Title:	Circulating microRNAs and hypertension – from new insights into blood pressure regulation to biomarkers of cardiovascular risk
Short Title:	Circulating microRNAs and hypertension
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Abstract

Hypertension is a leading cause of cardiovascular morbidity and mortality worldwide, yet the molecular mechanisms underpinning the development of high blood pressure remain incompletely understood. MicroRNAs are small, non-coding RNA molecules approximately 22 nucleotides in length that act as post-transcriptional regulators of gene expression. We highlight through a review of recent literature, that studies on circulating microRNAs have provided novel insights into blood pressure regulation. They have also complemented tissue- and animal-based experiments in shedding new light on our understanding of established pathways in hypertension, such as the renin-angiotensin system. Despite a number of challenges, we believe microRNAs herald particular potential in becoming effective biomarkers of target-organ damage in hypertension.

Highlights

- MicroRNAs have highlighted new regulatory aspects of blood pressure control
- Array-based studies identified many microRNAs that are dysregulated in hypertension
- Circulating microRNAs are excellent candidate biomarkers in hypertension
- MicroRNA biomarkers of target-organ damage show the greatest potential
- Technical challenges remain before microRNA biomarkers can enter clinical practice

Circulating microRNAs and hypertension – from new insights into blood pressure regulation to biomarkers of cardiovascular risk

Introduction

Hypertension is a leading cause of cardiovascular disease, including coronary artery disease, heart failure, chronic kidney disease, peripheral vascular disease, and stroke.[1] In the United States it affects over 30% of the population and for 2015, direct medical costs alone have been estimated at almost 100 billion dollars.[2] The pathophysiology of hypertension is complex and represents a combination of environmental and genetic factors. Unfortunately, despite considerable research, the molecular mechanisms remain incompletely understood. Furthermore, persistently elevated blood pressure manifests as pathological changes in organs throughout the body, known as 'target-organ damage'.[3]

MicroRNAs are small, non-coding RNA molecules approximately 22 nucleotides in length that act as post-transcriptional regulators of gene expression.[4] Commonly, although not exclusively, through binding to complimentary sequences in the 3' untranslated region (UTR) of target messenger RNA (mRNA), microRNAs induce translational repression or mRNA degradation, the exact mechanisms of which remain unclear.[5] A crucial feature of microRNA biology is that a single microRNA can target multiple genes, and a single gene can be targeted by multiple microRNAs. Thus, microRNAs represent a complex regulatory network that controls both physiological and pathological processes fundamental to a wide variety of cardiovascular diseases, including hypertension.[5,6] In this review, we focus on circulating microRNAs as emerging biomarkers in essential hypertension, as well as their ability to provide novel insights into pathology, and to identify targets for novel therapeutic agents; studies relating to gestational hypertension and preeclampsia were not considered.

Circulating microRNAs

In 2008, microRNAs were found in human serum and plasma.[7,8] They have subsequently been detected in a wide range of bio-fluids, including urine, saliva and cerebrospinal fluid.[9] These circulating microRNAs have a number of characteristics that make them attractive targets for biomedical research – they are remarkably stable in the blood, appear in concentrations measurable by current techniques and often show tissue specific expression; furthermore, they are likely to become dysregulated prior to physical symptoms of disease becoming present.[10] These factors, paired with limited availability and difficulty in obtaining human tissue samples, have meant that circulating microRNAs studies (rather than tissue microRNAs) have predominated in the search for microRNA-based biomarkers in hypertension. The two most common approaches utilised in this search are comparisons of microRNA expression in hypertensive versus normotensive individuals

using either unbiased screening with microarrays (see Table 1) or focussed investigations employing candidate microRNAs and quantitative polymerase chain reaction (qPCR; see Table 2).

