Circulating long non-coding RNAs NRON and MHRT as novel predictive biomarkers of heart failure

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Abstract

This study sought to evaluate the potential of circulating long non-coding RNAs (lncRNAs) as biomarkers for heart failure (HF). We measured the circulating levels of 13 individual lncRNAs which are known to be relevant to cardiovascular disease in the plasma samples from 72 HF patients and 60 non-HF control participants using real-time reverse transcription-polymerase chain reaction (real-time RT-PCR) methods. We found that out of the 13 lncRNAs tested, non-coding repressor of NFAT (NRON) and myosin heavy-chain-associated RNA transcripts (MHRT) had significantly higher plasma levels in HF than in non-HF subjects: 3.17 ± 0.30 versus 1.0 ± 0.07 for NRON (P < 0.0001) and 1.66 ± 0.14 versus 1.0 ± 0.12 for MHRT (P < 0.0001). The area under the ROC curve was 0.865 for NRON and 0.702 for MHRT. Univariate and multivariate analyses identified NRON and MHRT as independent predictors for HF. Spearman’s rank correlation analysis showed that NRON was negatively correlated with HDL and positively correlated with LDH, whereas MHRT was positively correlated with AST and LDH. Hence, elevation of circulating NRON and MHRT predicts HF and may be considered as novel biomarkers of HF.

Keywords: heart failure, LncRNA, NRON, MHRT, plasma

Introduction

HF is a major public health problem afflicting a large population (>25 million patients) in the world [1] and an intricate pathophysiological syndrome consequent to feeble cardiac contraction and inadequate blood ejection [2]. The clinical manifestations of HF mainly arise from myocardial infarction (MI), hypertension, myocarditis and inherited cardiomyopathy [3, 4]. Without successful intervention within a certain timeframe, HF can cause sudden cardiac death or severe disability, being the most devastating cardiovascular disease in terms of mortality, morbidity and the quality of life. One of the difficulties for timely treatment of HF is our current dearth of sensitive and specific biomarkers for early diagnosis of the malady. A number of clinically validated biomarkers such as cardiac troponin, natriuretic peptide, B-type natriuretic peptide (BNP) and N-terminal proBNP (NT-proBNP) have been used in the diagnosis of HF [5–10]. Nonetheless, these traditional biomarkers have some limitations in defining the aetiology or prognosis of HF [5–7]. For example, none of these markers are...
specific to HF, but their serum/plasma levels can rise in a number of other diseases such as cardiopulmonary disease, kidney failure and hepatic cirrhosis. Quest for more reliable biomarkers is therefore highly desirable. It is known that aberrant changes in the expression of multiple genes in myocardium are a major cause, as well as useful predictors of the pathologic remodelling in failing heart. Identification of such genes, particularly those that are highly sensitive and specific to HF, may be the key step towards reliable early prediction of HF.

Non-coding RNAs (ncRNAs), including microRNAs (miRNAs) and IncRNAs, have recently been found to play important regulatory roles in the development and progression of cardiovascular diseases [11–14]. These RNAs have also been implicated in the diagnosis of cardiovascular diseases owing to the characteristic alterations of their circulating levels with different categories and grades of pathological processes. IncRNAs belong to a newly discovered class of functional mRNA-like transcripts that lack significant open reading frames or protein-coding capacity [14] and have emerged as an important player in cardiovascular diseases, including a number of cardiac-specific or cardiac-related IncRNAs such as SRA, DIO3OS, SAF, NESPAS, MIAT, NRON, CARL, HCG22, FENDRR, MHRT, aHIF, ZFAS1 and CDR1AS [15–26] (http://cmbi.bjmu.edu.cn/lncrnadisease). Recent research data have also suggested the roles of IncRNAs in HF [15–18]. Most prominently, circulating IncRNAs are exceptionally stable in the bloodstream and readily detectable in human subjects, such as in patients with cancers or acute kidney injury, implying that circulating IncRNAs might be a non-invasive and rapid diagnostic tool for disease diagnosis and prognosis [15]. However, studies on circulating IncRNAs for the prediction of HF have been sparse. A comprehensive study using microarray analysis compared expression alterations of IncRNAs in the heart, whole blood and plasma in a mouse model of acute HF [17]. Their results revealed 32 differentially expressed IncRNAs with changes greater than twofold. Another study conducted with serum samples from HF patients suggested the potential of LIPCAR (the mitochondrial IncRNA uc022bq5.1) to predict survival in patients with HF. Yet, none of these deregulated IncRNAs belong to the cardiac-specific or cardiac-related ones mentioned above.

