

Use of Buffy Coat miRNA Profiling for Breast Cancer Prediction in Healthy Women

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Abstract

MicroRNAs are key regulators of different biological processes and their deregulation is associated with the occurrence of many diseases among which cancer. Due to the higher stability of microRNAs and to the easiness in their detection both in organs than in biological fluids, many studies are turned toward potential use of this small molecules as biomarkers for the prediction and diagnosis of different types of cancer. Here we describe the experiment protocol that we have used for microRNA profiling analysis in buffy coat samples of women who developed breast cancer versus women who remained healthy during a 20 year follow-up period, with the aim to identify predictive microRNAs of breast cancer occurrence.

Key words microRNA, microRNA profiling, Buffy coat, Breast cancer, RNA extraction

1 Introduction

MicroRNAs (miRNAs) are a class of small noncoding RNAs able to modulate gene expression at posttranscriptional level degrading mRNA and/or impairing translation [1]. miRNAs constitute about 3 % of the human genome, indicating that thousands of human genes can be target of miRNA-mediated regulation.

MiRNA activity has been correlated to the pathogenesis of cancer, since miRNAs were identified as a new class of genes with tumor-suppressor and oncogenic functions [2–7]. Moreover, the localization of nonrandom chromosomal abnormalities and other types of genetic alterations at miRNA genomic regions observed in several types of cancer cells furthermore underline the contribution of the deregulation of miRNA expression to malignancies process [8].

Advance in expression technologies has facilitated the high-throughput analysis of small RNAs, showing that these genes may be aberrantly expressed in various human tumors. MiRNA profiling is emerging as a useful tool in the characterization of a variety of human cancers, potentially being of even greater predictive/

prognostic value than the analysis of the expression of messenger RNAs [9–11].

The higher stability of miRNAs is clearly in contrast to the distinctly lower stability of mRNAs. This difference is due principally to the dissimilarity in their length: miRNAs are only 20–22 nucleotides in length, so also in degraded RNA preparation from human tissue they maintain their stability [12, 13]. This feature makes miRNAs excellent potential biomarkers; for this reason many studies are designed to identify differences in the expression of miRNAs between normal tissue and tumoral tissue.

In our study, we aimed to test the hypothesis that miRNAs may represent early indicators of future breast cancer incidence. In particular we compared leucocyte miRNA profiles of healthy women who subsequently became affected with breast cancer, versus women who remained healthy. This was performed using a case–control study design nested in the ORDET (hORMones and Diet in the ETiology of Breast Cancer) prospective cohort over a follow-up period of 20 years [14].

The analysis identified 20 differentially expressed miRNAs, 15 of them were down-regulated. Among the 20 miRNAs, miRNA-145-5p and miRNA-145-3p, each derived from another arm of the respective pre-miRNA, were consistently and significantly down-regulated in all databases we surveyed. For example, analysis of more than 1500 patients (the UK Metabric cohort) indicated that high abundance of miRNA 145-3p and miRNA-145-5p was associated with longer, and for miRNA-145-3p also statistically significant, survival. The experimental data attributed different roles to the identified microRNAs: while the 5p isoform was associated with invasion and metastasis the other isoform appears related to cell proliferation [15].

Here we describe how to obtain good-quality RNA from leucocytes preserved at $-80\text{ }^{\circ}\text{C}$ for 20 years that can be used for miRNA profiling on Agilent Platform.

2 Materials

2.1 RNA Extraction

1. TRIzol Reagent (Life Tec Rockville, MD) Chloroform.
2. Isopropyl alcohol.
3. 475 % ethanol (in DEPC-treated water).
4. RNase-free water (to prepare RNase-free water, draw water into RNase-free glass bottles). Add diethylpyrocarbonate (DEPC) to 0.01 % (v/v). Let stand overnight and autoclave.)

2.2 RNA Quality Control

1. Spectrophotometers (NanoDrop ND-1000, Thermo Fisher Scientific, Waltham, MA, or similar).
2. Agilent 2100 bioanalyzer.

2.3 RNA Hybridization

1. Human microRNA Microarray (Agilent).
2. miRNA Labeling and Hybridization Kit (Agilent).
3. Agilent DNA Microarray Scanner (P/N G2565BA).

3 Methods

3.1 Buffy Coat Collection

1. Blood samples were drawn after overnight fasting between 7:30 and 9:00 AM in the morning from each woman and stored at -80°C .
2. Samples from each case and related control were handled identically and assayed together on the same laboratory session. Laboratory personnel were blinded to case-control status.
3. A total of 20 mL of blood was collected in two 10 mL heparin vacutainers:
 - (a) 1–10 mL vacutainer, containing sodium heparin as anticoagulant.
 - (b) 1–10 mL vacutainers, containing no additives.
4. Each vacutainer was identified by a different color.
5. The filled vacutainers were immediately protected from direct light using aluminum foil, then kept on ice at -4°C until the samples were transferred to the central laboratory for processing and aliquoting. The time of the blood drawing was recorded.
6. From the heparin vacutainer, the buffy coat (0.8 mL) was collected from the most superficial part of the tube's corpuscular portion by moving the top of the pipette gently around the surface of the clot.

