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EXPERIMENTAL BIOLOGY

Detection of Low-Abundant MicroRNAs with Hybridization Microchips

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The effect of low concentrations of miRNA on the ability of GeneChip miRNA 4.0 hybridization chips to evaluate their representation in the sample was studied. It is shown that the evaluation of the expression of 61 miRNAs is statistically significantly associated with the multiplicity of plasma dilution. Only 12 miRNAs showed very high Pearson correlation coefficient (>0.95) and they all decreased in response to dilution. High abundance of has-miR-4532 miRNA in plasma was demonstrated. This miRNA was never detected during sequencing of similar samples. It was concluded that in case of miRNA expression <1.12±0.33 units in log2 scale, dilution was not followed by further decrease in the signal intensity in GeneChip miRNA 4.0 chips.

Key Words: *hybridization chips; miRNA; sensitivity*

Preclinical drug testing are often performed on human cell cultures *in vitro*. In this area, new technologies are constantly being developed and are introduced, especially in the field of tissue engineering. A new approach in *in vitro* modeling appeared with the development of microphysiological models, for example, microfluidic "organs-on-chips" systems that allowed studying physiological functions of organs and pharmacological effects of drugs [10]. Relevant models of human diseases were created. For instance, "lung-on-chip" model was successfully used to model chronic obstructive pulmonary disease [3], and "liver-on-chip" model was used to study non-alcoholic steatohepatitis [8]. However, systemic human diseases affect several organs and require modeling of a combination of different organs. To simulate this systemic interaction, microphysiological platforms have been developed with several types of cells from different organs placed in separate compartments connected with microfluidic channels that simulate the circulatory system [10].

A problem associated with using "organs-on-chip" systems consists in difficulty of tracking the current state of models of individual organs. The multi-organ platform allows sampling and analyzing culture medium that circulates through microchannels between the organ-like structures (organoids) and is analogous to blood; however, cells from organoids cannot be sampled for analysis without disturbing system integrity.

As only tens to hundreds of thousands of cells are used in the "organs-on-chip" models, the assessment of the state of individual organ models based on protein molecules is extremely difficult. A promising parameter for assessment of the state of the models

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is circulating nucleic acids, in particular microRNA, short non-coding RNAs that regulate the expression of at least 60% of the known human genes. A correlation was found between the level of different miRNAs and normal and pathological processes in the body. microRNA are characterized by high stability, due to which they persist in the extracellular space for a long time [9]. The spectra of blood miRNA specific of blood cells and extracellular miRNAs of healthy people were determined [13].

Despite nucleic acids are detected in significant quantities in human tissue samples, their concentration in physiological fluids is rather low. Thus, the concentration of total RNA in the plasma is 6-300 ng/ml [6,15] and miRNA accounts for only a few percent [12]. The human body consists of ~3.72×10^{13} cells [4] and the volume of plasma, lymph, and interstitial fluid is ~5-6 liters [5]. Thus, in a human body there are about 7 mlns cells per 1 μl of these fluids. In the "organs-on-chip" models, this ratio is 350 cells per 1 μl of culture medium, which is almost by 20 thousand times less. Thus, it is necessary to evaluate how existing methods of miRNA detection are applicable for samples with a low concentration of miRNA.

There are three main approaches to quantifying the level of miRNA in physiological fluids: quantitative real-time PCR, hybridization technologies, and next-generation sequencing [11]. It should be noted that the use of quantitative PCR and sequencing involves modification of miRNA molecules, as well as about reverse transcription, which introduces significant distortions in their quantitative assessment. In addition, comprehensive screening with PCR is very laborious, and sequencing is still expensive and difficult task. Hybridization technologies do not have these disadvantages.

The goal of this study was to assess the ability of hybridization microchips of the new generation GeneChip miRNA 4.0 Array to determine low levels of miRNA.

MATERIALS AND METHODS

The study used a plasma sample from a healthy volunteer who signed informed consent. The blood was taken with S-Monovette EDTA-KE 8 ml (Sarstedt) vacuum tubes.

The plasma was separated by centrifugation in a 5810 R centrifuge (Eppendorf) in two stages: 10 min at 2000*g*, transfer of the upper phase to a new tube, 10 min at 4000*g*. All stages were conducted at room temperature, the speed of acceleration and deceleration of the centrifuge was set at an average level.

The plasma was diluted 1:1 and 1:3 with PBS (pH 7.2) (cat.# 70013-016, Gibco). For RNA isolation, two 400-μl aliquots from each dilution and two 400-μl aliquots of the whole plasma were prepared. Thus, 3 types of samples were prepared: aliquots of whole plasma (S1.1 and S1.2) and aliquots of 2-fold (S2.1 and S2.2) and 4-fold (S4.1 and S4.2) diluted plasma.