Circulating microRNAs and novel mechanisms of blood pressure regulation

One of the greatest advantages of array-based techniques is that they can simultaneously assess all currently known microRNAs in the human genome (the miRNome). Consequently, they do not require an *a priori* hypothesis and thus have the potential to identify previously unknown players in the development of hypertension. This was first employed by Li *et al*. to identify a novel link between human cytomegalovirus (HCMV) and essential hypertension.[11] By screening 1700 microRNAs in the plasma of 13 hypertensive patients and five control participants, they identified 27 microRNAs that were differentially expressed, of which 14 were validation using qPCR (see Table 1).[11] After validation in larger cohorts, they were able to confirm that expression of an HCMV-encoded microRNA (hmcv-miR-UL112) was 2.5 times greater in hypertensive patients than healthy controls and demonstrate that HCMV seropositivity, viral copies and hmcv-miR-UL122 expression were all independently associated with an increased risk of hypertension.[11] Finally, they identified interferon regulatory factor 1 (IRF1) as a direct target of hmcv-miR-UL112, thereby identifying a potential regulator of blood pressure. Whilst the above finding has yet to be fully validated, it nicely illustrates how microRNA 'screening' can offer new insights into the biology and pathology of hypertension.

More recently, Yang *et al*. used a microarray to screen approximately 1350 microRNAs in six hypertensive and six healthy individuals.[12] They identified increased expression of miR-210, miR-425 and miR-505 in hypertensives compared to controls; the finding for miR-505 was replicated in two larger validation cohorts. Interestingly, a non-significant trend towards increased expression in pre-hypertensives vs. controls was also reported. This suggests that miR-505 could represent a biomarker of pre-hypertension, as well as established hypertension. Yang *et al*. also undertook further *in silico* and *in vitro* analyses to demonstrate that increased miR-505 levels impaired migration and tube formation in cultured endothelial cells, possibly through regulation of FGF18 and HMGB1, suggesting a role for miR-505 in angiogenesis.

Karolina *et al*. used the same microarray methodology and qPCR validation to identify 15 microRNAs that were differentially expressed in the blood of normotensive controls (N = 29) compared with both hypertensives (N = 16) and those with metabolic syndrome (N = 32; see Table 1).[13] Whilst a cluster of three microRNAs (miR-92a, miR-130a and miR-195) were reported to be uniquely dysregulated in hypertension and metabolic syndrome but not type 2 diabetes mellitus (T2DM) or hypercholesterolaemia, the study highlights the significant interplay in the pathology of these

conditions. Whilst this makes identifying microRNAs that are dysregulated specifically in hypertension challenging, it raises the potential for microRNA-based therapeutics to target multiple cardiovascular pathologies. It is also noteworthy that miR-92a was found to be present (and differentially expressed) in exosomes (isolated from serum),[13] which have been postulated to play a key role in facilitating microRNA-mediated cell-to-cell communication.[14,15] Pertinently, miR-92a was predicted to target *AGTR1*, the gene encoding the angiotensin II type I receptor (AT_1R) – a key component of the renin-angiotensin system. Unfortunately, no *in vitro* validation was performed.

Circulating microRNAs and existing mechanisms of blood pressure regulation

In contrast to the above unbiased screens, many studies of circulating microRNAs have targeting microRNAs already proposed to play a role in hypertension, usually through tissue-based studies, in order to provide a new perspective. In an illustration of this, our group utilised circulating miR-181a to gain a deeper understanding of its association with blood pressure.[16] In 2011, Marques *et al*. identified differential expression of miR-181a in kidneys of hypertensive and normotensive individuals.[17] Importantly, binding of miR-181a to the 3'UTR of the renin gene was demonstrated using luciferase reporter assays transfected into HEK293 kidney cells. This clearly explains how miR-181a may lead to decreased renin mRNA levels. More recently, we demonstrated that in two independent cohorts, serum levels of miR-181a correlated with systolic blood pressure.[16] Interestingly however, this association was opposite to that expected – increased miR-181a levels were associated with elevated blood pressure. Furthermore, this association was independent of circulating renin levels, suggesting that the effects of miR-181a may be mediated by mechanisms other than renin inhibition. Indeed, we utilised RNA-sequencing data to explore the signature of miR-181a on the global renal transcriptome. This analysis implicated miR-181a in pathways associated with mitochondrial respiratory function, immunity and inflammation.[16]

