

## **Circulating lncRNA ANRIL level positively correlates with disease risk, severity, inflammation level and poor prognosis of coronary artery disease.**

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### **Abstract**

**Objective:** The purpose of this study was to investigate the association of long non-coding RNA (lncRNA) ANRIL expression with disease risk, severity, inflammation level and prognosis of coronary artery disease (CAD).

**Methods:** A total of 169 patients with unexplained chest pain or CAD-like symptoms underwent coronary angiography were consecutively recruited, among whom 92 patients diagnosed with CAD were included in CAD group while the other 77 patients were included in control group. Plasma lncRNA ANRIL level of all patients was detected by quantitative polymerase chain reaction, Gensini score was evaluated *via* Gensini criterion. In CAD patients, plasma level of inflammatory factors was evaluated by enzyme linked immunosorbent assay, and overall survival (OS) was calculated.

**Results:** Plasma level of lncRNA ANRIL was increased in CAD group compared to control group ( $P<0.001$ ) and disclosed a good predictive value for CAD risk (AUC=0.806, 95% CI: 0.741-0.871). In CAD patients, lncRNA ANRIL was positively correlated with Gensini score ( $r=0.270$ ,  $P=0.009$ ), high-sensitivity C-reactive protein level ( $r=0.293$ ,  $P=0.005$ ), tumor necrosis factor- $\alpha$  level ( $r=0.271$ ,  $P=0.009$ ) and interleukin (IL)-6 level ( $r=0.464$ ,  $P<0.001$ ) while negatively associated with IL-10 ( $r=-0.274$ ,  $P=0.008$ ) level. Additionally, the OS was poorer in CAD patients with high lncRNA ANRIL expression compared to patients with low lncRNA ANRIL expression ( $P=0.025$ ).

**Conclusion:** Circulating lncRNA ANRIL expression associates with increased disease risk, severity, inflammation level and poorer prognosis of CAD.

**Keywords:** lncRNA ANRIL, Plasma, Coronary artery disease, Disease risk, Disease severity.

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### **Introduction**

Coronary artery disease (CAD) is one of the most common diseases in the world which affects approximately 110 million people and causes 8.9 million deaths every year [1,2]. Due to the unhealthy life type and the increased lifespan, CAD is becoming more and more prevalent both in developing countries and developed countries [1-3]. Although great improvement has been achieved in CAD diagnosis and treatment in the past decades, there still lack sensitive and specific biomarkers for early CAD diagnosis and disease monitoring [3-5].

Long non-coding RNA (lncRNA) is a sort of RNAs which have more than 200 nucleotides but possess little or no open reading frame [6]. According to previous studies, lncRNA is widely distributed in eukaryotic cells and is implicated in various biological activities through epigenetic regulation, transcriptional regulation and post-transcriptional regulation [7-9]. Among the numerous lncRNAs, lncRNA antisense non-coding RNA in the INK4 locus (lncRNA ANRIL) is discovered to be involved in many complicated diseases such as cancers, diabetes and inflammatory diseases [10-12]. More

interestingly, there are increasing evidence showing that lncRNA ANRIL plays an important role in cardiovascular diseases [13-15]. For instance, in CAD patients who have received drug-eluting stents (DESs) treatment, lncRNA ANRIL is upregulated in those patients with in-stent restenosis (ISR) compared with patients without ISR [16]. In another study, lncRNA ANRIL expression is also increased in type 2 diabetes mellitus (T2DM) patients with CAD compared to T2DM patients without CAD [11]. However, the role of lncRNA ANRIL in CAD remains largely unclear. To this end, we conducted the current study to investigate the association of lncRNA ANRIL expression with disease risk, severity and prognosis of CAD.

