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Original Article

Roles of lncRNA H19 and MALAT1 as biomarkers in patients with white-coat hypertension

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Abstract: Background and aim: White-coat hypertension (WCH), characterized by elevated clinic blood pressure (BP) but normal home or ambulatory BP, is a clinical observation that requires recognition and timely diagnostic follow-up. Until now, the role of long non-coding RNAs (lncRNAs) in WCH has been unclear. In this study, we have identified deregulated lncRNAs in patients with WCH and assessed their diagnostic value. Methods: The expression levels of lncRNAs in subjects with WCH (n=35) as well as those with hypertension (HT; n=35) and normotension (NT) were acquired by microarray analysis and validated by quantitative PCR. The relationship between the lncRNA expression and clinical BP or 24-hour ambulatory blood pressure monitoring (ABPM) was analyzed using the Pearson correlation test. Receiver operating characteristic analysis was performed to assess the diagnostic value of the selected lncRNAs. Results: Microarray data revealed 29 aberrantly expressed lncRNAs between the groups. Among these, *H19* and *MALAT1* were markedly upregulated in the WCH group compared to those in the NT and HT groups ($P<0.01$). The expression of circulating *H19* and *MALAT1* was negatively associated with ABPM, whereas there was no rectilinear correlation between their expression levels and clinical BP. The values for area under the curve (AUC) (95% CI) for *H19* and *MALAT1* were 0.76 (0.65-0.86) and 0.80 (0.69-0.89), respectively, for distinguishing WCH from HT. Conclusion: The expression levels of *H19* and *MALAT1* were significantly increased in subjects with WCH compared to those with NT and HT, suggesting their potential role as novel noninvasive biomarkers.

Keywords: White-coat hypertension, diagnosis, lncRNAs

Introduction

Hypertension (HT) is the most important risk factor for death and disability worldwide, affecting more than one billion individuals and causing an estimated 9.4 million deaths every year [1]. Chronic exposure to increased blood pressure (BP) induces several changes in the structure and function of tissues and organ systems [2], and under these circumstances, medical treatment should be provided without delay [3]. Hypertension is defined as systolic blood pressure (SBP) ≥140 mmHg and/or diastolic blood pressure (DBP) ≥90 mmHg, which are conventionally measured at the doctor's office. On the other hand, guidelines recommend ruling out white-coat hypertension (WCH) before starting antihypertensive treatment [4]. WCH is defined by the occurrence of elevated BP at the doctor's office and low ambulatory or home BP, and is widely regarded as a condition reflecting a pronounced pressor response to BP measurement in the clinic in normotensive (NT) individuals [5]. However, it remains controversial as to whether WCH has the same cardiovascular risk as sustained HT [6-8]. Since the use of 24-hour ambulatory blood pressure monitoring (ABPM) is limited in the clinic environment, it would be useful to find new noninvasive biomarkers that could distinguish WCH from sustained HT.

Long non-coding RNAs (lncRNAs) are transcribed RNA molecules >200 nucleotides in length, that have no protein-coding potential [9]. Accumulating evidence indicates that lncRNAs play important roles in various physiological processes, including gene imprinting, modulation of apoptosis and invasion, cell differentiation, and organogenesis [10-12]. Recent studies have indicated that lncRNAs also function in embryonic heart development as well as the development of heart disease [13-15]. LncRNAs are measurable in blood and urine, which makes them ideal potential diagnostic and prognostic biomarkers for diseases such as heart failure and myocardial infarction

[16] and myocardial infarction [17]. However, whether circulating lncRNAs could become promising biomarkers for the diagnosis of WCH or essential HT remains elusive.

This study indicates the feasibility of lncRNA expression quantification in the peripheral-blood mononuclear cells (PBMCs) of patients with elevated clinical BP, and shows that the lncRNAs *H19* and *MALAT1* could serve as novel noninvasive diagnostic biomarkers for differentiating WCH from HT. Aberrantly expressed lncRNAs in the PBMCs of subjects were determined using microarray analysis and were subsequently validated by quantitative PCR (qPCR). LncRNAs, *H19* and *MALAT1*, which were previously demonstrated to have cardiac disease involvement, were explored as candidate circulating biomarkers for the diagnosis of WCH by performing receiver operating characteristic (ROC) analysis.

