LncRNA NEAT1 correlates with Th17 cells and proinflammatory cytokines, also reflects stenosis degree and cholesterol level in coronary heart disease patients

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Abstract
Background: Long non-coding RNA nuclear enriched abundant transcript 1 (Inc-NEAT1) regulates endothelial cell functions, CD4+ T cell regulation and chronic inflammation related to coronary heart disease (CHD). Then this case-control study measured Inc-NEAT1 expression in CHD patients, aiming to explore its clinical value in CHD management.

Methods: Totally, 120 documented CHD patients and 120 suspected subjects without CHD diagnosis as controls were enrolled. Plasma Inc-NEAT1 was detected by RT-qPCR in all participants, plasma inflammatory cytokines were assessed by ELISA, T helper (Th) 1, Th2, Th 17 cell proportions in CD4+ T cells were analyzed by flow cytometric analysis in CHD patients, respectively.

Results: Lnc-NEAT1 was higher in CHD patients than in controls (p < 0.001). In CHD patients, Inc-NEAT1 positively associated with Gensini score (r = 0.323, p < 0.001). Besides, Inc-NEAT1 positively correlated with tumor necrosis factor-α (r = 0.271, p = 0.003), interleukin (IL)-1β (r = 0.216, p = 0.018), IL-6 (r = 0.217, p = 0.018) and IL-17 (r = 0.292, p = 0.001); meanwhile, it was positively associated with the percentage of Th 17 cells (r = 0.384, p = 0.002). However, no correlation was found in Inc-NEAT1 with the percentage of Th1 or Th2 cells (all p > 0.05). Moreover, Inc-NEAT1 was correlated with higher hyperuricemia prevalence (p = 0.028), increased total cholesterol (r = 0.263, p = 0.004) and low-density lipoprotein cholesterol (r = 0.261, p = 0.004), but was not associated with other characteristics (all p > 0.05).

Conclusion: Lnc-NEAT1 correlates with Th17 cells and proinflammatory cytokines, also reflects stenosis degree and cholesterol level in CHD patients, which potentially improves the management of CHD patients.

Key words
CD4+ T cell, coronary heart disease, Gensini score, inflammatory cytokines, nuclear enriched abundant transcript 1
Coronary heart disease (CHD) is the most common cardiovascular disease with atherosclerosis as its leading cause. As a chronic disease, CHD mainly results from a progressive narrowing of blood vessels, interrupting or blocking blood supply to the heart, which gradually gives rise to ischemia when oxygen demand increases. Nearly 80% of CHD can be prevented by reasonable nutrition, regular exercise, coupled with avoiding alcohol intake and smoking. Besides, there are some appropriate medical treatments and surgical therapies, such as anticoagulants, percutaneous coronary intervention, coronary bypass surgery, etc. for CHD patients. Although these prevention and treatment methods have greatly decreased CHD risk and improved CHD management, CHD still appears with high mortality rate and is a major cause of death for adults worldwide. Long non-coding RNA nuclear enriched abundant transcript 1 (Inc-NEAT1) functions as a structural component of paraspeckles, which modulates various gene expressions. Besides, it is reported that Inc-NEAT1 plays critical roles in CHD. For instance, Inc-NEAT1 expression increases human coronary endothelial cells viability and inhibits apoptosis by activating miR-140-3p/mitogen-activated protein kinase 1 (miR-140-3p/MAPK1) signaling; overexpression of Inc-NEAT1 increases the proliferation and migration of human umbilical vein endothelial cells (HUVECs) by regulating miR-181d-5p/cyclin-dependent kinase inhibitor 3 (miR-181d-5p/CDKN3) axis. Meanwhile, it is also suggested that the Inc-NEAT1 expression promotes CD4+ cell differentiation, the latter is also implicated in the chronic inflammatory infiltration of CHD. For example, the Inc-NEAT1 expression promotes Th17 cell differentiation by elevating the signal transducer and activator of transcription 3 (STAT3) protein level, facilitates Th17 cell differentiation and migration through regulating miR-144-3p/rho-associated protein kinase 2 (miR-144-3p/ROCK2) axis. Besides, it is also suggested that Inc-NEAT1 has relations to Th2 cell, one study reveals that by inhibiting STAT6 ubiquitination, Inc-NEAT1 promotes Th2 cell activation; meanwhile, Inc-NEAT1 affects Th1/Th2 balance. Moreover, Inc-NEAT1 promotes inflammation in various inflammation-related diseases. For instance, Inc-NEAT1 elevates the levels of inflammatory cytokines and aggravates inflammation in hepatic ischemia injury; Inc-NEAT1 might promote sepsis-induced myocardial injury by modulating the toll-like receptor 2/nuclear factor-kappaB signaling pathway. Except for the above-mentioned information, the clinical value of Th1/2/17 cells determination for inflammation or stenosis in coronary artery disease had been reported previously. For instance, Th1/Th2 functional imbalance exists in coronary arterial inflammation; the Th1 cell proportion and the Th1/Th2 ratio elevate with the increase of the number of affected arteries and the degree of stenosis. However, the clinical data about Inc-NEAT1 measurement in CHD control is seldom reported.