### **Methods**

#### **Participants**

One hundred and sixty-nine patients with unexplained chest pain or CAD-like symptoms who were admitted to The Affiliated Hospital of Guizhou Medical University for elective coronary angiography between January 2013 and December 2014 were consecutively included in this study. All enrolled

patients were over 18 years old without congenital heart disease, heart valvular disease, vasospastic angina, cardiomyopathy or coronary artery bypass graft surgery history. The following patients were excluded: (1) contraindication of coronary angiography; (2) serious heart, lung or kidney dysfunction; (3) history of cancer, malignant blood disease, severe infection or autoimmune diseases; (4) pregnant or lactating women. After coronary angiography, 92 patients diagnosed with CAD were included in CAD group while the other 77 patients were included in control group. CAD was defined as at least one coronary artery occurred stenosis ( $\geq 50\%$ ) according to coronary angiography in this study. All patients or their guardians signed informed consents and the study was approved by the Ethics Committee of the Hospital.

### **Data collection**

Clinical data of patients were collected after enrolment which consisted of (1) baseline characteristics: age, gender, body mass index (BMI), smoke, hypertension, diabetes, and the history of CAD; (2) routine laboratory testing: triglyceride (TG), total cholesterol (TC), high-density lipoprotein cholesterol (HDL-C) and low-density lipoprotein cholesterol (LDL-C).

### **Coronary artery damage assessment**

Gensini score was used to assess the severity of coronary artery damage. Firstly, gave a basic score to each coronary as follows: 1 point for  $\leq 25\%$  narrowing, 2 points for 26 to 50% narrowing, 4 points for 51 to 75% narrowing, 8 points for 76 to 90% narrowing, 16 points for 91 to 99% narrowing, and 32 points for 100%. Secondly, a multiplier factor was determined according to the importance of the lesion localization in the coronary arterial system as follows: 5 for the left main coronary, 2.5 for the proximal left anterior descending (LAD) and left circumflex (LCX), 1.5 for the mid segment LAD, 1 for the distal segment of LAD and LCX, first diagonal branch, first obtuse marginal branch, right coronary artery, posterior descending artery and intermediate arteries and 0.5 for the second diagonal and second obtuse marginal branches. Finally, the score of each lesion vessel equalled to the basic score times the factor of same location, and the sum of each lesion vessel score was the final Gensini score in this patient.

### **Sample collection**

Blood sample of patients were collected in the anticoagulation tube and centrifuged at 4°C for 15 minutes at 1800 g. Then the supernatant was transferred to the EP tube and centrifuged at 4°C for another 10 minutes at 2500 g. Finally, the supernatant was transferred to the cryogenic vials (400~500  $\mu\text{L}$ /vial) and stored at -80°C for further detections.

### **Measurements of inflammation markers and cytokines**

The levels of high-sensitivity C-reactive protein (hs-CRP) and erythrocyte sedimentation rate (ESR) of CAD patients were measured by the PA8800 particular globin analyser (Perlong Medical, China) and PUC-2068A ESR analyser (Perlong Medical, China), respectively. Human Enzyme-linked immunoassay (ELISA) kits (R&D, USA) were used to determine the concentrations of plasma inflammatory cytokines according to the manufacturer's instructions, including tumor necrosis factor (TNF- $\alpha$ ), interleukin-1 $\beta$  (IL-1 $\beta$ ), IL-6, IL-8, IL-10 and IL-17.

### **Measurement of lncRNA ANRIL**

All the patients' plasma samples were collected and stored at -80°C after enrolment. Then total RNA extractions were performed using TRIzol reagent (Invitrogen, Carlsbad, CA, USA) according to the manufacturer's recommendations. cDNA was then synthesized using the transcription kit (TOYOBO, Japan), and the relative expression of lncRNA ANRIL was detected by real-time quantitative polymerase chain reaction (RT-qPCR) with the use of SYBR Premix Ex Taq II (Takara, Japan) and Applied Biosystems 7500 HT PCR system (Applied Biosystems, USA). Glyceraldehyde 3-phosphate dehydrogenase (GAPDH) was utilized as normalized internal reference and the relative expression of lncRNA ANRIL was calculated using the  $2^{-\Delta\Delta C_t}$  method. The primers were as follows: ANRIL: forward: 5'-TGCTCTATCCGCCAATCAGG-3', reverse: 5'-GGGCCTCAGTGGCACATAACC-3'; GAPDH: forward: 5'-GAGTCAACGGATTTGGTCGT-3', reverse: 5'-TTGATTTTGGAGGGATCTCG-3'.

