Serum MicroRNAs Are Promising Novel Biomarkers

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Abstract

**Background:** Circulating nucleic acids (CNAs) offer unique opportunities for early diagnosis of clinical conditions. Here we show that microRNAs, a family of small non-coding regulatory RNAs involved in human development and pathology, are present in bodily fluids and represent new effective biomarkers.

**Methods and Results:** After developing protocols for extracting and quantifying microRNAs in serum and other body fluids, the serum microRNA profiles of several healthy individuals were determined and found to be similar, validating the robustness of our methods. To address the possibility that the abundance of specific microRNAs might change during physiological or pathological conditions, serum microRNA levels in pregnant and non pregnant women were compared. In sera from pregnant women, microRNAs associated with human placenta were significantly elevated and their levels correlated with pregnancy stage.

**Conclusions and Significance:** Considering the central role of microRNAs in development and disease, our results highlight the medically relevant potential of determining microRNA levels in serum and other body fluids. Thus, microRNAs are a new class of CNAs that promise to serve as useful clinical biomarkers.

Introduction

Specific clinical biomarkers have the potential to revolutionize diagnosis and treatment of various medical conditions, ranging from abnormal pregnancies to myocardial infarctions and cancer. In particular, a theme of current cancer research is the quest for sensitive biomarkers that can be exploited to detect early neoplastic changes. Ideally, biomarkers should be easily accessible such that they can be sampled non-invasively. Therefore biomarkers that can be sampled from body fluids, such as serum or urine, are particularly desirable. In recent years it has become clear that both cell-free DNA and mRNA are present in serum, as well as in other body fluids, and that these CNAs represent potential biomarkers [1–3]. Accordingly, we considered it likely that microRNAs might be present in bodily fluids.

MicroRNAs are a recently discovered class of small non-coding RNAs that regulate gene expression and have a critical role in many biological and pathological processes [4,5]. In general, microRNAs are regulated and transcribed like protein coding genes. Subsequent microRNA biogenesis involves discrete processing and transport steps, whereby the active moiety of 20–22 nucleotides is excised from a longer RNA precursor that exhibits specific hairpin structure. Finally, these 20–22 nucleotides are incorporated into a composite machinery, which promotes partial duplex formation between the short RNA and the 3′ untranslated regions (UTR) of targeted mRNAs, resulting typically in mammals in translational silencing [6,7]. A relevant, though at present enigmatic, feature of microRNA biology is their remarkable stability. For example, microRNAs are preserved well in tissue samples even after formalin-fixation and paraffin-embedding and can be efficiently extracted and evaluated [8]. Therefore, if microRNAs are indeed circulating, we hypothesized further that they should preserve their stability in body fluids. Monitoring the typically small amounts of CNAs in body fluids requires complex and sensitive extraction and detection methods, which until now have been prohibitive either practically or economically [9]. However, we anticipated that the stability of microRNAs should allow development of practicable detection methods, such that they can realistically serve as clinical biomarkers.

Although the total number of microRNAs remains controversial [4,10,11] and the roles of specific microRNAs are only beginning to be defined, microRNA expression analyses indicate that diverse tumors display microRNA expression profiles (for mature and/or precursor microRNAs) significantly different from normal tissue [12,13]. Furthermore, microRNAs are emerging as highly tissue-specific biomarkers [14,15] with potential clinical applicability for defining the cancer origin of metastases, as we have shown recently [16]. These data led us to expect that the microRNA abundance profile of bodily fluids might reflect physiological and/or pathological conditions and, furthermore, might do so more
additional synthetic RNAs were spiked-in as controls. For RNA extraction, the pellet was re-suspended in DDW and two fragments. Finally, after a second acid phenol:chloroform extraction and then RNA was ETOH precipitated ON at 0.65 mg/ml Proteinase K (Sigma P2308). Two synthetic RNAs complementary to the consensus 3′ of the specific microRNA sequence as well as to part of the polyA adaptor sequence, and a universal reverse primer complementary to the consensus 3′ sequence of the oligoDT tail.