In a similar approach, the Vardas laboratory focussed on microRNAs linked to vascular smooth muscle modulation,[18] hypertrophy and endothelial dysfunction[19] in animal and tissue models. Across both studies, which employed the same participants, the group showed increased expression of miR-1 and miR-21 and decreased expression of miR-9, miR-126, miR-133, miR-143 and miR-145 in peripheral blood mononuclear cells (PBMCs) of hypertensive individuals versus healthy controls.[18,19] Amongst hypertensive individuals, miR-21, miR-143, and miR-145 negatively correlated with 24-hour ambulatory mean blood pressure, mean DBP and mean pulse pressure; miR-133 positively correlated with each of these measures.[18] Whilst associations for 24-hour mean blood pressure and mean DBP were not provided for miR-9 or miR-126, both correlated positively

with mean pulse pressure.[19] Finally, miR-9 (but not miR-126) also correlated positively with left ventricular mass index.[19]

Elsewhere, Ceolotto *et al*. [20] measured miR-155 levels in PBMCs of 64 young (18-45 years) hypertensive individuals, selected according to genotype, to further elucidate the mechanisms underpinning the well-demonstrated association between the +1166A>C SNP in the 3' UTR of *AGTR1* and hypertension.[21] Finally, Cengiz *et al*. measured circulating microRNAs previously linked to hypertension in an attempt to provide a new perspective on the pathogenesis of 'white-coat hypertension' (WCH) and how it relates to established hypertension.[22] They identified let-7e, miR-21 and miR-296-5p as differentially expressed between those with hypertension and WCH. However, whilst some of the microRNAs previously shown to be up-regulated in hypertensive versus normotensives were replicated (let-7e, miR-21, miR-122, miR-296-5p, miR-637), others were not (miR-125a, miR-126, miR-130a, miR-155, miR-195).[22]

Circulating microRNAs as biomarkers of target-organ damage

The potential for microRNAs to become biomarkers for target-organ damage has recently received increasing attention.[3] In an excellent example of this, Kontaraki *et al*. focussed on left ventricular hypertrophy (LVH), a known consequence of persistently elevated blood pressure and example of subclinical organ damage.[23] After identifying six microRNAs shown to have either hypertrophic (miR-21, miR-208b, miR-499) or anti-hypertrophic (miR-1, miR-26b, miR-133a) effects in animal models, they measured their levels in PBMCs of either healthy or hypertensive individuals. Statistically significant increased expression of miR-1, miR-21, miR-208b and miR-499 and decreased expression of miR-26b and miR-133a were observed. Additionally, amongst hypertensive individuals, miR-1 and miR-133 negatively correlated with left ventricle (LV) mass index, whereas miR-miR-21, miR-26b, miR-208b, and miR-499 correlated positively with LV mass index. Importantly, these correlations remained significant after correction for 24-hour systolic or diastolic blood pressure.[23] Whilst further validation is clearly required, these findings suggest that microRNAs (or panels of microRNAs) herald great potential to identify target-organ damage in hypertension, independent of blood pressure.

Similarly, Dickinson *et al.* used a salt-sensitive rat model to identify six microRNAs (miR-16, miR-20b, miR-93, miR-106b, miR-223, and miR-423-5p) that were up-regulated in response to hypertension-induced heart failure.[24] Interestingly, it was also noted that these microRNAs increased during disease progression and were reduced in response to treatment with ACE inhibitor.[24] Whilst the limitations of an animal model must be accepted, this work highlights great potential for microRNAs

to not only act as biomarkers of target-organ damage, but also of disease progression and response to treatment. More recently, Sanchez-de-la-Torre *et al*. screened 84 'cardiovascular' microRNAs to identify a panel of three plasma microRNAs whose pre-treatment levels could accurately predict blood pressure lowering response to continuous positive airway pressure (CPAP) in patients with resistant hypertension and obstructive sleep apnoea.[25] After identification of eight microRNAs that were differentially expressed in a discovery phase, a training set was used to identify the three microRNAs with the highest statistical associations (miR-378a-3p, miR-486-5p, miR-100-5p) – these were added to a logistic regression model that was shown to have excellent discriminatory power in a validation phase (ROC AUC = 0.92 [95% CI = 0.79 to 1.00]). Whilst clearly in its infancy, these studies provide early evidence that microRNAs have potential utility as predictors of treatment response in hypertension.