This study was therefore designed to explore the possibility of the known cardiac-specific and cardiac-related IncRNAs in plasma samples from patients with HF as circulating biomarkers for HF. Quantitative RT-PCR was employed to determine the plasma levels of the test IncRNAs. Our results identified NRON and MHRT as possible novel biomarkers for predicting HF.

Materials and Methods

Participants

Between February 2014 and January 2015, 104 HF patients and 109 non-HF control participants presented to the First Affiliated Hospital, the Second Affiliated Hospital, the Third Affiliated Hospital and the Fourth Affiliated Hospital of the Harbin Medical University (Harbin, China). Diagnosis of HF and the criteria for inclusion of patients were as previously described in detail [27, 28] (see Supplementary Methods). The clinical characteristics of the study population are summarized in Table 1 and Tables S1 and S2.

Ethical approval of studies and informed consent

The study protocols and the procedures for handling human samples were approved by the Institutional Research Board of the Harbin Medical University (No.HMUIRB-20140027). The written informed consents were obtained from all subjects recruited to our study.

Collection and handling of human blood samples

Whole blood samples (1 ml per patient) were drawn from the study subjects via a direct venous puncture into the tubes containing sodium citrate. The human whole blood samples in sodium citrate vacuum tubes were kept at 4 °C and then centrifuged at 2000 × g/min. at 4 °C for 10 min. to obtain plasma samples.

RNA extraction and quantitative real-time reverse transcription (RT)-polymerase chain reaction (qPCR)

Total RNA was extracted from the prepared plasma samples using Trizol LS reagent (Invitrogen, Carlsbad, California, USA) according to the manufacturer’s instructions. In brief, each plasma sample (0.25 ml) was mixed well with 1 ml Trizol reagents in a tube. Chloroform (0.2 ml) was added into the sample and shaken vigorously by hand for 15 sec. The sample was incubated at room temperature for 5 min. and then centrifuged at 12000 × g/min. at 4 °C for 15 min. The supernatant was transferred to a new tube, and an equal volume of isopropanol was added to the aqueous phase. After mixing and incubation at room temperature for 10 min., the sample was again centrifuged at 12000 × g/min. at 4 °C for 10 min. After removal of the supernatant, the pellet was washed with 1 ml of 75% ethanol for the initial homogenization. Then, the sample was centrifuged at 10,600 r.p.m./min. at 4 °C for 5 min. The RNA pellet was dissolved in DEPC water. The quality of our RNA samples was first measured by NanoDrop ND-8000 (Thermo Fisher Scientific, Waltham, MA, USA). To ensure the RNA/DNA ratio 1.8–2.0. Then, the integrity of the RNA samples was assessed by standard denaturing agarose gel electrophoresis and confirmed by discrete 28 s and 5 s bands without smear.

The SYBR Green PCR Master Mix Kit (cat#: 4367659, Life technology, USA) was used for qPCR for relative quantification of IncRNAs (see Supplementary Materials online for detail). The qPCR primer pairs used in our study are listed in Table S4 online.

