Down-regulated miRNAs in PTC open new diagnostic perspectives

Belgian Thyroid Club 50th MEETING

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Dr Carine Maenhaut’s lab IRIBHM Erasme (ULB)
miRNA: definition and biogenesis

- Small non-coding RNA family (± 22 nucleotides)
- miRNAs regulate gene expression at a post-transcriptional level
- Exist first as nonfunctional primary forms

miRNA: naming conventions

miR-146b-5p

miRNA number

selected arm

miRNA

position in the group
miRNA: implication in cancer

- There are more than 2000 human miRNAs

- Each of them may regulate hundreds of mRNAs (Bartel, *cell*, 2009)

- More than 50% of mRNAs present at least one miRNA hybridization site (Friedman *et al.* *Genome Research*, 2009)

- miRNAs have become major recognized actors in molecular cell biology in general and in cancer physiopathology and diagnosis in particular
miRNA: implication in cancer

- miRNA genes are frequently observed in cancer-associated altered genomic regions (Calin et al. 2004)

- miRNA expression profiles have been shown to differentiate histological and pathological types (Lu et al. 2005)

- In vitro data and transgenic mouse models showed that miRNAs have roles in the initiation and propagation of cancer (Costinean et al. 2006; Callegari et al. 2012; Lin & Gregory 2015; Liu et al. 2015)
miRNA: role in tumorigenesis

- Tumor suppressor miRNAs target oncogenes
- Oncogenic miRNAs target tumor suppressors genes

Numerous studies have described the role of miRNAs in the pathogenesis of thyroid cancer
- But the complex regulation network between miRNAs and mRNAs is rarely explored

Thyroid cancer and miRNA: state of the art in 2012

- Expression variation between tumor and normal samples

<table>
<thead>
<tr>
<th>microARN</th>
<th>Modulation</th>
<th>microARN</th>
<th>Modulation</th>
<th>microARN</th>
<th>Modulation</th>
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<td>miR-181b-5p</td>
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<td>miR-222-3p</td>
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<td>miR-222-3p</td>
<td>+</td>
</tr>
</tbody>
</table>

Aim of our project

- to characterize the miRNA expression profiles of 3 primary tumors and their associated normal samples and lymph node metastases (LNM)
  - Samples selection based on cellular content
  - Using one of the most recent and sensitive miRNA quantification technology: miRNA next generation sequencing
• miRNA extraction with affinity columns

• Size selection

• Adapters ligation et retro-transcription

• Sequencing by synthesis (Illumina)

• Alignment of the sequences on the reference genome and miRNAs quantification
Differential expression

Multidimensional scaling (398 miRNAs)

Differential expression PTC / normal

<table>
<thead>
<tr>
<th>A precursor</th>
<th>microRNA</th>
<th>logFC</th>
<th>PValue</th>
</tr>
</thead>
<tbody>
<tr>
<td>hsa-mir-372</td>
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Differential expression Lymph node metastasis / PTC

<table>
<thead>
<tr>
<th>B precursor</th>
<th>microRNA</th>
<th>logFC</th>
<th>PValue</th>
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</table>
Differential expression

- Quantitative RT-PCR validation on normal samples, primary tumors and LNM from 14 independent patients

- \textit{In silico} validation using the dataset from The Cancer Genome Atlas: miRNA next generation sequencing of 59 normal samples, 495 PTC, and 8 LNM

- Results:
  - We identified down-regulated miRNA, such as miR-7-5p or miR-30c-2-3p
  - Some of these miRNA are less expressed in LNM than primary tumors
Differential expression

- Loss of expression is stronger in aggressive tumors

*: t-test; expression modulation significant between normal simples and primary tumors or LNM (p<0,05)

**: t-test; expression modulation significant between primary tumors and LNM (p<0,05)
Conclusions:

- These observations contradicted previous studies which suggested that PTC present mainly up-regulated miRNAs.