Results
qRT-PCR can be used to monitor low microRNA levels specifically and sensitively

We have developed a proprietary qRT-PCR based platform for evaluating microRNA molecules that detects specifically mature microRNA levels that differ by only a single nucleotide. Three such microRNAs were generated synthetically (hsa-let 7a, c & d) and each one subjected to three independent qRT-PCR reactions, where in each reaction there were present PCR primers specific to only one of the 3 family members. In most of these reactions, we observed amplification only of the appropriate family member matching the specific primer, indicating that this qRT-PCR platform detects microRNA with accuracy at the single nucleotide level (Figure 1B). Next, we mixed the three synthetic microRNAs and subjected the mixture to qRT-PCR in the presence of the let-7d primer-probe set. In parallel, we took the same amount of synthetic let-7d microRNA as used in the mixture but subjected it alone to qRT-PCR. These parallel PCR reactions were repeated two further times, using reducing concentrations of let-7d synthetic microRNA. We observed that the qRT-PCR amplified let-7d equivalently whether it was alone or in the presence of homologous family members at all tested concentrations of let-7d (Figure 1C). In an additional experiment, we wished to confirm the capability of our platform to detect specific microRNAs in a biologically relevant complex background. Decreasing concentrations (from 100% down to 0%) of total RNA extracted from liver tissue were diluted into total RNA extracted from brain tissue and 0.1 ng of these mixtures subjected to PCR reaction, each reaction containing primers specific for hsa-miR-122a, a liver specific microRNA [18]. Hsa-miR-122a was detected, even when only 0.03% of liver RNA was spiked into brain RNA (Figure 1D). Importantly, hsa-miR-122a detection was linear and there was no detection in the absence of liver RNA (100% brain RNA).

Finally, we validated the reproducibility of our methods, at two independent times, two different researchers subjected the same mix of 32 microRNAs to qRT-PCR. Similar levels of abundance were determined for each microRNA on both occasions, such that the two abundance profiles (for all 32 microRNAs) were within less than 1 Cq difference of one another (Figure 1E). Summarily, our qRT-PCR based platform is sensitive, reproducible and specific, capable of detecting accurately a few molecules of microRNA present in a complex RNA background. Such sensitivity makes it possible to use this platform to monitor the minute amount of microRNA present in cell-free body fluids.

microRNAs are present in cell-free bodily fluids

When we initiated this study, there had been no demonstration that microRNAs are present in cell-free body fluids. While this manuscript was in preparation, it was reported that microRNAs are detectable in serum [19]. Here, a reliable protocol is presented that we developed for extracting microRNAs from body fluids, microRNA that is cell-free and DNA-free (see Materials and Methods) and that can serve as the template in our qRT-PCR based platform.

To validate that microRNAs are indeed reproducibly detectable in serum, RNA was extracted from the sera of two healthy unrelated individuals and the levels of highly abundant microRNAs examined (Figure 2A). We observed that microRNAs are present at similar levels in both serum samples. This finding not only establishes the reproducible presence of microRNAs in serum, but also indicates that in general microRNA levels are similar among individuals. Moreover, this finding supports our premise that changes in the levels of specific microRNAs might allow detection of clinical conditions. In parallel, using the same methods we confirmed that microRNAs are also detectable in other body fluids, such as urine, saliva, amniotic fluid and pleural fluid (Figure 2B and data not shown). Of note, serum and urine display different microRNA abundance profiles as might be expected for two dissimilar biological fluids, further supporting our hypothesis that bodily fluid microRNA profiles reflect physiology.
microRNAs in serum are sufficiently stable to serve as clinical biomarkers

In order for microRNAs in serum to be useful as clinical biomarkers, they must be stable for reasonable periods of time, and preferably during freeze-thaw cycles, to allow for routine processing of clinical samples. Therefore, we investigated the stability of serum microRNAs at room temperature and checked the influence of freeze-thaw cycles on serum microRNA levels. We found that the levels of different microRNAs in unfrozen serum do not change substantially over a 4 hour period at room temperature (Figure 3A). Moreover, serum microRNA levels are little affected by twice freezing and re-thawing of serum samples (Figure 3B). Thus, microRNAs in serum are sufficiently robust to serve as practicable clinical biomarkers.
Serum microRNA profiles reflect physiological conditions

