Early biomarker discovery using microRNA profiling

microRNA

microRNAs are small regulating RNAs that have been shown to play important regulatory roles in most cellular and developmental processes and have been implicated in a large number of human diseases including cancer. Due to their wide-ranging biological potential and the fact that miRNAs seem to be relatively stable in readily available clinical samples, these small 20-22 nt molecules are prime candidates for use as biomarkers in molecular diagnostics. Indeed, small sets of microRNAs have already been shown to enable classification of different disease states and conditions.

However, microRNAs are challenging targets for analysis due to their short length and highly divergent sequences with large variation in GC content. This variability leads to very different hybridization properties between different miRNA sequences and makes simultaneous measurement of all miRNAs challenging. Exiqon's miRCURY LNA™ microRNA array overcomes these challenges by using LNA™ -enhanced Tm normalized capture probes that allow sensitive profiling of all microRNAs regardless of sequence.

Diagnosing thyroid cancer

It is estimated that 4-5% of the American population has palpable thyroid lesions. According to the National Cancer Institute more than 50,000 new cases of thyroid cancer are reported in the US annually. Thyroid cancer is a diverse array of cancers with markedly different course of progression, ranging from almost indolent cases of papillary and follicular cancer to some of the most aggressive anaplastic cancers with a median survival time of less than a year from diagnosis.

Differential diagnosis of thyroid cancer is difficult because the assessment of thyroid nodules is problematic. Consequently, inconclusive fine needle biopsies lead to more than 100,000 operations annually to rule out cancer. Given that most nodules are benign, and that most cancers are slowly progressing, pre-operative diagnostic procedures aimed at reducing the number of surgeries is mainstay. Lesions with follicular morphology are particularly difficult to categorize accurately on cytology alone, and a significant proportion of cytologies (15-30%) are classified as 'Indeterminate', while still carrying a 5-30% risk of malignancy. There is therefore an unmet need to identify biomarkers that can be used to diagnose the nodule in order to reduce the number of surgeries, and to separate the various subcategories of thyroid cancers.

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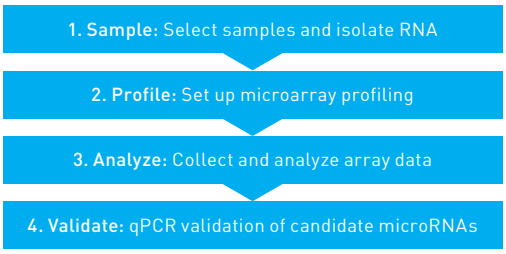
Discovery of microRNA biomarkers for thyroid cancer

We used the miRCURY LNA™ microarray platform to profile the global miRNA expression in thyroid cancer tissue, benign tissue and normal adjacent tissue in order to identify putative microRNA biomarkers for carcinoma. The miRCURY LNA™ 7th gen miRNA array platform supports global gene expression analysis as it contains capture probes for all registered and annotated human miRNAs from miRBase V18.0 as well as human miRPlus sequences not yet annotated in miRBase.

Our analysis enabled identification of microRNAs that are significantly over-expressed in carcinoma tissue compared to the adenoma tissue. Thus we propose that the microRNA profile of tissue can be used as pre-operative diagnostic tool and provides a basis for differential diagnosis of thyroid cancer versus benign neoplasm in glandular thyroidea.

Figure 1. From samples to microRNA biomarkers in 4 working days. The miRCURY LNA™ microRNA array and microRNA PCR systems enable rapid progression through the first stages of biomarker discovery and validation. Each of the four steps below can be performed in 1 day when all the reagents are available.



Methods

Samples: 19 formalin fixed, paraffin embedded (FFPE) sections from different thyroid patients (ProteoGenix) were analyzed and profiled. The samples covered both cancer (papillary carcinoma), benign diseases (follicular adenoma and nodular goiter), and normal adjacent tissue (follicular carcinoma and medullary carcinoma) (table 1).