RNA was isolated for each aliquot separately by a modified protocol [14]: 400 μl sample of whole or diluted plasma was added to 1.2 ml Qiazol Lysis Reagent (Qiazol), thoroughly mixed for 15 sec, and incubated for 10 min at room temperature for complete dissociation of nucleoprotein complexes. After that, 1.6 μl of Ambion GlycoBlue Coprecipitant (15 mg/ml; Life Technologies) was added to the mixture to increase the efficiency of RNA precipitation during isolation on columns. Then, 320 μl chloroform was added to the mixture, the sample was stirred for 45 sec and incubated at room temperature for 5 min; then, the mixture was centrifuged at 16 000*g* and 4°C for 20 min and total RNA was isolated using miRNeasy Serum reagent/Plasma Kit (Qiagen) according to manufacturer's protocol.

Analysis of miRNA in RNA samples isolated from plasma aliquots was performed for each aliquot separately on GeneChip miRNA 4.0 microchips (Affymetrix) according to manufacturer's protocol. The results of microchip analysis were preprocessed by the RMA method using Affymetrix Expression Console software (version 1.4.1.46) at RMA+DABG-NO-NORM-Human-only setting.

Pearson correlation analysis was performed using R statistical computation environment. The correlation of microRNA with the degree of blood plasma dilution by 2 and 4 times was considered statistically significantly at Pearson's correlation coefficient $r<0$ and $p<0.05$.

RESULTS

In initial plasma samples, the total number of microRNAs with expression above the background value [1] was 264 (the chip contains probes to 2578 human miRNAs), in 2- and 4-fold diluted samples, the corresponding values were 283 and 253. This agrees with our previous data [1]. We will discuss only miRNAs expressed above the background values.

We evaluated the convergence of technical repeats (miRNA samples isolated from paired aliquots). The coefficient of variation 10% was taken as the threshold. In aliquots S1.1 and S1.2 (whole plasma), the number of miRNAs with a coefficient of variation less than the threshold was 145; in aliquots S2.1 and S2.2 (2-fold dilution) — 112; in aliquots of S4.1 and S4.2 (4-fold dilution) — 95 (Fig. 1). However, only 42 miRNAs corresponded to the specified criteria in all aliquots (expression above the threshold level and