In this study, we investigated the association of Inc-NEAT1 with disease risk, general conditions, inflammatory cytokines and Th1/Th2/Th17 cells of CHD, aiming to assess Inc-NEAT1 as a potential biomarker for reflecting disease severity and inflammation in CHD, which might improve the management of CHD patients.

2 | METHODS

2.1 | Subjects

After the approval of the Institutional Review Board of The Second Affiliated Hospital, Zhejiang University School of Medicine, a total of 120 patients with CHD confirmed by coronary angiography (CAG) for unexplained chest pain or suspected CHD symptoms in the hospital from May 2018 to September 2020 were included in the case-control study. The inclusion criteria were as follows: (a) diagnosed as CHD, which was based on typical angina symptoms and confirmed by CAG (at least one major epicardial vessel with >50% stenosis); (b) aged older than 18 years; (c) willing to provide blood samples for this study. The exclusion criteria were: (a) complicated with other heart diseases apart from CHD; (b) concomitant with autoimmune disease, inflammatory diseases or severe infections; (c) severe abnormality in liver or kidney function; (d) had history of cardiac surgery including minimally invasive surgery and open-heart surgery; (e) had history of cancers or hematologic malignancies; (f) pregnant and lactating women. During the same period, another 120 subjects without CHD excluded by CAG were also enrolled in the study as controls. All controls presented with unexplained chest pain or suspected CHD symptoms at admission and were excluded from CHD by CAG examination. Controls were ineligible for enrollment if they were pregnant and lactating women, concomitant with autoimmune disease, inflammatory diseases, severe infections, severe abnormal liver or kidney function, or had history of cancers or hematologic malignancies. Meanwhile, to match the age and sex of controls and patients, the age of controls was limited within 50 to 80 years, and the sex ratio of male vs. female in controls was set as 4:1. All subjects signed the informed consents.

2.2 | Data recording and sample collection

After diagnostic tests, clinical data of subjects were documented, including demographics, medical histories and biochemical indexes. The degree of coronary artery stenosis was quantified using Gensini score. The whole blood samples of all subjects were collected before CAG. Within 30 min, the collected samples were centrifuged at 2000 g for 10 min at 4°C, next, the resulted plasma samples were further centrifuged at 16000 g for 10 min to remove the impurities. Finally, the plasma samples were stored at −80°C for the following determination of Inc-NEAT1 expression and inflammatory cytokines. In addition, the whole blood samples from 63 of 120 CHD patients were collected again after CAG, and these 63 samples were used for quantitative analysis of Th1, Th2, and Th17 cell proportion in the CD4+ T cells.
2.3 | Reverse transcription quantitative polymerase chain reaction (RT-qPCR)

The expression of Inc-NEAT1 in plasma was assessed by RT-qPCR. The sample was processed by RNeasy Protect Mini Kit (Qiagen, Duesseldorf) to extract total RNA, which was subsequently submitted to perform reverse transcription using PrimeScript™ RT reagent Kit (Takara). Thereafter, qPCR was carried out with QuantiNova SYBR Green PCR Kit (Qiagen). Glyceraldehyde-3-phosphate dehydrogenase (Becton, Dickinson and Company).