RNA Isolation: RNA was extracted from macro dissected FFPE sections. Total RNA was isolated by using the miRNEasy FFPE kit from Qiagen. RNA concentrations were evaluated by Nanodrop.

microRNA profiling: For each sample 500ng RNA was labeled using miRCURY LNA™ Hi-Power labeling kit and hybridized on miRCURY LNA™ 7th gen microRNA Array (according to the manual). A sample consisting of RNA from a mixture of multiple human tissues was used as a hybridization reference sample.

Table 1. Overview of the sample material used in this study. The 19 FFPE samples are grouped in Normal adjacent tissue (NAT), Nodular goiter, benign (NGB), Follicular adenoma, benign (FAB), and Papillary carcinoma (PC).

FFPE samples analyzed	Abb.	# of samples
Papillary carcinoma	PC	5
Follicular adenoma, benign	FAB	5
Nodular goiter, benign	NGB	4
Normal adjacent tissue	NAT	5

Data analysis: Array analysis was performed with Imagene/Nexus Expression software (available from Exiqon), using quantile normalization. PCA plot was generated using GenEx.

qPCR validation: Array data were validated using individual qPCR assays for the relevant microRNAs from the miRCURY LNA™ Universal RT microRNA PCR system according to the manual. The miRCURY LNA™ microRNA and microRNA PCR system enables a rapid workflow of only 4 working days from samples to identified microRNA biomarkers (figure 1).

Figure 2. PCA plot of top 10 miRNAs with the largest variation across all the samples. Normal adjacent tissue (yellow), follicular adenoma (grey), nodular goiter (blue), and papillary carcinoma (red). The cancer samples clearly cluster separately from the remaining sample groups.

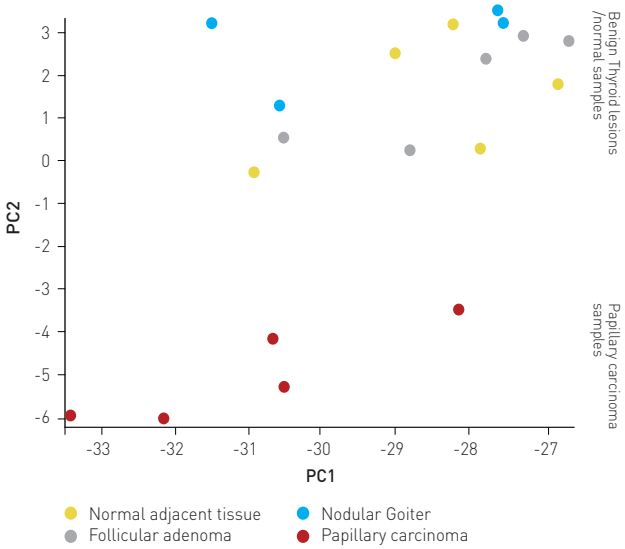
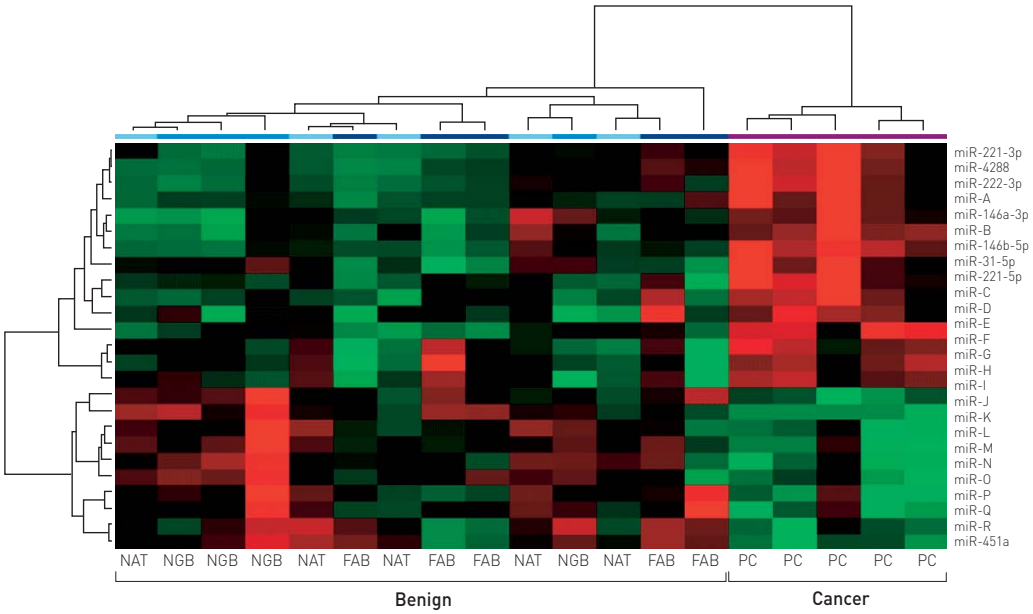


