MicroRNA profiling of breast cancer using miRCURY™ LNA Arrays

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Introduction

- MicroRNAs (**miRNAs**) comprise a recently identified class of small, non-protein-coding regulatory molecules that play important roles in many physiologic and pathologic processes, including differentiation, viral infection, and oncogenesis.
- In cancer, abnormal miRNA expression suggests that these molecules may serve as valuable diagnostic and prognostic molecular signatures.
- To study the global miRNA expression profiles in breast cancer, we use the **miRCURY™ LNA Array** platform, which is based on locked nucleic acid (**LNA**)-modified capture probes that have uniquely high affinities for miRNA.
- For quantitative validation of new miRNA biomarkers, we apply a novel **miRCURY™ LNA qPCR** assay.

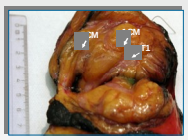


Fig. 1. An invasive ductal carcinoma, 19 mm in width. The three samples, primary tumor (T1), and two normal adjacent tissues (1CM and 5CM) were dissected and frozen at -78 °C within one hour post surgery.

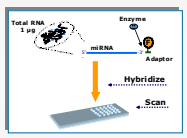


Fig. 2. Procedure for miRNA profiling with **miRCURY™ LNA Arrays**, which are Tm normalized to 72 °C with LNA-enhanced capture probes

1. Prepare total-RNA
2. Label RNA with Hy5 / Hy3
3. Hybridize overnight
4. Scan and analyze

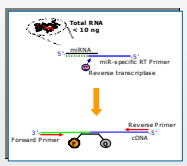


Fig. 3. 2-step **miRCURY™ LNA qPCR** procedure for detection of miRNA:

1. miRNA-specific first strand synthesis
2. Quantitative real-time PCR amplification of the cDNA

Less than 10 ng total-RNA is required for the qPCR reaction

Methods

Samples

- Biopsies from primary tumors (PT) and from normal adjacent tissue (1 cm and 5 cm from the tumor) were collected from eight female patients undergoing surgery for invasive ductal carcinoma (**Fig. 1**).

microRNA extraction

- RNA was isolated by guanidinum isothiocyanate / phenol:chloroform extraction. From 50 mg breast tissue, ca. 5-10 µg total-RNA was routinely retrieved.

Microarray expression profiling

- 1 µg total RNA was analyzed for miRNA expression on **miRCURY™ LNA Array** containing capture probes for 491 miRNAs[1]. The miRNA labeling and hybridization procedure is outlined (**Fig. 2**).

Quantitative RT-PCR measurement of microRNA

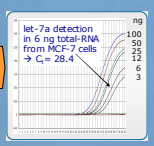
- A number of selected miRNAs are currently being quantified with the gene-specific **miRCURY™ LNA qPCR** assay (**Fig. 3**).

Identification of microRNAs by 454 sequencing

- To corroborate the results obtained by microarray screening (and to identify novel miRNAs), we also undertook a 454 pyrosequencing approach, counting the 15-30 nt population of RNAs extracted from a pool of five breast cancers.

Data analysis

- The miRNA expression data were analyzed with dChip 2006[2]. Unsupervised hierarchical clustering was applied to both samples and genes using the centroid linkage method and (1 – Pearson correlation) distance metric.



Identification of novel breast-cancer associated microRNAs
In addition to the known miRNAs, we identified **numerous novel** (i.e. not previously reported in humans) breast-cancer associated miRNAs. LNA probes for these new miRNAs are currently being designed.

Results – microRNA profiling

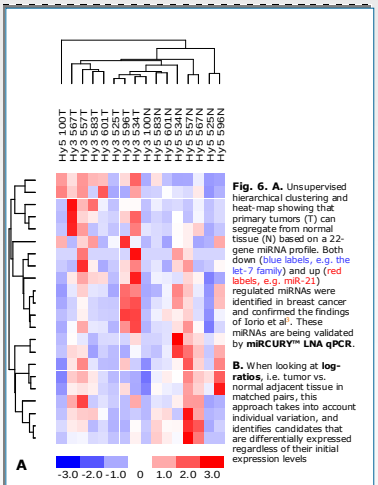


Fig. 6. A. Unsupervised hierarchical clustering and heat-map showing that primary tumors (T) can segregate from normal tissue (N) based on a 22-gene miRNA profile. Both down (blue labels, e.g. the let-7 family) and up (red labels, e.g. miR-21) regulated miRNAs were identified in breast cancer and confirmed the findings of Iorio et al[3]. These miRNAs are being validated by **miRCURY™ LNA qPCR**.

B. When looking at **log-ratios**, i.e. tumor vs. normal adjacent tissue in matched pairs, this approach takes into account individual variation, and identifies candidates that are differentially expressed regardless of their initial expression levels

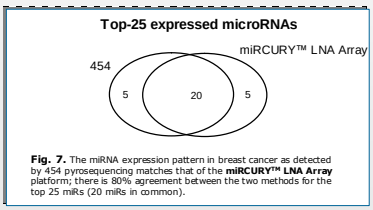


Fig. 7. The miRNA expression pattern in breast cancer as detected by 454 pyrosequencing matches that of the **miRCURY™ LNA Array** platform; there is 80% agreement between the two methods for the top 25 miRs (20 miRs in common).

Results – technology

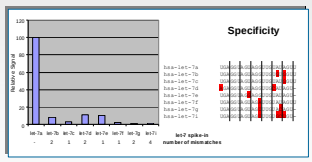


Fig. 4.
The signal for capture probe let-7a does not cross-react with other members of the let-7 family. Thus, single-nucleotide mismatch discrimination is obtained with this probe-set.

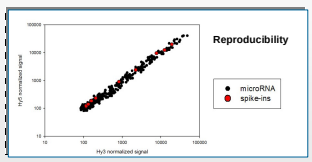


Fig. 5.
Self-self hybridization with 2 µg lung total-RNA + spike-in mix

$R_{spike\text{-}in}$ = 0.99

R_{miRNA} = 0.97

(R , correlation coefficient)

Conclusions

miRCURY™ LNA Array

- Superior **sensitivity**, < 50 amol miRNA detected (< 1 µg total-RNA)
- Excellent **specificity**, single mismatch discrimination
- High **reproducibility**, both intra-array, and inter-batch
- **Fast** and **simple** RNA labeling
- No need for amplification or miRNA enrichment

microRNA profiles

- Known, breast-cancer associated miRNAs were confirmed with the **miRCURY™ LNA Array** platform and validated by pyrosequencing.
- A number of novel miRNAs not previously connected with breast cancer were identified with the **miRCURY™ LNA Array** approach.
- Some of these miRNAs may represent novel diagnostic signatures
- We are currently validating the new potential biomarkers with **miRCURY™ LNA qPCR** and on the Luminex platform

References
1. www.exiqon.com 2. biosun1.harvard.edu/complab/dchip/ 3. Iorio et al. Cancer Res 2005; 65: 7066.

For further information
Please contact support@exiqon.com
More information on other miRCURY™ LNA Products can be found at www.exiqon.com

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