Detecting gene amplifications in NGS data: from lab data to clinical report

Recommendations for routine diagnostics

Astrid Eijkelenboom, PhD, Clinical Scientist in Molecular Pathology
Radboud university medical centre, Nijmegen, The Netherlands
Disclosures

Nothing to declare.
Recommendations for the clinical interpretation and reporting of copy number gains using gene panel NGS analysis in routine diagnostics

Astrid Eijkelenboom¹ · Bastiaan B. J. Tops² · Anke van den Berg³ · Adrianus J. C. van den Brule⁴ · Winand N. M. Dinjens⁵ · Hendrikus J. Dubbink⁵ · Arja ter Elst³ · Willemina R. R. Geurts-Giele⁵ · Patricia J. T. A. Groenen¹ · Floris H. Groenendijk⁵ · Daniëlle A. M. Heideman⁶ · Manon M. H. Huibers⁷ · Cornelis J. J. Huijsmans⁴ · Judith W. M. Jeukën⁸ · Léon C. van Kempen³ · Esther Koropershoek⁵ · Leonie I. Kroeze¹ · Wendy W. J. de Leng⁷ · Carel J. M. van Noesel⁹ · Ernst-Jan M. Speel¹⁰ · Maartje J. Vogel¹¹ · Tom van Wezel¹² · Petra M. Nederlof¹¹ · Ed Schuuring³ · Marjolijn J. L. Ligtenberg¹,¹³

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1) Department of Pathology, Radboud university medical center, Nijmegen, The Netherlands
2) Princess Máxima Center for Pediatric Oncology, Utrecht, The Netherlands
3) Department of Pathology, University of Groningen, University Medical Center Groningen, Groningen, The Netherlands
4) Pathology-DNA, Location Jeroen Bosch Hospital, Den-Bosch, The Netherlands
5) Department of Pathology, Erasmus MC Cancer Institute, University Medical Center Rotterdam, Rotterdam, The Netherlands
6) Amsterdam UMC, Vrije Universiteit, Pathology Department of Pathology, Cancer Center Amsterdam, Amsterdam, The Netherlands
7) Department of Pathology, University Medical Center Utrecht, Utrecht, the Netherlands
8) Department of Pathology, PAMM, Eindhoven, The Netherlands
9) Department of Pathology, Amsterdam UMC, University of Amsterdam, Amsterdam, The Netherlands
10) Department of Pathology, Maastricht University Medical Center, Maastricht, The Netherlands
11) Department of Pathology, Netherlands Cancer Institute, Amsterdam, The Netherlands
12) Department of Pathology, Leiden University Medical Center, Leiden, The Netherlands
13) Department of Human Genetics, Radboud university medical center, Nijmegen, The Netherlands
From diagnostic experience to recommendations

- March 2018: National meeting of Dutch Clinical Scientist (PATH project)

- 6 centres shared experiences in detection CNVs in diagnostics using NGS panels

- Determine requirements for pathology report

- Manuscript with recommendations, co-authored by 12 centres
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Detection of gene amplifications in NGS data: from lab-data to clinical report

• What are gene amplifications (copy number gains)?

• Approaches to detect gene amplifications in NGS data

• Implementation into routine diagnostics
Detection of gene amplifications in NGS data: from lab-data to clinical report

• What are gene amplifications (copy number gains)?

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Gene amplifications

Potential therapeutic target and/or differential diagnosis

Gene amplifications: *just one of many aberrations*

Untreated NSCLC:

- **Unknown** (~40%)
- **ALK rearrangement** (3–7%)
- **RET rearrangement** (1–2%)
- **NTRK1 fusion** (<1%)
- **ROS1 rearrangement** (1–2%)
- **MET amplification, exon 14 skipping** (~5%)
- **KRA rearrangement** (10–15%)
- **Other**

NGS for mutation detection

<table>
<thead>
<tr>
<th>Inhibitors</th>
<th>EGFR</th>
<th>ALK</th>
<th>ROS</th>
<th>RET</th>
</tr>
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<tbody>
<tr>
<td><strong>Inhibitors</strong></td>
<td></td>
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<td></td>
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<tr>
<td>Crizotinib*</td>
<td>Erlotinib*</td>
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<td>Crizotinib*</td>
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<td>Brigatinib</td>
<td>EGFR816</td>
<td>Entrectinib</td>
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<td>APSE273</td>
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<tr>
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<td>Entrectinib</td>
<td>HM61713</td>
<td>DS-601b</td>
<td>Entrectinib</td>
</tr>
</tbody>
</table>

**Less tissue, but more aberrations to screen for**

https://www.cell.com/trends/cancer
Detection of gene amplifications in NGS data: from lab-data to clinical report

• What are gene amplifications (copy number gains)?