### **Follow up**

Regularly follow up for all CAD patients was performed by telephone until the last follow-up data of March 31<sup>st</sup>, 2018. The median follow-up duration was 43.5 months (range: 5.0-54.0 months). A total of 14 CAD patients lost follow up and they were excluded from the overall survival (OS) analysis. OS was defined as the data from enrolment to the data of death from any cause.

### **Statistical analysis**

SPSS 22.0 statistical software (IBM, USA) and Graphpad Prism 6.01 software (GraphPad Software Inc., USA) were used for statistical analysis and chart making process. Data were expressed as mean  $\pm$  SD, count (percentage) or median (25<sup>th</sup>-75<sup>th</sup> quartile). Comparison was determined by Chi-square test, t test or Wilcoxon rank sum test. The diagnostic value of lncRNA ANRIL relative expression for CAD risk was analysed by receiver operating characteristic (ROC) curve; correlation analysis was performed using Spearman's rank correlation test; the OS difference between lncRNA ANRIL high and low expression patients was determined by Kaplan-

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Meier method and Log-rank test. P value<0.05 was considered significant.

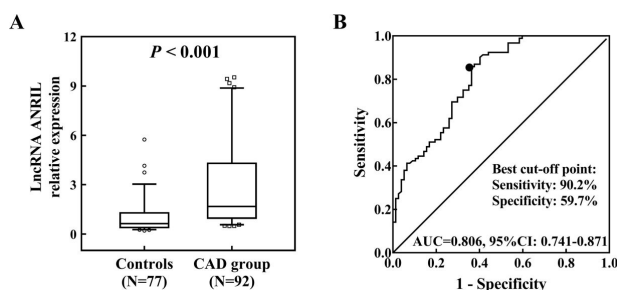
### Results

#### Characteristics of CAD patients and controls

In CAD group, there were 71 male patients and 21 female patients, and the mean age was  $60.7 \pm 9.3$  years; in control group, there were 58 male patients and 19 female patients, and the mean age was  $58.8 \pm 8.1$  years (Table 1). No difference of demographic characteristics between the two groups was observed ( $P>0.05$ ). Meanwhile, HDL-C was  $1.08 \pm 0.34$  mmol/L in CAD group and was  $1.23 \pm 0.38$  mmol/L in control group ( $P=0.007$ ), Gensini score was 45.0 (20.9-69.8) in CAD group and was 1.0 (1.0-2.0) in control group ( $P<0.001$ ). Other characteristics were depicted in Table 1.

#### Comparison of lncRNA ANRIL expression between CAD patients and controls and the ROC curve

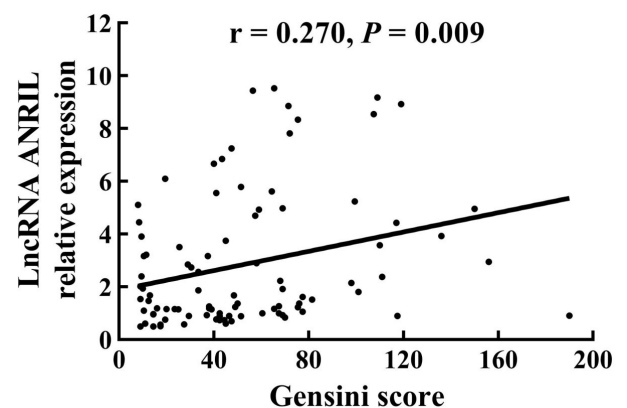
Plasma level of lncRNA ANRIL in CAD group was increased compared with control group ( $P<0.001$ , Figure 1A). ROC curve showed that it well distinguished CAD patients from controls with an AUC of 0.806 (95% CI: 0.741-0.871), and the sensitivity and the specificity at the best cut-off point (lncRNA ANRIL expression: 0.733) was 90.2% and 59.7%, respectively (Figure 1B). The best cut-off point was defined as the point that the value of sensitivity plus specificity was the largest. These data indicated that lncRNA ANRIL might be served as a biomarker for predicting CAD risk.