3.2 RNA Isolation

RNA isolation from buffy coat was performed by using TRIzol Reagent.

TRIzol Reagent is based on RNA separation from DNA after extraction with an acidic solution containing guanidinium thiocyanate, sodium acetate, phenol, and chloroform, followed by centrifugation [16]. Under acidic conditions, total RNA remains in the upper aqueous phase, while most of DNA and proteins remain either in the interphase or in the lower organic phase. Total RNA is then recovered by precipitation with isopropanol.

Here is the procedure that we have used for buffy coat samples that is quite similar to the original protocol but with some changes to enhance RNA yield:

1. Lyse buffy coat cells by adding 3 mL of TRIzol Reagent to 1.5 mL of buffy coat. Vortex vigorously and incubate samples for 15 min at room temperature to permit the complete dissociation of nucleoprotein complexes.

2. Add 0.6 mL of chloroform. Vortex vigorously and incubate samples for 5 min at room temperature. Centrifuge at $12,000\times g$ for 20 min at 4 °C (*see Note 1*).
3. Remove the aqueous phase of the sample by angling the tube at 45° and pipetting the solution out. Avoid drawing any of the interphase or organic layer into the pipette when removing the aqueous phase.
The volume amount should be ~2.5 mL to be divided into two 2 mL tubes (~1250 mL each) (*see Note 2*).
4. Add 0.75 mL of 100 % isopropanol to the aqueous phase, shake tubes by hand and incubate at room temperature for 30 min. Centrifuge at $12,000\times g$ for 30 min at 4 °C (*see Note 3*).
5. Remove the supernatant from the tube, leaving only the RNA pellet.
6. Wash the pellet with 0.5 mL of 75 % ethanol. Vortex the sample briefly, and then centrifuge the tube at $7500\times g$ for 10 min at 4 °C. Discard the wash.
7. Air-dry the RNA pellet for 10–20 min (*see Note 4*).
8. Resuspend the RNA pellet in RNase-free water or (20–50 µL) by vortexing in heat block set at 55–60 °C for 15 min.
9. Proceed to downstream application, or store at –80 °C.

To preserve RNA integrity avoid frequent freeze and thaw. It is suggested to aliquot RNA.

3.3 RNA Quality Control

The long-term effect of cryopreservation may be a factor which affects the miRNA arrays. We matched cases and controls on the date of sample collection to allow for potential cryopreservation effect. Furthermore, the fact that samples may have been exposed to the long term effects of cryopreservation means that the quality of total RNA derived from samples makes it difficult to carry out gene expression analyses. This was one of the reasons for us to focus on miRNA because this RNA population is less sensitive to degradation and, as analyzed by Agilent bioanalyzer and Northern blot analysis, miRNA population resulted suitable for further experimental evaluation.

To proceed with miRNA expression profile analysis it is important to check the quantity and quality of the RNA.

To assess the concentration and purity of total RNA use a Nanodrop™ 1000 spectrophotometer (Nanodrop Technologies, Wilmington, DE, USA) or similar. The amount of RNA yield from 1.5 mL of buffy coat sample should be between 25 and 120 µg.

It is also suggested to check the amount of small RNA fraction (<200 nucleotides, including microRNAs) in the total RNA isolated from buffy coat samples.

We analyzed the small nucleic acids ranging in size from 6 to 150 nucleotides running the small RNA assay on the Agilent 2100 bioanalyzer.

The small RNA assay can (a) visualize miRNA, small RNA, oligo nucleotides from 6 to 150 nt for verifying sample integrity; (b) quantify miRNA component (in the concentration range of 50–2000 pg/ μ L) among all small RNAs (pre-miRNA, 5S, ribosomal RNA, etc.) relative to an external standard, for verifying sample enrichment and purity; and (c) automate sample quantization, sizing, and purity determination.

The resulting miRNA component in our samples was about 20–30 % (Fig. 1).

To verify the hybridization ability of the miRNAs present in the preparations we also performed a northern blot assay on a pull of samples (Fig. 1). The blot filter was hybridized with a [32 P] γ ATP radiolabeled LNA oligonucleotide complementary to miRNA-223 sequence. miR-223 is highly specific for hematopoietic cells and constitutes a regulator of myelopoiesis [15, 16].

The RNA samples displayed a good hybridization ability, where human promyelocytic HL60 cells treated with retinoic acid 10⁻⁶ M, a known inducer of miR-223, were included as positive control.