Statistical analysis

Categorical data are presented as count and percentile. Continuous variables are described as mean ± S.E.M. (standard error of measurement), min, max, median or interquartile range, as specified in the data descriptions. The statistical analyses are described in detail in supplementary methods. All analyses were carried out with SAS 9.1 (Serial No. 989155) except that ROC was carried out with SPSS v17.0 software. The significant level was set at 0.05, and two-tailed P values <0.05 were considered statistically significant.
| Characteristics | Non-HF | HF | P value |
|-----------------|--------|----|---------|
| Age             |        |    |         |
| N (missing)     | 60 (0) | 72 (0) | 0.8710 |
| Mean (Std)      | 60.08 (11.97) | 59.31 (11.19) | |
| Min, max        | 36, 88 | 28, 83 | |
| Median          | 58     | 60.50 | |
| Range           | 52–67.50 | 51–67 | |
| Gender          |        |    |         |
| Male            | 37     | 47 | 0.6676 |
| Female          | 23     | 25 | |
| Total (missing) | 60 (0) | 72 (0) | |
| Hypertension    |        |    |         |
| Yes             | 17     | 39 | 0.2858 |
| No              | 19     | 28 | |
| Total (missing) | 36 (24) | 67 (5) | |
| Diabetes        |        |    |         |
| Yes             | 7      | 17 | 0.4480 |
| No              | 29     | 48 | |
| Total (missing) | 36 (24) | 65 (7) | |
| CHOL            |        |    |         |
| N (missing)     | 57 (3) | 63 (9) | 0.0344 |
| Mean (Std)      | 4.72 (0.70) | 4.40 (1.11) | |
| Min, max        | 3.24, 6.33 | 1.98, 8.84 | |
| Median          | 4.69 | 4.31 | |
| Range           | 4.33–5.21 | 3.71–5.03 | |
| TG              |        |    |         |
| N (missing)     | 57 (3) | 63 (9) | 0.3930 |
| Mean (Std)      | 1.32 (0.52) | 1.49 (0.74) | |
| Min, max        | 0.66, 2.43 | 0.49, 3.65 | |
| Median          | 1.16 | 1.26 | |
| Range           | 0.91, 1.51 | 0.92–1.89 | |
| Characteristics | Non-HF | HF | \( P \) value |
|-----------------|--------|----|---------------|
| **HDL**         |        |    |               |
| \( N \) (missing) | 57 (3) | 63 (9) | 0.00739 |
| Mean (std)      | 1.20 (0.23) | 1.12 (0.30) |   |
| Min, max        | 0.79, 1.88 | 0.79, 2.85 |   |
| Median          | 1.20   | 1.07   |   |
| Range           | 1.06–1.31 | 0.95–1.21 |   |
| **LDL**         |        |    | 0.6399       |
| \( N \) (missing) | 57 (3) | 63 (9) |   |
| Mean (Std)      | 2.84 (0.56) | 2.95 (0.92) |   |
| Min, max        | 1.70, 4.31 | 0.93, 6.36 |   |
| Median          | 2.86   | 2.75   |   |
| Range           | 2.46–3.23 | 2.28–3.44 |   |
| **Glycemia**    |        |    | 0.13911      |
| \( N \) (missing) | 57 (3) | 67 (5) |   |
| Mean (Std)      | 5.99 (1.90) | 6.93 (4.25) |   |
| Min, max        | 3.98, 14.20 | 2.89, 36.6 |   |
| Median          | 5.46   | 5.86   |   |
| Range           | 5.11–6.04 | 5.03–7.34 |   |
| **ALT**         |        |    | 0.0737       |
| \( N \) (missing) | 26 (34) | 65 (7) |   |
| Mean (Std)      | 23.27 (12.23) | 30.49 (20.31) |   |
| Min, max        | 11.00, 59.00 | 0.26, 108.00 |   |
| Median          | 21.50  | 25     |   |
| Range           | 15.00–27.00 | 17.00–35.00 |   |
| **AST**         |        |    | 0.02176      |
| \( N \) (missing) | 26 (34) | 65 (7) |   |
| Mean (Std)      | 20.73 (3.81) | 28.14 (15.27) |   |
| Min, max        | 14.00, 28.00 | 7.00, 92.00 |   |
| Median          | 20.50  | 24.00  |   |
| Range           | 18.00–23.00 | 19.00–32.00 |   |
| Characteristics | Non-HF | HF | P value |
|-----------------|-------|----|---------|
| **AST/ALT**     |       |    |         |
| N (missing)     | 23 (37)| 65 (7)| 0.2975 |
| Mean (Std)      | 1.06 (0.36)| 1.39 (3.02)|         |
| Min, max        | 0.50, 1.80| 0.40, 25.00|         |
| Median          | 1.00  | 0.85 |         |
| Range           | 0.80–1.30| 0.70–1.20|         |
| **BUN**         |       |    | <0.0001|
| N (missing)     | 55 (5)| 67 (5)|         |
| Mean (Std)      | 5.76 (1.46)| 7.36 (2.65)|         |
| Min, max        | 3.30, 10.34| 3.07, 18.04|         |
| Median          | 5.50  | 6.94 |         |
| Range           | 4.53–6.85| 5.64–8.77 |         |
| **Cr**          |       |    | 0.00215|
| N (missing)     | 57 (3)| 68 (4)|         |
| Mean (Std)      | 72.96 (16.64)| 90.11 (34.60)|         |
| Min, max        | 8.20, 103.60| 6.31, 239.30|         |
| Median          | 75.00 | 81.55|         |
| Range           | 62.20–85.00| 67.70–100.80|         |
| **Bun/Cr**      |       |    | <0.0001|
| N (missing)     | 23 (37)| 68 (4)|         |
| Mean (Std)      | 84.25 (24.31)| 48.90 (40.44)|         |
| Min, max        | 45.92, 159.08| 0.05, 124.58|         |
| Median          | 83.69 | 58.73|         |
| Range           | 69.00–93.00| 0.12–78.58|         |
| **UA**          |       |    | <0.0001|
| N (missing)     | 56 (4)| 68 (4)|         |
| Mean (Std)      | 316.21 (79.21)| 422.88 (144.25)|         |
| Min, max        | 156.40, 516.00| 88.75, 799.70|         |
| Median          | 304.30| 387.15|         |
| Range           | 257.10–374.30| 320.45–532.95|         |
Results
Clinical characteristics of the study population

