MicroRNA profiling of breast cancer using novel Locked Nucleic Acid (LNA™) based technologies

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Introduction

- Abnormal expression of microRNAs (miRNAs) in cancer implies that these small molecules play a role in oncogenesis. Therefore miRNAs may comprise a novel class of diagnostic and prognostic signatures.
- Here, we study the global expression profiles of miRNAs in breast cancer and normal adjacent tissue in order to identify possible new biomarkers for breast cancer.
- To study the global miRNA expression profiles in breast cancer, we use the miRCURY™ LNA Array platform 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 LNA-enhanced real-time PCR detection methods.

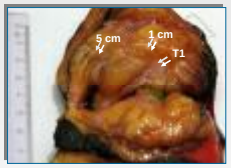


Fig. 1. An invasive ductal carcinoma, 19 mm in width. The three samples, primary tumor (T1), and two normal adjacent tissues (1 cm and 5 cm) were dissected and frozen at -78 °C within one hour post surgery.

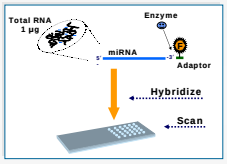


Fig. 2. Procedure for miRNA profiling with miRCURY™ LNA Arrays, which are Tm normalized to 72 °C with LNA-enhanced capture probes

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

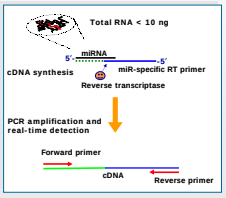


Fig. 3. 2-step LNA-enhanced real-time PCR procedure for detection of miRNA:

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

Less than 10 ng total-RNA is required for the real-time PCR reaction

Results – technology miRCURY™ LNA Arrays

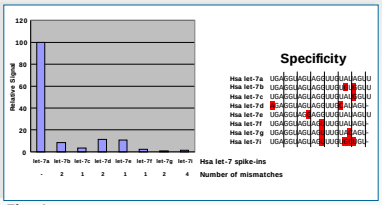


Fig. 4. The signal for capture probe hsa let-7a does not cross-react with other members of the hsa let-7 family. Thus, single-nucleotide mismatch discrimination is obtained with this probe-set.

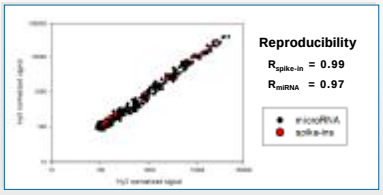


Fig. 5. Self-self hybridization with 2 µg lung total RNA + spike-in mix (R, correlation coefficient)

Methods

Samples

- Biopsies from primary tumors and from normal adjacent tissue (1 cm and 5 cm) from the tumor were collected from 11 female patients undergoing surgery for invasive ductal carcinoma (Fig. 1).

microRNA extraction

- Total 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 Arrays containing capture probes for 491 miRNAs[1]. The miRNA labeling and hybridization procedure is outlined (Fig. 2).

Confirmation of expression pattern with real-time PCR

- The expression pattern of a number of selected miRNAs was confirmed with the gene-specific LNA-enhanced real-time PCR assays. The real-time PCR detection were either based on hydrolysis probes or Sybr green intercalating dye (Fig. 3, 7 and 8).

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.

Conclusions

miRCURY™ LNA Arrays

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

LNA-enhanced real-time PCR

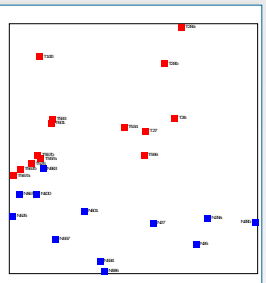
- High sensitivity, < 10 ng total-RNA
- Unique specificity, single mismatch discrimination
- Good reproducibility

miRNA profiles

- Known, breast-cancer associated miRNAs were confirmed with the miRCURY™ LNA Arrays and validated by LNA-enhanced real-time PCR.
- A number of novel miRNAs not previously connected with breast cancer were identified with the miRCURY™ LNA approach.
- Some of these miRNAs may represent novel diagnostic signatures.
- We are currently validating the new potential biomarkers with a 454 pyrosequencing approach, LNA-enhanced real-time PCR methods, and on the FlexmiR™ microRNA Luminex platform.

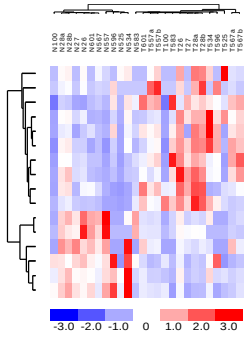
Results – miRNA profiling

Fig. 6.
A. Principal Component Analysis (PCA) performed on the miRNAs that vary across the analyzed samples. A distinguishable separation of tumor samples (red) and normal tissue (blue) is achieved based on the miRNA expression levels.



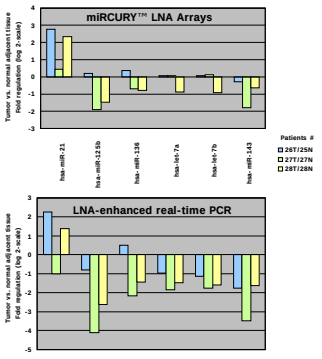
B. Unsupervised hierarchical clustering and heat-map showing segregation of primary tumors (T) and normal tissue (N) based on the miRNA profile. Samples consisted of paired samples from eleven patients. Both down (blue labels) and up (red labels) regulated miRNAs were identified in breast cancer and confirmed the findings of Iorio *et al.*[3]

Findings were confirmed by LNA–enhanced real-time PCR (see Fig. 7).



Identification of novel breast-cancer associated miRNAs
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.

Fig. 7. Confirmation of array data (upper panel) with LNA-enhanced real-time PCR (lower panel). Fold regulation was determined using the $\Delta\Delta C_t$ method using reference assay normalization. Samples consisted of paired samples from three patients.



Results – technology

Fig. 8. Relative detection (in %) with LNA-enhanced real-time PCR using 1 fmol synthetic RNA spike-in and 10 ng yeast total RNA as complex background. The calculation is based on the threshold value difference between perfectly matched and mismatched RNA templates for each hsa let-7 assay.

LNA-enhanced real-time PCR assays \ Synthetic RNA targets	let-7a	let-7b	let-7c	let-7d	let-7e	let-7f	let-7g	let-7i
let-7a	100	0.1	14.9	0.3	0.1	1.4	0.0	0.0
let-7b	0.8	100	5.7	0.0	0.2	0.0	0.0	0.0
let-7c	6.2	33.5	100	0.0	0.1	0.2	0.0	0.0
let-7d	46.4	1.5	6.9	100	0.1	2.9	0.0	0.0
let-7e	0.4	0.0	0.2	0.0	100	0.0	0.0	0.0
let-7f	0.0	0.0	0.0	0.0	0.0	100	0.0	0.0
let-7g	0.0	0.0	0.0	0.0	0.0	0.0	100	0.2
let-7i	0.0	0.0	0.0	0.2	0.0	0.0	0.3	100

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 jacobsen@exiqon.com
More information on related projects can be obtained at www.exiqon.com

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