Materials and methods

Study population

From 2013 to 2015, 35 untreated patients with WCH (23 males, 12 females; Age 44.2±8.4 years) and 35 newly diagnosed and untreated patients with HT (25 males, 10 females; Age 45.3±5.6 years) as well as 35 NT volunteers (25 males, 10 females; Age 46.1±4.7 years) were recruited from Gansu Provincial Hospital. Subjects were instructed to sit in a quiet room for at least 5 min prior to checking their blood pressure, which was taken using a mercury sphygmomanometer on three occasions within a span of five days. Home or office BP measurements were taken twice in the morning and twice in the evening for five to seven consecutive days [18]. The aveage of the three measurements was calculated as the SBP and DBP. ABPM (TM-2430, A&D Medical, Japan) was then performed on the left arm. Daytime BP was recorded at 20-min intervals from 8:00 AM to 10:00 PM and was considered for analysis. WCH was diagnosed by the following critria: Clinical systolic BP≥140 mmHg and diastolic BP≥90 mmHg; Elevated office BP≥140/90 mmHg and 24-h ABPM<130/80 mmHg; Daytime AB-PM<135/85 mmHg; nighttime ABPM<120/70 mmHg [19]. Exclusion criteria were as follows: (a) Patients with malignant tumors and other severe systemic diseases (such as renal failure or hepatic disease); (b) Unavailable baseline information on conventional BP, ABPM, and cardiovascular disease risk factors; (c) Antihy pertensive drug treatment at baseline; and (d) A history of stent or surgery. The Research Ethics Committee of the Gansu Provincial Hospital approved this study, and informed consent was obtained from each patient. Whole peripheral venous blood samples were drawn into commonly used test tubes containing EDTA and stored at -80°C within one hour.

RNA extraction and complementary DNA synthesis

One milliliter (mL) of peripheral blood was carefully transferred to a centrifuge tube containing equal amounts of lymphocyte separation medium (Haoyang Biological Manufacture CO. LTD, Tianjin, China) followed by centrifugation at 1500×*g* for 15 min. PMBCs were then transferred to a new tube for RNA extraction. Total RNA was extracted from PMBCs using TRIzol reagent (Invitrogen, Carlsbad, CA), strictlyaccording to the manufacturer's protocol. The reaction mixture (20 μL) containing 2 μg of total RNA was reversely transcribed to complementary DNA (cDNA) using a Reverse Transcription Kit (Takara, Dalian, China). Reverse transcription was performed at 65°C for 1 min, 30°C for 5 min, 37°C for 15 min, and 90°C for 5 min, and the cDNA obtained was stored at -20°C.

Microarray analysis and data analysis

To screen the differential lncRNA expression in WCH and HT patients, we performed lncRNA expression profiling in PMBC samples from the WCH and HT groups, and 35 healthy donors using the Arraystar lncRNA Array (V2.0) system. Samples with an RNA integrity number >8 were processed for hybridization. After isolation, RNA samples were labeled using the Quick Amp Labeling kit (Arraystar, Rockville, MD) and hybridized on a Human LncRNA Array (version 2.0) station. Scanned images were imported into an Axon GenePix 4000B microarray scanner (KangChen Bio-tech, Shanghai, China). Quantile normalization and data processing were performed using the GeneSpringGX v. 11.0 software package (KangChen Bio-tech) for data analysis. The threshold value for significance used to define upregulation or downregulation of lncRNA expression was a fold change >1.5, with a value of $P<0.05$ calculated by the Student's *t* test.

Table 1. Demographic characteristics, clinical blood pressure, and abpm of study groups

Parameters	NT (n=35)	WCH (n=35)	HT (n=35)	*P* value
Ratio of women/men (%)	25/10	23/12	25/10	0.531
Age, years	46.1±4.7	44.2±8.4	45.3±5.6	0.101
BMI, kg/m^2	23.6±1.6	24.5±2.0	24.8±1.4	0.464
Glucose, mg/dL	85.2±5.6	86.7±6.8	87.2±7.6	0.117
Hemoglobin a1c, %	5.0±0.5	5.6±0.7	5.5±0.6	0.272
T. cholesterol, mg/dL	180.0±27.4	182.4±28.1	185.1±28.8	0.099
HDL, mg/dL	57.5±12.4	53.9±10.5	52.2±11.7	0.101
LDL, mg/dL	109.7±20.5	114.1±24.9	117.3±22.3	0.069
Thyroid stimulating hormone, mIU/mL	1.9±0.7	2.1±0.4	2.0±0.3	0.551
Creatinine clearance, mL/min	121.0±30.1	120.4±32.5	119.2±29.7	0.220
NT-proBNP pg/mL	35.3±4.6	52.2±6.4	79.6±9.6	0.027*
Clinical SBP, mmHg	119.2±7.1	142.5±4.8	149.3±5.0	0.002*
Clinical DBP, mmHg	73.5±5.6	93.7±4.2	95.1±6.7	0.013*
24-hour ABPM				
SBP, mmHg	117.0±4.2	119.8±5.1	145.6±3.2	<0.001*
DBP, mmHg	71.9±7.4	75.3±4.2	92.8±4.0	<0.001*

Note: ABPM = ambulatory blood pressure monitoring, BMI = body mass index, HDL = High-density lipoprotein, LDL = Low-density lipoprotein, NT-proBNP = N-terminal pro brain natriuretic peptide, HT = hypertension, WCH = white coat hypertension, NT = normotensive, SBP = systolic blood pressure, DBP = diastolic blood pressure. *P<0.05.

