Introduction

MicroRNAs (miRNAs) are short, non-coding, single-stranded oligonucleotides (19 – 25 mers) that play a critical role in the cellular process such as the repression of mRNA translation and degradation. Several reports have been published on determining the biological function of miRNAs.1–4 The circulating serum miRNAs have been used as molecular signatures for identifying breast cancer,5 retinoblastoma,6 and colorectal cancers.7 The accurate assessment of miRNAs related to the disease condition in the serum samples would help in early diagnosis.8,9 The existing standard methods for quantifying miRNAs are northern blotting and qRT-PCR methods. These methods require high amount of input RNA template and lengthy processing time, which is not suitable for routine clinical diagnostics on serum samples. Earlier reports on the rapid and sensitive detection of miRNA used a molecular beacon probe with an additional step for removing any non-hybridized probe.10 To overcome these technical challenges, the molecular beacon probes modified with locked nucleic acid (LNA) bases were used for better sensitivity and specificity of hybridization.10 Here, we show an extended application of LNA beacon probes for the direct detection, quantification of well-studied miR-18a in human serum samples.10,11 To the best of our knowledge, this application has not been reported for direct serum hybridizations. Moreover, in this method a minimum volume of 0.1 μL healthy human serum and retinoblastoma serum was used to show the biological variation of the miRNA copy number.

Keywords
Locked nucleic acid (LNA), serum, miRNA, retinoblastoma

Experimental

Materials
Vacutainer (BD, USA), circulating RNA isolation kit (Qiagen, Germany), Synthetic hsa-miR-18a (Origene, USA), hsa-miR-129 (Eurofins), Human Serum and commercial fetal bovine serum (Sigma Aldrich, India), Biotinylated (FITC) LNA miR-18a probe (M/s Exiqon, USA Design ID: 201793, 5′/6-FAM/CCGAGCTATCTGCACTAGATGCACCTTAC/iBiodT/CGG/3′Dab), Fluorescent microscopy (Nikon Eclipse TS100) with fluorescein isothiocyanite (FITC) emission filter (Nikon Intense light, C-HGFI pre-centered fiber illuminator, Germany) and camera (Q imaging Retiga Exi, Fast 1394), Spectrophotometer (SpectraMax M4 MultiMode Microplate Reader, Molecular Devices LLC, USA).

Methods

Serum sample collection. A volume of 1.0 mL blood sample was collected directly into a vacutainer from healthy and Retinoblastoma (RB) individuals. The whole blood was allowed to stand for 30 min at room temperature (RT) and centrifuged at 1800g for 20 min at RT. The resultant serum was aliquoted into sterile diethylpyrocarbonate (DEPC) treated, RNAase free 1.5 mL tubes and stored in a –80° C freezer. A total of fifteen non-retinoblastoma age-matched healthy and fifteen retinoblastoma samples were used for this study. Commercial fetal bovine serum was used for in vitro studies. Our institution...
ethics review board approved the collection of serum samples to study the serum miRNA profile in retinoblastoma (Ethics code: 49B-2011-P; 298A-2011-P).

MiR-18a beacon probe design. The design of the miR-18a probe was 5′/6-FAM/CCGAGCTATCTGCACTAGATGCACCTTAC/iBiodT/CGG/3′Dab/. Briefly, a microRNA probe designed for mature miR-18a (MIMAT0000072) or the hairpin loop region of precursor miR-18a contained a total number of 33 nucleotides in length. It comprises the middle ‘23’ nucleotides complementary to the miR-18a seed sequence; (underlined), a 6-carboxylfluorescein (FAM) fluorophore at the 5′ end, a Biotin (iBio) moiety, and a 4-dimethylaminoazobenzene 4 carboxylic acid (Dab or Dabsyl) quencher at the 3′ end. The seed sequence was flanked at both ends by 5 residues; CCGAG/CTCGG to form the stem structure of the hair-pin loop. The approximate number of LNA modifications for the seed sequence were calculated based on given DNA/RNA Tm of the synthesized probe, and was found to be equivalent to 10 numbers (Exiqon Oligo Tm prediction tool, www.exiqon.com/RNA-tm; Underlined in the probe sequence). The synthesized probes were HPLC purified and re-constituted in nuclease-free water, and stored at –20°C in a freezer.

Fluorescence spectra of LNA probe hybridization with synthetic miRNA. Initial experiments were conducted in PBS spiked with synthetic miR-18a and miR-129 in order to study the specificity and sensitivity of the custom miR-18a LNA probe hybridization. The molecular beacon probe designed with sequence complimentary to miR-18a, was labeled with 5′-FAM and 3′-Dabcyl reporter dye molecules (Fig. 1). A different concentration of synthetic miR-18a and miR-129a (non-specific) starting from 500, 1000, 1500, 2000 to 2500 nM was hybridized with 500 nM of a custom LNA probe, standard miR-18a template ranging from 500 to 2500 nM and unknown concentration of 0.1 µL (LOD) human serum in a final volume of 20 µL each. The emission intensity was measured and used to calculate the miRNA copy number from a standard graph. The standard graph was obtained by calculating the area under the curve by measuring the pixel of the image using ImageJ software. The difference in the fluorescence intensity between healthy serum and RB serum (n = 10) was found to be significant (Fig. 2D).

