td>
<td>3</td>
<td>~8,5ml verse liquor, DNA uit celpellet, ~8ml supernatant liquor, cfDNA</td>
</tr>
<tr>
<td>4</td>
<td>NI, Onvoldoende wt druppels</td>
<td>Metastase melanoom</td>
<td>4</td>
<td>~1,6ml (spijt LKCH) supernatant liquor, cfDNA</td>
</tr>
</tbody>
</table>

3. Hiemcke-Jiwa et al. CSF cfDNA unpublished data
Extracellular vesicles

- Particles shed by all cells
- Increased release by tumor cells
- Are believed to contain microRNA, mRNA and proteins (and targeted drugs)

Kanada et al, Trend in Cancer [2016]
Exosomes to screen for BRAF V600E mutation on RNA

- Exosomes of a BRAF mutated cell line showed to contain the V600E mutation

- Exosomes of BRAF mutated cell line spiked in healthy donor blood
  - BRAF V600E mutation was still detected when spiked in EDTA and citrate blood
  - < 1,50E+09 exosomes quantitative detection (confirmed with ddPCR)
  - 1 BRAF V600E mRNA per 144E+06 exosomes (confirmed with ddPCR)
  - 4,2E+12 exosomes/mL serum (confirmed with literature)

- Work in progress... thus far, no positive signal by ddPCR measured on BRAF+ patient EDTA plasma

Figure 1 – The BRAF V600E mutation is detected in cDNA from an A375 cell line, isolated from cells and EVs.
Take home message...

In pathology, tissue is still the golden standard...

However... There is a lot of information available in less invasive patient samples!

cfDNA in liquid biopsies could inform us about tumor behavior (non-invasive)

Molecular techniques are improving making it possible to take the next step and...

... implement liquid biopsies in the diagnostic setting
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