Real-time PCR

To confirm the findings obtained by analyzing the lncRNA profiles, quantitative realtime PCR was performed using the Kapa SYBR fast qPCR kit (Kapa Biosystems, MA, USA), according to the manufacturer's instructions. *GAPDH* was used as an endogenous control for data normalization. Sequences of primers used are as follows: *H19* forward, 5'-ATCGGCTCTGGAAGGTGAAG-3'; *H19* reverse, 5'-TGGTGGCTGGTGGTCAAC-3'; *MALAT1* forward, 5'-CAGCAGTTCGTGGTGAAGATAG-3'; *MALAT1* reverse, 5'-GCCTCCTCCGTGTGGTTG-3'; *β-actin* forward, 5'-AGTTGCGTTACACCCTTTCTTG-3'; and *β-actin* reverse, 5'-CACCTTCACCGTTCCAGTTTT-3'. Quantitative PCR was performed at 95°C for 2 min, folowed by 40 cycles at 95°C for 15 s and 60°C for 1 min, using the Corbett Research 6000 Detection System (Applied Biosystems, CA). All samples were analyzed in triplicate. The fold change for lncRNA expression was obtained using the 2-ΔΔCt method and results are presented as the mean ± standard error of samples.

Statistical analysis

The data were analyzed using SPSS software version 20.0 (SPSS Inc., Chicago, IL). A *P*-value of <0.05 was considered statistically significant. Data for continuous variables are presented as mean ± standard deviation. Categorical variables were expressed as percentages. The Student's *t*-test or non-parametric ANOVA (Mann-Whitney test) were used to compare the differences in lncRNA levels between the groups. Associations between lncRNA expression and AMPB levels were determined using the Pearson correlation test. The Spearman correlation test was used for nominal data. To evaluate the diagnostic accuracy of lncRNA for separate WCH from sustained HT or healthy individuals, we built ROC curves. The AUC was used to assess the diagnostic values of lncRNAs.

Results

Clinical characteristics of study subjects

As shown in **Table 1**, among several clinical parameters compared between the groups, except for BP, N-terminal pro brain natriuretic peptide (NT-proBNP) was expressed at higher levels in the WCH group than in the NT group (P=0.017) and NT-proBNP was significantly upregulated in the HT group compared to that in the WCH group (P=0.004). This phenomenon was in accordance with a previous finding that NT-proBNP in plasma is a new biomarker that differentiates WCH from sustained hyperten-