Plasma samples were collected from a total of 104 HF patients and 109 control participants for measuring lncRNAs. To have more rational comparisons between HF and control participants, we filtered the plasma samples based upon the clinical or demographic characteristics of the patients recruited. We identified 32 HF patients and 49 control participants who did not have matched clinical or demographic characteristics between the two groups, and we therefore discarded these samples leaving 72 HF patients and 60 control participants for detailed statistical analysis. Of the 72 HF patients, 65 had an elevated NT-proBNP at enrolment during the study period. Table 1 shows the clinical and demographic characteristics of the patients enrolled in this study (also see Tables S1 and S2 online for the complete data sets of all 104 HF patients and 109 control participants). There were no age and gender differences between the test patients and control participants, nor was any difference in blood pressure.

Reciprocal changes in NRON and MHRT blood levels in AMI patients

Our initial quantitative real-time RT-PCR (qPCR) analysis included 13 known cardiac-specific or cardiac-related lncRNAs: SRA, DIO3OS, SAF, NESPAS, MIAT, NRON, CARL, HCG22, FENDRR, MHRT, aHIF, ZFAS1 and CDR1AS. As illustrated in Figure 1A, of 13 IncRNAs tested, only two, NRON and myosin heavy–chain-associated RNA transcripts (MHRT), demonstrated significant differences in plasma samples between HF and non-HF. Specifically, the circulating level of NRON was significantly higher in HF than in non-HF subjects (3.17 ± 0.30 versus 1.00 ± 0.07; P < 0.0001) (Fig. 1B and C; Table 2). Similarly, the plasma level of MHRT was also markedly elevated in HF (1.66 ± 0.14) relative to that in non-HF subjects (1.00 ± 0.12; P < 0.0001). The median Ct value for NRON was 26.3 by 40 cycles of qPCR with standard deviation of 1.6, indicating that these two lncRNAs are fairly abundant in plasma. Similar elevations of the circulating levels of NRON and MHRT were consistently observed when all plasma samples (104 HF patients and 109 control participants) were included in our analysis (Table S3 online).

