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ORIGINAL ARTICLE

Expression and methylation of circulating microRNA-510 in essential hypertension

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Hypertension (HTN) is one of the most common emerging disease in developing countries. It alters endothelial cell structure and function, resulting in several diseases, such as cardiovascular disease, peripheral vasculopathy, cerebrovascular disease and nephropathy. Although much progress has been made in researching HTN in recent years, early diagnosis and treatment of HTN are not yet satisfactory, and progression control/treatment is still poor. MicroRNAs are well-known regulators of the physiological and developmental processes of HTN. Our results revealed that miR-510 was upregulated in blood samples from HTN patients, whereas no significant differences were observed in the control samples. Methylation analyses corroborated the miR-510 upregulation in patient samples. These results suggested that miR-510 can be used as a novel biomarker for diagnosis and as a new therapeutic target for HTN.
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INTRODUCTION

Hypertension (HTN) is one of the most common noncommunicable diseases worldwide and is becoming increasingly common in developing countries, such as India. It results in a substantial public health burden on cardiovascular health and health-care systems in most countries.[1] HTN is responsible for 57% of all stroke deaths and 24% of all coronary heart disease deaths in India.[2] Interestingly, the World Health Organization stated that HTN is one of the most significant causes of premature death worldwide.[3] HTN has caused ~7.5 million deaths, which accounts for 12.8% of the global mortality, and 57 million disability-adjusted life years or 3.7% of total disability-adjusted life years have been attributed to HTN.[4] HTN can be inhibited by lifestyle modification and medical involvement. Moreover, screening and early management of HTN through periodic surveillance will decrease disease progression.[5]

The molecular mechanisms underlying the genetic predisposition to HTN have been extensively researched. The protein-coding regions of the genome account for <2% of the entire human DNA, suggesting that genetic mechanisms besides protein-coding genes are likely to contribute to HTN regulation.[6]

MicroRNAs (miRNAs) or circulating miRNAs are important post-transcriptional regulators of gene expression in many types of diseases and they are associated with various diseases.[7,8] Recent studies have shown that miR-9 and miR-126 are strongly related to essential HTN in humans, suggesting that they may be potential biomarkers and candidate therapeutic targets for essential HTN.[9] A recent study showed that miR-510 is involved in cell proliferation, migration and apoptosis,[10] and it is a major regulatory circuit in cells.[11] Interestingly, another study revealed that miR-510 directly binds to the 3′UTR of peroxiredoxin 1 and blocks its protein expression, thereby suppressing migration of human breast cancer cells.[12]

Several studies have examined miR-510 in other diseases, such as cancer, but there is no evidence showing that miR-510 is involved in HTN. MiRNA silencing via methylation of CpG islands may be an important mechanism for disease progression.[13] Although DNA methylation is an important mechanism for miRNA upregulation, this has not been explored in HTN. Thus, our study predominantly focused on epigenetic regulation and expression of circulating miR-510 in HTN, which could be useful for elucidating the molecular mechanism of miR-510 in HTN and its role in disease progression. Circulating miRNAs are known to be an important factor involved in the initiation and progression of HTN. On the basis of the above information, in the present study, we studied the mechanism of circulating miR-510 in blood samples from HTN patients, and we found that miR-510 was a diagnostic and prognostic marker for HTN.

MATERIALS AND METHODS

Clinical samples

A total of 428 genetically unrelated patients were selected and divided into two groups. Group I consisted of 208 essential hypertensive patients and Group II consisted of 220 healthy volunteers (age- and sex-matched controls) who were randomly selected. Data collected from each subject included age, height,

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weight, body mass index and family history. HTN was defined as a systolic blood pressure >140 mm Hg and sustained diastolic blood pressure >90 mm Hg. Blood pressure was measured in subjects who were seated and resting for 5 min, and was taken twice to calculate the mean systolic blood pressure and diastolic blood pressure. No patients were receiving antihypertensive drugs, and patients with secondary HTN, renal, liver and cardiac abnormalities were excluded from the present study.