- Down-regulated miRNAs in PTC could be used as diagnostic and prognostic markers and could have a function in thyroid tumorigenesis.
Bethesda classification

- Hundreds of thousands of fine needle aspiration biopsies (FNAB) per year in Europe for thyroid cancer suspicion
- The cytological diagnosis of 20 to 30% of FNAB are indeterminate (Bethesda categories III to V)
- Patients presenting indeterminate nodules often go to lobectomy / thyroidectomy, but the majority of these nodules are benign

<table>
<thead>
<tr>
<th>category</th>
<th>cytology</th>
<th>% of risk of malignancy</th>
<th>% of the total FNAB</th>
</tr>
</thead>
<tbody>
<tr>
<td>I</td>
<td>non diagnostic</td>
<td>1 - 4</td>
<td>12,9</td>
</tr>
<tr>
<td>II</td>
<td>benign</td>
<td>0 - 3</td>
<td>59,3</td>
</tr>
<tr>
<td>III</td>
<td>follicular lesion of undetermined significance</td>
<td>5 – 15</td>
<td>9,6</td>
</tr>
<tr>
<td>IV</td>
<td>suspicion for a follicular neoplasm</td>
<td>15 – 30</td>
<td>10,1</td>
</tr>
<tr>
<td>V</td>
<td>suspicious for malignancy</td>
<td>60 – 75</td>
<td>2,7</td>
</tr>
<tr>
<td>VI</td>
<td>malignant</td>
<td>97 – 99</td>
<td>5,4</td>
</tr>
</tbody>
</table>

Molecular test for FNAB diagnosis

- Commercially available molecular tests for indeterminate thyroid fine needle aspirations, in USA:
  - Afirma (Veracyte company): mRNA gene expression microarray analysis (Alexander et al. 2012)
  - ThyroSeq (University of Pittsburgh): mutational panel
  - ThyGenX/ThyraMIR (Interpace Diagnostics): miRNA expression and detection of specific mutations

- Each of them presents advantages and drawbacks

“Altogether, only a benign GEC result has meaningful impact on patient management, but a suspicious GEC result merely confirms the indeterminate nature of the nodule.”

Nishino, *Cancer Cytopathology*, 2015

*An Independent Study of a Gene Expression Classifier (Afirma) in the Evaluation of Cytologically Indeterminate Thyroid Nodules*

Bryan McIver, M. Regina Castro, John C. Morris, Victor Bernet, Robert Smallridge, Michael Henry, Laura Kosok, and Honey Reddi
Molecular test for FNAB diagnosis

- Commercially available molecular tests for indeterminate thyroid fine needle aspirations, in USA:
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- Each of them presents advantages and drawbacks

Evaluation of ThyroSeq v2 performance in thyroid nodules with indeterminate cytology

“However, the PPV of the test was lower than expected; and its performance particularly in B-III specimens may be variable among centers”

Valderrabano et al., Endocrine-Related Cancer, 2017
Molecular test for FNAB diagnosis

- Commercially available molecular tests for indeterminate thyroid fine needle aspirations, in USA:
  - Afirma (Veracyte company): mRNA gene expression microarray analysis (Alexander et al. 2012)
  - ThyGenX/ThyraMIR (Interpace Diagnostics company): miRNA expression and detection of specific mutations (Labourier et al. 2015)
- Each of them presents advantages and drawbacks
- miRNAs used by ThyGenX/ThyraMIR:
  miR-29b-1–5p, miR-31–5p, miR-138–1–3p, miR-139–5p, miR-146b-5p, miR-155, miR-204–5p, miR-222–3p, miR-375 and miR-551b-3p

What could be the diagnostic utility of recently discovered down-regulated miRNAs?
Diagnostic utility of down-regulated miRNA

- 51 per-operative biopsies have been obtained from 14 malignant nodules (13 PTC and 1 FTC) and 37 benign nodules.
- After RNA purification, miRNA expression of biomarker candidates have been evaluated on the cytological samples by quantitative RT-PCR and a computational analysis classifies the samples as benign or malignant.
- The postoperative histological status of the samples have been used as gold standard reference in order to define the test performance.