Table 1. Continued

| Characteristics | Non-HF | HF | P value |
|----------------|-------|----|---------|
| Co2CP          |       |    |         |
| N (missing)    | 52 (8) | 48 (24) | 0.4233  |
| Mean (Std)     | 36.48 (47.05) | 37.44 (67.67) | |
| Min, max       | 23.20, 281.00 | 10.00, 495.70 | |
| Median         | 27.35 | 27.75 | |
| Range          | 26.10–28.50 | 26.00–30.00 | |
| NT-proBNP      |       |    |         |
| N (missing)    | 0 (60) | 65 (7) | NA      |
| Mean (Std)     | 3786.62 (6091.64) |          | |
| Min, max       | 104.00, 35,000.00 |          | |
| Median         | 2144.00 |          | |
| Range          | 635.00, 3788.00 |          | |

CHOL, total cholesterol; TG, triglyceride; HDL, high-density cholesterol; LDL, low-density cholesterol; ALT, alanine aminotransferase; AST, aspartate aminotransferase; BUN, blood urea nitrogen; Cr, creatinine; UA, uric acid; Co2CP, carbon dioxide combining power; NT-proBNP, amino-terminal pro-brain natriuretic peptide.

Evaluation of circulating NRON and MHRT as new biomarkers for HF

Having established that NRON and MHRT are present in the peripheral circulation and their plasma levels are anomaly altered in HF patients, we sought to determine the potential utility of circulating NRON and MHRT as diagnostic biomarkers of HF. To this end, ROC analysis was performed to evaluate the predictive power of circulating NRON and MHRT alone for HF. Our results showed that the area under ROC curve was 0.865 (95% CI = 0.805–0.926) for NRON alone (Fig. 2A), 0.702 (95% CI = 0.612–0.791) for MHRT alone (Fig. 2B).
The univariate analysis with logistic regression showed that the odds ratios (OR) were 4.505 (95% CI: 2.393–8.478) for NRON \((P < 0.0001)\), and 1.701 (95% CI: 1.225–2.363) for MHRT \((P = 0.0015)\) between HF and non-HF (Table 3).

The multivariate logistic regression analysis further verified NRON and MHRT as independent predictors for HF (Table 4): The OR values were 3.377 (95% CI: 1.441–7.915) for NRON \((P = 0.0051)\) and 1.679 (95% CI: 1.068–2.639) for MHRT \((P = 0.0248)\) between HF and non-HF (Table 4).

**Relation of NRON and MHRT to conventional prognostic markers**

To further evaluate the usefulness of circulating NRON and MHRT as HF biomarkers, we tested whether their levels were correlated with cardiac risk factors, conventional HF markers and cardiac function parameters. The data summarized in Table 5 show that NRON was negatively correlated with HDL and positively correlated with LDL, whereas MHRT was positively correlated with AST and LDH (Table 6). Neither NRON nor MHRT was correlated with age, gender, diabetes mellitus, hypertension, smoking history, total cholesterol, triglyceride (TG), cardiac troponin I (cTnI), aspartate aminotransferase \([29]\), creatine kinase (CK), creatine kinase-myocardial band (CKMB), NT-proBNP or cardiac function parameters.