Th17 cell detected by a flow cytometer, and the proportions of Th1/Th2/Th17 cells were analyzed by FlowJo 7.6 software (Becton, Dickinson and Company).

2.4 | Quantitative analysis of inflammatory cytokines

Enzyme-linked immunosorbent assay (ELISA) was applied for testing the levels of tumor necrosis factor-α (TNF-α), interleukin (IL)-1β, IL-6, and IL-17 in plasma of CHD patients. The kits, including Human TNF-α Quantikine ELISA Kit, Human IL-1β Quantikine ELISA Kit, Human IL-6 Quantikine ELISA Kit, and Human IL-17 Quantikine ELISA Kit, were all purchased from the Bio-Techne China Co., Ltd., and the assay procedures were completed referring to the experiment approach provided by the producer.

2.5 | Quantitative analysis of Th1/Th2/Th17 cells

For the 63 whole blood samples collected after CAG, the peripheral blood mononuclear cells (PBMC) were isolated by density gradient centrifugation, then Human Th1/Th2/Th17 Phenotyping Kit (Becton, Dickinson and Company, Franklin Lakes, New Jersey, USA) was used to identify the functionally polarized CD4+ T cell subsets (Th1/Th2/Th17 cells) based on their distinctive patterns of cytokine secretion (Th1 cells selectively produced large amounts of interferon-gamma (IFN-γ); Th2 cells selectively produced IL-4; Th17 cells expressed high levels of IL-17A). The BD Human Th1/Th2/Th17 Phenotyping Kit provided an easy-to-use four-color cocktail of fluorescent antibodies-specific for human CD4, IFN-γ (for Th1), IL-4 (for Th2) and IL-17A (for Th17). In brief, firstly, the PBMCs were stimulated with PMA/Ionomycin in the presence of BD GolgiStop™ Protein Transport Inhibitor (provided in the kit), then the stimulated cells were harvested and transferred to flow test tube, followed by cell fixation using cold BD Cytofix™ Fixation Buffer (provided in the kit); secondly, the fixed cells were permeabilized by BD Perm/Wash™ buffer (provided in the kit); thirdly, the fixed/permeabilized cells were suspended in BD Perm/Wash™ buffer and Human Th1/Th2/Th17 Phenotyping Kit, followed by incubation in the dark; finally, after resuspending the stained cells in flow cytometry staining buffer, the cells were detected by a flow cytometer, and the proportions of Th1/Th2/Th17 cell in CD4+ T cells were analyzed by FlowJo 7.6 software (Becton, Dickinson and Company).

2.6 | Statistical analysis

SPSS 26.0 (IBM Corp.) and GraphPad Prism 7.02 (GraphPad Software Inc.) were applied for data analysis and graph construction. The distribution of continuous variables was determined by Kolmogorov-Smirnov test. The mean and standard deviation (SD) were applied to describe the characteristics of normally distributed variables, and the median with interquartile range was used to describe the characteristics of skewed-distributed variables. Frequency was used to describe the categorized variables. Comparison was determined by chi-square test, Student’s t test or Wilcoxon rank sum test. Association between two continuous variables was checked by Spearman rank correlation test. Receiver operating characteristic (ROC) analysis was used to illustrate the performance of variables in distinguishing CHD from controls, and the sensitivity and specificity at the selected cut-off points were also calculated. A p value < 0.05 indicated statistical significance.

3 | RESULTS

3.1 | Clinical characteristics of CHD patients and controls

A total of 120 patients with CHD and another 120 subjects without CHD as disease controls were enrolled in the study. In brief, the mean ages of CHD patients and controls were 63.5 ± 9.4 years and 61.5 ± 7.0 years, respectively. Besides, there were 97 (80.8%) male CHD patients and 96 (80.0%) male controls. According to comparison analysis, the number of patients with hypertension (p = 0.032) and diabetes mellitus (p = 0.015) was higher in CHD patients than in controls; as to biochemical indexes, the mean fasting blood glucose (p = 0.045), median C-reactive protein (p < 0.001) were elevated, while the median high-density lipoprotein cholesterol was decreased (p = 0.005) in CHD patients than in controls. Moreover, the median Gensini score in CHD patients was greatly higher than that in controls (p < 0.001). However, no difference was found in other clinical features between CHD patients and controls (Table 1).