The sample is collected in RNA conservative solution. miRNA extraction with affinity columns. Quantification of biomarker candidates. Computational analysis.
Diagnostic utility of down-regulated miRNA

Malignant
Benign

* : p<0.05;
** : p<0.01;
*** : p<0.001

Mann and Whitney U test
Diagnostic utility of down-regulated miRNA

Up-regulated miRNAs in thyroid cancer compared to normal sample

- miR-1
- miR-2
- miR-3
- miR-4
- miR-5
- miR-6
- miR-7
- miR-8
- miR-9
- miR-10
- miR-11
- miR-12
- miR-13
- miR-14

* : p<0.05;
** : p<0.01;
*** : p<0.001
Mann and Whitney U test
Diagnostic utility of down-regulated miRNA

Down-regulated miRNAs in thyroid cancer compared to normal sample

- mir-1
- mir-2
- mir-3
- mir-4
- mir-5
- mir-6
- mir-7
- mir-8
- mir-9
- mir-10
- mir-11
- mir-12
- mir-13
- mir-14

Malignant
Benign

* : p<0.05;
** : p<0.01;
*** : p<0.001

Mann and Whitney U test
Diagnostic utility of down-regulated miRNA

Objective: to compare the diagnostic utility of a selection of down-regulated miRNAs to previously characterised miRNA biomarkers of thyroid malignancy

Protocol used: expression levels of the 14 miRNAs in each sample have been used by a classification algorithm. The samples have been classified as benign or malignant by the algorithm. This classification has been compared to the postoperative histological status

Results: we obtained a list of miRNAs which maximize the efficiency of the prediction of the status for each sample independently

<table>
<thead>
<tr>
<th>Gold standard</th>
</tr>
</thead>
<tbody>
<tr>
<td>Positive</td>
</tr>
<tr>
<td>Positive</td>
</tr>
<tr>
<td>Negative</td>
</tr>
</tbody>
</table>

Sensitivity: TP/ (TP+FN); the probability that a patient with the disease receives a positive result.

Specificity: TN/ (TN+FP); the probability that a patient without the disease receives a negative result.

Positive predictive value (PPV): TP/ (TP+FP); the probability that a patient with a positive result actually has the disease.

Negative predictive value (NPV): TN/ (TN+FN); the probability that a patient with a negative result is actually disease free.
First potential diagnostic signature

- Signature 1: algorithm has been designed to promote the correct classification of malignant nodules

<table>
<thead>
<tr>
<th>Molecular test</th>
<th>Malignant (n=14)</th>
<th>Benign (n=37)</th>
<th>PPV: 47% (prevalence 27% )</th>
<th>NPV: 100% (prevalence 27%)</th>
</tr>
</thead>
<tbody>
<tr>
<td>Positive (n=30)</td>
<td>14</td>
<td>16</td>
<td></td>
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<tr>
<td>Negative (n=21)</td>
<td>0</td>
<td>21</td>
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</table>

**Sensibility: 100%**  
**Specificity: 57%**

miRNAs selected by the algorithm to maximize the efficiency of the prediction:  
miR-13 (100%); miR-12 (96%); miR-14 (41%); miR-1 (37%)

Down-regulated miRNAs  
Up-regulated miRNAs
**Second potential diagnostic signature**

- **Signature 2:** algorithm has been designed to promote the correct classification of benign nodules

<table>
<thead>
<tr>
<th>Molecular test</th>
<th>Gold standard</th>
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</thead>
<tbody>
<tr>
<td></td>
<td>Malignant (n=14)</td>
</tr>
<tr>
<td>Positive (n=9)</td>
<td>7</td>
</tr>
<tr>
<td>Negative (n=42)</td>
<td>7</td>
</tr>
<tr>
<td>Sensibility: 50%</td>
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</table>

miRNAs selected by the algorithm to maximize the efficiency of the prediction:
- miR-13 (100%); miR-12 (98%); miR-14 (66%); miR-1 (64%); miR-11 (60%); miR-6 (60%); miR-3 (60%); miR-2 (50%); miR-9 (33%); miR-7 (33%); miR-5 (33%); miR-8 (33%); miR-4 (33%); miR-10 (33%)

Down-regulated miRNAs
Up-regulated miRNAs
Conclusions:

- Some down-regulated miRNAs between primary thyroid tumors and normal tissues show the same down-regulation in malignant FNAB compared to benign FNAB.

- Our data suggest that down-regulated miRNAs in malignant FNAB may be better biomarkers than previously characterised up-regulated miRNAs.

- miRNA signatures have the potential to discriminate benign from malignant FNAB with a high PPV or NPV.
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Ligia Craciun
Alex Spinette
Denis Larsimont