**Discussion**

In the present study, we analysed the levels of a selected set of lncRNAs in the plasma samples of HF patients for their potential as biomarkers for the diagnosis of HF. These lncRNAs were selected for our study because they have been documented to play important

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**Table 2 Statistical analysis of the circulating NRON and MHRT**

| LncRNA | Non-HF | HF | P value |
|--------|--------|----|---------|
| **MHRT** |        |    |         |
| \(N\) (missing) | 60 (0) | 72 (0) | <0.0001** |
| Mean (Std) | 1.00 (0.89) | 1.66 (1.2) | |
| Min, max | 0.06, 5.85 | 0.10, 7.08 | |
| Median | 0.86 | 1.27 | |
| Range (Q1, Q3) | 0.38–1.43 | 0.90–2.15 | |
| **NRON** |        |    |         |
| \(N\) (missing) | 60 (0) | 72 (0) | <0.0001** |
| Mean (Std) | 1.09 (0.54) | 3.17 (2.58) | |
| Min, max | 0.09, 8.14 | 0.13, 9.86 | |
| Median | 1.20 | 1.77 | |
| Range (Q1, Q3) | 0.54–1.26 | 1.26–2.99 | |

\(P\) values are for comparisons between HF patients versus non-HF control participants. * \(P < 0.001\) vs. Non-HF.

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The univariate analysis with logistic regression showed that the odds ratios (OR) were 4.505 (95% CI: 2.393–8.478) for NRON \((P < 0.0001)\), and 1.701 (95% CI: 1.225–2.363) for MHRT \((P = 0.0015)\) between HF and non-HF (Table 3).
roles in shaping developmental process of the heart and in the pathogenesis and progression of cardiac diseases [15-26]. Our results identified two lncRNAs, NRON and MHRT, out of 13 known cardiac-relevant lncRNAs examined, as promising candidate biomarkers for HF in the light of the significant elevations of their circulating levels in HF patients relative to non-HF control participants and the close correlation between the circulating levels of NRON and MHRT.

Published studies on circulating LncRNAs as HF biomarkers

NRON (Non-coding RNA repressor of NFAT) is enriched in muscles (including cardiac muscle), placenta, spleen, thymus and lymph nodes and has been denoted as a repressor of the nuclear factor of activated T cells (NFAT) by influencing its nuclear trafficking [30, 31]. NFAT is known to be a critical protein in the regulation of intracellular Ca\(^{2+}\) homeostasis and of gene expression as a transcription factor in the heart, and its expression and activity are tremendously increased in HF [32, 33]. It is conceivable that by regulating NFAT, NRON can participate in the genesis and development of HF. Yet, such a notion requires rigorous experimentation to verify. MHRT was initially identified as a cardiac-specific and cardiac enriched protective lncRNA by Han et al. [21]. It acts to protect the heart against pathological hypertrophy; yet, pathological stress such as hypertrophy and HF inhibits MHRT transcription in the heart [21]. Recently, MHRT was found to suppress cardiomyocyte apoptosis induced by H\(_2\)O\(_2\) to simulate the acute ischaemic condition [26], and under such context, MHRT expression in cardiomyocytes was activated by oxidative stress. In this same study, the authors found that the plasma MHRT level is markedly elevated in patients with acute MI. Nevertheless, the potential usefulness of NRON and MHRT as biomarkers of HF has not been previously evaluated.

Here, we revealed that NRON and MHRT were both elevated in their plasma levels in patients with HF relative to non-HF control participants. We also found that these two lncRNAs are fairly abundant in the plasma of HF patients relative to non-HF control participants.

![Fig. 2](image-url)

**Fig. 2** Receiver operator characteristic analysis of circulating NRON and MHRT for predicting HF. The area under ROC curve was determined to evaluate the predictive power of circulating NRON (A) and MHRT (B) levels for HF using non-HF participants as control.