**Figure 1.** lncRNA ANRIL levels in two groups and the ROC curve. lncRNA ANRIL expression was increased in CAD group compared with control group (A) ROC curve disclosed that it well discriminated CAD patients from controls with an AUC of 0.806 (95% CI: 0.741-0.871), and the sensitivity and the specificity at the best cut-off point was 90.2% and 59.7%, respectively (B) Comparison between two groups was determined by Chi-square test. ROC curve was utilized to evaluate the diagnostic value of lncRNA ANRIL expression for CAD risk. P value<0.05 was considered significant. lncRNA: long non-coding RNA; ANRIL: Antisense Non-Coding RNA in the INK4 Locus; CAD: Coronary Artery Disease; ROC: Receiver Operating Characteristic Curve; AUC: Area Under the Curve.

#### Association of lncRNA ANRIL expression with disease severity and inflammation level in CAD patients

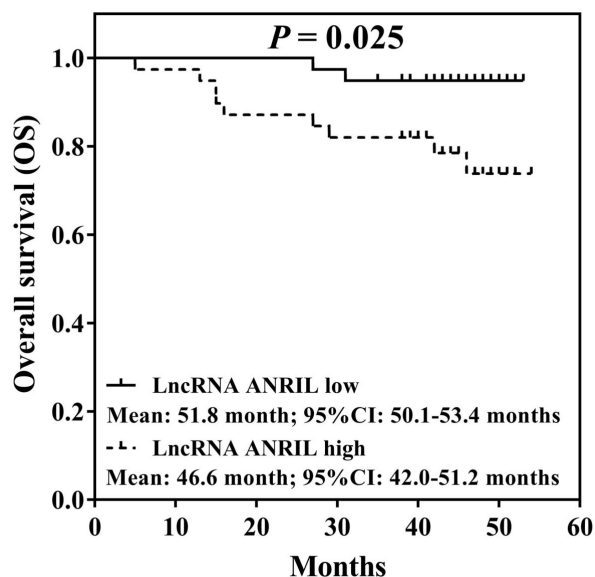
Plasma lncRNA ANRIL expression was positively associated with Gensini score ( $r=0.270$ ,  $P=0.009$ ), suggesting that lncRNA ANRIL expression was associated with increased severity of CAD (Figure 2). Besides, lncRNA ANRIL was also positively correlated with hs-CRP ( $r=0.293$ ,  $P=0.005$ ), TNF- $\alpha$  ( $r=0.271$ ,  $P=0.009$ ) and IL-6 ( $r=0.464$ ,  $P<0.001$ ) while negatively associated with IL-10 ( $r=-0.274$ ,  $P=0.008$ ), implying that it was also correlated with elevated inflammation level of CAD (Table 2). As for ESR ( $r=0.198$ ,  $P=0.059$ ), IL-1 $\beta$  ( $r=0.099$ ,  $P=0.346$ ), IL-8 ( $r=0.185$ ,  $P=0.078$ ) or IL-17 ( $r=0.098$ ,  $P=0.351$ ), no correlation of lncRNA ANRIL level with them was observed.



**Figure 2.** Correlation of lncRNA ANRIL expression with disease severity in CAD patients. lncRNA ANRIL level was positively correlated with Gensini score. Correlation analysis was performed using Spearman's rank correlation test. P value<0.05 was considered significant. lncRNA: long non-coding RNA; ANRIL: Antisense Non-Coding RNA in the INK4 Locus; CAD: Coronary Artery Disease.

