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**NGS and Thyroid FNA**

Large-scale NGS

Targeted NGS

Utility, implementation examples, pitfalls

Fresh FNA samples

Fixed FNAs

Cell blocks

**Next Generation Sequencing Approaches**

- Large-scale NGS: whole genome, exome, transcriptome (RNA-Seq)
  - Discovery tool, used in tissues, requires large amount of NA, expensive, time-consuming
  - Targeted NGS: sequencing of multiple preselected genes or gene regions
- Small amount of NA (fewer number of cells), fast, less expensive, used clinically

**Targeted NGS allows for detection of all types of genetic alterations in a single workflow**

- DNA
- RNA
- Point mutations
- Copy number alterations
- Gene fusions
- Gene expression
**Molecular Alterations in Thyroid Cancer**

TCGA study of PTC

- **Point mutations**: 79%
- **Gene fusions**: 15%
- **Copy number**

PTC >95% with known alterations

**Pan-cancer Targeted NGS for Thyroid FNA**

Pan-cancer NGS panels can be used in thyroid FNAs for detection of individual genetic alterations

- Report individual mutation findings (e.g., BRAF), high PPV, low NPV

**ThyroSeq Workflow**

Pre-Analytical

- FNA collection

Analytical

- Next Generation Sequencing
- Cytology Diagnosis
- BI Computation Analysis

Interpretation

- ThyroSeq® v.2
  - 14 genes for mutations, >1000 hotspots
  - 42 fusion types, 16 genes for expression

- Ion Torrent/Proton based targeted NGS, <300 cells, 5-10ng of NA

**Custom Targeted NGS Panel: ThyroSeq**

- ThyroSeq v.1: 15-gene panel
- ThyroSeq v.2: 56-gene panel
- ThyroSeq v.3: >100-gene panel

**Molecular Alterations for Prognostics: BRAF V600E + TERT**

- BRAF V600E
- TERT

**ThyroSeq® v.2**

- 14 genes for mutations
- 42 fusion types

**ThyroSeq® v.2**

- 14 genes for mutations
- 42 fusion types

**ThyroSeq® v.2**

- 14 genes for mutations
- 42 fusion types
**FNA Sample Types**

- **Freshly collected** (preferred type)
  - Pros: excellent preservation of DNA/RNA, 98% works
  - Cons: separate pass, unknown cell representation
- **Fixed cytology slides and cell blocks**
  - Pros: fits into cytology workflow, easy to ship and store
  - Cons:
    - works well for DNA, less optimal for RNA (90% works)
    - destroying cytology slide
    - scraping from slides is time consuming
    - loss of cells during NA isolation

**Step 1: Assessment of FNA Sample Adequacy and Cell Lineage**

- Parathyroid lesions and c-cells medulary thyroid carcinomas may mimic thyroid follicular cells-derived nodules
- Diagnostic difficulty on imaging and fine-needle aspiration cytology

### Examples of Thyroid Cell Aneuploidy markers

- **PGK1** — pan-cell marker
- **TTF1**
- **NIS**
- **KRT20** — metastatic
- **PTH** — parathyroid
- **Calcitonin** — MTC

### Expression Profile: Parathyroid cells

- Low quantity (no cells)
- Low quality (degraded)

### Expression Profile: Medullary Thyroid Ca

- High gene expression of other cells (lymphocytes, macrophages, etc.)
- Limited number of thyroid follicular cells
- High gene expression of thyroid follicular cells

**0.6% of Thyroid FNAs (Bill-V) are Parathyroid Lesions**

0.4% of Thyroid FNAs (Bill-V) Diagnosed as MTC

### 4,789 FNA samples (Bethesda Bill-V) analyzed by ThyroSeq® Genomic Classifier

- 29 (64%) FNAs showed MTC profile
- 5 FLU/LUS
- 6 FN/SSN
- 10 SMC

### Surgery (n=10)

- 13/13 (100%) diagnosed as MTC
- 10/10 (100%) diagnosed as parathyroid lesions

### Importante of NGS Data Interpretation:

- Correct Annotation, mutations have different functional significance

- **TERT**
  - Driver, clinically significant
  - Mutation Type (somatic vs. germline)
  - Mutant Allele Frequency (MAF)
  - Combination of Mutations