### Table 3

| Variable | B      | S.E. | \(z^2\) | \(P\)   | OR Low | 95% CI Low | OR High | 95% CI High |
|----------|--------|------|---------|---------|--------|------------|---------|-------------|
| NRON     | 1.5051 | 0.3226 | 21.7608 | <0.0001 | 4.505  | 2.393      | 8.478   |
| MHRT     | 0.5313 | 0.1676 | 10.0523 | 0.0015  | 1.701  | 1.225      | 2.363   |
| Age      | -0.00592 | 0.0153 | 0.1504  | 0.6982  | 0.994  | 0.965      | 1.024   |
| Gender   | 0.1558 | 0.3630 | 0.1843  | 0.6677  | 1.169  | 0.574      | 2.381   |
| HDL      | -1.1380 | 0.7586 | 2.2509  | 0.1335  | 0.320  | 0.072      | 1.417   |
| LDL      | 0.1928 | 0.2424 | 0.6326  | 0.4264  | 1.213  | 0.754      | 1.950   |
| TG       | 0.4278 | 0.2985 | 2.0546  | 0.1518  | 1.534  | 0.855      | 2.754   |
| CHOL     | -0.3756 | 0.2072 | 3.2842  | 0.0699  | 0.687  | 0.458      | 1.031   |
Table 4 Multivariate regression analysis for the association of \textit{NRON} and \textit{MHRT} with demographic characteristics between HF patients and non-HF control participants

| Variable | B     | S.E. | \( \chi^2 \) | \( P \) | OR    | 95% CI  |
|---------|-------|------|-------------|--------|-------|--------|
|         |       |      | Low | High       |       |       |
| \textit{NRON} | 1.2170 | 0.4346 | 7.8412 | 0.0051 | 3.377 | 1.441 – 7.915 |
| \textit{MHRT} | 0.5182 | 0.2308 | 5.0395 | 0.0248 | 1.679 | 1.068 – 2.639 |
| Age     | 0.00199 | 0.0315 | 0.0040 | 0.9496 | 1.002 | 0.942 – 1.066 |
| Gender  | -0.6181 | 0.7135 | 0.7505 | 0.3863 | 0.539 | 0.133 – 2.182 |
| HDL     | 7.6350 | 2.0206 | 14.2775 | 0.0002 | >999.999 | 39.437 – >999.999 |
| LDL     | 6.1932 | 1.4468 | 18.3247 | <0.0001 | 489.417 | 28.720 – >999.999 |
| TG      | -6.3369 | 1.4537 | 19.0036 | <0.0001 | 0.002 | <0.001 – 0.031 |
| CHOL    | 2.6408 | 0.7249 | 13.2708 | 0.0003 | 14.024 | 3.387 – 58.066 |

RNA species in human serum samples based on the relatively low Ct values of qPCR experiments (the median Ct value for \textit{NRON} was 26.3, ranging from 22.3 to 31.4; and the median Ct value for \textit{MHRT} was 27.0 with a range from 23.4 to 30.4 in patients with HF). These facts prompted us to propose that either of these two IncRNAs is a reasonable predictor of HF. For years, NT-proBNP has been believed to be an established risk marker for HF. Our analysis indicated that the predictive power of \textit{NRON} is comparable to that of NT-proBNP: The reported value of AUC is 0.844 for NT-proBNP [34], and the value for \textit{NRON} was 0.865. By comparison, the AUC value for \textit{MHRT} is lower (0.702); yet, it still falls into the ‘good’ category for clinical applications according to the guide for classifying the accuracy of a diagnostic test with the traditional academic point system (0.9–1.0 excellent; 0.8–0.9 very good; 0.7–0.8 good; 0.6–0.7 sufficient; 0.5–0.6 bad; < 0.5 test not useful) [35, 36].

In an earlier study, Kumarswamy et al. [16], conducted global transcriptomic analyses in plasma RNA from patients with or without left ventricular remodelling after MI with three independent patient cohorts developing cardiac remodelling and HF. The authors found that \textit{LIPCAR} is down-regulated early after MI but up-regulated during later stages. Plasma levels of \textit{LIPCAR} can predict patients developing cardiac remodelling and future cardiovascular deaths. Li et al. [17] analysed the expression levels of IncRNAs in whole blood, tissue and plasma in a mouse model of acute HF. The study revealed that 518 IncRNAs are up-regulated while 908 are down-regulated in the heart with microarray-based analyses with 32 differentially expressed IncRNAs with changes greater than twofold. Greco et al. [29] profiled and validated IncRNAs in left ventricle biopsies of 18 patients affected by non-end-stage dilated ischaemic cardiomyopathy and 17 matched controls. Fourteen IncRNAs were significantly modulated in non-end-stage HF patients, identifying a HF IncRNA signature. In particular, \textit{CDKN2B-AS1/ANRIL} (antisense non-coding RNA in the INK4 locus), \textit{HOTAIR} (HOX transcript antisense RNA) and \textit{LOC285194/TUSC7} (tumour suppressor candidate 7) showed similar modulation in peripheral blood mononuclear cells and heart tissue, suggesting a potential role as disease biomarkers. Yan et al. [37] identified an IncRNA \textit{UCA1} (urothelial carcinoma-associated 1) as a biomarker for acute myocardial infarction (AMI) with its plasma level significantly decreased in AMI patients, compared with non-AMI subjects.