Finally, as a proof of concept, we investigated whether circulating microRNAs can be used to identify clinical conditions. It has been established that circulating maternal RNA contains placental embryonic RNA [2]. Therefore, we chose to compare the serum microRNA abundance profiles of non pregnant versus pregnant women, the latter in either their first or third trimester. We measured the serum levels of 28 microRNAs, including microRNAs reported to be placenta-specific [10,20] as well as broadly expressed microRNAs. Box plots show relative microRNA levels in the sera of 10 non pregnant women, 10 women in the first trimester and 10 women in the third trimester (Figure 4A). The median fold changes in microRNA levels comparing third trimester pregnant women to non pregnant women are detailed in Table 1. MicroRNAs expressed equivalently across all samples were used for normalization. All of the placental microRNAs are found at higher levels in the sera from pregnant women, their levels rising with gestational age, and the levels of 12 microRNAs increased by more than 5-fold.
Specifically, amounts of hsa-miR-526a and hsa-miR-527 are dramatically higher in the sera of third trimester pregnant women (elevated by more than 600 fold). Indeed, we found that the levels of three placental microRNAs (hsa-miR-526a, hsa-miR-527 and hsa-miR-520d-5p) could be used to accurately distinguish pregnant from non pregnant women (Figure 5).

Figure 4. Differential amounts of four microRNAs in the sera of pregnant vs. non pregnant women. Box plots comparing microRNA levels in the sera of 10 non pregnant women (A), 10 women in the first trimester (B), and 10 women in the third trimester (C). microRNA level is specified as 50-CT, where CT is the cycle threshold of the PCR reaction. Results were normalized by subtracting the global microRNA level in the sample (average CT of the 6 microRNAs chosen for normalization) from the level (CT) of each microRNA. A) The three placental microRNAs (miR-527, miR-520d-5p and miR-526a) are highly abundant in the sera of pregnant women and their levels rise as pregnancy progresses. Hsa-let-7d levels are also shown; this was one of the 6 microRNAs chosen for normalization as this microRNA exhibits similar abundance across the three groups. B) microRNA miR-141 and miR-149 levels are mildly upregulated during pregnancy.

doi:10.1371/journal.pone.0003148.g004
imported clinical biomarkers. Importantly, we have developed reliable microRNAs are present in bodily fluids and represent useful amounts during pregnancy, which are insufficient to discriminate pregnancy and this may explain the small changes in their amounts in serum and display increased abundance during pregnancy \[19\]. Therefore, we examined also the levels of these two microRNAs in the sera of pregnant vs. non pregnant women. The location of each symbol in the plot represents the collective expression of all three microRNAs in a given serum. The y axis indicates the amount of mir-527, and the x axis indicates the average level of mir-520d-5p and mir-526a.

We identified previously a large subset of microRNAs that are expressed almost exclusively in the placenta \[10,20\] and found that a sizeable number of these are primate-specific \[10\]. The biological role of these placental microRNAs remains unclear, although it is tempting to speculate that they contribute to specific morphological placental features characteristic of primates and key to their evolution \[21\]. Future studies may reveal how these placental microRNAs influence primate physiology. Here we show that many of these microRNAs are found in maternal serum, at levels that increase with gestational age. Easily accessible biomarkers for pregnancy complications and for various diseases are an urgent goal. In particular, preeclampsia is a disorder affecting 3–8% of pregnancies that is a leading global cause of maternal and infant illness or death \[22,23\]. The recently reported, distinctive expression of microRNAs in the placenta in association with preeclampsia \[24\] highlights the possibility that serum levels of particular microRNAs may serve as future diagnostic biomarkers for preeclampsia.

This proof of concept study reveals the potential of body fluid microRNAs to serve as practicable molecular markers for diverse physiological and pathological conditions, especially those where microRNAs have already been found to play a critical role, such as cancer. Moreover, we demonstrate here the ease and reliability of determining body fluid microRNA profiles and thus, pave the way for their wide application, both in the research laboratory and in the clinic.

Table 1. Serum microRNA levels - comparison between non pregnant women and pregnant women in their third trimester.