Venous blood samples (5 ml) were collected from each subject for the analyses. A portion of each blood sample was stored in an EDTA tube (Becton Dickson, New Jersy, NJ, USA) for genomic DNA extraction, and the remaining sample was left to clot to obtain serum and stored at −20 °C for further analysis. Serum samples were analyzed by a HUMASTAR-300 autoanalyser (HUMAN Gesellschaft für Biochemica und Diagnostica mbH, Wiesbaden, Germany) using kits supplied by Human Diagnostics GmbH (HUMAN Gesellschaft für Biochemica und Diagnostica mbH) to determine the levels of fasting blood glucose, serum urea, creatinine, total cholesterol, high-density lipoprotein cholesterol and triglycerides. Low-density lipoprotein cholesterol was calculated by the Friedewald formula. The Third Report of the National Cholesterol Education Program guidelines (NCEP report 2001) were followed for the classification of lipid profiles. Serum sodium, potassium and chloride levels were determined using a HUMLYTE electrolyte analyzer (ion-selective electrode method, HUMAN Gesellschaft für Biochemica und Diagnostica mbH).

RNA isolation and quantification of miRNAs

Total RNA was extracted using a RNeasy Mini Kit (Qiagen, New Delhi, India), and the expression pattern of miR-510 was analyzed as described by Balcells *et al.*,[14] where poly (A) tailing of the miRNA was followed by reverse transcription with a tagged poly (T) primer. Quantification of miRNAs was performed by PCR using the following primers: forward: 5′-AGTATGGCCCG GCCGTGA-3′; reverse: 5′-AGGTCCATTTTTTTTTTTTTTTCCT-3′. miR-510 levels were normalized to 18s RNA levels using the $2^{\Delta\Delta Ct}$ model.

Bisulfite conversion and methylation analysis

DNA and bisulfite-treated DNA were extracted from HTN blood samples and normal blood samples using a QIAamp Mini Kit (Qiagen) as described previously.[15] Briefly, DNA was treated with bisulfite using the EZ DNA Methylation Gold Kit (Zymo Research, Chennai, India) following the manufacturer's instructions.

DNA methylation analysis

Methylation status of the miR-510 promoter was detected using the methods described in the study by Vogelsang *et al.*,[15] which consisted of a two-stage methylation-specific PCR (MSP) or nested MSP that involved a first-round amplification using primers unbiased toward the methylation status of the analyzed region followed by conventional MSP. During the first round, the amplification primers MSP-miR-510-fw (5′-ACA ATCACT CTT TCC ATA TAC-3′) and MSP-miR-510-rv (5′-GCC CAG TCA CAT CTA GTT-3′) were used to amplify a 562 bp fragment including a portion of CpG-rich promoter region from bisulfite-treated DNA templates. We used the amplification protocol and MSP analysis described in the study by Vogelsang *et al.*[15] Specific primer sets to selectively amplify unmethylated and methylated alleles were designed according to previous references as follows:[16] miR-510-U-fw 5′-ATC TCG TTT TTA TTT AAA CGT-3′, miR-510-U-rv 5′-AGT GAC TCA CCCTAT TCT CTG ATG-3′ (unmethylated amplicon size: 144 bp) and miR-510-M-fw 5′-ACA AAA GCC GCC TAT CCC TGT CCC CTA-3′, miR-510-M-rv 5′-TTA GAA TGG ACC TCT AAACTC AAA-3′ (methylated amplicon size: 147 bp).