• Approaches to detect gene amplifications in NGS data

• Implementation into routine diagnostics
Detection of gene amplifications using NGS

normal cell

Gene A  
Gene B  
Gene C  

Gene A  
Gene B  
Gene C  

Gene A  
Gene B  
Gene C  

Gene A  
Gene B  
Gene C  

tumor cell

Gene A  
Gene B  
Gene C  

Gene A  
Gene B  
Gene C  

Gene A  
Gene B  
Gene C  

Gene A  
Gene B  
Gene C  

Radboudumc
Detection of gene amplifications using NGS...

NGS reads:

...by coverage analysis (I)
NGS based detection of gene amplifications

Coverage based detection

1) Normalization

Different approaches can be used for normalization
NGS based detection of gene amplifications

Coverage based detection

2) Comparison with other samples

Comparison with reference pool

Comparison with internal or external reference pool, or mixed approach
NGS based detection of gene amplifications

Coverage based detection

3) Relevant quantitative (fold change) & statistical (z-score) measures
NGS based detection of gene amplifications

Coverage based detection

3) Note: statistical measures depend on variation and can differ per gene...

\[ z = \frac{\text{change}}{\text{stdev}} \rightarrow \text{statistical measure, reliability} \]

Relative coverage or fold change: quantitative measure
NGS based detection of gene amplifications

Coverage based detection

3) Note: statistical measures such as z-scores can be different per gene/batch/lab. Z-scores are therefore not a proper measure for the level of amplification.
Detection of gene amplifications using NGS...

NGS reads:

...by SNP allele frequency (II)
Detection of gene amplifications using NGS...

...by SNP allele frequency (II)
NGS based detection of gene amplifications

SNP based detection: B allele frequency (BAF)

Requires sufficient SNPs at/near each gene locus

Does is represent gain or loss? → coverage information required
Detection of gene amplifications in NGS data: from lab-data to clinical report

• What are gene amplifications (copy number gains)?

• Approaches to detect gene amplifications in NGS data

• Implementation into routine diagnostics
Implementation routine diagnostics

What about:

- the magnitude of amplification?
- the influence of tumorload?
- analytical sensitivity?
- the clinical report?
Implementation routine diagnostics

What about:

- the magnitude of amplification?
- the influence of tumorload?
- analytical sensitivity?
- the clinical report?
NGS based detection of gene amplifications is influenced by magnitude of amplification

*normal*  
*‘low’ level*  
*‘high’ level*

NGS based detection of gene amplifications is influenced by magnitude of amplification.

- **normal**
- ‘low’ level (3 copies)
- ‘high’ level (9 copies)
NGS based detection of gene amplifications is influenced by magnitude of amplification

- **normal (1+1)n**
  - coverage 1x

- **‘low’ level (1+3)n**
  - coverage 2x

- **‘high’ level (1+9)n**
  - coverage 5x
NGS based detection of gene amplifications is influenced by magnitude of amplification.

- **normal**
- ‘low’ level (3 copies)
- ‘high’ level (9 copies)
NGS based detection of gene amplifications is influenced by magnitude of amplification

**normal**
- $1/2 \rightarrow 50\%$
- $1/2 \rightarrow 50\%$

**‘low’ level**
- $1/4 \rightarrow 25\%$
- $3/4 \rightarrow 75\%$

**‘high’ level**
- $1/10 \rightarrow 10\%$
- $9/10 \rightarrow 90\%$
NGS based detection of gene amplifications is influenced by magnitude of amplification

Resolution of coverage vs SNP-based detection:
Implementation routine diagnostics

What about:

- the magnitude of amplification?
- the influence of tumorload?
- analytical sensitivity?
- the clinical report?
NGS based detection of gene amplifications is influenced by tumor load:

Copy number estimation with 2x increased coverage:

- 100% tumor load $\rightarrow \sim 3$ copies
- 50% tumor load $\rightarrow \sim 5$ copies
- 25% tumor load $\rightarrow \sim 9$ copies
NGS based detection of gene amplifications is influenced by tumorload

\[
(1+1)n \times 100\% \rightarrow \text{coverage 1x}
\]

4 cells $\rightarrow$ 8 copies of \(\text{GeneB}\)

\[
(1+3)n \times 100\% \rightarrow \text{coverage 2x}
\]

4 tumour cells $\rightarrow$ 16 copies of \(\text{GeneB}\)
NGS based detection of gene amplifications is influenced by tumorload

\[(1+1)n \times 100\% \rightarrow \text{coverage } 1x\]

4 cells $\rightarrow$ 8 copies of GeneB

\[\left((1+1)n \times 50\%\right) + \left((1+5)n \times 50\%\right) \rightarrow \text{coverage } 2x\]