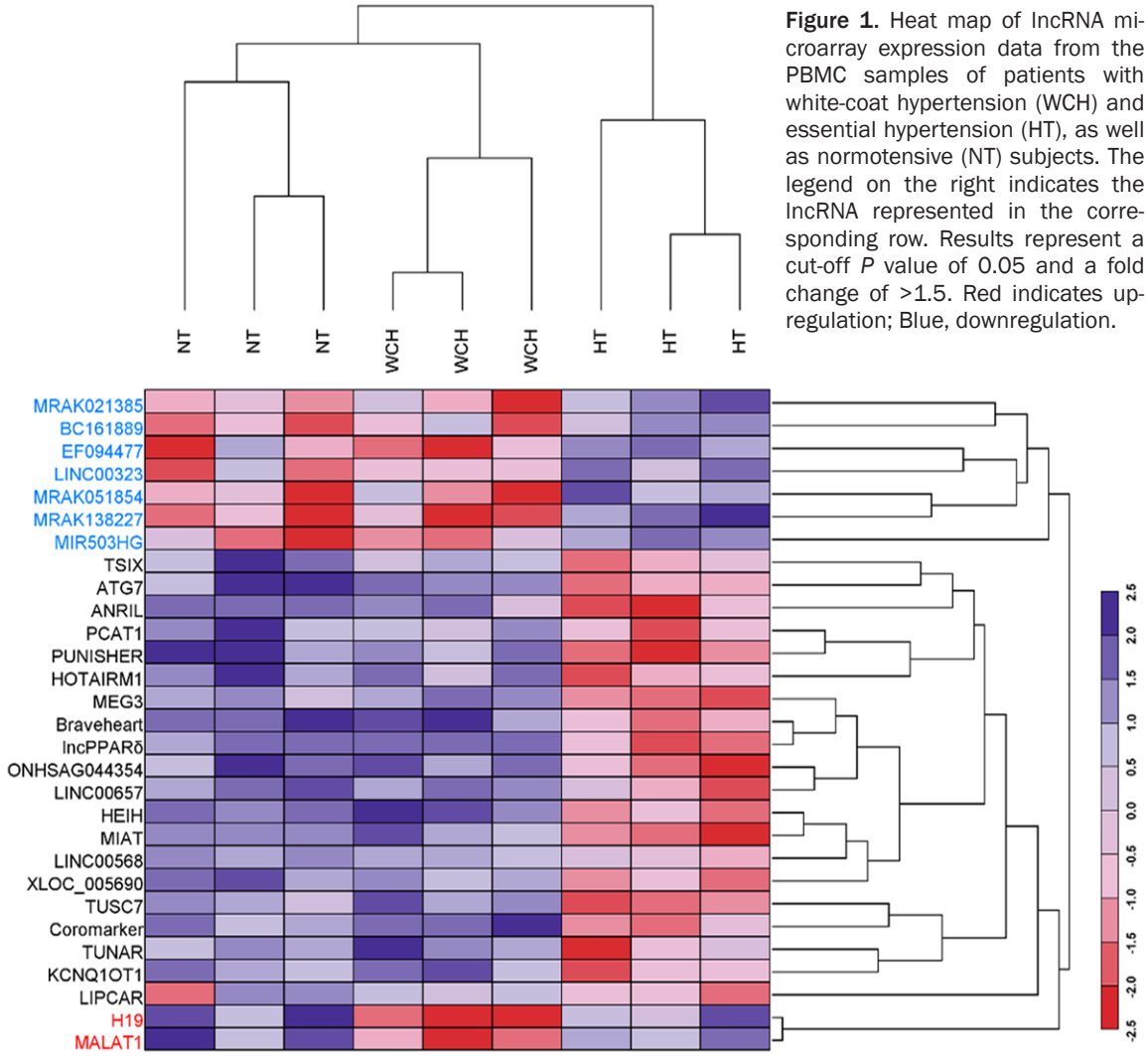

Figure 1. Heat map of lncRNA microarray expression data from the PBMC samples of patients with white-coat hypertension (WCH) and essential hypertension (HT), as well as normotensive (NT) subjects. The legend on the right indicates the lncRNA represented in the corresponding row. Results represent a cut-off *P* value of 0.05 and a fold change of >1.5. Red indicates upregulation; Blue, downregulation.

sion [20]. However, there were no statistically significant differences detected in other selected clinical indexes among the groups. The clinical BP of each subject is listed in **Table 1**. The results of 24-hour ABPM showed that subjects in the NT and WCH groups had lower BP than patients in the HT group (P<0.01 for all).

LncRNA expression profiles in PMBC of patients with WCH and HT

To obtain an lncRNA signature from the PMBCs of patients with WCH and HT, we performed microarray analyses of healthy volunteers and patients with WCH and HT. Interestingly, microarray data revealed 29 aberrantly expressed lncRNAs among the three study groups. **Figure 1** shows seven lncRNAs were downregulated in the HT group compared with those in other groups, and 20 lncRNAs were upregulated in patients with HT compared with those measured in patients with WCH as well as healthy volunteers (fold change, ≥1.5, P<0.05). However, only lncRNA *H19* and *MALAT1* showed high expression in the WCH group and low expression in the HT and NT groups, thus prompting us to explore their potential for differentiating WCH from HT. Expression levels of lncRNA *H19* and *MALAT1* were determined in the PMBCs of 70 patients with elevated clinical BP and 35 healthy individuals by using quantitative real-time PCR. In agreement with the microarray data, the relative expression of *H19* and *MALAT1* was significantly higher in the WCH group than in the NT and HT groups (P<0.01 for all; **Figure 2**). The expression level of *H19* was higher in the HT group than in the NT group (P<0.01; **Figure 2A**), but *MALAT1* did not exhibit a significant difference between the HT and NT groups (**Fig-ure 2B**).