Table 1 Baseline characteristics of the study population

Variable	*Hypertension (n = 20)*	*Controls (n = 20)*
Age (years)	42.6 ± 5.2	45.89 ± 5.9
Gender (male/female)	12/8	13/7
BMI (kg m^{-2})	25.12 ± 3.1	24.3 ± 4.0
SBP (mm Hg)	150 ± 9.7[a]	129.34 ± 1.9
DBP (mm Hg)	91.56 ± 8.6[a]	80.89 ± 5.0
BGL (mg dl^{-1})	92 ± 6[a]	85 ± 3
Serum urea (mg dl^{-1})	21.22 ± 4.11	25.8 ± 4.7
Serum creatinine (mg dl^{-1})	0.96 ± 0.21	0.99 ± 0.24
Serum sodium (mmol l^{-1})	141.1 ± 3.1[a]	139.54 ± 2.61
Serum potassium (mmol l^{-1})	3.62 ± 0.21	4.76 ± 0.31
Serum chloride (mmol l^{-1})	88.32 ± 4.21	89.63 ± 3.2
Total cholesterol (mg dl^{-1})	205 ± 4.5[a]	159 ± 6.9
LDL cholesterol (mg dl^{-1})	124.19 ± 6.2[a]	89 ± 4.6
HDL cholesterol (mg dl^{-1})	39.21 ± 4.2[a]	55 ± 3.4
Triglycerides (mg dl^{-1})	169 ± 8.2[a]	136 ± 6.9

Abbreviations: BGL, blood glucose level; BMI, body mass index; DBP, diastolic blood pressure; HDL, high-density lipoprotein; LDL, low-density lipoprotein; SBP, systolic blood pressure.
[a]Unpaired *t*-test data, *P*-value <0.005 highly significant.

Statistical methods

All clinical data are expressed as the mean ± s.d. All statistical analyses were carried out using SPSS software version 15.0 for Microsoft Windows (SPSS software, IBM, India). Continuous variables were compared between the cases and control groups using two-tailed Student's *t*-tests. $P<0.05$ was considered statistically significant.

RESULTS AND DISCUSSION

In general, miRNAs control a wide range of biological functions, such as proliferation, differentiation, apoptosis and other gene functions.[17–19] Recent reports have shown that miRNAs can act as a repressor or inducer, and have key roles in the initiation and progression of HTN.[9] MiRNAs have been detected in body fluids, such as serum, plasma and urine, and can be readily used as noninvasive biomarkers.[20] Our study group included 20 essential HTN patients and 20 controls. Table 1 shows the baseline demographic, clinical and laboratory data of the study population. Among the patients, 12 (58.2%) were men and 8 (41.8%) were women. There was no significant difference between patients and controls with respect to age and body mass index ($P>0.005$). Salt-sensitive-hydroxylase (SBH) and dopamine beta-hydroxylase (DBH) were higher in patients compared with controls ($P=<0.005$).

Serum total cholesterol, HDL, LDL, very low density lipoprotein (VLDL) and triglyceride levels were significantly higher in patients than the controls ($P=<0.005$). Blood glucose and serum sodium levels also showed differences between patients and controls ($P=<0.005$). There were no significant differences between patients and controls with respect to serum creatinine, urea, potassium and chloride ($P=>0.005$). To elucidate the mechanisms underlying abnormal miRNA expression in HTN, an increasing number of

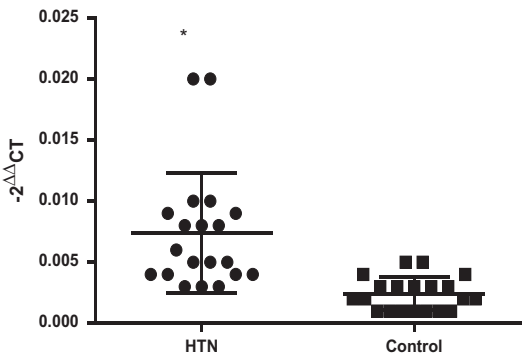

Figure 1 Expression level of circulating miR-510 in blood samples from hypertension patients (HTN) by quantitative real-time PCR. Unpaired *t*-tests were used to assess the data. *P-value <0.005 *vs.* control samples.