Fig. 1 Schematic representation of LNA hybridization with miRNA. The LNA probe binds to target miRNA, which results in hybridization by opening of the beacon loop and the quenching of FAM dye.

Results and Discussion

A gradual increase in the fluorescence was observed by fluorescent microscopy for 500 nM custom LNA probe hybridization with different concentrations of synthetic miR-18a ranging from 500, 1000, 1500, 2000 to 2500 nM (Fig. 2A) in a buffer solution. The image intensity pixel values were measured using ImageJ software ($R^2 = 0.94$) (Fig. 2B). An increased fluorescence was observed for an increased volume of healthy serum such as 0.1, 0.2, 0.5, 1, and 5 µL with 500 nM when hybridized with a custom LNA-18a probe (Fig. 2C). The difference in the fluorescence intensity between healthy serum and RB serum (n = 10) was found to be significant (Fig. 2D). Similarly, a gradual increase in fluorescence was observed.
with known standard concentrations of synthetic miR-18a (500, 1000, 1500, 2000, and 2500 nM) by spectroscopic method ($R^2 = 0.97$) (Fig. 3A). There was no gradual increase in the fluorescent spectra of the LNA beacon miR-18a probe with non-target miR-129 (Fig. 3A) indicating the specificity of the probe to the target miR-18a. The copy numbers of miRNA in serum samples were calculated by using the above standard graph. A spectral analysis of varying volume (0.1 to 0.5 $\mu$L) of healthy serum (Fig. 3B) had shown a difference in the fluorescence intensity (Fig. 3B). Further, it was validated in fifteen healthy samples for copy number/concentration variation (Fig. 3C).

The copy number of miRNA in healthy serum was calculated for both microscopy and spectroscopy methods using standard curves (Figs. 2B and 3A). The copy number of healthy serum was found to be 349808.4 ± 3375 copies (1399.23 ± 13.5 nM) for the microscopic method ($n = 10$) (Fig. 2C), whereas 356743.3 ± 18300 copies (1426.92 ± 73 nM) were obtained by the spectroscopic method ($n = 15$) (Fig. 3A) in 0.1 $\mu$L. Similarly, for RB samples, the microscopic method ($n = 10$) showed 528815.3 ± 9975 copies (2115.2 ± 39.9 nM) and the spectroscopic method ($n = 15$) showed 595862.3 ± 24970 (2383.22 ± 99 nM) copies. Significant differences were obtained in both methods ($p < 0.01$) (Figs. 2D and 3C).

In a detailed analysis of this study, we used the LNA miR-18a probe directly on to the serum samples for miR-18a detection. Further, we could detect miRNA directly from a minimum volume of 0.1 $\mu$L serum despite serum complexities, such as interference from other proteins and coagulation factors. In contrast, the conventional methods require 200 – 500 $\mu$L of serum for the extraction of miRNA.14,15 This method showed that hybridization was more specific, and there was no enhancement in the fluorescence or cross hybridization with miR-129 as well as with bovine originated microRNAs which might be present in FBS.16 The copy number determination method can be performed in 384 well plates, to also enable the method to be more robust and economic in quantifying miRNA from serum samples. It was very evident from the results that the copy number detected by microscopic and spectroscopy methods was greater in number, indicating that it is more sensitive than the qRT-PCR methods. The relative fold change difference between healthy and RB individuals was the same in the spectroscopy ($n = 15$) and microscopic ($n = 10$) methods, which showed a copy number ≥ 1 fold difference. Although both methods showed a uniform relative difference between healthy and RB, the spectroscopy method seems to be more sensitive in determining the copy number compared to the microscopic method. This suggest that the labeled LNA method is robust for the quantification of miRNAs.
The usual lower yield of the miRNA copy number from serum or plasma by qRT-PCR is due to multiple steps involved in the process. This LNA molecular beacon approach can overcome any technical difficulties such as improper copy number estimations, through indirect hybridization which will not be suitable for the diagnostic purpose. Moreover, in clinical settings it is important to measure any intact miRNAs from biological samples to find out the actual amount of miRNAs without any processing artefacts. Thus, the LNA molecular beacon approach is very rapid and can detect microRNA in intact 0.1 μL healthy serum samples within 30 min. This approach may thus open new avenues for developing point-of-care device platforms.

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