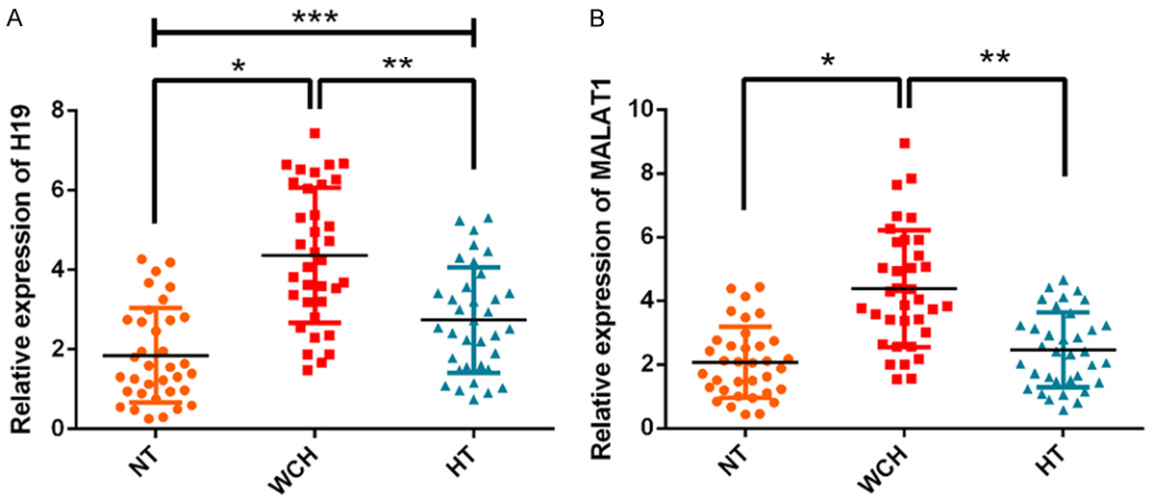

Figure 2. Relative expression levels of *H19* (A), and *MALAT1* (B) in the PBMC samples of healthy individuals (NT), patients with white-coat hypertension (WCH), and patients with essential hypertension (HT). *P<0.05 versus NT group, **P<0.05 versus HT group, ***P<0.05 NT group versus HT group.