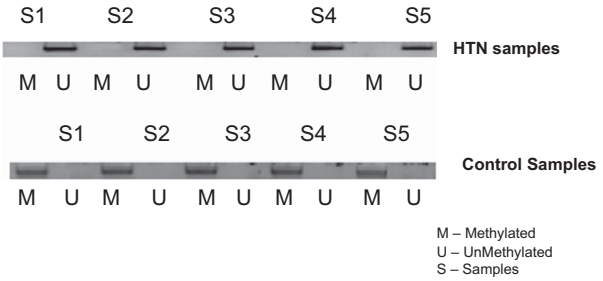

Figure 2 The methylation status of the miR-510 promoter region in hypertension (HTN) samples and the control samples by methylation-sensitive PCR (MSP).

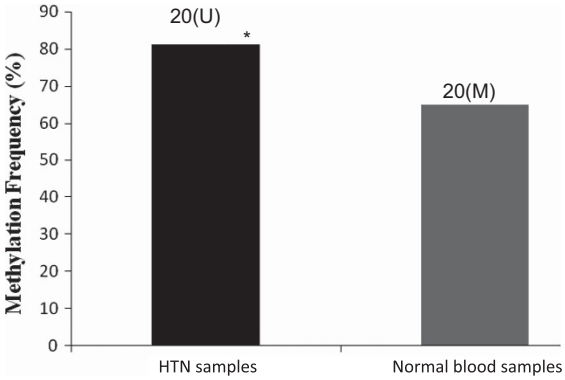

Figure 3 Methylation frequency for the miR-510 promoter was compared between hypertension (HTN) blood and normal blood samples. The number of methylated (M) and unmethylated (U) samples is indicated above the bars. Unpaired *t*-tests were used to assess the data. **P*-value <0.005 *vs.* control.

studies have investigated how miRNAs are regulated, and it is now widely accepted that miRNAs undergo the same regulatory mechanisms as any other classical protein-coding genes, including epigenetic regulation. We hypothesized that epigenetic modification of genes encoding the miRNAs is a key factor in inducing HTN, and we assessed this hypothesis in detail. To test this hypothesis, we examined the expression of mature miR-510 in blood samples from HTN patients and control samples. Our results suggested that the miR-510 level in blood samples from HTN patients was significantly higher than that in the normal control samples (Figure 1).

The expression of miR-510 in patient blood samples suggested that the upregulation of miR-510 is relevant to the initiation and development of HTN. On the basis of the results from the gene expression study, we assessed the molecular mechanisms underlying the upregulation of miR-510. A recent study by Formosa *et al.*[13] reported the role of the methylation status of CpG islands upstream of a subset of miRNAs using MSP. Additional reports also have demonstrated that the epigenetic regulation of miRNAs is a widespread phenomenon, as shown, for example, for miR-9-1, miR-107, miR-127, miR-193a, miR-137, miR-342, miR-203, miR-34b/c and miR-1.[21,22]

We used MSP or nested MSP to investigate the status of miR-510 promoter methylation in HTN blood and normal blood samples. Our hypermethylation study revealed that the miR-510 promoter was unmethylated in HTN blood samples, but in the control sample, it was found to be methylated. Representative examples of MSP analyses are shown in Figure 2. The methylation frequency for the miR-510 promoter was compared between the HTN blood and normal blood samples (Figure 3). Overall, we found that the methylation frequency was significantly different between these samples (81.0 *vs.* 65.0%, $P=0.005$). Our data suggested that hypermethylation is corroborated with miR-510 expression in blood samples. In this study, we found that miR-510 was unmethylated in hypertensive patient blood samples, whereas in control samples, their expression levels significantly correlated with methylation status of the miR-510 promoter. Moreover, this is the first report to show the methylation of the promoter of circulating miR-510, and further investigations are needed to confirm that miR-510 is a potential circulatory miRNA for HTN.

CONFLICT OF INTEREST

The authors declare no conflict of interest.

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