Emergence of new ALK mutations at relapse of neuroblastoma

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Background

- Cancer: frequent secondary progression, resistance to conventional chemotherapy: therapeutic challenge
- Selection for genetic alterations during treatment
- Subclonal driver mutations might play a role in tumor progression
- Presence of driver mutation-harboring subclones at diagnosis, which might expand at relapse, has been linked to adverse outcome in hematological malignancies (Landau et al, 2013)
- Different models of clonal evolution (Ding et al, 2011):
  - Dominant clone evolves into relapse clone
  - A minor clone carrying the vast majority of primary tumor mutations escapes and expands at relapse
Hallmark of neuroblastoma: clinical heterogeneity

Treatment of neuroblastoma:
- Observation only
- Surgery
- Chemotherapy

Chemotherapy
- High dose chemotherapy with autologous stem cell rescue
- Surgery
- Radiotherapy
- Immunotherapy
- Maintenance treatment

Improvement of EFS in NB (all stages) over the last decades
But: despite significant progress for lower stages, survival in stage 4 disease does still not exceed 40% (mortality linked to metastatic tumor progression)

Moroz et al, 2011
ALK TRK plays a role in NB oncogenesis

Germline Mutations of ALK in neuroblastoma families

- Activating ALK mutations in 8 – 10% of all NB at diagnosis (Mosse et al, Janoueix-Lerosey et al, Chen et al, George et al 2008; De Brouwer et al, 2010)
- Objectives of this study: Determine the frequency of ALK mutations at relapse and their role in clonal evolution of NB
Methods

- 54 paired diagnosis – relapse NB tumor samples (France, Sweden, Belgium)
- 2 cell lines with corresponding tumor sample from which cell lines were established
- Sanger sequencing

- Deep sequencing (IonTorrent PGM®, LifeTechnologies) when an ALK mutation was seen in only one of the paired samples (7 cases)
  - Resequencing of hotspots exon 23 and exon 25
  - Depth of coverage: 100,000x
  - 4 control cell lines (CLB-Car, SKNDZ, SJNB12, SKNAS)
Heterogeneity of ALK mutations in NB

Ganglioneuroblastoma
Local progression,
Treatment by surgery

Michel Peuchmaur

Alk mutation R1275Q (CGA>CAA)

No Alk mutation
### Results (Sanger sequencing): 14/54 Alk mutations

<table>
<thead>
<tr>
<th>Patient N°</th>
<th>Age at diag (months)</th>
<th>stage (INSS)</th>
<th>Interval diagnosis-relapse (months)</th>
<th>relapse type</th>
<th>FU (months from diagnosis)</th>
<th>Outcome</th>
<th>Genomic profile</th>
<th>ALK mutation</th>
<th>ALK Detection by Sanger</th>
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<td>23</td>
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</table>

All pts with ALK mutations at diagnosis also had ALK mutations at relapse

5/54 new ALK mutations at relapse

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Methods

• 54 paired diagnosis – relapse NB tumor samples
• 2 cell lines and tumor sample from which cell lines were established
• Sanger sequencing
• Deep sequencing (IonTorrent PGM®) when an ALK mutation was seen in only one of the paired samples (7 cases)
  • Resequencing of hotspots exon 23 (hotspot F1174) and exon 25 (hotspot R1275)
  • Depth of coverage: 100,000x
  • 4 control cell lines (CLB-Car, SKNDZ, SJNB12, SKNAS)
Analysis of noise (control cell lines):

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### PGM® analysis: cases with discordant Sanger results

<table>
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<tr>
<th>Chr position / patient n°</th>
<th># reads</th>
<th>A</th>
<th>C</th>
<th>G</th>
<th>T</th>
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<td>chr2:29432655 (A) Y1278S</td>
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<td></td>
<td></td>
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<td>NB1382_D</td>
<td>226371</td>
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<td>0.011</td>
<td>1.00E+00</td>
<td>0.031</td>
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<td>NB1382_R</td>
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<td>&lt;1E-016</td>
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<td>Controls (n=4)</td>
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<tr>
<td>Total of all reads</td>
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<td>0.012</td>
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<td>Controls (n=4)</td>
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<td>Mean</td>
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<td>2.49E-003</td>
<td>0.012</td>
<td>4.67E-003</td>
<td>99.921</td>
</tr>
</tbody>
</table>

Cases studied by PGM® in case of discrepancies of Sanger sequencing in different samples
- 5 patients: 13 samples
- 2 established cell lines: 4 samples

Analysis: comparison of base frequencies observed in a given sample, at a given position, to that observed in controls (Fisher exact test)

In some cases: no evidence of ALK mutated subclones

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PGM® analysis: evidence of subclonal events

NBG17: Presence of an ALK mutated subclone (TTC> TTA; 0.798%) at diagnosis

NB0308: ALK mutation switch (TTC> TTA/TTG) leading to the same AA change at D and R
Results (NB cell lines)

<table>
<thead>
<tr>
<th>Cell line</th>
<th>Age at diag (months)</th>
<th>stage (INSS)</th>
<th>Primary sample</th>
<th>Cell line established from</th>
<th>FU (months)</th>
<th>Outcome</th>
<th>Genomic profile</th>
<th>AA change</th>
<th>Primary sample</th>
<th>Cell line</th>
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<tbody>
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<td>4</td>
<td>Abdominal tumor</td>
<td>bone marrow</td>
<td>16</td>
<td>DOD</td>
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<td>F1174L</td>
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<tr>
<td>CLB_Ba</td>
<td>27</td>
<td>4</td>
<td>bone marrow</td>
<td>bone marrow</td>
<td>117</td>
<td>NED</td>
<td>MNA</td>
<td>F1174L</td>
<td>neg</td>
<td>F1174L</td>
</tr>
</tbody>
</table>

Presence of an ALK mutated subclone (6.6%) in the Sample from which the cell line was established
Discussion

- **Sensitivity of the PGM® deep sequencing technique:**
  - Mean overall coverage (control cell lines): >175,000X
  - Errors in PGM:
    - vary strongly according to the genome structure: homopolymers;
    - possible link to sequencing errors due to polymerase slippage, errors in the chemistry
  - Mean overall background variability: 0.034%+/-0.035% for each base
  - Number of reads would be considered statistically different from the background (Bonferroni correction): a variation supported by 296 reads, or observed with a frequency of 0.17%, would result in a statistically significant difference from the controls (two-sided Fisher’s exact test)
  - **Sensitivity 0.17%** (100 fold that of Sanger sequencing, 20%)

- **Limits in sensitivity: quantity of analyzed material**
  - PCR of exon 23/25 amplicons: 50 ng of genomic DNA
  - ~5000 (diploid) cells
  - Limit of detection: 1/5000 cells; 1/10 000 haploid genomes
ALK in tumor progression

- In NB, *ALK* mutations might occur as subclones at diagnosis with secondary expansion
  - selective advantage during tumor progression
  - *ALK*-mutated and non-mutated cells might co-exist in an equilibrium
  - expansion of an *ALK*-mutated clone upon treatment: preferential cytotoxic effect on *ALK* non-mutated clones?

Clinical implications
Encourage new biopsy at relapse!

Search for subclones:
- Evaluation of frequency of *ALK* mutated subclones at diagnosis
- *ALK* targeted treatment for cases with subclonal *ALK* mutations?
  - further investigations in vitro and in vivo

When considering biomarker based *ALK* targeted therapy:
- Search for genetic alteration of *ALK*
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[Logos and graphics]