#### Association of lncRNA ANRIL expression with OS of CAD patients

Among the 92 CAD patients, 14 patients lost to follow up, hence a total of 78 CAD patients were recruited to survival analysis. Then the 78 CAD patients were divided into lncRNA ANRIL high expression group (n=39) and lncRNA ANRIL low expression group (n=39) based on the median level of lncRNA ANRIL (1.675). The median OS was 46.6 months (95% CI: 42.0-52.2 months) in lncRNA ANRIL high expression group, which was lower than that of 51.8 months (95% CI: 50.1-53.4 months) in lncRNA ANRIL low expression group ( $P=0.025$ ), indicating that lncRNA ANRIL was associated with worse OS of CAD patients (Figure 3).



**Figure 3.** Correlation of lncRNA ANRIL level with OS of CAD patients. The median OS was 46.6 months (95% CI: 42.0-52.2 months) in lncRNA ANRIL high expression group, which was lower than that of 51.8 months (95% CI: 50.1-53.4 months) in lncRNA ANRIL low expression group. OS analysis was determined by Kaplan-Meier method and Log-rank test.  $P$  value < 0.05 was considered significant. lncRNA: long non-coding RNA; ANRIL: Antisense Non-Coding RNA in the INK4 Locus; CAD: Coronary Artery Disease; OS: Overall Survival.

**Table 1.** Characteristics of CAD patients and controls.

Characteristics	CAD patients (N=92)	Controls (N=77)	P value
Age (years)	60.7 ± 9.3	58.8 ± 8.1	0.149
Gender (male/female)	71/21	58/19	0.778
BMI (kg/m <sup>2</sup> )	23.9 ± 3.0	23.6 ± 2.8	0.506
Hypertension (n/%)	81 (88.0)	61 (79.2)	0.119
Diabetes (n/%)	19 (20.7)	14 (18.2)	0.687
Smoke (n/%)	41 (44.6)	40 (51.9)	0.339
Family history of CAD (n/%)	30 (65.2)	22 (28.6)	0.571
TG (mmol/L)	1.79 ± 0.89	1.68 ± 0.81	0.406
TC (mmol/L)	4.72 ± 1.50	4.58 ± 1.61	0.56
HDL-C (mmol/L)	1.08 ± 0.34	1.23 ± 0.38	0.007
LDL-C (mmol/L)	2.85 ± 1.22	2.61 ± 1.09	0.183
Gensini score	45.0 (20.9-69.8)	1.0 (1.0-2.0)	<0.001

Data were presented as mean value ± standard deviation, median (25<sup>th</sup>-75<sup>th</sup> quantiles) or count (percentage). Comparison was determined by t test, Wilcoxon rank sum test or Chi-square test.  $P$  value < 0.05 was considered significant. CAD: Coronary Artery Disease; BMI: Body Mass Index; TG: Triglyceride; TC: Total Cholesterol; HDL-C: Fasting High-Density Lipoprotein Cholesterol; LDL-C: Fasting Low-Density Lipoprotein Cholesterol.

**Table 2.** Correlation of lncRNA ANRIL relative expression with systematic inflammation markers and inflammatory cytokines.

Items	LncRNA ANRIL relative expression	
	Correlation coefficient (r)	P value
hs-CRP	0.293	0.005
ESR	0.198	0.059
TNF- $\alpha$	0.271	0.009
IL-1 $\beta$	0.099	0.346
IL-6	0.464	<0.001
IL-8	0.185	0.078
IL-10	-0.274	0.008
IL-17	0.098	0.351

Correlation was determined by spearman correlation analysis.  $P$  value < 0.05 was considered significant. hs-CRP: hypersensitive C-Reactive Protein; ESR: Erythrocyte Sedimentation Rate; TNF: Tumor Necrosis Factor; IL: Interleukin.