Challenges and limitations

Despite this early promise, there are a number of challenges and limitations to measuring the circulating microRNAs in hypertension. A number of technical issues remain, including difficulties in measuring absolute levels of microRNAs – at present, relative levels vs. 'controls' are often used. Additionally, there is a lack of standardised protocols for quantification, significant intra- and inter-laboratory variation and no established endogenous 'normalisation' controls.[26] From a pathophysiological perspective, a significant challenge exists in determining the biological relevance of circulating microRNAs. Whilst initially postulated simply to be reflective of disease states in tissues, there is mounting evidence that microRNAs may play a key role in cell-to-cell communication.[14,15] In hypertension, unbiased screens have identified a large number of microRNAs that appear dysregulated in hypertension, but these frequently vary between studies and lack validation – indeed, only a few microRNAs have been replicated across multiple studies (see Figure 1). There are many reasons why this may be the case: firstly, it could well be a reflection of the highly complex nature of hypertension and its multifactorial aetiology; secondly, and similarly, it could result from the wide-ranging clinical manifestations of persistent hypertension in multiple organs; thirdly, and perhaps more simply, these differences may be explained by inter-study variation of populations and methodologies or by the frequently small sample sizes employed. At present, this is likely a result of the high cost of microRNA-arrays. However, we hope that in time, much like with genotyping arrays, if the cost of these begins to fall sample sizes will increase.

Conclusion

In the relatively short time since their discovery, microRNAs have already shown great promise as circulating biomarkers. Although other cardiovascular disorders such as acute myocardial infarction

and heart failure have received much greater attention,[5] biomarkers of hypertension-induced target-organ damage appear particularly promising. Whilst there remains much work to do before such biomarkers are ready for clinical practice, particularly robust validation, by providing insights into novel regulatory processes in the pathogenesis of hypertension, the seeds have been sown for microRNAs to play a key role in the development of innovative treatments of hypertension.

Table 1: Microarray studies comparing circulating microRNAs in hypertensive and normotensive individuals

	Study Phase	Study Population	Source	Technique	Notable MicroRNAs	Summary Results
Li *et al*. [11]	Discovery	HC = 5; HTN = 13	Plasma	Microarray & qPCR	↑ = hcmv-miR-UL122, let-7e, miR-516b, miR-600, miR-605, miR-623 ↓ = miR-18b, miR-30d, miR-296-5p, miR-324-3p, miR-486-5p, miR-518b, miR-1227, miR-1236	In a microarray screen (N = 1700) of 13 hypertensive vs. five control participants, 46 microRNAs were found to be differentially expressed. Of these, 27 were present in miRbase and 14 were successfully validated (in the same cohort) using qPCR.
	Validation	HC = 67; HTN = 127	Plasma	qPCR	↑ = hcmv-miR-UL122, let-7e ↓ = miR-296-5p	Three microRNAs (hcmv-miR-UL122, let-7e, miR-296-5p) were validated in a much larger cohort.
Karolina *et al*. [13]	Discovery	HC = 17; HTN = 14; MS = 18; T2DM = 21; HCL = 41	Blood	Microarray	↑ = miR-92a, miR-103, miR-130a, miR-195 ↓ = miR-150, miR-17, miR-183, miR-192, miR-197, miR-23a, miR-27a, miR-320a, miR-509-5p, miR-584, miR-652	In a microRNA screen (N=1769), 19 microRNAs were found to be dysregulated in MS and one of T2DM, HCL or HTN.
	Validation	HC = 29; HTN = 16; MS = 32; T2DM = 29; HCL = 48	Blood	qPCR	↑ = miR-92a, miR-103, miR-130a, miR-195 ↓ = miR-150, miR-17, miR-183, miR-192, miR-197, miR-23a, miR-27a, miR-320a, miR-509-5p, miR-584, miR-65	In a qPCR validation study, 15 of these microRNAs were replicated, all of which showed differential expression between healthy controls and hypertension in univariate analyses. Following cluster analysis, miR-92a, miR-130a, and miR-195 clustered uniquely with MS and HTN.
Yang *et al*. [12]	Discovery	HC = 6; HTN = 6	Plasma	Microarray	↑ = miR-210, miR-425, miR-505	In a microarray screen (N = 1347) of six hypertensive vs. six control participants, miR-210, miR-425, and miR-505 showed greater expression in hypertensives.
	Validation 1	HC = 11; PH = 20; HTN = 19	Plasma	qPCR	↑ = miR-505, miR-210	In a validation cohort, increases in miR-505 in hypertensives and pre-hypertensives were observed compared to controls; a non-significant trend was observed for miR-210.
	Validation 2	HC = 91; HTN = 101	Plasma	qPCR	↑ = miR-505	In a second validation cohort, a 2.09 fold increase in plasma miR-505 was observed in hypertensives vs. healthy controls.