3.2 | Inc-NEAT1 expression in CHD patients and controls

Inc-NEAT1 expression was higher in CHD patients than in controls (p < 0.001). Meanwhile, the ROC curve showed that Inc-NEAT1 expression had good potential in discriminating CHD patients from controls with area under curve (AUC) of 0.866 (95%CI: 0.822–0.910) (Figure 1A,B). Moreover, the sensitivity and specificity of Inc-NEAT1 expression for discriminating CHD patients from controls were explored, which showed that sensitivity and specificity were 99.2% and 25.0% at cut-off value of 0.678, 90.8% and 50.0% at cut-off value of 1.000, as well as 79.2% and 75.0% at cut-off value of 1.306, respectively (Table 2).
3.3 Correlation of lnc-NEAT1 expression with Gensini score in CHD patients

In the present study, the CHD patients were divided as the age >60 years group and the age ≥60 years group. Subsequently, Gensini score was assessed in both groups, which disclosed that lnc-NEAT1 expression was positively associated with Gensini score in the age >60 years group (p = 0.049) (Figure 2A) and the age ≥60 years group (p = 0.019) (Figure 2B).

3.4 Association of lnc-NEAT1 expression with inflammation in CHD patients

The association of lnc-NEAT1 expression with inflammatory cytokines were investigated subsequently, then data showed that lnc-NEAT1 expression positively correlated with TNF-α (r = 0.271, p = 0.003) (Figure 3A), IL-1β (r = 0.216, p = 0.018) (Figure 3B), IL-6 (r = 0.217, p = 0.018) (Figure 3C) and IL-17 (r = 0.292, p = 0.001) (Figure 3D).

### TABLE 1 Clinical characteristics

| Items                        | Controls (N = 120) | CHD patients (N = 120) | p value |
|------------------------------|--------------------|------------------------|---------|
| Age (years), mean ± SD       | 61.5 ± 7.0         | 63.5 ± 9.4             | 0.073   |
| Male, No. (%)                | 96 (80.0)          | 97 (80.8)              | 0.871   |
| BMI (kg/m²), mean ± SD       | 23.6 ± 3.1         | 23.9 ± 2.9             | 0.445   |
| Smoke, No. (%)               | 43 (35.8)          | 55 (45.8)              | 0.115   |
| Family history of CHD, No. (%)| 22 (18.3)         | 27 (22.5)              | 0.423   |
| Complications                |                    |                        |         |
| Hypertension, No. (%)        | 78 (65.0)          | 93 (77.5)              | 0.032   |
| Hyperlipidemia, No. (%)      | 55 (45.8)          | 62 (51.7)              | 0.366   |
| Hyperuricemia, No. (%)       | 39 (32.5)          | 45 (37.5)              | 0.417   |
| DM, No. (%)                  | 16 (13.3)          | 31 (25.8)              | 0.015   |
| Biochemical indexes          |                    |                        |         |
| FBG (mmol/l), mean ± SD      | 5.5 ± 0.9          | 5.7 ± 1.1              | 0.045   |
| Scr (umol/l), mean ± SD      | 74.6 ± 13.7        | 76.4 ± 15.3            | 0.333   |
| SUA (umol/l), mean ± SD      | 359.6 ± 56.0       | 357.3 ± 63.4           | 0.761   |
| TG (mmol/L), median (IQR)    | 1.5 (0.9–2.3)      | 1.7 (0.9–2.3)          | 0.348   |
| TC (mmol/L), mean ± SD       | 4.6 ± 1.0          | 4.8 ± 1.0              | 0.122   |
| LDL-C (mmol/L), mean ± SD    | 2.8 ± 0.7          | 3.0 ± 0.7              | 0.068   |
| HDL-C (mmol/L), median (IQR) | 1.0 (0.8–1.2)      | 0.9 (0.8–1.0)          | 0.005   |
| CRP (mg/L), median (IQR)     | 6.5 (2.6–10.1)     | 9.0 (6.8–12.1)         | <0.001  |
| Gensini score, median (IQR)  | 1.0 (0.0–2.0)      | 42.0 (22.5–60.0)       | <0.001  |