- **NRAS**
  - Germline Variant
  - No Functional Significance

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[Image 3/23/2017]
Important of NGS Data Interpretation:

Single vs. multiple mutations, early (initiating) mutations vs. late (progression) mutations, mutation allele frequency (AF)

<table>
<thead>
<tr>
<th>Mutation</th>
<th>Early sub-clonal lesion, likely benign</th>
<th>NIHFT or low risk cancer</th>
<th>High risk cancer</th>
</tr>
</thead>
<tbody>
<tr>
<td>NRAS Q61R (3% AF)</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>NRAS Q61R (15% AF)</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>NRAS Q61R (35% AF)</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>TERT C228T (28% AF)</td>
<td></td>
<td></td>
<td></td>
</tr>
</tbody>
</table>

60 year-old female with incidentally noted 1.5 cm thyroid nodule and another 0.6 cm nodule

Cytology:
- Isthmus nodule
  - Benign
- 0.6 cm Right lobe nodule
  - AUS/FLUS (Bethesda III)

**ThyroSeq:**

**Mutations detected:**

<table>
<thead>
<tr>
<th>Gene</th>
<th>Mutation</th>
<th>Allele Frequency</th>
</tr>
</thead>
<tbody>
<tr>
<td>BRAF pV600E</td>
<td>p.V600E</td>
<td>37%</td>
</tr>
<tr>
<td>AKT1 pE17K</td>
<td>p.E17K</td>
<td>21%</td>
</tr>
<tr>
<td>PIK3CA pH1047R</td>
<td>p.H1047R</td>
<td>21%</td>
</tr>
<tr>
<td>TERT C228T</td>
<td>C228T</td>
<td>77%</td>
</tr>
</tbody>
</table>

Co-occurrence of BRAF V600E, TERT, PIK3CA, AKT1 mutations:
- ~100% probability of cancer
- Increased risk of aggressive disease

Diagnostic and prognostic application

**ThyroSeq:**

**FNA adequacy:** GOOD
**FNA Cellular composition:** follicular cell nodule

**Right Lobe:** 0.6 cm hypoechoic nodule

**Isthmus:** Mixed solid and cystic nodule

60 year-old female with incidentally noted 1.5 cm thyroid nodule and another 0.6 cm nodule

**Total Thyroidectomy:**
- Papillary carcinoma, 0.6 cm
- Extrathyroidal extension
- Positive resection margin

Large Scale NGS: Feasibility in thyroid FNA samples

- RNA-Seq used as discovery tool in tissue specimens
- Gene expression and gene fusions
- Requires large amount (80 ng - 1 ug) of RNA

**Library Preparation:**
- cDNA to cRNA

**Cluster Generation:**
- cRNA to single-stranded DNA

**Sequencing:**
- Illumina MiSeq
- 1.6-8 days

**Analysis:**
- 3 days
- 5 days
- 10 days
RNA-Seq for Detection of Novel Genomic Alterations in Thyroid FNA samples

- Optimized RNA-Seq analysis for small RNA input (30 ng) from freshly collected FNA samples
- Analyzed 24 clinical FNA samples, negative for mutations but with suspicious expression profile on ThyroSeq
- 21/24 (88%) worked
- Novel driver fusions or new breakpoints were detected in 15 (71%) of cases
- RNA-Seq can be successfully performed on many thyroid FNA samples (good discovery tool)

NGS Performance in Fixed FNA Samples

- International study designed and coordinated by Dr. Giancarlo Troncone, University of Naples Federico II
- Study goal: To evaluate performance of fixed FNA samples for isolation of DNA and detection of mutations by targeted NGS
- 14 institutions received 4 unstained air dried FNA slides with cell mixture harboring EGFR, BRAF, KRAS, PIK3CA, NRAS mutations at 10%, 5%, 1% AF and wild type

SUMMARY

- Thyroid FNA samples can be successfully analyzed by targeted NGS
- All types of genetic alterations can be detected in freshly collected FNA samples
- Fixed FNA samples can be used for DNA-based targeted NGS analysis
- Large-scale sequencing (RNA-Seq) can be performed on cellular freshly collected thyroid FNA samples (discovery use)

THANK YOU