Figure 3. Heatmap of the 25 most differentially expressed microRNAs in cancer and benign tissue. Clustering is performed using the complete-linkage method together with the euclidean distance measure. Each row represents a microRNA and each column represents a sample. The microRNA clustering tree is shown on the left. The color scale illustrates the relative expression level of microRNAs. Red color represents an expression level below the reference channel, and green color represents expression higher than the reference.



Results

In total, 47 microRNAs on the array showed significant differential expression (Log 2 ratio > 0,5 and p value < 0,05) between the cancer sample (papillary carcinoma) and the three benign sample groups as whole. The Principal Component Analysis (PCA) plot in figure 2 illustrates how the 19 samples cluster according to their biological groups. It is clearly seen that the papillary carcinoma samples are separated from the three benign sample groups – follicular adenoma, nodular goiter, and normal adjacent tissue – which cluster together.

The heat map diagram (figure 3) shows the result of a two-way hierarchical clustering of microRNAs and samples of the 25 most differentially expressed microRNAs in cancer (on the right) and normal tissue (on the left). The heat map demonstrates a different expression pattern in the cancer tissue samples compared to the samples from the benign tissue. Several of the differentially expressed microRNAs have already been described in the literature. However, we also identified a number of new potential biomarkers including microRNAs recently added to miRBase.

Several putative microRNA biomarkers for papillary carcinoma were thus identified by using miRCURY LNA™ microRNA array profiling. Significant differential expression in follicular adenoma and papillary carcinoma for a subset of these microRNAs is shown in figure 4. Human microRNAs miR-146-5p, mirR-221 and miR-222 were all found to be over-expressed in the five papillary carcinoma samples (represented by red dots in figure 4) compared to the five follicular adenoma samples (grey dots in figure 4). This is complementing what has previously been reported in the literature (de la Chapelle et al., 2011). Two microRNAs recently added to miRBase (miR-4288 and miR-4324) were also identified as over-expressed in the cancer sample.

A subset of the biomarkers identified by the miRCURY LNA™ 7th gen microRNA Array were subsequently validated using the miRCURY LNA™ Universal RT microRNA PCR platform (figure 5). Eight of the 10 microRNAs were available for qPCR analysis. The differential expression observed on the microarray platform was confirmed by qPCR. Figure 5 illustrates how the available qPCR assays show the same differential expression pattern as in the array analysis.

As expected, the qPCR results show greater differential expression for many microRNAs due to the larger dynamic range afforded by this method.

Figure 4. Significant differential expression in follicular adenoma and papillary carcinoma for a subset of microRNAs. Log 2 expression signals for 10 microRNAs with the differential expression between papillary carcinoma (red) and follicular adenoma (grey), all with P-values < 0,01 (apart from miR-451a; P-value 0,0318).

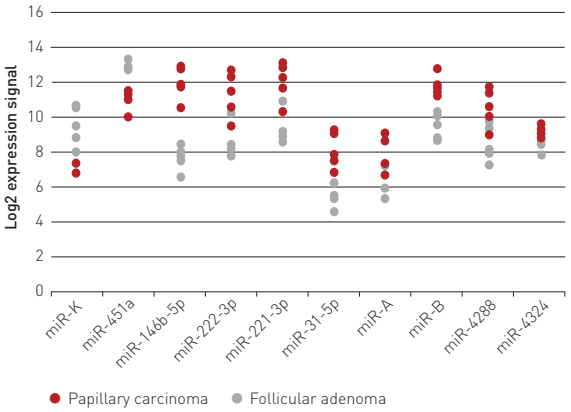
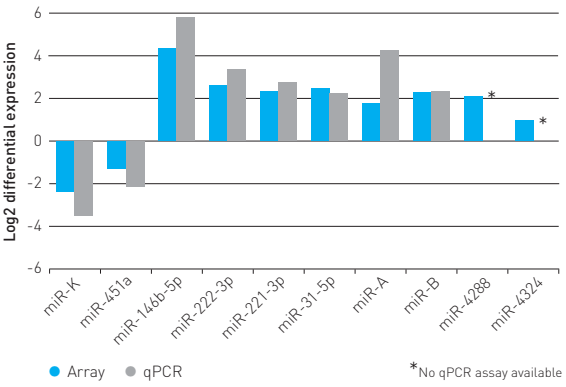