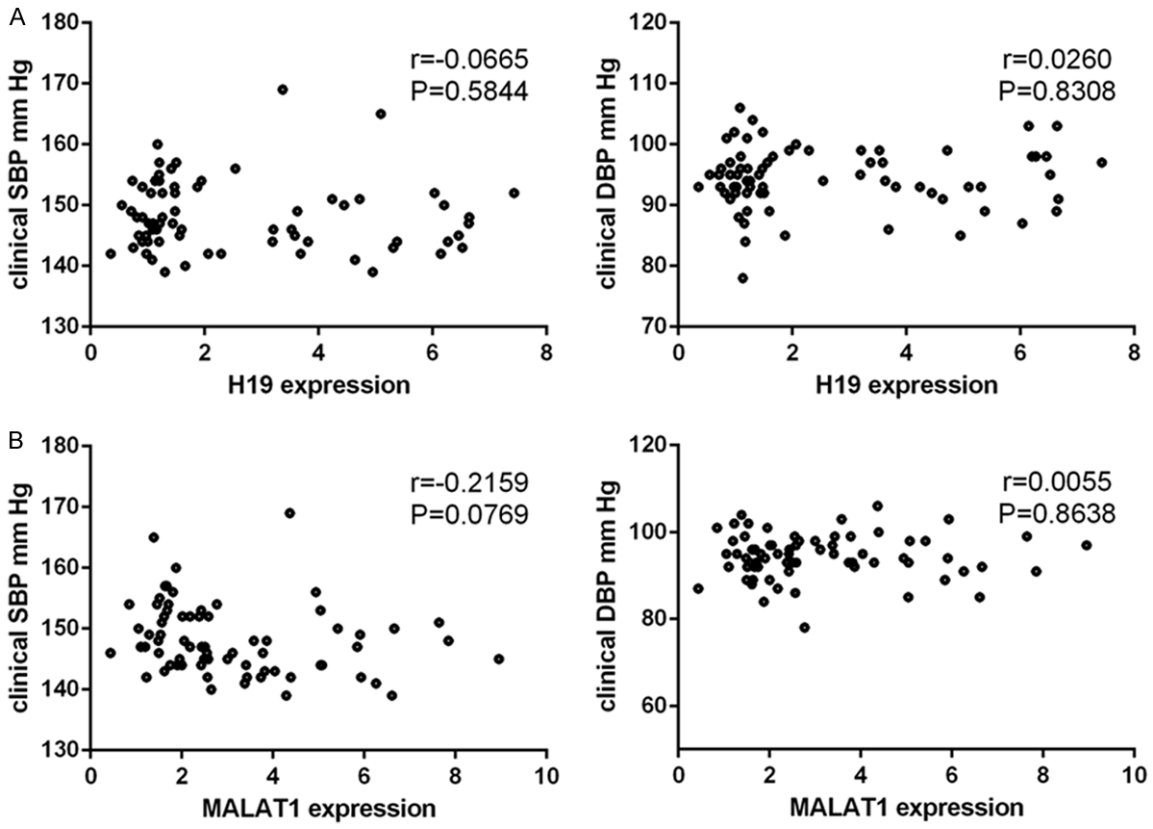

Figure 3. Pearson correlation test demonstrating the relationship between lncRNA *H19* (A) and *MALAT1* (B) relative expression levels with clinical systolic blood pressure (SBP) and clinical diastolic blood pressure (DBP).

The value of lncRNAs for distinguishing WCH from NT and HT

To explore the diagnostic value of lncRNAs in differentiating patients with WCH from HT or NT subjects, correlation analyses between lncRNA expression levels and BP levels were peformed. Results of the Pearson correlation test did not show rectilinear correlation between the expression of these two lncRNAs and clinical

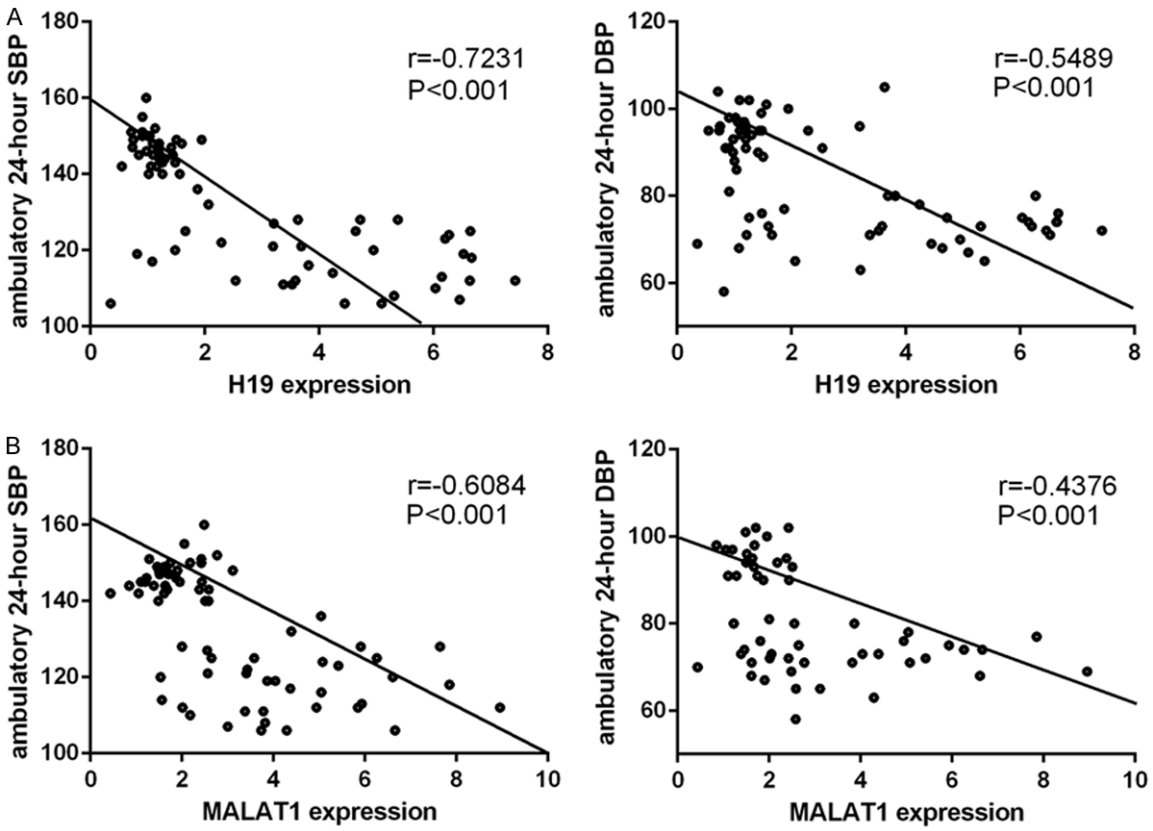

Figure 4. Pearson correlation test demonstrating the relationship between lncRNA H19 (A) and MALAT1 (B) relative expression levels with ambulatory 24-hour systolic blood pressure (SBP) and diastolic blood pressure (DBP). P<0.001 for all, r = coefficient of product-moment correlation.

