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Relationship between circulating microRNA-30c with total- and LDL-cholesterol, their circulatory transportation and effect of statins

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ABSTRACT

Background: Small non-coding microRNAs (miR) have important regulatory roles and are used as biomarkers of disease. We investigated the relationship between lipoproteins and circulating miR-30c, evaluated how they are transported in circulation and determined whether statins altered the circulating concentration of miR-30c.
Methods: To determine the relationship between lipoproteins and circulating miR-30c, serum samples from 79 subjects recruited from a lipid clinic were evaluated. Ultracentrifugation and nanoparticle tracking analysis was used to evaluate the transportation of miR-30c in the circulation by lipoproteins and extracellular vesicles in three healthy volunteers. Using archived samples from previous studies, the effects of 40 mg rosuvastatin ($n = 22$) and 40 mg pravastatin ($n = 24$) on miR-30c expression was also examined. RNA extraction, reverse transcription-quantitative real-time polymerase chain reaction was carried out using standard procedures.
Results: When stratified according to total cholesterol concentration, there was increased miR-30c expression in the highest compared to the lowest tertile ($p = 0.035$). There was significant positive correlation between miR-30c and total- ($r = 0.367$; $p = 0.002$) and LDL-cholesterol ($r = 0.391$; $p = 0.001$). We found that miR-30c was transported in both exosomes and on HDL3. There was a 3.8-fold increased expression of circulating miR-30c after pravastatin treatment for 1 year ($p = 0.005$) but no significant change with atorvastatin after 8 weeks ($p = 0.145$).
Conclusions: This study shows for the first-time in humans that circulating miR-30c is significantly, positively correlated with total- and LDL-cholesterol implicating regulatory functions in lipid homeostasis. We show miR-30c is transported in both exosomes and on HDL3 and pravastatin therapy significantly increased circulating miR-30c expression adding to the pleiotropic dimensions of statins.

1. Introduction

Small non-coding microRNAs (miR) are currently a subject of intense research; however, their exact roles in different diseases and in physiological regulation remains incompletely understood. miR are small (~22 nucleotides), non-coding RNA which regulate gene expression at the post-transcriptional level via inhibition of the translation of messenger RNA (mRNA) or by inducing the degradation of specific mRNA [1,2]. In the circulation they are resistant to degradation due in part, to being transported by carrier molecules including lipoproteins such as HDL, proteins such as argonaute-2 and exosomes, which render them highly stable and they can therefore be readily detected in blood samples [3]. Thus, they are currently being studied as potential biomarkers of diseases. Circulating levels of specific miRs have been used as biomarkers to diagnose cancers [4]; they have been implicated in the pathogenesis of obesity [5], diabetes mellitus [6], coronary artery disease [7] and used in the diagnosis of myocardial infarction [8].

High plasma cholesterol concentration is a known major risk factor for atherosclerosis. Recently, miRs have been linked to cholesterol homeostasis [9]. In one study, lentiviral mediated hepatic expression of miR-30c was shown to induce the degradation of microsomal triglyceride transfer protein (MTP) mRNA, which is the apparatus required for the assembly and secretion of apolipoprotein-B containing lipoproteins such as LDL [10]. This in turn reduced hyperlipidemia and atherosclerosis in mice without causing hepatosteatosis [10]. More recently, the delivery of a miR-30c mimic to the liver diminished diet-induced hypercholesterolemia in C57BL/6J mice with long term dose escalation

Abbreviations: Cq, quantification cycle; miR, microRNA; mRNA, messenger RNA; MTP, microsomal triglyceride transfer protein; RT-qPCR, reverse transcription-quantitative real time polymerase chain reaction.

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studies showing that miR-30c mimic caused sustained reductions in plasma cholesterol with no obvious side effects [11]. In addition, miR-30c mimic significantly reduced hypercholesterolemia and atherosclerosis in Apoe −/− mice [11]. These studies show that miR-30c lowers plasma cholesterol and mitigates atherosclerosis by reducing MTP expression and lipoprotein production without hepatosteatosis. Furthermore, there is emerging evidence that increasing hepatic miR-30c levels may be a viable treatment option for reducing hypercholesterolemia and atherosclerosis.

Whether specific circulating miRs are dysregulated in lipid disorders in humans such as familial hypercholesterolemia (FH) has not been studied before. We therefore hypothesized that circulating miR-30c could reflect tissue level expression and therefore serve as a biomarker of cardiovascular and metabolic diseases. To study this we examined whether circulating miR-30c was correlated with established circulating biomarkers of cardiovascular disease including lipoproteins and cholesterol. In addition, in an effort to understand how miR-30c is transported in circulation as this would affect any observed associations with lipoproteins, we used ultracentrifugation to separate the various lipoprotein fractions to determine in which subfraction they were carried. We used nanoparticle tracking analysis to evaluate the role of extracellular vesicles, specifically exosomes, in trafficking miR-30c. Previous studies have shown that circulating miRs are carried on HDL [12] and to a lesser extent on LDL [13], on proteins such as argonaute 2 (14) and in exosomes [15]. However, these studies did not specifically examine miR-30c and there is therefore paucity of data with regard to this. Finally, an emerging concept is whether treatments and interventions alter circulating miR concentrations. Indeed one study by Ortega et al. [5] demonstrated that specific circulating miRs changed (some increased whereas others decreased) with bariatric surgery-induced or diet-induced weight loss. To date there is limited data regarding the impact of treatment with statins on specific circulating miR concentrations in various population groups. We therefore sought to delineate the effect, if any, of statin therapy on circulating miR-30c.