| microRNA      | delta C\(_T\) | fold change | p-value |
|---------------|---------------|-------------|---------|
| hsa-miR-526a  | 9.44          | 694         | 2.10E-07|
| hsa-miR-527   | 9.28          | 622         | 1.20E-14|
| hsa-miR-515-5p| 9             | 511         | 6.90E-08|
| hsa-miR-521   | 7.36          | 164         | 8.10E-09|
| hsa-miR-523   | 4.81          | 28          | 2.20E-06|
| hsa-miR-524*  | 4.81          | 27          | 2.80E-03|
| hsa-miR-518a-3p| 3.61        | 12          | 1.80E-04|
| hsa-miR-520d-5p| 3.1           | 8.6         | 3.30E-07|
| hsa-miR-525-3p| 2.73          | 6.6         | 5.60E-04|
| hsa-miR-526c  | 2.47          | 5.5         | 1.10E-01|
| hsa-miR-519e* | 2.38          | 5.2         | 1.30E-04|
| hsa-miR-518d  | 2.35          | 5.1         | 7.60E-03|
| hsa-miR-524  | 2.27          | 4.8         | 3.80E-03|
| hsa-miR-512-3p| 2.16          | 4.5         | 1.90E-03|
| hsa-miR-141  | 2             | 4.0         | 3.90E-04|
| hsa-miR-519d  | 1.9           | 3.7         | 2.60E-02|
| hsa-miR-517*  | 1.82          | 3.5         | 5.80E-02|
| hsa-miR-518e  | 1.5           | 2.8         | 3.50E-02|
| hsa-miR-145  | 0.98          | 2.0         | 3.20E-02|
| hsa-miR-149  | 0.92          | 1.9         | 6.00E-01|
| hsa-miR-141  | 0.59          | 1.5         | 5.20E-01|
| hsa-miR-16    | 0.39          | 1.3         | 6.90E-01|
| hsa-miR-126  | 0.16          | 1.1         | 1.60E-01|
| hsa-miR-572  | 0.11          | 1.1         | 8.70E-01|
| hsa-miR-202  | 0.1           | 1.1         | 4.80E-01|
| hsa-miR-451  | 0.13          | 0.91        | 8.40E-01|

For each microRNA, “delta C\(_T\)” indicates the difference in median C\(_T\) between the serum of pregnant women in the third trimester (n = 10) and non-pregnant women (n = 10). For each sample, the relative amount of the microRNAs was normalized by subtracting the average C\(_T\) of the non-placenta-specific microRNAs. The fold change is the ratio of the median abundance in linear space, equal to the exponent (base 2) of the delta C\(_T\). P-values are calculated by a two-sided unpaired t-test.

doi:10.1371/journal.pone.0003148.t001

While we were preparing these results for publication, another research group reported that hsa-miR-141 and hsa-miR-149 are present in serum and display increased abundance during pregnancy \[19\]. Therefore, we examined also the levels of these two microRNAs in our serum samples. We found hsa-miR-141 and hsa-miR-149 levels to be higher by 4 and less than 2 fold, respectively, in third trimester pregnant women relative to non-pregnant women (Table 1 and Figure 4B). These two microRNAs are associated with epithelial tissues \[16\] that are not unique to pregnancy and this may explain the small changes in their amounts during pregnancy, which are insufficient to discriminate pregnant from non pregnant women.

Discussion

The results of this study clearly uphold our basic hypothesis that microRNAs are present in bodily fluids and represent useful clinical biomarkers. Importantly, we have developed reliable methods for extracting microRNAs from bodily fluids and for evaluating their abundance. Moreover, we have demonstrated that microRNA serum levels reflect physiological conditions, such as pregnancy, and can even be used to determine pregnancy stage.

We identified previously a large subset of microRNAs that are expressed almost exclusively in the placenta \[10,20\] and found that a sizeable number of these are primate-specific \[10\]. The biological role of these placental microRNAs remains unclear, although it is tempting to speculate that they contribute to specific morphological placental features characteristic of primates and key to their evolution \[21\]. Future studies may reveal how these placental microRNAs influence primate physiology. Here we show that many of these microRNAs are found in maternal serum, at levels that increase with gestational age. Easily accessible biomarkers for pregnancy complications and for various diseases are an urgent goal. In particular, preeclampsia is a disorder affecting 3–8% of pregnancies that is a leading global cause of maternal and infant illness or death \[22,23\]. The recently reported, distinctive expression of microRNAs in the placenta in association with preeclampsia \[24\] highlights the possibility that serum levels of particular microRNAs may serve as future diagnostic biomarkers for preeclampsia.

This proof of concept study reveals the potential of body fluid microRNAs to serve as practicable molecular markers for diverse physiological and pathological conditions, especially those where microRNAs have already been found to play a critical role, such as cancer. Moreover, we demonstrate here the ease and reliability of determining body fluid microRNA profiles and thus, pave the way for their wide application, both in the research laboratory and in the clinic.

Author Contributions

Conceived and designed the experiments: SG EM ZB MH AC. Performed the experiments: SG EM YY SB DL NY HC AC. Analyzed the data: SG EM SB DL HB MK YG AC. Contributed reagents/materials/analysis tools: XM. Wrote the paper: SG ZB MH AC.
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