HC = healthy control; HCL = hypercholesterolaemia; HTN = hypertension; MS = metabolic syndrome; N = number; PH = pre-hypertension; qPCR = quantitative polymerase chain reaction; T2DM = type 2 diabetes mellitus

Table 2: Candidate-based studies comparing circulating microRNAs in hypertensive and normotensive individuals

Study	Study Population	Source	Technique	Notable MicroRNAs	Summary Results
Kontaraki *et al*. [18]	HC = 29; HTN = 60	PBMC	qPCR	↑ = miR-1, miR-21 ↓ = miR-133, miR-143, miR-145	Amongst hypertensive individuals, miR-21, miR-143, and miR-145 negatively correlated with 24-hour mean blood pressure, mean DBP and mean pulse pressure; miR-133 positively correlated with 24-hour mean blood pressure, mean DBP and mean pulse pressure.
Mandraffino *et al*. [27]	HC = 64; HTN & AS = 53; HTN & LVH = 41	CD34+ CPC	qPCR	↑ = miR-221, miR-222	miR-221 and miR-222 expression was higher in hypertensives than controls and higher in hypertensives with LVH than hypertensives with AS.
Kontaraki *et al*. [19]	HC = 29; HTN = 60	PBMC	qPCR	↓ = miR-9, miR-126	Amongst hypertensive individuals, miR-9 and miR-126 correlated positively with 24-hour mean ambulatory pulse pressure. Additionally, miR-9 (but not miR-126) correlated positively with LV mass index.
Park *et al*. [28]	HC = 13; HTN = 13; CA = 11; ARAS = 13	Plasma	qPCR	↑ = miR-126, miR-155, miR-210 ↔ = miR-21, miR-124a	Plasma levels of miR-126, miR-155 and miR-210 were elevated in those with EH vs. HC in unadjusted analyses. However, only miR-126 remained differentially expressed after correction for eGFR. None of the examined miRs correlated with SBP or DBP in a group containing HC, EH, CA and ARAS patients.
Cengiz *et al*. [22]	HC = 30; HTN = 30; WCH = 30	Plasma	qPCR	↑ = let-7e, miR-21, miR-122, miR-637 ↔ = miR-125a, miR-126, miR-130a, miR-155, miR-195 ↓ = miR-296-5p	miR-122, miR-296-5p and miR-637 were also up-regulated in WCH vs. HC; miR-21, miR-122, miR-637 and let-7e were positively correlated with clinic SBP. miR-21 and let-7e correlated positively with ambulatory daytime and night-time SBP; miR-296-5p correlated negatively with 24-hour SBP and DBP.
Cengiz *et al*. [29]	HC = 28; HTN = 28	Plasma	qPCR	↑ = miR-21	Plasma miR-21 expression was significantly higher in HTN vs. HC, correlated positively with clinic SBP and DBP and also CIMT. There was no correlation with ABPM.
Kontaraki *et al*. [23]	HC = 30; HTN = 102	PBMC	qPCR	↑ = miR-1, miR-21, miR-208b, miR-499 ↓ = miR-26b, miR-133a	Amongst hypertensive individuals, miR-1 and miR-133 negatively correlated with LV mass index, whereas miR-21, miR-26b, miR-208b, and miR-499 correlated positively with LV mass index
Marques *et al*. [16]	TRANSLATE: NIRC = 200	Serum	qPCR	↑ = miR-181a	miR-181a expression was positively associated with SBP and DBP in multiple linear regression models. The associations of miR-181a with SBP and DBP remained after correction for circulating levels of renin.
	GRAPHIC: HC = 100; HTN = 99	Serum	qPCR	↑ = miR-181a	miR-181a expression was positively associated with SBP and showed a non-significant trend towards a positive association with DBP in multiple linear regression models.