Table 2. Sensitivity, specificity, AUC, cut-off, and asymptomatic significance of ROC analysis of lncRNAs

	AUC (95% CI)	Sensitivity (%)	Specificity (%)	Cut-off	Youden	*P* value
NT vs WCH						
H19	0.88 (0.78-0.95)	82.7	77.2	3.00	0.61	<0.001
MALAT1	0.85 (0.76-0.93)	83.0	77.1	2.95	0.60	<0.001
WCH vs HT						
H19	0.76 (0.65-0.86)	74.29	68.57	3.52	0.43	<0.001
MALAT1	0.80 (0.69-0.89)	77.14	74.29	3.13	0.52	<0.001

ROC = receiver operating characteristic, AUC = area under the curve, CI = confidence interval, HT = hypertension, NT = normotensive, WCH = white coat hypertension.

SBP (**Figure 3B**) or DBP (**Figure 3B**). The ambulatory 24-hour SBP and DBP were negatively correlated with *H19* expression levels (r=-0.7231, P<0.01 and r=-0.5489, P<0.01, respectively; **Figure 4A**) and with *MALAT1* expression levels (r=-0.6084, P<0.01 and r=-0.4376, P<0.01, respectively; **Figure 4B**). ROC curves were then constructed and AUC values were generated to assess the power of the lncRNA *H19* and *MALAT1* for distinguishing between the NT, WCH and HT groups. As illustrated in **Table 2**, lncRNA *H19* and *MALAT1* had AUC values of 0.88 (0.78-0.95) and 0.85 (0.76-0.93), respectively, which demonstrates their sufficiency in distinguishing WCH from NT individuals (**Figure 5A**). When differentiating patients with WCH from HT, lncRNA *H19* and *MALAT1* had AUC values of 0.76 (0.65-0.86) and 0.80 (0.69-0.89), respectively (**Figure 5B**). Detailed information on the ability of lncRNA *H19* and *MALAT1* to differentiate between patients with or without real HT is presented in **Table 2**.

Discussion

In this study, we investigated lncRNA expression signature in the PBMCs of patients with

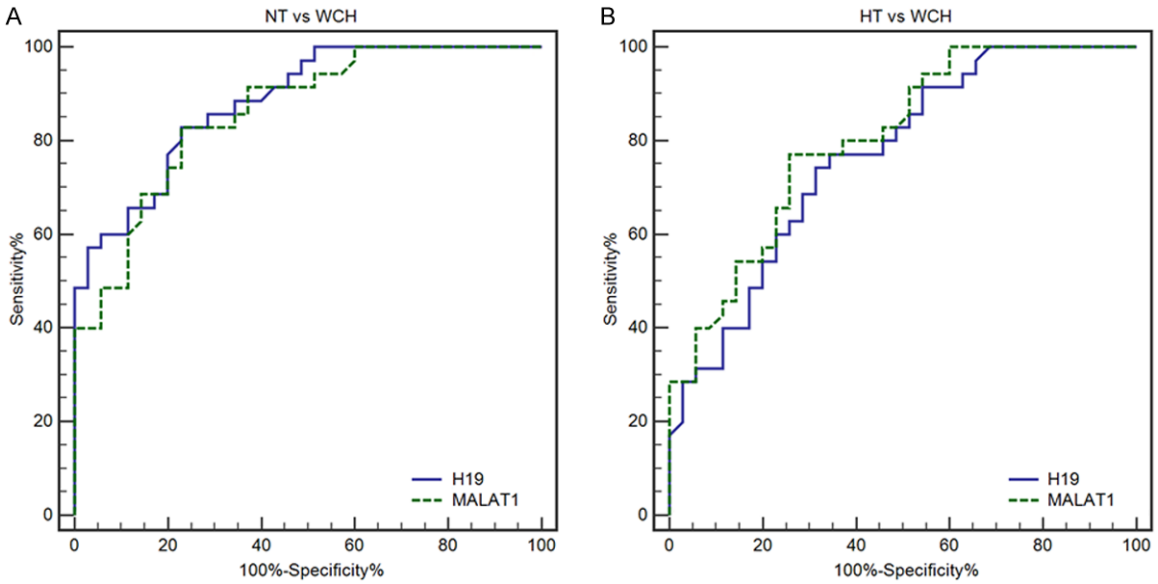

Figure 5. Receiver operating characteristic curve analysis demonstrating the diagnostic performance of lncRNA *H19* and *MALAT1* in the PBMC samples to distinguish normotensive (NT) subjects from patients with white coat hypertension (WCH) (A) and to distinguish WCH patients from those with hypertension (HT) (B).

sustained hypertension and white-coat hypertension, compared with healthy subjects. The data revealed that the expression levels of *H19* and *MALAT1* were significantly increased in subjects with WCH compared to those with NT and HT and further exploration find these two lncRNAs showed good performance to distinguish WCH from HT individuals. To our knowledge, we are the first to report a link between lncRNA and WCH.