Figure 5. Differential expression between follicular adenoma and papillary carcinoma samples for 10 microRNAs analyzed by microRNA array and qPCR. The blue bars represent the array data and the grey bars the qPCR data. microRNAs with negative log2 differential expression are down-regulated in the cancer samples compared to the benign samples whereas positive log2 differential expression indicates over-expression in cancer. The asterix indicates miRs where no qPCR assay is available.



Conclusion

We have illustrated the application microRNA profiling by miRCURY LNA™ 7th gen microRNA Array for early biomarker discovery. We suggest that expression profiles of miRNAs could be used to improve the diagnostic accuracy of thyroid biopsies, as the malignant samples cluster away from the benign samples and possess a unique microRNA expression signature.

We have identified previously known as well as novel microRNA biomarkers for carcinoma. The putative biomarkers can be validated by miRCURY LNA™ microRNA qPCR.

microRNA profiling is a promising preoperative diagnostic tool to enable differential diagnosis of thyroid cancer versus benign neoplasm based on fine needle biopsies.

Products/ Ordering information

miRCURY LNA™ microRNA Array, 7th gen, hsa/mmu/rno	Product description	Product no.
3, 6 or 24 slides	Microarray slides, hyb & wash buffer and spike-in miRNA	208500 (3 slides) 208501 (6 slides) 208502 (24 slides)
miRCURY LNA™ microRNA Hi-Power Labeling Kits	**Product description**	**Product no.**
Hy3™/Hy5™	Fluorescent labeling of microRNAs from total RNA samples. 2x12 rxns	208035
miRCURY LNA™ microRNA Power Labeling Kits	**Product description**	**Product no.**
Hy3™/Hy5™	Fluorescent labeling of microRNAs from total RNA samples. 2x12 rxns	208032-A
miRCURY LNA™ microRNA Array Analysis Software	**Product description**	**Product no.**
ImaGene®/Nexus™ - Perpetual license	Microarray Analysis Software	208220
ImaGene®/Nexus™ - 30 day license/24 slides	Microarray Analysis Software	208221
miRCURY LNA™ microRNA PCR Reagents	**Product description**	**Product no.**
Universal cDNA Synthesis Kit	Polyadenylation and cDNA synthesis kit (16 to 32 rxns)	203300
SYBR® Green master mix, Universal RT, 2,5ml	250 rxns of 20µl or 500 rxns of 10µl	203450
SYBR® Green master mix, Universal RT, 25ml	250 rxns of 20µl or 500 rxns of 10µl	203400
Individual assays	**Product description**	**Product no.**
xxx-miR-xxx, LNA™ PCR primer set, UniRT	microRNA primer set, 200 rxns	204000
Reference gene PCR primer set, UniRT	Reference gene primer set, 200 rxns	203901-203912
Exiqon GenEx Software	**Product description**	**Product no.**
Pro Industrial	Exiqon GenEx, qPCR analysis software, industrial license	207005
Pro Academic	Exiqon GenEx, qPCR analysis software, academic license	207006
Enterprise Industrial	Exiqon GenEx, qPCR analysis software, industrial license	207007
Enterprise Academic	Exiqon GenEx, qPCR analysis software, academic license	207008

Contact information

Outside North America
Phone: +45 45 65 09 29 · Fax: +45 45 66 18 88
North America
Phone: +1 781 376 4150 · Fax: +1 781 376 4152

www.exiqon.com

920555 - v1.0 - 09/2012