Abbreviations: BMI, body mass index; CHD, coronary heart disease; CRP, C-reactive protein; DM, diabetes mellitus; FBG, fasting blood glucose; HDL-C, high-density lipoprotein cholesterol; IQR, interquartile range; LDL-C, low-density lipoprotein cholesterol; Scr, serum creatinine; SD, standard deviation; SUA, serum uric acid; TC, total cholesterol; TG, triglyceride.

**FIGURE 1** Comparison of lnc-NEAT1 expression between CHD patients and disease controls in CHD patients. Lnc-NEAT1 expression in CHD patients and controls (A) the potential of lnc-NEAT1 expression in discriminating CHD patients from controls (B) Lnc-NEAT1, long non-coding RNA nuclear paraspeckle assembly transcript 1; CHD, coronary heart disease; AUC, area under curve; CI, confidence interval; CHD, coronary heart disease.
3.5 | Correlation of lnc-NEAT1 expression with Th1, Th2 and Th17 cells in CHD patients

Lnc-NEAT1 expression was positively associated with the percentage of Th17 cells \((r = 0.384, p = 0.002)\) (Figure 4C). However, no association was found in lnc-NEAT1 expression with the percentage of Th1 cells \((r = 0.162, p = 0.203)\) (Figure 4A) or the percentage of Th2 cells \((r = -0.120, p = 0.350)\) (Figure 4B).

3.6 | Association of lnc-NEAT1 expression with chronic complications and biochemical indexes in CHD patients

As to chronic complications, lnc-NEAT1 expression was only positively correlated with hyperuricemia \((p = 0.028)\) (Table 3). Regarding the biochemical indexes, lnc-NEAT1 expression was positively associated with total cholesterol (TC) \((r = 0.263, p = 0.004)\) and low-density lipoprotein cholesterol (LDL-C) \((r = 0.261, p = 0.004)\), while negatively correlated with HDL-C/LDL-C \((p = 0.037)\) (Table 4).

### Table 2

| Cut-off value | Sensitivity | Specificity |
|--------------|-------------|-------------|
| Lnc-NEAT1 = 0.678 | 99.2% | 25.0% |
| Lnc-NEAT1 = 1.000 | 90.8% | 50.0% |
| Lnc-NEAT1 = 1.306 | 79.2% | 75.0% |

Abbreviations: CHD, coronary heart disease; Lnc-NEAT1, long non-coding RNA nuclear paraspeckle assembly transcript 1.

The cut-off value was set according to the 1/4 quantile, 2/4 quantiles, and 3/4 quantiles of lnc-NEAT1 expression in controls.

### Discussion

In this study, we observed that (a) lnc-NEAT1 expression was increased in CHD patients; (b) lnc-NEAT1 expression was positively associated with Gensini score, which represented more advanced disease severity in CHD patients; (c) lnc-NEAT1 expression positively correlated with TNF-α, IL-1β, IL-6, and IL-17 in CHD patients; (d) lnc-NEAT1 expression was positively associated with the percentage of Th17 cells in CHD patients.
As to the correlation of lnc-NEAT1 expression in inflammation-related disease, lnc-NEAT1 expression increases in patients with sepsis compared to healthy control.\textsuperscript{16} Additionally, lnc-NEAT1 relative expression is elevated in allergic rhinitis patients than in controls.\textsuperscript{17} Moreover, lnc-NEAT1 expression is upregulated in asthma patients in exacerbation compared with healthy controls.\textsuperscript{18} Our study found that lnc-NEAT1 expression was higher in CHD patients than in disease controls. The reason could be that lnc-NEAT1 expression might increase coronary endothelial cell viability by activating the miR-140-3p/MAPK1 pathway, then affecting the development and pathogenesis of CHD.\textsuperscript{7} In addition, we also found that cut-off points of lnc-NEAT1 expression at the 1/4 quantile, 2/4 quantiles and 3/4 quantiles in controls showed good sensitivity and specificity, which suggested lnc-NEAT1 expression might be used as a predictive biomarker for CHD risk.