2 normal cells $\rightarrow$ 4 copies of GeneB
2 tumour cells $\rightarrow$ 12 copies of GeneB
NGS based detection of gene amplifications is influenced by tumor load

\[(1+1)n \times 100\% \Rightarrow \text{coverage } 1x\]

\[4 \text{ cells } \Rightarrow 8 \text{ copies of } GeneB\]

\[\frac{(1+1)n \times 75\%}{100\%} + \frac{(1+9)n \times 25\%}{100\%} \Rightarrow \text{coverage } 2x\]

\[3 \text{ normal cells } \Rightarrow 6 \text{ copies of } GeneB\]
\[1 \text{ tumour cell } \Rightarrow 10 \text{ copies of } GeneB\]
NGS based detection of gene amplifications is influenced by tumorload

Copy number estimation with 2x increased coverage:

100% tumorload $\rightarrow$ ~ 3 copies
50% tumorload $\rightarrow$ ~ 5 copies
25% tumorload $\rightarrow$ ~9 copies

Does it represent a clinically relevant / targetable gene amplification?

Note:
- approximation of tumorload is error prone
- based on 2n genomes of normal and tumor cells
Quantitative interpretation of NGS based detection of gene amplifications

Clinically relevant? Comparison with FISH required?
Implementation routine diagnostics

What about:

- the magnitude of amplification?
- the influence of tumorload?
- analytical sensitivity?
- the clinical report?
Analytical sensitivity

Analytical sensitivity depends on cut-offs and tumour cell%
Implementation routine diagnostics

What about:

- the magnitude of amplification?
- the influence of tumorload?
- analytical sensitivity?
- the clinical report?
NGS based detection of CNVs in routine diagnostics: the report

1) Gene name

2) Type CNV (amplification, deletion, LOH)

3) Relative coverage (fold change) and approximation of the number of gene copies (based on tumorload)

4) Statement about assay sensitivity (false negative for low level gene amplifications or in case of low tumorload)

Optional:

Statistical measures (z-score etc).
To summarize: technical considerations

Table 1  Technical considerations for detection of copy number gains (gene amplifications) using panel NGS data

<table>
<thead>
<tr>
<th>Technical issue</th>
<th>Why relevant?</th>
<th>Considerations</th>
</tr>
</thead>
</table>
| Panel content       | Panel size and selection of genomic loci can affect detection of copy number runs | (i) Contains amplicons/probes sufficiently spread throughout the genome  
(ii) Includes loci likely to not be affected by copy number variation in tumor of interest  
(iii) Minimal number of amplicons/probes per gene, preferably throughout gene locus  
(iv) For BAF, include sufficient number of heterogeneous loci for sufficient “SNP-density” |
| Normalization       | Required to correct for differences in gDNA input quality/quantity           | Choose method that is not/minimally affected by copy number variation                                                                                                                                     |
| Reference pool      | Is required to detect coverage outliers indicative of copy number gains       | (i) Internal and/or external reference pool  
(ii) Includes samples without copy number variation (e.g., normal tissue)  
(iii) Processed using identical protocols                                                                                                           |
| Thresholds          | Required to distinguish genuine copy number gains from technical noise       | (i) Validated by positive/negative controls using other methods  
(ii) Includes minimal coverage thresholds to prevent false positive calls from poor quality gDNA  
(iii) Include positive and negative controls on a regular basis, to ensure assay stability and test validated thresholds |
| Sensitivity         |Awareness of assay limitations is critical for routine diagnostics            | (i) Affected by thresholds and neoplastic cell percentage  
(ii) Should be included in clinical report                                                                                                           |

Eijkelenboom et al., Virchows Arch. 2019 Jun;474(6):673-680
To summarize: relevant biological phenomena

<table>
<thead>
<tr>
<th>Biological phenomena</th>
<th>Why relevant?</th>
<th>How does it affect detection of copy number gains?</th>
</tr>
</thead>
</table>
| Neoplastic cell content | Measurements are obtained from a mixture of tumor-derived and non-neoplastic gDNA | (i) The actual detected increase in coverage/deviation in BAF increases with neoplastic cell content  
(ii) Influences the estimation of the allele copy number  
(iii) Determines assay sensitivity (in combination with thresholds used to identify statistically significant gains) |
| Allele copy number/magnitude of amplification | Clinical consequences are based on cutoffs in allele copy number of gene amplification | (i) The detected increase in coverage/deviation in BAF increases with allele copy number  
(ii) Assay sensitivity should match the clinically relevant cutoffs in allele copy number |
| Aneuploidy | Can affect normalization and allele copy number estimation | (i) Results in underestimation or overestimation depending on the nature and extend of aneuploidy and the number of genomic regions that are included in the gene panel  
(ii) High-level copy number gains are likely less affected compared to low copy number alterations |

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