Thus, the aims of this study were: a) determine the relationship between lipoproteins, triglycerides and circulating miR-30c; b) determine how they are transported in circulation; and c) investigate whether statin therapy led to alterations in the circulating concentrations of miR-30c.

2. Materials and methods

2.1. Patients

To determine the relationship between lipoproteins, triglycerides and circulating miR-30c, serum samples from 79 fasted subjects attending the lipid clinic at the Glasgow Royal Infirmary were studied. As this was a proof-of-principle study, we did not analyse outcomes in these patients in any detail and samples were provided to the investigators anonymised. We stratified our cohort into tertiles based on total cholesterol concentrations with those <5 mmol/L in the first tertile ('normal' group), those between 5.0 and 7.5 mmol/L in the second tertile ('indeterminate group') and those with raised cholesterol > 7.5 mmol/L in the third tertile ('hypercholesterolemia' group) [16].

To evaluate the transportation of miR-30c in circulation by lipoproteins, we studied serum samples from three healthy volunteers. Samples from two groups of subjects were used to assess the impact of statin therapy on circulating miR-30c concentration. For the study involving rosuvastatin all the available remnant samples (n = 22 from the original 29) were used [17]. In this study, subjects with raised cholesterol were used in a cross-over trial to evaluate rosuvastatin 40 mg/d. After a 5 week dietary run-in subjects were randomized to treatment or placebo for 8 weeks, followed by a 4 week washout period and then crossed over to the opposite arm of the study for a further 8 weeks. The miR concentrations were determined in stored (frozen at −80 °C) samples from baseline and the end of each treatment period. The second group was 24 men from the active arm (i.e. on pravastatin 40 mg/d) of the West of Scotland Coronary Prevention Study (WOSCOPS) [18], from whom stored samples were available for the baseline and one-year time-points. Again the samples had been stored at −80 °C. For this study, samples were randomly selected by the laboratory technician from the available samples stored in our institution using the random number generator function of Excel.

The local Research Ethics Committee approved this study and all volunteers in the original studies gave informed written consent. All procedures were conducted in accordance with the guidelines of The Declaration of Helsinki.

2.2. Lipid measurements

All analysis was carried out on the ILAB 600 Clinical Chemistry Analyser, using Roche reagents (Burgess Hill, UK). Total-cholesterol, HDL-cholesterol and triglyceride were measured by enzymatic-colorimetric methods whereas LDL was calculated using the Friedewald equation, where LDL-cholesterol = Total cholesterol − HDL cholesterol − (Triglycerides / 2.2) and is only valid when the triglyceride concentration is <4.6 mmol/L or Triglycerides / 5.0 if cholesterol and triglycerides are reported in mg/dL (to convert Triglyceride mg/dL to mmol/L, multiply by 88.5). VLDL-cholesterol (mmol/L) was estimated by dividing the triglycerides by 2.2.

2.3. Separation of lipoprotein subfractions by centrifugation

To evaluate how miR-30c is transported in the circulation, serum samples were collected into clot activator tubes with gel separator from three healthy volunteers. The samples were centrifuged at 10,000*g* for 10 min at a temperature of 4 °C. The resultant supernatant was then separated into lipid subfractions by sequential centrifugation using density gradient solutions on the Beckman TLA 100.2 ultracentrifuge (Beckman Coulter, UK). Briefly, VLDL was first separated by overlaying 500 μL of sample with 500 μL of 1.006 g/mL density solution and centrifuged at 100,000*g* for 2.5 h at 25 °C. To isolate IDL, 500 μL of the remaining infranatant from the previous step was mixed with 40 μL of 1.182 g/mL density solution and overlaid with 460 μL of 1.019 g/mL density solution and centrifuged at 100,000*g* for 2.5 h at 25 °C. To isolate LDL, 500 μL of the remaining infranatant was mixed with 184 μL of 1.182 g/mL density solution and then overlaid with 316 μL of 1.063 g/mL of density solution. This was centrifuged as above. To isolate HDL2, 500 μL infranatant was mixed with 88 μL of 1.478 g/mL of density solution and then overlaid with 412 μL of 1.125 g/mL density solution, centrifuged at 53,000*g* for 18 h at 23 °C. Finally to isolate HDL3, 500 μL infranatant was mixed with 160 μL of 1.478 g/mL density solution and then overlaid with 340 μL of 1.21 g/mL of density solution. This was then centrifuged at 100,000*g* for 5 h at 23 °C. The remaining infranatant was deemed to be lipid depleted (LPDS), which was confirmed by the demonstration of negligible concentrations of lipids and apolipoproteins in them (Supplemental Table 1). At each of the above steps, 500 μL supernatant was stored at −80 °C for the subsequent extraction of RNA.

2.4. Nanoparticle tracking analysis to determine presence of extracellular vesicles

Given that a large percentage of the miRs studied were detected in the lipid-depleted subfraction (LPDS), we inferred that they contained extracellular vesicles. To confirm this we used nanoparticle tracking analysis (NTA) performed with a NanoSight LM10 instrument (NanoSight Ltd., Amesbury, UK). NTA captures real-time videos, showing particles moving by Brownian motion [19]. A 635 nm laser is used to illuminate nanoparticles in liquid suspension. The light scattered from the particles can be visualized using a ×20 objective lens of a light microscope that is attached to a camera running at 30 fps. The