ABPM = ambulatory blood pressure monitoring; ARAS = atherosclerotic renal artery stenosis; AS = arterial stiffening; CA = coronary atherosclerosis; CIMT = carotid intima-media thickening; CPC = circulating progenitor cells; DBP = diastolic blood pressure; eGFR = estimated glomerular filtration rate; EH = essential hypertension; HC = healthy control; HTN = hypertension; LV = left ventricle; LVH = left ventricular hypertrophy; N = number; NIRC = non-invasive renal cancer; PBMC = peripheral blood mononuclear cells; qPCR = quantitative polymerase chain reaction; SBP = systolic blood pressure; WCH = white coat hypertension

Figure

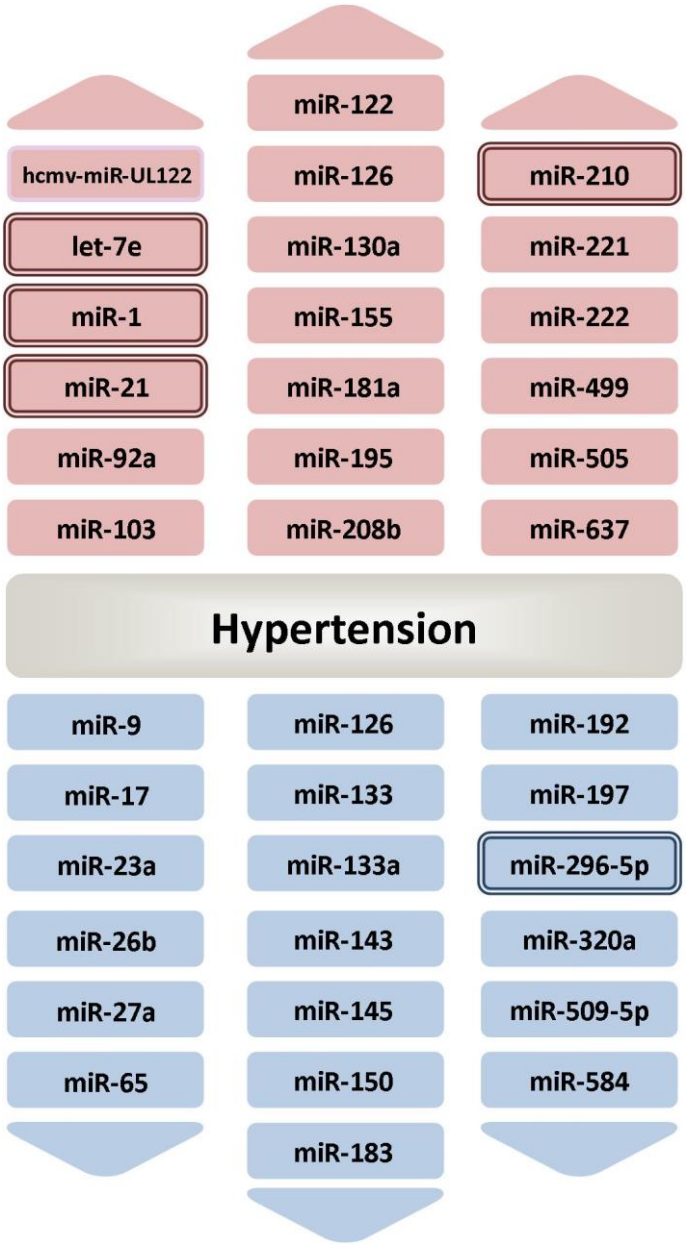

Figure 1: Circulating microRNAs with increased (red) or decreased (blue) expression in studies of human hypertension

MicroRNAs with a double border have been linked to hypertension by more than one study – for details see Tables 1 and 2.

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Authorship statement

The topic for the review was proposed by the Editors. SPRR performed the literature search and drafted the manuscript, which was revised by MT. Additional revisions were made by FJC and NJS. All authors approved the final version for publication.

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