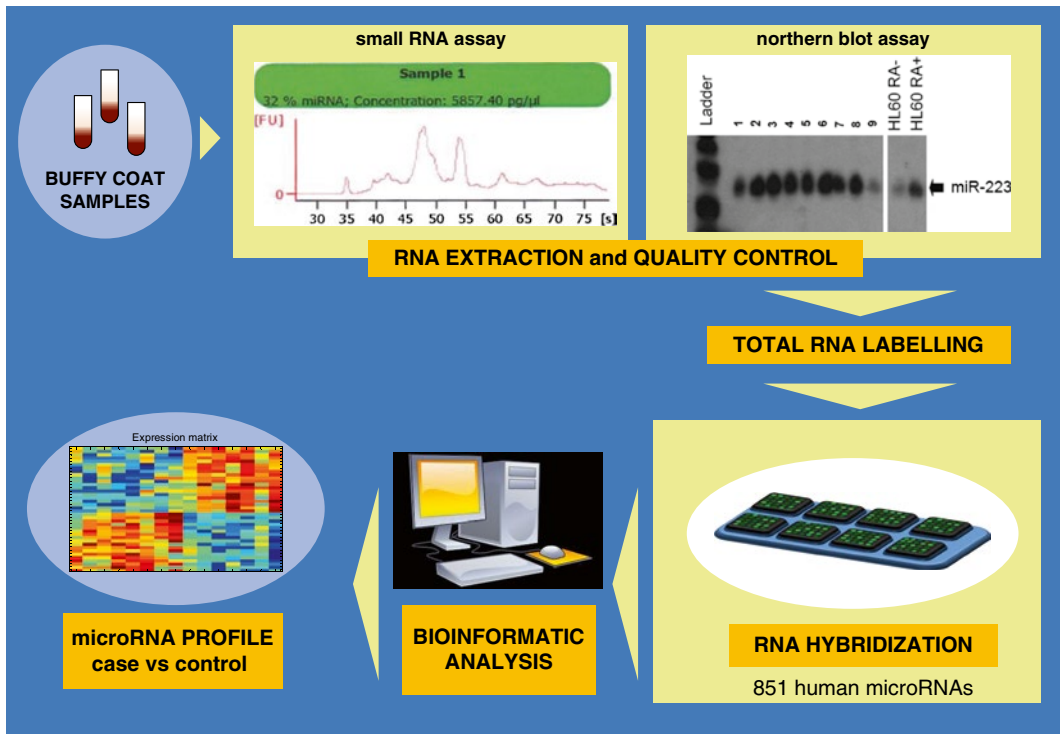


Fig. 1 Schematic representation of the experimental workflow

3.4 RNA Hybridization

Total RNA (100 ng) was labeled and hybridized to human microRNA Microarray V.3 (Agilent) containing probes for 866 human and 89 human viral miRNAs from the Sanger database release 12.0.

Each slide is an 8×15K format (~15,000 features printed in an 8-plex format, eight individual microarrays on a 1"×3" glass slide) printed using Agilent's 60-mer Inkjet Technology, which, unlike competitive platforms, synthesizes 40–60-mer oligonucleotide probes directly on the array, resulting in high-purity, high-fidelity probes.

This miRNA platform requires small input amounts of total RNA—in the 100 nanogram range—because it uses a high-yield labeling method, and does not require size fractionation or amplification steps that may introduce undesired bias during miRNA profiling.

Scanning and image analysis were performed using the Agilent DNA Microarray Scanner (P/N G2565BA) equipped with extended dynamic range (XDR) software according to the Agilent miRNA Microarray System with miRNA Complete Labeling and Hybridization Kit Protocol manual.

Feature Extraction Software (Version 10.5) was used for data extraction from raw microarray image files using the miRNA_105_Dec08 FE protocol.

3.5 Microarray Data Analysis

Data were verified and extracted by Agilent Extraction 10.7.3.1 software and analyzed using an in-house built routines by Matlab (The MathWorks Inc.).

All arrays were quantile-normalized, assuming that all samples were measured and analyzed under the same condition, enforcing all the arrays to assume the same mean distribution. Pearson's coefficient was calculated to assess correlation between technical replicates of some randomly chosen samples.

We fitted a linear model to the expression values for each miRNA, to assess the significance of differential expression between case and control. In addition, we used empirical Bayes methods implemented in the LIMMA package to construct moderated-t statistics and incorporated the statistical tools to adjust for the multiplicity of the tests. The Benjamini and Hochberg's method (1995) was used to control for false discovery [17].

We considered the liner model including the matched case-control study design, the case-control status and the error term.

4 Notes

1. The mixture separates into a lower red phenol chloroform phase, an interphase, and a colorless upper aqueous phase. RNA remains exclusively in the aqueous phase. The upper aqueous phase is ~50 % of the total volume.

2. Save the interphase and organic phenol chloroform phase if isolation of DNA or protein is desired. The organic phase can be stored at 4 °C overnight.
3. The RNA is often invisible prior to centrifugation, and forms a gel-like pellet on the side and bottom of the tube.
4. Do not allow the RNA to dry completely, because the pellet can lose solubility. Partially dissolved RNA samples have an A 260/280 ratio <1.6.

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