In terms of the association of lnc-NEAT1 expression with inflammation-related disease severity, a previous study shows that lnc-NEAT1 high expression associates with increased National Institute of Health Stroke Scale score, indicating a worse disease condition in acute ischemic stroke.\textsuperscript{19} Another study suggests that lnc-NEAT1 expression is positively associated with exacerbation severity in asthma.\textsuperscript{18} In our study, we discovered that lnc-NEAT1 expression was positively associated with Gensini score, which represented more advanced disease severity. The reason might be that: through modulating miR-181d-5p/cyclin-dependent kinase inhibitor 3 (miR-181d-5p/CDKN3) axis, lnc-NEAT1 increased the proliferation of HUVECs,\textsuperscript{20} which could contribute to coronary artery stenosis and pathophysiological process in CHD,\textsuperscript{21} thus resulting in unfavorable disease severity. Furthermore, a study reveals that elevation of TC is closely correlated with coronary artery stenosis.\textsuperscript{22} In our study, lnc-NEAT1 had a relation to TC, which might contribute to stenosis in CHD.

Regarding the correlation of lnc-NEAT1 expression with inflammatory cytokines in patients with inflammation-related disease, lnc-NEAT1 expression positively correlates with TNF-α, IL-1β, IL-6, and IL-8 in sepsis patients.\textsuperscript{23} Besides, lnc-NEAT1 expression is positively
associated with TNF-α, IL-1β, and IL-17 in asthma patients. In our study, we found that Inc-NEAT1 expression positively correlated with TNF-α, IL-1β, IL-6, and IL-17. A possible explanation might be that: through activating NF-kB signaling, the Inc-NEAT1 expression enhanced inflammatory level and the production of inflammatory cytokines, thus Inc-NEAT1 expression was correlated with higher TNF-α, IL-1β, IL-6, and IL-17. Besides, we also found that Inc-NEAT1 expression was positively associated with the percentage of Th17 cells. The reason might be that: Inc-NEAT1 facilitated Th17 cell differentiation by increasing the STAT3 protein level.

Concerning the association of Inc-NEAT1 expression with hyperuricemia, TC or LDL-C, as far as we know, there was no study conducted before to explore the correlation between them. According to our study, Inc-NEAT1 expression positively correlated with hyperuricemia, TC and LDL-C. The above finding suggested that Inc-NEAT1 might affect the metabolism of uric acid and lipid: Inc-NEAT1 might regulate the formation of foam cells induced by ox-LDL, further causing the relation of NEAT1 to TC in CHD patients. However, further investigation should be performed. Hyperuricemia and high cholesterol were risk factors for CHD. By influencing the metabolism of uric acid and lipid, high expression of Inc-NEAT1 might indirectly cause the development of CHD.

Although a lot of findings were identified in this study, there were still some limitations. Firstly, the sample size could be further expanded to improve the statistical power. Secondly, our study evaluated the correlation of Inc-NEAT1 expression with general disease conditions of patients having CHD, while the association of Inc-NEAT1 expression with disease outcomes such as subsequent risk of major adverse cardiovascular events could be further evaluated. Thirdly, this study did not investigate the molecular mechanism of Inc-NEAT1 expression and its related inflammation involved in CHD progression, thus in vivo and in vitro experiments might be essential to further conducted.

Conclusively, Inc-NEAT1 correlates with Th17 cells and proinflammatory cytokines, also reflects stenosis degree and cholesterol level in CHD patients. It might potentially serve as a biomarker for CHD prognosis, which improves the management of CHD patients.

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CONFLICT OF INTEREST
The authors of this work have nothing to disclose.

DATA AVAILABILITY STATEMENT
The datasets used and/or analyzed during the current study are available from the corresponding author on reasonable request.

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