In one of our previous studies, we reported two IncRNAs zinc finger antisense 1 (\textit{ZFAS1}) and Cdr1 antisense (\textit{CDR1AS}) as novel biomarkers of acute MI, with their reciprocal changes in the whole blood samples (\textit{ZFAS1} was down-regulated, whereas \textit{CDR1AS} was up-regulated) independently predicting acute MI [38]. Intriguingly, in the context of HF as in the present study, these two IncRNAs did not show significant alterations in their circulating levels, indicating that they may be specific for predicting acute MI. To the best of our knowledge, there have been no other published studies on the circulating IncRNAs in HF patients. Our study therefore represents the first of such efforts to identify biomarkers with the potential to predict HF in humans.

\textbf{Significance of our findings}

\textit{NRON} and \textit{MHRT} as biomarkers could offer a number of advantages. First, IncRNAs have been found to be overall more stable than protein markers in circulation and can be easily detected in blood samples (whole blood, plasma and serum); thus, it is possible that \textit{NRON} and \textit{MHRT} might also be more stable in the blood than the traditional protein markers [15, 39, 40]. Second, \textit{NRON} and \textit{MHRT} can be detected in a quantitative manner by highly sensitive methods such as real-time PCR. And finally, changes in \textit{NRON} and \textit{MHRT} in the bloodstream may reflect alterations of cardiac function and structure during the development of heart disease thereby helping us to infer the underlying molecular mechanisms. This is in resembling miRNAs as biomarkers of heart disease. For example, the early elevation of circulating \textit{miR-1} during acute myocardial infarction can be interpreted as increased apoptotic cardiomyocyte death [41–43].

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Limitations of our study

In the present study, we focused on only a subset of lncRNAs that are known (at the time we initiated our study) to be relevant to cardiac disease without dealing with the global transcriptome profiling. Thus, our findings do not provide a panorama for comprehensive understanding of all lncRNAs identified thus far, but might have missed out many other important lncRNAs that were not included in the present study for their potential as HF biomarkers. Nonetheless, the lncRNAs selected for our study are those that have been shown to be able to cause cardiac disorders or are abundantly expressed in heart cells.

Another limitation of the study is the unknown sources of NRON and MHRT in the bloodstream: Are they released from dead cells in the failing heart or are they secreted by blood cells in response to the damaged heart?

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Conflict of interest

None declared.

Author contributions

LNX, LHS, YZ, YJL and BFY designed, performed study and supervised all aspects of the research and analysis. LNX, YJL and BFY finalized the manuscript. LNX, YJL and BFY assisted in research, data analysis and interpretation. BY, YT, SW, ZMD and YJL were responsible for collect blood samples and for the final approval of the manuscript. YH was responsible for the statistical analysis involved in the study. BY, YT, SW and ZMD reviewed the clinical aspects and writing of manuscript.

Supporting information

Additional Supporting Information may be found online in the supporting information tab for this article:

Table S1 The demographic characteristics and HF-relevant indicators in HF patients, non-HF control participants for NRON

Table S2 The demographic characteristics and HF-relevant indicators in HF patients, non-HF control participants for MHRT

Table S3 The Statistical Analysis of Circulating NRON and MHRT

Table S4 Human gene-specific primers for real-time PCR

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