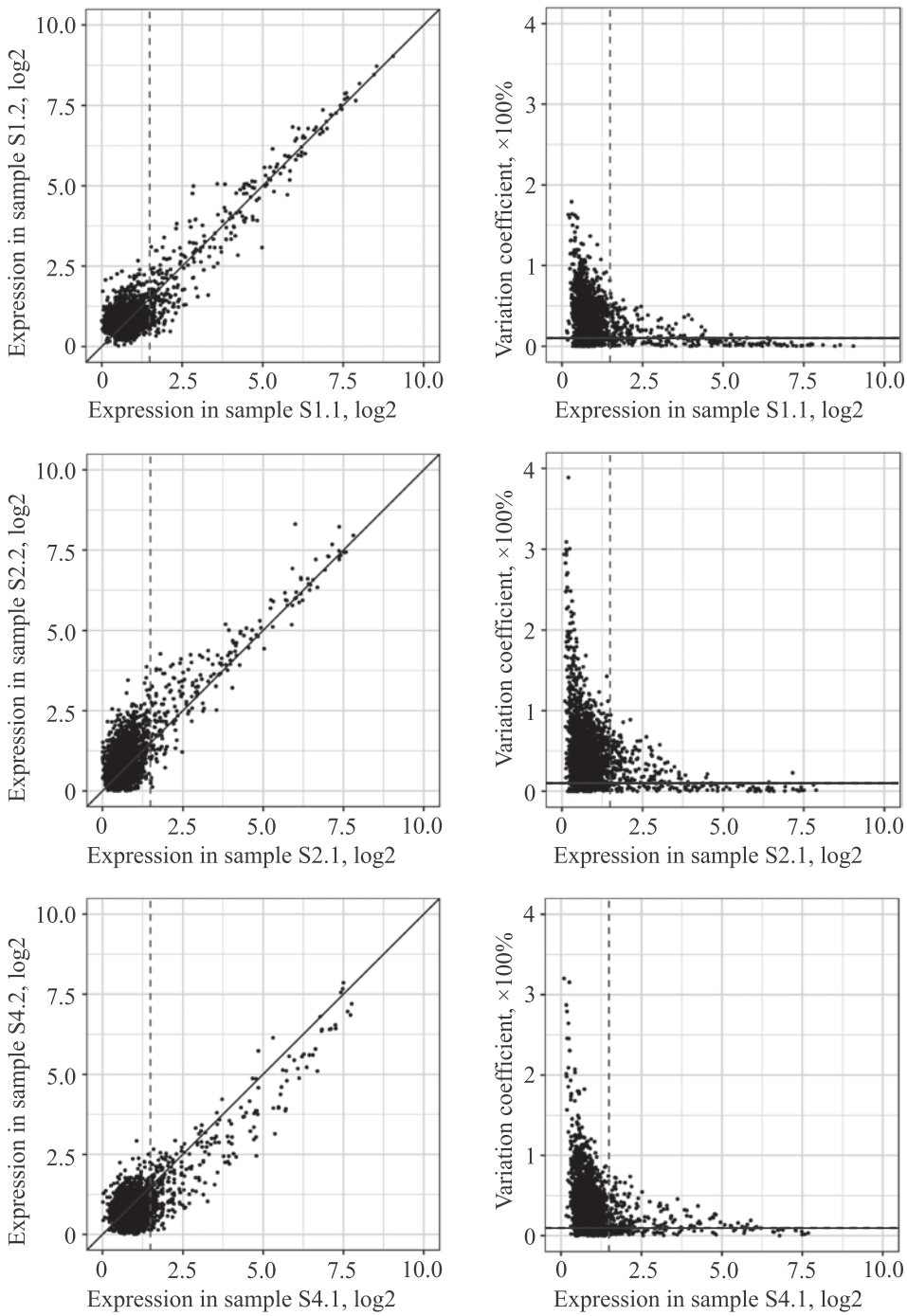

Fig. 1. Analysis of the number of miRNAs correlating with sample dilution. Dashed line: previously determined [2] background threshold equal to 1.49 units in log2-scale. Straight line: threshold value for variation coefficient 10%.

coefficient of variation <10%). With increasing the degree of plasma dilution, *i.e.* with decreasing RNA concentration, the number of miRNAs with low coefficients of variation decreased.

Analysis of Pearson linear correlation coefficients showed that expression of 61 miRNAs significantly correlated to multiplicity of plasma dilution. Of these, expression of 58 miRNAs decreased and expression of only 3 miRNAs (has-miR-6732-5p, has-miR-3921, and has-miR-3619-5p) increased. These 3 miRNAs had the lowest representation and, obviously, could not be adequately measured on the chip. It should be noted that only 12 miRNAs had very high Pearson's correlation coefficient (>0.95) and their signals decreased after dilution (Table 1). According to sequencing data, these miRNAs are among the most abundant

TABLE 1. Abundance of MicroRNA Highly Correlating with Sample Dilutions (*M±SD*)

miRNA	Expression, log2			Spearman's correlation coefficient
	S1	S2	S4	
hsa-miR-4532	9.04±0.01	7.89±0.11	6.85±0.59	0.96
hsa-miR-486-5p	8.63±0.13	7.17±0.21	5.95±0.45	0.98
hsa-miR-16-5p	8.46±0.00	6.93±0.40	5.71±0.37	0.98
hsa-miR-92a-3p	8.10±0.12	6.39±0.29	5.42±0.43	0.97
hsa-miR-22-3p	6.03±0.11	4.37±0.45	2.12±0.16	0.99
hsa-let-7b-5p	5.88±0.02	4.83±0.02	4.14±0.25	0.98
hsa-miR-185-5p	5.50±0.11	4.16±0.22	1.90±0.70	0.97
hsa-miR-106a-5p	5.09±0.06	3.09±0.40	2.26±0.39	0.96
hsa-miR-19b-3p	5.09±0.37	2.25±0.31	1.21±0.17	0.96
hsa-miR-619-5p	4.32±0.54	3.12±0.19	2.26±0.10	0.96
hsa-miR-320e	4.04±0.04	2.07±0.13	1.41±0.11	0.96
hsa-miR-4454	4.03±0.00	2.59±0.14	2.16±0.13	0.95

in the plasma [2]. In general, at expression of miRNA below 1.12±0.33 units in log2-scale with dilutions, the reduction of the signal on the chip is not observed. This result is consistent with the previous data on the sensitivity threshold 1.49 in the log2-scale for these microchips [1].

Has-miR-4532 is of particular interest. This miRNA was not detected previously during sequencing of blood plasma RNA. However, its expression according to the results of microchips roughly corresponds to miR-486-5p, the most abundant miRNA in plasma. miR-4532 microRNA is characterized by extremely high cytosine and guanine content (94%), which can determine high dissociation constant with the complementary probe on a microchip. In addition, 28S ribosomal RNA contains full-length sequence of miR-4532.

The problem of the limited detection capacity of hybridization microchips for small amounts of microRNA was addressed in previous studies [7,11]. The data on blood plasma miRNA profiling obtained by us confirmed the lower threshold of sensitivity of hybridization microchips GeneChip miRNA 4.0. To increase the reliability of the assay, it is advisable to analyze additionally at least one (two) dilution of the sample.

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