## Discussion and Conclusion

In the current study we discovered that (1) lncRNA ANRIL expression was increased in CAD patients compared with controls and it well discriminated CAD patients from control. (2) lncRNA ANRIL level was associated with increased disease severity and inflammation level as well as worse OS of CAD patients.

lncRNA ANRIL locates within *p15/CDKN2B-p16/CDKN2A-p14/ARF* gene cluster, it could bind to polycomb repressive complex (PRC) 1 and PRC2 then exerts its epigenetic regulations [17,18]. In a recent study, lncRNA ANRIL is overexpressed in atherosclerotic systemic lupus erythematosus (SLE) patients compared with non-atherosclerotic patients and it presents with good predicting value for atherosclerosis in SLE patients [19]. In another study, lncRNA ANRIL is also found to be upregulated in T2DM patients with CAD compared to T2DM patients without CAD [18]. And ROC curve discloses that it might be served as a novel biomarker for predicting CAD in T2DM patients [11]. In addition, CAD patients who have received DESs treatment are more likely to occur ISR in lncRNA ANRIL high expression patients than that in lncRNA ANRIL low expression patients, and lncRNA ANRIL high expression could be a risk factor for ISR [16]. These studies illustrate that lncRNA ANRIL might be involved in cardiovascular diseases especially CAD. However, whether lncRNA ANRIL expression associates with CAD risk is rarely known. In the present study, we discovered that lncRNA ANRIL expression was higher in CAD patients than that in controls, and it pronouncedly distinguished CAD patients from controls, suggesting that lncRNA ANRIL was correlated with increased CAD risk. The possible reasons for our result might be that: (1) lncRNA ANRIL binds to PRC1 and PRC2, then promotes myocardial infarction and increases CAD risk through epigenetic regulations [17,18]. (2) lncRNA ANRIL might also upregulate inflammatory cytokine levels via sponging microRNAs (miRNAs) then elevates myocardial infarction and inflammation injury, which further cause CAD [20].

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Accumulating data have suggested that lncRNA ANRIL is correlated with increased disease severity and inflammation level of many diseases [15,19,20]. In atherosclerotic SLE patients, lncRNA ANRIL is positively associated with SLE duration, complement 3 level, SLE disease activity index and systemic lupus international collaborating clinics (SLICC) index, indicating that lncRNA ANRIL is correlated with increased disease severity and inflammation level of atherosclerotic SLE [19]. In patients with atherosclerosis, lncRNA ANRIL expression is positively associated with disease severity [15]. lncRNA ANRIL is also observed to be highly expressed in human coronary endothelial cells (HCAECs) and CAD mice models than that in human umbilical vein endothelial cells and control mice, respectively [20]. Besides, upregulating lncRNA ANRIL levels in HCAECs enhances IL-6, IL-8, TNF- $\alpha$ , inducible nitric oxide synthase, intercellular cell adhesion molecule 1, vascular cell adhesion molecule 1, vascular endothelial growth factor and heat shock protein 70 expressions [20]. More importantly, elevated lncRNA ANRIL expression in CAD patients associates with higher blood pressure, cholesterol, triacylglycerol and homocysteine levels [20]. Partly in accordance to these studies, our study showed that lncRNA ANRIL expression was positively correlated with Gensini score, hs-CRP, TNF- $\alpha$ , IL-6 and IL-10 levels as well as shorter OS, indicating that lncRNA ANRIL level was associated with increased severity, inflammation level and poorer prognosis. The possible explanation might be due to that: lncRNA ANRIL binds to PRC1 and PRC2 then modulates inflammatory cytokine levels, which further contribute to elevated inflammatory injury, disease severity and poorer OS [17,18]. (2) lncRNA ANRIL might also act as a miRNA sponge (such as miR-181) thereby increasing inflammatory cytokine levels and disease severity, and causing a poor prognosis [20].

There were some limitations in this study. To begin with, some characteristics between CAD group and control group were different, including HDL-C level and Gensini score, which might cause selection bias. However, it was justified that HDL-C level was lower whereas Gensini score was higher in CAD group than that in control group. Secondly, the underlying roles of lncRNA ANRIL in CAD pathogenesis have not been explored in this study. Lastly, this was a small-sample study with only 92 CAD patients enrolled, thus future studies with larger sample size needed to be conducted.

In conclusion, circulating lncRNA ANRIL expression associates with increased disease risk, severity, inflammation level and poorer prognosis of CAD.

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### **Conflicting Interests**

The authors declare that they have no competing interests.

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