Emerging evidence suggests that lncRNA signatures of normal cancer tissues and metastases are used to classify different cancer types, indicating the potential of these lncRNAs as biomarkers for diagnosis, prognosis, and therapy [21-23]. Recent studies also revealed that lncRNAs are potential biomarkers and therapeutic targets in cardiovascular disease [24]. The *H19* gene, discovered 25 years ago, produces a non-coding RNA, which is abundantly expressed during embryonic development and is down-regulated after birth [25]. A recent study suggested that the physiological role of *H19* is to limit growth of the placenta prior to birth by regulating the Insulin-like growth factor 1 receptor through miR-675 [26]. Emerging evidence had highlighted the important roles of *H19* during the complex process of tumorigenesis [27]. Meanwhile, it might predict progression and metastasis in cancers, such that the expression of *H19* was associated with histological grade TNM and tumor invasion depth in gastric cancer [28]. Furthermore, studies are gradually uncovering the function of *H19* in the heart. Liu et al detected the RNA level of *H19* and its encoded miR-675 expression level in both normal and diseased hearts with pathological cardiac hypertrophy in mice, and verified their upregulation in pathological cardiac hypertrophy and heart failure. Further experiments revealed a novel function of H19-miR-675 axis that targets CaMKIId, a multifunctional serine/threonine protein kinase mainly found in the heart, as a negative regulator of cardiomyocyte hypertrophy [29]. Greco et al found lncRNA *H19* was significantly upregulated in the left ventricular biopsies of 18 non-end-stage ischemic dilated cardiomyopathy patients compared with 17 control subjects, and confirmed its upregulation in a mouse model of cardiac hypertrophy [30]. In the present study, we determined the upregulation of lncRNA *H19* in the PBMCs of patients with increased clinical BP, and ROC analysis suggested it had enough power to distinguish WCH from HT individuals.

Metastasis associated lung adenocarcinoma transcript 1 (*MALAT1*), which was first associated with metastasis of lung tumors [31], is a typical multifunctional gene that is important in a wide array of cancers [32, 33], and *MALAT1* silencing may be an effective therapeutic

approach against tumors [34]. A recent study has revealed that *MALAT1* deregulation is implicated in the pathogenesis of diabetic retinopathy and its knockdown could regulate retinal endothelial cell proliferation, migration, and tube formation and ameliorate diabetic retinopathy in vivo [35]. In addition, Katharina et al elucidated that genetic ablation or pharmacological inhibition of *MALAT1* inhibited proliferation of endothelial cells and reduces vascular growth in mice [36]. Furthermore, *MALAT1* was significantly upregulated in human umbilical vein endothelial cells subjected to transforming growth factor-1 treatment [37]. However, *MALAT1* lncRNA has not yet been studied in HT or WCH. We revealed that circulating *MALAT1* expression was higher in patients with WCH than in NT or HT subjects, and identified its diagnostic value for differentiating WCH from NT and HT. To our knowledge, we are the first to demonstrate deregulated expression of lncRNA *H19* and *MALAT1* in the PMBCs of patients with elevated BP, and to establish them as novel potential noninvasive biomarkers for differentiating between WCH and HT. This is important as misdiagnosis of WCH can contribute to inappropriate anti-hypertensive treatment and adverse events. Furthermore, similar observations have been made for WCH, suggesting a complementary role of out-of-office and office BP values in the determination of patients' prognoses [38]. At present, ABPM, which may accurately grade the severity of hypertension and predict the cardiovascular risk of patients, is the gold standard in the identification of WCH. However, the disadvantages of ABPM are its limited availability and discomfort as well as the reluctance of some patients to use it.

In summary, our data suggest that lncRNA *H19* and *MALAT1* could serve as novel noninvasive biomarkers for distinguishing WCH from HT individuals, and provide crucial insights into the diagnosis of WCH and could extend the understanding of the role of lncRNAs in cardiovascular disease. However, this study has limitations. We explored the diagnostic value of lncRNA *H19* and *MALAT1* in a small cohort and did not confirm our findings in another group with a larger number of subjects. In addition, we did not investigate the expression levels of these lncRNAs in samples after anti-hypertensive treatment and compare the differences, which may help to discover more differential lncRNAs during this process. Thus, further studies are required to understand the exact mechanisms underlying these observations in WCH and HT.

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Disclosure of conflict of interest

None.

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